Nous reactius d'acoblament per a la síntesi de pèptids basats en 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma) com a additiu

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Als meus pares, l'àvia i la Lídia

Agraïments

Finalment ha arribat l'hora d'escriure els famosos agraïments… la primera (i potser única) pàgina que s'obre d'una Tesi Doctoral, no ens enganyéssim!! I per alguna estranya raó és l'última part que es sol escriure! Potser perquè són tantes coses a recordar i les paraules a escollir que un ho vol fer quan la resta de la tesi ja està coll avall…el cas és que després de llegir tantes tesis (perdó, agraïments de tesi), de vegades pensava en què escriuria jo i la veritat és que arribada l'hora de la veritat no sé per on començar! Han passat 4 anys…increíble!! Sembla que fos ahir quan començava amb la beca de col·laboració com a pinche del Jan amb el PyClock…déu n'hi do si ha plogut! Moltes coses han canviat durant aquests anys….

El primer apartat li correspon al *Fernando*, no pas per protocol, sinó perquè realment m'ha ajudat moltíssim en la meva curta carrera científica. Tot va començar arran d'un exàmen de Química Orgànica II, on em va posar Matrícula d'Honor. Encara recordo que quan vaig veure el seu mail un dissabte d'estiu (vaig pensar: que fa, un dissabte i treballant??), molt escuet, en què em felicitava per la nota i em deia que em passés pel seu despatx, el primer que em va venir el cap és que els meus amics de la uni m'estaven fent una broma…però per sort va resultar ser verídic i em va comentar si volia fer un Passa l'Estiu al Parc! Pocs mesos després d'aquella reunió em va convèncer per embarcar-me en l'aventura anglesa a GSK (si no fos per aquella trucada recordant-me l'entrevista amb la Viqui i el Harry Kelly…quin despistat!). Ja com alumne de Passa l'Estiu al Parc, un dia (plujós) li vaig preguntar de què servia fer un doctorat i em va dir: "per trobar menys feina però millor". Amb aquesta enigmàtica frase al cap, l'estiu del 2006 li vaig preguntar si tindria lloc al grup i em va obrir les portes. Gràcies per donar tantes oportunitats a la gent jove que vol començar en investigació! En el meu cas particular has tingut un pes enorme. Tot i que els principis no van ser fàcils i han hagut de passar molts dissabtes, algun diumenge i sobretot moltes nits llargues, la Tesi ha valgut molt la pena i si hagués de tornar-me a decidir per un director de Tesi i un grup, la elecció seria la mateixa. Hi han hagut molt moments d'estrès i pressió (en especial al principi), però també m'ha servit per aprendre a portar un, dos o *n* tasques a la vegada (Luxembourg forever and for everybody!) i per superar més i més reptes, així com créixer científicament: primer escriure un Chimica Oggi, després un article sencer, més endavant un capítol de llibre (la historia interminable) i així fins a la llibertat dels últims anys per desenvolupar els projectes que m'has donat, amb millors o pitjors resultats, però en qualsevol cas agafant independència.

I also would like to thank *Ayman* for all the guidance and e-mail help during these years (and some summer face-to-face synthetic advice and comments on coupling reagents). Once, at the beginning of the PhD, you said that I would be better than you when I completed the four years. Well, I don't think that premonition has become true, but your proximity and knowledge had great impact in what I've learned in all these years! També voldria agraïr a la *Mercedes Álvarez* l'acollida al 100 (…o era Pharmamar?) durant el Passa l'Estiu al Parc 2005, al *Gerardo* i al *Antonio* pel que em van ensenyar aquells dos mesos (si, vaig fer química també, malas lenguas!!).

Arribem al 300! Ha passat molta gent per aquest laboratori en 4 anys, i voldria destacar aquells amb els que he compartit més moments, sobretot fora d'aquestes quatre parets. El primer de tots el *titu Albert*, al qui personalment admiro pel seu optimisme inesgotable, la seva vàlua científica i perquè sempre ha tingut un moment per dedicar-me, especialment al principi quan es necessita algú a prop amb actitud pedagògica, que et resolgui dubtes químics. Gràcies també per ajudar-me i aconsellar-me en molts aspectes, inclosos els interrogants futurs, i per tantes converses que hem tingut aquest temps, en persona o via mail (sovint parlant del barça!). La teva tesi ha estat tot un exemple per mi! De la gent que ja no hi és, recordo especialment la sempre simpàtica i agradable *Martina*, la *Gemma Comellas*, del poc temps que vam coincidir, el *Frank*, el primer company de poyata que vaig tenir (l'alemany no convencional), que muntava unes enormes festes al seu pis i portava la guitarra a les cases rurals, la *Steph* i el *Renaud*, els francesos més encantadors que mai he conegut (i no són molts). Sembla que fa mil anys de quan anàvem als menjadors en massa…(com diu la dita: *La venganza es un plato que se sirve (servia) en los comedores*); en aquella època vaig coincidir amb el *Manu*, el hombre de las mil despedidas. No em voldria oblidar dels *malakas Thanos* i *Kate*, no sé com encara recordo la majòria d'insults en grec que em va ensenyar! (*thanuklasis*

starjidiamus), dels millors companys de poyata que he tingut, l'home dels acoblaments en aigua, al microones i amb Boc (lo normal…), amb qui vaig debutar com espectador del Palau en un Barça-Panathinaikos, el *Hiroshi*, amb qui vam conèixer una mica més la cultura japonesa de no-sabem-dir-que-no-ni-vull-entendre-que-em-parles-de-bitch-i-no-de-beach, com oblidar els seu mmm, aaa,mmm, ra-ra-ra-ramon o les converses surrealistes dels cafès. De l'època com a Passa l'Estiu al Parc tinc bons records del *Jesús Vázquez*, que tot i no tenir temps d'interaccionar molt, em va ajudar força enviant fax a Stevenage per llogar el pis (la primera tesi que vaig veure, cantant al pica-pica el siete hoooras….). Del grup de l'Ernest Giralt, la *Susana*, una científica excel·lent i companya de laboratori els dissabtes al Parc el primers anys, molt agradable tot i que de primeres podia semblar el contrari, que va emigrar a Boston fa uns anys, la *Silvia*, japonesa d'adopció, amb qui vaig fer els *pinitos* (¿pinets?) en la tasca d'encarregar material a la botiga (nunca fue tan difícil).

Encara ronden per aquí la *Marta Pelay*, alias Pelayo o Nanomarta, constant rialla al 300, que estic segur que treurà endavant la tesi (lo que no mata engorda), la *Rubí*, el talent mexicà del laboratori, va començar com alumne de Passa l'Estiu al Parc i es convertirà sens dubte en un gran sintètica, el *Miguel*, estrella del tiki-taka dels Brutal, ha introduit un nou personatge al joc del Lobo, una mica esbojarrat, de conversació fàcil i amena a l'altre costat de l'escriptori, sempre té un HUAHUA o un TODO MAL quan passes pel costat, el *Bernat* (petit), la nova fornada de doctorands del Giralt, científic, dansaire, productor de cervesa, davanter habilidós…, la *Sol* i la *Laura Nevola*, expertes sintètiques (aquestes de debò) però que potser es passen al ball després dels últims videos que han sortit a la llum…, la *Judit*, l'essència gironina del grup, investigadora capaç de portar el seu projecte i els de *n*-mil doctorands a la vegada, espectacular! la primera en arribar pels matins, el *Jan*, tota una font d'inspiració personal…va ser el pioner, i finalment el *Roger*, això és una tesi multidisciplinar i lo demés son tonteries! Has passat una travesia al desert en molts moments però seràs un dels doctors més complets que sortiran d'aquest laboratori, un exemple d'esforç i dedicació, de moment ja hi ha algú que ha vist el teu talent (mira qué talento!), sort al futur, ho mereixes!, i la resta de membres actuals i passats amb els qui en algún moment he interaccionat.

Encetem el capítol 100-Pharmamar, que també promet ser llarg (a aquest pas no imprimiré la tesi…). Del territori de la Mercedes vaig coincidir amb el *Roger Soley*, que va ser prou llest com per canviar un treball que li aportava poc per un altre de comercial amb el que se'l veu infinitament més feliç, *el Marc*, vam compartir molts breaks durant els pocs mesos que va estar aquí abans de passar-se a la industria (un que és llest…), la *Delia*, companya de vitrina l'estiu del 2005, un exemple de com l'entorn de treball pot influir en el teu humor, el *Samuel*, guitarrista semi-professional de CV molt extravagant, que va deixar empremta del seu pas pel grup. Entre els membres actuals, me n'enduré un bon record del *Paolo*, postdoc amb ànima de concertista de bombardino, un altre habitual dels dissabtes, una mica tocat del bolet, impossible avorrir-se, el *Xavi*, successor a GSK, químic amb un cert punt *friki*, un gran cuiner de menjar oriental, que amaga un gran potencial com a investigador i la *Sveta*, de la qui sempre he obtingut bones cares i ajuda quan ho he necessitat. A l'altra banda del laboratori està Pharmamar, on em vaig passar mig Passa l'Estiu al Parc i part de la tesi, de visita rutinària. No em podria oblidar de la *Leti*, química nocturna i investigadora de gran recorregut, la visió nano del grup, la *Yesi*, alias la chunga de Badalona, tot i que en els darrers anys la interacció ha estat més aviat nul·la, m'emporto els moments dels principis on era l'element esbojarrat de Pharmamar. Els *pero a ver, una cosa te voy a decir, vas a pillar y no va a ser cacho*, etc, son marca registrada seva en cases rurals i sopars, la *Fayna*, l'altre japonesa, de carrera meteòrica, una cara sempre simpàtica a Pharmamar, la *Camila,* che boludo! argentina compromesa, la *Hortensia*, tranquila que las fotos de la noche de los 42 euros nunca las verá tu marido! (Lidia, no es lo que parece), ben aviat de tornada, la *Hilda*, l'alegria cubana, mama de Pharmamar, la *Eli*, exalumna del Maragall com un servidor, sempre disposada a donar un cop de mà, sort a Bellaterra! el *Padi*, l'home de les mil carreres, màsters i doctorats, quin gran aniversari aquell al Chiquipark!, el *Carles* (o Cal·la), investigador fet a si mateix, de caràcter directe i molt afable, un fiestero de mucho cuidado, l'EPI de Santiago està ple de grans moments, com la sortida de la discoteca, la sala del vicio…me n'alegro que hagis tornat, a veure si podem compartir més estones aquests propers mesos! Encara aquí, trobem el *Gerardo*, el cubà veterà del lab, expert en síntesis a escala multigram i terror del sintetitzador, el seu gest picant amb els tres dits i EL RAMON són mítics!, la *Marta Paradís*, una altra experta, la primera a qui acudir en cas de dubte peptídic, una de les principals motivacions per tornar al Parc després del Passa l'Estiu del 2005 va ser el bon rollo que hi havia al lab i especialment amb tu, aquells *te gusta camela, los otros i els gomets*, etc…em van marcar! Hi ha moltes anècdotes que conservaré d'aquests anys, això segur!, les colombianes *Ximena*

i *Vida*, amb les seves històries de la machaca, el *Jordi*, aquest en si no era ni d'aquí ni d'allà, ni de Biosyner ni de la Maria Macias, sempre trobàvem moments per parlar del patiment comú culé.

Menció especial mereixen la *Lorena*, el *Pau*, el *Dani*, la *Myriam* i el *Nessim*, sens dubte aquells amb els que he compartit més moments, en incomptables esmorçars, dinars, cafès, sopars, congressos, etc que han fet molt més agradable el dia a dia d'aquesta etapa de la meva vida. La *Lorena*, que ha hagut de superar molts obstacles a la seva Tesi, inclòs personatges que no cal ni nombrar, però que trobarà recompensa molt aviat i ja veuràs com sortiràs molt preparada. Molt directa i sincera, sempre aportes el punt caòtic i assimètric necessari al grup com a contrapunt, disposada a sortir de festa, fer unes birres o lo que surja. Pocs s'han treballat més la seva tesi que el *Pau*, però el camí (llarg i sinuós) ja s'acaba i el teu títol de doctor tindrà un mèrit encara major. Has estat un gran company on trobar recolzament quan era necessari o cap ajuda. Gràcies per la rebuda que vaig trobar en arribar a San Diego, sobretot les primeres setmanes, i per la companyia d'aquells mesos en que trobàvem a faltar el component mediterrani de la vida de casa. Amb el *Dani*, he passat moltes hores a la Terrassa i molts instants inoblidables de festa, de vicio de la Wii, a Santiago o a Lisboa, comentant la calorimetría una mica passats de voltes, un gran tio i químic computacional, gràcies per aquests moments i les converses que hem tingut aquests anys, és un dels records més bons que tinc. La *Myri*, es nota quan hi és i quan no hi és, una altra que sempre està a disposició per qualsevol dubte i amb la que també pots estar de conya una bona estona i a la vegada comentar el que sigui, amb el seu riure inconfudible. El *Nessim*, el partner del 300 a l'hora de fer cafès i de diverses sales del vicio, pel record queden frases com *no me vengan con que a chuchita la bolsearon, ya sabemos de que lado masca la iguana*, *coche negro oscuro, oríllese a la orilla*, etc.hem arribat juntets a la recta final i sense el teu consell hagués estat encara més difícil. No ho sé, es difícil condensar en unes poques línies, paraules que reflecteixin l'estima que se li agafa a algú amb qui has passat tants minuts al dia, durant tant de temps, només espero que sapigueu que els petits moments d'aquests darrers anys és segurament, el que recordaré més intensament d'aquest període.

Moltes estones al migdia he passat jugant al futbol del *ai-er-bi*, al costat de la resta de membres dels Brutal *Soft and Fluffy* Deluxe: el capitano *Tommaso*, l'etern doctorand i central expeditiu, el *Bernat Serra* (alias Bernat ros), d'actuacions esporàdiques però convincents, el *Claudio*, conducció amb accent argentí, *Àlex*, samba brasileira que et trenca quan menys t'ho esperes, el *Pep*, excel·lent porter que ens ha fet créixer com equip, el *Peter*, l'holandès combatiu, imprescindible a l'equip, el *Sean* (caràcter irlandés), el *Lluís* (gran central quan va estar disponible), el *Kay*, Kaiser del IBEC, quin personatge, *pon-la-mano-getca-roger!, chica òpticamente muy buena*, *el hombre misterioso*, etc…, la *Laura*, la periquita del equip, l'únic amb el qual pot aspirar a guanyar títols, ejem, i finalment el *Pau* Pollo, s'ho fa tot solet, un Cristiano en potència.

També he d'agraïr l'ajuda i assessorament del *Rafel Prohens* i el *Rafael Barbas* de la Plataforma de Calorimetria, en els assajos indirectes d'explosivitat de la Oxyma i el COMU, així com tot el que après del tema gràcies a les reunions i discussions científiques que vam tenir al principi de la Tesi. De UQC destacar la *Miriam Royo*, group leader atípica i de fàcil conversa, la organitzadora dels seminaris que tan bé han anat per preparar TAC's i congressos.

Una dedicatòria especial també es mereix la *Eva Poca*, sense la qual el grup no podria funcionar com ho fa en l'actualitat, gràcies per tot l'esforç que dediques perquè la nostra investigació tiri endavant, sempre amb una cara amable, tot i que vagis fins al capdamunt de feina.

Moving to San Diego, I need to thank *Phil Dawson* for allowing me to stay in his group in The Scripps Research Institute, La Jolla, from September to December, 2010. I had the opportunity to learn new techniques and, of more importance, how to work in a different research environment to the one I was used to. The informal discussions in our small office about Boc SPPS, Cys chemistry and ligations in general contributed to a high extent to my knowledge in the field. My thanks also to *Darren Thomas*, whose experience and constant help and advise was basic for accomplishing my objectives. You are a special one! Also thanks for driving me to San Diego airport on that night of the 23rd December, I appreciate that very much. A *Juan*, futuro Ramon y Cajal, por su ayuda durante el poco tiempo que coincidimos en el grupo, un crack de la metodologia de native chemical ligation y quantum dots. Espero que nos veamos pronto por aquí. También gracias a *Felipe y a su "bebé"*, los limpiadores de mi planta en Scripps, que pese a ser del Real Madrid, fue muy agradable escuchar una voz hispana todos los días y conversar sobre futbol y la vida en USA. De mi etapa en

San Diego, no me quiero olvidar de *Elena y Antonio*, (la mejor noche de Halloween la pasé en University Avenue y también quedarán muchos Taco Tuesdays!), *Xavi i Raquel*, companys de Pau al SIO i que molt aviat tindrem per aquí i a *Natalie*, que ens va acollir a Pau i a mi a Ensenada com uns membres més de la família, y amb la qual vam passar moltes nits de divendres, ja fos de sushi o al Island's de La Jolla. Thanks also to *Nancy and John* for the accomodation, and yes! It was cold there!!! (¿next time I come around maybe can I use the swimming pool?).

Cómo no, agradecer a *Cristhian, Miriam y Victor* por los ratos en que hemos quedado después del lab para olvidar el trabajo y echarnos unas risas o ver el partido de fútbol que tocase. Pese a que no nos vemos tanto como hace unos años, ya sabeis que os quiero mucho y estoy para lo que haga falta, campeones! *Victor*, aun me tienes que enseñar tu álbum de fotos de cuando te casaste, para cuándo los retoños?? (y yo que me lo perdí…un dia deberías de empezar a escribir tus memorias, porque daría para mucho!). Siempre te quedará pensar que te sacarás económicas, no como otros, jeje. De *Cristhian*, que me alegro mucho que superaras ese mal año y de que la nueva vida que empezasteis siga así durante mucho tiempo, os sienta muy bien. A ver si podeis conseguir un pisito ya para los dos pronto (por cierto, la cagaste, no fui el primero en casarme, juas, juas). Al *Lafu* a ver si se deja ver más el pelo y organiza más fiestas de fin de año (aunque se me de uno que no se apunta), que me lo pasé muy bien, ya celebraremos pronto que te has sacado la carrera. Y al *Enano*, agradecer todos los momentos en que nos hemos reído ~~de~~ con él, no tienen precio!! Profesor Garcia Palacios, quién lo iba a decir, seguro que va de líder. A *Germán*, el papá, siempre fuiste precoz en todo, a ver si te dejan escapar más a menudo para quedar y cenar, ¿para cuándo el bar Naya?,a *Alfredo* el extravagante, mago, prestidigitador, músico…todo un personaje. Als meus amics de la uni: els habituals a l'hora de menjar (i que van portar el Jorge Cham), el ja *doctor Bagán*, el *Marc Belenguer* (famós pel seu flautón), l'*Abel*, el *Marc Puigmartí* (aka Tatiana, un nou doctorand al CSIC), la *Liria* (futura general manager de Serrano Ceña, ¿Cómo va la Coca Cola?), la *Miriam* (et dic pel teu nom vaig progressant), la *Sara* (on pares ara? Madrid, Albacete?), el *Cayo* (te debe faltar poco para ser tecnólogo alimentario, no? Queda muy fashion..pero exactamente que es??)les *Montse*, la *Mònica*, el *Fernando* (un businessman de mucho cuidado, te vuelves a Barcelona?), i el *Carlos* i la *Carol* (esto es…cuando tu vas, yo vengo de allí…). Una menció també als amics de Palamós i La Fosca, la *Mar*, el *Jacobo*, el *Pere*, la *Mariona*, la *Jimena*, el *Jesús*, la *Floreta* i el *Raimon* per les nits d'estiu al Castellet, la platja o el que caigués.

Per descomptat tinc molt que agraïr als meus pares, *Roser* i *Pere*, entre d'altres haver-se assegurat que mai em faltés de res i haver-me subvencionat la majoria de la carrera de Química. Un record molt especial per *l'àvia Rosita*, a qui estic segur li hagués fet especial il·lusió estar present el dia que em faran doctor. En part t'ho dec a tu, per haver-me ensenyat el valor de l'esforç i la importància de no parar mai d'aprendre. Al tiet *Jordi* i la tieta *Angelina*, per haver fet dels estius i altres vacances a Palamós els moments més feliços que puc recordar, junt amb l'àvia. A tots tres, us trobo molt a faltar, espero que pogueu veure el nou doctor des d'algun lloc. No em puc deixar la *Luisa-Yeye, Juan-Ai i la Pilar*, a qui considero la meva segona família després de tants anys i anys junts dia rere dia, ara veient-nos més esporàdicament.

Finalment, dedicatòria especial per la *Lidia*. No sé que puedo decir que no sepas ya, aparte de que a estas alturas no me imagino la vida sin ti, y que me hace mucha ilusión empezar una nueva vida a tu lado y con el tiempo (no mucho…) formar una família. Si he llegado a ser doctor también es gracias a tu incansable apoyo y cariño (vale, y también por tu ayuda en la portada…); no creo que llegue a ser un gran hombre, ni lo busco, me sobra y me basta con estar al lado de una gran mujer, porque es lo que eres, ya va siendo hora de que te convenzas de ello. Estoy seguro de que alguien apostará por ti en Alemania y no me cabe duda de que no les defraudarás y te darás cuenta de que vales y mucho. Curiosamente, nuestra historia empezó una semana después de empezar el doctorado pero, a diferencia de este, no ha hecho nada más que empezar.

Índex

ÍNDEX i

ABREVIATURES, ACRÒNIMS I ANNEXOS iii

Abreviatures i acrònims iii

Annex I: Aminoàcids proteinogènics emprats vii

Annex II: Aminoàcids no proteinogènics emprats vii

Annex III: Grups protectors del *N*-terminal emprats viii

Annex IV: Grups protectors de cadena lateral emprats viii

Annex V: Additius i agents d'acoblament emprats ix

Annex VI: Carbodiimides i base emprades ix

Annex VII: Resines emprades x

RESUM DE LA MEMÒRIA 1

INTRODUCCIÓ I OBJECTIUS 3

Introducció General 5

Objectius 13

CAPÍTOL 1: ***Paper de les N-hidroxilamines en metodologia de síntesi peptídica***. 15

Publicació I. *N*-hydroxylamines for peptide synthesis 17

CAPÍTOL 2: ***Oxyma com a additiu per carbodiimides***. 127

Publicació II. *Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion* 129

CAPÍTOL 3: ***Carbonats d'Oxyma com a reactius per la introducció dels grups protectors Fmoc i Alloc*** 141

Publicació III. *Oxime Carbonates, Novel Reagents for the Introduction of Fmoc and Alloc Protecting Groups, Free of Side-Reactions* 143

CAPÍTOL 4: ***Oxyma en la prevenció de reaccions secundàries catalitzades per base*** 151

Publicació IV. *Aspartimide formation in peptide chemistry: occurrence, prevention strategies and the role of N-hydroxylamines* 153

Publicació V. *Use of Oxyma as pH modulatory agent to be used in the prevention of base-driven side reactions and its effect on 2-chlorotrityl chloride resin* 167

CAPÍTOL 5: ***Oxyma com a bloc de construcció dins de sals d'oni*** 189

Publicació VI. *COMU: A Safer and More Effective Replacement for Benzotriazole-Based Uronium Coupling Reagents* 191

Publicació VII. *Microwave irradiation and COMU: a potent combination for solid-phase peptide synthesis* 241

Publicació VIII. *PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts* 247

DISCUSSIÓ GLOBAL 267

CONCLUSIONS GLOBALS 277

APÈNDIXS 281

Apèndix I: EROS Oxyma 283

Apèndix II: IR d'Oxyma 293

Apèndix III: RMN d'Oxyma 297

Apèndix IV: EROS COMU 301

Apèndix V: Impacte actual dels reactius basats en Oxyma 311

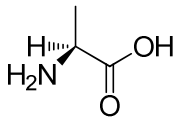
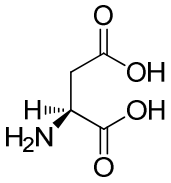
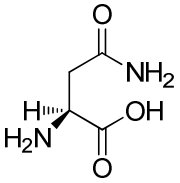
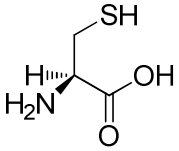
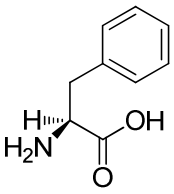
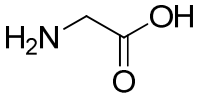
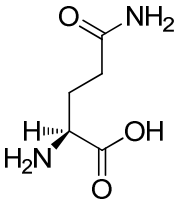
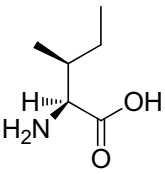
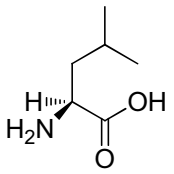
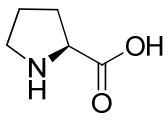
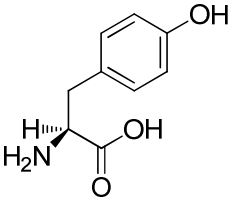
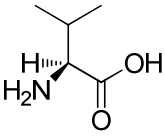
Abreviatures i acrònims

AA	aminoàcid
Ac	acetil
ACP	Proteina Portadora de grup Acil (*Acyl Carrier Protein*)
Aib	àcid α-aminoisobutíric
Alloc	al·liloxicarbonil
API	Ingredient Farmacèutic Actiu (*Active Pharmaceutical Ingredient*)
ARC	Calorimetria d'Índex Acceleratiu (*Accelerating Rate Calorimetry*)
Boc	*tert*-butiloxicarbonil
Bzl	benzil
^{13}C-RMN	Ressonància magnètica nuclear de carboni
6-Cl-HOBt	6-cloro-1-hidroxibenzotriazol
Cp	capacitat calorífica a pressió constant
COMU	hexafluorofosfat de (1-Ciano-2-etoxi-2-oxoetilidenaminooxi) dimetilaminomorfolinocarbeni
DCC	*N*,*N*-diciclohexilcarbodiimida
DCM	clorur de metilè
des-aa	pèptid deleció en el qual no s'ha incorporat l'aminoàcid aa
DEPBT	3-(Dietoxifosforiloxi)-1,2,3-benzotriazin-4(3*H*)-ona
DIC	*N*,*N*-diisopropilcarbodiimida
DIEA	*N*,*N*-etildiisopropilamina
DMF	*N*,*N*-dimetilformamida
DSC	Calorimetria d'Escombratge Diferencial (Differential Scanning Calorimetry)
EDC·HCl	clorur de *N*-etil-*N'*-(3-dimetilaminopropil) carbodiimida
Eq	equivalent
Fmoc	9-fluorenilmetoxicarbonil
FT-IR	espectròmetria d'infrarroig amb transformada de Fourier
ΔH	Variació d'entalpia
HATU	hexafluorofosfat de 3-òxid-*N*-[(dimetilamino)-1*H*-1,2,3-triazolo[4,5-b]piridin-1-il-metilen]-*N*-metilmetanamini
HBTU	hexafluorofosfat de 3-òxid-*N*-[(1*H*-benzotriazol-1-il)-(dimetilamino)metilen]-*N*-metilmetanamini

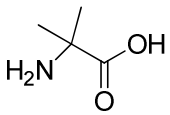
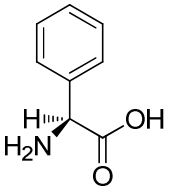
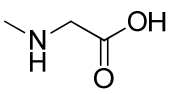
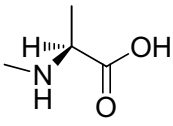
HCTU	hexafluorofosfat de 3-òxid-*N*-[(1*H*-6-clorobenzotriazol-1-il) (dimetilamino) metilen]-*N*-metilmetanamini
HDMA	hexafluorofosfat de 3-òxid-1-(dimetilamino)-morfolino)metilen]-1*H*-[1,2,3]triazolo [4,5-b]piridini
HDMB	hexafluorofosfat de 3-òxid-1-[(dimetilamino)-morfolino)metilen]-1*H*-benzotriazoli
HDMC	hexafluorofosfat de 3-òxid-6-cloro-1-[(dimetilamino)-morfolino)metilen]-1*H*-benzotriazoli
HECO	veure Oxyma
HIV-1	Virus d'Immunodeficiència Humana tipus 1 (*Human Immunodeficiency Virus-1*)
HMMU	hexafluorofosfat de 1-[1-(2,2-dimetil-4,6-dioxo-1,3-dioxan-5-ilidenaminooxi)dimetilaminomorfolinometilen]metanamini
HOAt	7-aza-1-hidroxibenzotriazol
HOBt	1-hidroxibenzotriazol
HOCt	1-hidroxi-1*H*-1,2,3-triazol-4-carboxilat d'etil
HODhat	3-hidroxi-3,4-dihidro-4-oxo-5-azabenzo-1,2,3-triazina
HODhbt	3-hidroxi-3,4-dihidro-4-oxo-1,2,3-benzotriazina
HONM	5-(hidroxiimino)-2,2-dimetil-1,3-dioxan-4,6-diona
HOPO	veure HOPy
HOPy	*N*-òxid de 2-hidroxipiridina
HOSu	*N*-hidroxisuccinimida
HOTU	Hexafluorofosfat de *O*-[Ciano(etoxicarbonil)metiliden)amino]-1,1,3,3-tetrametiluroni
HPLC	Cromatografía de líquids d'alta resolució
HPLC-MS	Cromatografía de líquids d'alta resolució acoblat a espectrometría de masses
J	constant d'acoblament
2-MBT	2-mercaptobenzotiazol
NMP	*N*-metil-2-pirrolidinona
NpsOXY	2-ciano-2-(naftalen-2-ilsulfoniloxiimino)acetat d'etil
Oxyma	2-ciano-2-hidroxiiminoacetat d'etil
ΔP	Variació de pressió
pH	logaritme decimal negatiu de l'activitat del ió hidrogen en solució
Phg	fenilglicina

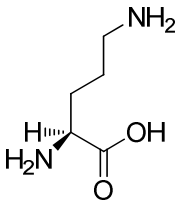
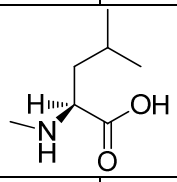
pK_a	logaritme decimal negatiu de la constant de dissociació àcida
PyAOP	hexafluorofosfat de 7-azabenzotriazol-1-il-*N*-oxi-tris(pirrolidino)fosfoni
PyBOP	hexafluorofosfat de benzotriazol-1-il-*N*-oxi-tris(pirrolidino)fosfoni
PyClock	hexafluorofosfat de 6-clorobenzotriazol-1-il-*N*-oxi-tris(pirrolidino)fosfoni
PyOOP	veure PyOxP
PyOxB	tetrafluoroborat de *O*-[(1-ciano-2-etoxi-2-oxoetiliden)amino]-oxitris (pirrolidin-1-il) fosfoni
PyOxim	veure PyOxP
PyOxP	hexafluorofosfat de *O*-[(1-ciano-2-etoxi-2-oxoetiliden)amino]-oxitris (pirrolidin-1-il) fosfoni
q	quadruplet
t	triplet
T	temperatura
ΔT_{ad}	Variació de temperatura adiabàtica
TATU	tetrafluoroborat de 3-òxid-*N*-[(1*H*-7-azabenzotriazol-1-il) (dimetilamino)metilen]-*N*-metilmetanamini
TBTU	tetrafluoroborat de 3-òxid-*N*-[(1*H*-benzotriazol-1-il)(dimetilamino)metilen] *N*-metilmetanamini
^{t}Bu	*tert*-butil
TCTU	tetrafluoroborat de 3-òxid-*N*-[(1*H*-6-clorobenzotriazol-1-il)(dimetilamino) metilen]-*N*-metilmetanamini
TFA	àcid trifluoroacètic
TFFH	hexafluorofosfat de tetrametilfluoroformamidini
TMP	2,4,6-trimetilpiridina
TOTU	Tetrafluoroborat de *O*-[Ciano(etoxicarbonil)metiliden)amino]-1,1,3,3-tetrametiluroni
t_R	temps de retenció
Trt	tritil
TsOXY	2-ciano-2-(tosiloxiimino)acetat d'etil
Z	benziloxicarbonil
δ	desplaçament químic
ν	freqüència

ANNEX I: Aminoàcids Proteinogènics emprats

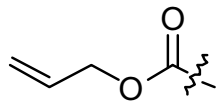
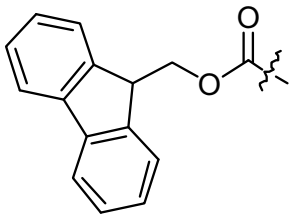
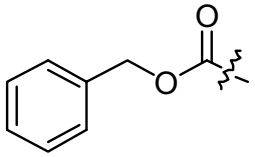
	alanina	Ala	A		àc.aspàrtic	Asp	D		asparagina	Asn	N		cisteína	Cys	C
	fenilalanina	Phe	F		glicina	Gly	G		glutamina	Gln	Q		isoleucina	Ile	I
	leucina	Leu	L		prolina	Pro	P		tirosina	Tyr	Y		valina	Val	V

ANNEX II: Aminoàcids no proteinogènics emprats

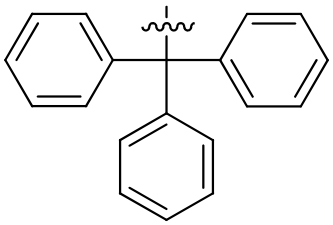
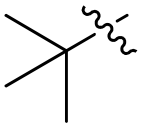
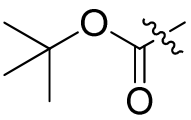
	àcid α-aminoisobutíric	Aib		fenilglicina	Phg
	*N*-metilglicina	*N*MeGly		*N*-metilalanina	*N*MeAla

	Ornitina	Orn
	*N*-metilleucina	*N*MeLeu

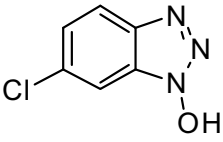
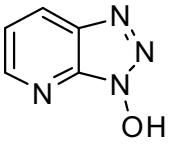
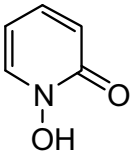
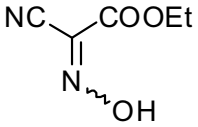
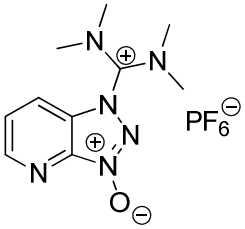
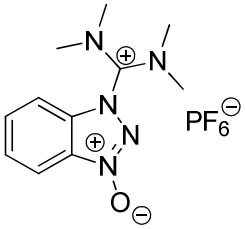
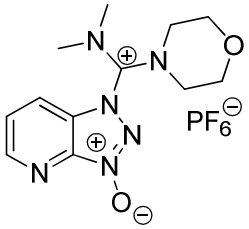
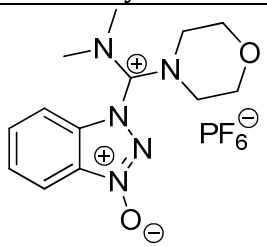
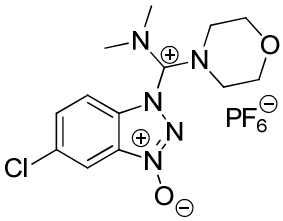
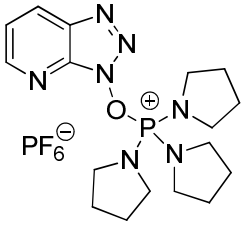
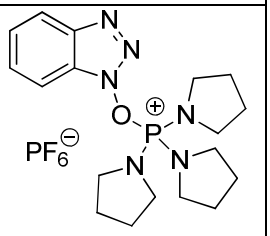
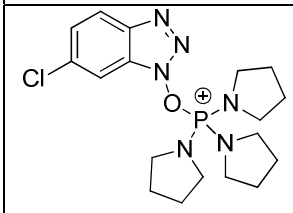
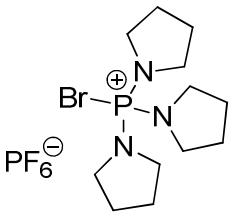
ANNEX III: Grups protectors del *N*-terminal emprats

		
Alloc	Fmoc	Z

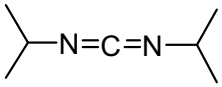
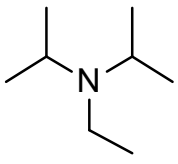
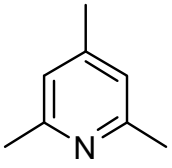
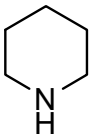
ANNEX IV: Grups protectors de cadena lateral emprats

AMINOÀCID	*ESTRUCTURA*	*ABREVIATURA*
Asn Cys Gln		Trt
Asp Tyr		^{t}Bu
Orn		Boc

ANNEX V: Additius i agents d'acoblament emprats[†]

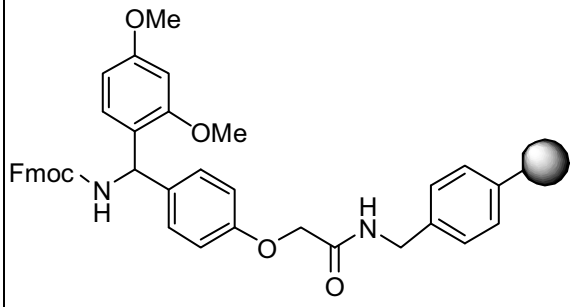
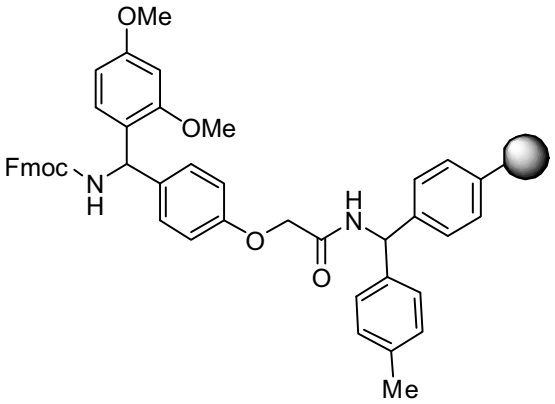
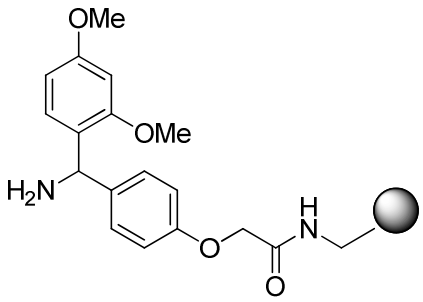
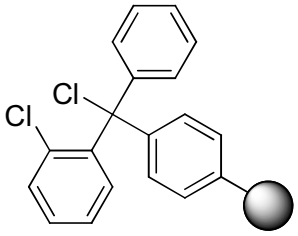
			
HOBt	6-Cl-HOBt	HOAt	HOPO
			
Oxyma	HATU	HBTU	HDMA
			
HDMB	HDMC	PyAOP	PyBOP
			
PyClock	PyBroP		

ANNEX VI: Carbodiimides i base emprades

			
DIC	DIEA	TMP	piperidina

[†] Es presenten els additius i reactius d'acoblament que s'han adquirit de fonts comercials o eren disponibles al laboratori, fruit d'anteriors treballs.

ANNEX VII: Resines emprades

	
Fmoc-RinkAmida-AM-PS	Fmoc-RinkAmida-MBHA-PS
	
H-RinkAmida-AM-ChemMatrix	2-Clorotritil

Resum de la memòria

Els resultats obtinguts a la present Tesi Doctoral es presenten com a compendi de vàries publicacions, englobades en diferents capítols.

El **primer capítol** correspon a un capítol de llibre de la sèrie editorial Patai (que tracta de la química de compostos heterocíclics) on es descriuen la síntesi, propietats i rellevància de diverses *N*-hidroxilamines utilitzades com a additius o dins d'agents d'acoblament autosuficients, en la formació de l'enllaç peptídic. Aquesta publicació s'inclou com a introducció extensa de la temàtica general de la Tesi.

El **segon capítol** és un estudi sobre la utilització d'Oxyma com a additiu de carbodiimides. A la publicació recollida es tracta la seva capacitat de reduir l'epimerització del carboni-α, eficiència en diversos models peptídics i perfil de seguretat tèrmica en comparació amb benzotriazols, així com la investigació de l'estabilitat a nucleòfils del grup carbonil.

Al **tercer capítol** es tracta la síntesi i evaluació de carbonats actius de vàries oximes àcides, d'entre les quals Oxyma, d'aplicació en la introducció dels grups protectors Fmoc i Alloc. Es presenta l'estratègia sintètica escollida per la síntesi d'aquests reactius, així com la seva caracterització i capacitat de reduir l'impacte de dipèptids en la protecció de glicina.

El **quart capítol** es divideix en dos treballs que tracten sobre la reacció secundària de formació d'aspartimides i subproductes derivats:

- En primera instància es presenta un article de revisió sobre la formació d'aquesta reacció secundària, així com els principals factors que la originen i les estratègies per minimitzar-ne l'impacte o prevenir-la. D'entre aquests mètodes es dedica una atenció especial al paper que juguen les *N*-hidroxilamines.

- El segon treball és un estudi sobre l'efecte de l'acidesa d'Oxyma i alguns benzotriazols en la reducció de reaccions secundàries catalitzades per base en l'estratègia Fmoc/tBu, com ara aspartimides o sobreacoblament d'aminoàcids causat per prolina. També s'investiga el percentatge d'escissió de pèptids ancorats a la resina clorotitil amb aquestes *N*-hidroxilamines.

El **cinquè capítol** es composa de tres publiacions sobre sals d'oni basades en Oxyma:

- Al primer article es sintetitzen diverses sals d'uroni d'Oxyma i d'altres oximes àcides, d'entre les quals destaca el derivat de tipus dimetilmorfolino basat en Oxyma, COMU. La solubilitat, estabilitat i altres característiques com retenció de configuració òptica i habilitat de fomentar la unió entre aminoàcids impedits es compara amb derivats de HOBt i HOAt. Per últim s'analitza el perfil de descomposició, tant de COMU com dels anàlegs de tipus benzotriazol, en assajos calorimètrics.

- El segon treball és un estudi breu sobre la combinació de COMU i sintetitzadors automàtics assistits per irradiació de microones, en la preparació del pentapèptid Aib-encefalina, tot investigant la presència de subproductes provinents d'addició nucleòfila a Oxyma.

- En el tercer article es presenta la síntesi i evaluació de diverses sals de fosfoni que contenen Oxyma a la seva estructura, tot investigant l'efecte del contranió (tetrafluoroborat o hexafluorofosfat). Ambdòs derivats es comparen, junt amb anàlegs de tipus benzotriazol, en relació a l'eficiència en sistemes impedits lineals i cíclics, reducció d'epimerització, solubilitat i estabilitat.

Per donar una visió global de la Tesi Doctoral, s'inclouen una introducció general que justifica la investigació realitzada entorn a Oxyma, una discussió general sobre els resultats obtinguts i una conclusió general de la idoneitat dels reactius basats en Oxyma en síntesi peptídica.

Finalment, en els apèndixs s'inclou informació sobre els espectres de IR i RMN d'Oxyma, així com una fitxa dels reactius Oxyma i COMU, incloses a l'enciclopedia EROS, i una perspectiva actual i futura dels reactius d'Oxyma en la metodologia de síntesi peptídica

Introducció
i
Objectius

Introducció General

Pèptids i proteïnes són biopolímers amb una funció essencial dins la complexa biomàquina que constitueixen els éssers vius. A diferència de les proteïnes, de caire eminentment estructural i enzimàtic, l'àmbit d'actuació dels pèptids presenta una àmplia diversitat: neurotransmissors, hormones, endorfines, etc. En les darreres dècades, l'enorme potencial dels pèptids en ciència s'ha anat descobrint, extenent-se a camps tan diversos com la biotecnologia, cosmètica, administració de fàrmacs i la química medicinal, bé extrets de fonts naturals o provinents de modificacions químiques. Del primer cas, cal destacar el paper que juguen els pèptids trobats en organismes marins, com Kahalalide F (**1**) i Dolastatin-10 (**2**), els quals presenten una elevada activitat antitumoral i han arribat a fases clíniques (**Fig. 1**).[1,2] Les propietats antibacterianes, antiparasitàries, antiobesitat o inhibidores del HIV-1 d'alguns pèptids també s'han descobert recentment. Per tal de millorar el perfil farmacocinètic i reduir la rapidesa de biodegradació dels pèptids, s'intenten introduir elements estructurals que generalment impliquen major dificultat sintètica. En aquest escenari, resulta imprescindible disposar d'eines químiques adequades per assolir en el menor temps possible, la major quantitat de pèptid objectiu en alt grau de puresa, per tal d'avançar al mateix ritme que ho fan les investigacions biomèdiques i biotecnològiques que se'n deriven. La present tesi doctoral s'emmarca dins la necessitat de disposar de reactius d'acoblament adients que aconsegueixin la obtenció d'un pèptid determinat de forma ràpida, segura, eficient i econòmica.

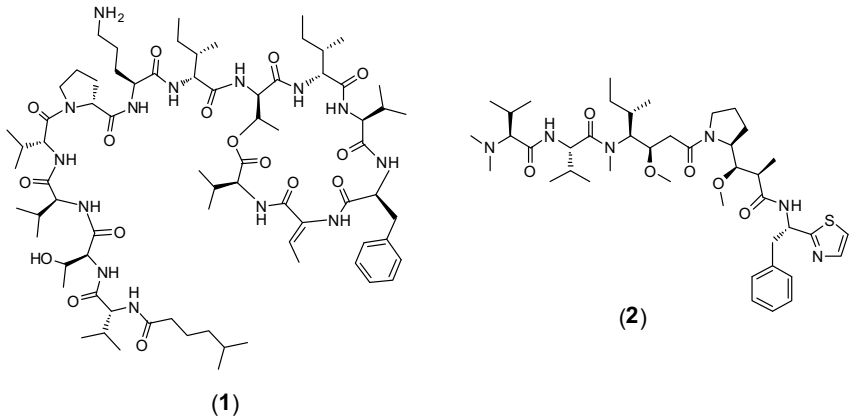


Figura 1. Estructura dels pèptids anticancerígens Kahalalide F i Dolastatin-10.

En gran mesura, la presència creixent dels pèptids en aquests àmbits es pot atribuïr a l'implementació de la fase sòlida en la síntesi peptídica per part del laboratori de Merrifield a mitjans del segle passat.[3] Mitjançant aquesta tècnica, el pèptid creix ancorat a un suport polimèric, originàriament clorometilestirè copolimerizat amb divinilbenzè, en sentit C→N terminal, contràriament al que succeeix en la biosíntesi ribosòmica. La gran avantatge d'aquesta metodologia recau en la simple separació de subproductes solubles per filtració, possibilitat d'automatització del procés i addicció de grans excessos de reactius per aconseguir acilacions quantitatives. D'aquesta manera, la obtenció de pèptids amb més de 4 o 5 residus passava de suposar un esforç sintètic considerable i una gran inversió de temps, a esdevenir un procediment rutinari i fàcilment accessible. En anys posteriors, la fase sòlida es començaria a utilitzar també en la síntesi d'altres

[1] Rademaker-Lakhai, J. M.; Horenblas, S.; Meinhardt, W.; Stokvis, E.;de Reijke,T. M.; Jimeno, J. M.; Lopez-Lazaro, L.; Lopez Martin, J. A.; Beijnen, J. H.; Schellens, J. H. M. *Clin. Cancer Res.* **2005**,*11*, 1854.

[2] Vaishampayan, U.; Glode, M.; Du, W.; Kraft, A.; Hudes, G.; Wright, J.; Hussain, M. *Clin. Cancer Res.* **2000**, *6*, 4205.

[3] Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

compostos orgànics lineals o heterocíclics, aprofitant mètodes espectroscòpics o colorimètrics per detectar la compleció de reaccions directament en resina.[4,5] En el context de la metodologia de síntesi peptídica, es poden considerar altres fites rellevants el desenvolupament de l'esquema de protecció Fmoc/tBu, de condicions de desproteccions més suaus que Boc/Bzl i completament ortogonal, i de la resina 2-cloro-clorotritil, la qual permet l'escissió del pèptid objectiu amb els grups protectors laterals intactes.[6,7] Més recentment, la tècnica de la lligació química nativa ha suposat una revolució en la obtenció de pèptids de mida mitjana-llarga o fins i tot de proteines, a partir d'un fragment pèptid-tioèster *C*-terminal i un altre Cisteína-pèptid *N*-terminal totalment desprotegits i de manera quimioselectiva.[8,9]

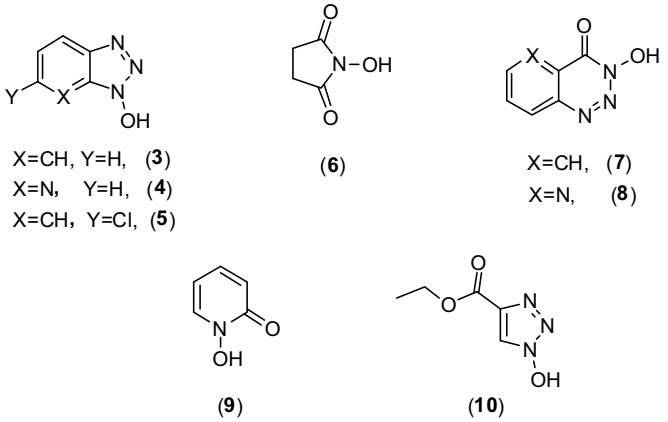


Figura 2. Estructura dels compostos que presenten el grup *N*-hidroxilamina més rellevants en síntesi peptídica

No obstant, si unes mol·lècules han esdevingut protagonistes en les últimes dècades pel que fa al camp metodològic peptídic, són certes *N*-hidroxilamines i fenols/triazines electrònicament desactivats, els quals presenten una elevada acidesa (pK_a=2-10) en el context dels compostos orgànics, el qual els hi confereix unes propietats molt especials.[10] Dintre dels compostos que contenen grups *N*-hidroxilamina àcids a la seva estructura, podem trobar-ne diverses subfamílies: heterocicles aromàtics polinitrogenats (benzotriazols, triazols o tetrazols), àcids hidroxàmics heterocíclics (benzotriazines, piridones o tiopiridones) i imides heterocícliques (succinimides o phtalimides) (**Fig. 2**).[10,11,12] Indubtablement, els membres més rellevants de la familia de les *N*-hidroxilamines la componen els *N*-hidroxibenzotriazols, la versatilitat dels quals en quant a reactivitat, afegit a l'absència de reaccions secundàries greus i un preu generalment assequible, les ha col·locat al capdamunt del sector durant molts anys.[10] HOBt (**3**, pK_a=4.60, **Fig. 2**) fou el pioner en trobar aplicació en el camp de la metodologia de síntesi de pèptids, gràcies al treball de König i Geiger, en primera instància com a additiu en la supressió d'epimerització del C_α.[13] Dècades més tard el seguiren tot d'anàlegs aza o 6-substituïts, d'entre els quals destaquen el 7-HOAt (habitualment referit simplement com a HOAt, **4**, pK_a =3.28, **Fig. 2**), el derivat més potent i car, i el 6-Cl-HOBt (**5**, pK_a=3.35, **Fig. 2**) de reactivitat intermitja i amb relació potència/preu més accessibles.[14,15] Un altra *N*-hidroxilamina amb molt de pes històricament en aquest camp es la imida

[4] Nicolaou, K. C.; Pfefferkorn, J. A. *Biopolymers (Pept. Sci.),* **2001**, *60*, 171.
[5] Cironi, P.; Álvarez, M.; Albericio F. *QSAR Comb. Sci.* **2004**, *23*, 61.
[6] Atherton, E.; Cameron, L.; Meldal, M.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun*. **1986**, (*24*), 1763.
[7] Barlos, K.; Gatos, D.; Kapolos, S.; Papaphotiu, G.; Schäfer, W.; Yao, W. *Tetrahedron Lett*. **1989**, *30 (30)*, 3947.
[8] Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science*, **1994**, *266 (5186)*, 776.
[9] Mende, F.; Seitz, O. *Angew. Chem. Int. Ed.* **2011***, 50 (6)*, 1232.
[10] Valeur, E.; Bradley, M. *Chem. Soc. Rev.,* **2009**, *38*, 606.
[11] Spetzler, J. C.; Meldal, M.; Felding, J.; VedsØ, P.; Begtrup, M. *J. Chem. Soc., Perkin Trans. 1,* **1998,** 1727.
[12] Bailén, M. A.; Chinchilla, R.; Dodsworth, D. J.; Nájera, C. *J. Org. Chem.* **1999,** *64,* 8936.
[13] a) König, W.; Geiger, R. *Chem. Ber*. **1970**, *103*, 788; b) König, W.; Geiger, R. *Chem. Ber.,* **1970**, *103*, 2034.
[14] Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.

cíclica HOSu (**6**, pK_a=6.0, **Fig. 2**), de menor reactivitat que HOBt (**3**) però compatible amb acoblaments en medi aquós.[16] D'altra banda, certes benzotriazines, com HODhbt (**7**, pK_a =3.97, **Fig. 2**), de reactivitat propera a HOAt (**4**), han posat en compromís la superioritat dels benzotriazols, fins que es va demostrar que podien reaccionar amb nucleòfils donant subproductes d'obertura d'anell.[13,17] El aza-anàleg d'aquest últim, HODhat (**8**, **Fig. 2**), proposat per Carpino, tot i mostrar un increment en potència, pot patir la mateixa reacció secundària.[18] Altres *N*-hidroxilamines a destacar són HOPy (o HOPO, **9**, pK_a=5.9, **Fig. 2**), que es mostra eficient en sistemes bifàsics DCM/H_2O i el triazol HOCt (**10**, pK_a =2.16, **Fig. 2**), proposat per Ramage i colaboradors a finals del segle passat.[19,20]

L'aplicació més notable de les *N*-hidroxilamines en química de pèptids correspon a assistir la formació de l'enllaç peptídic, bé com a additius de carbodiimides, inclosos en reactius d'acoblament "autosuficients" o en èsters actius preformats.[10] Les estratègies que s'apliquen en química de pèptids per unir dos aminoàcids o pèptids mitjançant un enllaç amida es desmarquen d'aquelles que es fan servir en altres branques de la química orgànica, més agressives per la integritat quiral de l'aminoàcid activat. Així com la transformació d'un àcid en el corresponent clorur d'àcid, per exemple, és rutinària en síntesi orgànica estàndar per obtenir un grup amida, l'aplicació d'aquest mètode en pèptids resulta, sens dubte en un èster molt reactiu, però amb el gran inconvenient de promoure l'epimerització del carboni-α.[10] El mateix es pot extrapol·lar per a les azides d'àcid, que a més, tenen associat un enorme risc explosiu quan estan seques. Els diferents agents dedicats a l'acoblament entre dos aminoàcids i/o pèptids han d'aconseguir, doncs, un compromis de reactivitat per ser eficients però alhora evitar reaccions secundàries. Les estratègies més conegudes les podem classificar en:

A. Èsters preformats

Conceptualment, es relacionen amb grups sortints (*N*-hidroxilamines) de reactivitat més aviat baixa, ja que han de poder ser aïllables i prou estables com per romandre intactes durant el temps en que es pretenguin utilitzar (normalment unes quantes semanes a temperatura ambient). La principal avantatge recau en què, donat que s'evita l'activació *in situ*, s'elimina la presència i separació dels subproductes derivats. D'aquesta manera, s'utilitzen èsters de pentafluorofenol, *p*-nitrofenol, HOSu (**6**) o altres imides cícliques, abans que els èsters de HOBt (**3**), poc aconsellables per la precària estabilitat que mostren.[21,22]

→*Especialment recomanats per obtenir acoblaments nets i eficients en solució*.

B. Reactius de formació d'èster actiu in situ

Representa una estratègia més general, potent i de ràpid accés que l'anterior i consequentment és la opció preferida en els laboratoris d'investigació peptídica.[10] Se'n distingeixen dues grans subclasses: les carbodiimides i els agents d'acoblament "autosuficients".

B.1. Carbodiimides

La metodologia tradicional per la generació d'èsters actius *in situ* correspon a les carbodiimides (**11**, **Fig. 3**), bé utilitzades com a únic agent d'acoblament o en presència d'un

[15] Ueki, M.; Yanagihara, T. *Peptides 1998, 25th Proceedings of the European Peptide Symposium (Eds.: S. Bajusz, F. Hudecz), Akademiai Kiado*, Budapest, **1999**, pp. 252
[16] Anderson, G . W.; Zimmerman, J. E.; Calahan, F. M. J. *Am. Chem. Soc.* **1963**, *85*, 3039.
[17] Carpino, L. A.; El-Faham, A.; Albericio, F. *Tetrahedron Lett.* **1994***, 35*, 2279.
[18] Carpino, L. A.; Xia, J.; Zhang, C.; El-Faham, A. *J. Org. Chem*. **2004**, *69*, 62.
[19] Ho, G.-J.; Emerson, K. M.; Mathre, D. J.; Shuman, R. F.; Grabowski, E. J. J. *J. Org. Chem*. **1995,** *60***,** 3569.
[20] Jiang, L.; Davison, A.; Tennant , G.; Ramage, R. *Tetrahedron* **1998**, *54*, 14233.
[21] Fujino, M.; Kobayashi, S.; Obayashi, M.; Fukuda, T.; Shinagawa, S.; Nishimura, O. *Chem. Pharm. Bull.* ***1974****, 22*, 1857.
[22] Kitada, C.; Fujino, M. *Chem. Pharm. Bull*. **1978**, *26,* 585.

additiu (*N*-hidroxilamina de forma gairebé exclusiva).[10,23] La segona opció va suposar la entrada en escena per primer cop de les *N*-hidroxilamines, principalment per evitar la transformació de la *O*-acilisourea reactiva en una *N*-acilisourea inactiva i reduir l'epimerització del carboni-α, tot formant el corresponent èster actiu *in situ*.[13,24] Amb el temps, els acoblaments amb carbodiimides (**11**) (habitualment DCC per estratègia Boc/Bzl, DIC per estratègia Fmoc/tBu, o EDC·HCl en solució), han anat inevitablement associats a l'addició de *N*-hidroxilamines.[25] Estudis recents demostren que l'ús de carbodiimida i additiu constitueix la opció preferent en sintetitzadors, pel compromís entre preu, reactivitat i absència de reaccions secundàries.[26]

→*Per acoblaments de dificultat estàndar o intermitja/en sintetitzadors automàtics*.

B.2. Agents d'acoblament "autosuficients"

La necessitat de disposar de reactius més potents que les carbodiimides en acoblaments lents o complicats va portar a la introducció dels reactius "autosuficients" (stand-alone coupling reagents en anglès).[10] Reben aquest nom degut a que la seva estructura es pot considerar com una carbodiimida unida a un additiu (LY, **Fig. 3**). No obstant, no solen incloure centres bàsics, en contraposició a les carbodiimides, i per tant requereixen base per formar l'èster actiu *in situ.*

B.2.1. Sals d'amini/uroni

Aquest tipus de sals d'oni (**12**, **Fig. 3**) ha rebut el major nombre de propostes al llarg dels anys, probablement degut a que constitueixen el grup d'agents d'acoblament més potent, com a conseqüència del marcat caràcter electròfil del carboni deficient central.[10] Tot i que la contribució de l'additiu (grup sortint) ha estat àmpliament estudiada, destacant els anàlegs de HOAt (**4**), l'esquelet carbocatiònic no ha rebut la mateixa atenció.[27,28,30,56] L'assignació amini o uroni depèn de l'àtom pel qual la *N*-hidroxilamina s'uneix a l'electròfil: nitrogen (amini, normalment pels benzotriazols) o oxigen (uroni, per la resta d'additius).[10,29] L'elevada reactivitat els fa adients per acoblaments impedits o difícils, tot i que sol anar acompanyat, com a contrapartida, de la guanidilació de l'extrem *N*-terminal en certes ocasions.[30,31]

→*Especialment recomanats en seqüències difícils/acoblaments impedits.*

B.2.2. Sals de fosfoni

Aquest tipus de sals d'oni es van introduïr fa un parell de dècades per esmenar el major inconvenient de les sals d'amini/uroni: la guanidilació de l'amino *N*-terminal, que bloqueja l'allargament de cadena peptídica. Tot i ser lleugerament menys reactives que les anteriors, les sals de fosfoni és mostren molt eficients i no originen la terminació del grup amino (**13**, **Fig. 3**).[30] La principal diferència vers les sals d'amini/uroni escau en la naturalesa del centre electròfil que rep el primer atac nucleòfil per part del carboxilat.[10] En un principi de tipus hexa(metilamino), la toxicitat dels subproductes d'acoblament (hexametilfosforamida) va conduïr a la reinvenció de l'esquelet electròfil cap a un de naturalesa tris(pirrolidino), la majoria dels quals introduits per Castro i colaboradors.[32,33,34,35]

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[23] Sheehan, J. C.; Hess, G. P. *J. Am. Chem. Soc.*, **1955**, *77*, 1067.
[24] König, W.; Geiger, R. *Chem. Ber.,* **1970**, *103*, 2024.
[25] Carpino, L. A.; El-faham, A. *Tetrahedron*, **1999**, *55*, 6813.
[26] Hachmann, J.; Lebl, M. *Biopolymers (Pept. Sci.),* **2006**, *84*, 340.
[27] El-Faham, A.; Albericio, F. *J. Org. Chem*. **2008**, ***73***, 2731.
[28] Dourtoglou, V.; Ziegler, J.-C.; Gross, B. *Tetrahedron Lett.* **1978**,*15*, 1269.
[29] Carpino, L. A.; Imazumi, H.; El-Faham, A.; Ferrer, F. J.; Zhang, C.; Lee, Y.; Foxman, B.M.; Henklein, P.; Hanay, C.; Mügge, C.; Wenschuh, H.; Klose, J.; Beyermann, M.; Bienert, M. *Angew. Chem. Int. Ed.* **2002**, *41 (3)*, 441.
[30] Albericio, F.; Bofill, J. M.; El-faham, A.; Kates, S. A. *J. Org.Chem.*, **1998**, *63*, 9678.
[31] Nájera, C. *Synlett*, **2002**, 1388.
[32] Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.*, **1990**, *31(2),* 205.
[33] Subirós-Funosas, S.; Moreno, J. A.; Bayó-Puxan, N.; Abu-Rabeah, K.; Ewenson, A.; Atias, D.; Marks, R. S.; Albericio, F. *Chimica Oggi*, **2008**, *26(4),* 10.
[34] Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C.*Tetrahedron Lett*. **1975**, *14*, 1219.

→ *Especialment recomanats en ciclacions o acoblaments lents amb poc excés d'àcid.*

B.2.3. Fosfats

Tot i no rebre la mateixa atenció que les sals d'oni des del punt de vista comercial, els fosfats (**14, Fig. 3**) mereixen una menció per la seva impressionant capacitat de reduïr la pèrdua de configuració del carboni quiral.[18,36] Una possible explicació es podria basar en la menor reactivitat de la primera espècie activa que es forma, la qual es considera la major responsable del grau d'epimerització obtingut.[10] El derivat de HODhbt (DEBPT) aconsegueix excel·lents resultats en acoblaments entre fragments peptídics.[37]

→*Especialment indicats per unir fragments peptídics*

B.2.4. Miscel·lània

Per últim, cal esmentar altres agents d'acoblament "autosuficients" que, tot i presentar una reactivitat molt marcada (fins i tot excessiva), no han rebut el mateix grau d'acceptació que les anteriors estratègies, en els laboratoris de recerca.[10] A tall d'exemple, els sulfonats (**15**, **Fig. 3**), els quals en presència de nucleòfils potents es poden fragmentar, bloquejant el grup amino, o les sals de formamidini (**16**, **Fig. 3**), bé com a fluorurs o clorurs, que tot i mostrar una gran eficàcia en acoblaments difícils de tipus Aib-Aib, poden generar com a subproducte àcids forts (HF, HCl) que ocasionen nombroses reaccions secundàries indesitjades.[38,39,40]

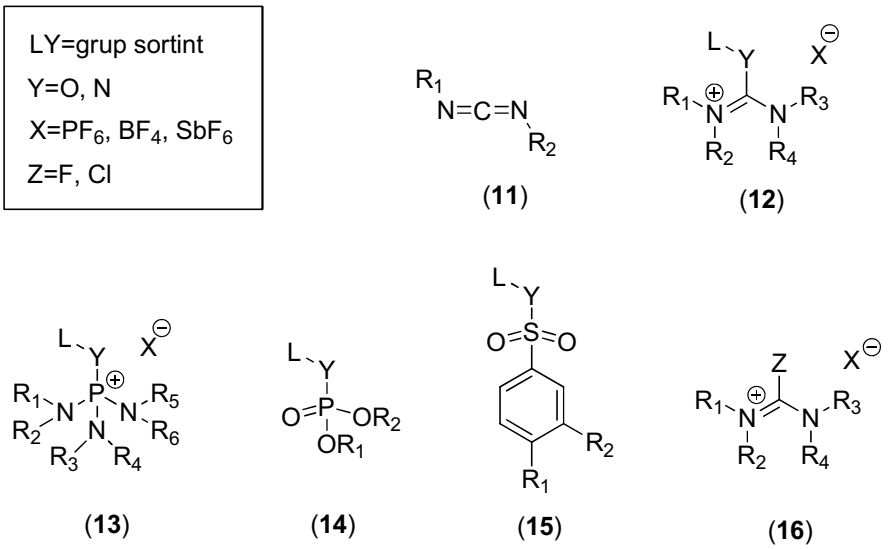

Figura 3. Estructura general de carbodiimides i agents d'acoblament "autosuficients" utilitzats en la formació de l'enllaç peptídic.

L'impacte de les *N*-hidroxilamines en síntesi peptídica va més enllà de l'assistència en la formació de l'enllaç peptídic entre aminoàcids. Gràcies a la seva marcada acidesa, s'utilitzen com a additius en les típiques mescles de desprotecció del grup Fmoc (20% piperidina en DMF), en concentració 2% o 0.1M, per minimitzar la formació d'aspartimides i subproductes derivats.[41] S'ha especulat amb un mecanisme de competició per la base present en el medi amb el protó àcid del grup amida pertanyent al residu precedent a l'àcid aspàrtic (o en menor mesura l'asparagina),

[35] Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. *Tetrahedron Lett.***1997**, *38*, 4853.
[36] Kiso, Y.; Miyazaki, T.; Satomi, M.; Hiraiwa, H.; Akita, T. *J. Chem. Soc., Chem. Commun*. **1980**, 1029.
[37] Li, H.; Jiang, X.; Yun-hua, Y.; Fan, C.; Romoff, T.; Goodman, M. *Org. Lett*., **1999***, 1*, 91.
[38] Carpino, L. A.; El-Faham, *J. Am. Chem. Soc*. **1995**, *117*,5401.
[39] Furukawa, M.; Hokama, N.; Okawara, T. *Synthesis* **1983**, 42.
[40] Itoh, M.; Nojima, H.; Notani, J.; Hagiwara, D.; Takai, K. *Tetrahedron Lett.,* **1974**, *35*, 3089.
[41] Dölling, R.; Beyermann, M.; Hänel, J.; Kernchen, F.; Krause, E.; Franke, P.; Brudel. M.; Bienert, M. *J. Chem. Soc., Chem. Commun*., **1994**, 853.

responsable de la ciclació intramol·lecular que inicia l'esmentada reacció secundària.[42] La *N*-hidroxilamina més emprada en aquest àmbit és HOBt (**3**), juntament amb HOSu (**6**) i fenols electrodeficients com ara el pentaclorofenol, pentafluorofenol o 2,4-dinitrofenol, postulant-se com una alternativa senzilla, eficient (pot augmentar la puresa del pèptid de 40% a 67%) i econòmica a altres estratègies més potents però menys accessibles.[41,42,43] La conversió de residus d'arginina sense protecció lateral en ornitina durant l'acoblament també es veu reduïda per addició de 0.1M HOBt (**3**), tot protonant el grup guanidino.

Les estratègies de protecció de l'amino N_α-terminal amb grups de tipus carbamat com Fmoc o Alloc, també s'han beneficiat del funcionament de certes *N*-hidroxilamines com a excel·lents grups de sortida, evitant els típics inconvenients que presenten mètodes excessivament reactius o poc segurs (**Fig. 4**).[44,45] Dintre de la primera categoria trobem els cloroformiats (**17**) espècies altament reactives amb cinètica de protecció elevada, però que a la vegada promouen la formació de dipèptids, que en el cas de residus poc impedits pot arribar a ser del 20%.[46] Amb la intenció de solucionar aquesta problemàtica es van introduïr els azidoformiats corresponents (**18**), els quals comparativament donen lloc a aquests subproductes en quantitat molt menor.[47] Les azides, no obstant, poden donar lloc a reaccions explosives quan estan seques, problema que s'agreuja en augmentar l'escala de síntesi. Per millorar les prestacions de l'arsenal de metodologies d'introducció de grups temporals carbamat, els carbonats actius d'algunes *N*-hidroxilamines es van implementar, sobretot els corresponents de HOSu (**19**), menys reactius que els cloroformiats i més segurs que els azidoformiats.[48] Malauradament, la obertura nucleofílica de la imida cíclica per part del residu a protegir dóna peu a la formació de *β*-Ala-OH i *β*-Ala-AA-OH en percentatges mínims (fins a 0.4%) però suficientment greus en la preparació de API.[49] Com a alternativa, s'han descrit metodologies que formen l'azida i la fan reaccionar in situ o l'ús dels de tiocarbonats de 2-MBT (**20**), el qual tot i ser segur i lliure de reaccions secundàries, dóna rendiments baixos.[50,51]

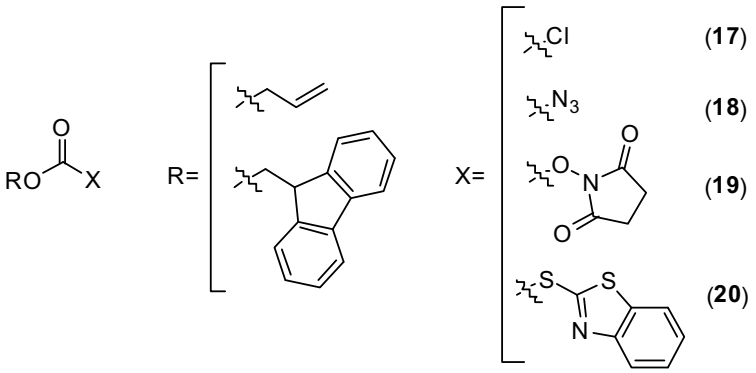

Figura 4. Estructura dels reactius més habituals en la introducció dels grups protectors Fmoc i Alloc.

Recentment, el monopoli dels *N*-hidroxibenzotriazols s'ha posat en seriós dubte com a conseqüència d'estudis publicats en referència al caràcter explosiu dels mateixos, el qual ha causat gran impacte en el camp de la síntesi peptídica, donada la rellevància d'aquests reactius.[52,53] De fet, ja al segle passat s'havia descrit l'explosivitat de les sals de plom de HOBt i derivats a 270ºC, però

[42] Martinez, J.; Bodanszky, M. *Int. J. Pept. Protein Res*. **1978**, *12*, 277.
[43] Mergler, M.; Dick, F.; Sax, B.; Stahelin, C.; Vorherr, T. *J. Pept. Sci.* **2003***, 9*, 518.
[44] Albericio, F. *Biopolymers (Pept. Sci.)* **2000**, (*55*), 123.
[45] Isidro-Llobet, I.; Álvarez, M.; Albericio, F. *Chem. Rev.* **2009,** *109,* 2455.
[46] Tessier, M.; Albericio, F.; Pedroso, E.; Grandas, A.; Eritja, R.;Giralt, E.; Granier, C.; Van-Rietschoten, J. Int. J. Pept. Protein Res.**1983**, *22*, 125.
[47] Carpino, L. A.; Han, G. Y. J. Org. Chem.**1972**, *37*, 3404.
[48] Sigler, G. F.; Fuller, W. D.; Chaturvedi, N. C.; Goodman, M.;Verlander, M. *Biopolymers* **1983**, *22*, 2157.
[49] Hlebowicz, E.; Andersen, A. J.; Andersson, L.; Moss, B. A. *J .Peptide Res.* **2005**, *65*, 90.
[50] Isidro-Llobet, I.; Just-Baringo, X.; Ewenson, A.; Álvarez, M.; Albericio, F. *Biopolymers (Pept Sci)*, **2007**, *88*, 733.
[51] Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. *Org. Proc. Res. Dev.* **2004**, *8*, 920.
[52] Wehrstedt, K. D.; Wandrey, P. A.; Heitkamp, D. *J. Hazard. Mater.*, **2005**, *126*, 1.
[53] Dunn, P. J.; Hoffmann, W.; Kang, Y.; Mitchell, J. C.; Snowden, M. J. *Org. Process Res. Dev*. **2005**, *9*, 956.

no va ser fins el 2005 que es va investigar en profunditat.[54] Dunn i col·laboradors van predir la inestabilitat tèrmica de HOBt monohidrat (**3**) i sobretot HOAt (**4**), tot i que no van trobar evidències de comportament explosiu en assajos de sensitivitat a impacte mecànic.[53] El mateix any, Wehrstedt va realitzar un estudi exhaustiu de la tendència de HOBt (**3**) (sec o hidratat al 50%), 6-Cl-HOBt (**5**) i sals d'amini com TBTU a propagar deflagracions, detonacions, així com la seva sensitivitat a impacte i a ser escalfats en recipient tancat (test Koenen), mostrant tots ells una alta perillositat, inclòs HOBt monohidrat (**3**) (**Fig. 5**).[52] Destacaven especialment HOBt (**3**) i 6-Cl-HOBt (**5**), l'elevada explosivitat dels quals va portar a clasificar-los com a substàncies explosives de Classe 1, el rang màxim dins dels compostos que presenten aquest comportament. Estudis de caire similar realitzats per organismes interns de la ONU van mostrar també el risc d'explosió de HOAt (**4**), etiquetant-lo de la mateixa manera.[55] Anys abans ja s'havia recomanat no secar TBTU a altes temperatures, degut al perill d'originar descomposicions violentes.[56] El perill real dels benzotriazols no recau en les condicions en que es realitzen acoblaments peptídics, normalment duts a terme a temperatura ambient, sinó en el transport d'aquestes substàncies a gran escala, donada la sensitivitat a impacte mecànic.[57] En conseqüència, s'han establert importants limitacions comercials (el transport per mar i aire està estrictament prohibit), que ha pràcticament apartat els *N*-hidroxibenzotriazols del mercat.

Figura 5. Receptacle d'acer per al test de König abans i després d'assajar HOBt 50% hidratat.†

Un buit en la metodologia de síntesi peptídica s'ha obert en veure les conseqüències dels estudis d'explosivitat en benzotriazols. En aquest marc, no només resulta necessària la implementació de noves famílies de *N*-hidroxilamines que siguin segures tèrmicament, sinó que idealment han de ser prou reactives per assolir les anomenades seqüències difícils o impedides que es segueixen resistint en síntesi peptídica. No obstant, la solució podría consistir en recuperar o rellançar un conjunt d'oximes que, tot i haver estat descrit el seu ús en metodologia de pèptids, han tingut nul o mínim impacte. En particular, els treballs de Itoh i després Izdebsky als 70 sobre oximes estables, amb substituents electroatraients (CN, COOEt, $CONH_2$ i 2-piridil) que n'augmentaven l'acidesa, van ser pioners (**21-25**, **Fig. 6**) en la utilització com a additius i també en la introducció del grup Boc en forma de carbonat.[58,59,60] La dicianooxima (**21**) presentava la major capacitat per reduïr l'epimerització, però malauradament també era a la vegada la més inestable (tan sols la sal de plata és sòlida) donant lloc a baixos rendiments. D'entre les altres, la més àcida (pK_a=4.60) i la que mostraba un millor balanç entre rendiment, reactivitat, estabilitat i reducció d'epimerització

[54] Searle, N. E. *Org. Synth*. **1956**, *36*, 25.
[55] *UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria*, *fourth revised ed*., United Nations, New York and Geneva, **2003**.
[56] Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30 (15)*, 1927.
[57] Tot i això, alguns casos d'accidents a gran escala s'han descrit en laboratoris de recerca, com el que es va produïr als laboratoris Lacamas a Portland, Oregon, USA.[53]
† Figura extreta amb permís de la referència 11.
[58] Itoh, M. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2219.
[59] Izdebski, J. *Pol. J. Chem.* **1979**, *53*, 1049.
[60] Itoh, M.; Hagiwara, D.; Kamiya, T. *Bull. Chem. Soc. Jpn.* **1977**, 50, 718.

(superior a HOBt i HOSu) era el 2-ciano-2-hidroxiiminoacetat d'etil (**22**).[58,59] Tot i aquests resultats esperançadors, no s'ha descrit a la literatura cap altre exemple de la seva utilització com a additiu per a carbodiimides en els darrers 30 anys, probablement degut al caràcter preliminar dels estudis de Itoh i Izdebsky, en els quals tan sols es va provar l'epimerització en un dipèptid model poc sensible. En canvi, la mateixa oxima ha adquirit en aquest temps notorietat en química inorgànica on ha estat molt estudiada pel seu caràcter coordinador bidentat, sent anomenada HECO (**22**) i aconseguint disponibilitat comercial.[61] Alguns reactius d'acoblament "autosuficients" basats en 2-ciano-2-hidroxiiminoacetat d'etil (**22**), tot i això, si que s'han descrit, com ara el metansulfonat corresponent, inferior a l'anàleg de 6-Cl-HOBt (**26**), la sal d'uroni TOTU (**27**), que no ha rebut massa acollida, o la sal de fosfoni PyOOP (**28**), que es va sintetitzar i caracteritzar, però inexplicablement, no es va investigar el seu comportament en la formació de l'enllaç peptídic (**Fig. 6**).[39,62,63]

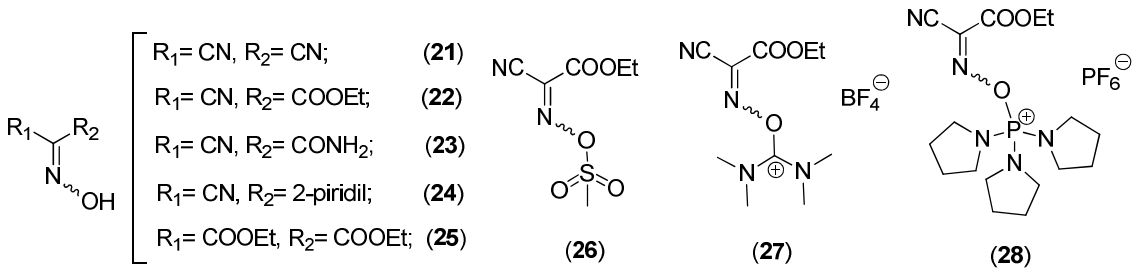

Figura 6. Estructura de diverses oximes àcides i de reactius d'acoblament basats en 2-ciano-2-hidroxiimino acetat d'etil.‡

[61] Eddings, D.; Barnes, C.; Gerasimchuk, N.; Durham, P.; Domasevich, K. *Inorg. Chem*. **2004**, *43*, 3894.
[62] Breipohl, G.; Koenig, W. *Ger. Offen.*, **1991,** *DE 90-4016596*.
[63] Hoffmann, F.; Jaeger, L.; Griehl, C. *Phosphorus, Sulfur Silicon Relat. Elem.*, **2003**, *178(2)*, 299.

‡ Com a resultes de la investigació presentada a la Tesi Doctoral, el 2-ciano-2-hidroxiiminoacetat d'etil s'ha reanomenat com a Oxyma (comercialment Oxyma Pure) i la sal de fosfoni de tipus trispirrolidino amb anió hexafluorofosfat com a PyOxP (comercialment PyOxim), per diferenciar-lo de l'anàleg tetrafluoroborat.

Objectius

La present Tesi Doctoral pretén realitzar un estudi exhaustiu de les prestacions del 2-ciano-2-(hidroxiimino)acetat d'etil, reanomenada aquí com Oxyma (**22**, **Fig. 6**, pàg. 12) i dels reactius anàlegs (i també en certs casos d'altres oximes àcides) en metodologia de síntesi peptídica, bé en l'assistència de la formació de l'enllaç peptídic o en d'altres aplicacions relacionades amb les seves propietats físiques. Paral·lelament, s'evaluarà la seguretat tèrmica que es deriva de la utilització dels reactius basats en Oxyma. Concretament, els objectius són:

- Investigar en profunditat el comportament d'Oxyma com a additiu per carbodiimides, tant en la retenció de la configuració òptica com en l'eficiència d'acoblament, tot comparant-lo amb els *N*-hidroxibenzotriazols més rellevants. De forma addicional, també es pretén evaluar el risc de descontrol tèrmic inherent a Oxyma, en comparació amb coneguts *N*-hidroxibenzotriazols explosius.

- Sintetitzar, caracteritzar i evaluar carbonats actius d'Oxyma i altres oximes àcides descrites anteriorment, com a reactius per a la introducció dels grups N_{α}-protectors de tipus carbamat Fmoc i Alloc, cercant la presència de possibles subproductes derivats d'aquesta metodologia, en especial la formació de dipèptids.

- Estudiar l'efecte obtingut amb la presència d'Oxyma, en relació a certs *N*-hidroxibenzotriazols, en la minimització de reaccions secundàries observades en l'estrategia Fmoc/tBu, originades per la utilització d'espècies marcadament bàsiques o nucleòfiles. Paral·lelament, també es busca analitzar la compatibilitat d'Oxyma i els *N*-hidroxibenzotriazols amb la resina 2-clorotritil, làbil a condicions àcides suaus.

- Preparar, caracteritzar i evaluar sals d'oni (uroni i fosfoni) basades en Oxyma com a agents d'acoblament "autosuficients" per a la formació de l'enllar peptídic, comparant les seves propietats i prestacions amb anàlegs *N*-hidroxibenzotriazòlics. La compatibilitat d'aquests reactius amb estratègies automàtiques combinades amb irradiació per microones s'estudiarà en detall, així com la observació del seu comportament de descomposició i risc tèrmic associat, en comparació a altres derivats de *N*-hidroxibenzotriazol.

Capítol 1. *Paper de les N-hidroxilamines en metodologia de síntesi peptídica*.

Publicació I

N-hydroxylamines for peptide synthesis†

N-hidroxilamines d'ús en síntesi peptídica

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Patai's Chemistry of Functional Groups, *DOI: 10.1002/9780470682531.pat0509, Ed. John Wiley & Sons*, **2011,** *2 (2)*, 623-730

† Ramon Subirós Funosas va realitzar la cerca a la literatura i l'elaboració corresponent a la síntesi i propietats de les *N*-hidroxilamines, així com la descripció d'altres aplicacions d'aquests compostos en síntesi peptídica.

Ayman El-Faham es va encarregar de cercar i redactar la part corresponent a l'aplicació de *N*-hidroxilamines i reactius d'acoblament derivats en la formacio de l'enllaç peptídic

Resum

Aquest capítol de llibre pretén fer les funcions d'introducció detallada i exhaustiva de la temàtica sobre la que tracta la present Tesi Doctoral.

En ell, es dóna una revisió de les *N*-hidroxilamines que s'han utilitzat al llarg dels anys en síntesi peptídica, centrant-se en les diverses aproximacions sintètiques descrites a la literatura, així com les seves propietats físiques i característiques estructurals. La primera part del capítol s'estructura en base a les diferents famílies de *N*-hidroxilamines que es coneixen d'ús en aquest camp: desde benzotriazols a piridones passant per benzotriazines, imides cícliques, triazols, etc. Més endavant, es tracta el seu ús com a additius per carbodiimides i la inclusió en reactius d'acoblament autosuficients per a la formació de l'enllaç peptídic, focalitzant l'atenció en descobriments recents realitzats en aquesta temàtica. Per últim, també es descriuen usos alternatius que han rebut les *N*-hidroxilamines en d'altres vessants de la síntesis de pèptids, com ara la prevenció de reaccions secundàries catalitzades per base (aspartimides majoritàriament) l'epimerització del centre quiral en alfa, etc.

CHAPTER **11**

N-Hydroxylamines for peptide synthesis

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I. ABBREVIATIONS 625
II. INTRODUCTION 631
III. PREPARATION AND PROPERTIES 632
A. Oxyma (Ethyl 2-cyano-2-(hydroximino)acetate) 632
B. Hydroxypyridines 634
1. HOPy, *N*-hydroxy-2-pyridinone 634
2. HOTPy, *N*-hydroxy-2-pyridinethione 637
C. HOI, *N*-Hydroxyindolin-2-one 639

The chemistry of hydroxylamines, oximes and hydroxamic acids, Vol. 2
Edited by Z. Rappoport and J. F. Liebman

D. *N*-Hydroxysuccinimides 641
1. HOSu, *N*-hydroxysuccinimide 641
2. HONB, *N*-hydroxy-3,6-endomethylene-Δ^4-tetrahydrophthalimide . . 643
3. HONOD, *N*-hydroxy-3,6-epoxy-1,2,3,6-tetrahydrophthalimide 644
4. HONP, *N*-hydroxyphthalimide 645
E. *N*-Hydroxy-2-phenylbenzimidazoles 647
1. HOBI, *N*-hydroxy-2-phenylbenzimidazole 647
2. 6-Cl-HOBI, 6-chloro-*N*-hydroxy-2-phenylbenzimidazole 649
F. HODhad, 3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene 652
G. Triazoles 653
1. 1-Hydroxy-1,2,3-triazole 653
2. HOCt, ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate 656
3. 1-Hydroxy-5-(methoxymethyl)-1,2,3-triazole 659
4. 5-Acetyl-1-hydroxy-1,2,3-triazole 661
5. 5-Chloro-1-hydroxy-1,2,3-triazole 662
H. Benzotriazoles 664
1. HOBt, 1-hydroxybenzotriazole 664
2. 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole 667
3. 6-CF_3-HOBt, 1-hydroxy-6-trifluoromethylbenzotriazole 668
4. 6-NO_2-HOBt, 1-hydroxy-6-nitrobenzotriazole 669
I. Azabenzotriazoles 672
1. 7-HOAt, 7-aza-1-hydroxybenzotriazole 672
2. 4-HOAt, 4-aza-1-hydroxybenzotriazole 676
3. 5-HOAt, 5-aza-1-hydroxybenzotriazole 678
4. 6-HOAt, 6-aza-1-hydroxybenzotriazole 680
J. Hydroxybenzotriazine 682
1. HODhbt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine 682
K. Hydroxyazabenzotriazine 685
1. HODhat, 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene . . 685
L. 2-Hydroxytetrazole 687
IV. *N*-HYDROXYLAMINES FOR CARBODIIMIDE-MEDIATED REACTIONS 689
V. *N*-HYDROXYLAMINES FOR THE PREPARATION OF ACTIVE ESTERS 693
VI. *N*-HYDROXYLAMINES FOR THE PREPARATION OF PHOSPHONIUM SALTS 694
A. HOBt Phosphonium Salts 694
1. BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate 695
2. PyBOP, benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate 696
3. Other HOBt phosphonium salts 697
B. HOAt Phosphonium Salts 697
C. HODhbt Phosphonium Salts 697
D. Oxyma Phosphonium Salts (PyOxP, PyOxB) 697
VII. *N*-HYDROXYLAMINES FOR THE PREPARATION OF URONIUM/IMINIUM SALTS 699
A. *N*-Hydroxylamines for the Preparation of Tetramethyl Uronium/Iminium Salts 699

11. *N*-Hydroxylamines for peptide synthesis 625

B. *N*-Hydroxylamines for the Preparation of bis-Pyrrolidino Iminium Salts . . . 702
C. *N*-Hydroxylamines for the Preparation of bis-Piperidino Iminium Salts . . . 702
D. *N*-Hydroxylamines for the Preparation of Imidazolium Uronium Salts . . . 702
E. *N*-Hydroxylamines for the Preparation of Pyrimidinium Uronium Salts . . . 705
F. *N*-Hydroxylamines for the Preparation of Unsymmetric Uronium/Iminium Salts . . . 705
G. *N*-Hydroxylamines for the Preparation of Morpholino-based Uronium/Iminium Salts . . . 706
H. Oxyma for the Preparation of Uronium Salts . . . 707
I. Isonitroso Meldrum's Acid Reagents for the Preparation of Uronium Salts . . . 708

VIII. *N*-HYDROXYLAMINES FOR THE PREPARATION OF PHOSPHORIC ACID DERIVATIVES . . . 712
IX. *N*-HYDROXYLAMINES FOR THE PREPARATION OF SULFONIC ACID DERIVATIVES . . . 712
X. *N*-HYDROXYLAMINES FOR THE PREPARATION OF POLYMER-SUPPORTED REAGENTS . . . 714
A. Polymer-bound HOBt Reagents . . . 714
B. Polymer-bound HOSu Reagents . . . 717
XI. OTHER APPLICATIONS IN PEPTIDE SYNTHESIS . . . 717
XII. ACKNOWLEDGMENT . . . 719
XIII. REFERENCES . . . 719

I. ABBREVIATIONS

AOMP	5-(7-azabenzotriazol-1-yloxy)-3,4-dihydro-1-methyl-*2H*-pyrrolium hexachloroantimonate
AOP	(7-azabenzotriazol-1-yl)oxy-tris(dimethylamino)phosphonium hexafluorophosphate
BDDC	bis[4-(2,2-dimethyl-1,3-dioxolyl)methyl]carbodiimide
BDMP	5-(*1H*-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl-*2H*-pyrrolium hexachloroantimonate
BDP	benzotriazol-1-yl diethylphosphate
BEC	*N*-*tert*-butyl-*N*′-ethylcarbodiimide
BOMP	2-(benzotriazol-1-yloxy)-1,3-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate
BOP	benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
BOP-Cl	*N*,*N*′-bis(2-oxo-3-oxazolidinyl)phosphinic chloride
BPMP	1-(*1H*-benzotriazol-1-yloxy)phenylmethylene pyrrolidinium hexachloroantimonate
BroP	bromo-tris(dimethylamino)phosphonium hexafluorophosphate

BTCFH	bis(tetramethylene)chlororformamidinium hexafluorophosphate
CF_3-BOP	[6-(trifluoromethyl)benzotriazol-1-yl]-*N*-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate
CF_3-HBTU	2-[6-(trifluoromethyl)benzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate
N-CF_3-HBTU	*N*-[6-trifluoromethyl(*1H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
6-CF_3-HOBt	6-trifluoromethyl-1-hydroxybenzotriazole
CF_3-NO_2-PyBOP	[4-nitro-6-(trifluoromethyl)benzotriazol-1-yl)oxy]tris(pyrrolidino) phosphonium hexafluorophosphate
CF_3-PyBOP	[6-(trifluoromethyl)-benzotriazol-1-yl]-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate
N-CF_3-TBTU	*N*-[6-trifluoromethyl(*1H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide
CIC	*N*-cyclohexyl, *N′*-isopropyl carbodiimide
CIP	2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate
6-Cl-HOBI	6-chloro-*N*-hydroxy-2-phenylbenzimidazole phosphonium hexafluorophosphate
6-Cl-HOBt	6-chloro-1-hydroxybenzotriazole
CloP	chloro-tris(dimethylamino)phosphonium hexafluorophosphate
CMBI	2-chloro-1,3-dimethyl-*1H*-benzimidazolium hexafluorophosphate
COMU	1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino morpholinomethylene)] methanaminium hexafluorophosphate
CPC	*N*,*N′*-dicyclopentylcarbodiimide
DCC	*N*,*N′*-dicyclohexylcarbodiimide
DEBP	diethyl 2-(3-oxo-2,3-dihydro-1,2-benzisosulfonazolyl) phosphonate
DEPAT	3-(diethoxyphosphoryloxy)-1,2,3-pyridino[*b*]triazin-4-(*3H*)-one
DEPB	diethyl phosphorobromidate
DEPBO	*N*-diethoxyphosphoryl benzoxazolone
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(*3H*)-one
DEPC	diphenyl phosphorochloridate
DIC	*N*,*N′*-diisopropylcarbodiimide
DIEA (DIPEA)	diisopropylethylamine
DKP	diketopiperazine
DNAs	*3H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-yl-2,4-dinitrobenzenesulfonate
DNBs	*1H*-benzo[*d*][1,2,3]triazol-1-yl-2,4-dinitrobenzenesulfonate
DOEPBI	phosphoric acid diethyl ester 2-phenylbenzimidazol-1-yl ester
DOMP	5-(3′,4′-dihydro-4′-oxo-1′,2′,3′-benzotriazin-3′-yloxy)-3,4-dihydro-1-methyl-*2H*-pyrrolium hexachloroantimonate
DOPBO	*N*-(2-oxo-1,3,2-dioxaphosphorinanyl)benzoxazolone
DOPBT	3-[*O*-(2-oxo-1,3,2-dioxaphosphorinanyl)oxy]-1,2,3-benzotriazin-4(*3H*)-one
DOPPBI	phosphoric acid diphenyl ester and 2-phenylbenzimidazol-1-yl ester
DPPA	diphenylphosphoryl azide
DPPAT	3-(diphenoxyphosphoryloxy)-1,2,3-pyridino[*b*]triazin-4-(*3H*)-one
DPPBI	diphenylphosphinic acid 2-phenylbenzimidazol-1-yl ester
DPP-Cl	diphenylphosphinic chloride
EDC	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride
Fmoc	9-fluorenylmethyloxycarbonyl

FOMP	5-(pentafluorophenyloxy)-3,4-dihydro-1-methyl-*2H*-pyrrolium hexachloroantimonate
HAE_2PipU	*O*-(*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-1,1-diethyl-3,3-pentamethyleneuronium hexafluorophosphate
HAE_2PyU	*O*-(*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-1,1-diethyl-3,3-tetramethyleneuronium hexafluorophosphate
HAMDU	*O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate
HAM_2PipU	*O*-(*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-1,1-dimethyl-3,3-pentamethyleneuronium hexafluorophosphate
HAM_2PyU	*O*-(*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-1,1-dimethyl-3,3-tetramethyleneuronium hexafluorophosphate
HAMTU	*O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-trimethyleneuronium hexafluorophosphate
HAPipU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HAPTU	(7-azabenzotriazol-yl)-1,1,3-trimethyl-1-phenyluronium hexafluorophosphate
HAPyTU	1-(1-pyrrolidinyl-*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene) pyrrolidinium hexafluorophosphate *N*-sulfide
HAPyU	1-(1-pyrrolidinyl-*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene) pyrrolidinium hexafluorophosphate *N*-oxide
HATeU	*O*-(*1H*-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
N-HATU	*N*-[(dimethylamino)-*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HATTU	*S*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
N-HATTU	*N*-[(dimethylamino)-*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-sulfide
N-HBPyU	(*1H*-benzotriazol-1-yl)(1-pyrrolidinylmethylene)pyrrolidinium hexafluorophosphate *N*-oxide
N-HBTU	*N*-[(*1H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl methanaminium hexafluorophosphate *N*-oxide
HBE_2PipU	*O*-(*1H*-benzotriazol-1-yl)-1,1-diethyl-3,3-pentamethyleneuronium hexafluorophosphate
HBE_2PyU	*O*-(*1H*-benzotriazol-1-yl)-1,1-diethyl-3,3-tetramethyleneuronium hexafluorophosphate
HBM_2PipU	*O*-(1*H*-benzotriazol-1-yl)-1,1-dimethyl-3,3-pentamethyleneuronium hexafluorophosphate
HBM_2PyU	*O*-(*1H*-benzotriazol-1-yl)-1,1-dimethyl-3,3-tetramethyleneuronium hexafluorophosphate
HBMDU	*O*-(benzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate
HBMTU	*O*-(benzotriazol-1-yl)-1,3-dimethyl-1,3-trimethyleneuronium hexafluorophosphate

HBPipU	*O*-(benzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HBPTU	(7-benzotriazol-yl)-1,1,3-trimethyl-1-phenyluronium hexafluorophosphate
HBPyU	*O*-(benzotriazol-1-yl)oxy-bis(pyrrolidino)uronium hexafluorophosphate
HBTeU	*O*-(*1H*-benzotriazol-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HDAPyU	*O*-(3,4-dihydro-4-oxo-5-azabenzo-1,2,3-triazin-3-yl)-1,1,3,3-bis(tetramethylene) uronium hexafluorophosphate
HDATU	*O*-(3,4-dihydro-4-oxo-5-azabenzo-1,2,3-triazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HDMA	1-(((dimethylamino)(morpholino)methylene)-*1H*[1,2,3]triazolo[4,5-*b*]pyridinium hexafluorophosphate-3-oxide
4-HDMA	3-(((dimethylamino)(morpholino)methylene)-*1H*[1,2,3]triazolo[4,5-*b*]pyridinium hexafluorophosphate-1-oxide
HDMB	1-(((dimethylamino)(morpholino)methylene)-*1H*-benzotriazolium hexafluorophosphate-3 oxide
HDMC	6-chloro-1(((dimethylamino)(morpholino)methylene)-*1H*-benzotriazolium hexafluorophosphate-3 oxide
6-HDMFB	6-trifluoromethyl-1-(((dimethylamino)(morpholino)methylene)-*1H*-benzotriazolium hexafluorophosphate-3-oxide
HDMODC	1-[(1-(dicyanomethyleneaminooxy)dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate
HDMODeC	1-[(1,3-diethoxy-1,3-dioxopropan-2-ylideneaminooxy)-dimethylaminomorpholinomethylene)] methanaminium hexafluorophosphate
HDMOPC	*N*-[(cyano(pyridine-2-yl)methyleneaminooxy)-(dimethylamino)methylene)]-*N*-morpholinomethanaminium hexafluorophosphate
HDMP	1-(((dimethyamino)(morpholino))oxypyrrolidine-2,5-dione methanaminium hexafluorophosphate
HDMPfp	1-(((dimethylamino)(morpholino))oxypentafluorophenyl-metheniminium hexafluorophosphate
HDmPyOC	1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethyl-amino pyrrolidinomethylene)] methanaminium hexafluorophosphate
HDmPyODC	1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino pyrrolodinomethylene)] methanaminium hexafluorophosphate
HDmPyODeC	1-[(1,3-diethoxy-1,3-dioxopropan-2-ylideneaminooxy)-dimethylamino pyrrolodinomethylene)] methanaminium hexafluorophosphate
HDPyU	*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate
HDTMA	1-(((dimethylamino)(thiomorpholino)methylene)-*1H*[1,2,3]triazolo[4,5-*b*] pyridinium hexafluorophosphate-3-oxide

HDTMB	1-((dimethylamino)(thiomorpholino)methylene)-*1H*-benzotriazolium hexafluorophosphate-3-oxide
HDTU	*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMPA	hexamethylphosphoramide
HMPyOC	1-((1-cyano-2-ethoxy-2-oxoethylideneaminooxy)-(morpholino)methylene) pyrrolidinium hexafluorophosphate
HMPyODC	1-((dicyanomethyleneaminooxy)morpholinomethylene)-pyrrolidinium hexafluorophosphoate
HOAt	1-hydroxy-7-azabenzotriazole
4-HOAt	4-aza-1-hydroxybenzotriazole
5-HOAt	5-aza-1-hydroxybenzotriazole
6-HOAt	6-aza-1-hydroxybenzotriazole
HOBI	*N*-hydroxy-2-phenylbenzimidazole
HOBt	1-hydroxybenzotriazole
HOCt	ethyl-1-hydroxy-*1H*-1,2,3-triazole-4-carboxylate
HODhad	3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene
HODhat	3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene
HODhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HODT	*S*-(1-oxido-2-pyridinyl)-1,3-dimethyl-1,3-trimethylenethiouronium hexafluorophosphate
HOI	*N*-hydroxyindolin-2-one
HONB	*N*-hydroxy-5-norbornene-*endo*-2,3-dicarboxyimide
HONP	*p*-nitrophenyl active ester
HOPy	1-hydroxy-2-pyridinone
HOSu	*N*-hydroxysuccinimide
HOTT	*S*-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium hexafluorophosphate
HOTU	*O*-[cyano(ethoxycarbonyl)methyleneamino]-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HPFTU	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate
HPTU	2-(2-oxo-1(2*H*)-pyridyl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPyONP	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-2-nitrophenyluronium hexafluorophosphate
HPyOPfp	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate
HPyOTCp	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate
HPySPfp	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*S*-pentafluorothiophenyluronium hexafluorophosphate
HSTU	2-succinimido-1,1,3,3-tetramethyluronium hexafluorophosphate
HTODC	*O*-[(dicyanomethylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate
HTODeC	*O*-[(diethoxycarbonylmethylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate
HTOPC	*N*-[(cyano(pyridine-2-yl)methyleneaminooxy)-(dimethylamino)methylene)-*N*-methyl methanaminium hexafluorophosphate

MPTA	dimethylphosphinothioyl azide
MPTO	3-dimethylphosphinothioyl-2(*3H*)-oxazolone
NAs	3-((naphthalen-2-ylsulfonyl)methyl)-*3H*-[1,2,3]triazolo[4,5-*b*]pyridine
2-Nas	*3H*-[1,2,3]triazolo[4,5-b]pyridin-3-yl 2-nitrobenzenesulfonate
4-Nas	*3H*-[1,2,3]triazolo[4,5-b]pyridin-3-yl 4-nitrobenzenesulfonate
NBs	1-((naphthalen-2-ylsulfonyl)methyl)-*1H*-benzo[*d*][1,2,3]triazole
2-NBs	*1H*-benzo[*d*][1,2,3]triazol-1-yl 2-nitrobenzenesulfonate
4-NBs	*1H*-benzo[*d*][1,2,3]triazol-1-yl 4-nitrobenzenesulfonate
NDPP	norborn-5-ene-2,3-dicarboximidodiphenylphosphate
NMM	*N*-methylmorpholine
6-NO_2-HOBt	1-hydroxy-6-nitrobenzotriazole
NO_2-PyBOP	(6-nitrobenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
NpsOPy	2-oxopyridin-1(*2H*)-yl naphthalene-2-sulfonate
NpsOXY	ethyl 2-cyano-2-(naphthalen-2-ylsulfonyloxyimino)acetate
Oxyma	ethyl 2-cyano-2-(hydroxyimino)acetate
P-DCT	polymer supported 2,4-dichloro-1,3,5-triazine
P-EDC	polymer supported 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide
P-HOBt	polymer supported 1-hydroxybenzotriazole
P-HOSu	polymer supported *N*-hydroxysuccinimide
P-SO_2-HOBt	polymer supported 1-hydroxy-6-disulfoxide benzotriazole
P-TBTU	*N*-[(*1H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl methanaminium tetrafluoroborate *N*-oxide
PTF	benzyltriphenylphosphonium dihydrogen trifluoride
PyAOP	[(7-azabenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate
PyBroP	bromotri(pyrrolidino)phosphonium hexafluorophosphate
PyCloK	(6-chlorobenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
PyCloP	chloro-tri(pyrrolidino)phosphonium hexafluorophosphate
PyDAOP	[(3,4-dihydro-4-oxo-5-azabenzo-1,2,3-triazin-3-yl]tris(pyrrolidino) phosphonium hexafluorophosphate
PyDOP	[(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)oxy]tris(pyrrolidino)-phosphonium hexafluorophosphate
PyFNBOP	[4-nitro-6-(trifluoromethyl)benzotriazol-1-yl)oxy]tris-(pyrrolidino)phosphonium hexafluorophosphate
PyFOP	[[6-(trifluoromethyl)benzotriazol-1-yl]oxy]tris-(pyrrolidino)phosphonium hexafluorophosphate
PyNOP	[(6-nitrobenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyOxB	*O*-[(1-cyano-2-ethoxy-2-oxoethyliden)amino]oxytri(pyrrolidin-1-yl)phosphonium tetrafluoroborate
PyOxP	*O*-[(1-cyano-2-ethoxy-2-oxoethyliden)amino]oxytri(pyrrolidin-1-yl)phosphonium hexafluorophosphate
PyPOP	(pentafluorophenyloxy)tris(pyrrolidino)phosphonium hexafluorophosphate

PyTOP	(pyridyl-2-thio)tris(pyrrolidino)phosphonium hexafluorophosphate
SOMP	5-(succinimidyloxy)-3,4-dihydro-1-methyl-*2H*-pyrrolium hexachloroantimonate
TAs	*3H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-yl 4-methylbenzenesulfonate
TATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
N-TATU	*N*-[(dimethylamino)-*1H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide
TBs	*1H*-benzo[*d*][1,2,3]triazol-1-yl 4-methylbenzenesulfonate
TBTU	*O*-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate
N-TBTU	*N*-[(*1H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide
TCFH	tetramethylchloroformamidinium hexafluorophosphate
TCP	2,4,5-trichlorophenyl active ester
TDATU	*O*-(3,4-dihydro-4-oxo-5-azabenzo-1,2,3-triazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TDBTU	2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TDTU	2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEMP	2,3,5,6-tetramethylpyridine
TMP	collidine
TMU	tetramethylurea
TNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
TODT	*S*-(1-oxido-2-pyridinyl)-1,3-dimethyl-1,3-trimethylenethiouronium tetrafluoroborate
TOPPipU	2-[2-oxo-1(*2H*)-pyridyl]-1,1,3,3-bis(pentamethylene)uronium tetrafluoroborate
TOTT	*S*-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium tetrafluoroborate
TOTU	*O*-[cyano(ethoxycarbonyl)methyleneamino]-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
TPFTU	*N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium tetrafluoroborate
TPhTU	2-phthalimido-1,1,3,3-tetramethyluronium tetrafluoroborate
TPTU	2-(2-oxo-1(*2H*)- pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TsOPy	2-oxopyridin-1(*2H*)-yl 4-methylbenzenesulfonate
TSTU	2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate

II. INTRODUCTION

Modern peptide chemistry, which has fuelled the renaissance of peptides as active pharmaceutical ingredients, shows three key cornerstones: (i) the solid-phase methodology developed by R. Bruce Merrifield; (ii) the fluorenylmethoxycarbonyl (Fmoc) group as temporary protecting group for the α-amino function developed by Louis A. Carpino; and (iii) 1-hydroxybenzotriazole (HOBt) for the coupling reaction by König and Geiger. Nowadays, solid-phase, Fmoc and HOBt (or an alternative *N*-hydroxylamine) are present

in any scientific paper or industrial process related to peptides. The first use of 1-hydroxybenzotriazole (HOBt) was just to reduce the reactivity of the active specie, *O*-acylisourea, formed by the reaction of the protected amino acid and the carbodiimide. The OBt active ester prepared *in situ* is more stable than the *O*-acylisourea and therefore the completion of the coupling step is more easily reached and reduces the loss of chirality of the protected amino acid due to the reduced activity. Although, the OBt active esters and related triazole analogues do not show enough stability for being prepared, isolated and stored; other *N*-hydroxylamine derivatives, mainly succinimide ones, can be used for this purpose. Later, *N*-hydroxylamine derivatives were the base for the development of stand-alone coupling reagents (phosphonium, aminium/uronium salts), which are being used as substitutes of the carbodiimides. Finally, as *N*-hydroxylamine derivatives show mild acid properties they are used during solid-phase peptide synthesis for minimizing side reactions, such as aspartiimide formation, or masking of the guanidine group of the arginine for masking its reactivity.

In this chapter, we will discuss the most frequent use for *N*-hydroxylamines in peptide chemistry. *N*-Hydroxylamine derivatives are summarized, first describing their preparation and physical properties, and then their application in peptide chemistry.

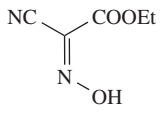
III. PREPARATION AND PROPERTIES

A. Oxyma (Ethyl 2-cyano-2-(hydroximino)acetate)

Ethyl 2-cyano-2-(hydroximino)acetate (Oxyma, **1**, Figure 1) is a water-soluble, white crystalline solid, obtained as needles, which allows easy handling and presents a melting point described in the range 128–133 °C, with little variation depending on the author[1–11]. Alternatively, **1** has been named as ethyl cyanoglyoxylate-2-oxime, and also abbreviated as HECO[6]. As a ketoxime, **1** shows high stability, in contrast to several oximes presenting α-carbon hydrogens, which prompted its commercial availability[2, 3, 12]. **1** is used as starting material in the preparation of 5-halo-1,2,3-triazoles, cyanopyrroles, 5-aminoimidazole nucleosides, phosphoesterase inhibitors or mGluR5a receptor antagonists[8, 13–15].

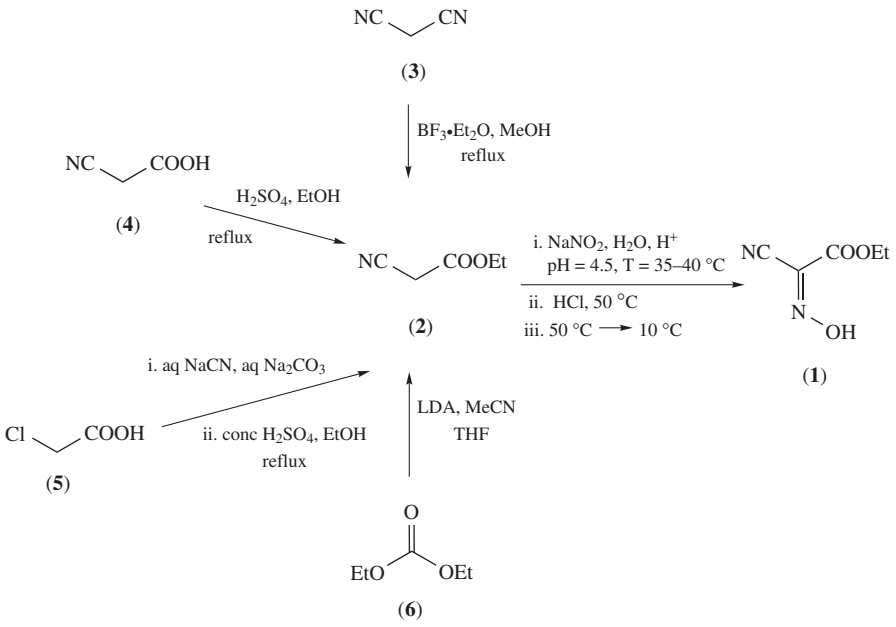
Most of the synthetic approaches used for **1** are based on the nitrosation of ethyl 2-cyanoacetate (**2**) in the presence of acetic acid and sodium nitrite, as an *in situ* source of nitrous acid (Scheme 1)[1, 6, 16]. Conrad and Schulze[16] were one of the first authors to propose this transformation for **2** and, since then, this method has been extensively used by other authors[1, 3, 5, 6, 14]. Slight changes have been introduced, such as the addition of sodium hydroxide or the use of ethyl nitrite[8, 13]. Phosphoric or sulfuric acid are also used to induce acidic pH, resulting in the yield being raised[17]. So far, the best optimization of the method was described by Parker, using phosphoric acid to set pH = 4.5; **1** was obtained almost quantitatively[10]. Other non-nitrosating strategies have been reported, by oxidizing **2** with nitrogen dioxide or forming an adduct with nitroprusside, which decomposes to **1**[7, 9]. These approaches, however, are associated with poor yields.

Ethyl 2-cyanoacetate (**2**) is readily and efficiently prepared from simple starting materials (Scheme 1). Malononitrile (**3**) can be converted to **2**, after reflux with boron trifluoride



(**1**)

FIGURE 1. Structure of ethyl 2-cyano-2-(hydroximino)acetate



SCHEME 1. Synthetic approaches for the preparation of Oxyma

etherate in methanol (MeOH)[18]. The reflux time is the key to the efficiency of this reaction, since one day-reflux renders 90% of the target activated-methylene compound, whereas 36 hours afford **1** quantitatively[18]. Alternatively, it can be obtained from 2-cyanoacetic acid (**4**), by esterification in refluxing ethanol and H_2SO_4 in 95–97% yield (Scheme 1)[19,20]. Remarkably, 2-chloroacetic acid (**5**) can be also transformed into **1** in a one-pot process with simultaneous introduction of the cyano group and esterification (80% yield) (Scheme 1)[21]. Finally, another interesting approach is the addition of diethyl carbonate (**6**) into a mixture of acetonitrile and lithium diisopropylamide (LDA) in THF, rendering **2** in 76% yield (Scheme 1)[22].

Unlike previously reported *N*-hydroxylamines, oximes such as **1** contain a nitrogen double-bonded to one single carbon, which accounts for some of its properties[1]. As a result, both types of compounds are known to possess similar acidity[23]. Consequently, **1** shows a high dissociation constant ($pKa = 4.60$), obtained by an automatic titration of an aqueous solution in 0.02M potassium hydroxide, which is almost identical to the value reported for 1-hydroxybenzotriazole (HOBt, **95**, see Section III.H.1)[1,3,4,24]. Achmatowicz and Szymoniak reported the IR of **1** in KBr analysis of **1**, showing a band for the hydroxyl group of the *N*-hydroxylamine, at high frequency of $\nu(O{-}H) = 3200{-}3100\,cm^{-1}$, and other bands at $\nu(C{=}N) = 2200\,cm^{-1}$, $\nu(C{=}O) = 1720\,cm^{-1}$, $\nu(C{-}O) = 1030\,cm^{-1}$ [7]. Additional bands were described by Cheng and Lightner at $\nu = 3600$, 3008, 2851 (C–H), 1636, 1594, 1452, 1319, 820, 763, 513 cm^{-1}.[8] **1** shows a characteristic absorption in the UV-visible region at $\lambda_{max} = 235{-}240\,nm$[2]. The conjugated anion, however, presents a yellow color, meaning a weak absorption in the visible area of the spectrum ($\log \varepsilon = 1.30{-}2.30$), due to a known transition corresponding to the nitroso group of deprotonated ethyl 2-cyano-3-isonitrosoacetate (**1′**, Figure 2), instead of the hydroximino species **1**[6,8,10,25–27]. This conclusion is supported by the IR spectra of the potassium salt

(**1**) (**1′**)

FIGURE 2. Tautomeric species of ethyl 2-cyano-2-(hydroximino)acetate

of **1**, reported by Eddings and coworkers[6], showing many similarities to the protonated **1**, except for the absence of *N*-hydroxylamine hydrogen and the presence of the characteristic nitroso band of the predominant anionic tautomer at ν(C=N) = 2209 cm^{-1}, ν(C=O) = 1674 cm^{-1}, ν(N=O) = 1280 cm^{-1} and ν(CNO) = 1140 cm^{-1} [6]. Additional bands were detected at ν(N=O) = 1265 cm^{-1} and ν(CNO) = 1115 cm^{-1}, corresponding to ^{15}N isotopomers[6]. GC-MS characterization, also described by Cheng and Lightner, show most fragmentations related to the ethoxy carboxylate and hydroxyl groups at (t_R = 7.2 min): (m/z) = 142 ($M^{+\bullet}$), 125 (M − OH), 114 (M − CH_2CH_3), 97 (M − OEt), 69 (M − CO_2Et)[8].

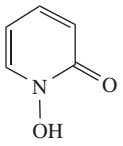
The ^{1}H-NMR spectra of **1** in DMSO-d_6 showed the *N*-hydroxylamine hydrogen at a considerably high frequency at δ 15.05[6]. In deuterated chloroform, this acidic proton was observed close to the methylene hydrogens at δ 4.833[8]. Regarding the ^{13}C-NMR (DMSO-d_6) spectra at room temperature, 5 carbon signals were detected[6]. A ^{13}C-NMR experiment, carried out in a range of temperatures from 20 to 110 °C, showed one set of 5 lines, which provides the information that the isomer composition of **1** is not a mixture of *syn* and *anti* isomers[6]. This result is in agreement with the findings of Conrad and Schulze[16] that **1** is present as the *Z* isomer[18]. The presence of intramolecular hydrogen bonding between the hydroxyl and the carbonyl groups might stabilize this isomer.

Recently, the stability of **1** has been evaluated and compared to that of the potentially explosive HOBt (**95**, Section III.H.1) and HOAt (**116**, Section III.I.1) by means of calorimetry assays (dynamic DSC and ARC)[2]. The outcome of both experiments showed that **1** decomposes in a slow and constant manner, releasing low pressure (61 bars), in contrast to **95** (Section III.H.1) and **116** (Section III.I.1), that showed a totally distinct behavior, decomposing very fast with 3-fold higher associated pressure, showing a typical explosive profile[33]. The onset of decomposition could be accurately set at 124 °C, after performing the ARC assay. Interestingly, **1** decomposed just after melting, unlike **95** (Section III.H.1) and **116** (Section III.I.1). As a result, it is recommended to keep the temperature <74 °C, in order to work safely[28, 29]. Nevertheless, microwave experiments using **1** at 80 °C proceeded without incidents[30].

B. Hydroxypyridines

1. HOPy, N-hydroxy-2-pyridinone

N-Hydroxy-2-pyridinone (HOPy, **8**, Figure 3) is a colorless crystalline solid, present in the form of needles or blades, soluble in hot water and ethanol, which melts at 148–151 °C, after recrystallization from ethyl acetate or methanol[31–38]. **8** (alternatively abbreviated as HOPO or Hhpno) contains a rather chemically stable hydroxamic acid moiety, being resistant to boiling aqueous acid or catalytic reduction[35, 39]. The presence of the cyclic hydroxamic acid is often confirmed after observing a characteristic deep red color in contact with a solution of ferric chloride[33, 34]. This acid moiety has also great impact on the significant antibactericidal properties exhibited by **8**, since the 3-hydroxy and 4-hydroxy isomeric pyridones do not have this activity[33, 34, 40]. Novel applications of **8** are



(**8**)

FIGURE 3. Structure of *N*-hydroxy-2-pyridinone

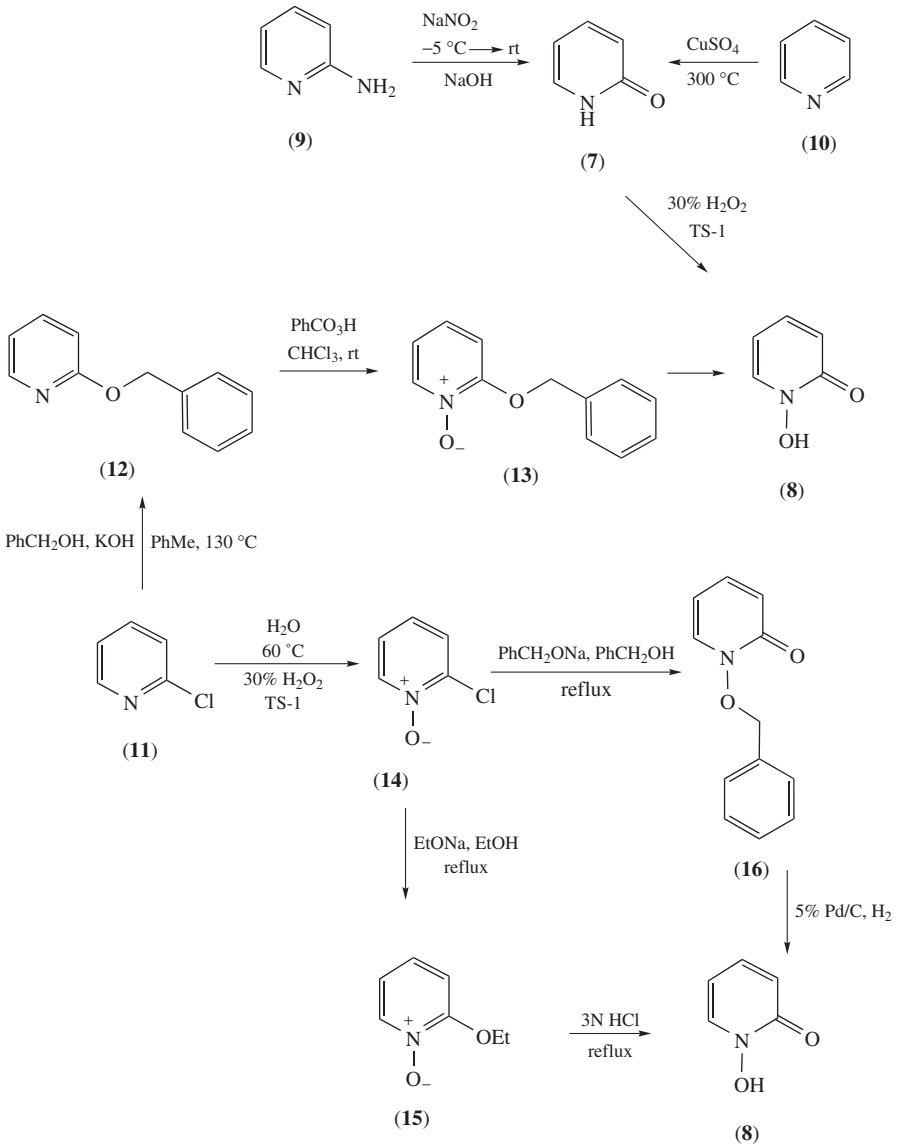
still discovered, such as the reduction of the percentage of ruthenium in the media, after performing Ring Closing Metathesis[39]. Recently, nanostructure silica materials functionalized with **8** (1,2-HOPO-SAMMS) have been shown to be effective sorbents for the abstraction of both free and chelated gadolinium-based contrast agents, in order to prevent nephrogenic systemic fibrosis[38]. Some derivatives of **8** have also been employed in obtaining hair preparations or the generation of guanine radicals[41].

Probably, the best method for preparation of **8** consists in the oxidation of 2-pyridinone (**7**), which is optimally conducted using green chemistry in the presence of hydrogen peroxide and zeolite catalysts to afford **8** in 92% yield (Scheme 2)[42]. Other selective oxidation methods include the action of *tert*-butyl hydroperoxide or molecular oxygen, using Mo or Ru catalysis (52–70% yield)[43,44]. Perbenzoic acid did not give satisfactory results[35]. **7** can be successfully obtained from 2-aminopyridine (**9**) by diazotization in sodium hydroxide at low temperature or by intramolecular nucleophilic hydroxylation of pyridine (**10**), although the latter strategy requires 300 °C to afford **8** in 95% yield[45–47]. Similar procedures were reported by hydrolysis of 2-fluoro, or demethylation of 2-methoxy to afford **8** in 47–81% yield[48–51].

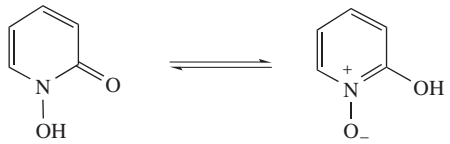
Alternatively, one of the other indirect routes to **8** from 2-chloropyridine (**11**) starts with the preparation of the 2-benzyl ether (**12**) from **11**, by treatment with benzyl alcohol and potassium hydroxide (96–97% yield)[52,53]. 2-Pyridinone, 2-fluoro or 2-bromopyridine, also lead to **12**, by means of microwave irradiation, silver catalysis or in solvent-free conditions[54–57]. **12** is then slowly oxidized to 2-benzyloxypyridine-1-oxide (**13**) in the presence of perbenzoic acid in moderate yield (45%), although other authors claim that this method yields mainly 1-benzyloxy-2-pyridinone[34,36]. Subsequent debenzylation by hydrochloric acid-mediated hydrolysis or catalytic reduction of **13** affords **8** in 68–69% yield[34]. A more efficient route results from the selective monooxidation of **11** using green chemistry and titanosilicate zeolites or *m*-chloroperbenzoic acid, to give 2-chloropyridine-1-oxide (**14**) in almost quantitative yield[37,58]. **14** could be converted to 2-ethoxy analogue (**15**) by refluxing in EtOH and EtONa (80% yield) or selectively *N*-benzylated with sodium benzyloxide, to give 1-benzyloxy-2-pyridinone (**16**) in 56% yield[36]. Pd-catalyzed reduction of **16** affords **8** in 83% yield, whereas hydrolysis of **15** by refluxing in hydrochloric acid is slightly less efficient and affords **8** in 73% yield[33,36,59].

8 is a highly acidic heterocycle ($pKa = 5.9$), as determined by potentiometric titration[34,36]. Infrared spectra of **8** in Nujol mulls showed a broad band at 3200–2200 cm^{-1} and additional signals at 3070, 2940 and 2400 cm^{-1} [36]. The same strong signal at 3200–2200 cm^{-1} was observed in chloroform[36]. A more detailed spectrum has been reported using KBr pellets[42]. **8** presents a strong UV absortion in ethanol at 228 nm and 305 nm (log $\varepsilon = 3.85$, 3.66 or 3.81, 3.60)[34,40]. Aromatic hydrogens can be detected by means of ^{1}H-NMR spectra[42].

The hydroxamic acid (**8**, *N*-hydroxy-2-pyridone) and *N*-oxide (**8′**, 2-hydroxypyridine-*N*-oxide) species is well reported from the time of the earliest synthesis (Figure 4)[33,34,39,40,42,60]. Some authors claim to obtain the *N*-oxide form **8′**, which is extraordinarily acidic ($pKa = -0.8$), whereas others find the *N*-hydroxy form



SCHEME 2. Synthetic strategies for preparation of *N*-hydroxy-2-pyridinone



(**8**) (**8′**)

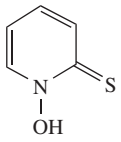
FIGURE 4. Tautomeric forms of *N*-hydroxy-2-pyridinone

(**8**) to be more consistent with the spectroscopic data received[33, 36, 42, 43]. Although Jaffe[61] found by calculations that the *N*-oxide (**8′**) is more stable than the *N*-hydroxy (**8**) by 20 kcal mol^{-1}, evaluation of **8** suggested the contrary[36]. Shaw[34] reported that 1- and 2-benzyloxy derivatives of **8** led, after hydrolysis in HCl, to the *N*-hydroxy (**8**) and that during this reaction, 2-benzyloxypyridine-1-oxide rearranges to 1-benzyloxypyrid-2-one, suggesting that both *N*-benzyloxy and *N*-hydroxy species are the most stable species[34]. Comparison with UV spectra in ethanolic mixtures containing sulfuric acid and sodium ethoxide[34, 36] showed the *N*-hydroxy tautomer (**8**) to be the preferred one. Moreever, ultraviolet spectra were similar to **7** and the 1-benzyloxypyridinone derivative[34, 40]. UV and IR spectroscopy showed the presence of a strong intramolecular hydrogen bonding, regardless of the predominant tautomeric form[32, 36].

2. HOTPy, N-hydroxy-2-pyridinethione

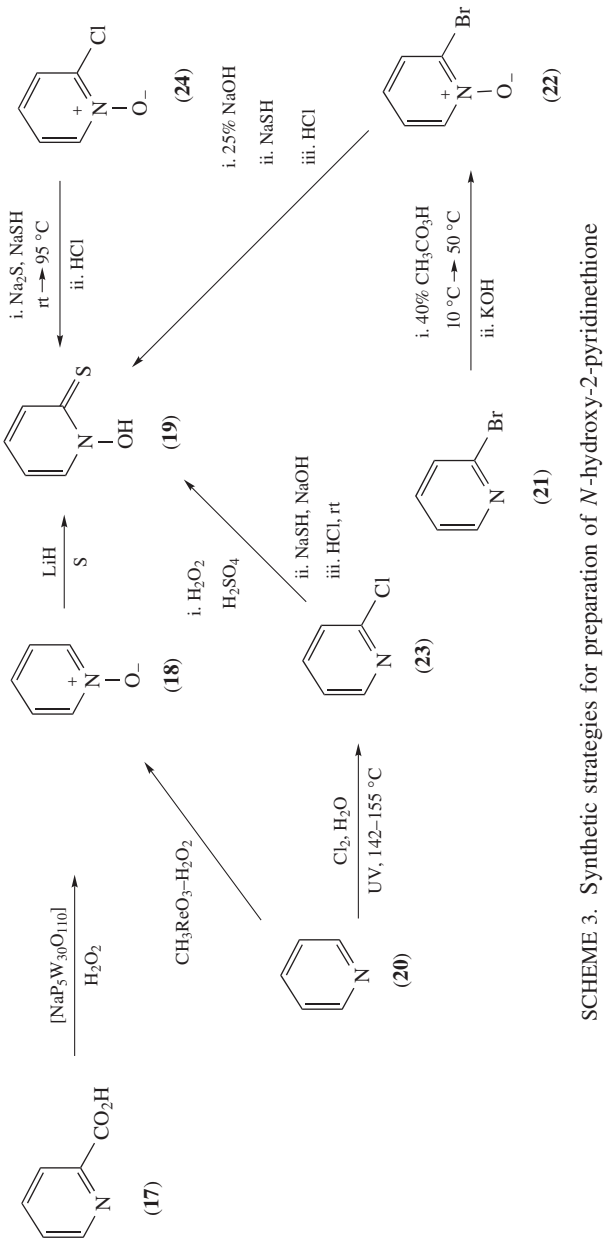
N-Hydroxy-2-pyridinethione (HOTPy, **19**, Figure 5) is a white monoclinic crystalline solid, with melting point 68–70 °C after recrystallization from aqueous ethanol. Some authors reported a lower melting point (63 °C)[62–67]. **19**, also referred to as pyrithione, is an allergenic compound, causing sneezing or sternutatory response[65, 66]. The esters formed with **19** are highly sensitive to decomposition under visible light irradiation, from a Tungsten lamp[63]. Some derivatives of **19**, such as Zn chelates, possess a marked antibacterial and antifungal activity[64–66].

One of the first designed routes to **19** consists in the regioselective direct lithiation of pyridine-*N*-oxide (**18**), followed by sulfurization with molecular sulfur, in a one-pot fashion (Scheme 3)[65, 68]. This method optimally renders **19**, when LiH is used instead of butyllithium or lithium amide, at room temperature, although the yields do not rise above 21%[64, 68]. One of the available methods enabling quantitative conversion to **19**, oxidative decarboxylation of 2-pyridinecarboxylic acid (**17**), using hydrogen peroxide as oxidant and Pressler's anion as green catalyst, affords **19** in 54–93% yield[69]. In addition, pyridine (**20**) transformed into **18** to treatment with methyltrioxorhenium-hydrogen peroxide, Keggin-type heteropolyoxo tungstates, perfluorinated ketones or magnesium monoperoxyphthalate as catalysts (Scheme 3)[70–73].



(**19**)

FIGURE 5. Structure of *N*-hydroxy-2-pyridinethione



SCHEME 3. Synthetic strategies for preparation of *N*-hydroxy-2-pyridinethione

Alternatively, moderate yields (60–61%) are obtained by nucleophilic substitution of 2-bromopyridine-*N*-oxide (**22**) with sodium sulfide or sodium hydrosulfide, under mild conditions (Scheme 3)[65, 74]. The percentage of **19** strongly depends on the purity of the sodium hydrosulfide employed, and the use of fresh reagent is recommended[65]. 2-Bromopyridine-*N*-oxide (**22**) is obtained by oxidation of 2-bromopyridine (**21**) with peracetic or perbenzoic acid in one day (60–70% yield) (Scheme 3)[65].

Excellent yields (71–81%) are obtained from 2-chloropyridine (**23**) by oxidation with H_2O_2 and H_2SO_4 or maleic anhydride, followed by nucleophilic substitution with sodium hydrosulfide (Scheme 3)[75]. Chlorination of pyridine (**20**) to 2-chloropyridine (**23**) is accomplished with high *o*/*p* regioselectivity under UV irradiation or high temperature (88–90% yield)[76]. However, the most efficient approach consists in the reaction of conversion of 2-chloropyridine-*N*-oxide (**24**) with sodium sulfide or hydorsulfide, followed by acidification to afford **19** in 94% yield (Scheme 3)[77].

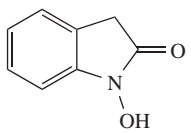
The *N*-hydroxy (*N*-hydroxy-2-pyridinethione, **19**) and *N*-oxide (2-mercaptopyridine-*N*-oxide, **19′**) species (Figure 6) are well documented, similarly to **8** (Section III.B.1)[65, 66, 74, 78]. **19** crystallizes as the *N*-hydroxythione tautomeric form **19**, the predominant species as established by UV and IR spectroscopy[66, 67]. The presence of the cyclic thiohydroxamic acid moiety of the *N*-hydroxy form (**19**) is confirmed by the appearance of a deep blue color with ferric chloride[63–65]. The intramolecular hydrogen bonding between the *N*-hydroxyl and 2-thione groups also stabilizes this structure[66].

C. HOI, *N*-Hydroxyindolin-2-one

N-Hydroxyindolin-2-one (HOI, **26**, Figure 7) is a pale yellow solid whose melting point has been described as 198–199 °C or 199–201 °C[79–81]. **26**, also named *N*-hydroxy-2-oxindole, is reported to melt at 200.5–202 °C when recrystallized from DCM–MeOH[80, 82, 83]. Remarkably **26**, having only one nitrogen atom, is not explosive and is regarded as a safer heterocyclic compound[79]. From a biomedical point of view, **26** is a weak inhibitor of mandelate racemase and is also used as template in the synthesis of agents to treat multiple sclerosis[82].

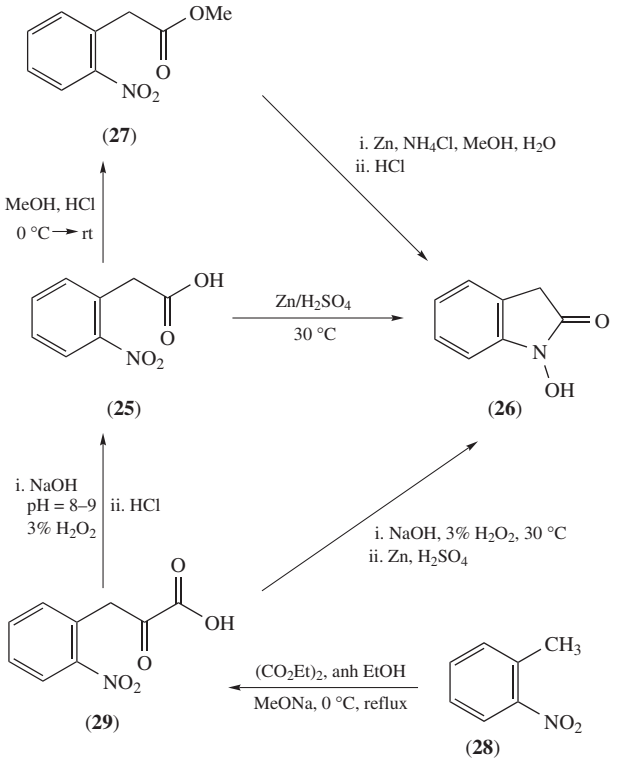
(**19**) (**19′**)

FIGURE 6. Tautomeric forms of *N*-hydroxy-2-pyridinethione



(**26**)

FIGURE 7. Structure of *N*-hydroxyindolin-2-one



SCHEME 4. Synthetic strategies for preparation of *N*-hydroxyindolin-2-one

26 is obtained by various routes, as shown in Scheme 4. It is obtained by reductive cyclization of *o*-nitrophenylacetic acid (**25**) in the presence of Zn and H_2SO_4, as described by Reissert many decades ago, along with some oxindole as byproduct (Scheme 4)[84]. Later, Wright and El-Faham and their coworkers adapted this methodology, affording **26** in 20–22% yield[79, 80]. Other Zn-based reducing agents were scrutinized by Wright and coworkers, such as Zn/Ca chloride and Zn/Ca sulfhydrate, with few or no percentage of **26** obtained[80]. Another strategy consists in the esterification of **25** with MeOH in the presence of HCl to afford **27**, and then subsequent reduction of **27** by treatment with Zn and ammonium chloride afforded **26** in 48% yield[83, 85, 86]. One of the other routes is treatment of *o*-nitrophenylpyruvic acid (**29**) with 3% hydrogen peroxide at a controlled basic pH, followed by acidification, to render **25** in 93% yield[80, 87]. An alternative is the direct conversion of **29** to **26** in 47% yield[80, 88]. Formation of **29** can be accomplished starting from *o*-nitrotoluene (**28**) in good yield, after reflux in the presence of diethyl oxalate and sodium methoxide, with subsequent acidification (51% yield)[80, 81, 87]. The direct transformation of **29** into *o*-nitrophenylacetic acid **25** is less efficient than the above-mentioned route[80].

The ^{1}H-NMR spectroscopic analysis of **26** showed the *N*-hydroxyl hydrogen resonating at high frequencies and the aromatic and aliphatic methylene protons[79]. In MeOD, the aromatic and, especially, the *N*-OH hydrogens are down-shifted[83]. The IR spectra of **26** in KBr displayed the hydroxamic acid carbonyl bands at 1675 and 1617 cm^{-1} [83].

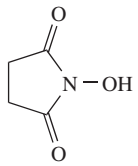
D. *N*-Hydroxysuccinimides

1. HOSu, N-hydroxysuccinimide

N-Hydroxysuccinimide (HOSu, **31**, Figure 8) is a colorless solid, obtained as crystalline plates with a melting point in the range 94–100 °C[31, 89–91]. **31** is highly soluble in H_2O, but decomposes on heating[31, 89, 92, 93]. Some derivatives or salts of **31** have found wide application in many fields, such as its esters, regarded as acylating reagents, especially for amino acids, certain silver complexes that are used to form silver film, or ethylenediamine-based analogues, which serve as crosslinking agents for the preparation of gels with wound-healing and nerve-regenerating properties[89, 91–95].

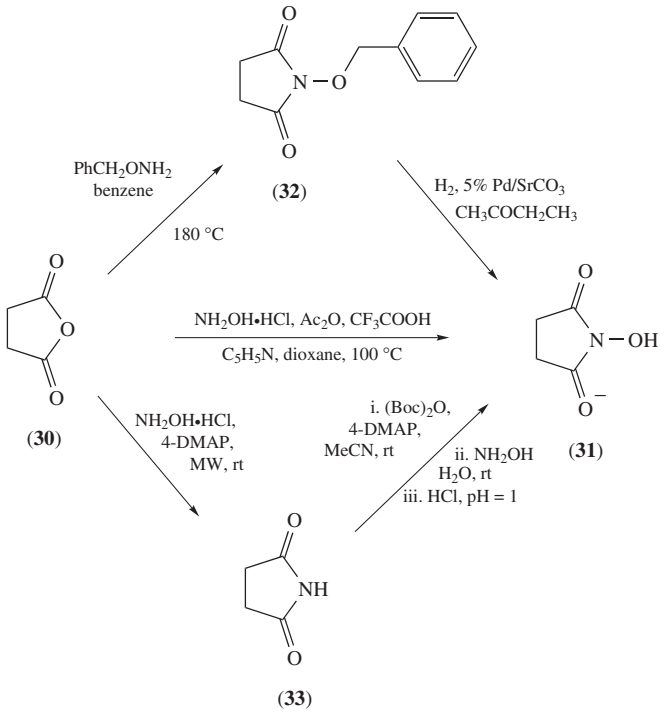
Various synthetic approaches to **31** have been proposed, many of which are based on succinic anhydride (**30**, Scheme 5). One of the earliest syntheses was described by Ames and Grey in 1955, which afforded **31** through catalytic hydrogenation of *N*-benzyloxysuccinimide (**32**) with palladium absorbed on strontium carbonate[31]. This intermediate is readily obtained from **30** by reaction with benzyloxyamine at high temperature (76% yield)[31]. An almost identical strategy was chosen by Chenavas, although few experimental details were given[96]. However, a more convenient direct conversion of **30** to **31** has been widely reported. Some approaches describe the use of free hydroxylamine to carry out such transformation, although usually higher yields are obtained with its hydrochloride salt[97]. The presence of an aqueous base, such as sodium hydroxide, is required in the first step to generate the free hydroxylamine *in situ*, followed by reflux in ethyl acetate[89, 91]. Alternatively, succinimide (**32**) can also lead to **33** (Scheme 5), adapting an analogous one-pot procedure for the synthesis of *N*-hydroxyphthalimide (HONP, **42**, see Section III.D.4) through formation of an intermediate *N*-carbamate, since direct oxidation of the imide is a difficult methodology[87, 98, 99]. In the first step, the intermediate *N*-(*tert*-butoxycarbonyl)succinimide is first formed in the presence of catalytic 4-*N*,*N*-dimethylaminopyridine (DMAP), which is rapidly transformed into the hydroxylammonium salt by addition of aqueous sodium hydroxide[87]. Final acidification to pH = 1 and recrystallization from EtOAc affords **31** in 83% yield (Scheme 5)[87].

The acidity of **31** in H_2O reported by Ames and Grey ($pK_a = 6.0$) is considerably high as an organic compound, and thus it can be considered as a suitable additive for peptide synthesis[31]. The work of Pop and coworkers, titrating a 9% MeOH in H_2O solution, led to the same dissociation constant[100]. A similar acidity at room temperature was also obtained by Koppel and coworkers ($pK_a = 6.09 \pm 0.03$) by using the standard Albert potentiometric technique[24]. However, at 54 °C, the acidity of **31** described by König and Geiger, in a 50:40 mixture of diethylene glycol dimethyl ether/H_2O, was higher ($pK_a = 5.1$) than that from the previous methods[101] with properties closely resembling the routinely used solvents in peptide synthesis. The dissociation constant determined by Koppel and coworkers, by potentiometric titration with Bu_4NOH in a solvent mixture benzene/isopropanol, was lower than in H_2O ($pK_a = 14.0 \pm 0.1$)[24]. Regarding ultraviolet spectra of a 95% ethanolic



(**31**)

FIGURE 8. Structure of *N*-hydroxysuccinimide



SCHEME 5. Synthetic pathways for preparation of *N*-hydroxysuccinimide

solution of **31**, strong absorption was detected around 200–210 nm ($\lambda_{max} = 265.5$ nm, log $\varepsilon = 2.79$; $\lambda_{max} < 205.0$ nm, log $\varepsilon > 3.89$), possibly due to the presence of a tautomeric *N*-oxide species (**31′**), which could be also involved in the high acidity of HOSu (Figure 9)[31].

Consistent with the reports on the acidity of **31**, ^{1}H NMR in C_6D_6 revealed that the *N*-OH proton resonates at high frequencies (11.0 ppm), a lower value than HOBt, **96** (Section III.H.1) (13.6 ppm)[100]. Methylene protons of **31** are clearly distinguishable from those of the sodium salt of **31** in D_2O (2.77 vs 2.63 ppm)[102]. The IR spectra of **31** resembles those of **32** and some *N*-alkyl derivatives, showing intense imide bands at 1789 and 1709 cm^{-1}, especially the latter[31]. Other bands are observed at lower frequencies (1692 cm^{-1} and 1511 cm^{-1})[31].

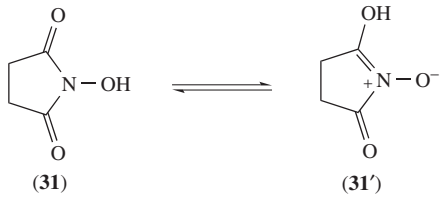
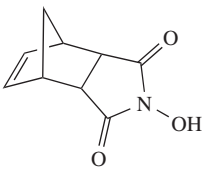


FIGURE 9. Tautomeric forms of *N*-hydroxysuccinimide

2. HONB, N-hydroxy-3,6-endomethylene-Δ^4-tetrahydrophthalimide

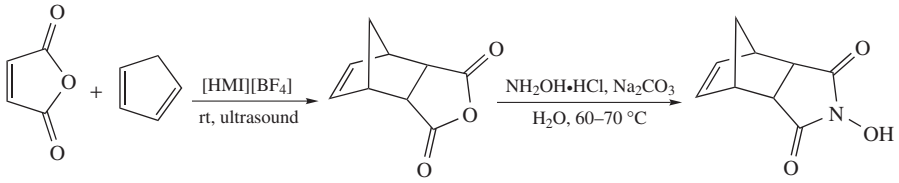
N-Hydroxy-3,6-endomethylene-Δ^4-tetrahydrophthalimide (HONB, **36**, Figure 10) is a white crystalline solid, obtained as colorless plates, melting at 165–166 °C from EtOAc[103–105]. Other authors describe a sligthly lower melting point (162–163 °C)[106]. **36** is also named *cis*-*N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide or *N*-hydroxybicyclo[2.2.1]hept-5-ene-2,3-dicarboximide[104, 106–109]. **36** is used for the preparation of primary *O*-alkoxyamines, a similar application to the one exhibited by HONOD (**39**, Section III.D.3)[106]. Its *O*-substituted derivatives possess herbicidal properties and show high phytotoxic activity even in small amounts[108].

The synthetic strategies for **36** are based on the reaction of *endo*-*cis*-3,6-endomethylene-Δ^4-tetrahydrophthalic anhydride (**35**, or 5-norbornene-*endo*,*cis*-2,3-dicarboxylic anhydride) with hydroxylamine (Scheme 6)[103]. This transformation was first accomplished by Stolberg and coworkers[103], who described the synthesis of **6**, after 24 hours, in the presence of MeONa at room temperature (80%). However, they claimed that they synthesized, in fact, the 'phthaloxime', in reference to the analogous structural debate that arose with *N*-hydroxyphthalimide (HONP, **42**, Section III.D.4)[103]. The titration of **36** with base suggested the absence of cyclic amide, although it gave a red color with ferric chloride[103]. Shortly thereafter, Bauer and Miarka improved the yield, by adapting the Orndorff methodology described for the synthesis of HONP (**42**, Section III.D.4), heating **35** with aqueous hydroxylamine hydrochloride and sodium carbonate (91% yield)[104]. Since then, similar procedures have been reported, altering the base (sodium hydroxide) or experimental details, but without further enhancement of the efficiency[105, 106]. **35** is obtained by stereoselective *endo* Diels–Alder cyclization of cyclopentadiene (**33**) and maleic anhydride (**34**) as dienophile (Scheme 6). This conversion can be quantitative and highly *endo* selective in the presence of imidazolium-based ionic liquids as solvent and under ultrasound[110]. Other highly efficient strategies which use SnW2-800 catalysts or Montmorillonite have been recently described[111, 112].



(**36**)

FIGURE 10. Structure of *N*-hydroxy-3,6-endomethylene-Δ^4-tetrahydrophthalimide



(**33**) (**34**) (**35**) (**36**)

SCHEME 6. Synthetic strategies for preparation of *N*-hydroxy-3,6-endomethylene-Δ^4-tetrahydrophthalimide

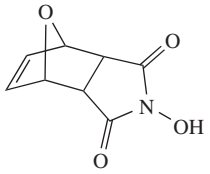
The pKa of 5.9 of **36** is one of the highest in the *N*-hydroxyl cyclic imide series, showing a higher dissociation constant than **42** (Section III.D.4)[103]. In the latter case, the lower acidity is due to the presence of the aromatic ring, which decreases the CO polarization and hence the *N*-OH dissociation constant[106]. IR analysis of **36** showed carbonyl bands with strong absorption at 1695, 1710 and 1770 cm^{-1} and the *N*-hydroxyl signal at high frequency (3100 cm^{-1})[103, 104]. Other bands were detected at 2850, 1610 and 720 cm^{-1} [106]. The expected ^{1}H-NMR spectra for the proposed structure was provided by Rougny and Daudon in DMSO-d_6[106]. **36** affords colorless anions when deprotonated, in contrast to the colorful anions of aromatic *N*-hydroxy imides[104].

3. HONOD, N-hydroxy-3,6-epoxy-1,2,3,6-tetrahydrophthalimide

N-Hydroxy-3,6-epoxy-1,2,3,6-tetrahydrophthalimide (HONOD, **39**, Figure 11) is a white crystalline solid, structurally derived from HOND (**42**) (Section III.D.4) or *N*-hydroxysuccinimide (**31**, Section III.D.1)[113, 114]. Alternatively, it has been abbreviated as HOEC (*N*-hydroxy-1,4-epoxy-5-cyclohexene-2,3-dicarboximide) and HONCE (*N*-hydroxy-1,4-epoxylcyclohex-5-ene-2,3-dicarboximide), or named as *exo*-*N*-hydroxy-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide or 4-hydroxy-10-oxa-4-azatricyclo[5.2.1.0]dec-8-ene-3,5-dione[115–120]. Some of its applications consist of the preparation of *N*-substituted maleimides by retro Diels–Alder reaction, which cannot be synthesized from maleamic acids due to detrimental isomaleimide formation, and molecularly imprinted polymers[113, 116, 121]. **39** also serves as a building block to form ring-opening metathesis polymers (ROMP), which are used as acyl transfer reagents in the synthesis of amides, carbamates, ureas or Mosher amides[117]. Arylantimony analogues of **39** show promising anticancer properties, whereas phosphates and *N*-aryloxyacetyl derivatives are useful herbicides, parasiticides and rodenticides[122, 123]. Conjugation of kidney imaging agents with antibodies has also been accomplished with the help of **39**[120].

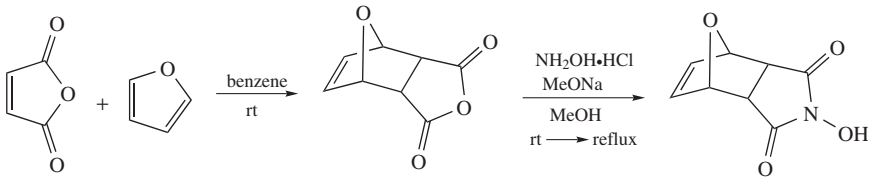
Narita and coworkers reported the one-pot synthesis of **39** from furan (**37**) and maleic anhydride (**33**) in 64% yield (Scheme 7)[113]. In the first step, the Diels–Alder cycloaddition of **33** and **37** yields the maleic anhydride-adduct of furan: 3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (**38**), which is present exclusively in the *exo* form[113, 124]. This intermediate is then converted to **39** by refluxing with hydroxylamine hydrochloride in the presence of NaOMe[113]. In the following decades, analogous strategies to that of Narita have been described, without significant variation or improvement[114, 125].

The melting point of **39** has given rise to some controversy, since some authors claim it melts at 187–188 °C, with decomposition at 150 °C, whereas others reported a considerably lower melting value (167–170 °C)[113]. **39** shows thermal instability, decomposing at 190 °C. However, in contrast with *O*-alkoxy derivatives, no reverse Diels–Alder cycloaddition products are observed[113]. **39** displays medium-to-strong infrared absorption at 1785



(**39**)

FIGURE 11. Structure of *N*-hydroxy-3,6-epoxy-1,2,3,6-tetrahydrophthalimide



SCHEME 7. Synthesis strategy for the synthesis of **39**

and 1725 cm^{-1}, corresponding to the cyclic imide moiety, and medium-absorbance *N*-hydroxyl bands at 3470 and 3300 cm^{-1} [113].

4. HONP, N-hydroxyphthalimide

N-Hydroxyphthalimide (HONP, **42**, Figure 12) is a colorless monoclinic crystalline solid, forming needles or rods, melting with decomposition at 232–233 °C[187, 99, 126–128]. Other authors reported a slightly lower melting point (230 °C)[99, 127]. The cyclic imide **42** is stable to air, causing allergenic response and dermatitis in contact with skin or eyes[129, 130]. **42**, which is also named phthalylhydroxylamine or abbreviated as NHPH, is poorly soluble in water, in contrast to the less hydrophobic *N*-hydroxysuccinimide (**31**, Section III.D.1) except in basic conditions, resulting in tedious work-ups[89, 99, 127, 131]. **42** is also rather insoluble in many organic solvents, such as benzene, chloroform, diethyl ether, toluene or petroleum ether[127]. Only in EtOH, acetone, EtOAc, HOAc and acetonitrile does **42** display a high solubility[127]. In spite of this general limitation **42** is widely used in the preparation of *O*-substituted hydroxylamines[132, 133]. Its radical also participates in hydrogen-abstraction mediated reactions, being considered as a suitable catalyst in the oxidation of hydrocarbons in mild conditions[134–137]. Even in biochemical oxidating processes, **42** is used as an initiator[138, 139]. Some of its derivatives have also been useful in electrocatalytic oxidations or as asymmetric catalysts[140–143].

During many years in the first part of the last century, it was considered that the reaction of phthalic anhydride with hydroxylamine yielded a compound regarded as a 'phthaloxime', the phthalic anhydride mono-oxime[144–146]. Brady and coworkers reported two isomeric versions for this phthaloxime, obtained as yellow and white solids, respectively[146]. Later, some authors, like Putokhin[127] or Ames and Grey[126], claimed that the above-mentioned white isomer was, in fact, **42**, the *N*-hydroxy cyclic imide of phthalic acid. Ames and Grey, who were unable to obtain the yellow isomer described by Brady, argued that the UV profile of the white isomer was very similar to phthalimide and also

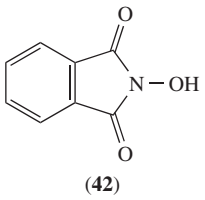
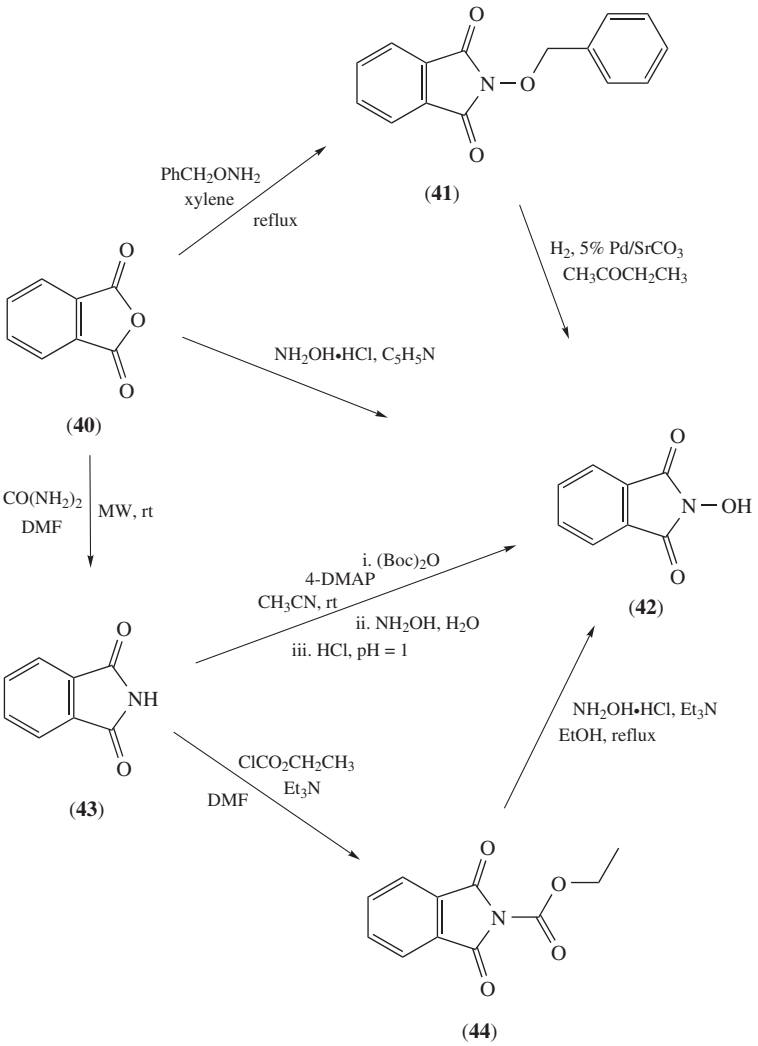


FIGURE 12. Structure of *N*-hydroxyphthalimide

that the pKa was off the range considered for oximes[126, 147]. A simultaneous report by Roderick and Brown concluded that both 'phthaloxime' isomers, including the yellow one, whose color is probably due to the presence of certain impurities, corresponded to the **42** structure[148].

Various methods to prepare **42** are given in Scheme 8. Ames and Grey adapted the method for preparing aliphatic cyclic imides to the synthesis of **42** (Scheme 8)[31]. First, phthalic anhydride (**40**) is converted to *N*-benzyloxyphthalimide (**41**) after reflux with *O*-benzyloxyamine in xylene[126]. Then, **41** is catalytically hydrogenated in the presence



SCHEME 8. Synthetic approaches for the synthesis of *N*-hydroxyphthalimide

of strontium carbonate over Pd, to afford **42** in 74% overall yield (Scheme 8)[126]. Later approaches have been designed to carry out the transformation of **40** to **42** by treatment with hydroxylamine hydrochloride[133, 149]. Sugamoto and coworkers recently reported a fast conversion using microwave irradiation[150]. Alternatively, **42** can also be efficiently synthesized from phthalimide (**43**). One of the first strategies consisted in the conversion of phthalimide or its potassium salt to *N*-(ethoxycarbonyl)phthalimide (**44**, the Nefkens reagent) by the action of ethyl chloroformate, followed by reflux with hydroxylamine hydrochloride (70% overall yield)[99, 127]. However, direct synthesis has also been reported by Einhorn and coworkers, through intermediate formation of *N*-(*tert*-butoxycarbonyl)phthalimide and subsequent reaction with aqueous hydroxylamine and acidification, in one-pot (92% yield)[90]. Alternatively, **43** can be slowly transformed into **42** with 350-nm UV irradiation in the presence of titanium dioxide[151]. Both pathways, starting from **41** or **43**, which is quantitatively obtained from the former after MW-assisted treatment with urea, lead to **42** in high yields[152].

The acidity of **42** reported by Ames and Grey in 50% aqueous methanol ($pK_a = 7.0$) was high enough to reconsider the initial assumption of the phthaloxime structure, since usually oximes show pK_a in the range 10–12 (except for those bearing electron-withdrawing substituents)[3, 126, 147]. Later, Koppel and coworkers also investigated the acidity of **43** in H_2O, showing that it was slightly less acidic ($pK_a = 6.32 \pm 0.03$) than the parent HOSu (**31**, Section III.D.1)[24]. In DMSO, a more similar solvent to those routinely used in peptide synthesis, the difference between the dissociation constants was higher ($pK_a = 14.0 \pm 0.1$), as determined by potentiometric titration with Bu_4NOH in the solvent mixture benzene/isopropanol[24]. Compared to **42**, **43** was 2 pK_a units more acidic in H_2O, confirming the impact of the *N*-hydroxylamine group in the acidity[24]. The ^1H- and ^{13}C-NMR analysis revealed that the *N*-hydroxyl hydrogen resonates at high frequencies close to those of the analogous group of HOSu (**31**, Section III.D.1)[100].

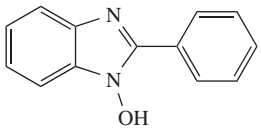
Ames and Grey also characterized **42** by means of IR spectroscopy, showing imide bands at 1745 and 1795 cm^{-1}, along with other signals (1721 and 1866 cm^{-1})[126]. Later, Wei and coworkers considered that bands appearing at 1780, 1720, 1375 and 1120 cm^{-1} were all related to the cyclic imide moiety[131]. The same authors also observed carbonyl and *N*-hydroxy signals at 1700 and 3120 cm^{-1} [131]. However, Krishnakumar and colleagues detected the *N*-hydroxy band at higher frequencies (3592 cm^{-1}), along with an additional intermolecular O–H bond (3442 cm^{-1})[128]. The ultraviolet spectrum of **42** resembles phthalimide (**43**), showing strong absorption at 220.5 nm ($\log \varepsilon = 4.48$) and at 298.0 nm ($\log \varepsilon = 3.20$)[126].

E. *N*-Hydroxy-2-phenylbenzimidazoles

1. HOBI, N-hydroxy-2-phenylbenzimidazole

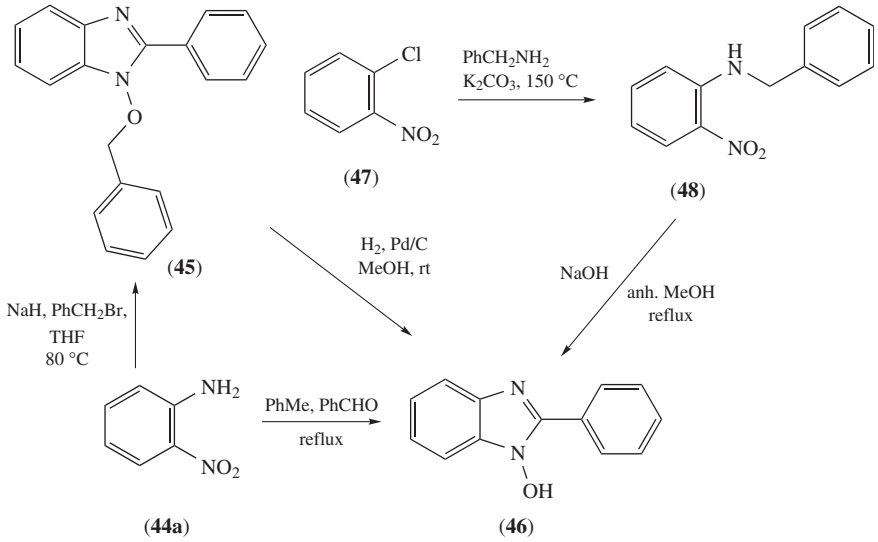
N-Hydroxy-2-phenylbenzimidazole (HOBI, **46**, Figure 13) is obtained as a white crystalline solid, with melting point 220 °C from ethanol[79, 153–155]. In contrast to benzotriazoles, which contain three consecutive nitrogen atoms in their heterocyclic core, **46** (alternatively abbreviated as HOPBI) displays carbon atoms between the two nitrogens, and therefore a safer decomposition profile is expected[79]. Benzimidazoles are included, on many occasions, in biologically active biomolecules[60, 156]. In particular, **46**, a photosensitive heterocyclic compound, serves as template for designing inhibitors of bacterial transcription factors[155, 157]. Its *N*-alkoxy derivatives show activity as central nervous system depressants in certain animals and also analgetics effects[158, 159].

The simplest way to **46** would consist in the oxidation of 2-phenylbenzimidazole. However, direct *N*-oxidations have not been reported for these aromatic systems and, consequently, the strategies designed to date have focused on indirect methods (Scheme 9).



(**46**)

FIGURE 13. Structure of *N*-hydroxy-2-phenylbenzimidazole



SCHEME 9. Synthetic strategies for the synthesis of *N*-hydroxy-2-phenylbenzimidazole

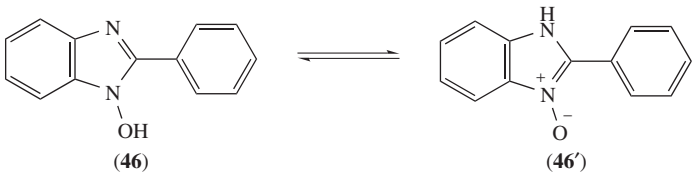
One method starts from *o*-nitroaniline (**44a**), which is converted into *N*-benzyloxy-2-phenylbenzimidazole (**45**) through a tandem cascade process, in which *N*-alkylation is followed by imidazole *N*-oxide formation and *in situ O*-alkylation, in the presence of a refluxing mixture of NaH and benzyl bromide (Scheme 9)[60]. A proposed mechanism is the generation of an α-amino carbanion, after the initial *N*-alkylation, with subsequent dehydration, prior to *N*-benzyloxy formation[61]. Surprisingly, benzimidazole *N*-oxide has never been found in the crude mixture, although the *N*-alkylated linear compound, coming from the first step of the reaction, is sometimes obtained as a byproduct[60]. Two distinct procedures have been developed for carrying out this one-pot preparation of **45**, as described by Gardiner and coworkers: in the first, the full amount of reagents are added almost simultaneously followed by reflux, whereas in the second, 1 equivalent is employed at the beginning, and the rest is poured over 12 hours[60, 158]. Gardiner and coworkers reported that the latter system was more efficient (79% yield), although some years later Kokare and colleagues reported a 97% yield of **45** with the former method[153, 154]. A careful evaluation of both procedures was recently performed by Albericio and coworkers, concluding that the former method leads to unreacted starting material, whereas slow addition of sodium hydride and benzyl bromide afforded 88% of **45**[79]. Subsequent Pd-catalyzed hydrogenation of **45** afforded **46** in an almost quantitatively manner[79, 153, 154].

An alternative strategy was designed by Stacy and coworkers from **44a**, which directly affords **46**, by treatment with benzaldehyde in anhydrous toluene, through intermediate formation of the *N*,*N*-benzylidene-*o*-nitroaniline Schiff base, in 86% yield, although prolonged reaction times (3 days) are necessary[155]. The *N*-alkylated byproduct from Gardiner's approach, *N*-benzyl-*o*-nitroaniline (**48**), might also be useful for the preparation of **46**, as outlined by Stacy and coworkers, by means of a base-catalyzed cyclization in the presence of sodium hydroxide in refluxing anhydrous methanol (71% yield) (Scheme 9)[155]. Nucleophilic attack of benzylamine onto *o*-chloronitrobenzene (**47**), in potassium carbonate-induced basic media and high temperature, renders **48**, in 80% yield, as reported by Stacy and coworkers, following the Gibson methodology[155, 160]. Bowser and coworkers recently envisioned an analogous synthesis of **46** based on *o*-fluoronitrobenzene, by treatment with benzylamine and sodium carbonate, followed by addition of sodium hydride, although no yields were reported[157].

IR and UV spectra of **46** revealed its similarity to 2-phenylbenzimidazole and 1-hydroxybenzimidazole[155]. The infrared spectra displayed *N*-hydroxyl and aromatic C=C bands at 3120 cm^{-1}, whereas ultraviolet spectra in EtOH showed a maximum at 298 nm ($\log \varepsilon = 4.33$)[155]. Regarding the ^{1}H-NMR spectra in DMSO-d_6 of **46**, they display the *N*-hydroxyl proton at high frequency at δ 12.22, indicating its high acidity[79, 153]. Finally, **46** can exist in two tautomeric forms, the *N*-hydroxy (**46**) and *N*-oxide (**46′**) species (Figure 14)[60, 155]. Initial studies on the parent *N*-hydroxybenzimidazole suggested the predominance of the *N*-oxide tautomer, due to the consistency of this structure with experimental results on the reactivity with various compounds[161]. A similar ratio of the *N*-hydroxy (**46**) and *N*-oxide (**46′**) forms could be envisaged in the case of **46**. Nonetheless, NMR spectra of **46** in TFA were consistent with the *N*-hydroxy species (**46**), as the more stable, preferred tautomer[155]. The tautomeric equilibrium of **46** has also been shown to be solvent-dependent[60].

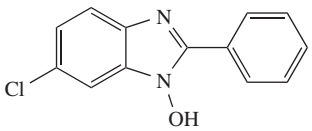
2. 6-Cl-HOBI, 6-chloro-N-hydroxy-2-phenylbenzimidazole

6-Chloro-*N*-hydroxy-2-phenylbenzimidazole (**53**, Figure 15) is an off-white solid, melting at 262–264 °C[79]. Surprisingly, a considerably lower melting point is reported by other



(**46**) (**46′**)

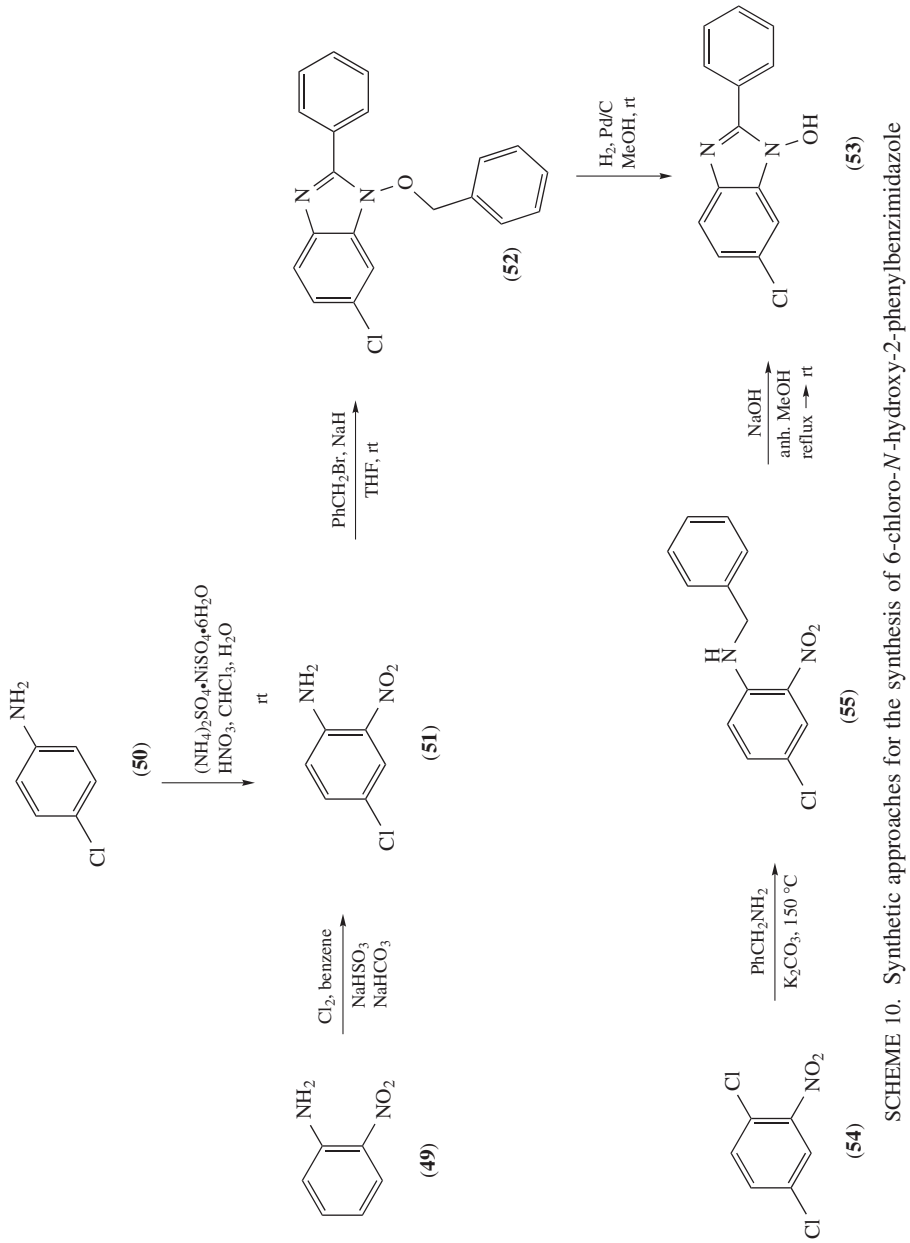
FIGURE 14. Tautomeric forms of *N*-hydroxy-2-phenylbenzimidazole



(**53**)

FIGURE 15. Structure of 6-chloro-*N*-hydroxy-2-phenylbenzimidazole

650



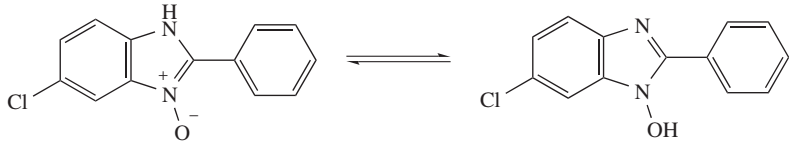
SCHEME 10. Synthetic approaches for the synthesis of 6-chloro-*N*-hydroxy-2-phenylbenzimidazole

authors (241 °C)[158]. The imidazole ring of **53** does not contain consecutive nitrogens, contrary to the triazole ring of benzotriazoles, and thus safer decomposition behavior is predicted. In general, benzimidazoles units are present in a wide range of pharmacologically active compounds[156]. *N*-Alkoxy derivatives of **53**, alternatively abbreviated as 6-Cl-HOPBI, induce a depressant effect on the central nervous system[129].

Direct oxidation of benzimidazoles to *N*-hydroxybenzimidazoles has never been achieved. As a result, synthetic approaches to **53** have been directed toward cyclization methods generating the *N*-hydroxy moiety (Scheme 10). **53** can be obtained from 4-chloro-2-nitroaniline (**51**), as described by Gardiner and coworkers, through a one-pot tandem process involving *N*-alkylation, ring formation and subsequent *O*-alkylation, in analogy to the synthesis of HOBI (**47**), followed by hydrogenation (Scheme 10)[60]. From the two described procedures for conducting this transformation, Gardiner recommended the one in which the reagents, sodium hydride and benzyl bromide, are added in small portions over many hours[60]. However, although high conversion to **52** was achieved in 81% yield with almost 50% impurties[60], El-Faham and Albericio used the same method successfully, obtaining **53** in 66% yield[79, 162]. The same authors reported the hydrogenation of this intermediate, catalyzed by Pd/C in methanol at room temperature, to give **53** in 73% yield (Scheme 10)[79]. The starting material, 4-chloro-2-nitroaniline (**51**), can be obtained either from **49** or **50**. The chlorination of **49** might be performed in the presence of chlorine and sodium bicarbonate (Scheme 10), although no yields were detailed by Chapman and coworkers[163]. The approach based on **50** was reported by Tasneem and coworkers, and efficiently yields **51** by nitration with ammonium nickel sulfate and nitric acid (92% yield)[164].

Similarly to the synthesis of **47** (Section III.E.1), an alternative strategy has been designed, consisting in the base-catalyzed cyclization of **55** by treatment with NaOH in anhydrous MeOH (Scheme 10)[158]. The conversion of **54** to **55** can be performed using the Gibson methodology, in the presence of benzylamine and potassium carbonate, above 100 °C (yields not described)[160].

^{1}H NMR in DMSO-d_6 data for **53** showed the presence of the highly acidic proton at δ 12.19 and aromatic protons[79]. Like **46** (Section III.E.1), two tautomeric species are found for **53**: the *N*-hydroxy (**53**) and *N*-oxide (**53′**) forms (Figure 16)[155]. Kew and Nelson[161] studied the composition of the parent *N*-hydroxybenzimidazole, concluding that the *N*-oxide tautomer might be favored, due to higher consistency with experimental results on its reactivity with various compounds. Thus, similar relative percentages of *N*-hydroxy (**53**) and *N*-oxide (**53′**) species could be envisioned for 6-Cl-HOBI (**53**). The tautomeric equilibrium of 6-Cl-HOBI is known to be solvent-dependent[60].



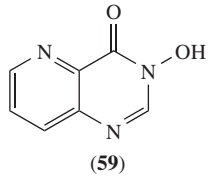
(**53′**) (**53**)

FIGURE 16. Tautomeric forms of 6-chloro-*N*-hydroxy-2-phenylbenzimidazole

F. HODhad, 3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene

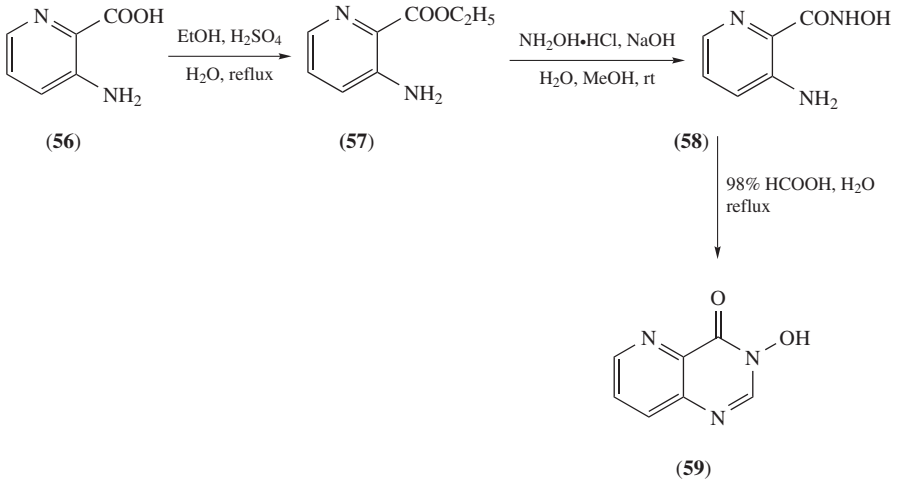
3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene (HODhad, **59**, Figure 17) is a yellow solid with melting point 318.5–320 °C from EtOH[165]. Its synthesis is essentially identical to that of the parent triazene HODhat (**158**, Section III.K) described by Carpino and coworkers, except for the last cyclization step, which was performed by refluxing 3-amino-2-picolinehydroxamic acid (**58**) with 98% formic acid, in 55% yield (Scheme 11)[165]. The hydroxamic acid (**58**) can be obtained quantitatively from ethyl 3-aminopicolinate (**57**), after treatment with hydroxylamine hydrochloride and NaOH in a H_2O–EtOH mixture (Scheme 11), during 2 days, improving the method designed by Harrison and coworkers[165, 166]. Carpino and coworkers also improved the esterification of **56** to **57**, by using an extensive reflux of the acid in absolute EtOH and concentrated H_2SO_4 (68% yield, Scheme 11)[165, 167, 168]. **56** was obtained from available precursors as described for HODhat (**158**, Section III.K)[165, 23, 169–174].

^{1}H-NMR data for **59** showed the hydrogens of the pyridine and diazine rings in the aromatic region[165]. The infrared spectra of **60** in KBr displayed a broad *N*-hydroxyl band at higher frequency than the corresponding signal of **158** (Section III.K): 2625 cm^{-1}, and another band corresponding to the hydroxamic acid amide moiety at 1683 cm^{-1} [165]. Other bands were observed at 1600, 1446, 1410, 1359, 1223, 990, 902 and 791 cm^{-1} [165].



(**59**)

FIGURE 17. Structure of 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene



(**56**) (**57**) (**58**) (**59**)

SCHEME 11. Synthetic approaches for the synthesis of 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,3-diazene

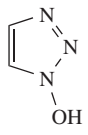
G. Triazoles

1. 1-Hydroxy-1,2,3-triazole

1-Hydroxy-1,2,3-triazole (**64**, Figure 18), initially known also as azimidole, is a solid melting at 92–93 °C, which represents the template of a family of additives in peptide synthesis, including many 5-substituted analogues[175–177]. The synthesis of **64** raised the interest of organic chemists more than a century ago, when Wolff reported its isolation from hydroxylamine and diazoketone, followed by partial reduction[177]. Since then, improved routes have been designed, which can be classified as these that obtain the triazole core by 1,3-cycloaddition of modified acetylenes and azides, followed by oxidation (Scheme 12), and those that directly obtain the hydroxytriazole core by a single oxidative cyclization step (Scheme 12).

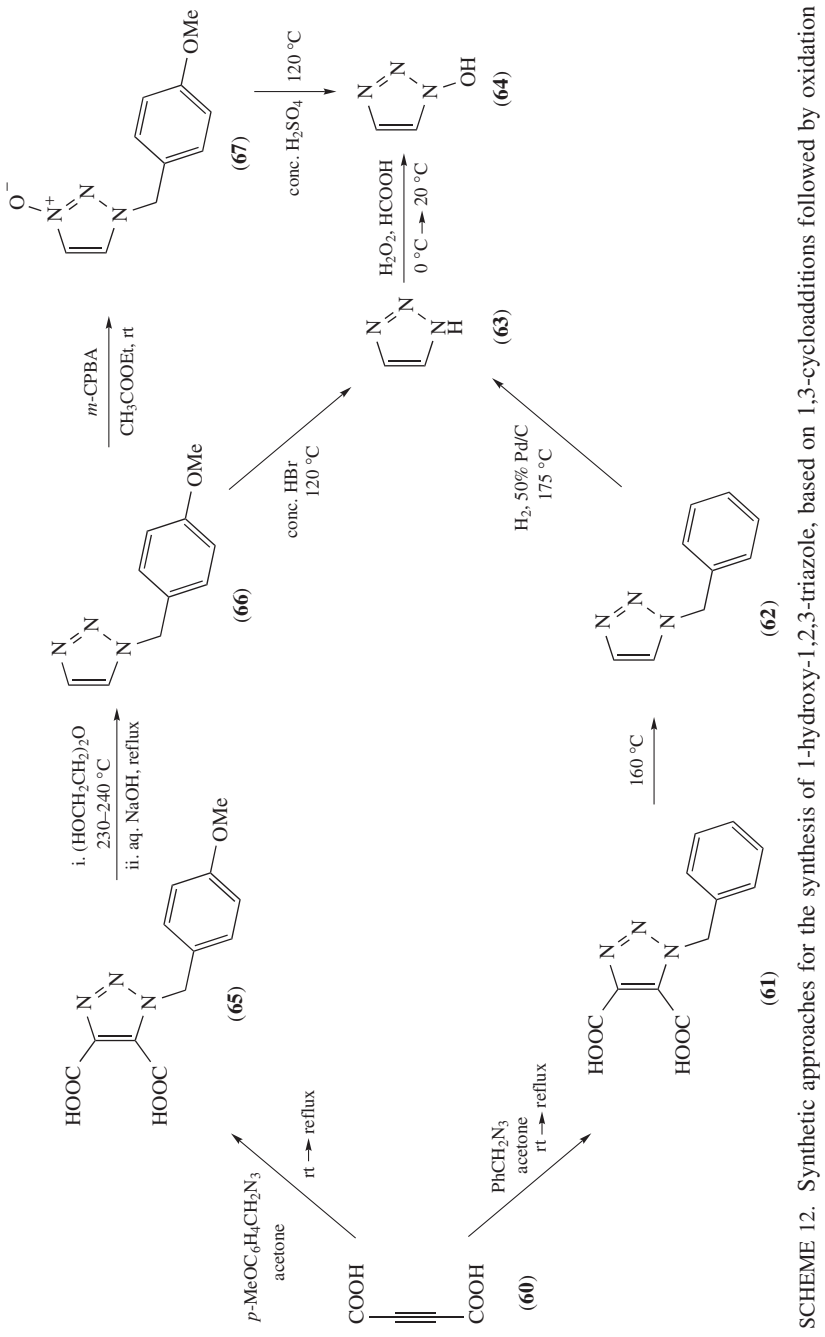
One of the approaches corresponding to the former class was developed by Wiley and coworkers, and affords the intermediate 1,2,3-triazole (**63**) via 1-benzyl-protection (Scheme 12)[178]. In the first step, acetylene dicarboxylic acid (**60**) is refluxed with benzyl azide to form a substituted triazole **61**, conversion that implies a lower risk than cyclization of unmodified acetylene and azide[178–180]. The route from **61** to **62** takes place preferably by decarboxylation at high temperature, followed by Pd-mediated debenzylation of **62** in 3–4 days (Scheme 12)[178]. The key step is the oxidation of **63**, which is most efficiently performed with stepwise addition of oxidant, although this optimization of yield (65%) is accompanied by a significant increase in the reaction time (up to 30 days)[181]. Many oxidants have been tested, such as 3-chloroperbenzoic acid or sodium perborate, but the reaction is best conducted in the presence of H_2O_2[175]. This approach has been improved by using 4-methoxybenzyl azide in the cycloaddition step, forming **65**, which leads to its decarboxylated derivative **66** (Scheme 12)[176, 182]. Subsequent debenzylation of **66** takes place with concentrated hydrobromic acid, in considerably shorter time than the above-mentioned synthesis. It also results in enhanced overall yield of **65** from **61** (30% vs. 28%)[176]. Alternatively, **66** can be transformed into its 1-oxide derivative (**67**) which, after treatment with concentrated sulfuric acid, also affords **64** in 23% overall yield (Scheme 12), although the long-time oxidation of **63** is avoided[176].

The above-mentioned strategies are based on the cycloaddition of acetylenes with azides; both, although substituted, present a high explosive potential[178]. Furthermore, the oxidation steps, even in the 4-methoxybenzyl modification, are only completed after a few days, which delays affording of the desired **64**. All these inconveniences can be avoided with the safer and quicker route starting from glyoxal (**65a**, Scheme 13)[176, 181, 183]. The success of this method partially lies on the optimally close to natural pH of the reaction medium. In the first step, **65a** is transformed into glyoxal *O*-benzyloxime (**66a**) by addition of benzyloxyamine hydrochloride[181, 184]. This intermediate then reacts with hydrazine hydrate to form *in situ* the derived hydrazone, which undergoes oxidative cyclization (presumably by a one-electron mechanism) with copper sulfate to yield **67a**[185, 186]. Final hydrogenolysis catalyzed by Pd in cold methanol affords the target **64** in a higher efficiency than the above-mentioned approaches (50% overall yield) (Scheme 13)[181]. Similar



(**64**)

FIGURE 18. Structure of 1-hydroxy-1,2,3-triazole



SCHEME 12. Synthetic approaches for the synthesis of 1-hydroxy-1,2,3-triazole, based on 1,3-cycloadditions followed by oxidation

SCHEME 13. Synthetic strategies for the synthesis of 1-hydroxy-1,2,3-triazole, based on oxidative cyclizations

strategies using benzylhydrazine or 4-methoxybenzylhydrazine lead to 2-benzyl-1,2,3-triazole-1-oxide (**68**) or 2-(4-methoxybenzyl)-1,2,3-triazole-1-oxide (**69**) as intermediates, followed by debenzylation with concentrated hydrobromic or sulfuric acid (iodotrimethylsilane might form iodine-based byproducts)[176, 181, 183, 187]. However, yields are lower than the 1-benzyloxy approach (26% and 27% vs. 50%).

The C4 and C5 hydrogens of the triazole ring of **64** are observed at similar frequencies, according to ^{1}H-NMR data of **64** in CD_3COCD_3[175]. The dissociation constant of **64** has not been reported, although **64** is known to show a strong acidic character, similar to the parent HOBt (**96**, Section III.H.1)[177]. In comparison with the latter, **64** permits easier tuning of acidity on introduction of substituents in the ring, due to its proximity to the *N*-OH group[178, 179]. The known explosive behavior of *N*-hydroxybenzotriazoles, *N*-hydroxytetrazoles and *N*-alkylimidazoles and pyrazoles could lead to the assumption that 1-hydroxy-1,2,3-triazoles share a similar decompostion behavior[2, 181, 188–191]. Nonetheless, **64** is regarded as non-sensitive to drop-weight impact of a hammer-head from 50 cm distance and therefore no safety precautions need to be taken in handling it[188].

Three main tautomers are considered for 1-hydroxy-1,2,3-triazole (**64**, **64′**, **64″**), excluding the possible non-aromatic species, which are not observed by ^{1}H-NMR spectra (Figure 19)[192]. The composition of the tautomeric equilibrium was studied by Guimon and coworkers using UV (HeI) photoelectron spectroscopy and semiemipiral quantum methods[192]. The composition of **64** in the gas phase was based on the similarity of spectra and ionization potentials with the *O*-methyl ester. This result was supported by the MNDO (modified neglect of diatomic overlap) semiempirical calculations, which found that **64** is the most stable tautomer in a broad range of temperatures. This method, using the Koopmans approximation, was generally in agreement with experimental values, except for the potential of the non-bonding nitrogen orbitals[193, 194]. Anders and coworkers extended the semiempirical study of this triazole system to the AM1 (Austin model 1) and PM3 (parametric method 3) calculations[195]. Whereas the former method agrees with the higher stability of **64** predicted by MNDO, the latter obtains similar energies of tautomers **64** and **64″**. However, the reliability of the PM3 method for these *N*-hydroxy/*N*-oxide tautomeric equilibria has been questioned[196]. The *N*-oxide form **64′**, which is slightly non-planar as calculated by *ab initio* methods (whereas **64** and **64″** are planar), is always calculated to be the least stable form. From *ab initio* calculations the bond lengths of 1-hydroxy-1,2,3-triazole are almost identical to those of 1,2,3-triazole and slightly longer than certain N−N bonds of HOBt (**96**, Section III.H.1)[195].

2. HOCt, ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate

Ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate (**75**, Figure 20) is one of the few 4-substituted derivatives of **64**, proposed as additive for peptide synthesis and also serves as starting material for building β-lactamase or tuberculosis inhibitors[197–199]. **75**, obtained as a colorless crystalline solid melting at 110–112 °C, is stable in air at room temperature

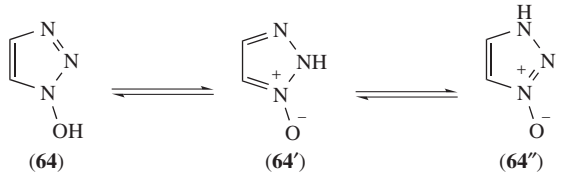
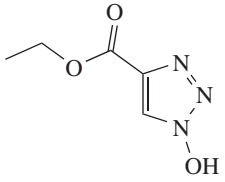


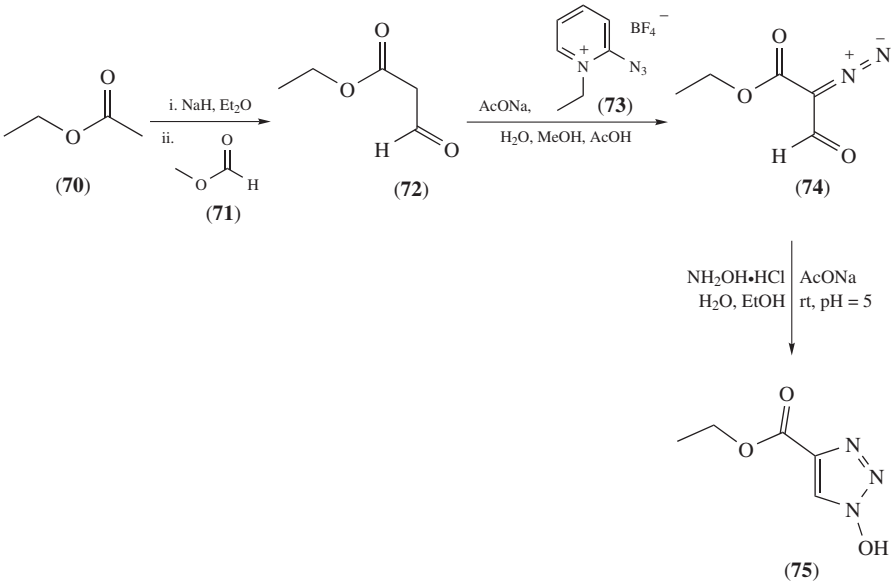
FIGURE 19. Tautomeric forms of 1-hydroxy-1,2,3-triazole



(**75**)

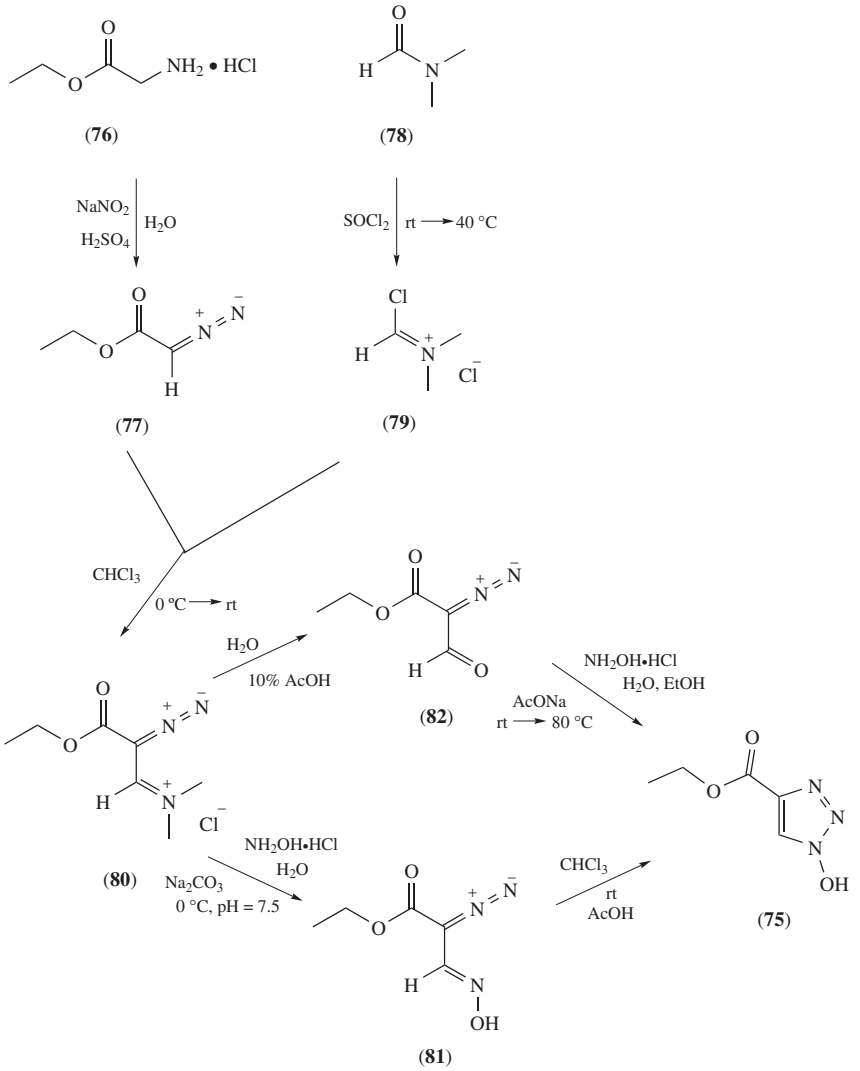
FIGURE 20. Structure of ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate

for years and is highly soluble in H_2O and many organic solvents, such as DMF, DCM or EtOH[198]. Unlike most 1,2,3-triazole derivatives, its synthesis does not involve heterocyclic ring formation by means of 1,3-cycloaddition of azides with acetylenes, but through condensation of 2-diazo-1,3-dicarbonyl compounds with hydroxylamine. An example of the latter approach is the route designed by Sezer and coworkers, affording **75** in 3 steps (Scheme 14)[200, 201]. Claisen condensation of ethyl acetate (**70**) with methyl formate (**71**) yielded ethyl 3-oxopropanoate (**72**), which was diazotized in the presence of 2-azido-1-ethylpyridinium tetrafluoroborate (**73**)[200, 201]. Apart from ethyl 2-diazo-3-oxopropanoate (**74**), the deformylated byproduct was also generated in considerable amounts, although it didn't interfere in the last cyclization step with hydroxylamine hydrochloride, performed at moderate acidic pH during 2 days[200, 201]. This methodology, however, rendered the target **75** in a poor 6% overall yield (Scheme 14) and a melting point (161–162 °C) differs significantly from the one reported by other authors[198, 201].



SCHEME 14. Synthetic approach for the synthesis of ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate

Jiang and coworkers attempted an alternative strategy from ethyl 2-oxo-3-oximinopropanoate, but initial transformation of ethyl 2-oxopropanoate to render this intermediate failed[198]. Re-evaluation of the synthetic design led to ethyl 2-diazo-3-dimethyliminium propanoate chloride (**80**) as key precursor of **75**, which was obtained by formylation of ethyl 2-diazoacetate (**77**) with dimethyl formamidinium chloride (**79**), using the Stojanovic and Arnold method (Scheme 15)[202]. Slow addition of the former reagent is advised, in order to modulate heat and gas release from the reaction vessel[198]. The route to **79** starts from ethyl glycinate hydrochloride (**76**),



SCHEME 15. Improved synthesis of ethyl 1-hydroxy-1,2,3-triazole-4-carboxylate

which is quantitatively diazotized in the presence of sodium nitrite[198, 203]. Replacement of H_2SO_4 with HCl did not improve the yield[198, 204]. The Vilsmeier reagent **79** is also used for the synthesis of nitriles, acyl and sulfonyl chlorides and is obtained by chlorination of *N*,*N*-dimethylformamide (**78**) with thionyl or oxalyl chloride[198, 205–208]. This transformation can be driven to completion and, in the absence of byproducts, by heating the mixture at 40 °C[198]. The product **80** obtained after formylation is highly unstable, and hydrolysis with 10% HOAc leads to ethyl 2-diazo-3-oxopropanoate (**82**), as reported by Stojanovic and Arnold[202]. Conversion of the latter to **75** takes place with hydroxylamine at 80 °C. Bennett and coworkers also reported the extension of the Stojanovic synthesis of **82** to **75**, although no reaction conditions were detailed[197]. The overall yield of **75** with this approach, however, was not enhanced (5%) with respect to the previous Sezer strategy. Alternatively, **80** could be transformed, dissolved in the minimum amount of H_2O, to **81** by treatment with hydroxylamine and sodium carbonate at a controlled pH = 7.5 (Scheme 15)[198]. This intermediate was quickly used, without drying, in the final cyclization step in chloroform, with a drop of acetic acid as catalyst[198]. After 2 days at room temperature and recrystallization from ethyl acetate, **75** was obtained in 37% overall yield[198]. Use of anhydrous ethanol in the synthesis of **81**, followed by reflux in benzene, resulted in enhanced yield (44%), although this approach also increases the risk of explosion of the diazo compound, due to evaporation of ethanol to almost dryness conditions and final treatment at high temperature[198].

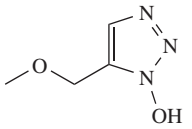
The *N*-hydroxylamine moiety of **75** shows a strong dissociation constant ($pKa = 2.16$), higher than the most acidic 1-hydroxybenzotriazole derivatives[198, 209, 210]. In agreement with these acidity measurements, the *N*-hydroxylamine proton resonates at high frequencies (12.5 ppm), according to ^{1}H-NMR spectroscopy[198]. IR spectra of **75** indicated the presence of a strong broad band at 3200–2000 cm^{-1}, corresponding to the *N*-hydroxyl group, and a characteristic ester band at 1730 cm^{-1}, apart from additional signals at 1447 and 1406[198, 201].

One of the main advantages of **75** for its use in peptide synthesis is that is doesn't present absorption at 302 nm ($\lambda_{max} = 260$ nm, $\log \varepsilon = 3.63$), which enables monitoring of the coupling process, prior to Fmoc removal[198]. EI-MS analysis has also been described[201]. **75** contains the N-N-N moiety, but although its decomposition profile has not been reported when drop-hammer assays were performed on the 1-hydroxy-1,2,3-triazole (**64**, Section III.G.1) and some 5-substituted derivatives (Section III.G)[188]. Therefore, a similar safety assessment of its parent triazole analogues is envisaged for **75**.

3. 1-Hydroxy-5-(methoxymethyl)-1,2,3-triazole

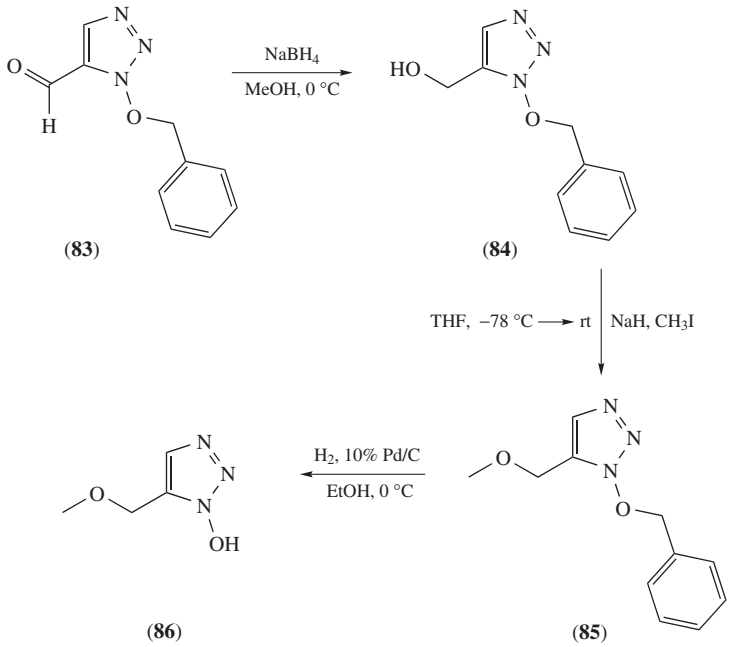
1-Hydroxy-5-(methoxymethyl)-1,2,3-triazole (**86**, Figure 21) was described, along with its 5-acetyl analogue, by Spetzler and coworkers as a solid, melting at 108–110 °C[188]. The synthetic strategy developed by these authors started from 1-benzyloxy-5-formyl-1,2,3-triazole (**83**), which was efficiently converted into the target **86** in 3 steps with high yield (82%, Scheme 16)[188]. In the first stage, 1-benzyloxy-5-(hydroxymethyl)-1,2,3-triazole (**84**) is obtained by reduction of the formyl moiety at C-5 of **83** with $NaBH_4$, and is then methylated to afford the 5-methoxymethyl derivative (**85**). Subsequent selective removal of the benzyl protecting group by Pd-mediated hydrogenolysis at low temperature yields the desired **86** (Scheme 16)[181, 188].

The starting material (**83**) for the above-mentioned strategy can be readily obtained from simpler compounds, as reported by Uhlmann and coworkers (Scheme 17)[181]. The C-5 formyl side chain is introduced into the triazole ring of 1-benzyloxy-1,2,3-triazole (**88**) by means of selective lithiation upon treatment with *n*-butyllithium at −78 °C, followed by rapid quenching with an excess of DMF as electrophile (87% yield)[181, 211].



(**86**)

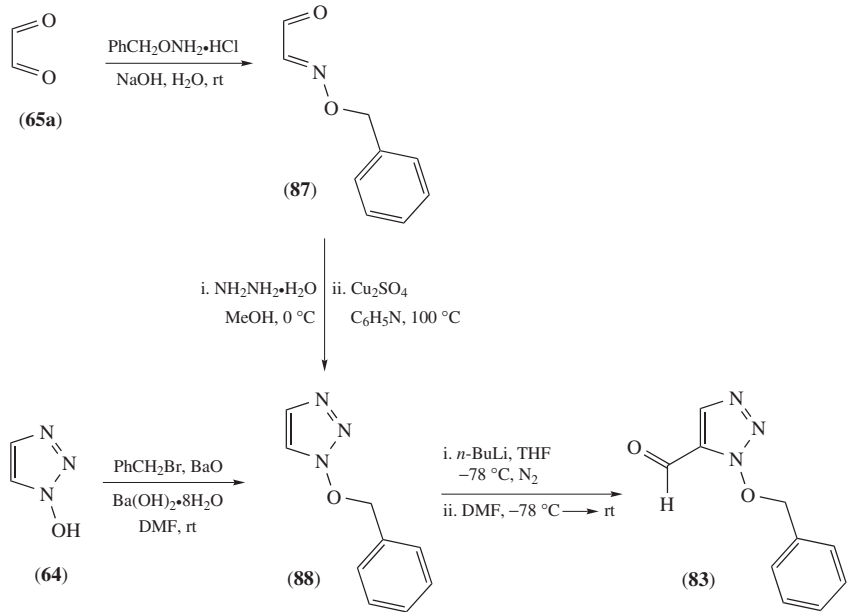
FIGURE 21. Structure of 1-hydroxy-5-(methoxymethyl)-1,2,3-triazole



(**83**) (**84**) (**86**) (**85**)

SCHEME 16. Synthetic strategy for the synthesis of 1-hydroxy-5-(methoxymethyl)-1,2,3-triazole

The *N*-benzyloxy group plays a key role in this directed *o*-metalation (DoM), since it is known to be strongly *o*-promoting, as previously observed in pyrazoles[211]. **88** can be obtained by two distinct methods. The first method, with 1-hydroxy-1,2,3-triazole (**64**, Section III.G.1), is *O*-protected under mild conditions with benzyl bromide in the presence of barium oxide and barium hydroxide octahydrate, conditions that minimize (<5%) unwanted side alkylation at *N*-3 (87% yield, Scheme 17)[176, 212]. The second method, and a more direct route, designed by Uhlmann and coworkers, successfully accomplishes the synthesis of 1-benzyloxy-1,2,3-triazole (**88**) from glyoxal (**65a**), after reaction with benzyloxyamine hydrochloride and subsequent oxidative cyclization of 2-hydrazonoglyoxal *O*-benzyloxime, formed *in situ* by the addition of hydrazine hydrate to glyoxal *O*-benzyloxime (**87**) (52–63% overall yield from **65a**, Scheme 17)[181, 184–186]. The latter method is preferred over the 1-hydroxy-1,2,3-triazole (**64**, Section III.G.1) protection approach, which is considerably more time-consuming, and moreover, its more convenient synthesis partially contains the described glyoxal **65** route (see Section III.G.1)[181].



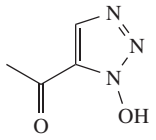
SCHEME 17. Synthetic approaches for preparation of **83**

86 presents good solubility in H_2O, in contrast to many benzotriazoles[188]. The ^{1}H-NMR spectra in D_2O clearly showed the presence of the methoxymethyl substituent at C-5 position and of H-4[188]. Bearing in mind the widely reported explosive character of *N*-hydroxybenzotriazoles, *N*-hydroxytetrazoles and *N*-alkylimidazoles and pyrazoles, a similar behavior would be expected for *N*-hydroxytriazoles due to its nitrogen-rich core[2, 181, 188, 190, 191]. Surprisingly, when subjected to drop-hammer tests, no incidents were observed for 1-hydroxy-1,2,3-triazole and some 5-substituted derivatives[188]. Therefore, a similar behavior would be envisaged in the case of **86**, based on structural similarity, although no experimental confirmation is reported.

4. 5-Acetyl-1-hydroxy-1,2,3-triazole

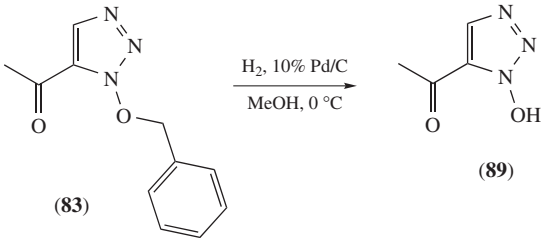
5-Acetyl-1-hydroxy-1,2,3-triazole (**89**, Figure 22) has been reported, along with other 5-substituted analogues, as a peptide additive by Spetzler and coworkers[188]. Decomposition occurs when heating **89** close to its melting point (143–145 °C), one of the highest for the triazole derivatives discussed in this section[188]. **89** can be obtained from structurally simple compounds by two distinct routes to afford **83**, as described in Scheme 18. Final hydrogenolytic debenzylation of **83** at low temperature affords the desired **89** in 93% yield[181].

After analysis by ^{1}H-NMR spectra in CD_3OD showed the presence of the acetyl moiety, the presence of H-4 was unequivocally confirmed[188]. The presence of three vicinal nitrogens in the heteroaromatic core of *N*-hydroxy-1,2,3-triazoles certainly could lead to the assumption of a similar explosive behavior to that observed for the parent *N*-hydroxybenzotriazoles, *N*-hydroxytetrazoles and *N*-alkylimidazoles and pyrazoles[2, 181, 188, 190, 191].



(**89**)

FIGURE 22. Structure of 5-acetyl-1-hydroxy-1,2,3-triazole



(**83**) (**89**)

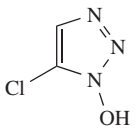
SCHEME 18. Synthetic strategy for the synthesis of 5-acetyl-1-hydroxy-1,2,3-triazole

5. 5-Chloro-1-hydroxy-1,2,3-triazole

5-Chloro-1-hydroxy-1,2,3-triazole (**91**, Figure 23) was described separately by Uhlmann and Begtrup, together with many 5-substituted triazole derivatives, as a solid presenting decomposition, after melting at 128 °C[176, 181]. Two alternative routes have been designed for the synthesis of **91** based on the use of **88** (Uhlmann and coworkers)[181] or **92** (Begtrup and coworkers)[176] (Scheme 19). Regardless of the pathway chosen, **91** is obtained in fewer steps than its analogues (**86** and **89**, see Sections III.G.3 and III.G.4)[188].

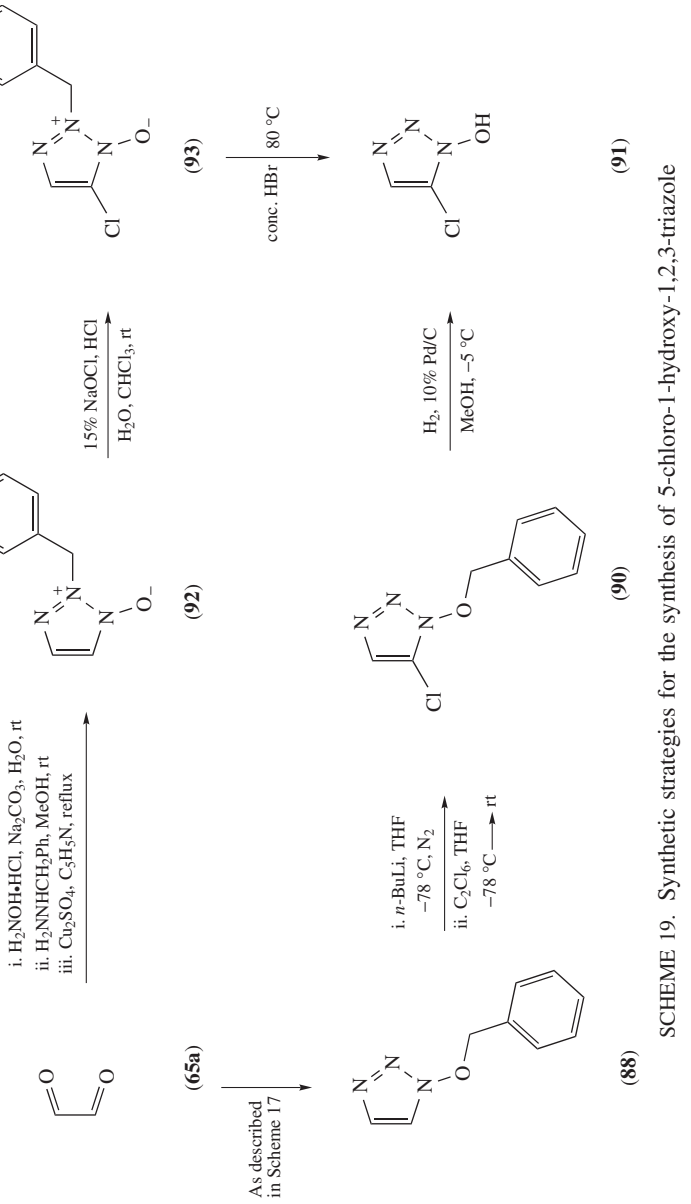
With regard to the synthesis of **88**, as mentioned for the synthesis of **83** (Section III.G.3, Scheme 17), the synthesis represents a more efficient and convenient approach, being achieved in fewer steps than the one starting from unsubstituted **64**[181]. Regioselective directed *o*-metalation (DoM) of **88** is accomplished with *n*-butyllithium at −78 °C, and upon quenching with hexachloroethane as electrophile **90** is isolated in 88% yield[181]. Subsequent benzyl removal with Pd catalysis, carried out at −5 °C in order to avoid detrimental dechlorination, affords the desired **91** in a quantitative manner (Scheme 19).

Alternative synthesis of **65a** also starts with **87**, which is converted in a one-pot fashion to 2-benzyl-1,2,3-triazole-1-oxide (**92**) via oxidative cyclization of the *in situ* generated α-oximinobenzylhydrazone, in a similar manner to the above-mentioned opposite strategy, but in lower yield (27%)[183]. Neutral conditions were found to be critical



(**91**)

FIGURE 23. Structure of 5-chloro-1-hydroxy-1,2,3-triazole



SCHEME 19. Synthetic strategies for the synthesis of 5-chloro-1-hydroxy-1,2,3-triazole

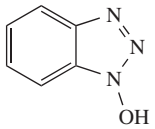
for the success of this method, since acidic or basic conditions led to side reactions. **92** was then selectively chlorinated at C-5 (even at 60 °C) by means of aqueous hypochlorite and hydrochloric acid at room temperature to render the 5-chloro derivative (**93**)[183]. Finally, debenzylation takes place with concentrated hydrobromic acid in 78% yield, affording **91** (Scheme 19)[176]. In comparison to the previously mentioned route, the target compound is obtained in considerably lower overall yield (15% vs. 46%). Moreover, the 1-(benzyloxy) methodology enables parallel synthesis of a wide variety of 5-substituted derivatives.

^{1}H-NMR spectra of **91** in $CDCl_3$ showed the proton of the hydroxyl group at δ 11 ppm, which indicates a strong acidic character[176]. Considering the presence of up to 3 vicinal nitrogens in the triazole ring, a similar decomposition behavior to *N*-hydroxybenzotriazoles, *N*-hydroxytetrazoles and *N*-alkylimidazoles and pyrazoles, which are known for its explosive nature, could be estimated[2, 181, 188, 190, 191]. However, when **91** was subjected to the drop-weight impact of a hammer-head from 50 cm distance, an explosive event did not occur, thereby indicating that this compound can be handled safely.

H. Benzotriazoles

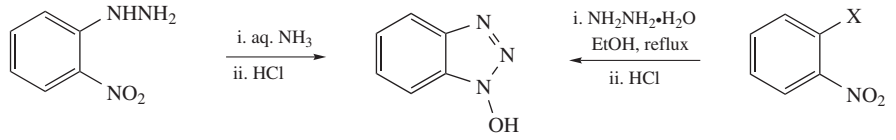
1. HOBt, 1-hydroxybenzotriazole

1-Hydroxybenzotriazole (**95**, Figure 24) is the template in which many analogues have been inspired, seeking to enhance its original properties and applications in many fields. **95** is a white crystalline powder, forming needles, whose melting point is described in the range 156–158 °C[190, 213–218]. **95** forms stable, semiconductor complexes with Cu, Co and Ni, which has been found to be linked to its anticorrosive properties when applied to metals[218]. Its synthesis was first accomplished separately by Nietzki and Zincke and their coworkers from *o*-nitrophenylhydrazine (**94**) by reaction with warm alkalis, such as aqueous ammonia (Scheme 20)[213, 214]. In those studies, **95** was named azimidolbenzene. A few years later, Brady and Reynolds obtained **95** in almost quantitative yield using the Zincke method (with 25% potassium hydroxide) from the monosulfonate sodium salt of **94**[219]. Alternatively, other routes starting from



(**95**)

FIGURE 24. Structure of 1-hydroxybenzotriazole



(**94**) (**95**)

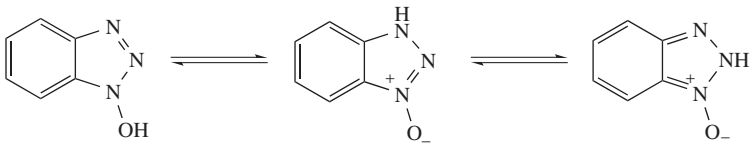
X = Cl, (**96**)
X = Br, (**97**)

SCHEME 20. Synthetic strategies for preparation of 1-hydroxybenzottriazole

o-chloronitrobenzene (**96**) or *o*-bromonitrobenzene (**97**) were developed by Müller and Brady, which under reflux with hydrazine hydrate afforded **95** in 88% and 60% yield, respectively (Scheme 20)[215, 216, 219, 220]. Interestingly, Brady and Reynolds found that when **97** was reacted with 50% hydrazine hydrate at 160 °C, **95** was not obtained but rather its reduction product (benzotriazole)[219]. In all these approaches **95** was usually obtained as the monohydrate, which is considerably unstable, losing water standing in air[219, 220].

95 is a strong acid ($pKa = 4.60$), compared with the average acidity of the majority of organic compounds[221]. The work of Pop and coworkers supported this value ($pKa = 4.64$)[100]. Koppel and coworkers also investigated the acidity of **95** at room temperature, obtaining the same value ($pKa = 4.60 \pm 0.03$), with a standard potentiometric technique[24, 200]. A slightly higher dissociation constant was reported by König and Geiger[101] ($pKa = 4.3$), in a 50:40 mixture of diethylene glycol dimethyl ether/H_2O at 54 °C, and also by Martinez and Bodanszky[221]. The acidity in DMSO, which closely resembles solvents used in standard peptide synthesis, was also determined by Koppel and coworkers ($pKa = 9.3 \pm 0.1$), by potentiometric titration of **95** with Bu_4NOH in a solvent mixture benzene/isopropanol[24]. In both solvents, **95** is more acidic than benzotriazole (by 2.6 and 3.8 pKa units in DMSO and H_2O, respectively), due to the replacement of *N*-H by *N*-OH[24]. Recently, the acidity of **95** was also determined in EtOH–H_2O and dioxane–H_2O mixtures at various temperatures (20–40 °C), observing an increase of pKa with decreasing temperature or increasing percentage of organic solvent[218]. In 100% H_2O, a similar pKa to these reported previously was obtained ($pKa = 4.53 \pm 0.01$ at 20 °C)[218]. In agreement with these dissociation constants, ^1H NMR in C_6D_6 showed a chemical shift of 13.6 ppm for the *N*-OH of **95**. The R_f in CH_2Cl_2 has been described (0.77)[218]. **95** shows an IR band at 3273 cm^{-1}, corresponding to OH stretching and also ν(C=C) = 1616.2 cm^{-1}, ν(O–N) = 1394.4–1357.8 cm^{-1} and ν(N=N) = 1222.8–1110.9 cm^{-1} [218]. This high acidity confers on **97** ideal properties for its use as additive to carbodiimides in peptide synthesis[189].

Tautomerism between *N*-OH **95** and *N*-oxide form **95′** (Figure 25) was first described by Brady and Day in 1923 after discovering that, during methylation of the benzotriazole, two isomers with distinct UV profile were formed[23]. Shortly after, Brady and Reynolds reported that experiments with methyl iodide or methyl sulfate in basic water yielded mainly the *N*-oxide **95′** derived product[219]. The same conclusion was extracted by Macbeth and Price, although in alcoholic solutions the *N*-hydroxy form **95** appeared to be the most favored one[222]. A deeper investigation on this tautomeric equilibrium was conducted by Boyle and Jones by means of basicity measurements, acidic function correlations and UV spectroscopy[215]. Mason equations suggested a mixture of the tautomers in water, although H_a correlations and UV spectra comparisons of **95** ($\lambda_{max} = 265$ nm, log $\varepsilon = 3.5$; $\lambda_{max} = 280$ nm, log $\varepsilon = 3.6$; $\lambda_{max} = 310$ nm, log $\varepsilon = 3.8$) and the methylation products clearly indicated the predominance of the *N*-oxide form **95′** (84%). In ethanolic solution, UV spectra of **95** ($\lambda_{max} = 265$ nm, log $\varepsilon = 3.6$; $\lambda_{max} = 285$ nm, log



(**95**) (**95′**) (**95″**)

FIGURE 25. Tautomeric forms of 1-hydroxybenzotriazole

$\varepsilon = 3.5$; $\lambda_{max} = 315$ nm, log $\varepsilon = 3.1$) confirmed the main presence of the *N*-hydroxy form (**95**)[215]. The same tautomer was found to be predominant in DMSO, suggested by ^{13}C-NMR spectra[223]. In DMF, **95** also showed λ_{max} at 265 and 280 nm[218]. In 1997, Horiki investigated the effect of solvent polarity on a mixture of acylated *N*-hydroxy and *N*-oxide tautomers once equilibrium was reached, observing that increasing percentages of acetone in H_2O gave rise to higher amounts of the less polar *N*-hydroxy acylated derivative[224]. In the solid state, forms **95** or **95′** were obtained as monoclinic crystals, depending on the polarity of the recrystallization solvent[192, 225]. The distinct ratio of forms **95** to **95′** based on solvent polarity has been previously observed in the parent benzimidazoles and imidazoles[226–228]. In the acyl-transfer process between both tautomers, intramolecular or intermolecular mechanisms have been proposed[195, 224, 229, 230]. Semiempirical methods have also been employed to analyze the composition of the tautomeric equilibrium[195]. The AM1 approach clearly indicates the higher stability of *N*-hydroxy form **95** over **95′**; but the controversial PMS method as well as *ab initio* calculations suggest a similar stability of both forms[195, 196]. All the methods agree that the *N*-oxide form **95″**, which has not been experimentally detected, is the least stable among the tautomers, probably due to its high energy owing to loss of the benzene ring aromaticity[195]. Carpino and coworkers achieved an easier analysis of the tautomeric equilibrium by means of ^{1}H NMR, using the integration of the methyl group of both methylation products (4.39 ppm vs. 4.11)[231].

The acidity of **95**, considering the percentages of the tautomers in water, was determined potentiometrically using the Albert method (pKa = 7.88)[232]. Later, a separate dissociation constant for each tautomer was calculated (pKa of *N*-oxide = 8.05; pKa of *N*-hydroxy = 7.39)[220]. These are considerably higher values than those obtained by titration methods[100].

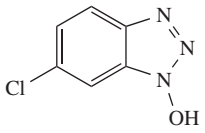
In 2005, Dunn and coworkers[191] predicted the explosive behavior of monohydrate **95**[227] by using a Yoshida plot[233], which is based on results of thermal instability. **95** was expected to be shock-sensitive, although the authors did not find experimental proof after drop-hammer tests[191]. Nevertheless, other studies found evidence of its explosivity when **95** was heated close to its melting point, which had caused some relevant incidents when handled on a large scale[191]. Actually, more than a century ago, the lead salt of **95** was already reported to explode at 270 °C, and so did other substituted derivatives[203, 234–236]. Shortly after, Dunn and coworkers investigated extensively the explosive properties of **95** and some derivatives[190]. These benzotriazoles were not only sensitive to heating in a closed confinement or under mechanical stimulus, but also were able to propagate a detonation or deflagration, regardless of the percentage of water in the sample, which acts as a desensitizer[190]. Special attention should be taken when handling **95**, dry or hydrated with <10% of water, which are considered Class 1 explosives, thereby compromising its transport[237]. Even samples containing >10% of water, placed in soft packages, are regarded as sensitized explosives, according to Division 4.1 of UN Recommendations[238]. The presence of the *N*-hydroxyl/*N*-oxide mixture might play a key role in the hazardous nature of **95**, since its parent 1*H*-benzotriazole does not display explosive properties, although it must be handled with precaution due to the high exothermic decomposition energy that is released above 100 °C[239]. Lately, an assessment of the thermal safety of **95** monohydrate (12.8% of water) has been conducted calorimetrically by dynamic DSC and ARC assays[2]. The combination of both techniques shows a decomposition behavior that matches the profile of an explosive compound: fast degradation with high released pressure (178 bars)[27]. The onset of decomposition, accurately set at 145 °C after the ARC assay, took place before melting (mp = 157 °C). As a result, it is recommended to keep the temperature at values $T < 95$ °C, in order to work safely[28, 29].

2. 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole

6-Chloro-1-hydroxybenzotriazole (**99**, Figure 26) is obtained as a colorless crystalline powder, which decomposes after melting at a higher temperature than the unsubstituted 1HOBt (mp = 189–192 °C, 195 °C or 198 °C have been reported)[190, 240, 241]. Methods for obtaining **99** were developed by Booy and Dienske in 1926[240] and by Joshi and Derhoa in 1952[241] (Scheme 21). These reactions started by refluxing 2,5-dichloronitrobenzene (**98**) in the presence of hydrazine hydrate to afford the desired benzotriazole in rather low yields. An optimization of the process (90.1% yield) was accomplished by Hagedorn and coworkers in 1997, obtaining the sodium salt of **99** from **98** after cyclization of the hydrazino derivative in the presence of sodium hydroxide, as an intermediate for the synthesis of HOBt after dechlorination[242].

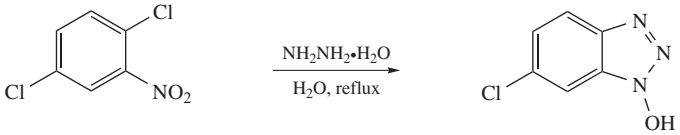
98 can be prepared from *p*-dichlorobenzene (**101**) by nitration with HNO_3 and H_2SO_4 in near-quantitative yields, although this transformation can take place in milder conditions in the presence of sieve catalysts mixed with MgO in good yields and 99–100% regioselectivity (Scheme 22)[243, 244]. The use of Sb-based catalysts in aprotic non-polar solvents also represents an efficient strategy for the nitration of **101**[245]. Several catalysts have been proposed for the *p*-chlorination of chlorobenzene (**100**), such as ferric chloride, kaolinitic clay or the mixture hydrogen peroxide-2,2,2-trifluoroethanol[246, 247]. Another approach to the synthesis of **98** consists in the chlorination of *o*-chloronitrobenzene (**102**), an intermediate for obtaining **95** (Section III.H.1), with chlorine and ferric chloride (Scheme 22)[248].

The acidity of **99** is one of the highest found among substituted 1-hydroxybenzotriazoles ($pK_a = 3.35$), along with that of the 6-nitro derivative (6-NO_2-HOBt, **112**, Section III.H.4)[24, 249]. Koppel and coworkers reported a slightly higher value ($pK_a = 4.15 \pm 0.03$) using the Albert potentiometrical technique[24, 232]. The same authors also determined, by means of potentiometric titration with Bu_4NOH in a mixture benzene/isopropanol, the acidity of **99** in DMSO ($pK_a = 8.6 \pm 0.1$), a solvent that closely resembles the conditions in which peptide synthesis is conducted[24]. Verma and Parmar characterized **99** by infrared spectroscopy in KBr; it is observed as a band for the acidic *N*-OH vibration at 1210 cm^{-1} and, as expected, with no sign of typical C=N absorption at 1650–1700 cm^{-1} [ν(N=N) = 1625 cm^{-1}, ν(O–N) = 1210 cm^{-1}, ν(C–Cl) = 670 cm^{-1}][210]. Vibrational frequencies of the ring system are observed in the range 910–1510 cm^{-1}. Surprisingly, the *N*-OH



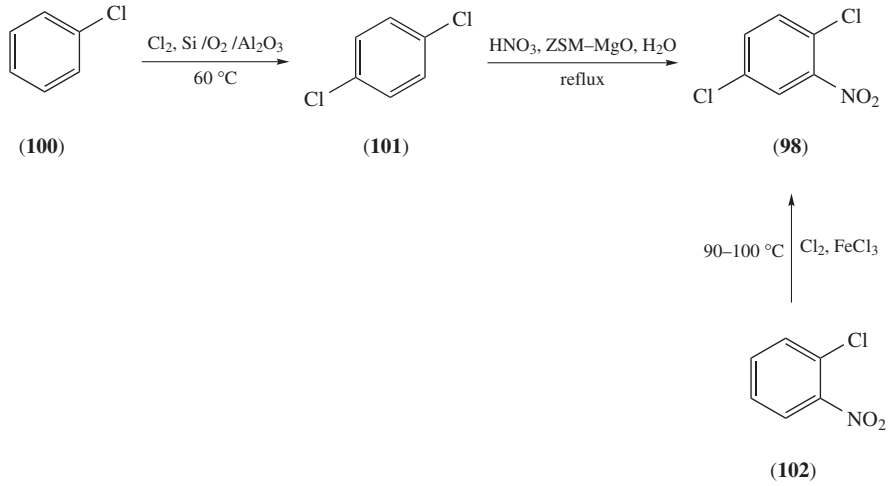
(**99**)

FIGURE 26. Structure of 6-chloro-1-hydroxybenzotriazole



(**98**) (**99**)

SCHEME 21. Synthetic strategy for preparation of 6-chloro-1-hydroxybenzotriazole



SCHEME 22. Synthesis of the intermediate **98**

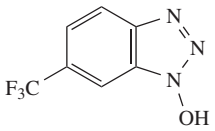
stretching band and C=C bands are not detailed, contrary to the IR spectra of **95** (Section III.G.1)[210, 218]. With regard to N=N absorption, it is observed around 1600 cm^{-1}, a considerably higher frequency than the one reported for **95**[210, 218].

99 has been reported to be safer than **95**[210, 218, 233, 250]. Dry **101** is regarded as a Class 1 explosive, compromising its transport in a similar way to **95**[237].

3. 6-CF_3-HOBt, 1-hydroxy-6-trifluoromethylbenzotriazole

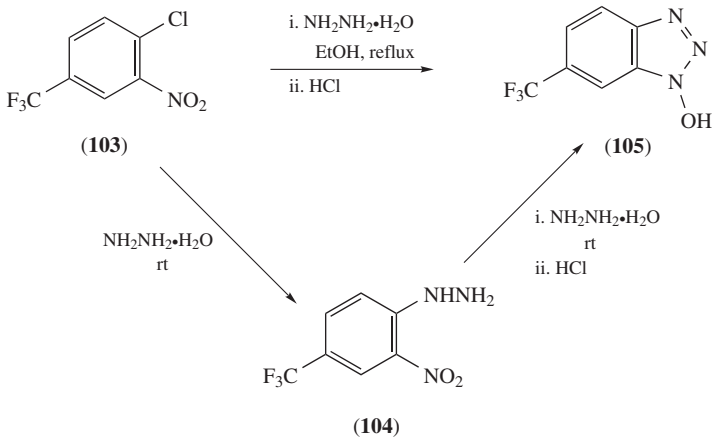
1-Hydroxy-6-trifluoromethylbenzotriazole (6-CF_3-HOBt, **105**, Figure 27) is a crystalline solid, with a lower melting point than its parent unsubstituted HOBt (**95**) (described in the range 143–149 °C)[189, 250–252]. Inspired by the method developed by Müller and Zimmermann in 1925,[216] König and Geiger[189] described the formation of **105** from 3-nitro-4-chlorotrifluoromethylbenzene (**103**) by the action of hydrazine hydrate in refluxing ethanol (Scheme 23)[250]. Later, Takeda and coworkers revised the former method and enhanced the yield (96%) by refluxing with 99% ethanol during 24 hours[251]. Alternatively, **105** can be obtained from **104** by treatment with hydrazine hydrate under mild conditions (Scheme 23)[149].

The starting material for the above-mentioned synthetic strategy, i.e. **103**, is obtained after nitration of *p*-chlorotrifluoromethylbenzene (**108**) (Scheme 24). This nitration can be achieved regioselectively in the absence of solvent with concentrated nitric acid (95%



(**105**)

FIGURE 27. Structure of 1-hydroxy-6-trifluoromethylbenzotriazole



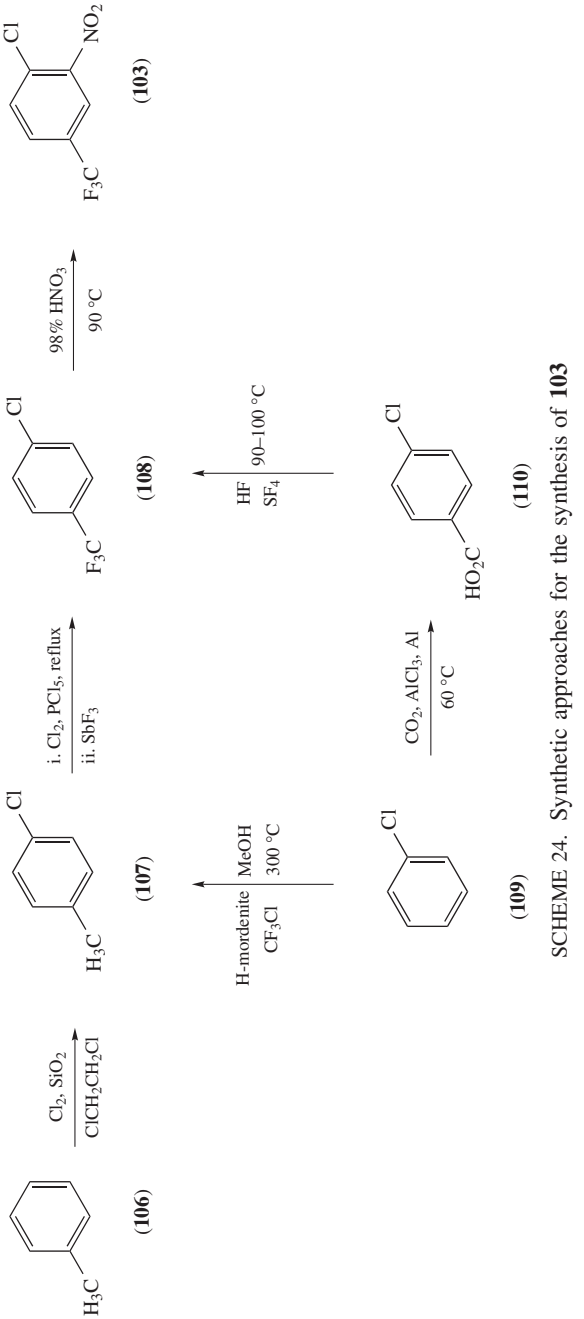
SCHEME 23. Synthetic approaches for the synthesis of 1-hydroxy-6-trifluoromethylbenzotriazole

yield), although classical nitration in the presence of H_2SO_4 was also described[149, 253]. **108** can be efficiently synthesized from *p*-chlorotoluene (**107**) or *p*-chlorobenzoic acid (**110**). The latter **110**, obtained in high regioselectivity by carboxylation of chlorobenzene (**109**) with aluminum trichloride in reasonable yields, affords **108** by fluorination in the presence of sulfur tetrafluoride at high temperature (70% yield) (Scheme 24)[253–255]. The former is transformed into **109** in two steps, after chlorination with phosphorus pentachloride catalysis and subsequent fluorination with tetrafluoroantimonate or hydrogen fluoride (88% yield) (Scheme 24)[256]. **107** is synthesized either from chlorobenzene (**109**), by treatment at high temperature with methanol and H-mordenite as catalyst (in low regioselectivity), or from toluene (**106**) in regioselectivity close to 90% using zeolites as catalysts (best yields using K-L type ones) (Scheme 24)[257, 258]. Interestingly, some methodologies have been developed to obtain **108** directly from **109** by trifluoromethylation, using distinct catalysts, such as trifluoromethyl iodide, bis (trifluoromethyl) telluride or bis (trifluoroacetyl) peroxide[259–261].

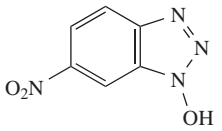
The inclusion of the trifluoromethyl group confers on **105** a higher acidity ($pKa = 3.80 \pm 0.03$) than that of the parent **95**, as determined by Koppel and coworkers by means of a standard potentiometric technique at room temperature[24, 232]. The acidity was also measured in DMSO ($pKa = 7.4 \pm 0.1$) by potentiometric titration of **105** following the tendency observed in H_2O relative to **95**[24]. Regarding IR spectra of **105** in KBr, a strong band is observed at $1630\,cm^{-1}$, corresponding to the aromatic benzotriazole core[251]. ^{1}H-NMR spectra in acetone-d_6 showed the aromatic hydrogens and the highly acidic *N*-hydroxylamino hydrogen at δ 7.50 and 10.94[251].

4. 6-NO_2-HOBt, 1-hydroxy-6-nitrobenzotriazole

1-Hydroxy-6-nitrobenzotriazole (6-NO_2-HOBt, **112**, Figure 28) is a dark yellow solid, arranged in large prisms or leaflets, and presents solubility in hot H_2O and EtOH[215, 262]. **112** has been described to melt at 190–192 °C, with decomposition, or at 200–204 °C[261, 262]. Other authors claim that no melting point is observed, the heating causing a violent detonation or deflagration at 206 °C[23, 242, 263]. A synthetic route to **112** was achieved separately by Brady, Curtius and their coworkers almost a century ago (Scheme 25)[23, 262]. Brady and Day

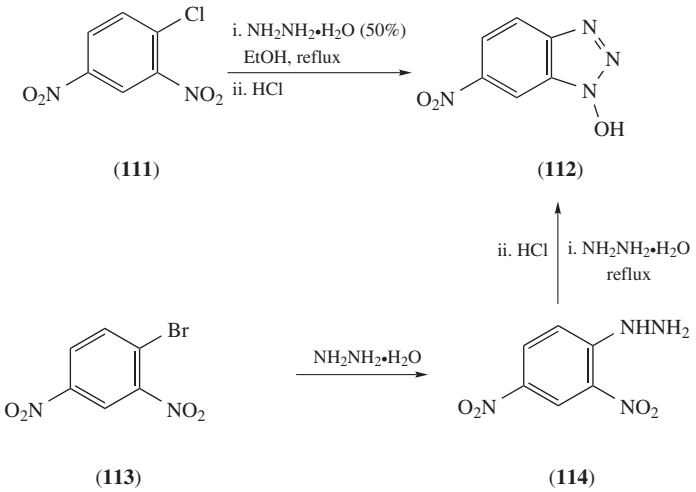


SCHEME 24. Synthetic approaches for the synthesis of **103**



(**112**)

FIGURE 28. Structure of 1-hydroxy-6-nitrobenzotriazole



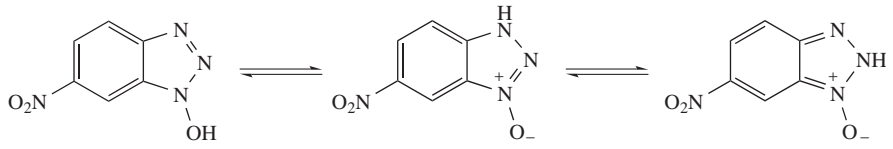
(**111**) (**112**) (**113**) (**114**)

SCHEME 25. Synthetic approaches for the synthesis of 1-hydroxy-6-nitrobenzotriazole

successfully obtained **112** in a few hours, after refluxing 2,4-dinitrochlorobenzene (**111**) with 50% hydrazine hydrate in alcohol with subsequent acidification, in 50–60% yield (Scheme 25)[23]. Earlier, Curtius and coworkers similarly used pure or aqueous hydrazine to afford **112** in 80% yield from 2,4-dinitrophenylhydrazine (**114**), which can be obtained from 2,4-dinitrobromobenzene (**113**) with hydrazine hydrate (Scheme 25)[262, 264]. Several bases have been tried as alkali catalysis for the transformation of **114** into **112**, such as potassium, barium and sodium hydroxide or aqueous ammonia, the yield strongly depending on the concentration of base[214, 215, 265, 266]. The preparation of **114** from **113** has also been reported.

The presence of the nitro group has a great impact on the acidity of **112** ($pKa = 2.75$), a similar effect to that introduced by Cl (see **98**, Section III.G.2)[24, 215]. The IR spectra of **112** in KBr has been reported by Verma and Parmar [ν(N=N) = 1610 cm^{-1}, ν(O−N) = 1210 cm^{-1}, ν(C−NO$_2$) = 1510 cm^{-1}][210]. Similarly to **98** (Section III.G.2), a band corresponding to the acidic N−OH vibration was observed at 1210 cm^{-1} and, as could be expected, no sign of typical C=N absorption at 1650–1700 cm^{-1} was detected[210]. Typical vibrational frequencies of the aromatic ring system are observed in the range 850–1490 cm^{-1} and a strong *C*-NO$_2$ band is located in the range 1510–1525 cm^{-1} [210], unlike the reported IR spectra of **95**[218].

Tautomerism between *N*-hydroxy and *N*-oxide species (**112**, **112′**, **112″**) and other substituted benzotriazoles (Figure 29) was first envisaged by Brady and Day in 1923, after



(**112**) (**112′**) (**112″**)

FIGURE 29. Tautomeric forms of 1-hydroxy-6-nitrobenzotriazole

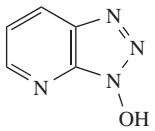
observing two isomeric methylation products[23]. However, this study was not conclusive concerning the predominant form **112**, due to the unavailability of a suitable method to separate the different tautomers[23].

In 1936, Macbeth and Price reported the tautomeric equilibrium by means of UV spectroscopy[222], revealing that 6-NO_2-HOBt was mainly in the *N*-hydroxy form **112** in EtOH and H_2O, although in H_2O solvent it was presented as the anion due to its high dissociation constant. Many decades later, Boyle and Jones[215] re-evaluated this equilibrium, based not only on UV specroscopy, but also on basicity parameters and acidic function correlations. Although tautomeric predictions using Mason equations suggested that *N*-hydroxy species **112** were the most stable in H_2O, the correlation of H_o and H_a values indicated the contrary. The UV spectra supported the hypothesis of predominance of the *N*-oxide form **112′** (75%) in H_2O ($\lambda_{max} = 260$ nm, log $\varepsilon = 4.2$; $\lambda_{max} = 335$ nm, log $\varepsilon = 3.7$) at strongly acidic pH and confirmed the predominance of the *N*-hydroxy form **112** (85%) in EtOH ($\lambda_{max} = 250$ nm, log $\varepsilon = 4.0$; $\lambda_{max} = 280$ nm, log $\varepsilon = 3.9$; $\lambda_{max} = 320$ nm, log $\varepsilon = 3.4$). The dependence of the *N*-hydroxy/*N*-oxide ratio on the solvent polarity is consistent with the analogous reports in benzimidazoles and imidazoles[226–228]. The least stable 2*H*-tautomer (**112′**) has not been observed.

I. Azabenzotriazoles

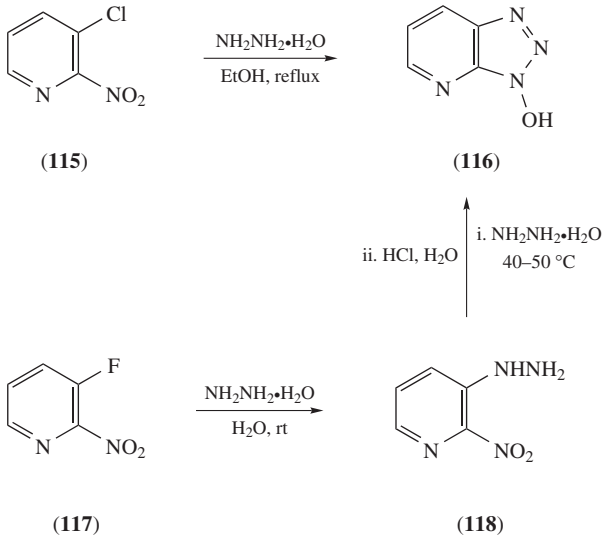
1. 7-HOAt, 7-aza-1-hydroxybenzotriazole

7-Aza-1-hydroxybenzotriazole (7-HOAt, **116**, Figure 30), also named 3-hydroxytriazolo[4,5-*b*]pyridine, is a pale yellow (nearly colorless) crystalline solid, which melts at 215–217 °C with decomposition[252, 257, 258]. Its synthesis was first described in 1973 by Sacher and coworkers from 3-chloro-2-nitropyridine (**115**) adapting the Müller methodology[216] for benzotriazoles (Scheme 26)[252]. Shortly after this study, Mokrushina and coworkers reported an alternative route to **116** from 3-fluoro-2-nitropyridine (**117**, Scheme 26)[267, 268]. Its enhanced reactivity toward aromatic nucleophilic substitution with respect to **117** precludes the need for strong reaction conditions, which in the case of **115** are known to cause unwanted substitution in the 2-nitro position[229]. The



(**116**)

FIGURE 30. Structure of 7-aza-1-hydroxybenzotriazole



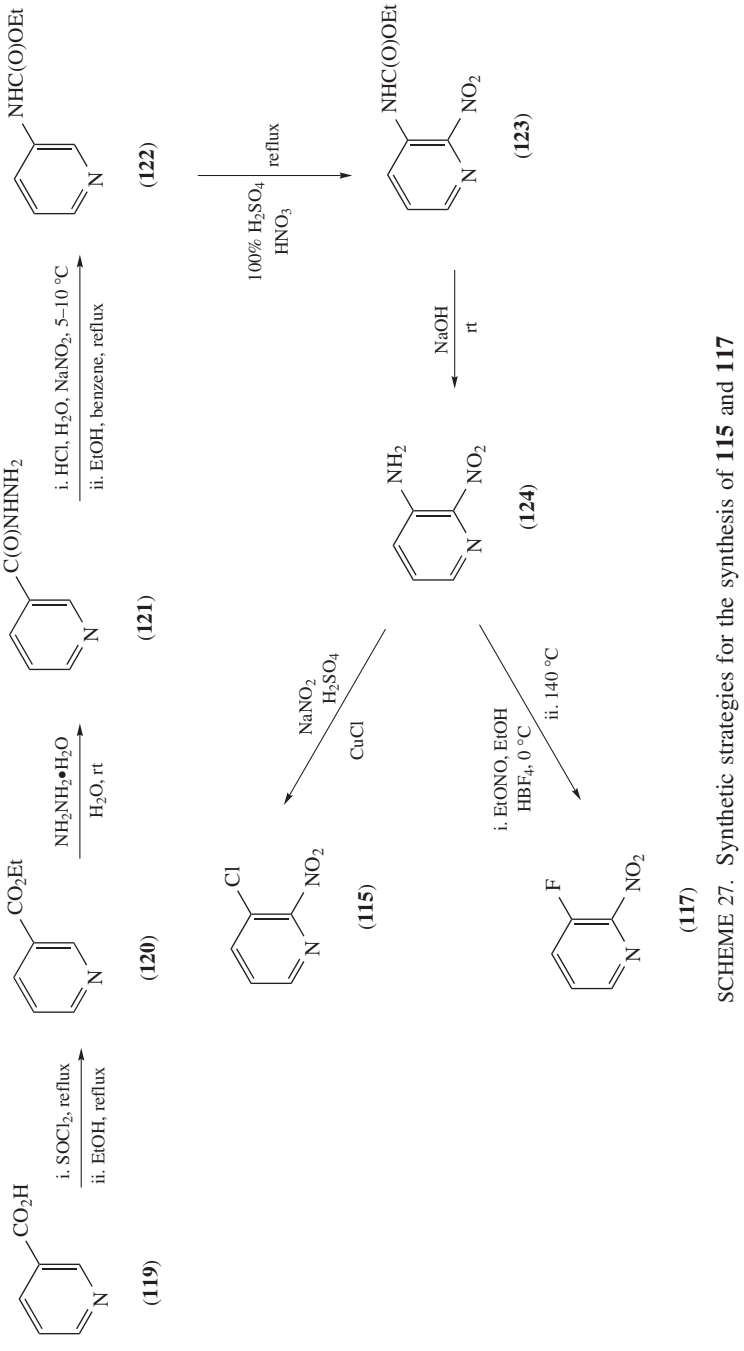
SCHEME 26. Synthetic approaches for the synthesis of 7-aza-1-hydroxybenzotriazole

authors found that in the presence of hydrazine hydrate, **117** is transformed into 3-hydrazino-2-nitropyridine (**118**) or the hydrazinium salt of **116**, depending on the conditions. Thus, at 20 °C mainly **118** is obtained, although the cyclized product is also detected. Reaction of **118** with a large excess of hydrazine hydrate (or other bases like morpholine or sodium carbonate) or at 40–50 °C affords, after acidification of the hydrazinium salt, **116** in 70% yield.

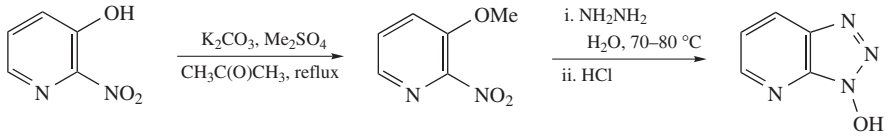
Both 3-halo-2-nitropyridines are easily obtained from the 3-amino derivative **124** (Scheme 27). The 3-chloro analogue (**115**) is formed via the Sandmeyer reaction in sulfuric acid (65% yield), whereas the Schieman method adapted to heterocycles, forming the diazonium tetrafluoroborate in the presence of fluoroboric acid with subsequent decomposition, afforded **117** in 25% yield[269, 270]. Starting from nicotinic acid (**119**), Clark-Lewis reported facile and high yield synthesis of **122** (Scheme 27)[271]. The first step consists of formation of ethyl nicotinate (**120**), after refluxing the *in situ*-formed acyl chloride in ethanol, which is then converted into nicotinhydrazide (**121**) with hydrazine hydrate[272, 273]. Then, **121** is transformed into 3-ethoxycarbonylaminopyridine (**122**) through intermediate formation of the acyl azide in an acidic aqueous solution of sodium nitrate[274]. Optimization of the Curry and Mason nitration method by refluxing **122** in 100% H_2SO_4 and HNO_3 gave **123**, and subsequent removal of the carbamate after a 2-day treatment with sodium hydroxide afforded **124** in 35% yield[275].

The above-mentioned routes were shortened by Carpino few decades later, accomplishing an efficient synthesis of **116** in 2 steps from the commercially available 3-hydroxy-2-nitropyridine (**125**, Scheme 28)[276]. In the first step, **125** is methylated with dimethyl sulfate in the presence of base, generating 3-methoxy-2-nitropyridine (**126**) in 95% yield. This electron-deficient pyridine (**126**) is heated with an excess of hydrazine hydrate for 24 h affording the desired **116** in 47% yields.

The vicinity of the nitrogen at position 7 to the *N*-hydroxylamino moiety confers on **116** one of the highest acidities found in benzotriazoles and their derivatives (pKa = 3.28), one pKa unit more acidic than HOBt (**95**, Section III.G.1), yielding a yellow



SCHEME 27. Synthetic strategies for the synthesis of **115** and **117**



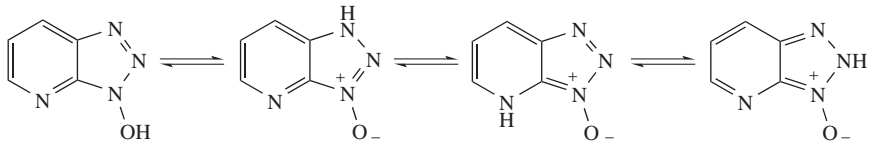
(**125**) (**126**) (**116**)

SCHEME 28. Carpino's approach for the synthesis of 7-aza-1-hydroxybenzotriazole

colored anion[267, 276]. A similar dissociation constant in H_2O was reported by Koppel and coworkers[24] using the Albert potentiometric method[232]. The acidity in DMSO was also determined ($pKa = 3.47 \pm 0.03$), which is more relevant than H_2O to the solvents regularly used in peptide synthesis, by potentiometric titration[267]. Aromatic protons at δ 7.35–8.66 in $CDCl_3$–DMSO-d_6 are identified by ^{1}H-NMR spectroscopy[276].

As found for other benzotriazoles, tautomerism between *N*-hydroxy and *N*-oxide forms of 7-HOAt (**116**, **116′**, **116″**, **116‴**) is envisaged, only that in this particular compound the 7-nitrogen permits the presence of two additional *N*-oxide species (Figure 31). The equilibrium in alcohol solution was studied by comparing the UV spectra of **116** ($\lambda_{max} =$ 260 nm, log $\varepsilon = 2.82$; $\lambda_{max} = 295$ nm, log $\varepsilon = 2.89$) with its methylated product and that of HOBt, which was previously reported, suggesting that in this media the *N*-hydroxy form was predominant[262, 267]. Mass spectrometric analysis[267] of the fragmentations of **116** led to the same conclusion. Carpino and coworkers investigated this equilibrium based on the ^{1}H-NMR chemical shift of the methyl group after methylation ($\delta = 4.49$), also indicating the predominance of the *N*-hydroxy specie (**118**)[231]. The intensity of the electronic absorption spectra in water ($\lambda_{max} = 220$ nm, log $\varepsilon = 4.10$; $\lambda_{max} = 280$ nm, log $\varepsilon = 3.78$; $\lambda_{max} = 325$ nm, log $\varepsilon = 3.40$) depends on the pH, showing an isosbestic point at 300 nm[268]. The presence of a maximum near 330 nm indicates a strong contribution of the anion, further supporting the high acidity attributed to **116**.

The triazole ring of the aromatic core is responsible for the explosive character of *N*-hydroxybenzotriazoles, and **116** is not an exception[190]. Some reports state that the high shock sensitivity of **116** is even stronger than that of the parent HOBt (**95**)[191]. Dunn and coworkers[191] found that this behavior resembles the one predicted with the Yoshida plot[233], based on thermal instability experiments. Its sensitivity to high temperatures when placed in a closed confinement, as extracted from the parameters of the Koenen test, is high enough to be labeled as Class 1 explosive substance, according to U.S. Department of Transportation and U.N. regulations[190, 237]. Recently, a thermal evaluation of **116** using two different calorimetry techniques (dynamic DSC and ARC) provided more details on its handling safety[2]. After careful analysis of the results obtained in both assays, it can be concluded that **116** behaves as an explosive compound, due to the extremely quick degradation associated with high pressure (167 bars)[27]. ARC assay also enabled a



(**116**) (**116′**) (**116″**) (**116‴**)

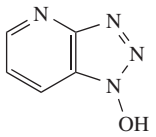
FIGURE 31. Tautomeric forms of 7-aza-1-hydroxybenzotriazole

precise determination of the onset of decomposition (178 °C), which is connected with the maximum temperature ($T = 128$ °C) at which working conditions can be certainly considered safe[28, 29].

2. 4-HOAt, 4-aza-1-hydroxybenzotriazole

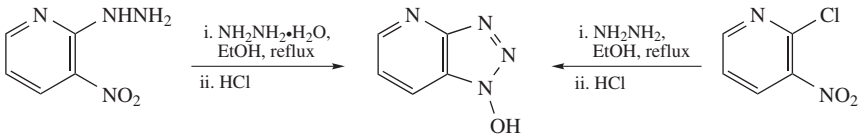
4-Aza-1-hydroxybenzotriazole (4-HOAt, **129**, Figure 32) is a yellow crystalline solid, which melts at 203–211 °C (after recrystallization) according to Carpino, although it has also been reported to melt at 220–222 °C and 209–210 °C[267, 168]. **129** can be obtained from 2-hydrazino-3-nitropyridine (**128**) or 2-chloro-3-nitropyridine (**127**) (Scheme 29) by refluxing with an alcoholic solution of hydrazine or its hydrate, followed by acidification (90% from **128**, Scheme 29), as described by Azev and coworkers[267, 268]. However, synthetic procedure from **127** requires a large excess of hydrazine in order to afford **129**.

Two decades later, Carpino accomplished the synthesis of **129** from **127** in two steps, using anhydrous ethanol as solvent (Scheme 30)[276]. Addition of hydrazine to **127** at room temperature caused in a short time the precipitation of the 2-hydrazino analogue (**128**) as a straw-yellow solid, a reaction already reported in 81% yield by Lewis and Robert in 1971[277]. Once **128** is isolated, this intermediate is readily transformed into the desired **129** upon reflux with 95% hydrazine and subsequent acidification, in 31% overall yield after recrystallization.



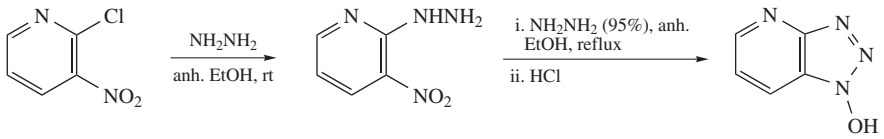
(**129**)

FIGURE 32. Structure of 4-aza-1-hydroxybenzotriazole



(**128**) (**129**) (**127**)

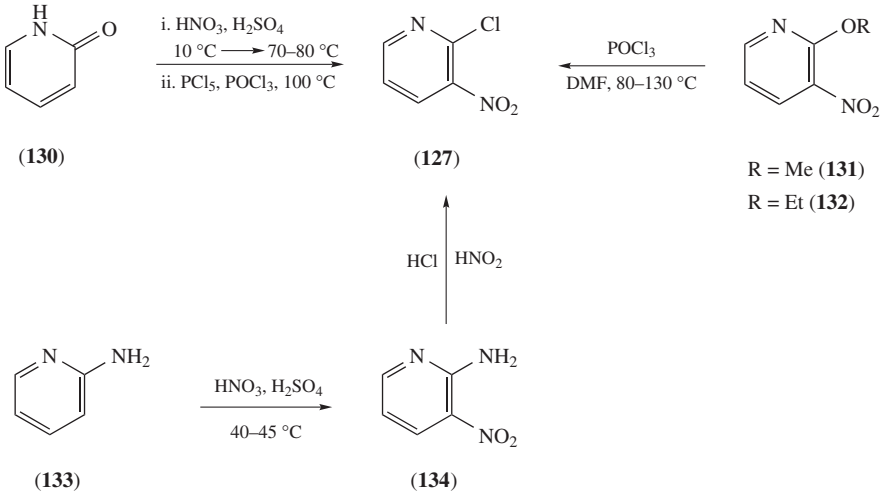
SCHEME 29. Synthetic approaches to 4-aza-1-hydroxybenzotriazole



(**127**) (**128**) (**129**)

SCHEME 30. Carpino's strategy for preparation of 4-aza-1-hydroxybenzotriazole

Few synthetic routes have been reported toward **127** (Scheme 31), the critical intermediate for obtaining **129**. The strategy proposed by Zakhs and coworkers[278] starts from pyridin-2(*1H*)-one (**130**), which is selectively nitrated by means of concentrated nitric and sulfuric acid in an ice–salt mixture, followed by 1-hour heating (14% yield) (Scheme 31)[278–280]. An alternative route has been described by Lai and coworkers from some 2-alkoxy-3-nitropyridine derivatives (**131**, **132**), after treatment under Vilsmeyer–Haack conditions (Scheme 31)[281]. High yields are obtained with this procedure from either the 2-methoxy (**131**, 90%) or 2-ethoxy (**132**, 87%) analogues. Finally, diazotization of 2-amino-3-nitropyridine (**134**) also leads to **127** (Scheme 31), as reported by Chichibabin and Builinkin[59]. Nonetheless, nitration of 2-aminopyridine (**133**) to yield **134** is not completely selective, as various percentages of the 5-nitro analogue are described[59, 282]. Selectivity issues also occur during introduction of chlorine to 3-nitropyridine *N*-oxide, a less efficient approach to afford **127**.



SCHEME 31. Synthetic pathways for the synthesis of **127**

The ionization constant of **129** is slightly higher than that of the 7-isomer ($pKa = 3.02$) and is one of the highest found in *N*-hydroxybenzotriazoles[267]. Koppel and coworkers[24] also found a high acidity ($pKa = 3.14 \pm 0.03$) when using a standard potentiometric methodology at room temperature[232]. The same authors described the dissociation constant in DMSO ($pKa = 8.1 \pm 0.1$), a solvent that resembles more closely than H_2O the solvents used in standard peptide synthesis[24]. As observed with **116** (Section III.I.1), in a crystalline sample the intensity of the IR band from 2200 to 2700 cm^{-1} decreases when the *N*-hydroxy hydrogen is replaced by deuterium, which indicates a strong contribution of the polar form of **129**[267].

Tautomeric equilibrium between the *N*-hydroxy form **129** and the three *N*-oxide species (**129′**, **129″**, and **129‴**) may take place with 4-HOAt (Figure 33). Azev and coworkers studied the tautomery in alcohol solutions by analyzing the UV spectra of **129** ($\lambda_{max} = 280$ nm, $\log \varepsilon = 2.94$) and that of the methylation product[267]. Neither of these resembled the UV spectra of *O*- and *N*-methylated HOBt (**95**, Section III.H.1), suggesting that 4-HOAt exists predominantly in alcoholic solution as one of the *N*-oxide species involving

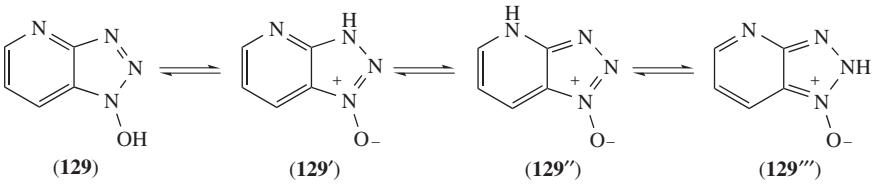


FIGURE 33. Tautomeric forms of 4-aza-1-hydroxybenzotriazole

participation of the pyridine ring due to its basic character as shown in forms **129′** and **129″**[222]. This was identified as **129′**, after analysis of the degradation fragments from mass spectroscopy. Consequently, 4-HOAt is also named triazolo[4,5-*b*]pyridine 1-oxide. In H_2O ($\lambda_{max} = 220$ nm, log $\varepsilon = 4.22$; $\lambda_{max} = 280$ nm, log $\varepsilon = 3.87$; $\lambda_{max} = 324$ nm, log $\varepsilon = 3.50$), different electronic curves are obtained depending on the pH, with an isosbestic point at 300 nm[268]. The spectra also show a maximum at 330 nm, indicating the high ionization constant of 4-HOAt.

Nevertheless, some controversy exists about the predominance of the *N*-oxide form in solution[231]. Carpino and coworkers claimed that the melting point of the methylation product of 4-HOAt does not coincide with the one reported by Azev and coworkers. Moreover, ^{1}H-NMR and X-rays analysis are consistent with the *N*-hydroxy species (**129**).

3. 5-HOAt, 5-aza-1-hydroxybenzotriazole

The synthesis of 5-aza-1-hydroxybenzotriazole (5-HOAt, **140**, Figure 34) has been reported by Carpino and coworkers, along with that of the 6-isomer[231]. The synthetic route starts from 3-fluoro-4-nitropyridine *N*-oxide (**135**), a highly electron-deficient heterocycle which can react with a wide variety of sulfur-, oxygen- or nitrogen-based nucleophiles, by displacing the 3-fluorine (Scheme 32)[283]. When mixing **135** with hydrazine hydrate, the 3-hydrazino-4-nitropyridine intermediate (**136**) is quickly formed, a reaction that was already described by Talik and Talik in 1966[283]. Cyclization to afford the benzotriazole core was accomplished upon refluxing **136** with excess hydrazine hydrate, followed by acidification. The *N*-oxide benzotriazolic intermediate **137** was transformed into **140** via **138** and **139**, after methylation of the 1-hydroxy group, 5-deoxygenation and demethylation (Scheme 32) for obtaining **140**.

The starting material of Carpino's strategy toward 5-HOAt, **135** can be obtained from 3-aminopyridine (**141**) in 35% yield, as described by Talik and Talik (Scheme 33)[284]. The fluorine at 3-position is introduced into the aromatic ring within a few hours upon reaction with the intermediate diazonium salt. The *N*-oxide is formed from 3-fluoropyridine (**142**) after extensive treatment with AcOH and H_2O_2 and this is then selectively converted into the desired 4-nitro analogue (**135**) (Scheme 33).

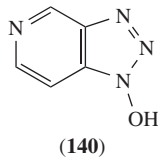
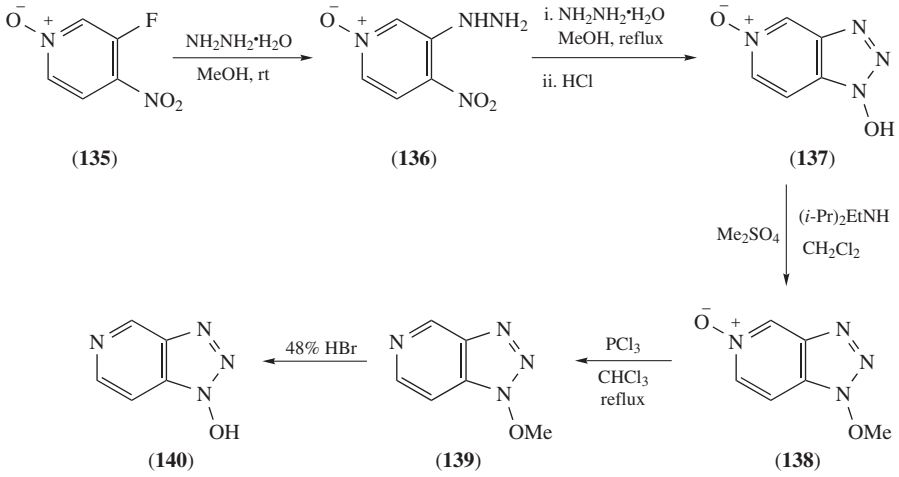
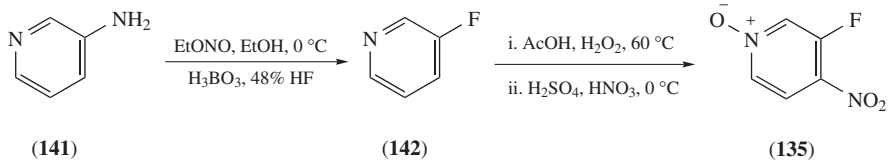


FIGURE 34. Structure of 5-aza-1-hydroxybenzotriazole



(**135**) (**136**) (**137**) (**138**) (**139**) (**140**)

SCHEME 32. Synthetic strategies for 5-aza-1-hydroxybenzotriazole

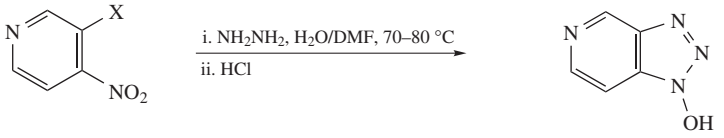


(**141**) (**142**) (**135**)

SCHEME 33. Synthetic strategies for the synthesis of **135**

Carpino proposed an alternative strategy for obtaining **140** with no need for the *N*-oxide pyridine, by extensively treating 3-methoxy-4-nitropyridine (**143**) or a range of 3-halo-4-nitropyridines (**144a–c**) with an excess of hydrazine hydrate, followed by acidification (Scheme 34)[231].

Unfortunately, an ionization constant for **140** has not yet been established, due to its instability under the conditions in which the Isaacs method is applied[285]. Nonetheless,



X = OMe (**143**)
X = F (1**44a**)
X = Cl (**144b**)
X = Br (**144c**)

(**140**)

SCHEME 34. Carpino's alternative strategy for 5-aza-1-hydroxybenzotriazole

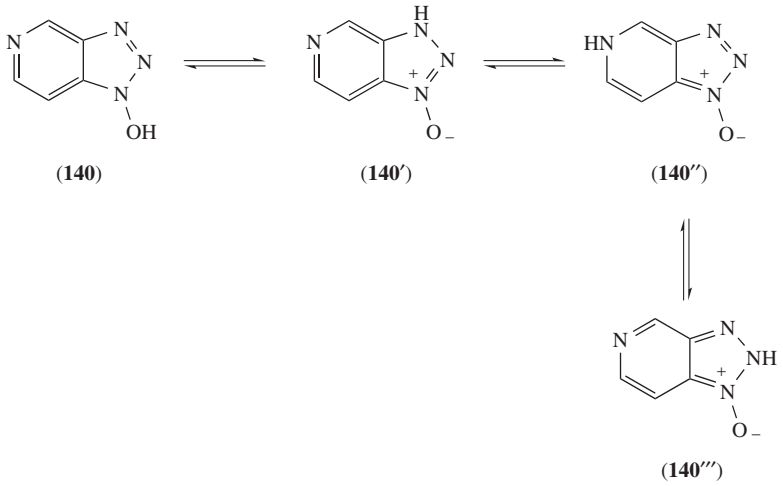


FIGURE 35. Tautomeric forms of 5-aza-1-hydroxybenzotriazole

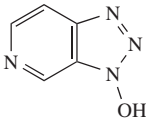
bearing in mind the relative reactivity as peptide additives for the 4-, 5-, 6- and 7-isomers (which is linked to their quality as leaving groups and so, to a great extent, with their acidity), an intermediate pKa between the 4- and 7-analogues can be estimated[231].

As for other benzotriazoles, tautomeric equilibrium between N-hydroxy (**140**) and N-species (**140′**, **140″**, **140‴**) has been studied (Figure 35)[231]. ^{1}H-NMR analysis of the methylation product in alcoholic solution showed a chemical shift for the methyl group ($\delta = 4.43$ ppm) that corresponds to its N-hydroxy form (**140**), indicating its predominance in this solvent[231].

4. 6-HOAt, 6-aza-1-hydroxybenzotriazole

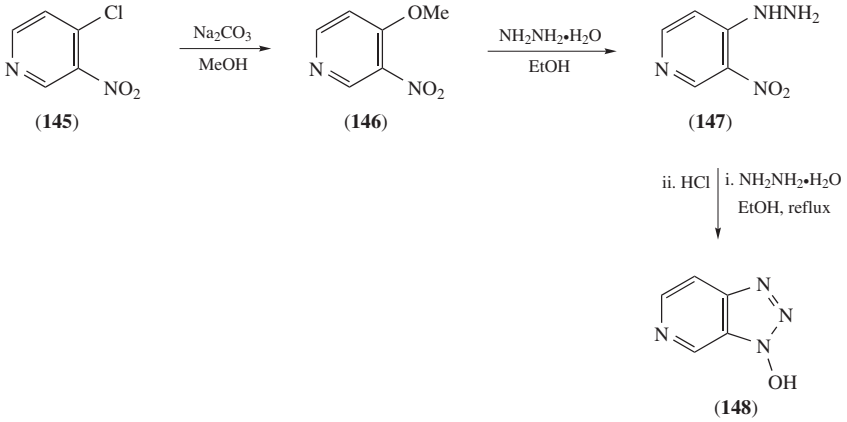
6-Aza-1-hydroxybenzotriazole (6-HOAt, **148**, Figure 36) is one of the four isomers of HOAt which find application as additives to carbodiimides in peptide synthesis. It was obtained in a few steps from 4-chloro-3-nitropyridine (**145**) by Carpino and coworkers (Scheme 35)[231]. **145** presents a marked activation toward nucleophilic aromatic substitution, which turns into high instability (self-reaction)[286]. Its lacrimatory and skin-irritant properties have also been described[287]. Reaction of **145** with MeOH yields the 4-methoxy derivative **146**, which is then converted into 4-hydrazino-3-nitropyridine (**147**) by adding hydrazine hydrate in EtOH, an improved preparation of the old method which was developed by Königs and Freter[288] and Hünig and Köbrich[289]. A refluxing mixture of **147** with hydrazine hydrate followed by acidification affords **148**. Carpino also described direct synthesis of **148** from **145** or its 4-fluoro/bromo analogues[231].

The hydrazino derivative **147** can be alternatively prepared from 4-hydroxy-3-nitropyridine (**150**), through intermediate formation of the 4-chloro (**145**) or 4-methoxy (**146**) activated species (paths **A** and **B**, Scheme 36). Path **A**, reported by Reich and coworkers, involves formation of the highly reactive **145** by heating **150** in the presence of phosphorus pentachloride and oxychloride[290]. **147** is then obtained after long treatment with hydrazine hydrate in ethanol, without isolating the 4-ethoxy intermediate, in 67% yield (Scheme 36). A few decades earlier, Fleet and Fleming described the synthesis

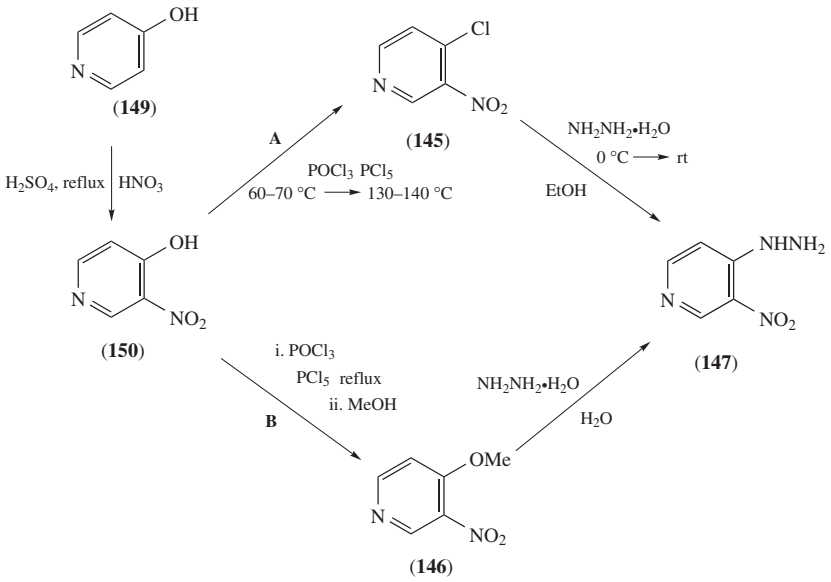


(**148**)

FIGURE 36. Structure of 6-aza-1-hydroxybenzotriazole



SCHEME 35. Synthetic strategy for 6-aza-1-hydroxybenzotriazole



SCHEME 36. Synthetic approaches for the synthesis of **147**

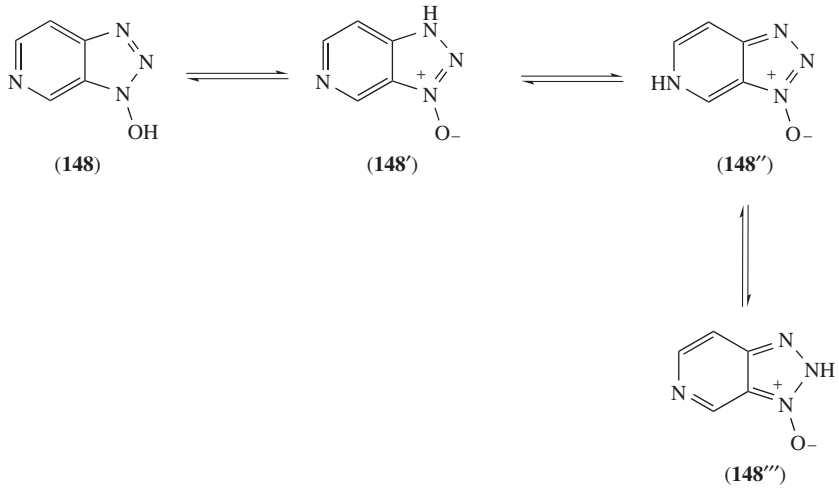


FIGURE 37. Tautomeric forms of 6-aza-1-hydroxybenzotriazole

of **147** from **146**, which is directly obtained in a short time from **150** by halogenation in methanol (75% overall yield, path **B**)[291]. Nitration of **149** in red fuming HNO_3 and fuming H_2SO_4 (18–24% SO_3) affords in a few hours the starting material for both routes (**150**) in 70–90% yield (Scheme 36)[288, 292].

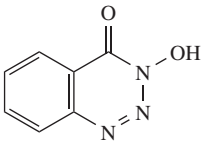
Similarly to the case of the 5-isomer, degradation of **148** under the conditions of the Isaacs method[285] for determining the pKa has lead to uncertainty about its ionization constant. Nevertheless, a pKa between those of the 4- and 7-analogues can be predicted considering the relative performance of 4-, 5-, 6- and 7-isomers as peptide additives (which is connected to their quality as leaving groups, and therefore with their acidity).

The tautomerism between *N*-hydroxy (**148**) and *N*-oxide (**148′**, **148″**, **148‴**) forms of 6-HOAt has been investigated, in a similar manner to those of most of *N*-hydroxybenzotriazoles (Figure 37)[271]. ^{1}H-NMR spectra of the methylation product indicated the predominance of the *N*-hydroxy form (**148**) in alcoholic solution, since the methyl group appeared in the typical area for this tautomer ($\delta = 4.49$ ppm)[231].

J. Hydroxybenzotriazine

1. HODhbt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine

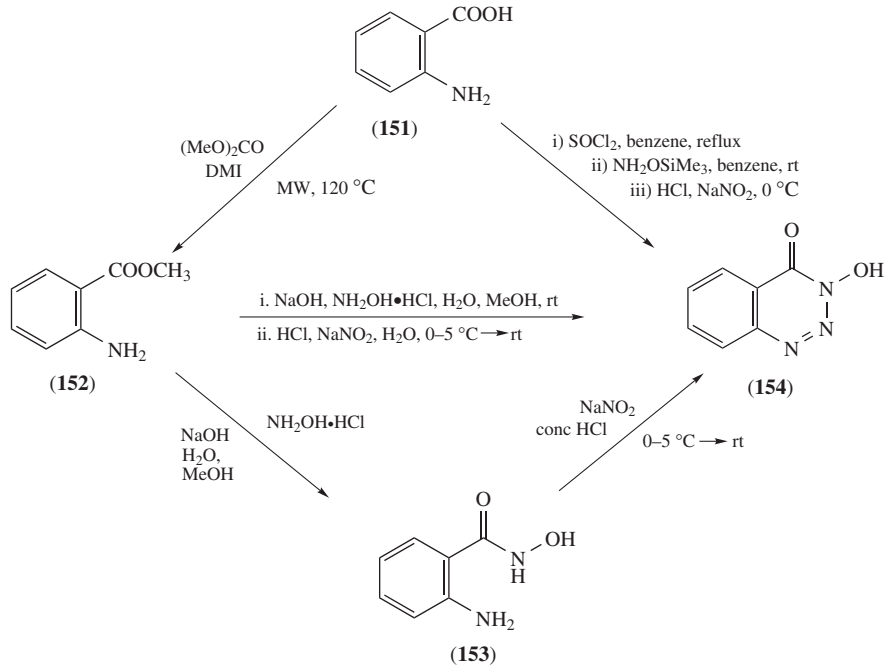
3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HODhbt, HOOBt, **154**, Figure 38) is a commercially available colorless solid (pale yellow before recystallization), which decomposes after melting at 180–181 °C, according to Harrison and Smith[293]. Some authors claimed a higher melting point for **156**: 182–185 °C or 186–187 °C[32, 294]. Alternatively, **154** has also been abbreviated as HOOBt[24, 101, 295]. It is a cyclic hydroxamic acid-containing compound, which gives a red color after reaction with ferric chloride. It is unstable in strong alkaline medium, leading to *o*-azidobenzoic acid (in 20% sodium hydroxide, complete degradation is observed after 1 h reflux), although it is stable in dilute conditions[32, 293]. The thermal breakdown byproducts have been widely studied. Unfortunately, such high reactivity might also lead to side reactions at room temperature, as a result of nucleophilic attack at the 4-carbonyl[32, 187, 276, 295–298].



(**154**)

FIGURE 38. Structure of 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine

Formation of the triazine ring of **154** from *o*-aminobenzhydroxamic acid (**153**) was already reported in 1960 by Harrison and Smith, adapting an old method (Scheme 37)[293]. Diazotization of the amino group of **153** in the presence of $NaNO_2$ and concentrated HCl, with subsequent heterocyclization, afforded **154** in 86% yield (Scheme 37)[293]. A similar process was reported by Ahern and coworkers in lower yield[32]. The synthesis of **153** can be achieved from methyl anthranilate (**152**) by reaction with hydroxylamine hydrochloride in a basic mixture of NaOH in H_2O/MeOH (70% yield) (Scheme 37)[299]. Previously, Vaughan and coworkers[300] separately proposed a similar, but less effective methodology. **152** can be obtained almost quantitatively from anthranilic acid (**151**), by selective methylation with dimethyl carbonate and 1,2-dimethylindole (DMI), forming an intermediate imidazolidium ion (98% yield) (Scheme 37)[301]. A similarly efficient approach

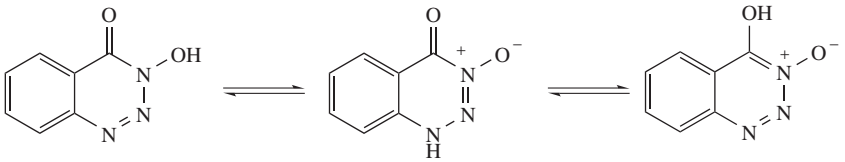


SCHEME 37. Synthetic strategies for the synthesis of 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine

consists in the reaction of **151** with diazomethane, rendering the target methyl ester **152** in mild conditions and high yield[302]. Possibly, the best strategy to afford **154**, although giving rise to lower yields than the above-mentioned method, was proposed by Jakobsen and coworkers in 1990[294]. They designed a one-pot method from **151** to the desired **154**, in which, in the first step, a ketenimine intermediate is formed after reflux with thionyl chloride[303]. This species has been found to be also present in the thermal breakdown of **154**[32, 294]. Next, the trimethylsilylated hydroxamic acid is generated *in situ* which, upon treatment with sodium nitrite in diluted hydrochloric acid, is hydrolyzed, diazotized and cyclized (68% overall yield) to afford pure **154** (Scheme 37) after recrystallization from H_2O/MeOH[294].

The presence of a cyclic hydroxamic acid contained within a triazine ring conferred on **154** a remarkable acidity. By means of a standard potentiometric technique, Koppel and coworkers measured the dissociation constant of **154** at room temperature ($pKa = 3.97 \pm 0.03$)[24, 232]. The acidity in DMSO was also investigated ($pKa = 8.9 \pm 0.1$), by potentiometric titration of **154** with Bu_4NOH in a solvent mixture benzene/isopropanol[24]. In both solvents, **154** was slightly more acidic than the parent benzotriazole HOBt (**95**, Section III.H.1). In a related study, the same dissociation constant was reported by König and Geiger[101] for both *N*-hydroxylamines ($pKa = 4.3$), in a 50:40 mixture of diethylene glycol dimethyl ether/H_2O at 54 °C, and also by Martinez and Bodanszky[221]. In spite of the almost identical acidities, **154** is regarded as a stronger nucleophile than **95**[101]. Infrared spectra of **154** in KBr showed a strong hydroxyl band at 2700 cm^{-1}, and a broad carbonyl signal at 1660 cm^{-1} [32]. The broadness of the two bands indicates the presence of an intramolecular hydrogen bond between those moieties[32]. Another signal at 1700 cm^{-1} was detected. Jakobsen and coworkers reported a slightly different IR spectra regarding the carbonyl region, and also identified a band corresponding to the triazine double-bond diaza group: ν(O−H) = 3200–2500 cm^{-1}, ν(C=O) = 1670 cm^{-1}, ν(N=N) = 1635 cm^{-1} [294]. The UV spectra of **154** has been studied in EtOH: λ_{max} (nm) = 221, 260, 303, 384. Most of the UV maxima, except the last one, were also observed in acylated derivatives[32]. In DMF, a band at 302 nm (log $\varepsilon = 3.76$) was also detected[294]. In the presence of a tertiary base (10 microliters of Et_2-NH), mimicking coupling conditions, strong absorptions had been observed near 400 nm: λ_{max} = 358 nm (log $\varepsilon = 3.87$), 430 nm (log $\varepsilon = 3.61$)[294]. Due to this UV absorbance, **154** shows a bright yellow color when ionized[276]. This enables practical detection of the final point in acylation reactions, such as peptide bond formation, since the absence of free amine results in negligible ionizaion[187, 276, 294, 296].

Jakobsen and coworkers reported the ^{1}H-NMR and ^{13}C-NMR spectra of **154** in DMSO-d_6, showing the proton of the N−OH at δ 2.51[294]. Vaughan and coworkers reported the fully decoupled, and without NOE, ^{15}N-NMR spectra of **156**: ^{15}N-NMR (DMSO-d_6) = δ 24.7 (*N*-2), −23.8 (*N*-1), −113.4 (*N*-3)[300]. The chemical shift for the three nitrogens lie in the typical area for the triazine ring[300]. Interestingly, the hydroxylamine-type *N*-3 is 40 ppm downshifted in comparison with the 3-methyl analogue of **154**. ^{15}N NMR also



(**154**) (**154′**) (**154″**)

FIGURE 39. Tautomeric forms of 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine

gave information about the tautomeric equilibrium of **154**[300] (Figure 39). The presence of the tautomeric form with a hydrogen bonded to *N*-1 (**154′**) or the *N*-3 oxide, hydroximic acid species (**154″**) is still unkown in solid-state or solution NMR[32, 293, 299]. However, a NOE study on the parent 4-oxobenzotriazine suggested the predominance of the hydroxylamide form **154** over the hydroxylimide one (**154″**), similarly to other *N*-hydroxy-α-oxo compounds[32, 61, 300]. Thus, a similar percentage of tautomers might be expected from **154**.

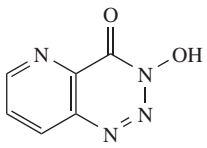
K. Hydroxyazabenzotriazine

1. HODhat, 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene

3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene (HODhat, **158**, Figure 40) is a yellow solid, which may cause an explosion after melting at 195 °C[165, 166, 293]. When **158**, also named 3-hydroxy-4-oxo-1,2,3,5-tetraazanaphthalene, is recrystallized from EtOH/H_2O (9:1), light orange-yellow needles are obtained, showing a higher melting point (203 °C)[165, 293], as a triazine-based cyclic hydroxamic acid **158** forms red-colored solutions upon reaction with ferric chloride[293]. The structural relationship to the parent **154** (Section III.J.1) translates into similar properties, like the possibility of clearer monitoring of the extension of acylation reactions (bright yellow to orange-red color)[165]. Unfortunately, **158** might also undergo ring-opening by a second molecule, when activated in acylations, leading to 3-(3′-azidopicolinoyloxy)-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazine[165].

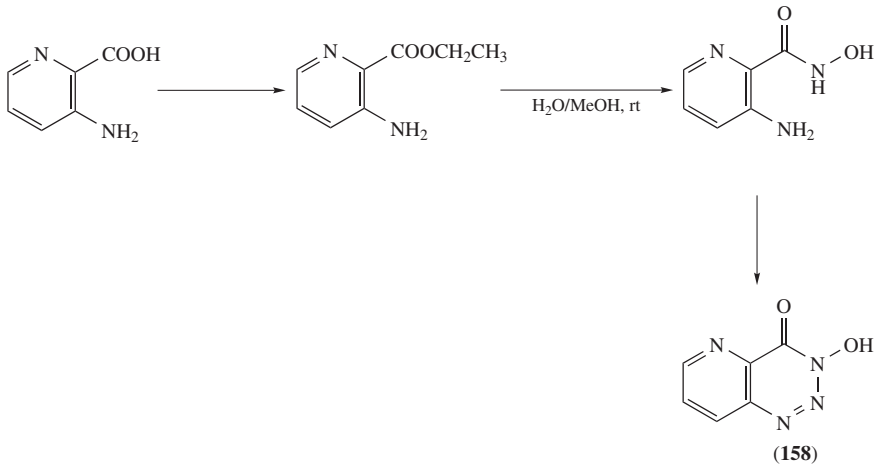
3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene (**158**) was first synthesized by Harrison and Smith[293] from 3-amino-2-picolinehydroxamic acid (**157**), by diazotization with $NaNO_2$ and HCl, followed by intramolecular cyclization, in 32% yield (Scheme 38)[293]. Interestingly, a trial reaction on the 2-aminonicotin analogue was unsuccessful, due to the low reactivity of the 2-aminopyridine group[293]. However, few experimental details were given, such as the formation of the linear hydroxamic acid (**157**)[293]. Later, Carpino and coworkers detailed a synthetic approach to **157**[165]. The conversion of ethyl 3-aminopicolinate (**156**) into **157** can be quantitative in the presence of hydroxylamine hydrochloride and sodium hydroxide, during 2 days, improving the yield obtained by previous work from Harrison and Smith[166]. Carpino and coworkers also optimized the esterification of 3-aminopicolinic acid (**155**), leading to **156** (Scheme 38), by extensively refluxing the acid in absolute ethanol and concentrated sulfuric acid (68% yield)[165].

Carpino and coworkers also reported an efficient strategy for obtaining **155** from pyridine-2,3-dicarboxylic acid (**160**, Scheme 39)[165]. The transformation of **160** to the cyclic 2,3-pyridine dicarboximide (**161**), by refluxing in acetic anhydride, followed by the addition of acetamide was already reported by Sucharda many decades before (75% yield) (Scheme 39)[170]. Recently, microwave-induced condensation of **160** with ammonia has been reported with identical efficiency, but in a shorter time[171]. Subsequent Hoffmann rearrangement of the cyclic imide **161** to **155**, in the presence of sodium hypobromite



(**158**)

FIGURE 40. Structure of 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene



SCHEME 38. Synthetic strategies for the synthesis of 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene

or sodium hydroxide and bromide, has been described by some authors, sometimes with side formation of 2-aminonicotinic acid (Scheme 39)[167, 169, 170]. The detailed procedure provided by Carpino afforded the highest yield, using sodium hypobromite (60% yield)[165]. Regarding the formation of **160**, various strategies have been reported from quinoline (**162**), 2-pyridinecarboxylic acid (**159**) or 8-hydroxyquinoline (**163**) (Scheme 39). Ozonolysis and ring cleavage of quinoline (**162**) with ozone and sulfuric acid has been studied, with the highest yield being 84%[304]. Similar efficiency, although avoiding sulfuric acid, can be achieved from **159** by regioselective lithiation, with subsequent carbonylation in lithium tetramethylpiperidide (LTMP)[174]. Higher yields are obtained from **163**, which can be quantitatively and chemoselectively oxidized to **160** by treatment with dilute nitric acid[170, 172, 173].

The expected structure of **158** was confirmed after ^{1}H-NMR analysis, showing the upshifted hydrogens of the pyridine ring as double doublets[210]. Infrared spectra of **158** in KBr displayed a broad *N*-hydroxyl band at 2600 cm^{-1} and a signal, corresponding to the amide moiety of the hydroxamic acid, at 1713 cm^{-1} [165]. Additional bands were detected at 1574, 1420, 1230, 1185, 1066, 974 and 794 cm^{-1} [165]. Similarly to **154** (Section III.J.1), the tautomeric equilibrium of the hydroxylamide form of **158** with the species presenting a hydrogen bonded to *N*-1 (**158′**) or the *N*-3 oxide (**158″**) has not been studied in solid state or solution (Figure 41)[32, 293, 299].

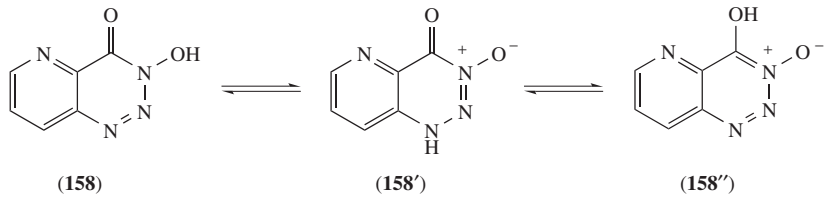
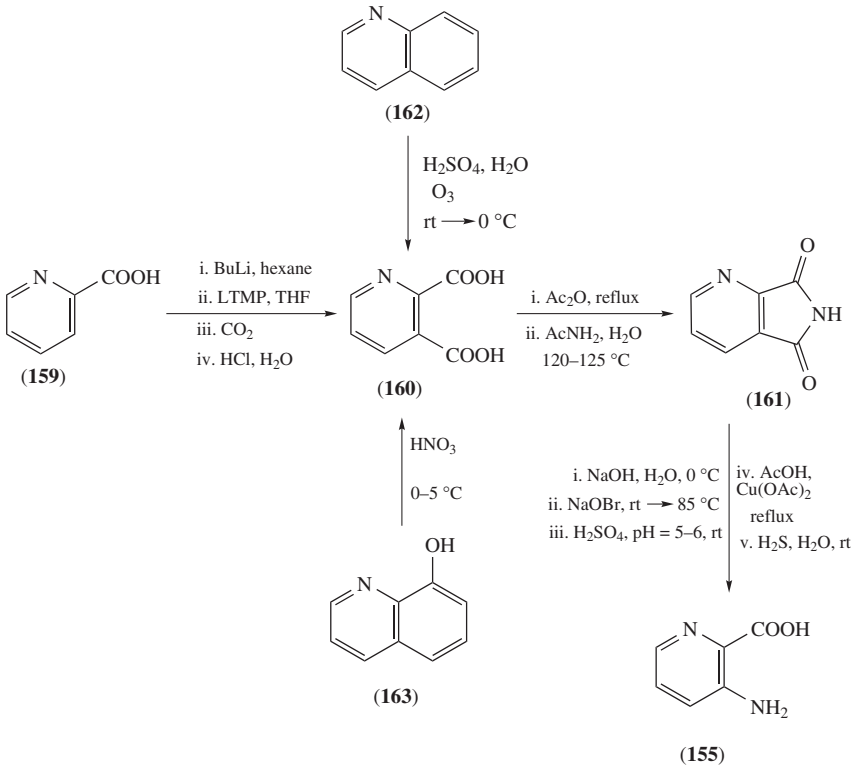


FIGURE 41. Tautomeric forms of 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene

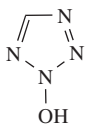


SCHEME 39. Synthetic approaches for the synthesis of **155**

Considering the NOE experiments performed on the parent 4-oxobenzotriazine, suggesting that the hydroxylamide form **158** predominates over the hydroxylimide one (**158′**), in agreement with other *N*-hydroxy-α-oxo compounds, a similar tendency is expected for **158**[32, 61, 300].

L. 2-Hydroxytetrazole

2-Hydroxytetrazole (**167**, Figure 42) is a non-natural heterocyclic compound, melting at 143–146 °C, after recrystallization from ethyl acetate[175, 176, 305]. **167** finds application as a

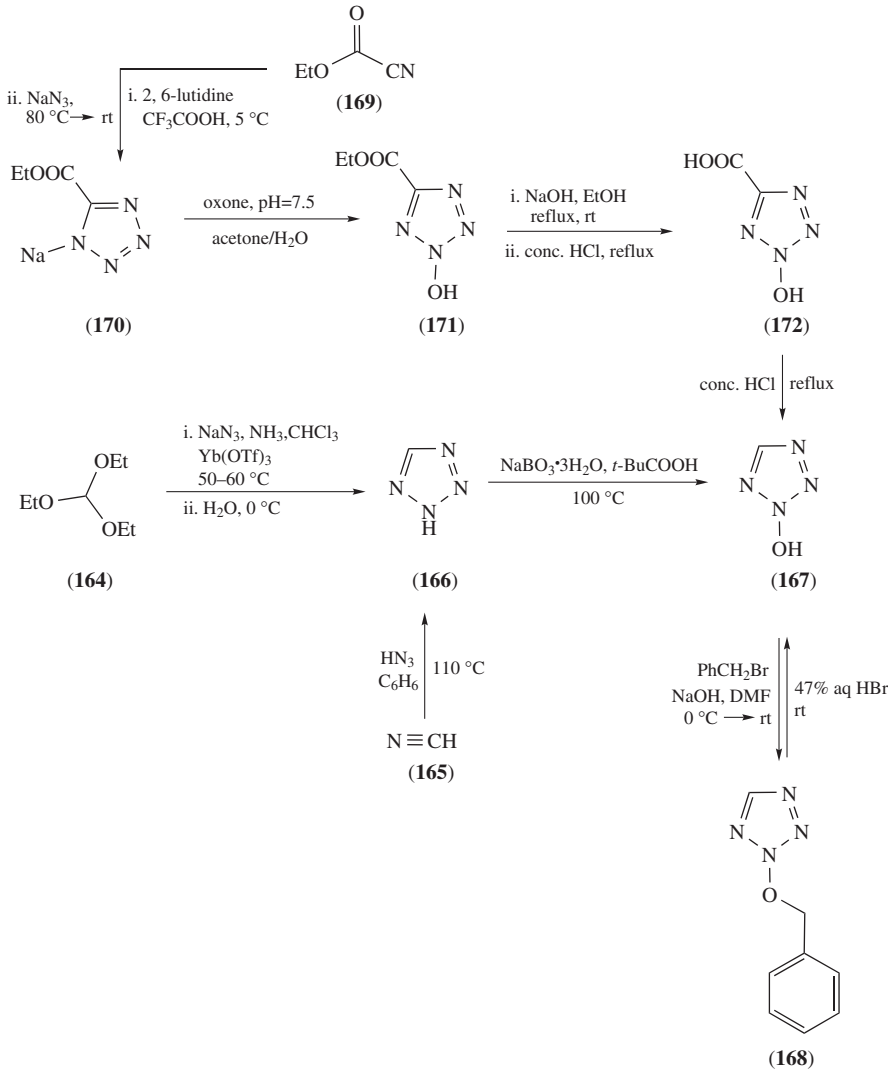


(**167**)

FIGURE 42. Structure of 2-hydroxytetrazole

propellant, apart from the high acylating character of its esters. Moreover, the remarkable stability of **167** to metabolic conditions combined with certain pharmacological activity results in an attractive drug-like profile[305–309].

Few synthetic strategies toward **167** have been reported, in contrast to its 1-hydroxy isomer, which was obtained in the 1950s[175, 310]. Its first synthesis was accomplished by Begtrup and Vedsoe fifteen years ago, by oxidation of tetrazole (**166**, Scheme 40)[175]. The analogous transformation in substituted tetrazoles is demanding, since the low HOMO of



SCHEME 40. Synthetic approaches for the synthesis of 2-hydroxytetrazole

these heterocycles poses difficulties in the oxidative process, even when strong oxidizing agents are used[305]. Nevertheless, *N*-3 oxides of 1,5- or 2,5-disubstituted tetrazoles have been recently reported, by action of hypofluorous acid[305]. The oxidation of **167** with 60% hydrogen peroxide and 3-chloroperbenzoic acid completely failed (0–3% conversion)[175]. However, **167** was observed with sodium perborate trihydrate in pivalic acid as solvent. Unfortunately, 1-hydroxytetrazole was also generated by this method (*N*-2/*N*-1 ratio = 2:1), due to its poor chemoselectivity[175]. The difficult separation of the two isomers forced the authors to carry out an extra *O*-benzylation step with sodium hydroxide and benzyl bromide, in order to facilitate the isolation of 2-benzyloxytetrazole (^{13}C NMR shows distinct C-5 signals for the *O*-benzylated derivatives)[175]. Subsequent debenzylation of 2-benzyloxy tetrazole (**168**) in the presence of aqueous hydrobromic acid afforded in 16 hours the desired **169** in a low 17% overall yield (Scheme 40)[230]. The starting **168** can be obtained from triethoxymethane (**164**), sodium azide and ammonium chloride as nitrogen source, in moderate yield and in a cost-friendly manner, or by cyclization of hydrogen cyanide (**165**) with hydrogen azide (Scheme 40)[311].

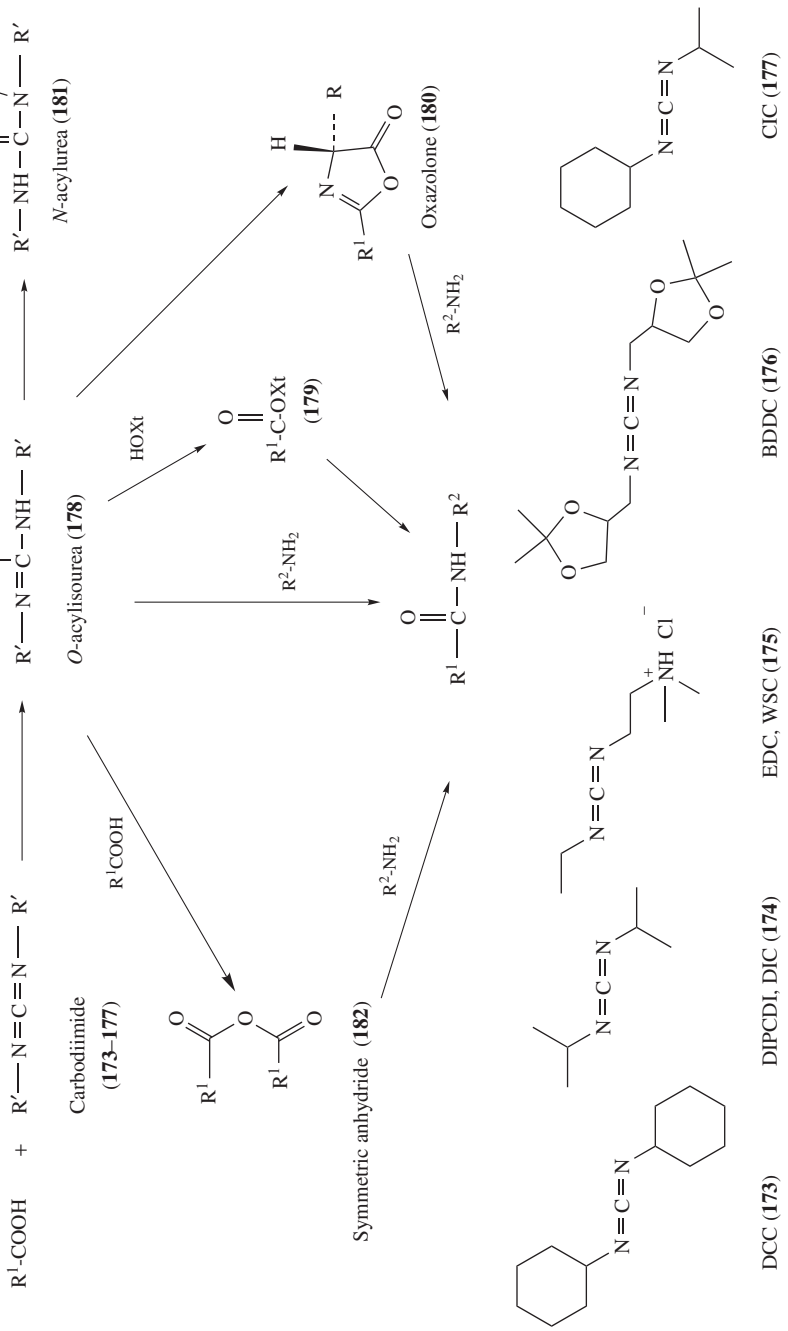
The low efficiency of Begrup's strategy prompted the search for alternative approaches. Giles and coworkers designed a facile, more efficient strategy, starting from the sodium salt of ethyl tetrazole-5-carboxylate (**170**), which can be obtained in 84% yield from ethyl cyanoformate (**169**) in the presence of 2,6-lutidine and TFA, followed by treatment with NaN_3 (Scheme 40)[312]. Oxidation of **170** takes place selectively, rendering almost exclusively ethyl *N*-2-hydroxytetrazole-5-carboxylate (**171**) by means of oxone (potassium peroxymonosulfate, $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$) in 80% yield (*N*-2/*N*-1 ratio = 70:1) (Scheme 40)[312]. Subsequent hydrolysis of **171** with NaOH in refluxing EtOH affords 2-hydroxytetrazole-5-carboxylic acid (**172**) in 65% yield (Scheme 40). **167** is obtained after decarboxylation of this intermediate **172** in refluxing, concentrated hydrochloric acid for 4 days in 40% yield[312].

^{1}H-NMR spectra of **167** in D_2O display the H-5 aromatic hydrogen at δ 8.67[174]. In general, *N*-hydroxytetrazoles are more stable than C-hydroxy analogues, in light of the shorter single bonds of the former, observed by DSC. Charge distribution of the molecule also supports this hypothesis[313]. In particular, the almost planar and aromatic **167** shows enhanced stability in comparison with the 1-hydroxy isomer, according to the corresponding heats of formation (HOF), 71.9 and. 75.4 kcal mol^{-1}, respectively, calculated by the density functional method (DFT-B3LYP)[309, 313]. In spite of this relative stability, the explosive character of **167** is well known, especially when carrying out melting-point determinations[174, 312]. As a result of this observed behavior, **167** is widely used as an explosive detonator[313].

IV. *N*-HYDROXYLAMINES FOR CARBODIIMIDE-MEDIATED REACTIONS

Nowadays, carbodiimides (**173**–**177**) are used with a HOXt additive as a trapping agent of the *O*-acylisourea (**178**) intermediate to form the corresponding active esters **179** (Scheme 41), which decrease the degree of racemization in numerous cases[314–321]. The highly reactive *O*-acylisourea can lead to oxazolone (**180**) formation, which facilitates the loss of chiral integrity (Scheme 41), or to an unreactive *N*-acylurea (**181**)[189, 276]. HOBt (**95**) or HOAt (**116**) and other HOXt (**8**, **31**, **46**, **53**, **64**, **75**, **86**, **89**, **91**, **99**, **105**, **112**, **129**, **140**, **148**, **154**) additives give the corresponding active esters after reaction with the *O*-acylisourea[189, 276]. The presence of a tertiary amine favors formation of the active ester[320]. Alternatively, the symmetric anhydride **182**, which is formed when 2 equivalents of *N*-protected amino acid are used with 1 equivalent of carbodiimide, can be employed as the active species (Scheme 41).

Compared with other additives, **116** forms superior active esters in terms of yield and degree of racemization in both solution and solid-phase synthesis, even when the coupling



SCHEME 41. Mechanism of peptide bond formation using carbodiimide and the most common additives

SCHEME 41. (*continued*)

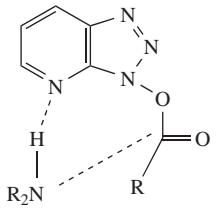
takes place with the hindered α-aminoisobutyric acid (Aib)[322–324]. The key behind the outstanding behavior of **116** is the nitrogen atom located at position 7 of the benzotriazole, which provides a double effect[276]. First, the electron-withdrawing influence of a nitrogen atom (regardless of its position) led to a better leaving group, thereby leading to greater reactivity. Second, placement of this nitrogen atom specifically at position 7 enables one to achieve a classic neighboring group effect (**183**, Figure 43), which can both increase reactivity and reduce the loss of configuration[322]. Compared to HOBt (**95**), the corresponding 6-HOAt (**148**), 5-HOAt (**140**) and 4-HOAt (**129**) all lack ability due to such a neighboring group participation, and have little influence on the extent of stereomutation during the segment coupling reaction[231, 325].

3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HODhbt, **154**) forms highly reactive esters, but their formation is accompanied by the byproduct 3-(2-azidobenzyloxy)-4-oxo-3,4-dihydro-1,2,3-benzotriazine (**184**), which can then react with the amino group to terminate chain growth (Figure 44)[189, 324].

Several coupling additives (**64**, **75**, **86**, **89**, **91**, **154**) have been evaluated in solid-phase Fmoc-based peptide synthesis in the presence of DIC (**174**)[188]. These reagents are advantageous because they don't have a UV absorption at 302 nm, thus allowing one to monitor the coupling process, a feature incompatible with Fmoc-methodology in the case of HOBt (**95**) or HOAt (**116**).

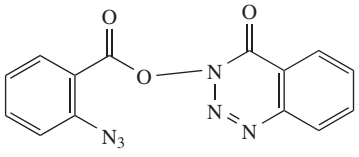
Recently, 6-Cl-HOBt (**99**) has been used in solid-phase synthesis. It is a good compromise between **95** and **116** in terms of reactivity and price[326]. Later, Carpino and coworkers described the aza derivative of **154** (HODhat, **158** and HODhad **59**, Section III.K.1)[165]. Active esters of **158** are slightly more reactive than OAt ones, which are considered the most reactive derivatives among these esters; however, the additive **158** gives the side product **185** (Figure 45), as occurs with HODhbt (**154**)[165, 324].

Because most of the benzotriazole derivatives, such as **95** and **116**, exhibit explosive properties, El-Faham and Albericio reported several *N*-hydroxylamine derivatives



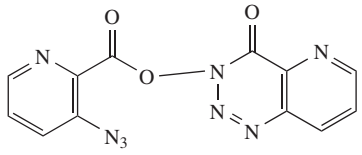
(**183**)

FIGURE 43. Neighboring group effect for HOAt



(**184**)

FIGURE 44. 3-(2-Azidobenzyloxy)-4-oxo-3,4-dihydro-1,2,3-benzotriazine as byproduct from HODhbt



(**185**)

FIGURE 45. Structure of the side product from HODhat

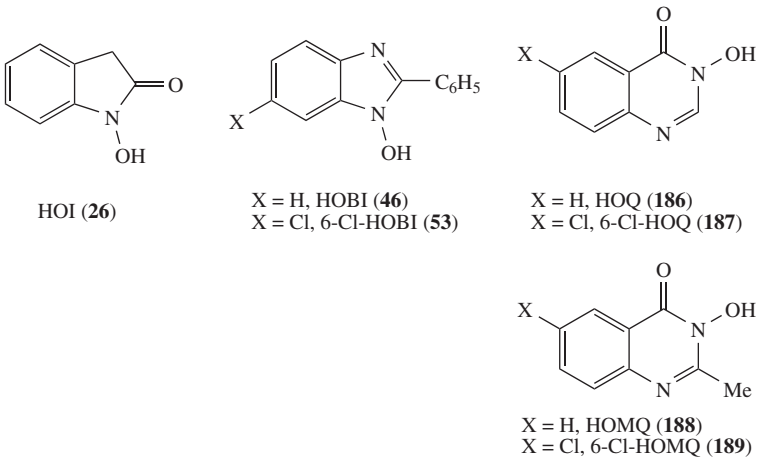


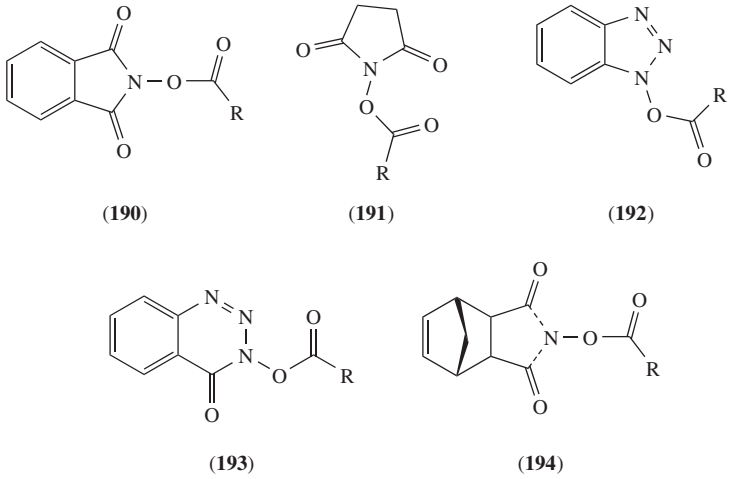
FIGURE 46. Structure of *N*-hydroxylamine derivatives reported by El-Faham and Albericio

26, **53** and **186**–**189** (Figure 46) to be used as substitutes for the **95** and **116**[79]. These *N*-hydroxylamines (**26**, **47**, **53**, **186**, **187**, **188** and **189**) performed well with DIC activation, but they did not show the same efficiency as **95** and **116** for racemization depression[79].

Later, Subirós-Funosas and colleagues reported a safe and highly efficient additive, ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, **1**, Section III.A), to be used mainly in the carbodiimide approach for forming the peptide bond[2]. **1** displays a remarkable capacity to suppress racemization and an impressive coupling efficiency in both automated and manual synthesis. These effects are superior to those shown by **95** and comparable to **116**. Stability assays show that there is no risk of capping the resin in standard coupling conditions. Finally, calorimetry assays (DSC and ARC) confirm the explosive potential of the benzotriazole-based additives and demonstrate the lower risk of explosion induced by **1**[2].

V. *N*-HYDROXYLAMINES FOR THE PREPARATION OF ACTIVE ESTERS

The substituted hydroxylamines are designed to render the carbonyl of the acyl moiety susceptible to nucleophilic attack by an amine at room temperature to afford the active esters. Activated esters undergo aminolysis due to the electron-withdrawing ability of the



(**190**) (**191**) (**192**)

(**193**) (**194**)

FIGURE 47. Structure of hydroxamic active esters

ester moiety. However, the esters formed from substituted hydroxamic acids are so highly activated that their reactivity cannot be explained on the basis of this property alone. An additional phenomenon is operative, i.e. neighboring atoms assist in the union of the two reactants. This neighboring group participation in the formation of a new chemical bond is referred to as *anchimeric assistance*. The reaction is, in fact, an intramolecular general base-catalyzed reaction[189, 327, 328].

The first activated esters derived from hydroxamic acids were the *o*-phthalimido (**190**) esters that liberate a water-insoluble side product, but these were soon replaced by the more versatile succinimido esters (**191**). The latter generate water-soluble *N*-hydroxysuccinimide that is easy to remove from target peptides. Additional examples of esters derived from hydroxamic acids are benzotriazolyl (**192**), 4-oxo-3,4-dihydrobenzotriazinyl esters (**193**) and *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboxyimide (HONB, **194**) esters (Figure 47)[329, 330]. These are activated not so much because of the electron-withdrawing effect of the ring moieties, but because of the nature of the heterocyclic rings. All the esters mentioned above can be used as shelf-stable reagents, except benzotriazolyl esters, which decompose too readily. In addition to their use as activated forms of the *N*-alkoxycarbonylamino acids, the esters derived from hydroxamic acids are implicated as intermediates in coupling reactions in which the *N*-hydroxy compounds have been added to promote efficient coupling between an acid and a primary or secondary amine. The aminolysis of activated esters generally occurs more readily in polar solvents and is catalyzed by mild acid or 1-hydroxybenzotriazole (**95**). Transesterification and using mixed anhydrides are other methods by which activated esters can be obtained[104, 123, 327, 331–335].

VI. *N*-HYDROXYLAMINES FOR THE PREPARATION OF PHOSPHONIUM SALTS

A. HOBt Phosphonium Salts

HOBt (**95**) is currently the most frequently used activating agent for the carboxyl group of amino acids[336]. The procedure is fast and suppresses racemization, but the

intermediate esters are moisture sensitive[336]. HOBt–DCC or DIC methodology can be used in all peptide couplings. DIC is often preferred because its urea byproduct is more soluble in organic solvents than that formed from DCC. Solid-phase peptide synthesis with HOBt–DCC is widely used in combinatorial peptide synthesis taking advantage of fast reactions.

95 has been found to be a useful tool, providing simplified workup and purification procedures[337–340]. Moreover, HOBt-based phosphonium, uronium or onium salts are more reactive and their preparation will now be discussed.

1. BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate

Kenner and coworkers were the first to describe the use of acylphosphonium salts as coupling reagents[341]. These species were widely adopted only after the extensive studies of Castro, Coste and coworkers, who introduced CloP (**195**) and BroP (**196**) as peptide-coupling reagents with noticeable racemization (Figure 48)[342–348]. After HOBt was discovered as a racemization suppressant, a new coupling reagent, known as BOP (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate) (**197**), was introduced in 1975 (Figure 48). **197** is a non-hygroscopic crystalline material that can be prepared effortlessly in large amounts, is easy to use and promotes rapid coupling[347, 349–351].

With regard to the mechanism, several authors have proposed that in the absence of the nucleophile that is incorporated in the reagent, for example HOBt in **197**, the active species is the acyloxyphosphonium salt[352–355]. Castro, Dormoy and coworkers have suggested that this salt is very reactive and even at low temperatures will react immediately with carboxylate ions present in the medium to give the symmetrical anhydride[342–344]. This pathway is supported by kinetic studies carried out by Hudson (Scheme 42)[356]. Several years later, Kim and Patel reported that this intermediate (**198**) could exist at −20 °C when **197** was used as a coupling reagent[357]. However, Coste and Campagne suggested that this species is very unstable and even at low temperatures undergoes conversion to an active ester[358]. In spite of this controversy, it is widely accepted that the active species is an active ester when phosphonium salts containing nucleophilic derivatives are used.

The formation of OBt ester is achieved in the presence of 1 equivalent of a tertiary base such as DIEA, NMM or TMP[343, 359, 360]. The presence of an extra equivalent of HOBt accelerates the coupling and also reduces the loss of configuration[344].

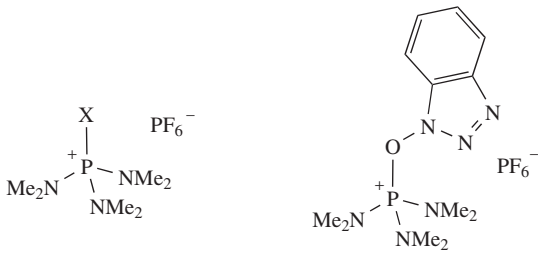
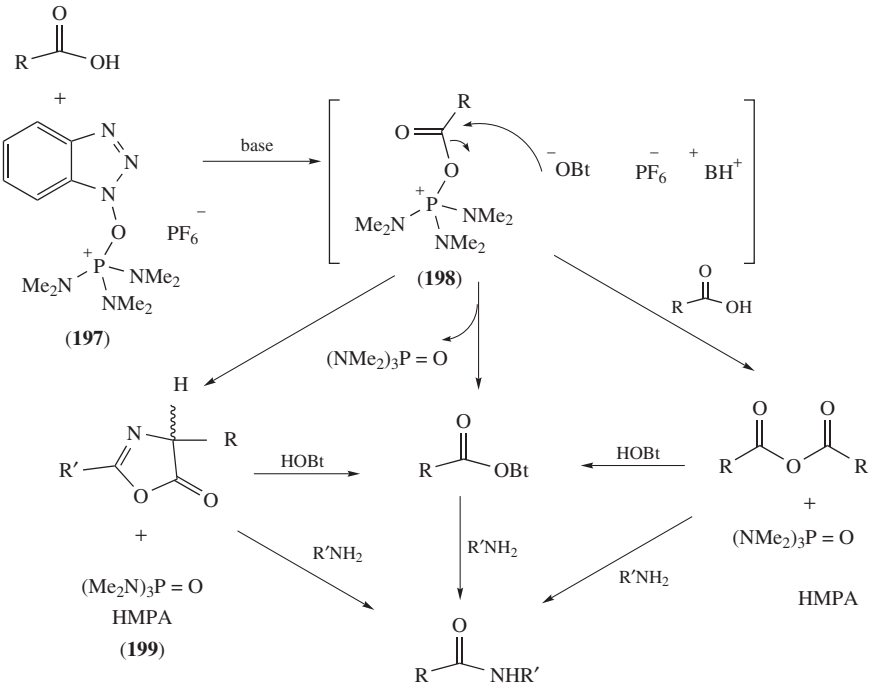


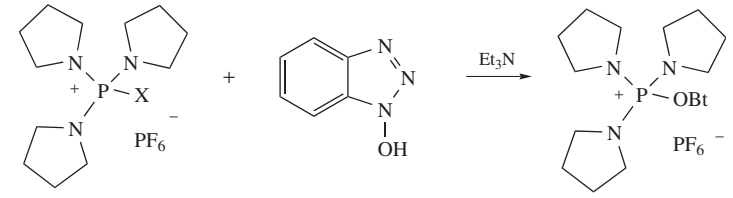
FIGURE 48. Structure of tris(dimethylamino)phosphonium salts



SCHEME 42. Mechanism of BOP-mediated reaction

2. PyBOP, benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate

PyBOP (**202**) was prepared by reaction of PyCloP (**200**) or PyBroP (**201**) with HOBt (**95**) in the presence of triethyl amine (Scheme 43). In these compounds the dimethylamino moiety is replaced by pyrrolidine[342, 361, 362]. These reagents prevent the generation of poisonous hexamethylphosphoramide (HMPA, **199**, Scheme 42) byproduct[356]. In a subsequent study Castro, Dormory and coworkers reported that halogenophosphonium reagents



SCHEME 43. Synthesis of PyBOP

often give better results than other phosphonium–HOBt reagents for the coupling of *N*-methylated amino acid[344].

3. Other HOBt phosphonium salts

Since the discovery of HOBt-mediated coupling reagents, many racemization suppressants have been exploited as a part of the composition of new peptide-coupling reagents. For example, 2-(benzotriazol-1-yloxy)-1,3-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP, **203**) was introduced as a useful reagent for peptide assembly[363]. PyNOP (**204**), PyFOP (**205**) and PyFNBOP (**206**), PyCloK (**207**) were prepared in this regard and serve as efficient peptide-coupling reagents for the synthesis of dipeptides bearing *N*-methyl amino acids (Figure 49)[165, 360–365].

B. HOAt Phosphonium Salts

Phosphonium salts derived from HOAt, such as (7-azabenzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (AOP, **208**) and (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP, **209**) (Figure 50), have also been prepared and are generally more efficient than BOP and PyBOP as coupling reagents (Figure 48)[323, 366–370]. The pyrrolidino derivative **209** is slightly more reactive than the dimethylamino derivative **208**, and does not release HMPA (**199**) in the activation step.

C. HODhbt Phosphonium Salts

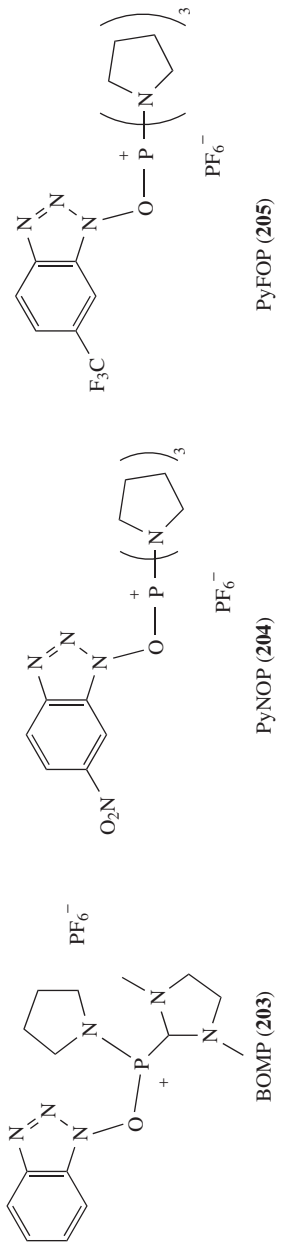
HODhbt (**154**) has been reported as a coupling additive that can indicate the completion of acylation by color change[101, 189]. Unfortunately, **154** has been reported to give a relatively higher degree of racemization than other azole derivatives in the presence of DIC when poly(ethylene glycol)-cross-linked polyamide (PEGA)-bound dipeptide was used for peptide synthesis[188]. Fully protected amino acid esters of HODhbt are stable crystalline solids, which can be stored for long periods at low temperature. However, a ring-opening side-reaction leading to 2-azidobenzoic acid Dhbt ester **184** was observed in DCC-mediated HODhbt conditions[296]. Fmoc-amino acid esters with HODhbt have been prepared in high yield[371, 372].

Recently, Carpino and coworkers described the phosphonium salts of HODhbt (PyDOP, **210**) and the aza derivative (PyDAOP, **211**) (Figure 51)[165]. The active esters of **211** additive are slightly more reactive than OAt ones, which are considered the most reactive derivatives among these esters; however, **211** gives the side product **185**, as occurs with **154** (Figure 45)[165].

D. Oxyma Phosphonium Salts (PyOxP, PyOxB)

Very recently, Subirós-Funosas, El-Faham and Albericio reported the phosphonium salts of Oxyma (**1**), *O*-[(1-cyano-2-ethoxy-2-oxoethyliden)amino]oxytri(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyOxP, **212**) and its tetrafluoroborate (PyOxB, **213**) (Figure 52) version, as an efficient, racemization-suppressing coupling reagent for the assembly of hindered peptides, performing better than classical benzotriazole aminium salts and similarly to the recently introduced uronium salt COMU (that will be discussed later)[373]. Cyclization models revealed the advantages of the use of **213**, which rendered a higher percentage of cyclic peptide than other known phosphonium salts[373].

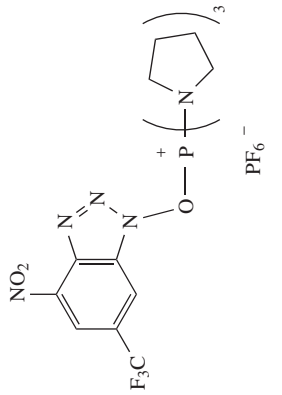
698



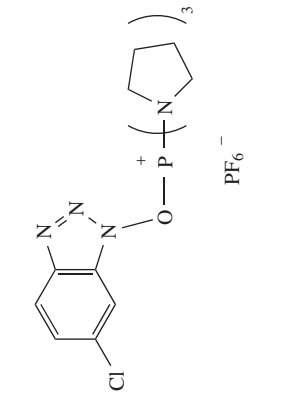
BOMP (**203**)

PyNOP (**204**)

PyFOP (**205**)

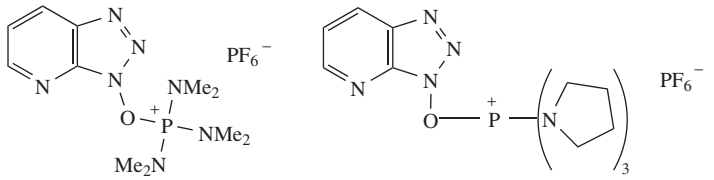


PyFNBOP (**206**)



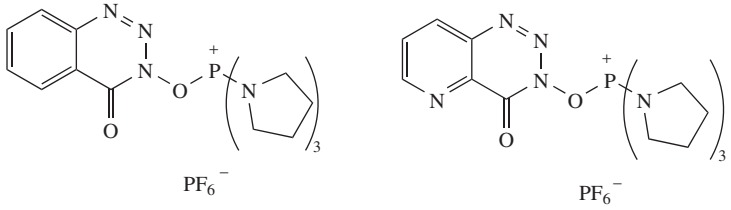
PyCloK (**207**)

FIGURE 49. Structures of other HOBt-derived phosphonium salts



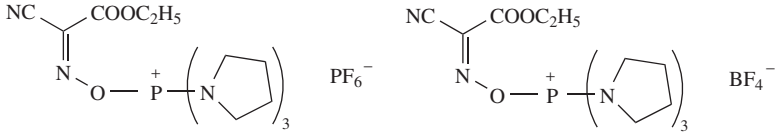
AOP (**208**) PyAOP (**209**)

FIGURE 50. Structure of HOAt-based phosphonium salts



PyDOP (**210**) PyDAOP (**211**)

FIGURE 51. Structure of HODhbt- and HODhat-based phosphonium salts



PyOxP (**212**) PyOxB (**213**)

FIGURE 52. Structure of Oxyma-based phosphonium salts

VII. *N*-HYDROXYLAMINES FOR THE PREPARATION OF URONIUM/IMINIUM SALTS

Iminium salts were initially assigned to be as uronium-type structure **214**, presumably by analogy with the corresponding phosphonium salts, which bear a positive carbon instead of the phosphonium residue[366]. Nevertheless, it has been shown by X-ray analysis that the salts crystallize as aminium salts (guanidinium *N*-oxides) (**215**) rather than the corresponding uronium salts (Figure 53)[374–384].

A. *N*-Hydroxylamines for the Preparation of Tetramethyl Uronium/Iminium Salts

The preparation of these commercially available reagents is achieved by treatment of tetramethylurea (TMU, **216**) with $(COCl)_2$ in toluene followed by transformation of the dichloro salts into the corresponding chlorouronium hexafluorophosphate salt (TMU-Cl, **217**, Scheme 44), by exchange with NH_4PF_6 or KPF_6 and then reaction of **217** with

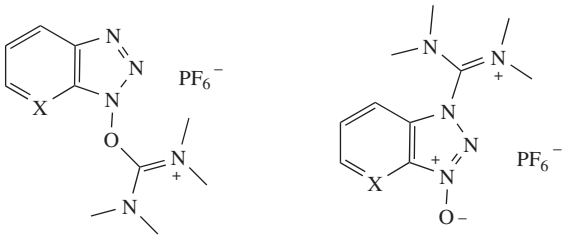
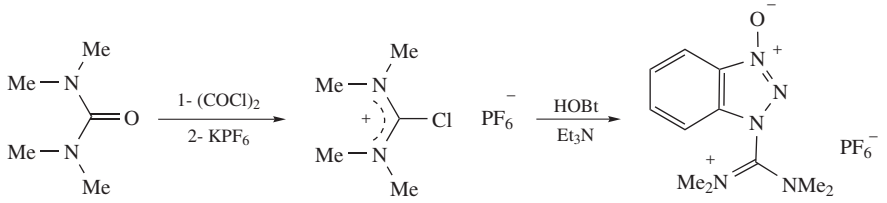


FIGURE 53. Structure of HOBt and HOAt-derived uronium/iminium salts



SCHEME 44. Synthesis of *N*-HBTU

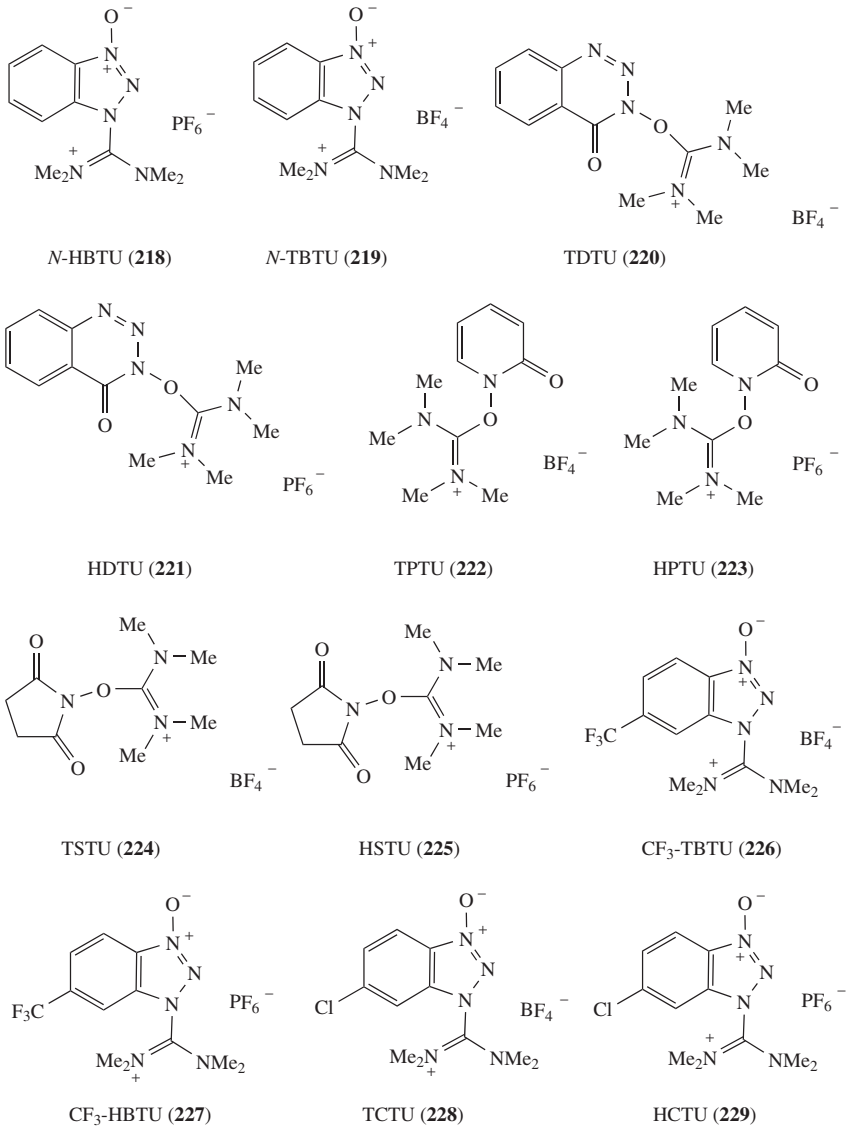
HOXt to afford the corresponding iminium/uronium salts **218**–**223** (Figure 54)[380, 381]. Chlorouronium salt **217** has also been prepared by replacement of the extremely toxic $COCl_2$ by oxalyl chloride or $POCl_3$[382–384]. *N*-HBTU (**218**) has been obtained using a one-pot procedure in organic solvents, and the analogous tetrafluoroborate reagent (*N*-TBTU, **219**) procedure was prepared similary (Scheme 44).

This one-pot method has also been applied to the preparation of the HODhbt derivatives 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TDTU, **220**) and HDTU (**221**), the pyridinone derivatives 2-(2-oxo-1(2*H*)-pyridyl-1,1,3,3-tetramethyluronium tetrafluoroborate (TPTU,**222** and HPTU, **223**) and the hydroxysuccinimide derivatives 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, **224**), which are also commercially available (Figure 54)[380]. The hexafluorophosphate analogue (HSTU, **225**) has also been prepared following the same strategy (Figure 54)[382, 383].

Other 1-hydroxybenzotriazole derivatives containing electron-withdrawing groups, such as the 6-trifluoromethyl derivative (CF_3-TBTU, **226** and CF_3-HBTU **227**) and 6-chloro derivative (TCTU, **228** and HCTU, **229**), have been prepared from tetrafluoromethyl chloroformamidinium hexafluorophosphate (Figure 54)[364].

The analogue uronium salts containing the HOAt structure (**116**) instead of HOBt (**95**) have been prepared from the TMU-Cl (**217**) salts to give the corresponding reagents *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate (*N*-HATU, **230**) and tetrafluoroborate (*N*-TATU, **231**)[379], which have been shown to be *N*-oxides with aminium structures (Figure 54)[101, 188, 369, 370]. The two tetramethylurea-derived thiouronium reagents, the HOAt derivative **232** (HATTU) and the *N*-hydroxy-2-pyridinethione derivatives **233** (HOTT), have been prepared,

both following Knorr's strategy (Figure 54)[382, 383,385–387]. The *O*-[(ethoxycarbonyl) cyanomethylene amino]-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TOTU **234** and HOTU **235**) has been reported and developed with other derivatives (**236**–**238**) by El-Faham, Albericio and coworkers (Figure 55)[162]. TNTU (**239**) and TPhTU (**240**) were prepared using the same strategy (Scheme 44)[388].



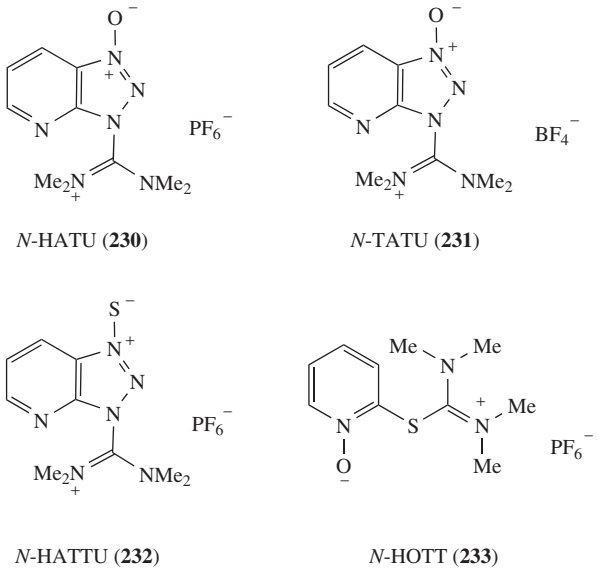
N-HBTU (**218**) *N*-TBTU (**219**) TDTU (**220**)

HDTU (**221**) TPTU (**222**) HPTU (**223**)

TSTU (**224**) HSTU (**225**) CF_3-TBTU (**226**)

CF_3-HBTU (**227**) TCTU (**228**) HCTU (**229**)

FIGURE 54. Structure of various uronium/iminium salts



N-HATU (**230**) *N*-TATU (**231**)

N-HATTU (**232**) *N*-HOTT (**233**)

FIGURE 54. (*continued*)

B. *N*-Hydroxylamines for the Preparation of bis-Pyrrolidino Iminium Salts

The chlorouronium salts (BTCFH, **242**) derived from dipyrrolidin-1-methanone (dipyrrolidinocarbonyl) **241** have been prepared by chlorination with phosgene, oxalyl chloride or $POCl_3$ followed by treatment with aqueous KPF_6[380–384]. HOBt (**95**) has been reacted with these chlorouronium reagents to give the corresponding iminium salt HBPyU **243** (Scheme 45).

The related HXPyU derived from HOAt (**116**), HODhbt (**154**) and HODhat (**158**) have been prepared by a similar method to that described above and afford HAPyU (**244**), HATPyU (**245**), HDPyU (**246**) and HDAPyU (**247**) (Figure 56). NMR spectral analysis showed that both **244** and **245** exist in the *N*-form (Figure 56)[375, 376].

C. *N*-Hydroxylamines for the Preparation of bis-Piperidino Iminium Salts

The chlorouronium salts derived from dipiperidinocarbonyl have been prepared by using the above method and then treated with HOXt to give the corresponding iminium salts HBPipU (**248**), HAPipU (**249**) and TOPPipU (**250**) (Figure 57)[323, 389, 390].

D. *N*-Hydroxylamines for the Preparation of Imidazolium Uronium Salts

The chloroimidazolium salt **252** (CIP) has been obtained by chlorination of the commercial 1,3-dimethylimidazolidin-2-one (**251**) with phosgene or oxalyl chloride followed by anion interchange with NH_4PF_6, followed by reaction with HOXt to afford the corresponding uronium salts HBMDU (**253**) and HAMDU (**254**) (Scheme 46)[390].

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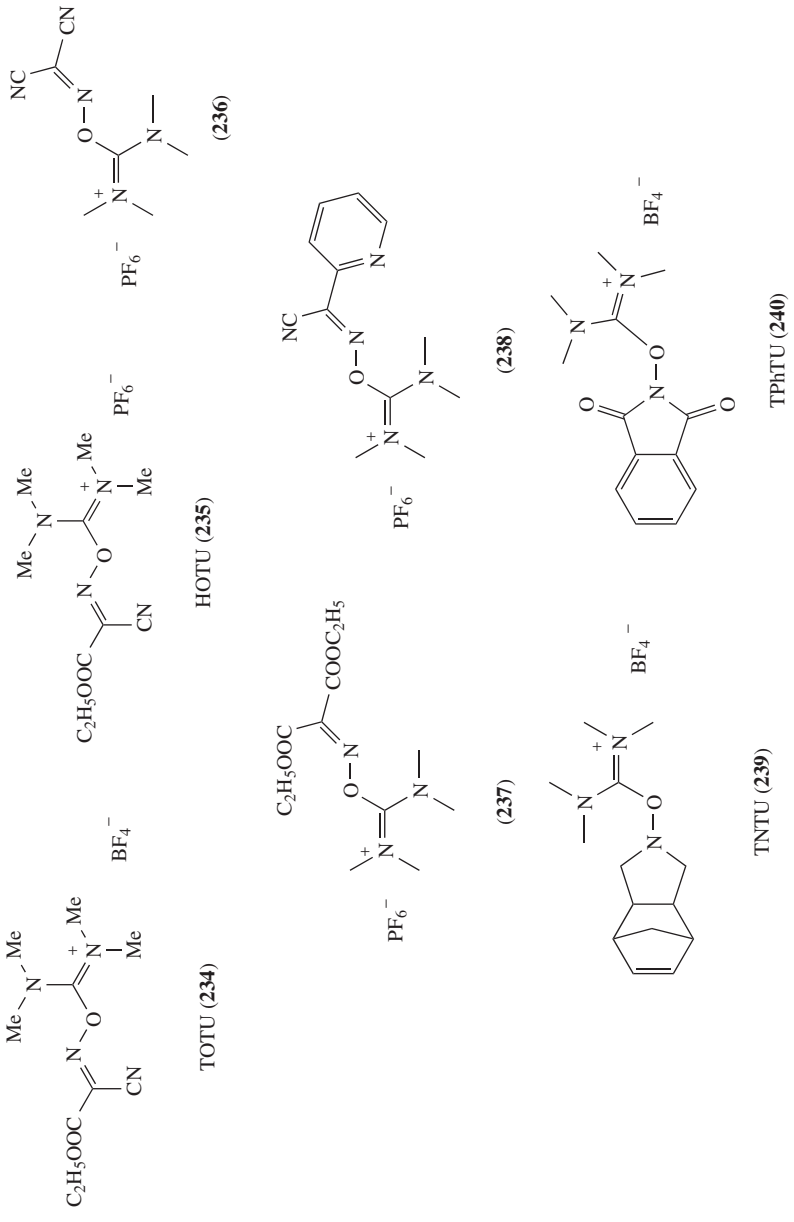
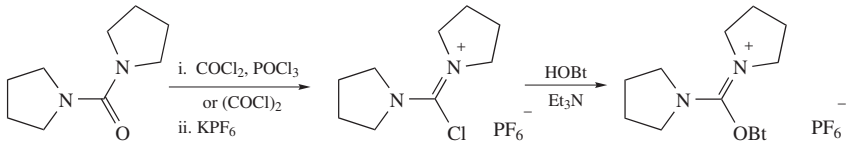
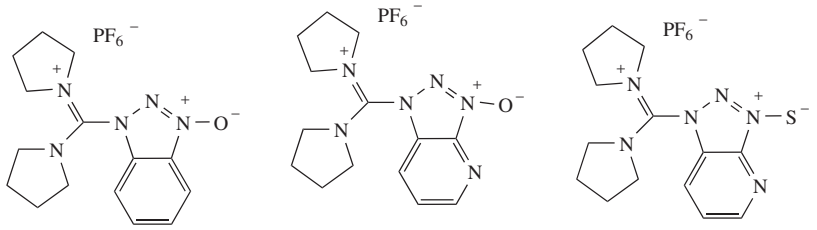


FIGURE 55. Structure of various uronium/iminium salts

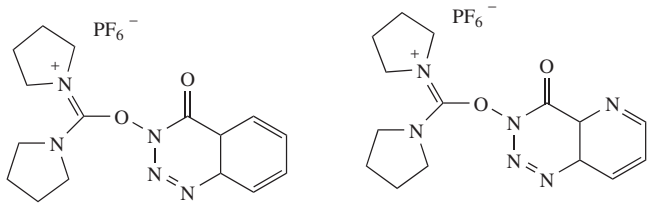


(**241**) BTCFH (**242**) HBPyU (**243**)

SCHEME 45. Synthesis of HBPyU

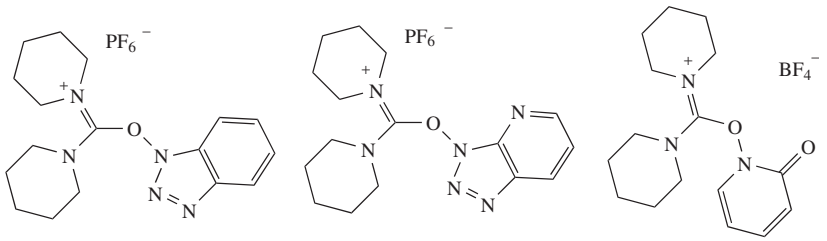


HBPyU (**243**) HAPyU (**244**) HATPyU (**245**)



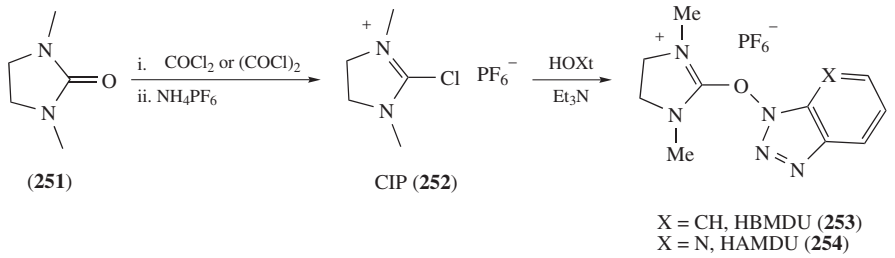
HDPyU (**246**) HDAPyU (**247**)

FIGURE 56. Structure of various bis-pyrrolidino iminium salts



HBPipU (**248**) HAPipU (**249**) TOPPipU (**250**)

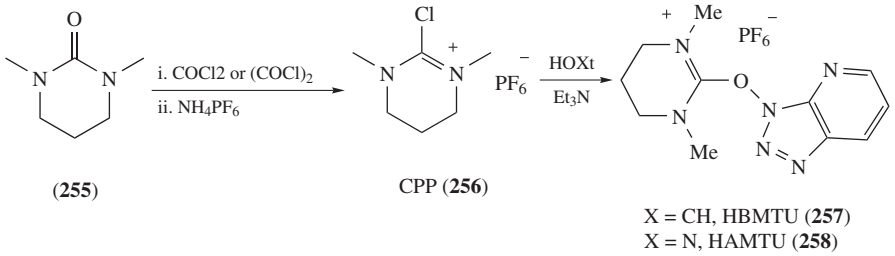
FIGURE 57. Structure of various bis-piperidino iminium salts



SCHEME 46. Synthesis of imidazolium uronium salts

E. *N*-Hydroxylamines for the Preparation of Pyrimidinium Uronium Salts

The chloro pyrimidinium salt (CPP, **256**) has been obtained by chlorination of the commercial 1,3-dimethyltetrahydropyrimidin-2(1*H*)-one (**255**) with phosgene or oxalyl chloride followed by anion interchange with KPF_6, followed by reaction with HOXt to afford the corresponding uronium salts HBMTU (**257**) and HAMTU (**258**) (Scheme 47)[390].

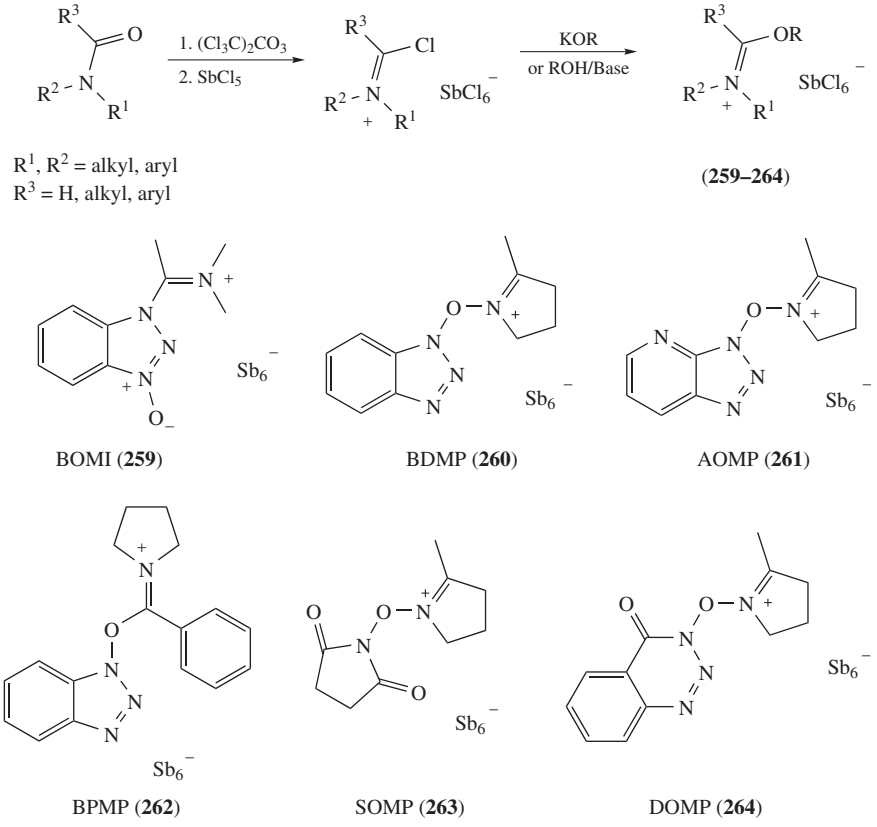


SCHEME 47. Synthesis of pyrimidinium uronium salts

F. *N*-Hydroxylamines for the Preparation of Unsymmetric Uronium/Iminium Salts

Several iminium salts derived from carboxamides have been prepared[391]. Thus, DMF has been transformed into an iminium chloride by reaction with triphosgene followed by stabilization with $SbCl_5$. Subsequent reaction with HOBt (**95**) gives benzotriazol-1-yloxy-*N*,*N*-dimethylmethaniminium hexachloroantimonate **259** (BOMI) (Scheme 48). Its structure was determined by X-ray analysis. The same methodology has been employed for the preparation of the immonium reagent **260** (BDMP) from *N*-methylpyrrolidine (NMP) and HOBt (**95**), in 80% overall yield (Scheme 48)[392]. When **95** is replaced by HOAt (**116**), the related reagent **261** (AOMP) is obtained[393]. The HOBt derived reagent **262** (BPMP), succinimide derived reagent **263** (SOMP) and the HODhbt derived reagent **264** (DOMP) have also been prepared from the more highly substituted *N*,*N*-tetramethylenebenzamide (Scheme 48)[393].

A few years ago, El-Faham and Albericio described a new family of iminium-type coupling reagents based on the differences in the carbocation skeletons of the coupling reagents, which correlated with differences in stability and reactivity[394, 395]. The unsymmetric tetra-substituted urea have been prepared from commercially available *N*,*N*-dialkylcarbamoyl chloride to afford the corresponding urea. The urea derivatives have been

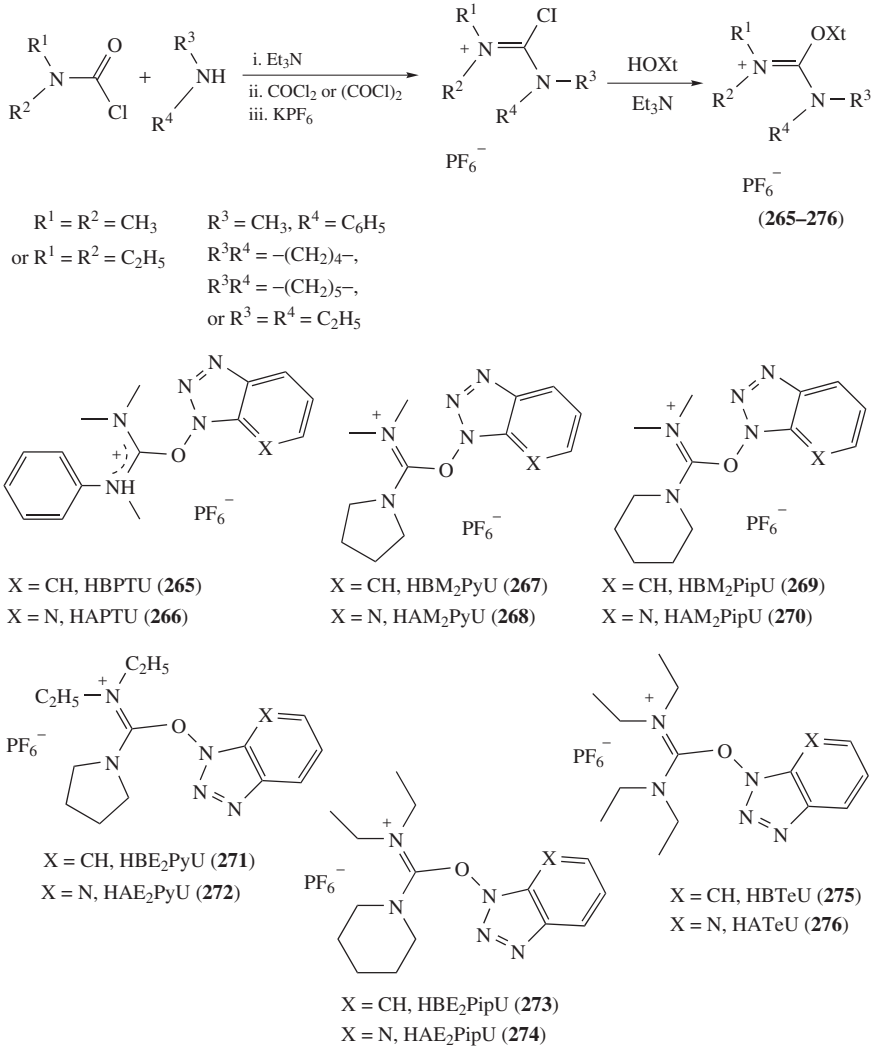


SCHEME 48. Synthesis of antimonate uronium salts

transformed into the corresponding chlorouronium salt by treatment with phosgene or oxalyl chloride followed by anionic interchange with KPF_6. Finally, reaction with HOBt (**95**) or HOAt (**116**) in the presence of triethylamine gave the corresponding aminium salts **265**–**276** (Scheme 49).

G. *N*-Hydroxylamines for the Preparation of Morpholino-based Uronium/Iminium Salts

Very recently, El-Faham and Albericio described a new family of immonium-type coupling reagents derived from dimethylmorpholino urea[396, 397]. The morpholine moiety has a remarkable effect on the stability and reactivity as well as decreasing the racemization extent. The recent uronium-type reagents can be readily prepared by treating *N*,*N*-methylcarbamoyl chloride (**277**) with morpholine (**278**) to give the corresponding urea derivatives (Scheme 50). The urea derivatives then react with oxalyl chloride to yield the corresponding chloro salts **279**, which are stabilized by the formation of a PF_6 salt (Scheme 50). Subsequent reaction with HOXt (HOBt, HOAt or 6-Cl-HOBt) in the

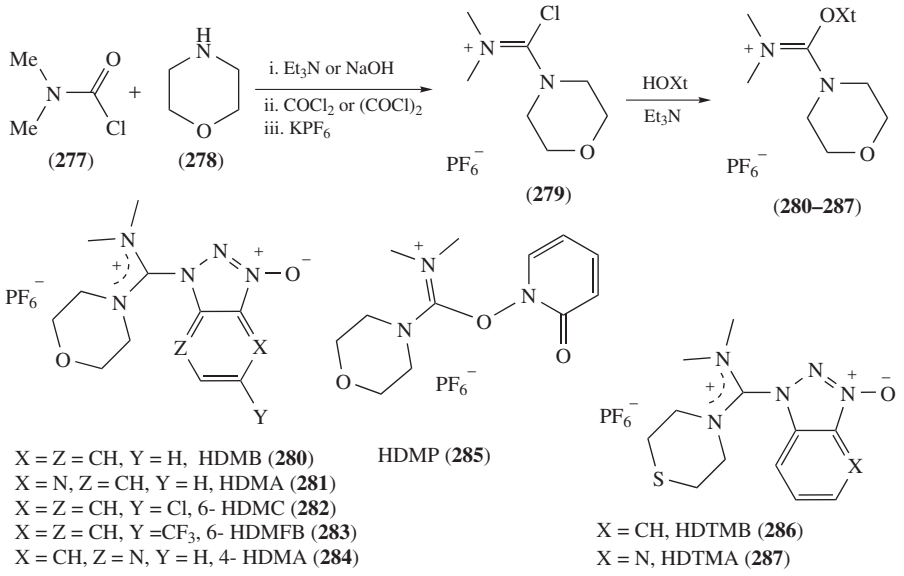


SCHEME 49. Synthesis of unsymmetrical uronium/iminium salts

presence of a tertiary amine such as Et_3N affords the desired compounds **280**–**287** as crystalline and shelf-stable solids (Scheme 50).

H. Oxyma for the Preparation of Uronium Salts

In a recent study, Subirós-Funosas, El-Faham and Albericio and coworkers showed that Oxyma (**1**) is an excellent replacement for HOBt (**95**) and its analogues[2]. Also, a third generation of uronium salt, COMU (**289**), which involves the combination of a



SCHEME 50. Synthesis of morpholino uronium/iminium salts

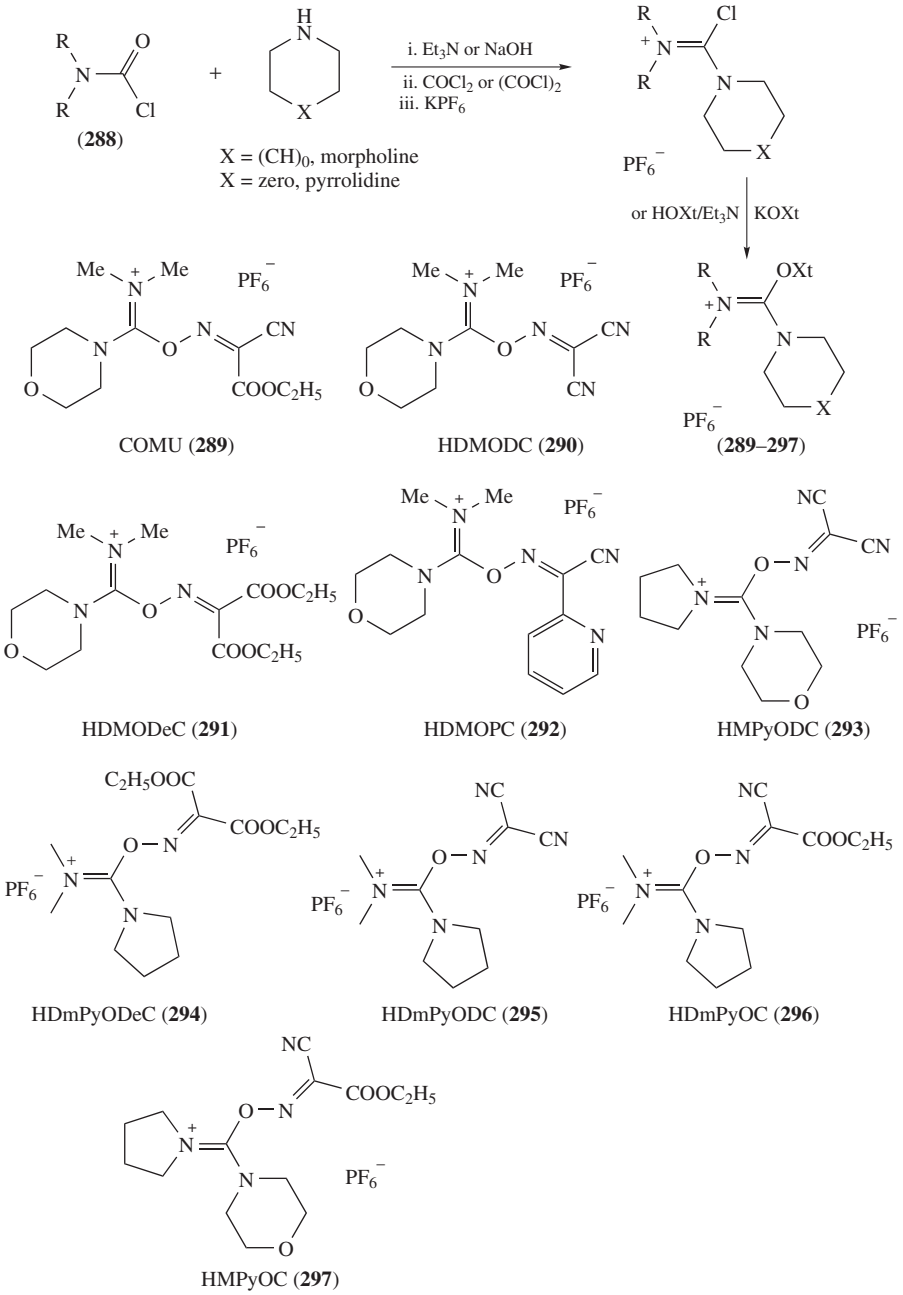
morpholinium-based immonium moiety as proton acceptor and **1** as leaving group, was devised by the same authors to provide a superior and safe coupling reagent for amide bond formation starting from **288** (Scheme 51)[162, 396, 397].

The oxygen in the iminium structure increased the stability of the coupling reagent compared with the tetramethyl derivatives. Furthermore, Oxyma (**1**) derivatives have higher stability than the benzotriazole derivatives, **230** and **218**. These observations are of practical relevance for both solid-phase and solution strategies. Although a typical protocol for the use of these reagents involves 2 equivalents of base, usually DIEA, the presence of the morpholinium moiety also allows good results with **289** when just 1 equivalent of DIEA is used. Alternatively, the less basic TMP (2 or even 1 equivalent) can be used instead of DIEA and provides good yields and reduces racemization[162].

Furthermore, **289** is compatible with microwave-assisted peptide synthesizers[30]. Consistent with previous reports, **290** displayed higher efficiency than **230** and **218** in the demanding synthesis of the Aib derivative of the Leu–Enkephalin pentapeptide and produced no Oxyma-based byproducts. Thus, the combination of microwave irradiation and **289** resulted in a similar performance to that observed by manual synthesis in a considerably shorter time. Also, the Oxyma-based salts showed much better results than other oxime derivatives (**290**–**297**, Scheme 51)[215].

I. Isonitroso Meldrum's Acid Reagents for the Preparation of Uronium Salts

Very recently, El-Faham, Subirós-Funosas and Albericio reported a new family of morpholino coupling reagents based on isonitroso Meldrum's acid (**298**), because of its structural similarity to Oxyma (**2**) with consideration that the introduction of two carbonyl groups in the six-member cyclic structure should enhance its reactivity due to the electron-withdrawing effect of the carbonyl group (Scheme 52)[398]. Although related to



SCHEME 51. Synthesis of Oxyma-based coupling reagents

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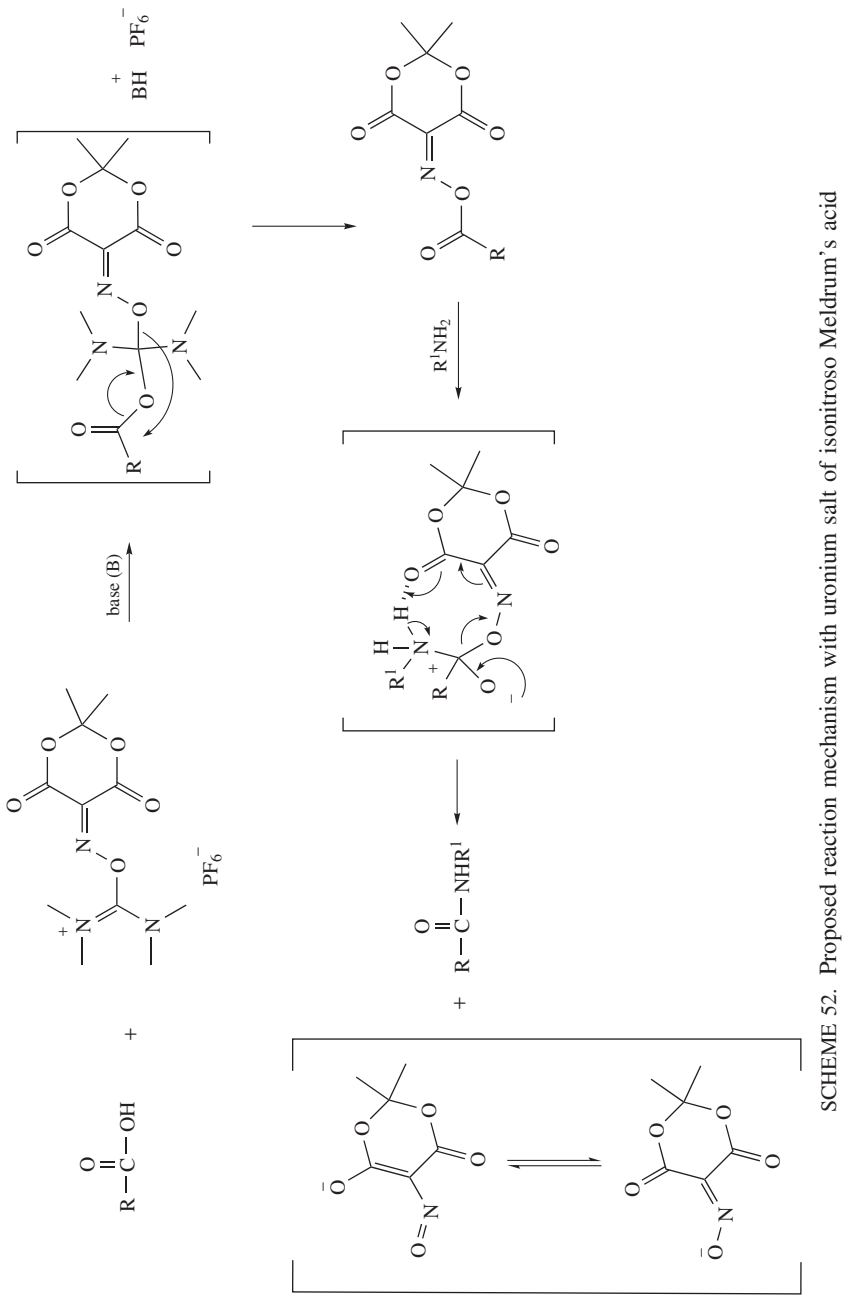
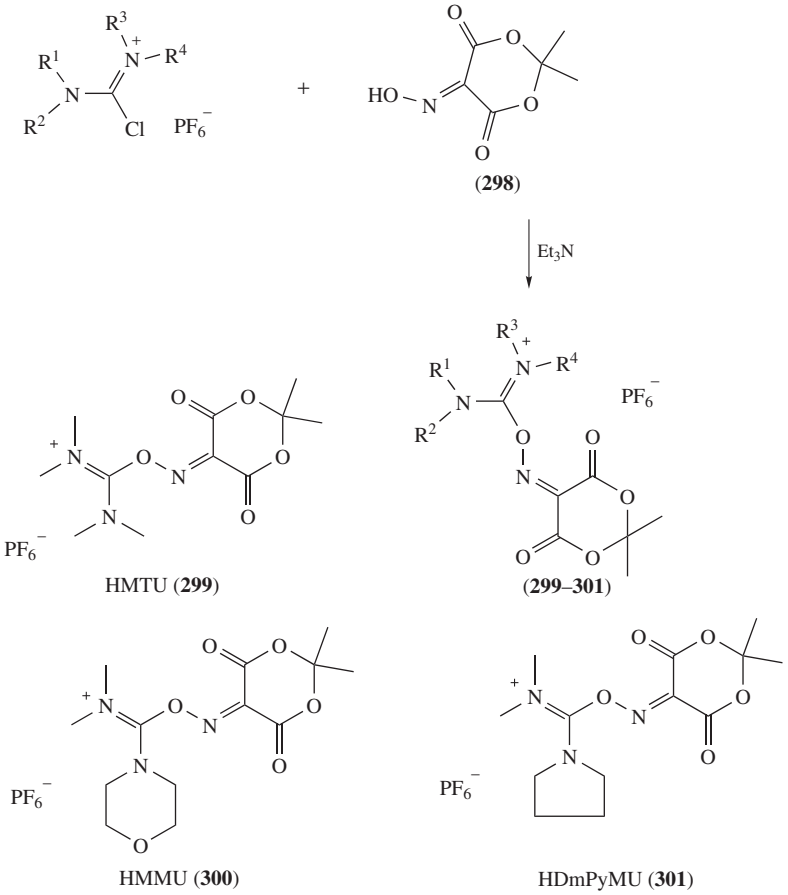


SCHEME 52. Proposed reaction mechanism with uronium salt of isonitroso Meldrum's acid



SCHEME 53. Synthesis of uronium salt of isonitroso Meldrum's acid

Oxyma in the same way, esters of Oxyma are not able to participate in the neighboring group effects commonly thought to enhance the effectiveness of cyclic Oxyma derivatives relative to those of Oxyma. Any neighboring group effect occurring in either case would involve the carbonyl function, as shown in the proposed mechanism in Scheme 52.

The novel uronium coupling reagents were prepared by reaction of the isonitroso Meldrum's acid (**298**) with the chloro salts in the presence of a tertiary amine such as Et_3N which afford the desired compound **299**–**301** as crystalline solids (Scheme 53).

The novel uronium coupling reagents were tested and compared to classical immonium salts HDMB (**280**) and HDMA (**281**) by using these models, which involve stepwise and also [2+1] segment coupling as well as solid-phase peptide synthesis[398].

VIII. *N*-HYDROXYLAMINES FOR THE PREPARATION OF PHOSPHORIC ACID DERIVATIVES

Since Takeuchi and Yamada[399] introduced the mixed carboxylic-phosphoric anhydride method using DPPA (**302**) from diphenylphosphorochloridate and sodium azide to peptide chemistry in 1972, various organophosphorus compounds have been developed as peptide-coupling reagents (Figure 58)[399–403].

On the basis of the earlier development of organophosphorus reagents, a great amount of effort has been focused on developing various coupling reagents of a similar kind, such as NDPP (**303**) and BDP (**304**) (Figure 58)[404–408].

A wider variety of phosphorous reagents, DEPAT (**305**), DPPAT (**306**), DEBPO (**307**), DOPBO (**308**), DOPBT (**309**) and DEPBT (**310**), have been prepared following a similar protocol (Scheme 54)[407–409]. **310** has been evaluated against other peptide-coupling reagents and gave good results in segment coupling reactions. Although the racemization-suppressing capacity of HODhbt (**154**) is greater than that of HOBt (**95**), its utility was limited due to side reactions.

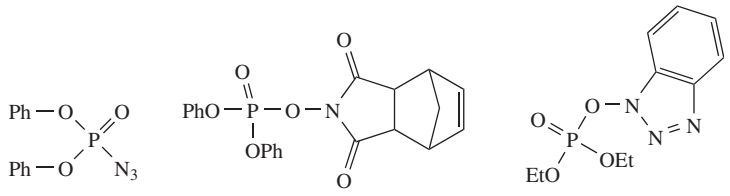
Carpino and coworkers introduced the organophosphorus reagents DEPAT (**305**) and DPPAT (**306**) (Scheme 54)[165]. In this case the neighboring group effects believed to be relevant to the properties of HOAt (**116**) are superimposed on the effects that enhance the efficiency of the phosphorus moiety. On the basis of the results described, these effects are related to the larger rate with which protected amino acids are converted to their active esters by the phosphorus derivatives.

Recently, phosphoric acid diethyl ester 2-phenylbenzimidazol-1-yl ester, diphenylphosphinic acid 2-phenylbenzimidazol-1-yl ester (Scheme 55) and phosphoric acid diphenyl ester and 2-phenylbenzimidazol-1-yl ester (**311**–**313**) have been reported as highly efficient coupling reagents[153, 156]. Their efficiency was evaluated through the synthesis of a range of amides and peptides, and the extent of racemization was found to be negligible[153].

IX. *N*-HYDROXYLAMINES FOR THE PREPARATION OF SULFONIC ACID DERIVATIVES

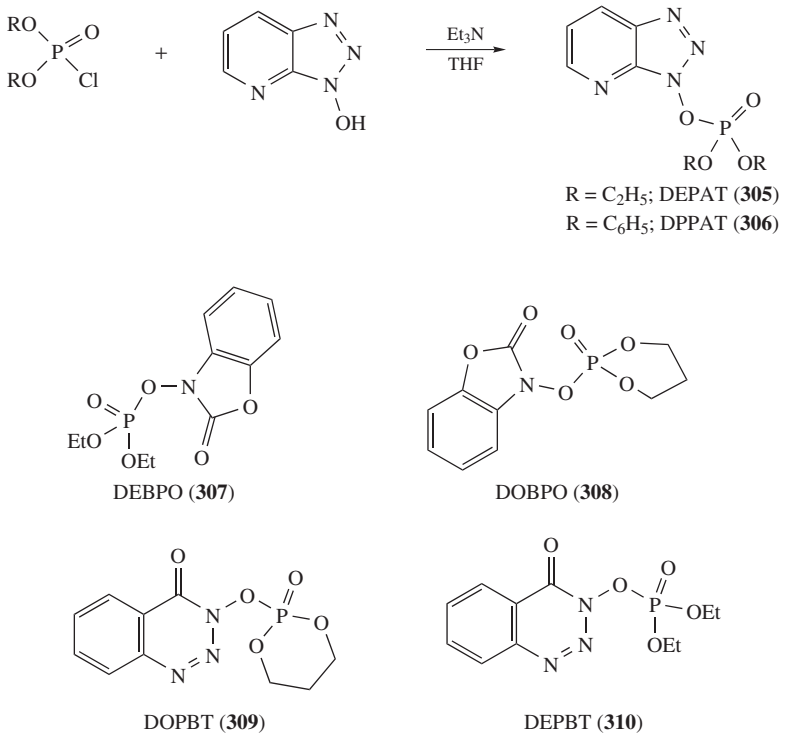
Esters of sulfonic acids and HOBt (**95**) related to those described for the phosphorus series have also been used for peptide coupling[4, 410–413]. The reactivity of such sulfonate esters was shown to be directly related to the presence of electron-withdrawing substituent in both **95** and the sulfonic acid moieties[414–417].

This methodology has seen little practical application however, since, depending on the basicity or reactivity of the amino component of the coupling process, the use of such sulfonate esters (**314**–**327**) for *in situ* coupling via the formation of an active ester is often compromised by formation of a sulfonamide byproduct (Scheme 56)[407, 408]. Such



DPPA (**302**) NDPP (**303**) BDP (**304**)

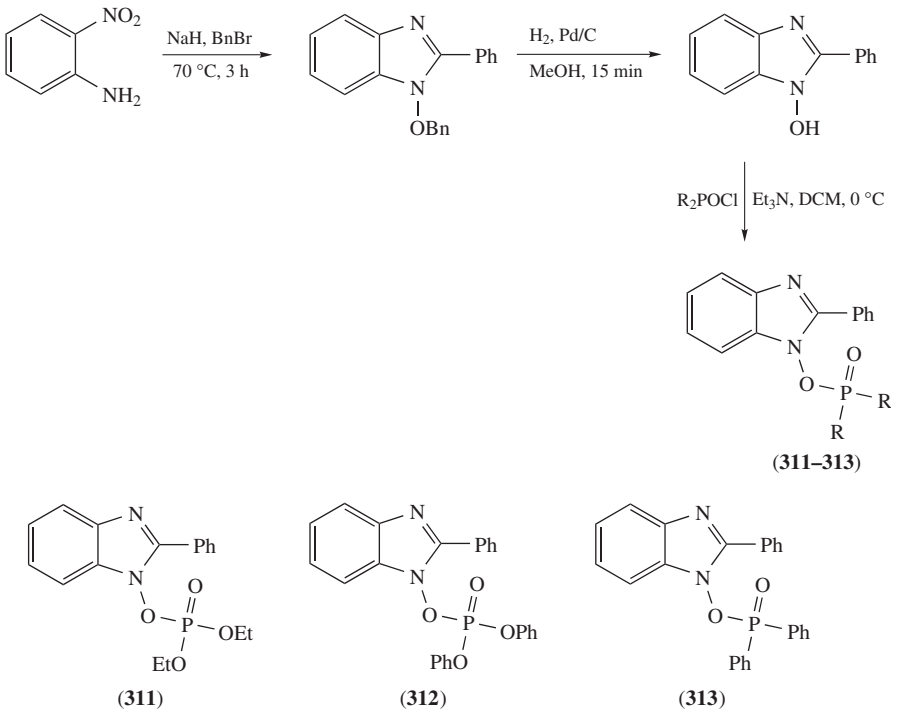
FIGURE 58. Stucture of DPPA, NDPP and BDP



SCHEME 54. Synthesis and structure of various organophosphorus coupling reagents

byproduct formation which, among amino acid derivatives, is particularly prominent with proline derivatives, can be avoided by preactivation of the reactive carboxylic acid component[418]. On the other hand, in the use of these reagents for segment coupling, especially if such conversions are slow, use of a preactivation step is counterproductive since loss of configuration at the C-terminal carboxylic acid residue directly increases with preactivation time[419]. As demonstrated in other systems, the substitution of HOAt (**116**) for **95** is expected to reduce the extent of configurational loss, although the advantages of **116** are lost at long preactivation times. The sulfonate esters were prepared by reaction of HOXt with the sulfonyl chloride in the presence of triethylamine (Scheme 56).

Khattab described a new family of sulfonate ester-type coupling reagents which differs in its leaving group[420]. The sulfonate ester coupling reagents ethyl 2-cyano-2-(naphthalen-2-ylsulfonyloxyimino)acetate (NpsOXY, **324**) and ethyl 2-cyano-2-(*p*-tosyloxyimino)acetate (TsOXY, **325**) are more efficient alternatives to the benzotriazole sulfonate esters in terms of racemization suppression and coupling effectiveness (Scheme 56). Both Oxyma (**1**) and its related sulfonate esters can be handled with a considerably lower risk than the explosive benzotriazole and its derivatives. 2-Oxopyridin-1(*2H*)-yl naphthalene-2-sulfonate (NpsOPy, **326**) and 2-oxopyridin-1(*2H*)-yl 4-methylbenzenesulfonate (TsOPy, **327**) sulfonate esters derived from 1-hydroxypyridin-2(*1H*)-one were also successfully used as new coupling reagents, requiring a longer preactivation time during the coupling process (Scheme 56). An improvement in yield



SCHEME 55. Synthesis of phosphorus reagents derived from 1-hydroxy-2-phenylbenzimidazole

and almost comparable optical purity to that of the 1-hydroxybenzotriazole (**95**) and 1-hydroxy-7-azabenzotriazole (**116**) analogues was observed[420].

X. *N*-HYDROXYLAMINES FOR THE PREPARATION OF POLYMER-SUPPORTED REAGENTS

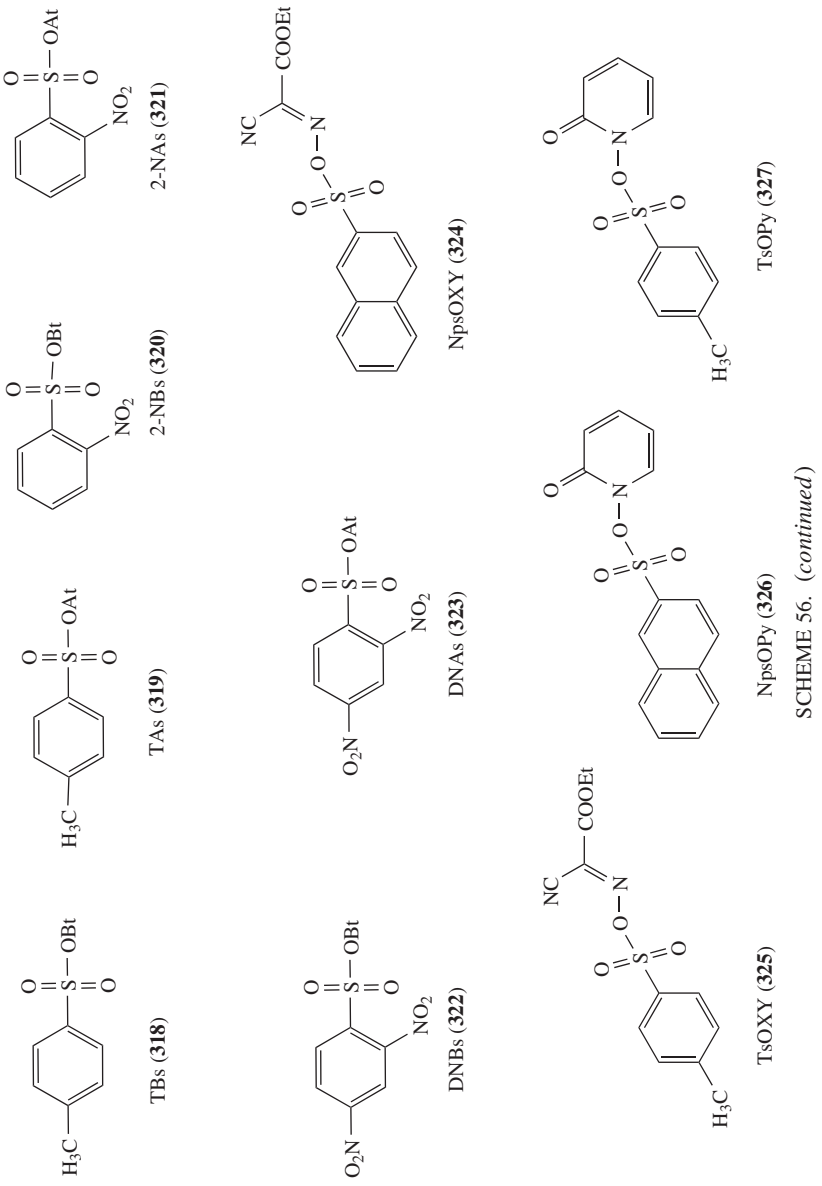
For the synthesis of peptides in solution phase, only a few solid-phase-supported reagents have been described.

A. Polymer-bound HOBt Reagents

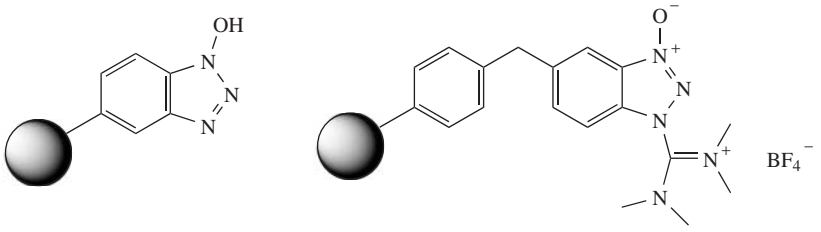
Polymer-bound HOBt (**328**) has been used for the formation of medium ring lactams with DCC in the presence of resin-bound esters and also for amides from primary and secondary amines (Figure 59). Polymer-bound TBTU (**329**) has also been used for the same purpose (Figure 59)[421–423].

The polymer-supported HOBt derivative **330**, bonded by a sulfonamide group to polystyrene, reacted with carboxylic acids in the presence of PyBroP (**201**) to give the corresponding active ester (Figure 60). Subsequent addition of amines afforded the corresponding amides in an automated procedure[100].

SCHEME 56. Synthesis and mechanism of peptide bond formation of organosulfur reagents

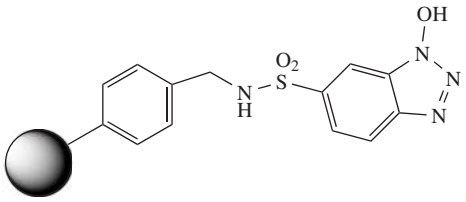


SCHEME 56. (*continued*)



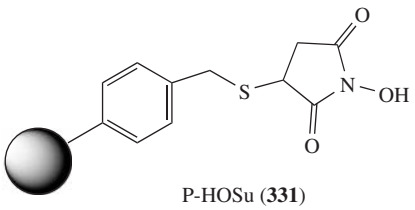
P-HOBt (**328**) P-TBTU (**329**)

FIGURE 59. Stucture of P-HOBt and P-TBTU



P- SO_2-HOBt (**330**)

FIGURE 60. Stucture of P-SO_2-HOBt



P-HOSu (**331**)

FIGURE 61. Stucture of P-HOSu

B. Polymer-bound HOSu Reagents

N-Hydroxysuccinimide bound to Merrifield and Argopore™ resins (**331**) also give resin-bound active esters, which are transformed into amides when reacted with primary, branched primary and secondary amines (Figure 61)[424].

XI. OTHER APPLICATIONS IN PEPTIDE SYNTHESIS

Peptide chemistry is often accompanied by the appearance of side reactions, occurring during activation of the *C*-terminal acid and coupling with the *N*-terminus. Acid or basic treatments include premature removal of the *N*-protecting group of a proresidue, and the deguanylidation of an Arg containing peptide leading to the corresponding Orn analogue when the side-chain of the Arg is unprotected[101, 425–431]. One of the most relevant undesired reactions is the intramolecular cyclization of aspartic acid (Asp) residue to give

amino-succinyl units, commonly called aspartimides (abbreviated as Asi, Asu or Asc), as a result of the nucleophilic attack of the amide nitrogen of the preceding residue to the β-carboxyl moiety of Asp[432, 433]. This amino-succinyl moiety can subsequently undergo opening by many nucleophiles, like water, obtaining a mixture of the isoaspartyl-β-peptide, the unmodified α-peptide and even the racemized versions[221, 434, 435].

Aspartimide formation is reported to arise under mild basic coupling conditions and after treatment of the peptide with strong acids, such as TFA or HF[432, 435–439]. In addition, the required piperidine treatment for removal of the Fmoc group also generates α- and β-piperidides[432, 437, 440, 441]. The extent of aspartimide formation and the ratio of byproducts is influenced by the type of base/acid used, β-carboxyl group, resin, temperature, solvent, sequence and conformation[434, 435, 437, 442–447]. Like most peptide-associated byproducts arising in peptide synthesis, formation of aspartimides and derivatives results in permanent modification of the peptide chain, posing significant difficulties in their purification. This event is especially troublesome in the case of APIs, even when such impurities are found in minimal amounts.

Besides its application in peptide bond formation and in the minimization of related side reactions, *N*-hydroxylamine-based additives are also beneficial in reducing the impact of other non-coupling derived byproducts. Thus, after the removal of the Fmoc group from the *N*-terminal of the peptide resin and the corresponding washings with the solvent, 3–4 washings with 0.1 M HOBt (**95**, Section III.H.1)/Oxyma (**1**, Section III.A) solution in DMF/NMP minimizes the removal of the Fmoc group of the next amino acid. Similarly, in a peptide resin containing an Arg residue with unprotected side-chain, before the incorporation of the incoming amino acids, washing with 0.1 M HOBt (**95**, Section III.H.1)/Oxyma (**1**, Section III.A) solution in DMF/NMP reduces Orn formation. The positive effect induced by *N*-hydroxyamines has also been observed in the base-catalyzed unwanted cyclization of Asp to generate aspartimide units[2, 162, 249, 276, 337, 382]. This strategy might not completely suppress aspartimide formation, but in syntheses of low- to mid-prone sequences it is usually sufficient. Alternative approaches have been developed, such as the use of sterically-hindered bases and protecting groups, mild-cleavable *N*-α-protecting groups, or the pseudo-proline approach, but they are rather expensive, difficult to synthesize/introduce, require additional steps and depend on the protection scheme[436, 440–452]. In contrast, *N*-hydroxylamines commonly used as additives in peptide synthesis are easily available, are of low cost and their reaction is faster.

The unique acidic properties of *N*-hydroxylamines ($pKa = 2–10$) are assumed to be responsible for this behavior, since they create a competition with the Asp-X amide backbone for the base, considered as the key step of aspartimide formation[221]. The positive effect induced by the presence of acidic *N*-hydroxylamines was first and unexpectedly observed during coupling in a Asp(OBzl)-Gly dipeptide model[280]. Addition of *N*-hydroxysuccinimide (HOSu, **31**, Section III.D.1) ($pKa = 5.1$) resulted in a 3-fold decrease in the cyclization rate of Asp to amino-succinyl residue, whereas 1-hydroxybenzotriazole (**95**, Section III.H.1) ($pKa = 4.3$) induced 20 times more inhibition than the experiment without additive[221]. It was also found that concentration of the additives is a key factor in the suppression of aspartimides, and that use of an equimolar amount of base and additive is the most efficient combination[221]. Interestingly, it was also noted that the DIC/HOBt approach gives rise to a lower percentage of byproducts than using HOBt-based TBTU and DIEA, presumably due to the absence of base in the coupling mixture[453]. In addition, an excess of coupling reagents leads to an increase in the extent of this side reaction[450].

Later, with the rise of the Fmoc/*t*-Bu scheme as the predominant protecting strategy, the impact of the inclusion of *N*-hydroxylamines in piperidine solutions was evaluated[442, 446, 447]. Similarly to what was observed during coupling in solution, equimolar amounts of weakly acidic additives, in respect to the base, substantially reduce aspartimide formation[442]. Thus, 2% of **95** (Section III.H.1) in 20% piperidine in DMF

resulted in an increase of the target peptide from 40% to 67%[447]. The presence of **95** was also beneficial in reducing to traces the amount of amino-succinyl peptide formed in peptides adopting conformations favorable toward this intramolecular cyclization[446, 447]. Increasing amounts of additive have been shown to induce higher suppression[447, 454].

Outstanding aspartimide-suppressant procedures have been described using *N*-hydroxylamines, like the combination of **95**, piperazine and microwave irradiation or the use of the cocktail hexamethyleneimine/*N*-methylpyrrolidine/HOBt/NMP/DMSO 4:50:4:71:71 (v/v/w/v/v), which minimizes to 1–5% the impact of aspartimide formation[453, 455–457]. Recently, HOAt (**116**, Section III.I.1), the 7-aza-analogue of HOBt (**95**, Section III.H), proved to be at least equally efficient as the parent benzotriazole[454]. The best results, however, are achieved by the presence of Oxyma (**1**, Section III.A)[454].

XII. ACKNOWLEDGMENT

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Capítol 2. *Oxyma com a additiu per carbodiimides*

Publicació II

Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion†

Oxyma: un additiu eficient per síntesi de pèptids reemplaçant els benzotriazols HOBt i HOAt amb un menor risc d'explosió

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† Ramon Subirós Funosas va realitzar els experiments d'epimerització de cisteína, d'eficiència del reactiu Oxyma en el sistema ACP (65-74) i en els diversos anàlegs de Leu-encefalina, així com els experiments d'estabilitat a nucleòfils. També va participar de la colaboració amb la Plataforma de Calorimetria en el disseny i discussió dels assajos calorimètrics DSC i ARC. El manuscrit va ser íntegrament elaborat pel doctorand.

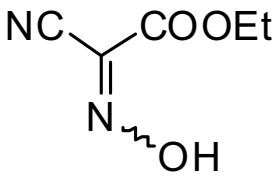
Ayman El- Faham es va encarregar de realitzar la resta d'experiments d'epimerització.

Rafel Prohens i Rafael Barbas, de la Plataforma de Calorimetria, van dur a terme els assajos calorimètrics, a més de aportar una valoració i discussió dels resultats.

Resum

El 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma) s'ha provat com additiu en l'estratègia que utilitza carbodiimides per a la formació de l'enllaç peptídic. Les prestacions d'Oxyma s'han estudiat i comparat, en relació a HOBt i HOAt, dels quals se n'ha descrit recentment el caràcter explosiu, cosa que ha accelerat la recerca d'una nova familia de substituts, que compleixin com a requisits promoure una alta eficiència d'acoblament, de manera segura.

Oxyma ha mostrat, en la publicació annexa, una excel·lent capacitat de reduir l'epimerització del carboni-α, unit a una excel·lent eficiència d'acoblament tant en síntesis automàtiques com manuals. En tots aquest experiments es va mostrar més eficaç que HOBt i com a mínim comparable a HOAt, superant aquest últim en els sistemes peptídics que impliquen un major impediment estèric. D'altra banda, els assajos d'estabilitat van demostrar que no hi ha perill de bloquejar el *N*-terminal del pèptid sota condicions habituals d'acoblament. Finalment, per mitjà dels assajos calorimètrics DSC i ARC es van observar perfils de descomposició per part de HOBt i HOAt consistents amb les propietats explosives descrites recentment a la literatura. En un extrem totalment oposat, els mateixos experiments van suggerir que el 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma) indueix un risc d'explosió considerablement menor.



2-ciano-2-hidroxiiminoacetat d'etil (Oxyma)

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Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion[1]

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Abstract: Oxyma [ethyl 2-cyano-2-(hydroxyimino)acetate] has been tested as an additive for use in the carbodiimide approach for formation of peptide bonds. Its performance in relation to those of HOBt and HOAt, which have recently been reported to exhibit explosive properties, is reported. Oxyma displayed a remarkable capacity to inhibit racemization, together with impressive coupling efficiency in both automated and manual synthesis, superior to those of HOBt and at least comparable to those of HOAt, and surpassing the latter coupling agent in the more demanding peptide models. Stability assays showed that there was no risk of capping the resin under standard coupling conditions. Finally, calorimetry assays (DSC and ARC) showed decomposition profiles for benzotriazole-based additives that were consistent with their reported explosivities and suggested a lower risk of explosion in the case of Oxyma.

Keywords: additives · calorimetry · oximes · peptide synthesis · solid-phase synthesis

Introduction

The use of additives to support various coupling methodologies is common practice in research laboratories devoted to peptide chemistry. Of these compounds, the most extensively used displayed a benzotriazole core: 1-hydroxybenzotriazole (HOBt), probably the most common reagent found in a peptide synthesis laboratory, was the first to be unveiled in the early 1970s,[2] whereas later on the use of the more powerful 1-hydroxy-7-azabenzotriazole (HOAt)[3] and, more recently, 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) for the same purpose was reported.[4] The presence of any of these compounds in the coupling medium induces the formation of an active ester, which then undergoes aminolysis to yield the desired peptide bond. The acidities of the additives (pK_a for HOBt: 4.60, pK_a for HOAt: 3.28, and pK_a for 6-Cl-HOBt: 3.35)[5,6] are key factors with regard to the stabilities and reactivities of their related active esters, because they are connected with their behavior as leaving groups. In addition, the nitrogen at the 7-position in HOAt provides a classic neighboring group effect that can both increase the reactivity and reduce racemization.[3]

The carbodiimide approach to formation of peptide bonds has long taken advantage of the properties of benzotriazole-based additives, because the active esters formed are less reactive but more stable than the *O*-acylisoureas. These intermediate species lead to lower levels of racemization and the suppression of other undesired side reactions such as the formation of inactive *N*-acylisoureas.[7] Other coupling strategies, such as the combination of base and stand-alone coupling reagents, such as immonium (HATU, HBTU/TBTU, and HCTU/TCTU) or phosphonium salts (PyAOP, PyBOP,

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and PyClock) have also been enhanced by the use of these additives.[8–10]

However, a potentially explosive character of HOBt and its related additives has recently been reported.[11] This observation has led to their reclassification under a Class 1 explosive category and has consequently increased transportation difficulties. In view of the relevance of these compounds in day-to-day peptide chemistry, it became evident that there was a need for another family of safe and efficient additives, based on a different template. This poses a difficult challenge if it is borne in mind that the performances of additives based on other compounds, such as *N*-hydroxysuccinimide (HOSu) or pentafluorophenol (HOPfp), are not comparable to those of HOBt and HOAt, because active esters of OSu or OPfp esters are less reactive than benzotriazole esters.[12–14]

Here we report an exhaustive study of ethyl 2-cyano-2-(hydroxyimino)acetate, an oxime first described in the 1970s with an acidity similar to those of the above additives (pK_a 4.60).[15] The suitability of this compound as a substitute for benzotriazole-based additives is discussed in terms of its capacity to control racemization, its effectiveness in difficult couplings either in manual or automated synthesis, and its stability in the presence of growing peptide chains. We have also evaluated the safety profile of this additive by calorimetric techniques.

Results and Discussion

In our search for a class of safe and efficient additives, we came across a family of strongly acidic oximes that showed properties of interest, reported by Itoh in the early 1970s and by Izdebsky a few years later.[15,16] Out of all the oximes tested in those studies, ethyl 2-cyano-2-(hydroxyimino)acetate (Figure 1, from now on referred to as Oxyma) displayed an appropriate balance of availability and ease of handling. Nonetheless, since the publication of those papers, which were not conclusive about its performance relative to those of benzotriazoles, Oxyma has not played an active role in day-to-day peptide chemistry, nor has it been used for general amide bond formation.

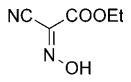


Figure 1. Structure of ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma).

Deeper investigation into racemization and the compound's effectiveness for coupling yields were therefore carried out. For study of racemization, peptide models (Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2 in solution and H-Gly-Cys-Phe-NH_2 on solid-phase) more reliable than those chosen in the previous papers were used for comparison of HOAt, HOBt, *N*-hydroxy-2-pyridinone (HOPO), and Oxyma.[17]

With regard to stepwise coupling (Z-Phg-OH onto H-Pro-NH_2), the non-benzotriazole-based additive HOPO (selected due to its association with low levels of racemization in two-phase systems),[18] although giving a higher yield of target dipeptide than HOAt and HOBt (Table 1, entry 5 vs entries 1 and 2), gave poorer retention of configuration (Table 1), especially when a 2 min preactivation time was used (Table 1, entry 6). In contrast, the performance of Oxyma (Table 1, entry 3) exceeded not only that of HOPO, but also those of HOBt and even HOAt (1.0%). Nevertheless, in the experiment conducted without preactivation, some of the starting acid remained unreacted. This inconvenience was overcome by use of a 2 min preactivation period (Table 1, entry 4). Moreover, the outstanding lack of racemization was maintained (1.1%).

Table 1. Yields and racemization during the formation of Z-Phg-Pro-NH_2 in DMF (stepwise solution-phase synthesis).[a]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	81.4	3.3
2	HOBt/DIC	81.9	9.3
3	Oxyma/DIC[b]	89.9	1.0
4	Oxyma/DIC[c]	88.2	1.1
5	HOPO/DIC	88.2[d]	17.4
6	HOPO/DIC[c]	83.4	26.1

[a] Couplings were conducted without preactivation, except for entries 4 and 6. LL and DL epimers of the test dipeptide have been described elsewhere.[17] The t_R values of LL and DL were identified by coinjection with pure samples of LL. [b] Unreacted Z-Phg-OH was detected. [c] A 2 min preactivation time was used. [d] Extra peak was found at 22.4 min (6.0%).

In the case of the more racemization-prone [2+1] segment coupling (Z-Phe-Val-OH onto H-Pro-NH_2), a similar trend was observed (Table 2). HOPO was the poorest racemization-suppressing additive out of the compounds tested, with a percentage of DL epimer of nearly 50% (Table 2, entry 4). In view of the results with Oxyma in the stepwise model, only the 2 min preactivation experiment was performed. In the segment model, the degree of racemization was clearly lower than that seen with HOBt and comparable to that achieved with HOAt (Table 2, entry 3 vs entries 1 and 2). Moreover, the yield obtained with Oxyma was higher (almost 90%).

Table 2. Yields and racemization during the formation of Z-Phe-Val-Pro-NH_2 in DMF (segment solution-phase synthesis).[a]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	86.1	2.1
2	HOBt/DIC	78.8	8.9
3	Oxyma/DIC[b]	89.8	3.8
4	HOPO/DIC	88.5	45.1

[a] Couplings were conducted without preactivation, except in the case of entry 3. LLL and LDL forms have been described elsewhere[17] and were coinjected with authentic and pure samples. [b] A 2 min preactivation time was used.

Further racemization experiments were carried out with regard to loss of configuration during elongation of Cys-containing peptides in the solid-phase approach (Table 3). For

that purpose, the model tripeptide H-Gly-Cys-Phe-NH_2 was assembled in stepwise fashion on a Fmoc-RinkAmide-MBHA-PS resin (0.45 mmol g^{-1}), with use of a 5 min preactivation time and Cys(Trt) as protecting group.[19,20] Levels of racemization were generally much lower than in the Z-Phg-Pro-NH_2 or Z-Phe-Val-Pro-NH_2 models, with $<1\%$ of DL epimer being obtained (Table 3). Comparison between HOAt, HOBt, and Oxyma showed the same trend as observed in solution phase: Oxyma performed with a level of inhibition of racemization similar to that of HOAt (Table 3, entry 1 vs entry 3) and superior to that of HOBt (Table 3, entry 2 vs 3 entry). Moreover, Oxyma afforded the tripeptide in a higher yield than the other additives (Table 3, entries 1 and 2 vs entry 3).

Table 3. Yields and racemization during the elongation of H-Gly-Cys-Phe-NH_2 in DMF (stepwise solid-phase synthesis).[a,b]

Entry	Coupling reagent	Yield [%]	DL [%]
1	HOAt/DIC	88.4	0.1
2	HOBt/DIC	84.1	0.2
3	Oxyma/DIC	90.8	0.1

[a] A 5 min preactivation time at room temperature was used in all experiments. The t_R values for LL and DL forms were identified by coinjection with authentic and pure samples. [b] An extra peak at 3.5 min, corresponding to the disulfide-bonded dimer (~1%), was found in all experiments.

The effectiveness of Oxyma in terms of yields in the solid-phase approach was tested by the assembly of the ACP (65–74) decapeptide (H-Val-Gln-Ala-Ala-Ile^{69}-Asp-Tyr-Ile^{72}-Asn-Gly-NH_2) on a Fmoc-RinkAmide-Aminomethyl-PS resin (0.63 mmol g^{-1}).[9c] For this purpose, an ABI 433 A peptide synthesizer and standard Fmoc/*t*Bu protocols were used. The relative effectivenesses of Oxyma, HOAt, and HOBt were tested in terms of percentage of target decapeptide and deletion peptides obtained. Before comparison of the additives, it was necessary to find suitable conditions under which differences would be clearly detectable. Five- or 10-fold excesses of reagents (Fmoc-amino acids, DIC, and additive) were not satisfactory for achieving this objective because nearly quantitative yields of ACP were obtained with HOBt. Use of twofold excesses, however, yielded mixtures of various deletion peptides, so relative potencies were more emphasized. No problems were encountered in the preparation of a solution of Oxyma in DMF.

Several deletion peptides were detected with use of the different additives, which made separation highly challenging. Those originating from misincorporation of a single amino acid (i.e., des-Ile^{72}, des-Ile^{69}, des-Val, des-Ala, des-Gly, or des-Asn) were the most common, but misincorporations of two amino acids also occurred (see Table 4). HOAt was the most efficient additive, with more than a 70% yield of ACP being obtained (Table 4, entry 1). Although the percentages of some deletion peptides were lower with HOBt, the decapeptide was produced only in about 60% yield (Table 4, entry 2). In contrast, the performance of Oxyma was clearly closer to that of HOAt than to that of HOBt (69%, entry 3, Table 4).

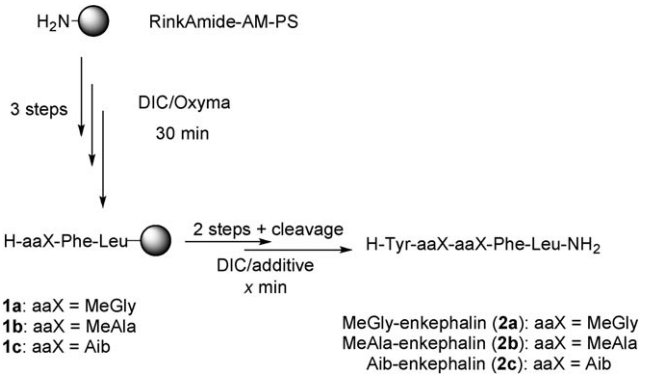
The performances of HOAt, HOBt, and Oxyma were further tested in the manual synthesis of Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-NH_2) analogues.[10] The modifications included the replacement of consecutive Gly amino acids by MeGly, MeAla, or Aib residues. These *N*-methylated or α,α-disubstituted residues were selected because they show steric hindrance suitable for emphasizing differences in reactivity between the active esters. The strategy followed in setting up the experiment is illustrated in Scheme 1.

Resin-bound tripeptides **1a**, **1b**, and **1c** of general structure H-aaX-Phe-Leu-resin were manually assembled on a Fmoc-RinkAmide-AM-PS resin (0.63 mmol g^{-1}) by means of 30 min couplings with use of DIC/Oxyma. A quantitative yield was verified by use of the Kaiser test for primary amines. There was no need for recoupling. Stepwise incorporation of the last two residues was then studied with each additive. Amino acids were preactivated for 3 min to ensure full formation of the active esters being compared. Coupling

Table 4. Percentages of ACP (65–74) decapeptide (H-Val-Gln-Ala-Ala-Ile^{69}-Asp-Tyr-Ile^{72}-Asn-Gly-NH_2) and various deletion peptides obtained during automated synthesis using different additives.[a]

Entry	Coupling reagent	des-2Ile [%]	des-Ile-Ala [%]	des-Ile^{72} [%]	des-Ile-Gly [%]	des-Ile^{69} [%]	des-Val [%]	des-2Ala [%]	des-Ala [%]	des-Gly [%]	ACP [%]	des-Asn [%]
1	HOAt/DIC	1.7	3.9	5.9	–[b]	5.8	1.8	2.9	4.2	0.5	71.8	1.5
2	HOBt/DIC	0.5	2.5	12.6	7.8[c]	4.9	3.2	–[b]	6.0[d]	0.2	62.3	–[b]
3	Oxyma/DIC	0.6	2.6	9.9	–[b]	7.9	2.1	2.7	5.1	0.1	68.7	0.3

[a] HPLC-MS showed the right mass for the ACP decapeptide at 1062.7. [b] No misincorporation was detected. [c] The observed mass could also correspond to that of the des-Ala-Val product. [d] Along with the mass of des-Ala, that of des-Gln was also detected.



Scheme 1. Strategy followed in the manual synthesis of Leu-enkephalin analogues to test the different additives.

times ranged from 5 min to 1 h depending on the Leu-enkephalin analogue, under conditions that afforded the best scenarios for comparing the additives. Pentapeptides **2a**, **2b**, and **2c** of general sequence H-Tyr-aaX-aaX-Phe-Leu-NH_2 were obtained after cleavage from the resin with concentrated TFA. The efficiency of each additive was measured in terms of percentages of pentapeptide and deletion tetrapeptides (and occasionally also starting tripeptide) obtained.

The pentapeptide model **2a** (H-Tyr-MeGly-MeGly-Phe-Leu-NH_2) was, compared to **2b** and **2c**, the least difficult to assemble. Therefore, it is not surprising that a 1 h couplings for the incorporation of both MeGly and Tyr were not the most suitable conditions to highlight the relative efficiencies of the different additives, because quantitative yields of **2a** were obtained. Coupling times were thus shortened to 5 min for this purpose, although even then high and similar percentages were detected with all three additives (see Table 5). Nevertheless, the deletion tetrapeptides des-MeGly and des-Tyr (and even starting tripeptide **1a**) were found in the crude products. The highest percentage of pentapeptide was obtained in the experiment with HOAt (94.9%, entry 1, Table 5), an excellent purity for such extreme conditions. HOBt was not as effective as its aza counterpart, but still provided the pentapeptide **2a** in an 84.8% yield (Table 5, entry 2). Under these conditions, the performance of Oxyma was impressive, surpassing that of HOBt (Table 5, entry 3), rising above 90% in percentage of pentapeptide, and showing a similar potency to HOAt. There was almost no starting tripeptide in the crude mixture (0.6%). In some cases, the percentage of des-Tyr was higher than that of des-MeGly, thereby confirming that this model pentapeptide was the least demanding.

Table 5. Percentages of H-Tyr-MeGly-MeGly-Phe-Leu-NH_2 (**2a**) and related deletion peptides obtained after solid-phase assembly with 5 min coupling times with the different additives.[a]

Entry	Coupling reagent	Pentapeptide [%]	des-MeGly [%]	des-Tyr [%]	Tripeptide [%]
1	HOAt/DIC	94.9	1.4	3.2	0.5
2	HOBt/DIC	84.8	7.5	6.6	1.1
3	Oxyma/DIC	91.4	3.8	4.2	0.6

[a] HPLC-MS showed the right mass for the pentapeptide at 583.8.

In view of our observations of the structural similarities between pentapeptide **2b** (H-Tyr-MeAla-MeAla-Phe-Leu-NH_2) and **2a**, we applied the same coupling times. In this system, however, very little pentapeptide was formed, but surprisingly Oxyma was almost ten times more effective than HOAt (0.6% for HOAt, 0.2% for HOBt and 5.3% for Oxyma). When 30 min couplings were used, considerable percentages of pentapeptide were observed and, unlike in the previous model, more significant differences arose (see Table 6). Synthesis with HOBt resulted in the least pure crude mixture (below 50%, entry 2, Table 6), whereas the other two additives afforded the pentapeptide **2b** in a much more efficient manner. Again, the performance of Oxyma was slightly better than that of HOAt (Table 6, entry 3 vs entry 1), with the purity being increased up to almost 80%. This superiority is attributed to the greater effectiveness of the incorporation of the MeAla residue, whereas the incorporation of Tyr was more even. The high percentage of the des-MeAla product relative to the des-Tyr product confirms the higher complexity of the sequence in relation to that of **2a**. The presence of a certain pentapeptide lacking the N-methyl group at the MeAla residues (about 1%), regardless of the additive used, should be mentioned.

Table 6. Percentages of H-Tyr-MeAla-MeAla-Phe-Leu-NH_2 (**2b**) and related deletion peptides obtained in solid-phase assembly with 30 min coupling times with the different additives.[a]

Entry	Coupling reagent	Pentapeptide [%]	des-MeAla [%]	des-Tyr [%]	Tripeptide [%]
1	HOAt/DIC	73.6	23.2	3.1	0.1
2	HOBt/DIC	46.1	38.1	15.2	0.6
3	Oxyma/DIC	79.0	16.9	4.0	0.1

[a] HPLC-MS showed the right mass for the pentapeptide at 611.5.

The coupling conditions that had afforded the pentapeptide **2b** in moderate yields were not valid for the **2c** analogue (H-Tyr-Aib-Aib-Phe-Leu-NH_2),[9c] because the purities were much lower (see Table 7). The enormous percentages of the des-Aib product present in the crude mixtures confirm that the synthesis of **2c** is the most demanding of those of the three analogues. Consistently with the trend observed in the previous tests, it was observed that HOBt was the least efficient additive, capable of affording the pentapeptide only in extremely low purity (3%, Table 7, entry 2). Use of its sister additive HOAt did not greatly increase the level of purity (11%, entry 1, Table 7). The superiority of Oxyma in this model was evident, its difference from HOAt in terms of reactivity being greater than that seen in the synthesis of the **2b** analogue (28%, entry 3, Table 7). In view of this general low purity profile, 1 h couplings were also carried out. The same trend in relative effectiveness was observed, although this time the percentages of pentapeptide were enhanced. Not only did HOBt still afford the lowest purity (9.8%, entry 5, Table 7), but it was the only reagent that still gave some of the des-Tyr product and the starting tripeptide under the new conditions. The relative performances of Oxyma and HOAt were maintained (Table 7, entry 6 vs entry 4).

In order to improve the level of coupling of the Aib residue further, double couplings of 30 min and of 1 h were performed. Although use of the former set of conditions did not result in any significant changes in the degrees of purity obtained, use of the latter resulted in enhanced percentages of **2c**, especially in the synthesis with Oxyma (69%, entry 12, Table 7). It is noteworthy that des-Tyr was still observed even in the longest coupling when HOBt was used (entry 11, Table 7).

Because Oxyma was clearly the additive that displayed the highest potency in the most difficult sequence, but the coupling to afford **2c** could still not be driven to completion under the conditions tested, a 4 h double coupling for the in-

Table 7. Percentages of H-Tyr-Aib-Aib-Phe-Leu-NH_2 (**2c**) and related deletion peptides obtained in solid-phase assembly under various sets of coupling conditions with the different additives.[a]

Entry	Coupling conditions	Coupling reagent	Pentapeptide [%]	des-Aib [%]	des-Tyr [%]	Tripeptide [%]
1	30 min	HOAt/DIC	11.3	86.1	1.8	0.8
2		HOBt/DIC	3.0	91.0	0.9	5.1
3		Oxyma/DIC	28.0	70.5	1.1	0.4
4	1 h	HOAt/DIC	28.7	71.3	–	–
5		HOBt/DIC	9.8	86.9	1.6	1.7
6		Oxyma/DIC	55.7	44.3	–	–
7	30 min[b]	HOAt/DIC	31.2	68.4	0.4	–
8		HOBt/DIC	8.0	90.6	0.8	0.6
9		Oxyma/DIC	46.5	53.5	–	–
10	1 h[b]	HOAt/DIC	55.0	45.0	–	–
11		HOBt/DIC	18.9	80.6	0.5	–
12		Oxyma/DIC	69.0	31.0	–	–

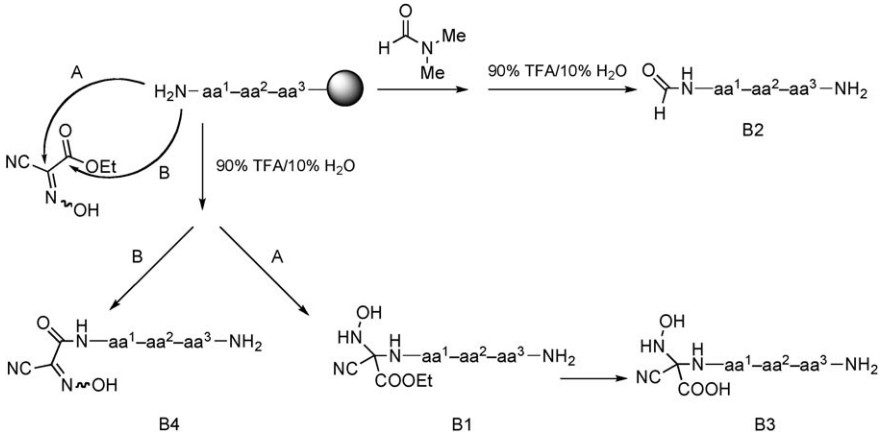
[a] HPLC-MS showed the right mass for the pentapeptide at 611.4. [b] A double coupling was performed.

corporation of Aib and a standard 1 h coupling for introducing Tyr was conducted with this additive. A near-quantitative yield (92%) was observed. It is worth noting that the coupling effectiveness of Oxyma in these pentapeptides can be considered similar to that of HOAt, one of the most efficient additives and classed among the most powerful coupling reagents.

The stability of Oxyma in relation to the N terminus of a resin-anchored peptide was studied. Our concern mainly related to the side-reaction involving nucleophilic attack of the N terminus amino group on the ethyl ester of Oxyma, which might generate a resin-bound byproduct. This unwanted reaction would lead to the termination of the peptide chain, one of the most undesirable events in solid-phase peptide synthesis. To check the viability of this side reaction, experiments involving two tripeptides (H-Gly-Phe-Val-resin and the less nucleophilic H-MeGly-Phe-Leu-resin) under various strong reaction conditions were carried out. In all experiments 10 equiv of a solution of Oxyma in DMF or NMP was mixed with the resin, without the rest of the reagents that would be added in a standard coupling, to provoke the appearance of side-reactions. Apart from the expected byproduct (named B4), others were also detected (see Scheme 2).

Experiments 1–3 were carried out with the H-Gly-Phe-Val-resin model tripeptide. Experiment 1 was assisted with microwaves for 10 min at 80°C, the resin being mixed with a solution of Oxyma in DMF. After cleavage from the resin with 90% TFA/10% H_2O, samples were analyzed by HPLC-PDA, revealing 43.2% of the unmodified peptide (Figure 2). The major byproduct found was **B2** (47.9%), a peptide formylated at the N terminus, which presumably involves the solvent. Byproducts **B1** and **B3** (2.5 and 2.4%, respectively, Path A, Scheme 2), and the predicted **B4** (4.0%, Path B) were also detected. The byproduct **B1** was the product of attack at the electrophilic carbon of the oxime group and **B3** appears to be its hydrolyzed derivative at the ethyl ester (either during microwave irradiation or in the cleavage step), although it should appear at a lower retention time than **B1** because of its higher polarity. The mass of **B4** was detected as two close peaks, suggesting that two isomers were present. The UV profiles of these additive-based byproducts each show a characteristic maximum of absorbance at 235–240 nm, like Oxyma. An unknown impurity with the same retention time as **B1** but with a +43 mu mass difference ($[M+H]^+ = 364.3$) with respect to the unmodified tripeptide ($[M+H]^+ = 321.2$) was present in the crude mixture, as detected by electrospray HPLC-MS.

In experiment 2, the same solution of the additive in DMF as used in experiment 1 was mixed with the resin at room temperature and left overnight. These considerably milder conditions afforded H-Gly-Phe-Val-NH_2 in 92.3% purity (Figure 2). Unlike in experiment 1, only the byproduct **B2** (2.1%), which is not additive-based, was observed, along with a compound (5.4%) showing an unusual UV profile that would suggest that it is not peptide-based. Coinjection of the products from experiment 1 and experiment 2 confirmed that this compound was not present in the products of experiment 1. The unknown compound with the +43



Scheme 2. Main byproducts found after cleavage of tripeptides used in Oxyma stability experiments from the resin.

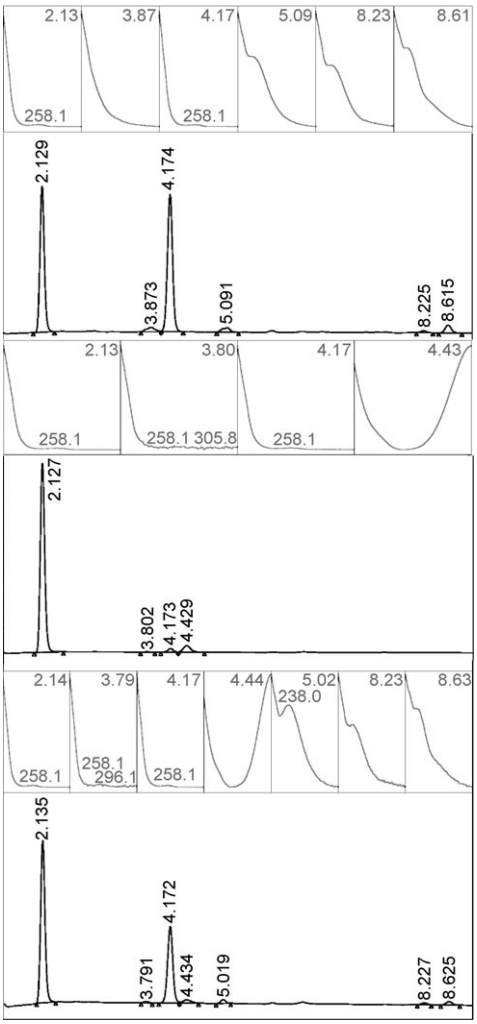


Figure 2. HPLC-PDA and UV profiles of crude mixtures from stability experiments: experiment 1 (above), experiment 2 (center), and coinjection of samples from experiment 1 and experiment 2 (below), showing the tripeptide H-Gly-Phe-Val-NH_2 (2.1 min, $[M+H]^+ = 321.2$) and byproducts **B1** (3.9 min, $[M+H]^+ = 463.3$, experiment 1 and coinjection), **B2** (4.2 min, $[M+H]^+ = 349.2$), **B3** (5.1 min, $[M+H]^+ = 435.3$), and **B4** (8.2 and 8.8 min, $[M+H]^+ = 417.2$). Unknown impurities are found at 3.9 min ($[M+H]^+ = 364.3$, experiments 1, 2, and coinjection) and at 4.4 min ($[M+H]^+ = 620.4$, experiment 2 and coinjection).

mass difference seen in experiment 1 was also detected (<0.2%) in experiment 2. The low mass increase and the different UV profile in relation to the additive-based byproducts suggests that this impurity was not generated by Oxyma.

In view of the effect of DMF in the stability experiments, experiment 3 was carried out in the microwave as in experiment 1 but this time with a solution of Oxyma in NMP. As expected, **B2** almost disappeared completely (3.6%), with the unmodified tripeptide being obtained in 64.0% purity. Comparison with experiment 1, which afforded only a 43.2% yield of tripeptide, illustrates the impact of the solvent on the purity obtained, although more byproducts were detected, including **B3**, **B4**, and the impurity with the +43 mass difference with respect to the unmodified tripeptide. As in experiment 2, no **B1** was detected. In all these experiments, Oxyma-based byproducts were detected only in those carried out under the more extreme conditions (Exps. 1 and 3).

Unlike Exps. 1–3, which were designed as the worst scenarios possible, including the presence of the less hindered (and therefore more prone to react) amino acid Gly at the N terminus, experiment 4 was conducted with the less nucleophilic H-MeGly-Phe-Leu-resin (which was also used in the coupling efficiency assays). The experiment was carried out in DMF, the regular solvent for SPPS, although the above results had been poorer. than those obtained in NMP, and with 10 min irradiation in the microwave. The unmodified tripeptide was obtained in 94.5% purity, much higher than the 43.2% achieved with the H-Gly-Phe-Val-resin tripeptide. Compound **B1** was the only additive-based byproduct detected (1.5%), together with **B2** (4.0%).

Having addressed the relative potencies of HOBt, HOAt, and Oxyma and the stabilities of the proposed additives, we next focused on the safety profiles of the additives. In view of the recent reports relating to the potentially explosive properties of benzotriazole-based additives, which restrict their transportation and commercial availability, it was crucial to ensure that Oxyma did not follow the classical pattern observed in explosive substances: fast decomposition with simultaneous large increase in pressure.[21]

To explore the thermal safety of the additives, dynamic Differential Scanning Calorimetry (DSC) and Accelerating Rate Calorimetry (ARC) assays were carried out. DSC allows the heat released by a certain compound to be measured, by comparing it to a reference when both are exposed to the same thermal treatment.[22] This experiment also provides valuable information about relative decomposition kinetics (which are connected with the explosive character of a given compound), when heated in a closed crucible under N_2 flow, from 30 to 300°C at a constant heating rate of 10°C min^{-1}. As a result, diagrams displaying the heat flow as a function of time/temperature are obtained. In addition to displaying higher normalized exothermic ΔH values (234 kJ mol^{-1} for HOBt hydrate and 226 kJ mol^{-1} for HOAt, vs 125 kJ mol^{-1} for Oxyma), the kinetic profiles of the benzotriazole-based derivatives differed significantly from that of Oxyma. Whereas the former compounds decomposed quickly, the latter decomposed in a much slower and constant manner (Figure 3). This decomposition profile cannot be labeled as non-explosive per se, because the study was not an explosivity test. However, it clearly does not coincide with the standard thermal behavior of an explosive substance, such as those observed for HOBt hydrate and HOAt. Interestingly, at 115°C, Oxyma, unlike HOAt and HOBt hydrate, melted before decomposing, and this endothermic phenomenon can be considered a possible safety barrier. Although this barrier is not very precise, because melting and decomposition processes occur almost simultaneously, it might moderate the exothermic released heat. In the experiment with HOBt hydrate, another endothermic

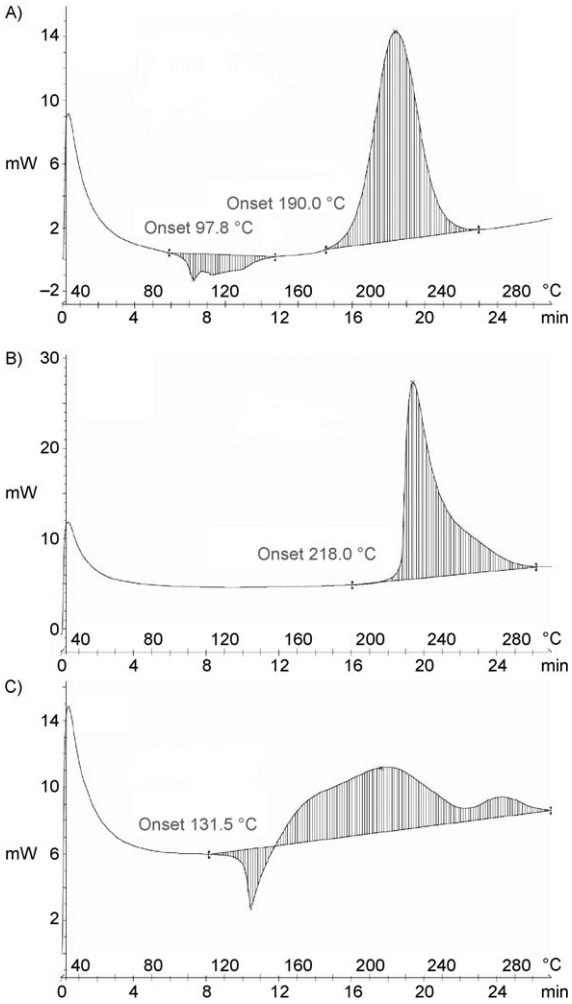


Figure 3. Thermograms showing heat flow versus temperature and time for DSC experiments with A) HOBt hydrate, B) HOAt, and C) Oxyma.

process, corresponding to the desolvation heat, can be observed. The approximate onset temperature at which decomposition began was 131 °C, considerably lower than in the cases of HOBt hydrate (190 °C) or HOAt (218 °C).

Complementary to the DSC assays is the ARC technique, which enables study of a given decomposition under adiabatic conditions.[23] The associated pressure rise can be measured and the onset temperatures accurately determined, unlike in DSC experiments, which suffer from uncertainty because of the low amount of sample.[21] The "heat-wait-seek" method is applied until self-heating of the sample is detected and then the experiment is changed to adiabatic mode. Once decomposition begins, pressure and temperature increase. The assay is stopped when the temperature rises above 300 °C.

Again, the benzotriazole-based additives showed markedly different behavior from that of Oxyma in terms of the pressure measured (Figure 4). In the case of the former compounds, high pressures were observed (178 and 167 bar for HOBt hydrate and HOAt, respectively). These results are not surprising, because benzotriazoles release N_2 when

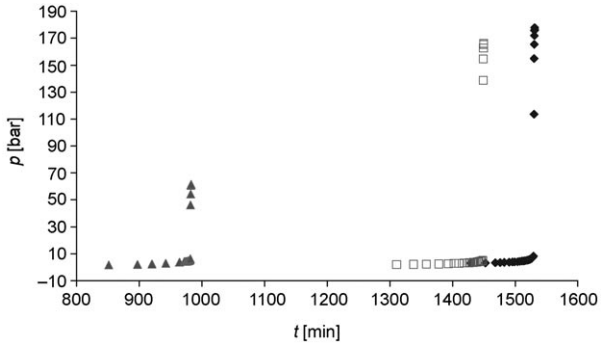


Figure 4. ARC experiments. Decomposition profiles of HOBt hydrate (◆), HOAt (□), and Oxyma (▲) showing released pressure (bar) as a function of time (min).

decomposing. In contrast, the associated pressure detected in the assay conducted with Oxyma was considerably lower (61 bar).

With regard to the onset temperatures, in the case of Oxyma decomposition began at 124 °C, whereas in the experiments with HOBt hydrate and HOAt it began at 145 °C and 178 °C, respectively (Figure 5). In order to work under safe conditions, it is recommended that the temperature of a given compound be kept at values at which the time to maximum rate under adiabatic conditions is greater than 24 h.[24] As a rule of thumb, this temperature value can be estimated in many cases by subtracting 50 K from the decomposition onset observed in an ARC experiment.[25] In the case of Oxyma, this safety value is at a lower temperature than is the case for HOBt hydrate and HOAt (74 °C vs 128 °C and 95 °C, respectively). Nonetheless, under standard conditions for peptide synthesis, the working temperature does not usually rise above 25 °C. Moreover, when stability assays were conducted with microwave irradiation at 80 °C, no incident was reported. Although in terms of thermal risk assessment Oxyma is less thermally stable, showing a lower decomposition onset temperature, the above results (mainly the pressure rise associated with decomposition) suggest that the risk of this process ending in a thermal runaway is less likely

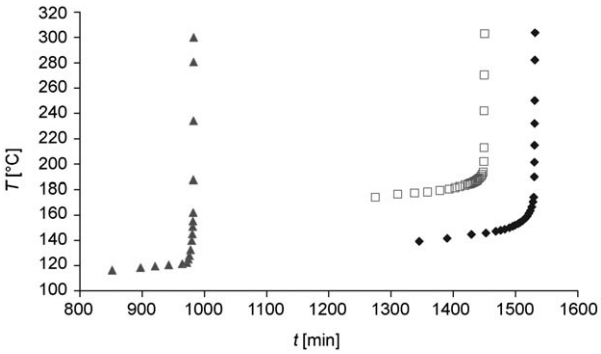


Figure 5. ARC experiments. Decomposition profiles of HOBt hydrate (◆), HOAt (□), and Oxyma (▲) showing temperature (°C) as a function of time (min).

when using Oxyma than in the cases of HOBt hydrate or HOAt.

Conclusions

To conclude, here we have extensively tested ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as an additive for peptide synthesis for use in combination with carbodiimides. Oxyma showed clear superiority to HOBt in terms of suppression of racemization and of coupling efficiency in all the experiments conducted. In some cases, such as the racemization assay carried out on the stepwise model or the demanding assembly of MeAla and Aib analogues of Leu-enkephalin, its performance was even superior to that of HOAt. The elongation of the ACP decapeptide in an ABI 433 A peptide synthesizer also demonstrated the compatibility and coupling efficiency of Oxyma in an automated synthesis. Stability assays of Oxyma with regard to N-terminal amino groups showed no peptide-bound byproducts when extremely strong conditions were avoided, even with use of 10 equiv in the absence of base, carbodiimide, and Fmoc-amino acid. Last but not least, two calorimetry techniques, DSC and ARC, showed decomposition profiles for HOAt and HOBt that resemble that of a explosive substance, because of their fast decomposition and high derived pressure rises. In contrast, Oxyma did not follow this pattern, and decomposed at a slower rate, releasing only one third of the pressure observed in the experiments with HOBt and HOAt. Therefore, Oxyma can be considered a potent replacement for benzotriazole-based additives showing a lower thermal risk.

Experimental Section

General: Oxyma (ethyl cyanoglyoxylate-2-oxime, 97%) was obtained from commercial sources (Aldrich). DMF was used in peptide grade purity. All peptides, des-amino acids, and byproducts were identified by HPLC-MS electrospray mass spectroscopy. The synthesis of ACP was carried out in an Applied Biosystems ABI 433 A automated peptide synthesizer by the Fmoc/*t*Bu protection strategy. Experiments involving side reactions related to Oxyma were conducted in a CEM Discover Microwave. Differential scanning calorimetry assays were performed in a Mettler–Toledo DSC-30 differential scanning calorimeter, with high-pressure crucibles of a capacity of 30 μL. In the adiabatic calorimetry experiment, an accelerating rate calorimeter from Thermal Hazard Technology was used, along with ARCTC-HC-MCQ (Hastelloy) test cells. The sensitivity threshold was set at $0.02\,^{\circ}C\,min^{-1}$.

Racemization tests with model peptides in solution phase: Test couplings were carried out as previously described for Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2.[17] Crude products were analyzed by reverse-phase HPLC (with a Waters Symmetry C18, 5 μm, 4.6×150 mm column), linear gradient over 30 min of 20 to 50% (Z-Phg-Pro-NH_2) or 20 to 80% (Z-Phe-Val-Pro-NH_2) CH_3CN/0.036% TFA in H_2O/0.045% TFA, detection at 220 nm. In the Z-Phg-Pro-NH_2 model, the t_R values of the LL and DL epimers were 26.01 and 27.40 min, respectively, whereas in the Z-Phe-Val-Pro-NH_2 case, the t_R values of the LLL and LDL epimers were 19.98 and 21.05 min, respectively.

Study of cysteine racemization during assembly of H-Gly-Cys-Phe-NH_2 on solid phase:[19,20] Experiments consisted of the study of the stepwise coupling of Cys and Gly residues onto previously formed H-Phe-RinkAmide-MBHA-PS-resin (0.45 mmol g^{-1}, 50 mg), with use of the Fmoc/*t*Bu and Cys(Trt) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than in the case of des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation of a solution of Fmoc-amino acid, Oxyma, and DIC (3 equiv excess) in DMF (0.3 M) at room temperature. Fmoc removal was carried out with piperidine/DMF (1:4, 2×5 min). The peptide chain was released from the resin by treatment with TFA/ethane-1,2-dithiol/H_2O/TIS (94:2.5:2.5:1) for 1 h at room temperature. The colorless solution was filtered and the resin was washed with CH_2Cl_2 (0.5 mL×3). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude pentapeptide was precipitated with cold Et_2O (3 mL×4) and, after being lyophilized, was analyzed by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6×100 mm), linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 3.37 and 3.60 min, respectively.

Solid-phase automated synthesis of ACP (65–74) (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2):[14] The ACP (65–74) decapeptide model was assembled on Fmoc-RinkAmide-Aminomethyl-PS-resin (0.63 mmol g^{-1}, 0.1 mmol) with use of an automated ABI 433 A peptide synthesizer. Twofold excesses of Fmoc-amino acids and solutions (0.2 M) of the corresponding additive and DIC in DMF were used (1 mL of the solution was added in each coupling step: 0.2 mmol). The peptide chain was cleaved from the resin by TFA/H_2O (9:1) treatment over 2 h at room temperature. After filtration of the solution containing the peptidic material, the resin was washed with CH_2Cl_2 (1 mL×2), which was removed under nitrogen along with TFA. The resulting crude peptide was purified with cold Et_2O (2 mL×3) and lyophilized. Peptide purity was analyzed by reversed-phase HPLC, with use of a Waters Symmetry C18 column (5 μm, 4.6×150 mm), linear gradient 12.5 to 17% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 30 min, with detection at 220 nm. Retention times were: t_R decapeptide = 26.88 min, t_R des-2 Ala = 24.48 min, t_R des-Ala = 24.50 min, t_R des-Asn = 32.01 min, t_R des-Gly = 26.33 min, t_R des-Ile^{69}, Ile ^{72} = 2.97 min, t_R des-Ile, Ala = 4.20 min, t_R des-Ile^{72} = 7.17 min, t_R des-Ile^{69} = 9.38 min, t_R des-Val = 20.22 min.

Solid-phase general synthesis of H-aaX-Phe-Leu-resin and H-Gly-Phe-Val-resin tripeptides: Peptides were manually assembled on a Fmoc-RinkAmide-Aminomethyl-PS resin (0.63 mmol g^{-1}, 2 g). Coupling times were 30 min, with 3 equiv excess of each Fmoc-amino acid, Oxyma, and DIC. During preactivation (1.5 min), a bright yellow color was observed immediately after addition of DIC. Fmoc removal was carried out with piperidine/DMF (1:4, 2×5 min). Tripeptide purity was checked after sample cleavage (10 mg) from the resin with TFA/H_2O (19:1) at room temperature for 1 h. The solution was filtered and the resin was washed with CH_2Cl_2 (0.5 mL×2). The solvent and TFA were removed under nitrogen flow. In all cases, reversed-phase HPLC analysis showed purity above 99%.

Solid-phase synthesis of H-Tyr-MeGly-MeGly-Phe-Leu-NH_2 (2a) with use of the different additives: The pentapeptide was manually elongated on a pure previously assembled H-MeGly-Phe-Leu-Rinkamide-Aminomethyl-PS-resin (0.63 mmol g^{-1}, 50 mg) with a preactivation time of 3 min and use of Fmoc-amino acids (3 equiv, excess), the corresponding additive (3 equiv), and DIC (3 equiv). The coupling time was 5 min for the introduction of both MeGly and Tyr. The pentapeptide was cleaved from the resin by treatment with TFA/H_2O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL×2), which was removed along with TFA under nitrogen. The crude pentapeptide was precipitated with cold Et_2O (2 mL×3) and, after being lyophilized, was analyzed by reversed-phase HPLC, with a Waters Symmetry C18 column (5 μm, 4.6×150 mm), linear gradient 16 to 16.5% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 30 min, with detection at 220 nm. The t_R value of the pentapeptide was 26.31 min, whereas the t_R values of des-MeGly, des-Tyr, and the tripeptide H-MeGly-Phe-Leu-NH_2 were 25.81, 9.62, and 8.58 min, respectively.

Solid-phase synthesis of H-Tyr-MeAla-MeAla-Phe-Leu-NH_2 (2b) with use of the different additives: The pentapeptide was manually assembled on a pure previously synthesized H-MeAla-Phe-Leu-Rinkamide-Amino-

methyl-PS-resin (0.63 mmol g^{-1}, 50 mg), with use of Fmoc-amino acids (3 equiv excess), the corresponding additive, and DIC. Preactivation and coupling times for the introduction of both MeAla and Tyr were 3 and 30 min, respectively. The peptide chain was cleaved from the resin by treatment with TFA/H_2O (9:1) over 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL × 2), which was removed along with TFA under nitrogen. The crude pentapeptide was purified with cold Et_2O (2 mL × 3) and lyophilized. Purity was analyzed by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 20 to 25% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R value of the pentapeptide was 8.21 min, whereas the t_R values of des-MeAla, des-Tyr, and the tripeptide H-MeAla-Phe-Leu-NH_2 were 8.81, 4.73, and 2.34 min, respectively.

Solid-phase synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 (2c) with use of the different additives:[14] The pentapeptide was manually elongated on a pure previously synthesized H-Aib-Phe-Leu-Rinkamide-Aminomethyl-PS-resin (0.63 mmol g^{-1}, 50 mg), with use of Fmoc-amino acids (3 equiv, excess), the corresponding additive (3 equiv), and DIC (3 equiv). Residues were preactivated for 3 min prior to addition to the resin. Coupling times are displayed in Table 7. The peptide chain was cleaved from the resin by treatment with TFA/H_2O (9:1) for 2 h at room temperature. The solution was then filtered and the resin was washed with CH_2Cl_2 (1 mL × 2), which was removed along with TFA under nitrogen. The crude peptide was purified with cold Et_2O (2 mL × 3) and, after lyophilization, purity was checked by reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6 × 100 mm) and a linear gradient of 20 to 35% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R value for the pentapeptide was 5.75 min, whereas the t_R values for des-Aib, des-Tyr, and the tripeptide H-Aib-Phe-Leu-NH_2 were 6.03, 3.93, and 2.25 min, respectively.

General procedure for stability assays with Oxyma: Stability experiments 1–4 were conducted on two resin-bound tripeptides: H-MeGly-Phe-Leu-resin and H-Gly-Phe-Val-resin. The resin was weighed into a 2 mL solid-phase syringe, swelled in CH_2Cl_2 (×5), and then conditioned in the reaction solvent, DMF or NMP (×5). In the overnight experiment, a solution of Oxyma in DMF or NMP (0.02 M, 10 equiv) was directly added to the resin. After 12 h at room temperature, the resin was filtered and washed with DMF or NMP (×10) and CH_2Cl_2 (×10). In contrast, in the microwave-assisted experiment, the resin was first transferred into a suitable microwave-compatible vial. The solution of Oxyma in DMF or NMP (0.02 M, 10 equiv) was then added to the resin and the mixture was irradiated at 80 °C for 10 min in a CEM Discover Microwave. The resin was then transferred back into the syringe and washed with NMP or DMF (×10) and CH_2Cl_2 (×10). In both types of experiments, the resin-bound compounds were cleaved from the resin by treatment with TFA/H_2O (9:1) for 1 h at room temperature. The solution was filtered and the resin was washed with CH_2Cl_2 (0.05 mL × 3), which was removed together with TFA under nitrogen. The crude peptide was purified with cold Et_2O (2 mL × 3) and lyophilized. The byproduct content of the samples was checked by reversed-phase HPLC. Exps. 1–3 (H-Gly-Phe-Val-resin) were analyzed by use of a SunFire C18 Column (3.5 μm, 4.6 × 100 mm), linear gradient 15 to 30% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. Retention times were: t_R unmodified tripeptide = 2.13 min, t_R byproduct **B1** = 3.87 min, t_R byproduct **B2** = 4.17 min, t_R byproduct **B3** = 5.09 min, t_R byproduct **B4** = 8.22 and 8.61 min, t_R impurity with detected mass M+43 = 3.80 min, t_R unknown impurity = 4.43 min. experiment 4 (H-MeGly-Phe-Val-resin) was analyzed with use of a Waters Symmetry C18 column (5 μm, 4.6 × 150 mm), linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 15 min, with detection at 220 nm. Retention times were: t_R unmodified tripeptide = 6.25 min, t_R byproduct **B1** = 6.88 min, t_R byproduct **B2** = 7.68 min.

General Procedure for dynamic differential scanning calorimetry assays: The thermal behavior of HOAt, HOBt hydrate, and Oxyma was tested. Samples (1 mg) were heated from 30 °C to 300 °C at a heating rate of 10 °C min^{-1} in a closed high-pressure crucible with N_2 flow in a Mettler–Toledo DSC-30 differential scanning calorimeter. Diagrams showing heat flow as a function of temperature and time were obtained.

General Procedure for ARC experiments: Assays were carried out on an Accelerating Rate Calorimeter (ARC) from Thermal Hazard Technology, in ARCTC-HC-MCQ (Hastelloy) test cells. Samples (2.083 g of HOBt hydrate, 1.605 g of HOAt, and 3.451 g of Oxyma) were introduced into the calorimetric test cell at room temperature, without stirring. The cell was heated at the initial temperature (30 °C) and the "heat-wait-seek" method was applied; this consisted of heating the sample by 5 °C and, after 15 min of equilibrium, measuring whether self-heating was occurring at a rate higher than 0.02 °C min^{-1} (default sensitivity threshold). When self-heating was detected, the system was changed to adiabatic mode. After decomposition, the assay was stopped when the temperature rose above 300 °C. The phi factors[26] were: 2.6058 (HOBt hydrate), 3.04373 (HOAt), and 1.95557 (Oxyma).

Acknowledgements

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[1] Abbreviations not defined in text: Aib, α-aminoisobutyric acid; ACP, acyl carrier protein decapeptide (65–74); AM-PS, aminomethyl-polystyrene resin; ARC, accelerating rate calorimetry; 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; DIC, *N,N*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DSC, Differential Scanning Calorimetry; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; HODhbt, 3-hydroxy-3,4-dihydro-4-oxo-l,2,3-benzotriazine; HOPO, 2-hydroxypyridine-*N*-oxide; HOPfp, pentafluorophenol; HOSu, N-hydroxysuccinimide; NMP, *N*-methyl-2-pyrrolidinone; PyAOP, azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyClock, 6-chloro-benzotriazol-1-yl-*N*-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl. Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977).

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Capítol 3. *Carbonats d'Oxyma com a reactius per la introducció dels grups protectors Fmoc i Alloc*

Publicació III

Oxime Carbonates, Novel Reagents for the Introduction of Fmoc and Alloc Protecting Groups, Free of Side-Reactions †

Carbonats d'oximes com a reactius novells per a la introducció dels grups protectors Fmoc i Alloc sense reaccions secundàries

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† Ramon Subirós Funosas va dur a terme la síntesi i evaluació dels reactius per a la introducció del grup protector Alloc, així com l'elaboració de la part corresponent del manuscrit i la introducció general.

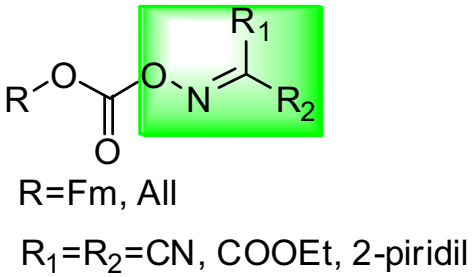
Sherine N. Khattab es va encarregar de la síntesi i evaluació dels carbonats de Fmoc de les diverses oximes i el HOPy, així com l'elaboració de la part proporcional del manuscrit.

Ayman El-Faham va realitzar l'elaboració de gran part de la introducció general.

Resum

Els grups protectors Fmoc i Alloc representen una alternativa consistent a la clàssica protecció amb el grup Boc en química de pèptids. El primer s'ha establert en anteriors dècades com el grup protector per excel·lència del grup amino *N*-terminal, mentre que el segon permet utilitzar un esquema de protecció completament ortogonal tant a Fmoc com a Boc.

Normalment la introducció dels grups Fmoc i Alloc té lloc a través dels halogenoformiats, azidoformiats o carbonats actius corresponents. Aquesta reacció, que pot semblar relativament trivial en primera instancia, ve acompanyada de reaccions secundàries preocupants, com ara la formació de Fmoc/Alloc-dipèptids i fins i tot tripèptids. El present treball descriu noves prometedores Fmoc/Alloc-oximes, reactius cristal·lins fàcils de preparar, estables i altament reactius, que donen lloc a Fmoc/Alloc-aminoàcids sense pràcticament subproductes i amb alts rendiments, seguint procediments convencionals. D'entre els anàlegs de tipus Fmoc-oxima cal destacar l'anàleg de tipus cianur de *N*-hidroxipicolinimidoïl, el qual va proporcionar els millors resultats en la preparació de Fmoc-Gly-OH, escollit ja que és el residu més predisposat a donar dipèptids. De la mateixa manera, es va sintetizar i caracteritzar la el Alloc-anàleg de la mateixa oxima, la qual va produïr Alloc-Gly-OH amb bons rendiments, puresa i quasi inexistent formació de dipèptid, després d'anàlisi per HPLC de fase reversa i RMN.



Carbonats de Fmoc i Alloc de diverses oximes amb substituents electroatraients

FULL PAPER

DOI: 10.1002/ejoc.201000028

Oxime Carbonates: Novel Reagents for the Introduction of Fmoc and Alloc Protecting Groups, Free of Side Reactions

Sherine N. Khattab,[a] Ramon Subirós-Funosas,[b,c] Ayman El-Faham,*[a,b,d] and Fernando Albericio*[b,c,e]

Keywords: Amino acids / Protecting groups / Peptides / Oximes

Fmoc and Alloc protecting groups represent a consistent alternative to classical Boc protection in peptide chemistry. The former was established in the last decades as the α-amino protecting group of choice, whereas the latter allows a fully orthogonal protection strategy with Fmoc and Boc. Usually, the introduction of the Fmoc and Alloc moieties takes place through their halogenoformates, azides, or activated carbonates. This rather simple reaction is accompanied by several side reactions, specially the formation of Fmoc/Alloc dipeptides and even tripeptides. The present work describes new promising Fmoc/Alloc-oxime reagents, which are easy to prepare, stable, and highly reactive crystalline materials that afford almost contaminant-free Fmoc/Alloc-amino acids in high yields by following a conventional procedure. Amongst the Fmoc-oxime derivatives, the *N*-hydroxypicolinimidoyl cyanide derivative (*N*-{[(9H-fluoren-9-yl)methoxy]carbonyloxy}picolinimidoyl cyanide) gave the best results for the preparation of Fmoc-Gly-OH, which is the most predisposed to give side reactions. The same Alloc-oxime analogue afforded the preparation of Alloc-Gly-OH in good yield, purity, and extremely low dipeptide formation, as analyzed by reverse-phase HPLC and NMR spectroscopy.

Introduction

An appropriate choice of the protection strategy is essential to the success of peptide[1] synthesis, conditioning the yield and purity of the desired product. To meet that purpose, several protecting groups have been proposed that offer a wide range of removal conditions, enabling the required orthogonality.[2,3] 9-Fluorenylmethyloxycarbonyl amino acids (Fmoc-amino acids) are potentially amongst the most versatile intermediates for peptide synthesis, especially when used in conjunction with acid-labile side-chain protecting groups, being considered as a milder alternative to the more classical Boc strategy.[4,5] The allyloxycarbonyl (Alloc) group has also been proposed for the preparation of *N*-urethane blocked amino acids, successfully allowing the synthesis of antitumoral peptides when used as temporary α-amino protecting group.[6,7] Due to its stability to general basic and acidic conditions, it introduces orthogonality towards Boc and Fmoc, being of special interest as side-chain protection in the synthesis of cyclic peptides, when the so-called three dimensional protection strategy is used.[8] Its removal can be easily and selectively achieved with tetrakis(triphenylphosphane)palladium(0) and a suitable nucleophile/scavenger.[9,10]

The use of chloroformates represents the most potent strategy for the *N*-protection with Fmoc and Alloc moieties.[11] Nevertheless, it has been reported that the high reactivity of these chlorides, such as Fmoc-Cl (**1**) or Alloc-Cl (**2**), might lead to the formation of amino-acid-based byproducts, including the protection of bulky residues, that become inserted into the peptide chain.[12–16] As result of this detrimental side reaction, considerable formation of Fmoc/Alloc-dipeptides and tripeptides (1–20%) has been observed, as established by HPLC, amino acid analysis, NMR spectroscopy, and the synthesis of a number of standards.[13] A previous "in situ" bis-trimethylsilylation step of protection of the residue, before conducting the reaction with the chloroformate, can prevent the formation of such byproducts.[15,17] In fact, Fmoc/Alloc-oligopeptides are likely formed through the intermediary formation of relatively stable mixed anhydrides when highly reactive chloroformates are used (Scheme 1, path B).[18,19]

The drawbacks associated to these powerful reagents prompted the evaluation of less reactive approaches for the introduction of *N*-blocking groups, like the 1,2,2,2-tetrachloroethyl,[20,21] 5-norbornene-2,3-dicarboximido,[22] pentafluorophenyl,[23] symmetrical pyrocarbonates,[24] and

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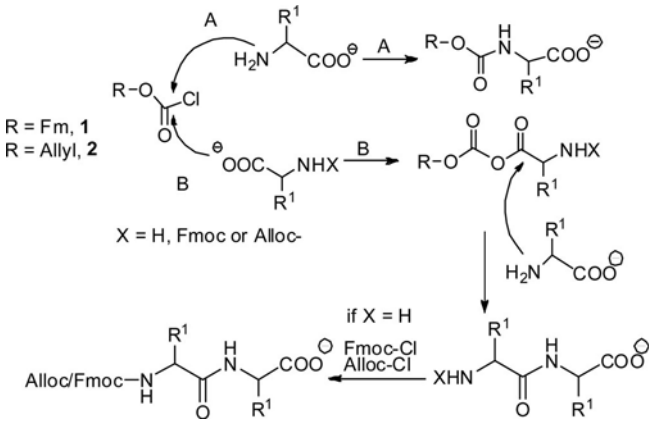
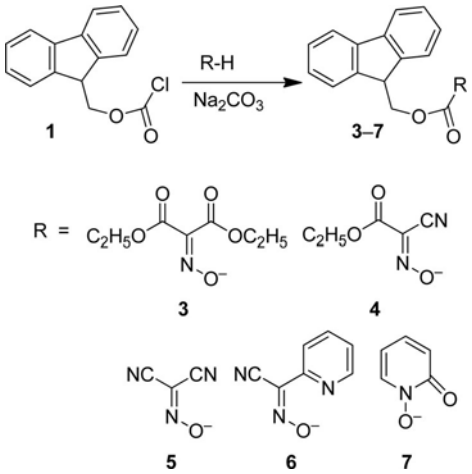


Scheme 1. Side reaction during Fmoc protection.



Scheme 2. Synthesis of Fmoc-oxime and *N*-hydroxypyridinone derivatives.

2-MBt (2-mercaptobenzothiazole),[25] although only the hydroxysuccinimido (Su) carbonate found general acceptance.[13–16,26,27] Nonetheless, the formation of up to 0.4% of some amino-acid-based byproducts like Fmoc/Alloc-β-Ala-OH and Fmoc/Alloc-β-Ala-AA-OH can occur when using this reagent for α-amino protection.[28] A detailed discussion of the mechanism of this side reaction has been proposed by Isidro-Llobet et al.[25] Azide derivatives, which can be used as solids after isolation from the chloroformates[12,29] or formed in situ before reacting with the amino acid,[30] have also been proposed as an alternative pathway to the N-protection of amino acids, although its explosive nature compromises its use in large-scale synthesis.

Our group has recently been interested in the search of a new family of *N*-hydroxy-containing compounds as replacements for 1-hydroxybenzotriazole (HOBt) and its derivatives. Some ketoximes, bearing various electron-withdrawing substituents were considered for that purpose, as they are rather stable compared to oximes with hydrogen atoms in the α-carbon position,[31] and they also contain a highly acidic *N*-hydroxy moiety, which results in an excellent leaving group. A deep study on ethyl 2-cyano-2-hydroxyiminoacetate (Oxyma) was undertaken, showing a high reduction of racemization and coupling efficiency in hindered sequences when this additive was employed.[32–35] Therefore, this oxime can be converted into promising carbonate-type reagents for the introduction of Fmoc and Alloc.

Results and Discussion

The suitability of these oximes as leaving groups in carbonate-type reagents for Fmoc/Alloc introduction was first tested in the synthesis and evaluation of Fmoc derivatives. Therefore, preparation of Fmoc-oximes **3**–**6** and (9*H*-fluoren-9-yl)methyl-2-oxopyridin-1(2*H*)-yl carbonate (**7**) was readily achieved by reaction of the corresponding oximes or *N*-hydroxy-2-pyridinone (HOPO)[36] with Fmoc-Cl (**1**) in the presence of sodium carbonate, following a well-reported method described for other reagents (Scheme 2).[26]

The Fmoc-introducing reagents were afforded by this method in high yields (>80% in all analogues synthesized) and purity, according to the found elemental analysis, compared to the calculated values (Table 1). Fmoc-oximes **3**–**6** and Fmoc-hydroxypyridinone **7** were obtained as white solids after recrystallization in CH_2Cl_2/hexane, except for diethylcarboxylate analogue **3**, which was an oil at room temperature, and therefore, its handling was tedious in comparison to the rest of the derivatives.

Table 1. Yield, m.p., and elemental analysis of the protecting groups.

	Yield	M.p.	Elemental analysis calculated (found)		
	[%]	[°C]	C	H	N
3	84	–[a]	64.23 (64.44)	5.14 (5.30)	3.40 (3.63)
4	91	174–175	65.93 (66.14)	4.43 (4.70)	7.69 (7.91)
5	87	150–151	68.14 (68.39)	3.49 (3.61)	13.24 (13.48)
6	93	165–166	71.54 (71.77)	4.09 (4.32)	11.38 (11.53)
7	89	195–196	72.06 (72.31)	4.54 (4.68)	4.20 (4.41)

[a] Ca. 20 °C; oil at room temperature.

Due to its size, H-Gly-OH is one of the amino acids that becomes most contaminated during its α-amino protection. When Fmoc-Gly-OH was synthesized by using Fmoc-Cl (**1**) in dioxane/aqueous sodium carbonate following the standard procedure described in the literature, the product was contaminated with a major (10–20%) amount of Fmoc-Gly-Gly-OH, as found by TLC.[13,18,19] For this reason, this residue has been chosen as a model for carrying out a careful in-depth study. The reaction was carried out overnight in water/dioxane, in the presence of sodium hydrogen carbonate.[37] In general, Fmoc-oxime reagents were quite stable to competitive hydrolysis by NaOH, Na_2CO_3, or triethylamine in the solvent mixture at room temperature. Compound **3** (diethylcarboxylate derivative) dissolves only partly in a solvent water mixture at an initial stage of the reaction, but the mixture usually becomes homogeneous within 1 h, whereas in the case of **6** (cyanopyridyl deriva-

tive) the reaction becomes homogeneous after 10–15 min. The results obtained in the preparation of Fmoc-Gly-OH with reagents **3**–**7** are summarized in Table 2.

Table 2. Yield of Fmoc-Gly-OH, m.p., formation of byproduct Fmoc-Gly-Gly-OH.

	Yield [%] Fmoc-Gly-OH	M.p. [°C][a]	Purity [%] Fmoc-Gly-OH[b]	Fmoc-Gly-Gly-OH [%][b]
3	82.9	166–167	99.7	0.17
4	92.1	164–165	99.4	0.57
5	48.5	158–159	99.7	0.22
6	91.6	165–166	99.9	0.01
7	92.8	166–167	99.5	0.41

[a] Authentic m.p. of Fmoc-Gly-OH = 166–167 °C. [b] HPLC analysis of the crude product.

Table 2 showed, after HPLC analysis of the crude Fmoc-Gly-OH obtained, that dipeptide formation with analogues **3**–**7** took place in the range 0.01–0.57% (Figure 1; the presence of the Fmoc-Gly-Gly-OH dipeptide in the crude mixture was identified after co-injection with a pure sample synthesized in solid-phase) with purities higher than 99% in all cases, confirming the first hypothesis about the reactivity of oxime derivatives. The most reactive oxime-based reagent **4** gave the highest percentage of the dipeptide (0.57%) and the least reactive one (i.e., cyanopyridyl, **6**) gave the best results (0.01%). Diester **3** and dicyano **5** also gave a very similar performance (0.17 and 0.22%, respectively). Yields obtained were reasonably high in general, with the exception of dicyano analogue **5**. The results obtained show that the Fmoc derivative of HOPO (**7**) is not recommended due to the formation of a considerable amount of Fmoc-dipeptide.

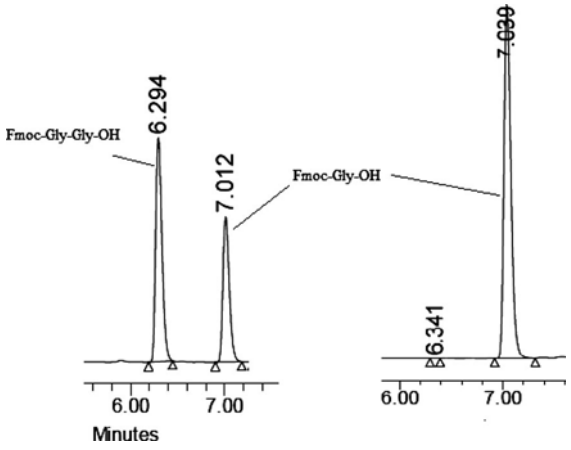
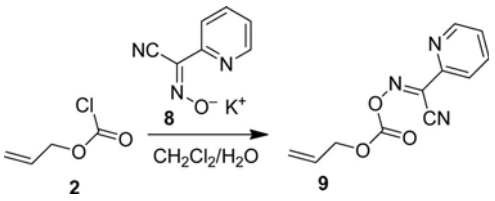


Figure 1. Fmoc-Gly-OH from **6** and co-injection with an authentic sample of Fmoc-Gly-Gly-OH.

To extend this methodology to other *N*-urethane-type protecting groups, the introduction of the Alloc moiety to the H-Gly-OH α-amino group was studied (allyloxime carbonates have been used in the past for allylation of active methylene compounds using Pd catalysis).[38] Considering the outstanding performance of cyanopyridyl reagent **6** in the Fmoc protection compared to other derivatives, only this analogue was selected to carry out the experiments regarding Alloc. The formation of the Alloc-cyanopyridyloxime reagent was accomplished by using the potassium salt of the oxime **8**, previously isolated by treatment with KOH in ethanol, and Alloc-Cl **2** (Scheme 3). This modified procedure should enhance the reaction rate over the use of the oxime plus addition of a base. Indeed, the reaction was complete in 3 h, affording Alloc-cyanopyridyloxime **9** as a pale-brownish solid after recrystallization (CH_2Cl_2/hexane) in 81% yield and high purity (>99%), as determined by HPLC and ^{1}H NMR spectroscopy.



Scheme 3. Synthesis of Alloc-cyanopyridyloxime reagent.

The performance of novel Alloc-oxime reagent **9** in preventing Alloc-based dipeptide formation was evaluated in the *N*-protection of H-Gly-OH. In comparison with Fmoc-introducing reagents, the Alloc-based analogues usually give rise to higher dimerization percentages as a result of the considerably lower steric hindrance of the allyl group than the fluorenylmethyl one, which favors this unwanted side reaction. The α-amino protection was complete after 1 h in 1%Na_2CO_3/dioxane at controlled pH 8, which was found to have great impact on the yield obtained. By using this methodology, Alloc-Gly-OH was afforded in excellent yield (83%) and purity (99.7%), as determined by HPLC and ^{1}H NMR spectroscopic analysis. The percentage of dipeptide in the crude mixture, although higher than in the analogue Fmoc-protection with compound **6**, was extremely low (0.02%), almost undetectable. This peptidic byproduct was identified after co-injection with a pure dipeptide reference, synthesized in the solid-phase by using the same Alloc-Gly-OH crude (Figure 2).

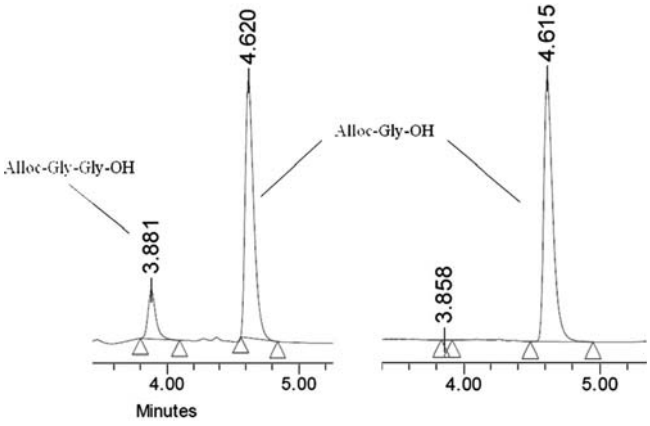


Figure 2. Alloc-Gly-OH obtained with Alloc-oxime **9** and co-injection with an authentic sample of Alloc-Gly-Gly-OH.

Conclusions

The use of the novel Fmoc-cyanopyridyloxime **6** and Alloc-cyanopyridyloxime **9** allows the preparation of Fmoc-amino acids and Alloc-amino acids free of side-products. Thus, Fmoc-Gly-Gly-OH and Alloc-Gly-Gly-OH dipeptides are formed in a negligible amount, and the absence of a succinimide moiety precludes the formation of β-alanine derivatives. Furthermore, the higher amount of dipeptide obtained with the cyanoethylester oxime (Oxyma) confirms our previous findings that this leaving group is the best substitution for hydroxybenzotriazole derivatives.[32,33] Interestingly and without studying the formation of dipeptides, Itoh and co-workers proposed a similar oxime, the phenyl one, for the introduction of the Boc group.[31]

Experimental Section

Materials: The solvents used were of HPLC reagent grade. Melting points were determined with a Mel-Temp apparatus. Magnetic resonance spectra (^{1}H NMR and ^{13}C NMR spectra) were recorded with a Joel 500 MHz (Fmoc-oxime experiments) and with a Mercury 400 MHz (Alloc-oxime experiments) spectrometer with chemical shift values reported in δ units (ppm) relative to an internal standard. Elemental analyses were performed with a Perkin–Elmer 2400 elemental analyzer, and the values found were within ±0.3% of the theoretical values. Follow-up of the reactions and checks of the purity of the compounds was done by TLC on silica-gel-protected aluminum sheets (Type 60 GF254, Merck), and the spots were detected by exposure to UV light at λ = 254 nm for a few seconds. The compounds were named by using ChemDraw Ultra version 11, Cambridge Soft Corporation. Exact masses were determined with a Waters Synapt HDMS mass spectrometer (ESI positive polarity, W analyzer, 3000 V capillary voltage, 150 and 100 °C desolvation and source temperature, 40 V sample cone, 100–1500 *m*/*z*) by introducing the sample by direct infusion. HPLC analysis was undertaken by using a reverse-phase Waters 2695 HPLC separation module, coupled to a Waters 2998 PDA UV detector, processing the chromatograms with Empower software. Separation was accomplished by using a Waters SunFire C_{18} (3.5 μ, 4.6 × 100 mm) column and linear gradients of solvent A [0.045% trifluoroacetic acid (TFA) in H_2O] in solvent B (0.036% TFA in CH_3CN) with flow = 1.0 mL min^{-1}. The mass of peptide materials was detected by using a HPLC-PDA system as the above described, coupled to a Waters Micromass ZQ mass detector, with MassLynx 4.1 software.

General Method for the Preparation of Fmoc-Oxime Derivatives 3–7: A solution of 9-fluorenylmethyloxycarbonyl chloride (10 mmol) in CH_2Cl_2 (30 mL) was added slowly to a solution of oxime or 1-hydroxypyridin-2(1*H*)-one (10 mmol) and sodium carbonate (20 mmol) in H_2O (20 mL) with stirring at 0 °C. The resulting clear mixture was stirred at 0 °C for 30 min and then at room temperature for 2 h. After dilution with CH_2Cl_2 (50 mL), the organic phase was collected and washed with water and saturated aqueous NaCl (30 mL) and then dried with Na_2SO_4 (anhydrous). After filtering, the solvent was removed under reduced pressure, and the residue was recrystallized (CH_2Cl_2/hexane) to give Fmoc derivatives **3**–**7**.

Diethyl 2-{[(9*H*-Fluoren-9-yl)methoxy]carbonyloxyimino}malonate (3): The product was obtained as an oil at room temperature (m.p. about 20 °C) in 84% yield (3.45 g). ^{1}H NMR ($CDCl_3$): δ = 1.30–1.46 (m, 6 H, 2 CH_3), 4.29–4.50 (m, 5 H, CH, 2 CH_2), 4.55 (d, *J* = 7.7 Hz, 2 H, CH_2), 7.31 (t, *J* = 6.9 Hz, 2 H, Ar-H), 7.38–7.43 (m, 2 H, Ar-H), 7.60 (t, *J* = 7.7 Hz, 2 H, Ar-H), 7.77 (d, *J* = 7.7 Hz, 2 H, Ar-H) ppm. ^{13}C NMR: δ = 14.04, 14.08, 46.51, 62.57, 62.74, 71.68, 106.71, 120.13, 120.27, 124.87, 127.69, 128.22, 141.41, 142.82, 144.37, 149.50, 151.95, 158.86, 160.22, 160.82 ppm. $C_{22}H_{21}NO_7$ (411.40): calcd. C 64.23, H 5.14, N 3.40; found C 64.44, H 5.30, N 3.63.

Ethyl 2-{[(9H-Fluoren-9-yl)methoxy]carbonyloxyimino}-2-cyanoacetate (4): The product was obtained as a white solid (m.p. 174–175 °C) in 91% yield (3.31 g). ^{1}H NMR ($CDCl_3$): δ = 1.43 (t, *J* = 6.9 Hz, 3 H, CH_3), 4.35 (t, *J* = 6.9 Hz, 1 H, CH), 4.49 (q, *J* = 6.9 Hz, 2 H, CH_2), 4.64 (d, *J* = 6.9 Hz, 2 H, CH_2), 7.35, 7.44 (2 t, *J* = 7.7 Hz, 4 H, Ar-H), 7.63, 7.80 (2 d, *J* = 7.7 Hz, 4 H, Ar-H) ppm. ^{13}C NMR: δ = 14.09, 46.44, 64.79, 72.62, 106.56, 120.38, 125.23, 127.51, 128.37, 131.23, 141.43, 142.50, 150.86, 156.65 ppm. $C_{20}H_{16}N_2O_5$ (364.35): calcd. C 65.93, H 4.43, N 7.69; found C 66.14, H 4.70, N 7.91.

{[(9*H*-Fluoren-9-yl)methoxy]carbonyloxy}carbonimidoyl Dicyanide (5): The product was obtained as a white solid (m.p. 150–151 °C) in 87% yield (2.76 g). ^{1}H NMR ($CDCl_3$): δ = 4.28–4.35 (m, 1 H, CH), 4.54, 4.62 (2 d, *J* = 7.7 Hz, 2 H, CH_2), 7.32–7.36 (m, 2 H, Ar-H), 7.41–7.45 (m, 2 H, Ar-H), 7.59 (t, *J* = 7.7 Hz, 2 H, Ar-H), 7.78 (t, *J* = 6.9 Hz, 2 H, Ar-H) ppm. ^{13}C NMR: δ = 46.41, 72.60, 106.25, 120.35, 120.39, 125.15, 127.47, 127.52, 128.34, 128.40, 132.63, 141.43, 142.44, 142.49, 150.85, 151.09, 157.36 ppm. $C_{18}H_{11}N_3O_3$ (317.30): calcd. C 68.14, H 3.49, N 13.24; found C 68.39, H 3.61, N 13.48.

***N*-{[(9*H*-Fluoren-9-yl)methoxy]carbonyloxy}picolinimidoyl Cyanide (6):** The product was obtained as a white solid (m.p. 165–166 °C) in 93% yield (3.43 g). ^{1}H NMR ($CDCl_3$): δ = 4.38 (t, *J* = 7.7 Hz, 1 H, CH), 4.62 (d, *J* = 7.7 Hz, 2 H, CH_2), 7.36, 7.44 (2 t, *J* = 6.9 Hz, 4 H, Ar-H), 7.48–7.51 (m, 1 H, Ar-H), 7.66, 7.79 (2 d, *J* = 7.7 Hz, 4 H, Ar-H), 7.84 (t, *J* = 6.9 Hz, 1 H, Ar-H), 8.14 (d, *J* = 8.4 Hz, 1 H, Ar-H), 8.79 (d, *J* = 4.6 Hz, 1 H, Ar-H) ppm. ^{13}C NMR: δ = 46.53, 72.00, 107.94, 120.34, 122.08, 125.32, 126.89, 127.48, 128.29, 137.35, 139.67, 141.43, 142.78, 147.16, 150.46, 151.97 ppm. $C_{22}H_{15}N_3O_3$ (369.37): calcd. C 71.54, H 4.09, N 11.38; found C 71.77, H 4.32, N 11.53.

(9*H*-Fluoren-9-yl)methyl 2-oxopyridin-1(2*H*)-yl Carbonate (7): The product was obtained as a white solid (m.p. 195–196 °C) in 89% yield (2.96 g). ^{1}H NMR ($CDCl_3$): δ = 4.37 (t, *J* = 7.7 Hz, 1 H, CH), 4.61 (d, *J* = 7.7 Hz, 2 H, CH_2), 6.20 (t, *J* = 6.9 Hz, 1 H, Py-H), 6.75 (t, *J* = 9.2 Hz, 1 H, Py-H), 7.33–7.44 (m, 6 H, Ar-H), 7.64 (d, *J* = 7.7 Hz, 2 H, Ar-H), 7.78 (d, *J* = 7.7 Hz, 2 H, Ar-H) ppm. ^{13}C NMR: δ = 46.51, 72.76, 105.27, 120.28, 123.20, 125.33, 127.45, 128.26, 134.89, 139.64, 141.43, 142.67, 152.40, 157.18 ppm. $C_{20}H_{15}NO_4$ (333.34): calcd. C 72.06, H 4.54, N 4.20; found C 72.31, H 4.68, N 4.41.

***N*-(9-Fluorenylmethyloxycarbonyl)glycine (Fmoc-Gly-OH):** A solution of Fmoc-OX derivative **3**–**7** (20 mmol) in acetone (100 mL) was added dropwise to a stirred solution of glycine (20 mmol) and $NaHCO_3$ (50 mmol) in water (100 mL). After stirring overnight, the reaction mixture was concentrated under reduced pressure and then extracted with CH_2Cl_2 (50 mL) to remove the unreacted Fmoc-OX derivatives. After cooling, the reaction mixture was acidified with 10% HCl to congo red litmus paper to give a white solid, which was filtered and washed with water several times, dried, and recrystallized (ethyl acetate/*n*-hexane) to give a white solid [m.p. 166–167 °C, authentic commercial sample m.p. 166–167 °C (Table 2)]. The purity of Fmoc-Gly-OH was determined by injection of 10 μL of a sample prepared from Fmoc-Gly-OH in acetonitrile onto HPLC by using the following conditions: linear

gradient 10 to 90% of solvent A in solvent B over 8 min; PDA detection at 254 nm. t_R (Fmoc-Gly-OH) = 7.03 min and co-injection with an authentic sample of Fmoc-Gly-OH.

***N*-(9-Fluorenylmethyloxycarbonyl)glycinylglycine (Fmoc-Gly-Gly-OH):** The synthesis was carried out in a plastic syringe, attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The 2-chlorotritylchloride resin with a loading 1.55 mmol/g (1 g) was washed with CH_2Cl_2 (3 × 50 mL), and then acylated with Fmoc-Gly-OH (2 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 14 mmol) in CH_2Cl_2 (10 mL). Then, the reaction mixture was stirred slowly for 1 min and let to couple for 15 min. Extra DIEA (6 mmol) was added, and the resin was stirred and left to stand with stirring from time to time for 45 min. MeOH (1 mL) was added to the resin, which was stirred for 5 min and then left to stand for an extra 5 min. The resin was filtered and then washed with CH_2Cl_2 and *N*,*N*-dimethylformamide (DMF, 2 × 50 mL each), and then deblocked by 20% piperidine in DMF for 7 min, washed with DMF, CH_2Cl_2, and DMF (2 × 50 mL each), and then coupled with the next Fmoc-Gly-OH (3 mmol), *N*,*N*-diisopropylcarbodiimide (DIC, 3 mmol), and HOBt (3 mmol) for 1 h. The dipeptide was cleaved from the resin by TFA/CH_2Cl_2 (2%, 50 mL) at room temperature for 5 min and then washed with extra solution of TFA/CH_2Cl_2 (2%, 20 mL). TFA and CH_2Cl_2 were removed in vacuo, and the crude dipeptide was precipitated with ether. The precipitate was collected and washed with ether (2×) and then dried under vacuum. The purity (99.7%) was determined by HPLC analysis by using a linear gradient 10 to 90% of solvent A in solvent B over 8 min; PDA detection at 254 nm. t_R (Fmoc-Gly-Gly-OH) = 6.29 min.

***N*-(Allyloxycarbonyloxy)picolinimidoyl Cyanide (9):** A solution of allyl chloroformate (**2**, 12 mmol) in CH_2Cl_2 (35 mL) was slowly added to a solution of the potassium salt of *N*-hydroxypicolinimidoyl cyanide (**8**, 12 mmol)[33] in H_2O (40 mL) with stirring at 0 °C. The resulting biphasic mixture was vigorously stirred at 0 °C for 30 min and then at room temperature for 3 h. After dilution with CH_2Cl_2 (50 mL), the organic phase was collected, washed with water (2 × 50 mL) and saturated aqueous NaCl (2 × 50 mL), and dried with anhydrous Mg_2SO_4. After filtering, the solvent was removed under reduced pressure, and the residue was recrystallized (CH_2Cl_2/hexane) to give desired Alloc-oxime compound **9** as a pale brownish solid (m.p. 102–103 °C) in 81% yield (2.24 g). ^{1}H NMR ($CDCl_3$): δ = 4.85–4.86 (dt, 2 H, CH_2), 5.38–5.40 (m, 1 H, CH_2), 5.46–5.50 (m, 1 H, CH_2), 5.97–6.07 (m, 1 H, CH), 7.47–7.50 (m, 1 H, Py-H), 7.82–7.86 (m, 1 H, Py-H), 8.12–8.15 (m, 1 H, Py-H), 8.77–8.79 (m, 1 H, Py-H) ppm. ^{13}C NMR (CCl_3): δ = 70.62, 107.98, 120.85, 122.16, 126.88, 130.48, 137.39, 139.63, 147.34, 150.52, 151.89 ppm. The purity of **9** was also determined after injection onto reverse-phase HPLC by using the following conditions: linear gradient 5 to 100% of solvent A in solvent B over 8 min; PDA detection at 220 nm. t_R [*N*-(allyloxycarbonyloxy)picolinimidoyl cyanide] = 6.84 min. A sample for exact mass determination was prepared, dissolving in $CHCl_3$ and diluting 1 to 100 in H_2O/MeOH (1:1): m/z = 254.0546 $[M + Na]^+$.

***N*-(Allyloxycarbonyl)glycine (Alloc-Gly-OH):** A solution of **9** (2.16 mmol) in dioxane (7 mL) was added dropwise to a stirred solution of H-Gly-OH (2.37 mmol) in 1% aqueous Na_2CO_3 (7 mL). The pH of the reaction mixture was controlled at 8 with addition of 10% aqueous Na_2CO_3. After stirring the reaction mixture for 1 h at room temperature, H_2O (50 mL) was added. The resulting mixture was acidified to pH 7 by addition of 2% aqueous HCl, and the white solid *N*-hydroxypicolinimidoyl cyanide was filtered. To remove the remaining oxime, the aqueous layer was washed with CH_2Cl_2 (3 × 60 mL) at pH 7 and pH 6.5. The aqueous layer was acidified to pH 1–2, and the product was extracted with AcOEt (4 × 60 mL). The organic layer was dried with Na_2SO_4 and filtered, and the solvent was removed under reduced pressure to render a yellow oil in 83% yield (284.1 mg). The purity of Alloc-Gly-OH was determined by injection onto reverse-phase HPLC by using the following conditions: linear gradient 5 to 40% of solvent A in solvent B over 8 min; PDA detection at 220 nm. t_R (Alloc-Gly-OH) = 4.61 min, t_R (Alloc-Gly-Gly-OH) = 3.86 min. The dipeptide was identified after co-injection with a pure sample, solid-phase synthesized by using the Alloc-Gly-OH prepared with this method. ^{1}H NMR spectroscopy showed a purity above 99%. ^{1}H NMR (CD_3COCD_3): δ = 3.88 (d, J = 6.2 Hz, 2 H, CH_2), 4.53 (dt, 2 H, CH_2), 5.14–5.17 (m, 1 H, CH_2), 5.28–5.32 (m, 1 H, CH_2), 5.89–5.94 (m, 1 H, CH), 6.53 (br. s, 1 H, NH) ppm.

***N*-(Allyloxycarbonyl)glycinylglycine (Alloc-Gly-Gly-OH):** The synthesis was carried out in a plastic syringe, attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The 2-chlorotritylchloride resin (500 mg, loading = 1.55 mmol/g) was swelled in CH_2Cl_2 (3 × 1 mL), conditioned in DMF (5 × 1 mL), and then acylated with Fmoc-Gly-OH (0.3 mmol, 1 equiv.) and DIEA (3 equiv.) in CH_2Cl_2 (0.8 mL). The reaction mixture was manually stirred for 10 min and then extra DIEA (7 equiv.) was added. The resin was left to stand for 45 min more with stirring. MeOH (0.4 mL) was added to the resin and this was manually stirred for 15 min. The resin was filtered and washed with CH_2Cl_2 (10 × 1 mL) and DMF (10 × 1 mL). After deblocking by treatment with 20% piperidine in DMF (1 + 5 + 5 min) and quantification of Fmoc, the new loading was calculated (0.57 mmol/g). This H-Gly-resin (101.6 mg) was washed with DMF, CH_2Cl_2, and DMF (10 × 1 mL each), and it was acylated by using a 0.4 M solution of the Alloc-Gly-OH prepared following the above-mentioned method (3 equiv.) and Oxyma (3 equiv.), preactivated with DIC (3 equiv.) for 3 min and left to stand for 1 h with stirring. The dipeptide was cleaved from the resin with 5% TFA/CH_2Cl_2 (1 mL, 5 × 5 min) at room temperature, washing the resin with CH_2Cl_2. The solvent and TFA were removed under an atmosphere of nitrogen, and the crude dipeptide was precipitated by adding cold Et_2O (3 × 3 mL). The resulting crude was lyophilized in H_2O (3 mL), obtaining a white solid powder in 92% yield (11.5 mg). The purity (>99%) was determined by HPLC analysis by using a linear gradient 5 to 100% of solvent A in solvent B over 8 min; PDA detection at 220 nm. t_R (Alloc-Gly-Gly-OH) = 3.39 min. HPLC-MS showed the expected mass for the dipeptide: m/z = $[M + H]^+$ = 217.04.

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Capítol 4. O*xyma en la prevenció de reaccions secundàries catalitzades per base*

Publicació IV

Aspartimide formation in peptide chemistry: occurrence, prevention strategies and the role of N-hydroxylamines †

Formació d'aspartimides en química de pèptids: causes, estratègies de prevenció i el paper de les *N*-hidroxilamines

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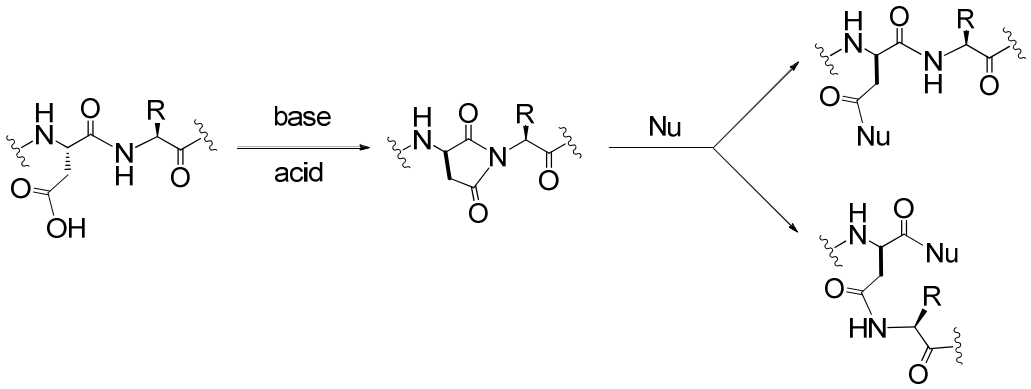
† Ramon Subirós Funosas va realitzar la recerca científica sobre l'impacte de les aspartimides en síntesi peptídica, els factors que influencien la seva formació i estratègies de prevenció existents. Així mateix, va elaborar el manuscrit.

Resum

L'article de revisió que s'inclou a continuació pretén ser una introducció de la problemàtica associada a la formació aspartimides i subproductes derivats, la qual s'investiga en detall, en especial pel que fa a l'efecte d'addicionar *N*-hidroxilamines, a la Publicació V.

La formació d'estructures de tipus aminosuccinil (aspartimides) a causa de la ciclació intramolecular de residus Asp, o en menor mesura Asn, és una gran problemàtica en químics de pèptids. Els subproductes produïts com a resultes de la obertura nucleòfila d'aquesta estructura cíclica provoquen la modificació permanent de l'esquelet peptídic, reacció que té un major impacte quan s'utilitza l'estratègia Fmoc/*t*Bu, ja que les successives desproteccions amb piperidina tenen efecte acumulatiu. S'han dedicat grans esforços a estudiar els efectes del grup protector de la cadena lateral, la base i la seqüència entre d'altres, així com també a evitar per mitjà de diverses estratègies aquesta reacció secundària. En aquest treball de revisió es recullen les bases del problema de l'aspartimides i es presenten les estratègies disponibles per minimitzar la formació d'aspartimides/piperidides. En particular, es centra l'atenció en l'efecte de l'addició de *N*-hidroxilamines, àmpliament usades com additius en la formació de l'enllaç peptídic, en aquesta reacció secundària de conseqüències greus.



Formació d'aspartimides i piperides a partir de la ciclació de residus d'àcid aspàrtic.

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Aspartimide formation in peptide chemistry: occurrence, prevention strategies and the role of *N*-hydroxylamines

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ABSTRACT

The formation of aminosuccinyl structures (aspartimides) during the intramolecular cyclization of Asp, or to a lesser extent Asn, is a major concern for peptide chemists. The byproducts produced from nucleophilic opening of this cyclic moiety results in permanent modification of the peptide backbone, which is more severe when using the event of using a Fmoc/tBu protection scheme. Considerable efforts have been devoted to studying the effect of the *β*-protecting group, base and sequence, among other factors, and also to circumventing this dramatic side reaction. Here we summarize the aspartimide problem and present the strategies available to minimize or prevent the formation of aspartimide/piperidides will be presented. In particular, attention will be focused on the effect of acidic *N*-hydroxylamines, widely used to assist peptide bond formation, in this detrimental side reaction.

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1. Introduction

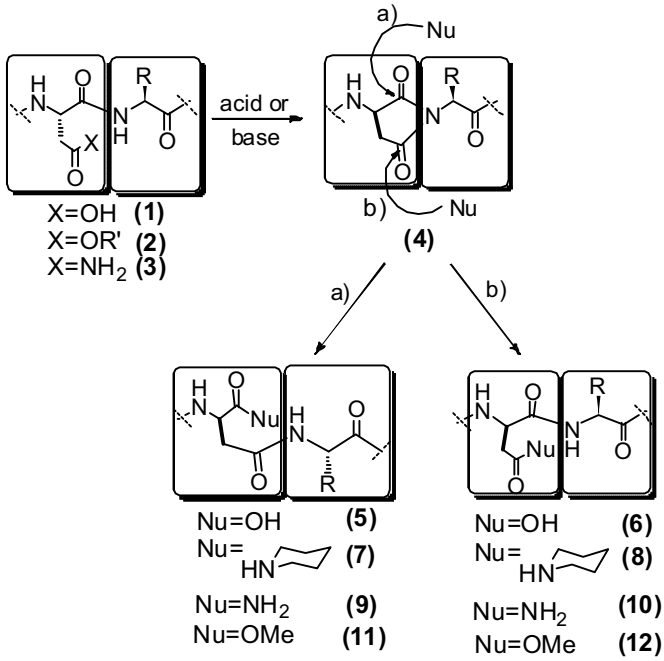
Many side reactions may occur at various stages of solid-phase or solution peptide synthesis.[1] Some of these take place during activation of the *C*-terminus and coupling with the *N*-terminus, such as epimerization of the α-carbon of the residue being activated, which is especially severe in the assembly of His and peptide fragments, or *N*-terminal guanidylation of the amino group when uronium/amminium salts are used, thereby blocking any further elongation of the peptide chain.[2,3,4,5,6] In these steps, certain amino acids also might undergo specific undesired reactions, like conversion to Orn, δ-lactam formation (Arg), dehydration to nitrile, or succinimide and glutarimide formation (Asn, Gln).[7,8,9,10,11] Strong acidic conditions (HBr, TFA, HF) used for the removal of Boc, Bzl and tBu protecting groups or final cleavage of the peptide from the resin often cause alkylation on residues with nucleophilic side chains (Met, Cys) or those activated towards electrophilic aromatic substitution (Trp, Tyr), unless suitable cocktails of scavengers are added.[12,13,14,15,16,17,18] Acidolysis occasionally gives rise to undesired cyclizations (Glu, Gln, Met) or, under treatment with HF, provokes fragmentation of the peptide chain in Met, Ser, Thr or Asp-Pro-containing sequences.[19,20,21,22,23,24,25] Depending on the resin and sequence involved, basic media required for Fmoc removal can lead to diketopiperazine formation at the dipeptide stage and also to elimination of the thiol group of Cys to give dehydroalanine and piperidinylalanine derivatives.[26,27,28,29] The oxidation of Trp, Met and Cys has also been observed.[30]

N-Hydroxylamine-based additives greatly contribute to the success of peptide synthesis.[31] In addition to assisting in the reduction or suppression of several side reactions that occur during peptide bond formation, such as *N*-acylisourea formation, amino acid epimerization and guanidylation of the peptide chain, the unique acidic character of *N*-hydroxylamine-based additives also minimizes the impact of other non-coupling-related undesired reactions. Here we focus our attention on what could be considered the most documented and studied side reaction in peptide chemistry, namely aspartimide formation. Like most of the above mentioned detrimental impurities, aspartimide and its derivatives remain attached to the peptidic core, thereby hindering their removal, especially during the assembly of long peptides. This event is of concern in the case of API's (, even when such impurities are encountered in very low amounts.

2. Appearance of aspartimide and derived byproducts

Aspartimides included amino-succinimide structures, formed or built as part of a peptide backbone (**4**, Scheme 1). Aspartimide units are often abbreviated as "Asu" (*A*mino-*su*ccinyl).[32,33] However, it is more appropriate to use the term "Asi" (*As*part*i*mide) or "Asc" (*A*mino-*su*ccinyl) when referrering to this structure, since "Asu" has also been used to denote *α*-aminosuberic acid (herein we use "Asi").[34,35] Asi-containing structures show properties and applications of interest in many fields. Given their biodegradability, polyaspartimides are promising solid supports for anchoring sialic acid linkers as inhibitors of viruses, like influenza.[36] Materials science also takes advantage of Asi-based compounds, like bis(*N*-silylalkyl)aspartimides, which have recently been used to prepare surfactants, viscosity modifiers, primers and adhesives.[37] The presence of Asi moieties has been recently observed to support cell adhesion of peptides *in vivo* and *in vitro*.[38] Alternatively, Asi structures have proved useful as peptidomimetics during the synthesis of 2,5-dioxopiperazines as peptidomimetics, upon nucleophilic ring opening.[39] Studies based on X rays, temperature dependence and Circular Dichroism on Boc-L-Pro-L-Asi-Gly-L-Ala-OMe concluded that the presence of Asi units induce type II'- *β*-turn in the peptide backbone conformation, as a result of stabilization by intramolecular hydrogen bonding.[40,41]

However, Asi (**4**) can be spontaneously generated during the elongation of Asp-containing peptides as result of nucleophilic attack from the amide nitrogen of the preceding residue to the *β*–carboxyl moiety of Asp (Scheme 1). Given the need for orthogonal protection schemes, the *β*–functional group in the side chain of Asp is normally masked as an ester (**2**), thereby acting as a leaving group in this side reaction. Nevertheless, under certain strongly acidic conditions, this unwanted cyclization is also reported to occur on the unprotected *β*–carboxyl group (**1**).[42,43,44,45] The early syntheses of Asp-containing peptides gave low yields and purities, although much effort was required to identify the source of the problem. It was only after ESI-MS detection of a $[M-18]^+$ ion in the crude mixture that Asi formation was envisaged, an event that was also supported by NMR.[46,47,48] Further proof of the Asp-based origin of the side reaction was obtained when the peptide fragment at the *N*-terminus of Asp was separately synthesized in a clean and efficient way.[48] The detection of Asi (**4**) formation can be troublesome in cyclic peptides, since the mass of this byproduct matches that of the desired cyclic material.[49]



Scheme 1. Formation of aspartimide and byproducts derived from nucleophilic ring-opening.

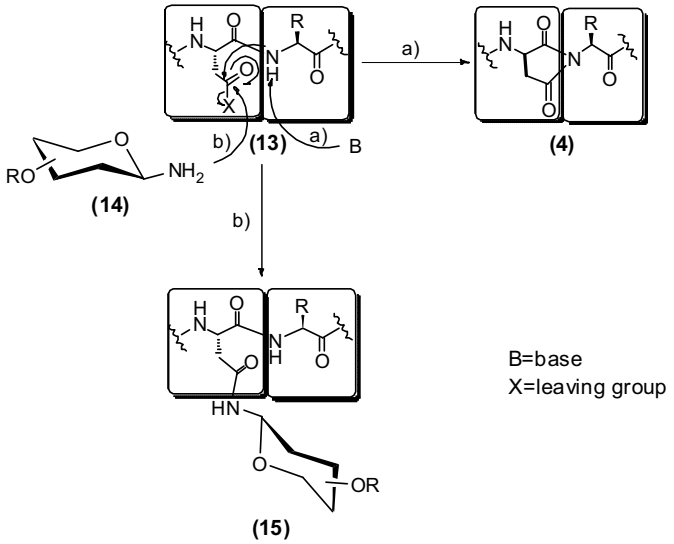
This byproduct (**4**) has been encountered in neutral, strongly acidic and basic media, either in solution or solid-phase synthesis.[33,43,45,48,50,51,52] It was originally found in the Boc/Bzl protection strategy, in the cleavage step of the peptide chain from the solid support using HF or CH_3SOOH.[42] In solution, this side reaction may take place during the removal of the Boc temporary protecting group and in the subsequent basic treatment to obtain the free amine, or during amide bond formation using tertiary amine catalysis.[32,43] Later on, with the increasing presence of Fmoc/tBu protection schemes, this side reaction was found during standard basic Fmoc deprotection conditions.[42] Asi units can also occur when using a base-labile linker attached to the

resin.[53] The wide variety of reaction conditions in which Asi are formed is not the major concern regarding this side reaction, but rather the additional byproducts derived from nucleophilic opening of the amino-succinyl moiety (**4**).[52] In aqueous media, either in the synthesis or purification step, hydrolysis of Asi (**4**), which derives from attack to the *β*-carboxyl of Asp (path B, Scheme 1), leads back to the unmodified *α*–peptide (**6**).[34] However, attack to the *α*-carboxyl group (path A, Scheme 1) generates a product with unnatural backbone, the isoaspartyl-*β* –peptide (**5**).[54] Unprotected hydrolyzed peptides **5** and **6** may go unnoticed after amino acid analysis, since both release Asp. Moreover their mass is identical.[42,44]The presence of the isoaspartyl- *β* –peptide (**5**) implies major difficulties in its separation from the target peptide.[34,55,56,57]

The impact of this detrimental side reaction is specially relevant in the Fmoc/tBu approach, the currently predominant choice of protecting strategy, not only because the basic treatment required for Fmoc removal takes place after each coupling/deprotection cycle, but also because this side reaction is faster in basic than in acidic media.[33,34,42,45,51,58] Moreover, in long sequences, stronger bases or extended reaction times are required for quantitative deprotection of the temporary group, thereby increasing the extent of Asi formation.[47] In addition, secondary nucleophilic amines, such as piperidine, are used to induce basic pH. Once an Asi ring (**4**) is present in the peptide backbone, piperidine can also open the cyclic structure in any of the carboxyl groups, thus resulting in a mixture of the piperidide of the *α*-peptide (**8**) and the piperidide of the *β*-peptide (**7**), in addition to the above mentioned hydrolyzed products (**5** and **6**).[45,47,48,51,52,54,58] Direct piperidine attachment to the *β*-carboxyl-protected group was considered by Yang and colleagues, although other authors rejected this mechanism.[45,55] These piperidide byproducts (**7** and **8**), which depend on the residue following Asp, can be easily identified by ESI-MS and show 47-mass units less than the target peptide.[45,47,48,59]

Thus, only 1 out of the 4 byproducts derived from the opening of the amino-succinyl ring leads to the desired *α*-peptide (**6**). This percentage of target peptide is even lower, bearing in mind that the content of piperidides **7** and **8** increases as does the number of deprotection steps performed. Moreover, the Asi ring (**4**) shows a remarkable activation towards epimerization of the *α*-carbon, which may produce additional aspartyl and piperidinyl derivatives.[47,52,55,57] Further studies also revealed that, as a result of electronic effects, the *β*-peptide byproducts (**5** and **7**) are more favored than *α* ones (**6** and **8**).[32,55,60,61] Steric and conformational contributions might also be involved.[55] Finally, Asn (**11** and **12**) and Asp-methyl ester (**9** and **10**) derivatives have also been reported to occur after bubbling with ammonia at 0ºC or may arise accidentally as a result of washings with methanol in the presence of traces of DIEA in solid-phase.[34,51,55,62]

Synthetic methods to afford *N*-glycopeptides (**15**) as an alternative to enzymatic strategies are also hampered by this undesired cyclization (Scheme 2).[54] The two most representative approaches to bind a *N*-glycosylamine (**14**) to a peptide chain (**13**) involve the activation of the *β*-carboxyl group of Asp (**1**, convergent approach) or Asn (**3**, building block approach).[56,63,64] However, this enhanced reactivity of the side chain often results in nucleophilic attack of the preceding backbone amide nitrogen, which is catalyzed by the presence of base such as DIEA to form the cyclic **4**, which occasionally represents the major product (Scheme 2). The competition between *N*-glycosylamine attachment to the *β*-carboxyl (**15**) and Asi formation (**4**) has been proposed by many authors.[54,63,64] Use of the sugar moiety as hydrogen-abstracting agent is one of the most efficient strategies to minimize this side reaction.[54]



Scheme 2. Base-mediated aspartimide formation during attachment of *N*-glycosylamines to Asp/Asn.

The generation of aspartimides (**4**) and derived byproducts during peptide synthesis causes a substantial decrease in yield and purity in addition to time-consuming purifications. Nevertheless, the effects of the appearance of these compounds in the peptide backbone are even more severe *in vivo*.[54] Although some exceptions are reported on the enhanced biological activity of Asi-containing sequences, the formation of the Asi (**4**) and, more important, the rearrangement to the *β*-peptide (**5**), which occurs in Asp (**1**) and Asn (**3**) residues, leads to dramatic events, such as the induction of flexible areas in proteins with second and tertiary structure and even their degradation.[54,60,65] The deamidation of Asn (**3**) to Asp (**1**), as a result of hydrolysis of the aspartimide on the *β*-carboxyl group, and racemization of Asp (**1**), are associated with protein aging and degradation and with Alzheimer's disease, since many proteins associated with fibril aggregation show a high percentage of these modified backbones.[66,67] Recent studies revealed a link between Asi (**4**) formation and protein dimerization.[68]

3. Factors influencing aspartimide formation

3.1. Base

The effect of alkalis on base-catalyzed aspartimide formation was first tested in solution, after reports of the appearance of this side reaction during coupling in this approach.[43] The stability of the Asp residue has been compared in the presence of various tertiary amines. The use of diisopropylethylamine (DIEA) has been shown to be safer than others, such as triethylamine (Et_3N). This result is attributed to its higher sterical hindrance.[61,42] However, even when DIEA is chosen as the basic pH inducer, a small percentage of Asi or its derivatives might be formed (Tam and coworkers calculated that 0.002% of Asi is generated in each coupling step). This formation is higher in the synthesis of *N*-glycopeptides (**15**).[54,61,63] The extent of this unwanted cyclization is dependent not only on the type but also on the total amount of base used in the coupling cocktail.[54,63]

Piperidine, the standard secondary amine applied in the removal of Fmoc in solid-phase, gives rise to a considerably

higher percentage of Asi (**4**) than previously mentioned tertiary amines, plus the additional presence of piperidides of the *α*- and *β*-peptide (**7** and **8**).[42] Even after long exposure to Asp-containing sequences, DIEA and Et_3N do not cause this side reaction.[42,69] The concentration of the secondary amine also affects the amount and ratio of byproducts.[45,47] Stronger bases, such as DBU, TBAF, aqueous NaOH and NH_3, further accelerate the rate of this cyclization, compared to piperidine.[46,47,52,60,62,70,71] For instance, 50% piperidine in DMF produces a similar amount of byproducts to 2%DBU-2% piperidine in DMF.[47]

3.2. Acid

Cleavage of the peptidic material from the solid support by means of the Boc/Bzl protection scheme requires strong acid. Treatment of the resin with hydrofluoric acid (HF) causes impurification with Asi (**4**) and *β*-peptide (**5**), an occurrence that is particularly severe when using the "low-high HF" cleavage strategy.[42] Other strong acids used to release the peptide chain, like trifluoromethanesulfonic acid (TFMSA) and concentrated TFA, also lead to these byproducts.[45,51,62,72] The latter organic acid has also been described to be problematic when simultaneously performing cleavage from the resin and removal of the Boc temporary group in the cyclic peptide argifin.[73] Diluted 1N hydrochloric acid (HCl) might solve this problem, although higher concentrations (6N) also give rise to Asi (**4**).[44,73]

Regarding the use of milder or less concentrated acids to remove the Boc temporary group in solution, acetic acid, hydrobromic acid (HBr) in acetic acid and 1:4 phenol/*p*-cresolv are not recommended since they may give rise to the undesired cyclization that produces Asi residues (**4**).[43] Alternatively, HBr-TFA mixtures or TFA alone render only traces of these byproducts.[43]

3.3. β –carboxyl Protecting Group

The nature of the *β*-carboxyl ester, acting as protecting group, is markedly influential on the impact of the aspartimide side reaction (Figure 1). In the acidolytic-catalyzed cyclization, Asp(OBzl) (**16)** gives rise to high percentages of Asi (**4**) peptide and thus offers the poorest protection against this unwanted process.[32,43] The *β*-protection of Asp as cyclohexyl ester (OChx, **17**) results in increased prevention of Asi (**4**), possibly because of its high bulkiness.[61,74,75]Although Asp(OChx) (**17)** does not completely suppress the appearance of aspartimide and *β*-peptide during final cleavage with HCl, its content is much lower than that of Asp(OBzl) (**16).**[51,61,72] Low efficiency of the *β*-benzyloxy protection (**16**) in preventing this side reaction has prompted its selection when the formation of Asi units (**4**) is desired, after Nicolas and colleagues found that conversion from Asp(OBzl) (**16)** to Asi (**4**) could be quantitative, only after 10 min. of acidic treatment.[34,42]

β-cyclohexyloxy protection (**17**) is also more effective than *β*-benzyloxy one in the prevention of base-catalyzed aminosuccinyl formation (**4**).[32,72] In the presence of tertiary amines, Asp(OBzl) (**16**) induces up to 50% undesired cyclization, whereas Asp(OChx) (**17**) only renders <1%.[59] Moreover, the presence of the benzyl ester is associated with additional side reactions, such as 1,4-diazepine-2,5-dione ring formation, which can be the major product.[59] Nevertheless, Asp(OChx) (**17**) might give rise to Asi (**4**) when basic cleavage of the resin is performed.[58] Protection with the *β*-allyloxy group (**18**) does not result in improved prevention of the side reaction and is comparable to that achieved by *β*-benzyl ester protection (**16**).[49,52] Protection with the bulky *tert*-butyl ester (**19**) has shown greater efficacy than the above mentioned strategies in minimizing this side reaction in basic media.[49,54,70,73,76,77]In Fmoc removal conditions (treatment with piperidine), Asp(OtBu) (**19**) gives rise to considerably lower conversion to Asi residues than Asp(OChx) (**17**) and even Asp(OBzl) (**16**).[42]

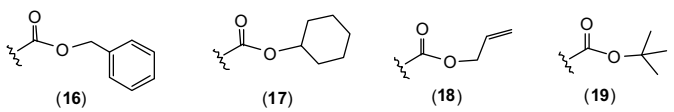


Fig. 1. Structure of some classical Asp β-carboxy protecting groups for Boc and Fmoc strategies

3.4. Solid support

The resin used as solid-phase support may sometimes contribute to decreasing the formation the formation of Asi (**4**). Thus, in certain syntheses, use of 2-chlorotritylchloride resin, which offers the possibility of mild acidolysis of the peptide chain, prevents this undesired cyclization to a greater extent than Tenta Gel-derived solid supports.[62,73] Further improvements are achieved when polystyrene-type resins are replaced by less hydrophobic supports, such as PEGA and CLEAR (cross-linked ethoxylate acrylate–type resin).[60,64] By using the latter resin and 20% piperidine in NMP, only 8% aspartimide-related byproducts are detected in multi-Asp sequences.[78] Cebrian and colleagues reported that, when using the latter resin, the purity of the peptide increased from 10-30% to 80%, because the disruption of peptide chain aggregation translates into more efficient Fmoc deprotection steps, and thus basic treatments can be shortened, leading to minimization of Asi (**4**) and the formation of derived byproducts (**5**-**8**) formation.[60]

3.5. Temperature

The temperature at which acidic or basic treatments are carried out contributes to a lower extent than previously mentioned factors in circumventing the formation of aminosuccinyl-containing peptide chains. However, high temperature alone (in the absence of solvent) also gives rise to Asi (**4**) formation, although at a slow rate.[68] However, a slight decrease in temperature might result in enhanced purity of target peptidic material.[54] Thus, Tam and coworkers observed that yield increased by10% when the reaction temperature was lowered from 0ºC to -20ºC.[61] In contrast, when the temperature of the treatment of Fmoc-protected peptides with piperidine is increased to 45ºC, a greater content of Asi (**4**) and piperidides (**7** and **8**) is detected.[52]

3.6. Solvent

The properties of the solvent in which acidic or basic steps are performed have a great impact on the rate of undesired cylicization of Asp (**1**-**3**) to Asi (**4**). Extremely influential is the solvent polarity, which increases the percentage of Asi (**4**) formation in the order HMPT>DMSO>DMF>>THF>DCM.[47] Addition of water to DMSO solutions results in additional instability of the Asp residue.[64] In the synthesis of *N*-glycopeptides, through the activation of *β*-carboxyl group of Asp/Asn (**1**/**3**), DCM gives rise to lower percentage of aspartimide (**4**).[35] Moreover, Dölling and colleagues reported a substantial decrease in piperidide (**7** and **8**) formation when DCM or THF is used instead of DMF in the Fmoc deprotection step (0.5% vs. 32%).[69] Among low-polarity solvents, the use of DCM

is more efficient in the prevention of this side reaction than THF.[56]

Protic solvents, like MeOH, EtOH or BuOH, show a faster rate of Asi (**4**) formation than non-protic ones like DMF [complete conversion of Asp (**1**) to Asi (**4**) takes place in 15 days in DMF and 1-2 days in the previous mentioned protic solvents].[55] DMF is also involved in the ratio of byproducts observed, since formation of *β*-peptide (**5**) and epimerized byproducts are favored in this solvent.[47,48,55] Finally, an efficient strategy for preventing Asi formation (**4**) consists of treating the Fmoc-peptide-resin with 30% piperidine in NMP (only 4 min. per cycle), in conjunction with a fluoride-labile linker (2% aspartimides).[53]

3.7. Sequence (Asp-X)

Undoubtedly, the nature of the neighboring amino acid located at the C terminus of the aspartic acid (Asp-X) determines the degree of aspartimide formation, since the cyclization of Asp (**1**) to Asi (**4**) is initiated by attack of the amide backbone nitrogen of the preceding residue.[47] Thus, Gly (the less sterically hindered amino acid) shows the highest tendency towards formation of this unwanted cyclic structure (**4**) both under acid or base catalysis.[33,45,79,80,81] The syntheses of many peptidic compounds fail due to this marked instability, such as partial sequences of coat protein phage MS2, CRH hormone or thrombospondin.[60] Thus, it is not surprising that Asp-Gly-containing sequences, such as the 1-6 fragment of toxin II of scorpion Androctonus australis Hector (H-Val-Lys-Asp-Gly-Tyr-Leu-NH_2) and H-Glu-Asp-Gly-Thr-OH, have been widely used as models for testing aspartimide formation.[32,33,42,44,45,50,52,54,57,71,79,82,83,84]

Many other residues have great influence on base-catalyzed Asi (**4**) formation. One of the most prone amino acids is Asn, either protected as Asn(Trt) or Asn(Mtt). Most sequences containing Asn(Trt), such as partial fragments of MS2 or CRH, might fail in an initial attempt, although some exceptions are reported.[45,47,48,69,70] Asn (Mtt) is also very sensitive to Asi (**4**) formation.[71,79] Gln(Trt) and Asp(OtBu) have been shown to favor the unwanted cyclization.[45,47,64] The tendency towards this cyclization in other residues depends on the protecting group. Thus, Cys(Acm) and Arg(Pbf) induce this side reaction to a higher extent than Cys(Trt) and Arg(Pmc).[52,69,70,71] Ala [even when Asp is protected as Asp(OChx)], Phe and Ile also favor undesired Asi (**4**) formation.[45,55,58,62,64,71,76] In contrast, His(Trt), Ser(tBu), Thr(tBu), Tyr(tBu), Leu and Val are relatively stable to this cyclization.[34,35,45,48,49,69,70,71,74] During glycosamine attachment on the Asp/Asn (**1**/**3**) side chain, Glu(OChx) or Ser(tBu) gave higher percentages of aminosuccinyl-peptide chain than other residues.[35,56]

In acid-catalyzed Asi (**4**) formation, some residues that induce stability under basic treatment, become markedly prone to this side reaction, thus proving that acid and basic-catalysis go through distinct pathways. This is the case of Ser, Thr and His.[42,45,50] Other residues, like Gly and Asn are as sensitive as in basic media.[42,45] Val is also stable under acidic catalysis.[32]

Revealing studies have been conducted on the tendency of unprotected amino acids preceding Asp residues (**1**) to give this undesired cyclization under basic conditions. On the one hand, Ser and Thr accelerate the rate of Asi (**4**) formation, compared to the average tendency of the overall residues.[32,70,71] It has been proposed that the favorable tendency towards this cyclization is due to the presence of a neighbouring group effect from the free *β*-hydroxyl group.[32] On the other hand, amino acids bearing acidic *β*-functional groups, such as Asp, Glu and Tyr show a lower towards this process because the presence of the negatively-charged side chain functional group precludes the formation of a second negative charge, which is necessary to initiate the cyclization.[32] Asn and Gln are not as sensitive as their protected analogues.[54,69] Surprisingly, Met does not favor the aspartimide (**4**).[32] Hypotheses have been made about the formation of a 6-membered cyclic structure with the Met side chain.[32]

3.8. Conformation

The conversion of Asp (**1**) to Asi (**4**) units is not only sequence- but also conformational-dependent.[47] It has been observed that replacement of L-aa by D-aa results in enhanced byproduct generation, depending on the residue that has been changed.[69] Thus, introduction of *D*-Asp(OtBu)-*D*-Gln(Trt) in positions 25 and 26 of the CRH hormone increases the percentage of Asi (**4**) and piperidides (**7** and **8**) after repetitive Fmoc deprotection, compared to *L*-Asp(OtBu)-*L*-Gln(Trt). This observation would indicate the formation of a more favored conformation.[47,48] The presence of *D*-amino acids in positions n+2 and n+3 induces a similar effect (n=Asp), whereas in more distant positions no increased tendency is detected.[48]

4. Current approaches to minimization/suppression of aspartimide formation

β-tertbutyloxy (**19**) and *β*-cyclohexyloxy (**17**) protection of Asp results in enhanced prevention of base- and acid-catalyzed Asi (**4**) formation respectively, compared to the more prone *β*-benzyloxy (**16**) and *β*-allyloxy (**18**) groups. Nevertheless, contrary to initial impressions after evaluating this kind of protection, it was observed that even when this choice of protecting group is supported by the presence of relatively non-strong base, like piperidine, considerable amounts of Asi (**4**) and derived byproducts can arise.[35,45,47,48,52,55,57,69] In particular, using Asp(OtBu) (**19**) in the Fmoc/tBu scheme, substantial formation of aspartimides (**4**) and piperidides (**7** and **8**) is detected. As a result of these observations, and with the aim to minimize or suppress this detrimental side reaction, efforts have been made to improve the tools available in peptide synthesis.

4.1. Sterically hindered protecting groups, bases and microwave irradiation

Initially, the so-called "temporary group" strategy was thought to be efficient in preventing the appearance of Asi (**4**) when following the Boc/Bzl scheme.[85] This approach consists of *β*-protection of Asp with a group showing orthogonality to the cleavage conditions, and the introduction of Fmoc-amino acids in the residues following Asp. Thus, these temporary *β* -protecting groups can be removed prior to HF treatment, which is envisaged as a safe scenario.[43,85] However, it was later discovered that acid-catalyzed Asi (**4**) formation also takes place in the free Asp (**1**).[42,44] Moreover, one of the first *β*-protecting groups proposed, the phenacyl ester, was found to be unstable to basic coupling conditions.[32,42] The introduction of aspartic acid as Asp(OtBu) (**19**) in this strategy results in similar or poorer performance than Asp(OBzl) (**16**) and Asp(OChx) (**17**).[42] Alternatively, the use of *β*-4-chloro-benzyloxy group in the standard Boc/Bzl strategy was not suitable, since this side reaction was enhanced in comparison with *β*-benzyloxy (**16**).[43]

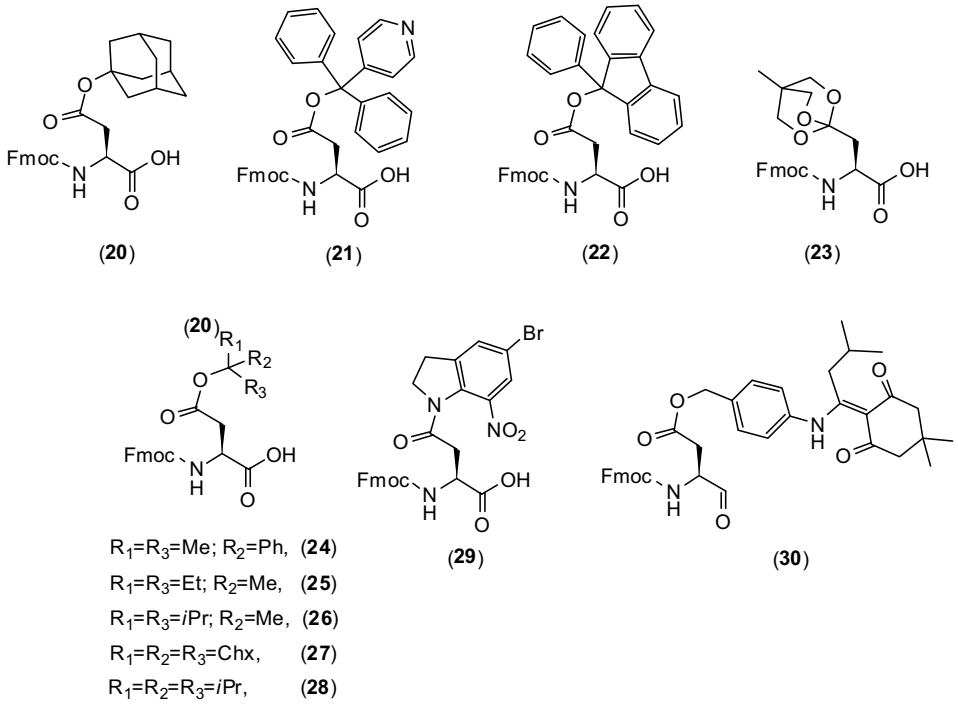


Figure 2. Structure of Fmoc-Asp-OH residues featuring sterically hindered *β*-carboxy protection.

In order to minimize the nucleophilic attack of the preceding amide backbone nitrogen atom to Asp, highly sterically hindered *β*-protecting groups for the Fmoc/tBu scheme have been designed (Figure 2). On the one hand, increase of the bulkiness does not result in lower percentage of Asi (**4**), when the rigidity of the protecting group is simultaneously enhanced.[52,57] Thus, *β*-protection as Asp[Oada=*β*-(1-adamantyl)] (**20**), Asp[OPyBzh=*β*-(4-pyridyl-diphenylmethyl)] (**21**), Asp[OPhFl=*β*-(9-phenyl-fluoren-9-yl)] (**22**), Asp[OBO=*β*-(4-methyl-2,6,7-trioxabicyclo[2.2.2]-oct-1-yl)] (**23**) or Asp[OPp=*β*-(2-phenylisopropyl)] (**24**) is less efficient than Asp(OtBu) (**19)** in preventing this undesired cyclization.[47,52,70,86] However, recent studies report that the latter approach, also referred to as Asp(OPhiPr) (**24**), dramatically reduces Asi (**4**) formation, in comparison with *β*-allyloxy protection (**18**).[64] The aromaticity of *β*-(4-pyridyl-diphenylmethyl) (**21**), *β*-(9-phenyl-fluoren-9-yl) (**22**) and *β*-(2-phenylisopropyl) (**24**) results in an excellent leaving group, which might favor the cyclization.[52] In contrast, the more bulky, although flexible, Asp[OMpe=*β*-(3-methylpent-3-yl)] (**25**) and Asp[ODie=*β*-(2,3,4-trimethyl-pent-3-yl)] (**26**) are considerably less sensitive than Asp(OtBu) (**19**) to Asi (**4**) formation.[52,57,71,79,87] Asp(ODie) is more efficient than Asp(OMpe) with prolongued exposure to piperidine treatment.[79] A further increase in bulkiness, as in the case of Asp[OTcm=*β*-(tricyclohexylmethanol-yl)] (**27**) and Asp[OTim=*β*-(triisopropylmethyl)] (**28**) results in extremely difficult syntheses. Moreover, this increased hindrance does not diminish the impact of this side reaction.[79] Regarding *N*-glycopeptide (**15**) chemistry, Asp[Bni=(*β*-5-bromo-7-nitroindoline)] (**29**) is the best choice for protection because it efficiently combines the prevention of Asi (**4**) formation with possible activation towards glycosamine attachment under UV light.[54,56,88] Finally, *β*-protection with the 2% hydrazine hydrate-labile Dmab [4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl] (**30**) increases the tendency to cyclization. Therefore, this protecting group is used when the formation of Asi (**4**) units is desired.[62]

The effect of using highly sterically hindered bases has also been studied. The addition of bulky tertiary amines in the coupling cocktail, such as protong sponge, methyldibenzylamine and tribenzylamine decrease the cyclization kinetics and also the rate of amide bond formation, thereby resulting in inefficient couplings.[43] Pyridine analogues, like collidine, 1-methyl-2-pyrrolidone and 4-pyrrolidino pyridine behaved similarly.[34] The use of the guanidine-based TMG (1,1,3,3-tetramethylguanidine) is not recommended, since conversion of Asp (**1**) to Asi (**4**) increases.[52] The prevention of aspartimide (**4**) formation can be enhanced by using bases structurally resembling piperidine.[69] Thus, 4-hydroxypiperidine, diethanolamine, piperazine and morpholine substantially decrease the byproduct content, compared to piperidine (0.5% vs. 32%).[35,58,60,89,90] In the synthesis of a CRH hormone analogue, piperazine does not give rise to Asi (**4**) or piperidides (**7** and **8**).[69,77] In addition, piperazine is less odorous than piperidine.[77,91]

By disrupting chain aggregation, microwave irradiation is a powerful tool for the synthesis of small organic molecules and peptides, especially in the assembly of hydrophobic sequences.[91,92] However, there is concern about the suitability of this technique in Asp-containing sequences, because the accomplishment of rapid coupling and Fmoc-removal steps has been associated with accelerated Asi (**4**) formation.[91]However, in fact microwave irradiation enhances the efficiency of weaker bases than piperidine, such as piperazine, which induce slow-rate deprotections in hydrophobic peptides.[77,91] In contrast, when Fmoc removal with piperazine was assisted by microwaves, deprotection was complete in only 3 min.[77,91]

4.2. Mild-cleavable linkers, backbone amide anchorage and N-α-protecting groups

Asi (**4**) formation can also be suppressed by using synthetic strategies that prevent strong acidic or basic conditions (Figure 3). Among these, linkers have been developed that allow cleavage from the resin under neutral or slightly basic conditions, which are particularly useful in the Boc/Bzl strategy, in which HF cleavage is the main source of Asi (**4**) formation.[53,58] For instance, Wagner and colleagues proposed the use of PTMSEL (2-Phenyl-2-trimethylsilylethyl) linker (**31**) which bears a weak benzylic C-Si bond and is labile to TBAF·$3H_2O$ in DCM, giving rise to only 2% of aspartimides (**4**).[53] Alternatively, the peptide chain can be anchored to the HMFS [*N*-[(9-hydroxymethyl)-2-fluorenyl] succinamic acid] handle (**32**), which can then be quickly cleaved in basic media, after treatment with anhydrous morpholine in DMF.[58,89] The absence of piperidine results in the prevention of aspartimide-based byproduct formation (**4**-**8**).

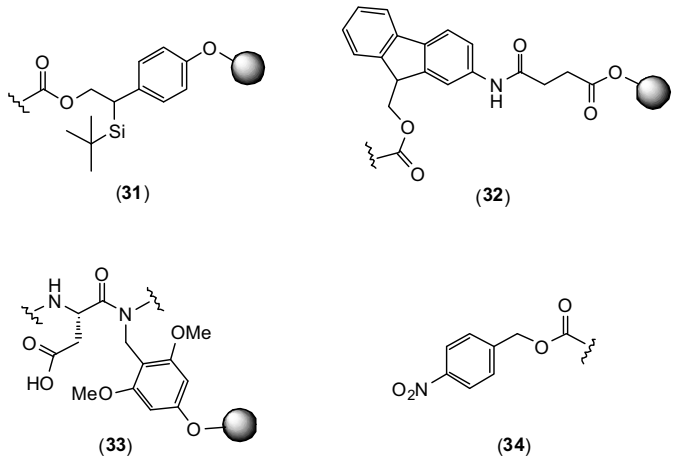


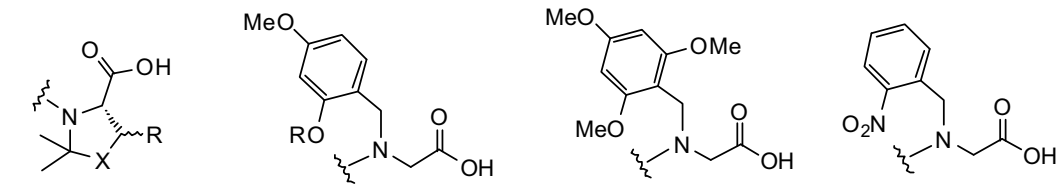
Figure 3. Structure of non-basic cleaved PTMSEL and HMFS linkers, backbone amide anchoring groups and Sn(II) labile pNZ N^{α}-protecting group.

In addition, amide backbone linkers (**33**) also have applications in the prevention of generation of Asi (**4**) units.[93] The advantage of this type of handle is that the growing peptide chain remains anchored to the resin by one of the backbone amide nitrogen atoms and therefore the Fmoc SPPS of C-terminal modified peptides can be achieved as result of the possibility of bidirectional growth.[93,94] If this nitrogen atom corresponds to the residue preceding Asp (which initiates the unwanted cyclization), aspartimides (**4**) are not formed.

Finally, some *N*α-amino protecting groups have been specifically designed to prevent this side reaction and are removed under safe conditions.[82] Thus, the pNZ (*p*-nitrobenzyloxycarbonyl) amino-protecting group (**34**), totally orthogonal to Fmoc, Boc and Alloc groups, is selectively eliminated under mild neutral conditions [hydrogenation or, more conveniently Sn(II) chloride], which do not give rise to Asi (**4**), isoaspartyl-*β*–peptide (**5**) or piperidides (**7** and **8**).[82] A recommended strategy consists of the introduction of pNZ-protected amino acids in the residues following Asp.[82]

4.3. Pseudo-Pro and Asp-X backbone amide protecting groups

Asp-Pro sequences do not undergo the undesired cyclization that leads to Asi (**4**) units because the backbone amide nitrogen atom of the preceding residue that initiates this reaction is *N*,*N*-alkylated and therefore not activated towards nucleophilic attack.[54] On the basis of this observation, pseudoproline (PP) building blocks featuring an oxazolidine ring were introduced, representing one of the most attractive approaches to completely suppress Asi (**4**) formation (Fig. 4).[95] These cyclic constructs, available as aa-PP dipeptides, which also disrupt secondary structures, are derived from Ser [Ser($\psi^{me,me}$pro), **35**] , Thr [Thr($\psi^{me,me}$pro), **36**] and Cys [Cys($\psi^{me,me}$pro), **37**] and function additionally as side chain-protecting groups, regenerating the original residue after peptide elongation under TFA treatment.[95] Alternatively, backbone amide-protecting groups, which mimic a pseudo-Pro effect, have been used for this purpose and to disrupt *β*-sheet aggregation (Fig. 4).[54,84,96] The first of these to be applied in the prevention of aminosuccinyl-peptide (**4**) generation was Hmb (2-hydroxyl-4-methoxybenzyl) (**38**), which in combination with Asp(OtBu) side chain protection (**19**) completely suppresses this side reaction.[52,54,97] Interestingly, the Asp-(Hmb)-Gly dipeptide building block can be directly introduced into the peptide chain.[52,84] However, many drawbacks have been associated with the use of Hmb, such as depsipeptide formation, incomplete removal after treatment with concentrated aq. TFA, low yield and purity, and high cost.[33,62,71,83,98,99,100] Furthermore Hmb is not compatible with the Boc/Bzl scheme and decreases the coupling rates once in the peptide chain as a result of its high bulkiness.[57,58]For this reason, the use of this compound is impractical for peptide synthesis.[77]



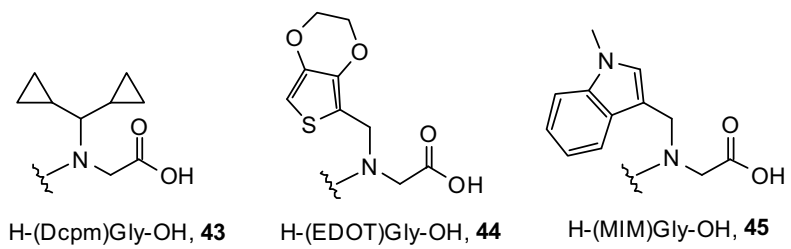


Figure 4. Pseudoproline and Gly-linked amide backbone-protecting groups designed to suppress aspartimide formation.

Some alternatives, such as AcHmb (**39**), Tmb (**41**), Nbzl (**42**) or Dmb (**40**), were proposed in the following years.[33,54,76] Of these, only Dmb showed significant advantages over its predecessor.[33] With this backbone-protecting group no Asi (**4**) was observed. Moreover, yields and acid lability were increased.[33] Dmb (**40**) is also reported to be more easily introduced.[81,84] Nevertheless, its removal (along with final cleavage) requires 95% TFA and sometimes prolonged treatments, conditions that are known enhance the formation of Asi (**4**).[80,83] Alternatively, backbone protection can be effected with Dcpm (**43**), EDOT (**44**) and MIM (**45**).[80,83,101] The former (*N*-dicyclopropylmethyl), introduced as synthon [H-(Dcpm)-Gly] (**43**) or as dipeptide, efficiently suppresses this side reaction and is more reactive than Hmb (**38**) and Dmb (**40**) analogues.[80,101] In contrast to Hmb (**38**), Dcpm is inert to acylations and can be removed with mild 5% TFA in chloform.[80] EDOT (3,4-ethylenedioxy-2-thenyl, **44**) and MIM (1-methyl-3-indolylmethyl, **45**) are, like Dcpm (**43**), more acid-labile than Dmb (**40**), and, in addition, they are commercially available and easily synthesized.[83] Remarkably, EDOT (**44**) is also introduced in higher yield than Dmb **40** (97% vs. 60%) because of its lower sterical hindrance.[83]

4.4. Asp(OMe) conversion

As previously mentioned, the aminosuccinyl-ring (**4**) can be opened with a variety of nucleophiles.[55] When methanol is used, then the methyl ester of the *α*- and *β*-peptide (**9** and **10**) is formed.[55,62] The appearance of such byproducts was envisaged after ESI-MS detection of a [M+14] molecular ion, which supposedly derives from the combination of the methanol used in the resin washings, and traces of DIEA from the coupling cocktail.[51,62] However, this ring opening of the Asi (**4**) can be turned into an advantage, as purification of the target peptide is facilitated when evolution to the *β*-peptide (**5**) is prevented.[34,51]Thus, complete conversion from Asi (**4**) to a mixture of the methyl esters (**9** and **10**) is achieved by treatment of the aspartimide-containing peptide chain with 2% DIEA in methanol.[51] The suitability of using secondary alcohols was rejected, since these require high temperature to yeild the esters.[34]

5. *N*-hydroxylamines as aspartimide-suppressants

N-hydroxylamine-based compounds and coupling reagents are widely used as amide bond-forming agents (Fig. 5).[31,102,103,104,105] In addition, additives are beneficial in order to reduce the extent of racemization and guanidylation of the *N*-terminus of the growing peptide chain and to increase coupling efficiency.[106,107] This substantial contribution to coupling strategies prompted their evaluation in the prevention of other non-coupling-derived side reactions. In some cases, like pyrrolidone carboxylic acid (pca) generation in Glu residues, the addition of *N*-hydroxylamines causes an increase in the rate of byproduct formation.[20] However, in other unwanted reactions, usually occurring under basic conditions, their addition can be advantageous. This is the case of Pro overcoupling or trifluoroacetylation of the amino group after Boc removal during the following coupling.[108,109]

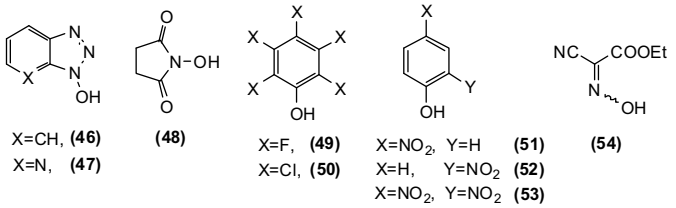
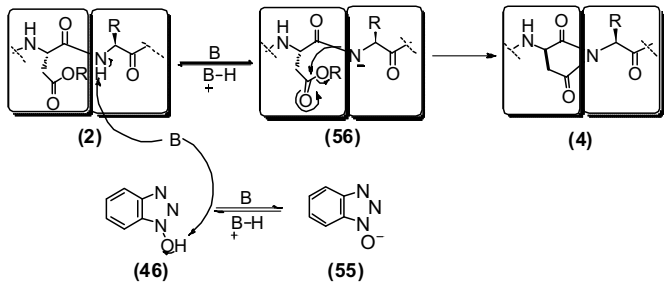


Fig. 5. *N*-hydroxylamine and phenol-type additives.

N-hydroxylamine-based additives also contribute to the wide arsenal of approaches to prevent the formation of Asi (**4**) and derived byproducts (**5**, **7**, **8**). This beneficial effect is observed in base-catalyzed Asi (**4**) cyclization, during coupling or Fmoc removal with secondary/tertiary amines. The common belief is that the unique acidic properties of *N*-hydroxylamines used as additives in peptide synthesis (pKa=2-10) are responsible for this behavior.[32] The abstraction of the amide backbone proton has been proposed as the crucial step in the cyclization that leads to Asi (**4**). Thus, addition of a relative strong acid, such as HOBt (**46**), results in competition with the Asp-X amide backbone for the base present in the medium (Scheme 3, exemplified with HOBt, **46**).[32] When HOBt (**46**) is used as additive, conversion to its anion (**55**) by the effect of the base, would decrease the percentage of negatively-charged amide backbone nitrogen (**56**), which is responsible for initiating Asi (**4**) formation (Scheme 3). Such minimization of the unwanted base-catalyzed cyclization, through acidic buffering of the medium is not surprising given the similar effect induced by unprotected Glu, Asp or Tyr when these precede Asp in the sequence. [50,58,70]Moreover, the acidity of *N*-hydroxylamines lies in a well-suited area, since lower pKa would result in complete abstraction by the base, thereby decreasing the efficiency of the coupling/deprotection reaction.[32]



Scheme 3. Competition between *N*-hydroxylamine and amide backbone proton abstraction.

5.1. Minimization during coupling in solution

When studying the extent of Asi (**4**) formation during coupling, the effect of additives to carbodiimides was examined in a Asp(OBzl)-Gly dipeptide model, a relatively prone sequence (Scheme 3).[32] Surprisingly, it was observed that these compounds, mainly *N*-hydroxylamines, do not accelerate the cyclization kinetics in the presence of tertiary amines, but delay them (see Scheme 3 for the proposed mechanism).[32] In detail, *N*-hydroxysuccinimide (**48**, pKa=5.1) resulted in a 3-fold decrease in the cyclization rate of Asp (**1**) to Asi (**4**), whereas 1-hydroxybenzotriazole (**46**, pKa=4.3) induced inhibition 20-fold times compared to the experiment without additive.[32]

Strongly acidic non-hydroxylamine additives, namely pentachlorophenol (**50**, pKa=5.3) and 2,4-dinitrophenol (**53**, pKa=4.1) are the most efficient in preventing the side reaction.[32] Unexpectedly, no direct correlation between acidity and suppression could be found. In example, pentafluorophenol (**49**, pKa=5.3) is as acid as its chloro analogue (**50**), but considerably less efficient. Similarly, HOBt (**46**) delays Asi (**4**) formation to a lesser extent than the less acidic pentachlorophenol (**50**), probably because of the higher bulkiness of the former.[32] Another hypothesis is that the presence of salt-like adducts when phenols are mixed with Et_3N or DIEA affects the side reacion.[32] Interestingly, an equimolar combination of any of the most acidic phenols (**50** and **53**) with HOBt (**46**) maintains the high efficiency.[32] The concentration of the additives is also is a key factor in the suppression of Asi (**4**). The optimal ratio is an equimolar amount of base and additive since a higher percentage of the latter does not result in improved performance.[32]

The evaluation of *N*-hydroxylamine and phenol-based additives was further tested in tri and tetrapeptide models including the Asp(OBzl)-Asn sequence.[32] As in the previous models, activated phenols (**50** and **53**) show enhanced inhibition in comparison to HOBt (**46**). Remarkably, the combination of pentachlorophenol (**50**) and HOBt (**46**) afford only traces of Asi (**4**), a stronger performance than any of the additives alone, and similar to the one of 2,4-dinitrophenol (**53**).[32] Other studies showed the advantages on the simultaneous use of HOBt (**46**) and triethylamine (Et_3N) as base. Although this strategy is less efficient in the prevention of Asi-peptide (**4**) formation than 4-DMAP, acylation is much faster (3 min. vs. hours), thus resulting in a more adequate choice.[43] Interestingly, it was also observed than DIC/HOBt (**46**) gives rise to a lower percentage of byproducts than HOBt-based TBTU/HOBt and DIEA, presumably due to the absence of base in the coupling mixture.[62] An excess of coupling reagents is also reported to increase the extent of this side reaction.[82]

5.2. Minimization during β-carboxyl activation.

As previously pointed out, during *β*-carboxyl activation of the side chain of Asp (**1**)/Asn (**3**) in *N*-glycopeptide (**15**) chemistry or cyclizations, significant aspartimide (**4**) formation is observed.[54,56] Many authors have proposed the presence of competition between the desired coupling and aspartic acid cyclization to Asi (**4**) units.[35,63.64] In this context, low-rate coupling reagents, such as PyBOP, result in enhanced percentage of byproducts, whereas more efficient agents, like DEPBT, are the most well-suited choice.[35,63]

Furthemore, in contrast to DCM, the addition of HOBt (**46**) partially solves the negative effect of THF, increasing the yield to 80% and the percentage of *N*-glycopeptide (**15**) from 29% to 66%.[56] Supposedly, the *N*-hydroxylamine accelerates the rate of glycosamine attachment, thereby decreasing the impact of Asi (**4**) formation. In contrast, the less acidic pentafluorophenol (**49**) increases the amount of byproducts (7% to 50% Asi).[56]

5.3. Minimization during Fmoc basic removal.

For the last ten years, efforts have been directed towards the prevention of this side reaction in Fmoc/tBu chemistry, the currently predominant protection strategy, because its repetitive removal substantially decreases the purity of the peptide.[47,77] Piperidides of the α- and *β*-peptide (**7**, **8**) are usually formed, plus racemized versions.[45] Furthermore, the formation of byproducts under base catalysis is more severe than in acidic media as a result of the enhanced kinetics.[34,42,51,58] In view of the positive effect of the additives in solution coupling, their inclusion in piperidine solutions has been evaluated.[47,48,69]

Similarly to the effect induced in solution, equimolar amounts of weakly acidic additives, with respect to the base, decreases Asi (**4**) formation.[47] Interestingly, 2% of the additives in 20% piperidine in DMF results in an increase in the target peptide from 40% to 60-71%.[69] Contrary to the relative performance in solution, the *N*-hydroxylamine HOBt (**46**) is more efficient than pentachlorophenol (**50**) (67% vs. 60% peptide).[48] The highly deactivated 2- or 4-mononitrophenol (**51** and **52**) and 2,4-dinitrophenol (**53**) are the most suitable choice of additive (70-71% peptide).[69] Other authors have reported the combination of HOBt (**46**) and 2,4-dinitrophenol (**53**) as a highly efficient methodology to prevent Asi (**4**) formation.[70]

During assembly of the *D*-Leu-*D*-Ala analogue of CRH hormone, which may adopt a favorable conformation towards cyclization of Asp, addition of 0.1M solution to the piperidine solution results in a decrease in the side reaction to only traces, as detected by ESI-MS.[48,69] Increasing amounts of additive have been shown to induce higher suppression.[69,109] Recently, HOAt (**47**), the 7-aza-analogue of HOBt (**46**), proved to be equally efficient.[109] Optimization of the inclusion of the additives is achieved by the presence of Oxyma (**54**).[109]

6. Conclusions

Aspartimide and piperidide formation is problematic in peptide synthesis. Although the inconveniences caused by these intramolecular cyclizations are well known, a solution has yet to be found. Many factors contribute to accelerating or delaying the extent of this side reaction, such as the type of base, acid, protecting group, resin and solvent used. Research on some of these influential parameters has been focused on enhancing the sterical hindrance of the base or protecting group, thus hampering amide backbone hydrogen abstraction, or on designing linkers/resin/protecting groups which do not require the use of a strong acid or base known to promote aspartimide formation. These strategies are generally simple and cost-saving but do not offer complete prevention in demanding sequences or experimental conditions. Thus, even if using Asp(ODie), piperazine or microwave irradiation, small percentages of byproducts are observed.[77,79] Hindering the size of the protecting group or base can also result in slower coupling and Fmoc-deprotection steps, thereby decreasing the global efficiency of the synthesis.[43,79] Moreover, the syntheses of these compounds might be complex and require two additional steps.[79,87] Furthermore, the introduction of non-conventional or unavailable protecting groups or linkers results in additional synthetic steps.

Full suppression of Asi (**4**) formation can be achieved by eliminating the amide backbone hydrogen of the residue preceding Asp, either using pseudoproline or amide backbone protectants. However, these building blocks also have substantial drawbacks that preclude their consideration as the ultimate solution to this side reaction.[62,80] To begin with, pseudoproline strategies are limited to Asp-Ser, Asp-Thr and Asp-Cys sequences. Similarly, the use of backbone amide-protecting groups as dipeptide building blocks is restricted to Asp-Gly sequences (the synthesis with chiral residues would result in a great extent of racemization).[80] Moreover, their introduction as synthons [H-(X)-Gly] is difficult and slow. The removal of these backbone protecting groups, with the exception of EDOT, MIM and Dcpm, often leads to strong acidic conditions, which might

cause the unwanted cyclization [62,84] In addition, most of these groups are rather expensive.Therefore, research efforts must continue in order to pursue straightforward, affordable and complete elimination of this detrimental side reaction and the derived ring-opening byproducts.

While this troublesome side reaction remains unsolved, an attractive, cost- and time-saving, and widely available alternative to previous mentioned strategies has emerged as result mainly of the work of Bodanszky and Dölling, namely acidic *N*-hydroxylamines and phenols. Consistently with reduction of aspartimide formation when acidic-side chain residues precede Asp, benzotriazoles and deactivated phenols cause a similar effect. A combination of some of these additives to carbodiimides in Fmoc-removal cocktails greatly reduces the appearance of aspartimides. Furthermore, this combination is independent of the synthetic strategy used and does not require additional introduction/removal steps for protecting groups. Excellent protocols including *N*-hydroxylamines have been reported, such as the combination of HOBt (**14**), piperazine and microwave irradiation or the use of the cocktail hexamethyleneimine/*N*-methylpyrrolidine/HOBt/NMP/DMSO 4:50:4:71:71 (v/v/w/v/v), which minimize Asi (**4**) formation to 1-5%.[62,70,71,77,91] Recently, novel oximes have been added to the arsenal of available *N*-hydroxylamines, thus contributing to improving options available to prevent aspartimide formation.

Acknowledgments

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Reference and Notes

Abbreviations not defined in text: Acm, acetamidomethyl; API, Active Pharmaceutical Ingredient; Boc, tert-butoxycarbonyl; CRH, Corticotropin-releasing hormone; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; DCM, Dicloromethane; DEPBT, 3-(Diethoxy-phosphoryloxy)-3H-benzo[d][1,2,3] triazin-4-one; DIEA, *N,N*-diisopropylethylamine; DIC, Diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI-MS, ElectroSpray Ionization Mass Spectrometry; Fmoc, Fluorenylmethoxycarbonyl; HMPT, Hexamethylphosphoramide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; NMP, *N*-methyl-2-pyrrolidone; NMR, Nuclear Magnetic Resonance; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane; PEGA, polyethylene glycol polyamide copolymer; Pmc, 2,2,5,7,8-pentamethylchroman; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SPPS, Solid-Phase Peptide Synthesis; TBAF, Tetrabutylammonium fluoride;TBTU, *N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl) uronium tetrafluoroborate;TFA, trifluoroacetic acid; THF, Tetrahydrofurane; UV, Ultraviolet.

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Publicació V

Use of Oxyma as pH modulatory agent to be used in the prevention of base-driven side reactions and its effect on 2-chlorotrityl chloride resin †

Utilització d'Oxyma com agent modulador del pH en la prevenció de reaccions secundàries catalitzades per base i l'efecte sobre la resina 2-clorotritil

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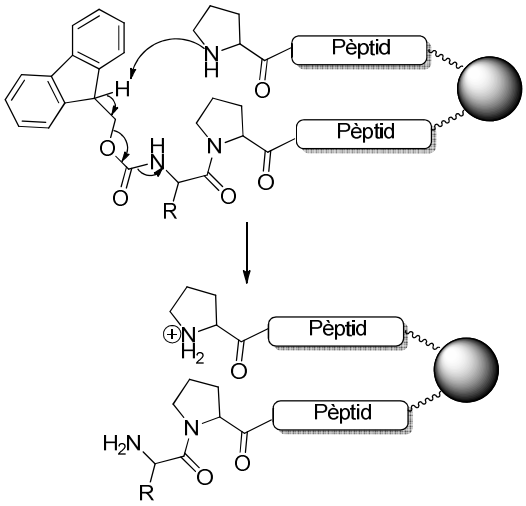
† Ramon Subirós Funosas va dur a terme el disseny i realització dels experiments per evaluar la reducció d'aspartimides, així com d'aquells que investiguen la minimització de sobreacoblament causat per prolina i la compatibilitat amb la resina 2-clorotritil. El mateix doctorand també va elaborar el manuscrit.

Resum

La presència de *N*-hidroxilamines àcides comporta grans beneficis en química de pèptids, degut a la reducció d'algunes reaccions secundàries promogudes per l'elevada basicitat d'espècies presents al medi, que a més actuen com a nucleòfils.

En el treball descrit a continuació s'han investigat en detall diverses aplicacions del 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma), a part de la utilització com additiu per carbodiimides, com ara en la prevenció de la formació d'aspartimides i piperidides. En aquest sentit, és possible obtenir una major minimització d'aquests subproductes que quan s'addiciona HOBt o HOAt, en gran part degut a un efecte modulador del pH per la considerable acidesa de les *N*-hidroxilamines. De forma similar, Oxyma ha demostrat ser més eficient que els benzotriazols en la reducció del sobreacoblament d'aminoàcids originat per Prolina, quan aquesta es troba al *N*-terminal, la qual constitueix una reacció secundària escassament estudiada. Finalment també s'ha examinat l'efecte que indueixen aquestes *N*-hidroxilamines quan resten en contacte per temps llargs amb la resina 2-clorotritil, làbil a condicions àcides suaus.



Desprotecció prematura de Fmoc originada per l'elevada basicitat de prolina, que causa sobreacoblament de l'aminoàcid entrant.

Use of Oxyma as pH modulatory agent to be used in the prevention of base-driven side reactions and its effect on 2-chlorotrityl chloride resin

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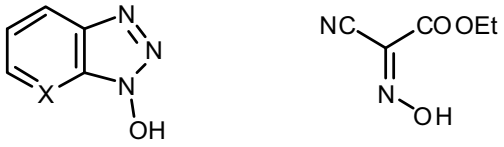
Abstract

The presence of low pKa *N*-hydroxylamines is beneficial in peptide chemistry as they reduce some base-mediated side reactions. Here we evaluated the applicability and buffering capacity of Ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) in the prevention of aspartimide/piperidide formation and Pro-based overcoupling and compared it with the performance of HOBt and HOAt. In addition, the compatibility of these additives with the highly acid-labile 2-chlorotrityl chloride resin is examined.

Introduction

N-hydroxylamines are among the most potent additives to carbodiimides in synthetic peptide chemistry.[1] Compounds containing such a moiety show unusually high acidity in the context of organic chemistry (pKa=3-10) and therefore are excellent leaving groups in acylation reactions, such as amide bond formation.[1] The inclusion of *N*-hydroxylamines in stand-alone coupling reagents, like onium salts, results in an even more powerful strategy.[2] Among the various templates proposed with this highly acidic

group (such as benzotriazines, succinimides or triazoles), benzotriazoles have undoubtedly been the most extensively studied and cited.[3] 1-hydroxybenzotriazole (HOBt, **1**) and 7-aza-1-hydroxybenzotriazole (HOAt, **2**), with a pKa of 4.60 and 3.28 respectively, have found general acceptance and are routinely used additives (Fig. **1** left).[3a,4] Nevertheless, safety issues associated with these heterocyclic compounds arose several years ago, thus prompting the search for additives based on alternative cores.[5] It has recently been demonstrated that stable and acidic oximes bearing electron-withdrawing substituents, like ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, **3**), developed in our group, are a safe and efficient approach for peptide bond formation (Fig. 1 right).[6]



X= CH, HOBt (**1**)

X= N, HOAt (**2**)

Oxyma (**3**)

Figure 1. Structure of *N*-hydroxylamine-type additives

However, the application of *N*-hydroxylamine-based compounds in peptide chemistry is not limited merely to assisting the assembly of amino acids and peptide fragments, but might also be beneficial in other steps of peptide/protein elongation, especially when using Fmoc/tBu protection. Although the Fmoc/tBu strategy is now the predominant protection approach due to its fully orthogonal character and milder α-amino deprotection and peptide-resin cleavage conditions, superseding the classical Boc/Bzl method, it is not exempt of detrimental side reactions.[7,8] Most of these occur as a result of the basic conditions required to remove the Fmoc temporary protecting group, such as dehydration of Cys to dehydroalanine or unwanted DKP formation at the dipeptide step.[9] Due to their unique acidic nature, *N*-hydroxylamines reduce the impact of some of these base-driven unwanted reactions, and thus act as pH regulators.[4a,10]

H-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 (**4**)

H-Tyr-Pro-Pro-Phe-Leu-NH_2 (**5**)

H-Gly-Phe-Leu-OH (**6**)

Figure 2. Model peptides used to perform comparative studies between the additives

Since Oxyma (**3**) and derivatives induce highly reactive ester formation, we sought to extend the scope of application of this outstanding additive to other methodological aspects of peptide chemistry, such as the prevention of some of the base-mediated undesired reactions in Fmoc/tBu chemistry. Thus, we focused our efforts on the minimization of one of the most critical and frequently encountered side reactions, namely aspartimide formation, and also on the rarely documented but troublesome Pro-based overcoupling.[11] Using the peptide models shown in Fig. 2, we evaluated the impact of the acidity of Oxyma (**3**) and compared it with the performance of classical benzotriazoles HOBt (**1**) and HOAt (**2**), not only in the reduction of base-driven impurities but also in the premature release of peptides bound to Barlos (2-chlorotrityl chloride) resin.[12]

Methods

Aspartimide/piperidide formation

The target Fmoc-hexapeptide **4** was manually elongated on 3 g scale of Fmoc-protected Rinkamide-MBHA-PS-resin (δ=0.45 mmol/g), using Orn(Boc), Asp(OtBu) and Tyr(OtBu) as side chain protected amino acids. 30 min-couplings were conducted by using 0.4M solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq) in DMF, with 3-min preactivation with DIC (3 eq), prior to addition to the resin. Fmoc removal cycles were performed by treatment of the peptide-resin with 20% piperidine in DMF for 1+5+5 min. Final test deprotections were conducted with 30 mg of Fmoc-hexapeptide **4**, with a double 6+6 h treatment with the corresponding piperidine-additive mixture in DMF (1 ml each). In most of the experiments a white solid appeared after prolonged exposure with the basic cocktail. After DMF and DCM washings, the peptide chain was cleaved from the resin after addition of a TFA/H_2O (19:1) cocktail for 2 h at room temperature. The solution was then filtered and the resin was washed with DCM (1 ml x 2), which was removed along with TFA under nitrogen flow. The crude peptide was precipitated with cold diethyl ether (2 ml x 3) and after lyophilization, purity of target peptide and percentage of aspartimide and derived byproducts was checked on reverse phase HPLC (see Fig. 3 and Table 1), with the following conditions: Waters SunFire C18 Column (3.5 μm, 4.6x100 mm), linear gradient 7 to 20% of

0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm; t_R (β-peptide)= 4.8 min, m/z=$[M+H]^+$=651.4; t_R (α-peptide)= 4.9 min, m/z=$[M+H]^+$=651.4; t_R (aspartimide)= 5.1 min, m/z=$[M+H]^+$=633.3; t_R (piperidide)= 7.9, 8.8 and 9.2 min, m/z=$[M+H]^+$=718.4. Byproducts were identified by coinjection with pure samples.

Pro-mediated overcoupling

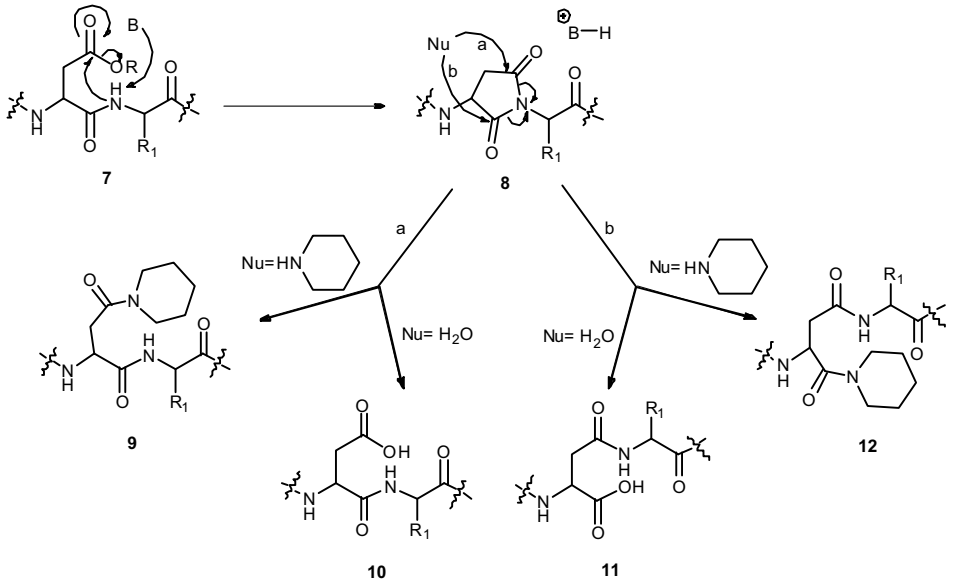
Tripeptide H-Pro-Phe-Leu-resin was assembled on Fmoc-Rinkamide-AM-PS resin (δ=0.59 mmol/g), using Tyr(OtBu) as side chain protected amino acid. 30 min-couplings were performed by preactivating 0.4M solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq) in DMF, with DIC (3 eq) for 5 min, prior to addition to the resin. Fmoc removal steps were carried out by treatment of the peptide-resin with 20% piperidine in DMF for 1+5+5 min. 100 mg of the tripeptide were swelled in DCM for 20min, conditioned in DMF and treated with 0.1M solutions of additives (1 mL x 2) for 1 min. Resins turned bright yellow with HOAt and bright yellow-orange with Oxyma. After DMF washings to eliminate the excess of additive solution (1 mL x 5; 30 sec.) a 0.5M solution of Fmoc-Pro-OH or Fmoc-Tyr-OH was added and stirred for 2 hours to promote early Fmoc removal. Then, DIC (3eq) and additive (3eq) in DMF were added to the mixture for a final concentration of reagents of 0.3M. After a 3-h coupling, resin was washed with DMF and DCM and the peptide chain was cleaved from the resin after addition of a TFA/H_2O (9:1) cocktail for 2 h at room temperature. The solution was then filtered and the resin was washed with DCM (1 ml x 2), which was removed along with TFA under nitrogen flow. The crude peptide was precipitated with cold diethyl ether (3 ml x 3) and lyophilized in H_2O, prior to HPLC analysis (see Table 2), using a Waters SunFire C18 Column (3.5 μm, 4.6x100 mm), linear gradient 15 to 21% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 30 min at 40ºC (to suppress the presence of different conformations), with detection at 220 nm; t_R (des-Pro)= 13.6 min, m/z=$[M+H]^+$=538.3; t_R (pentapeptide)= 17.6 min, m/z=$[M+H]^+$=635.3; t_R (+Pro)= 18.9 min, m/z=$[M+H]^+$=732.4; t_R (piperidide)=27.6 min, m/z=$[M+H]^+$=798.4. Byproducts were identified by coinjection with pure samples.

Compatibility with 2-chlorotrityl-chloride resin

The target tripeptide H-Gly-Phe-Leu-OH (**6**) was manually assembled on a 2 g scale on 2-chlorotritylchloride-PS-resin (δ=1.55 mmol/g). First residue was introduced with standard protocols with this resin in DCM, adding a large excess of DIEA with respect to Fmoc-Leu-OH (10 eq.) and let it mix for 55 min (3 eq of the base were added simultaneously with the amino acid, and extra 7 eq. 10 min. later). Then MeOH was added to effect capping of the resin. Loading was recalculated (0-48 mmol/g) after Fmoc removal with 20% piperidine in DMF (1+5+5 min treatment). Fmoc-Phe-OH and Fmoc-Gly-OH were introduced by means of 30 min-couplings, using 0.4M solutions of Fmoc-amino acids (3 eq. excess) and Oxyma (3 eq) in DMF, with 3-min preactivation with DIC (3 eq), prior to addition to the resin. Sample and reference experiments of compatibility of resin **15** with *N*-hydroxylamines were conducted with 25 mg of tripeptide-resin. A full cleavage reference was performed by treating the resin with 5% TFA in DCM (1 ml x 5; 5 min) , followed by DCM washings (1 ml x 2) and removal of solvent under nitrogen flow. To evaluate premature cleavage with various *N*-hydroxylamines, 0.1M solutions in DMF (1 ml) were mixed with resin **15**, previously swelled in DCM (20 min.) and conditioned in DMF (1 ml x 5). After treatment with the resin, filtrates were collected and resin was washed with DMF and DCM. Solvents were evaporated under reduced pressure (DMF coevaporated with toluene). The corresponding white solids (except the runs with Oxyma, in which a yellow oil was obtained) were disolved in H_2O/CH_3CN 1:1 (8 ml) and 23 ul were injected onto reverse-phase HPLC (see Fig. 5 and Table 3). A Waters SunFire C18 Column (3.5 μm, 4.6x100 mm) was employed, using a linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min, with detection at 220 nm; t_R (H-Gly-Phe-Leu-OH, **6**)= 4.0 min, m/z=$[M+H]^+$=336.3. Presence or absence of tripeptide **6** was confirmed after coinjection with a pure sample and by HPLC-MS analysis. The corresponding additives were also observed in the mixture.

Results and Discussion

Aspartimide formation



Scheme 1. Intramolecular base-catalyzed mechanism of aspartimide formation and subsequent breakdown by nucleophiles

In recent years, the advantageous properties of aspartimide-containing compounds have attracted increasing interest because of their application as chemical tools in biotechnology, chemical biology and material science.[13] However, the undesired formation of such cyclic constructs, originated in Asp residues, is still regarded as one of the major unsolved side reactions during peptide/protein synthesis. This unwanted intramolecular cyclization was originally observed in Boc SPPS, in the strong acidic media required for cleavage of the peptide from the resin (HF or TFMSA) or N^{α}-Boc removal (HBr or TFA) and under basic N^{α}-neutralization and coupling steps with tertiary amines.[4a,14,15] Nevertheless, the extent of this side reaction is dramatic in the Fmoc/tBu approach, since the repetitive piperidine treatments, required for Fmoc removal, increasingly reduce the percentage of unmodified peptide in each cycle after the introduction of Asp in the sequence.[11a,14] Base-mediated formation of aspartimide-peptide structures and derived byproducts in piperidine-containing cocktails is depicted in Scheme 1.

The internal cyclization of the Asp-containing peptide (**7**) is initiated by the nucleophilic attack of the backbone amide group of the preceding residue onto the *β*–carboxyl moiety of the side chain protecting group of aspartic acid. Basic-catalysis renders this aminosuccinyl-like structure (**8**) faster than in acidic conditions, although during HF cleavage of the peptide, aspartimides are reported to occur even in the unprotected side chain carboxylic acid.[11a,15,16] Therefore, aspartimide units (often referred to as Asi or Asc) result in backbone-modified structures that are at the same time reactive enough to undergo ring opening by nucleophiles.[17] Hydrolysis of Asi residues can take place in both succinimide carboxyl groups, either restoring the original *α*–peptide (**10**, path A, Scheme 1) or giving rise to the isoaspartyl-*β*–peptide (**11**, path B, Scheme 1), which permanently modifies the peptide backbone. Given the structural similarity of these two isomers, difficulties arise when attempting to separate them, especially when handling long peptides.[18] In addition, piperidine is nucleophilic enough to remain attached to the Asi cyclic structure, thereby giving rise to the corresponding piperidides of the *α*– and *β*-peptide (**9** and **12** respectively, Scheme 1).[10,11a,19] In summary, only 25% of the possible aspartimide-opening byproducts would lead to the unmodified target peptide. The true scenario is even worse, bearing in mind that the percentage of piperidides increases as does the number of Fmoc-removal cycles when Asp is included in the sequence and also that *β*-peptides (**11** and **12**) are preferentially formed in DMF, the regular solvent used in SPPS (Solid Phase Peptide Synthesis).[20] Moreover, the aspartimide cyclic ring is more sensitive towards racemization than the starting open sequence and thus even more detrimental impurities are obtained. The presence and amount of aspartimide and its derived opening byproducts are dependent on many factors, including the *β*–carboxyl group, temperature, solvent, resin, sequence and conformation. [10, 11b, 17, 19b]

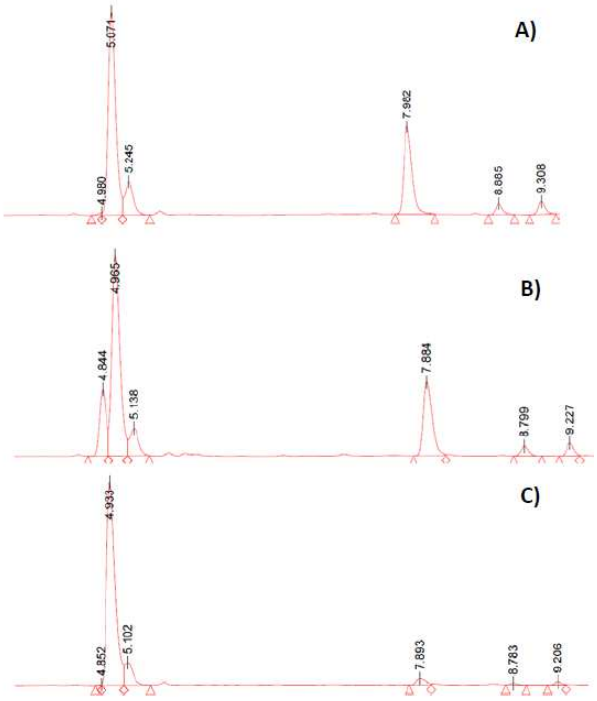


Figure 3. Reverse HPLC analysis on mixtures obtained after treatment of peptide model **4** with 20% piperidine in DMF for 6+6 hours at rt, without additive (A) or containing 1M Oxyma (C). A coinjection with the pure synthesized *β*-peptide was performed to indentify this byproduct (B). The desired hexapeptide is observed at 5.0 min (see Materials and Methods for more details)

Table 1. Reduction of aspartimide and derived byproducts during basic treatment of Fmoc-Ala-Orn-Asp-Gly-Tyr-Ile-Rinkamide-PS resin in the presence of various additives[a]

entry	concentration of additive in 20% piperidine/DMF	additive	target peptide and aspartimide-derived byproducts (%)			
			α-peptide	*β*-peptide[b]	aspartimide	piperidides[c]
1	--	--	55.62	0.23	11.06	33.09
2	0.1M	HOBt (**1**)	59.72	0.38	9.88	30.02
3		HOAt (**2**)	60.59	0.22	10.43	28.76
4		Oxyma (**3**)	62.75	0.26	9.36	27.62
5	0.5M	HOBt (**1**)	65.87	0.23	12.75	21.16
6		HOAt (**2**)	67.11	0.26	13.54	19.08
7		Oxyma (**3**)	74.74	0.51	8.08	16.67
8	1M	HOBt (**1**)	79.00	0.25	14.03	6.72
9		HOAt (**2**)	79.68	0.13	14.36	5.82
10		Oxyma (**3**)	85.59	0.10	9.60	4.67

[a]Fmoc-hexapeptide **4** was treated with 20% piperidine in DMF in the absence or presence of 0.1-1.0M additive for 6+6 hours at rt.

[b] The mass of the *β*-peptide would match that of the epimer of the *α*-peptide. However, the identity of the *β*-peptide is confirmed by coinjection with a pure sample, manually synthesized using Fmoc-Asp-OtBu.

[c]Up to 3 peaks containing the mass of the corresponding piperidide were observed in a ratio 10:1:1.5 (t_R = 7.9, 8.8 and 9.2 min, see Figure 3).

Several strategies have been envisaged to decrease or, at least prevent, the abovementioned cyclization of Asp residues. Some of these consist of enhancing the sterical hindrance of the base or *β*-carboxy side chain protecting group used, with the idea of impeding the initial attack of the amide backbone moiety.[19b,21] Alternative approaches are based on the pseudo Pro methodology, by the introduction of preformed Asp-X dipeptide building blocks, which avoid the risk of internal cyclization.[17,22] Mild-cleavable orthogonal N^{α}-protecting groups have also been described.[23] However, all these strategies are relatively expensive, time-consuming or require extra steps, thereby limiting their widespread application. In contrast, the addition of *N*-hydroxylamines in the N^{α}-amino deprotection cocktails is a readily available, efficient and cost-saving choice to prevent aspartimide formation. The advantages of the presence of HOBt (**1**) or HOSu (*N*-hydroxysuccinimide) in basic-catalyzed Asi formation were initially reported during coupling in the Boc SPSS approach and were later studied in Fmoc-removal

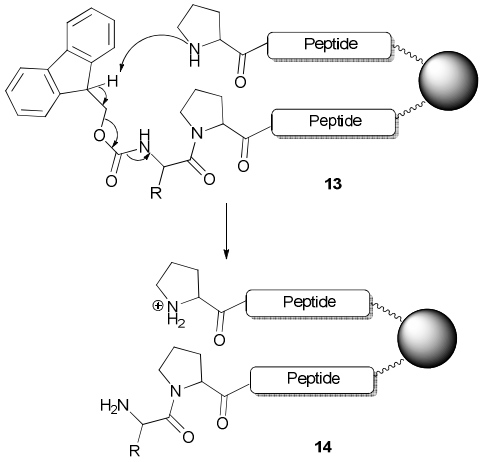
cocktails, using Fmoc/tBu protection.[4a] Substantial reduction of aspartimide-derived byproducts can be obtained by using 2% HOBt solutions in 20% piperidine in DMF, resulting in a remarkable increase of the target peptide.[10,19b] Such minimization of Asp cyclization is probably due to competition of the *N*-hydroxylamine with the acidic Asp-X backbone amide group for the base, which is thought to be the crucial step of aspartimide formation.[4a] Markedly acidic non-*N*-hydroxylamine additives, like deactivated phenols, are also known to induce a similar effect, although the mechanism is unclear.[10] In this regard, hypotheses have been proposed about the formation of salt-like adducts when phenols are mixed with tertiary amines, such as Et_3N.[4a]

In order to further test the capacity of *N*-hydroxylamines to decrease the internal cyclization of Asp residues in piperidine-based cocktails and the effect of the acidic group-containing template, here we compared the performance of Oxyma (**3**) with that of the benzotriazoles HOBt (**1**) and HOAt (**2**). On the basis of previous articles reporting their sensitivity towards this side reaction, hexapeptide Fmoc-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 (**4**, Fig. 2) was selected as the peptide model to conduct the comparison.[24] Aspartimide formation is severe in Asp-Gly sequences, since the absence of C^{α}-alkyl side chain in the preceding residue to Asp enhances the nucleophilicity of the amide backbone nitrogen.[11] Furthermore, the Asp-Gly-Tyr-Ile domain, originally contained within the 1-6 fragment of toxin II of scorpion *Androctonus australis Hector* (H-Val-Lys-Asp-Gly-Tyr-Leu-NH_2) gives extra propensity to this internal cyclization.[4a,14,16] The nature of the β-carboxy-protecting group also affects the extent of the side reaction, as this moiety is the leaving group on the determining step.[14,18a] In the present synthesis, Asp(OtBu) was used because it is easily available and known to produce a significant percentage of aspartimide-derived impurities, in comparison with the more bulky OAda and OPhiPr or with OMpe and ODie, which are highly flexible and efficient in preventing Asp to Asi conversion.[11b]

Synthesis of target peptide **4** took place by means of standard DIC/Oxyma (**3**), coupling cycles starting from Fmoc-Rinkamide-PS resin. Once the Fmoc-protected hexapeptide was successfully assembled, the extent of intramolecular cyclization of Asp (and consequently, the amount of derived impurities) was maximized in order to

achieve clearer comparison of the effects of the additives. Thus a 6+6 h double treatment of model peptide **4** with a 20% piperidine in DMF cocktail, mimicking the effect of repetitive Fmoc-removal steps, was found to cause up to 45% of aspartimide-related side products. Since increasing amounts of additive induced greater suppression of this undesired event, experiments conducted in the presence of various *N*-hydroxylamine concentrations, in the range 0.1M-1M, were performed. Results are summarized in Table 1.

Unexpectedly, under the experimental conditions, three piperidides were observed in the ratio 10:1:1.5 (see peaks at 7.9, 8.8 and 9.2 in Fig. 3). Taking into account the preferred ring-opening via path B in Scheme 1 when DMF is used as solvent, major piperidide is expected to be based on the isoaspartyl-β-hexapeptide (**11**). Epimerization on the C^{α} could lead to an extra form of piperidides. On the one hand, treatment of the Fmoc-hexapeptide **4** with 0.1M solutions of the additives induced a slight increase on the target α–peptide purity (4-7%, entries 2-4). On the other hand, higher concentrations (0.5M and especially 1M) achieved major improvements on the percentage of desired peptide, by substantially decreasing the amount of aspartimide and piperidides (entries 5-10). Experiments conducted in the presence of Oxyma (**3**) clearly produced a higher content of desired unmodified hexapeptide **4** than analogous tests with HOBt (**1**) and HOAt (**2**), which rendered similar purities. Oxyma (**3**) successfully reduced the severity of aspartimide and piperidide formation under all the concentrations tested (entries 4, 7 and 10). Of note was the minimization of internal Asp cyclization when 1M solutions of Oxyma (**3**) in 20% piperidine in DMF were used as Fmoc-removal cocktails (see Fig. 3), thus enhancing the α–peptide purity by 30% compared with the test conducted in the absence of additive (entries 1 vs. 10). It is also worth noting that the superiority of Oxyma (**3**) over benzotriazoles cannot be explained by the relative acidities of Oxyma (**3**, pKa=4.60), HOBt (**1**, pKa=4.60) and HOAt (**2**, pKa=3.28), suggesting the influence of additional, undisclosed factors on aspartimide prevention. In this regard, the lower bulkiness of Oxyma than the benzotriazoles could result in stronger competition with the acidic backbone peptide proton for piperidine.



Scheme 2. Mechanism of accidental Fmoc removal of recently incorporated residues by Proline.

Pro-based overcoupling

Fmoc N^{α}-amino protection is generally regarded as compatible with standard basic coupling conditions, generally carried out in the presence of DIEA (pKa=10.1), which is less basic than the piperidine required for the deprotection step (pKa=11.1).[25] However, the coupling cocktail may contain other basic amines that are also capable of removing the Fmoc protecting group to some extent. This is the case of proline, whose secondary amine character (pKa=10.6) confer it the highest α-amino basicity among proteinogenic amino acids. Although scarcely reported to date, accidental Pro-mediated overcoupling of the residue being incorporated may occur as result of the high basicity of this amino acid. This undesired deprotection is expected to be more pronounced when the Fmoc-amino acid is already introduced in the resin-bound peptide, due to the higher effective concentration of peptide and spatial proximity between growing peptide chains (Scheme 2). Once the corresponding activated amino acid has been incorporated into a proline, a vicinal, still unacyalted proline at the *N*-terminus of the elongated peptide might act as basic center substracting the 9-fluorenyl proton, thereby initiating the removal of Fmoc (**13**). Consequently, another molecule of activated Fmoc-amino acid can be introduced on the now N^{α}-amino unprotected H-aa-Pro-peptide (**14**), thereby resulting in overcoupling.

Given the role of Pro as basic center in this premature deprotection, and bearing in mind the remarkable basicity/nucleophilicity-masking properties exhibited by *N*-hydroxylamines **1-3** in the prevention of aspartimide formation, their capacity to reduce

the impact of Pro-mediated overcoupling was analyzed during elongation of H-Tyr-Pro-Pro-Phe-Leu-NH_2 (**5**, Fig. 2). This Leu-enkephaline modified peptide model presents two consecutive prolines in central positions and therefore, two distinct overcoupling-promoting sites. The experimental design was to quantitatively obtain the resin-bound H-Pro-Phe-Leu tripeptide, using Fmoc-Rinkamide-AM-PS and DIC/Oxyma standard couplings, and to study the extent of overcoupling at Pro^3 and Pro^4 after short treatment of the resin with 0.1M solutions of various *N*-hydroxylamines. In the absence of previous *N*-hydroxylamine washings, hexapeptides presenting extra Pro and Tyr were observed under regular coupling conditions (0.12% combining both byproducts). Although apparently insignificant, such a low percentage can cause major problems when synthesizing APIs (Active Pharmaceutical Ingredients). However, for comparison purposes, the percentage of +Pro and +Tyr was exaggerated by mixing the *N*-hydroxylamine-treated peptide-resin with a solution of the Fmoc-amino acid in DMF for 2 h, prior to addition of the coupling cocktail (DIC and corresponding *N*-hydroxylamine as additive). Results are shown in Table 2. The possible effect of the acidity of Fmoc-Pro/Tyr-OH in the reduction of Pro-overcoupling is included in the experiment without additive (entry 1), therefore results obtained in the experiments performed after treatment of the peptide-resin with 0.1M *N*-hydroxylamine are unequivocally attributted to the presence of these additives (entries 2, 3, 4).

Table 2. Minimization of Pro-driven overcoupling of activated residues by previous treatment of Pro-peptide-resin with various additives[a]

entry	additive	pentapeptide (%)	+Pro (%)[c]	+Tyr (%)[c]
1	--	96.86	0.56	2.58
2[b]	HOBt (**1**)	98.37	0.45	1.18
3[b]	HOAt (**2**)	98.69	0.44	0.87
4[b]	Oxyma (**3**)	98.44	0.39	1.17

[a] Misincorporation of Pro was observed in all experiments: 1.9% (no additive), 2.7% (HOBt), 1.2% (HOAt) and 0.9% (Oxyma).[b] Initial 1-min (x2) washings with a 0.1M solution of the corresponding additive in DMF were performed, carrying out the coupling in presence of the same additive.[c] In order to assure complete incorporation of any accidentally unprotected residues in solution into the peptide chain, 3-h couplings were performed and completion was monitored by chloroanyl visual tests.

An appreciable increase in the purity of the peptide was achieved with an initial double 1-min treatment of the Pro-peptide with HOBt (**1**), HOAt (**2**) or Oxyma (**3**), compared to a control experiment without any previous washing under DIC couplings (entry 1 vs. 2-4). Overcoupling of Tyr occurred to a higher extent than Pro in all the assays, but no simultaneous +Pro,Tyr heptapeptide was detected. Oxyma (**3**) was the most efficient *N*-hydroxylamine in reducing the presence of +Pro hexapeptide and presented a similar percentage of +Tyr to that detected in the experiment with HOBt (**1**). The 7-aza analogue of HOBt (**2**) afforded the lowest amount of the hexapeptide with an additional Tyr, with an overall percentage of pentapeptide close to 99%. Oxyma (**3**) showed an intermediate overall performance between HOBt (**1**) and HOAt (**2**). Nevertheless, a certain degree of des-Pro deletion peptide was observed in all cases, Oxyma (**3**) being the most remarkable agent in terms of coupling efficiency (see Table 2 footnote b) and consequently achieving a similar overall purity to that attained by HOAt (**2**), and at a lower cost. However, the exact mechanism of action of *N*-hydroxylamines remains unclear. Resins became brightly colored after initial washings (yellow with HOAt, yellow-orange with Oxyma), thereby suggesting the presence of the anion as result of acid-base exchange with Pro. However, this interaction did not completely suppress the nucleophilicity of Pro, since couplings were quantitative in 3 h or less.

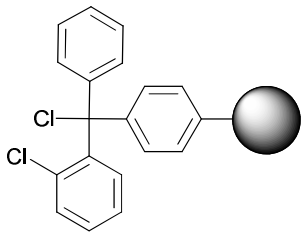


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Figure 4. Structure of polystyrene-based 2-chlorotrityl chloride resin

Compatibility with 2-chlorotrityl chloride resin

The convenience of the acidic nature of *N*-hydroxylamines discussed here might depend on the synthetic strategy chosen. Thus, a highly acid-labile resin or linker is

sometimes required to obtain a fully side chain-protected peptide upon cleavage, to be used in the next fragment coupling step or cyclization. For this purpose, 2-chlorotrityl chloride resin (**15**, Fig. 4, alternatively referred to as Barlos resin) has become popular as a *C*-terminus peptide acid-generating solid support, subject to cleavage upon diluted (1-5%) TFA treatment.[12] In this context, premature cleavage of the peptide from this resin, caused by the acidic pH induced by *N*-hydroxylamines, such as HOBt (**1**) or HOAt (**2**), has been generally accepted to occur to a low extent, but has never been tested or proved. Therefore, the additional goal of the present study was to study the ratio of undesired peptide release from the mild acid-cleavable resin **15**, when various *N*-hydroxylamines, including Oxyma (**3**), are present in the media

Tripeptide H-Gly-Phe-Leu-OH (**6**, Fig. 2), optimally assembled in Barlos resin (**15**) by means of DIC/Oxyma (**3**) couplings, was selected as peptide model to study the drawbacks associated with HOBt (**1**) and HOAt (**2**), and Oxyma (**3**), when such an acid-labile solid support is used. In order to maximize the potential adverse effects of these acidic additives, 0.1M solutions in DMF were mixed with resin **15** in the absence of other reagents commonly found in coupling cocktails such as Fmoc-aa-OH or carbodiimide. With the aim to monitor the progressive release of peptide **6**, various treatment times were examined (5 min, 1 h and 24 h), measuring the absorbance of filtrates at 220 nm, compared to a full cleavage reference. Table 3 shows the percentages of premature cleavage from 2-chlorotrityl chloride resin (**15**) with the distinct additives.

Table 3. Percentage of premature cleavage of peptide H-Gly-Phe-Leu-OH (**6**) from the highly acid labile 2-chlorotrityl chloride resin (**15**) induced by various acidic additives [a,b]

entry	additive	treatment time	peptide 6 released (%)
1		5 min	0
2	HOBt (**1**)	1 hour	0.29
3		24 h	5.64
4		5 min	0.20
5	HOAt (**2**)	1 h	0.33
6		24 h	6.30
7		5 min	0
8	Oxyma (**3**)	1 h	0.29
9		24 h	5.51

Percentage of cleavage is shown as relative absorbance, in reference to a sample obtained by treating resin **15** with 5% TFA/DCM. A blank of the treatment was also performed.[b] In order to minimize interference of HCl traces dissolved in DCM, solvent was purified under basic alumina (Al_2O_3).

For each *N*-hydroxylamine used, progressive cleavage was observed with time (see Fig. 5). After short 5-min treatments, only a minimum amount of peptide **6** was detected in the experiment conducted in the presence of HOAt (**2**) (entry 4 vs. 1, 7). Higher rates of cleavage were also observed with this additive than with HOBt (**1**) or Oxyma (**3**) after more prolonged exposure to Barlos resin (entries 5, 6 vs. 2, 3, 8, 9). This tendency is in agreement with the expected behavior regarding the relative pKa values, HOAt (**2**) being the most acidic *N*-hydroxylamine (3.28 vs. 4.60) and so the one rendering the highest percentage of released peptide **6**. The similar acidity of HOBt (**1**) and Oxyma (**3**) resulted in almost identical values. However, it must be noted that after 24 h of contact with 2-chlorotrityl chloride resin (**15**), percentages of undesired cleavage of >5% were observed, regardless of the type of additive used.

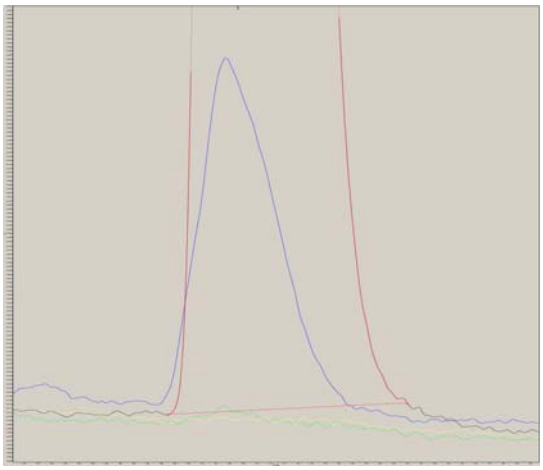

Figure 5. Reverse HPLC analysis on samples showing premature cleavage of tripeptide **6** from 2-chlorotrityl chloride resin (**15**), after 5 min (yellow), 1 hour (green) and 24 hours (blue) treatment with Oxyma (**3**), relative to 5% TFA cleavage (red) as reference.

Conclusions

N-hydroxylamines with pKa in the range 3-10 have been extensively used as additives to carbodiimides, assisting the formation of the native peptide bond. However, their capacity to reduce the impact of a wide range of base-driven side reactions, also as a result of their unusual high acidity, makes them a much more versatile tool than a simple catalyst in the context of peptide chemistry. Here we show that addition of *N*-hydroxylamines to a Fmoc-removal cocktail or short treatments, prior to couplings onto Pro-peptide resins, greatly minimizes the presence of undesired byproducts. It has been observed that more concentrated solutions further increase the potential of these additives, as noted in the case of aspartimide prevention, where the percentage of unmodified α–peptide can increase up to 30%. Thus, *N*-hydroxylamines are a cost-saving, easily available alternative to full suppression methods. In addition, we have demonstrated that the low pH induced by these *N*-hydroxylamines causes minimal loss of peptide during syntheses carried out in mild acid-labile resins, such as 2-chlorotrityl chloride. However, caution should be taken during long treatments with this solid support.

Among these *N*-hydroxylamines, the recently rediscovered ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) shows promising results as a suppressing agent of these base-derived side reactions. Furthermore, its low cost and safe decomposition make Oxyma an attractive alternative to classical benzotriazoles. The minimization

capacity of piperidides and other aspartimide-related byproducts supersedes that of HOBt and even HOAt, in the proposed hexapeptide model at all the concentrations tested. A substantial increase in the purity was achieved when using 1M solutions of Oxyma in 20% piperidine in DMF. With regard to the Pro overcoupling, Oxyma showed a similar performance to HOBt, with a much greater coupling efficiency. Interestingly, although the pKa of Oxyma is the same as that of HOBt (4.60) and higher than that of HOAt (3.28), the performances observed in the reduction of base-driven unwanted reactions did not follow this acidity trend. This observation suggests that other factors participate in the interaction between the *N*-hydroxylamine and the basic center. The sterical hindrance of the benzotriazole ring, in comparison to the much smaller *α,α*–disubstituted oxime, may be responsible for this behavior. However, in the premature cleavage of peptide from acid-labile Barlos resin, which depends only on the pH generated, the expected tendency between the additives is observed.

Acknowledgements

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Capítol 5. *Oxyma com a bloc de construcció dins de sals d'oni*

Publicació VI

COMU: A Safer and More Effective Replacement for Benzotriazole-Based Uronium Coupling Reagents †

COMU: un substitut de les sals d'uroni basades en benzotriazol amb perfil més segur i eficaç

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Chem. Eur. J. ***2009****, 15, 9404-9416*

† Ramon Subirós Funosas va participar en la preparació de mostres per realitzar raigs X, així com la realització dels experiments d'eficiència basats en el model *N*MeLeu-encefalina. Com en l'estudi anàleg sobre Oxyma, el doctorand va col·laborar amb la Plataforma de Calorimetria en el disseny i discussió dels assajos calorimètrics DSC dinàmic, DSC isotèrmic i ARC. El doctorand va participar de l'elaboració de la part corresponent del manuscrit.

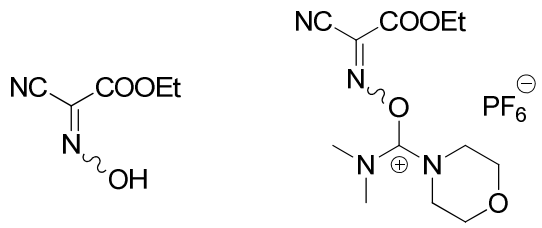
Ayman El-Faham es va encarregar de realitzar la síntesi de les diferents sals d'uroni, així com d'investigar la seva solubilitat, estabilitat, capacitat de reduir l'epimerització i eficiència d'acoblament en la resta de models.

Rafel Prohens, de la Plataforma de Calorimetria, va dur a terme els assajos calorimètrics, a més d'aportar una valoració i discussió dels resultats obtinguts.

Resum

En l'article que s'inclou a continuació es descriu una nova família de reactius d'acoblament de tipus sal d'uroni que difereixen en l'estructura carbocatiònica, així com en la naturalesa de la *N*-hidroxilamina actuant com a grup de sortida.

La presència del grup morfolino en combinació amb la oxima escollida com grup de sortida té una influència considerable en la solubilitat, estabilitat i reactivitat dels reactius derivats. D'entre totes les sals d'uroni preparades i evaluades, la nova sal d'uroni de tipus dimetilmorfolino basada en Oxyma (COMU) obté resultats excel·lents amb únicament 1 equivalent de base, confirmant l'efecte acceptor de pont d'hidrogen de l'oxigen del grup morfolino en la reacció d'acilació. D'altra banda, COMU també mostra un perfil de descomposició tèrmica molt menys perillós que el dels benzotriazols anàlegs HDMA i HDMB, els quals exhibeixen comportaments de descomposició típiques de reaccions autocatalítiques, caracteritzades per la seva impredecibilitat, el qual introdueix un element de perillositat. A més, el grup Oxyma contingut a COMU ja va mostrar en anteriors estudis un risc d'explosió molt menor que el de HOBt i HOAt.



Estructura del 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma) i la sal d'uroni de tipus dimetilmorfolino derivada (COMU).

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COMU: A Safer and More Effective Replacement for Benzotriazole-Based Uronium Coupling Reagents**

Ayman El-Faham,*[a, b, c] Ramon Subirós Funosas,[a, d] Rafel Prohens,[e] and Fernando Albericio*[a, d, f]

Abstract: We describe a new family of uronium-type coupling reagents that differ in their iminium moieties and leaving groups. The presence of the morpholino group in conjunction with an oxime derivative—especially ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma)—had a marked influence on the solubilities, stabilities, and reactivities of the reagents. Finally, the new uronium salt derived from Oxyma (COMU) performed extremely well in the presence of only 1 equiv of base, thereby confirming the effect of the hydrogen bond acceptor in the reaction. COMU also showed a less hazardous safety profile than the benzotriazole-based HDMA and HDMB, which exhibited unpredictable autocatalytic decompositions. Furthermore, the Oxyma moiety contained in COMU suggests a lower risk of explosion than in the case of the benzotriazole derivatives.

Keywords: coupling reagents · Oxyma · peptides · solid-phase synthesis · uronium salts

Introduction

Peptide synthesis is based on an appropriate combination of protecting groups and a suitable choice of coupling method.[1] Nowadays, almost all peptide bonds are formed in the presence of 1-hydroxybenzotriazole (HOBt, **1**, Figure 1, left)[2] or its derivatives (HOAt, **2**; 6-Cl-HOBt, **3**).[3,4] HOBt derivatives are therefore either used in combination with a carbodiimide or another coupling agent or are built into a stand-alone reagent such as an immonium [HATU (**4**), HBTU (**5**), HCTU, (**6**), Figure 1, right] or phosphonium (PyAOP, PyBOP, PyClock) salt.[5,6] An onium salt consists of two parts: a leaving group (YL) and the iminium moiety (Figure 1, center).

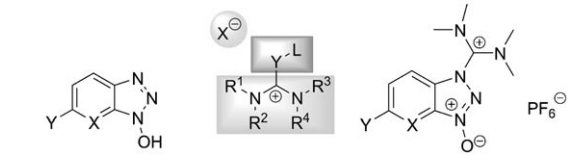

Figure 1. General structures of HOBt derivatives (left), immonium/uronium salts (center), and the most commonly used immonium salts (right).

Recently we showed that the incorporation of a hydrogen bond acceptor in the iminium part resulted in performances superior to those described previously.[7] As reported in our previous work, the presence of an oxygen in the iminium

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[**] All abbreviations used in the text are given in reference [1].

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moiety confers more solubility on the reagent, enhances coupling yields, and decreases racemization, thereby allowing the use of just 1 equiv of base. HDMA (**7**), HDMB (**8**), and 6-HDMCB (**9**) are thus more efficient in terms of coupling efficiency and reduction of racemization than their counterparts HATU (**4**), HBTU (**5**), and HCTU (**6**). Importantly, 6-HDMCB (**9**), which is consistently superior to HDMB (**8**), often performs in a similar manner to HATU (**4**), which is one of the most powerful commercially available immonium coupling reagents known to date.[7] It is important to note that all these reagents exist in their *N*-forms,[8] which are less reactive than the *O*-forms (Figure 2).[9]

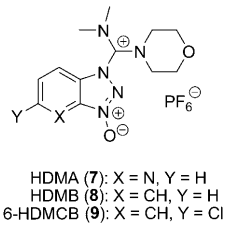


Figure 2. General structures of the dimethyl-morpholino immonium salts, which are superior to their tetramethyl counterparts.

Recent reports have confirmed the explosive properties of HOBt derivatives.[10] In our preceding paper,[11] we showed that Oxyma (**10**, Figure 3) is an excellent replacement for HOBt and its analogues. Here we report a new uronium salt, COMU (**11**, Figure 3), which represents the combination of a morpholonium-based immonium moiety, introduced in our previous work, and Oxyma (**10**) as leaving group, as a superior and safe coupling reagent for amide formation.

Results and Discussion

Scheme 1 shows the uronium salts prepared for the first screening. We tested four distinct oximes (**17a–d**) and several iminium moieties, including dimethyl-morpholino, pyrrolidino-morpholino, and dimethyl-pyrrolidino systems, the last of these as a reference for the role of the pyrrolidino moiety. The corresponding unsymmetrical uronium salts were prepared by treating *N,N*-dialkyl carbamoyl

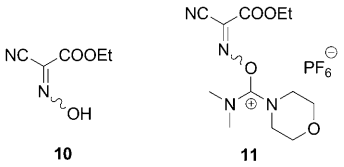
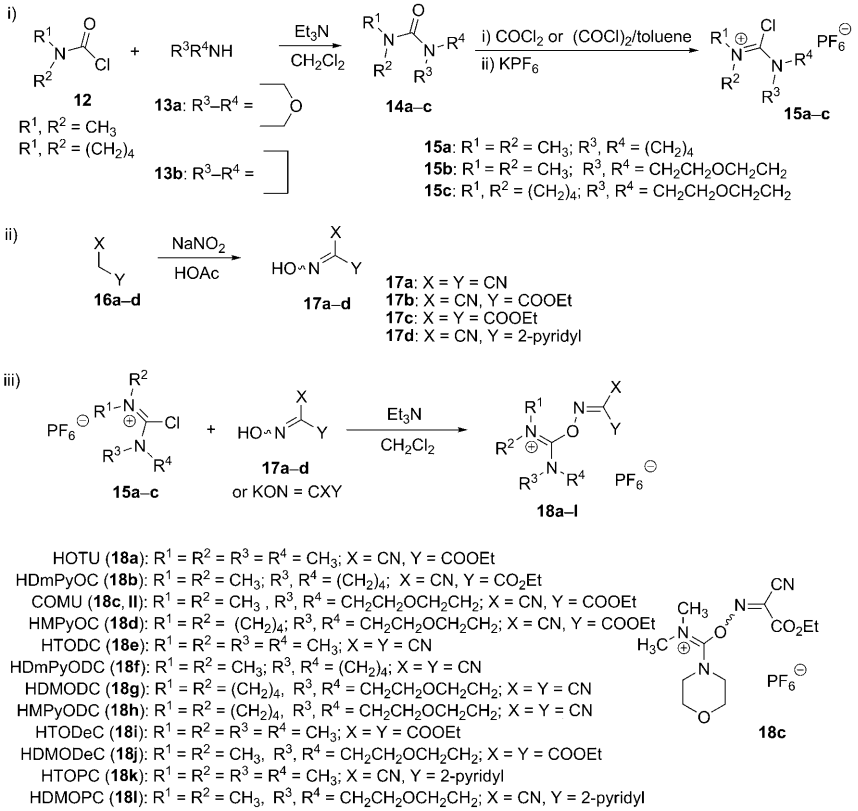


Figure 3. Structures of Oxyma and the new uronium salt.

chlorides **12a** or **12b** with morpholine (**13a**) or pyrrolidine (**13b**) to give the urea derivatives **14a–c** (Scheme 1). Urea derivatives (**14a–c**) were treated with phosgene or oxalyl chloride to yield the corresponding chloro salts, which were stabilized by the formation of hexafluorophosphate salts (**15a–c**). Subsequent treatment with oxime derivatives (**17a–d**), obtained by nitrosation from the active methylene compounds **16a–d**, in the form of their potassium salts or in the presence of Et_3N provided the target compounds (**18a–l**, Scheme 1).

Interestingly, the ^{13}C NMR spectra of these compounds indicated displacements of the carbocationic carbon of 156.11 ppm for HOTU (**18a**, the hexafluorophosphate counterpart of TOTU, already described in the literature)[12] and 156.14 ppm for COMU (**18c**). These displacements are consistent with those reported for this kind of compound in the *O*-form.[13,9] X-ray crystallography confirmed this hypothesis (Figure 4).



Scheme 1. Procedure followed for the preparation of the oxime-based uronium-type coupling reagents.

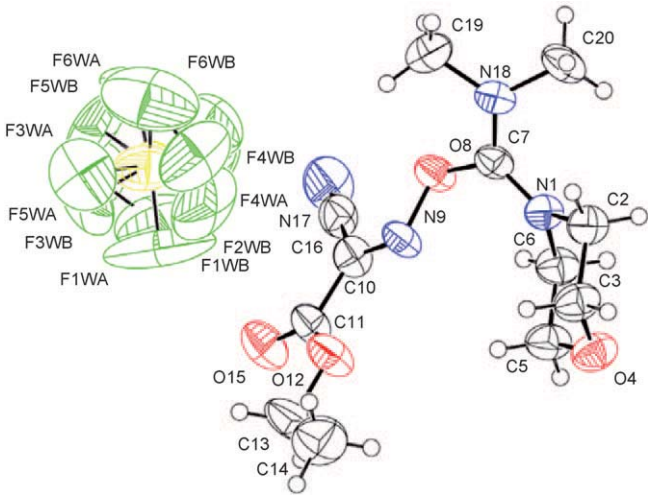


Figure 4. X-ray crystallographic structure of COMU.

To determine the compatibilities of the new coupling reagents with peptide synthesis in both manual and automatic modes, their solubilities and stabilities in solution and in the solid state were examined by ^{1}H NMR analysis. The presence of the oxygen in the iminium structure increased the stabilities of the coupling reagent relative to their tetramethyl derivatives (entries 3 vs. 4, Table 1). Furthermore, Oxyma

Table 1. Hydrolytic stabilities [%] of immonium/uronium-type coupling reagents in DMF (open vials).

Entry	Coupling reagent	5 h [%]	24 h [%]	48 h [%]
1	HATU (**4**)	99	95	76
2	HBTU (**5**)	100	98	86
3	HOTU (**18a**)	100	95	84
4	COMU (**18c**)	100	100	93

derivatives have greater stabilities than the benzotriazole derivatives HATU (**4**) and HBTU (**5**) (Figure 1). All these reagents showed stabilities greater than 95% in a closed vial. These observations are of practical relevance for both solid-phase and solution strategies: when the activation of a carboxylic acid is slow and the coupling reagent is not stable, it is degraded and no longer able to activate the carboxylic function. This feature is crucial in cyclization steps or for segment coupling steps in convergent strategies, in which the excess of the carboxylic function is either absent (cyclization) or low (segment coupling) and couplings are therefore very slow.

Table 2 indicates that the presence of the oxygen atom in the carbon skeleton and the leaving group are of marked relevance for the solubilities of the compounds. Thus, all dimethyl-morpholino derivatives (**18c**, **18g**, and **18l**) were more soluble than their tetramethyl counterparts (**18a**, **18e**, and **18k**) (Table 2, entries 3 vs. 4, 5 vs. 6, and 7 vs. 8). Furthermore, ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, **10**) derivatives were more soluble than dicyano and cyanopyridinyl ones (Table 2, entries 3, 4 vs. 5, 6 and 7, 8). Thus,

Table 2. Effect of oxygen on the solubility of the immonium-/uronium-type coupling reagents in DMF.

Entry	Coupling reagent	Wt/1 mL	Molarity
1	HATU (**4**)	0.165	0.43
2	HBTU (**5**)	0.175	0.46
3	HOTU (**18a**)	0.420	1.09
4	COMU (**18c**)	0.620	1.44
5	HTODC (**18e**)	0.410	1.20
6	HDMODC (**18g**)	0.520	1.36
7	HTOPC (**18k**)	0.320	0.79
8	HDMOPC (**18l**)	0.430	0.98

COMU (**18c**) and HDMODC (**18g**) were the most soluble. They were used to prepare solutions of up to 1.5 M and showed clearly higher solubilities than the benzotriazole derivatives **4** and **5** (Table 2, entries 4, 6 vs. 1, 2). This increased solubility can be used to prepare more concentrated solutions in order to enhance coupling yields and to facilitate the removal of the excess of coupling reagent and the urea side-products during the workup in a solution-mode approach.

A further characteristic of the Oxyma derivative **18c** is that the course of reaction can be followed due to a change in color, which depends on the type of base used. Thus, 2 min after the addition of the coupling reagent, a solution of **18c** has turned orange-red when DIEA is used as a base, and pink in the case of TMP. Once the reaction is complete, the solutions become colorless and yellow, respectively (Figure 5).

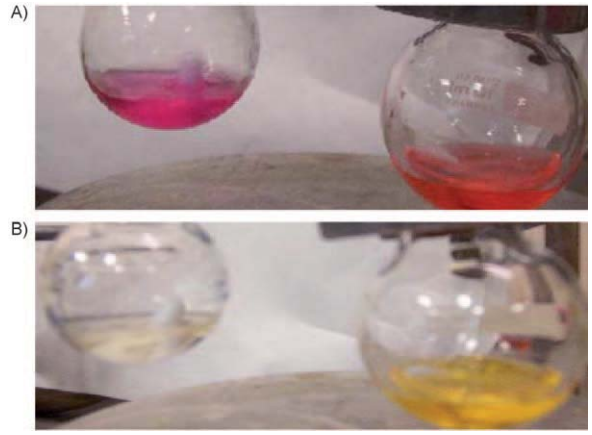


Figure 5. A) 2 min after the addition of COMU (pink with TMP and orange-red with DIEA). B) 1 h after the addition of COMU.

A preliminary screening on the efficiency of Oxyma-based coupling reagents **18a** and **18c**, in the coupling of hindered amino acids, was examined with two model systems (Fmoc-Val-OH + H-Val-NH_2 and Z-Aib-OH + H-Val-OMe) in solution. The reaction mixtures were followed by HPLC; the t_R values for the starting Fmoc-Val-OH and Z-Aib-OH were 20.89 min and 18.25 min, respectively, and those for the products (Fmoc-Val-Val- NH_2 and Z-Aib-Val-

OMe) were 19.88 min and 20.90 min, respectively. The Oxyma-based coupling reagents were more reactive than the benzotriazole derivatives (Tables 3 and 4, **18c**, **18a** vs. **7**, **4**). Again, the morpholine derivatives were superior to their tetramethyl counterparts (Tables 3 and 4, **18c**, **7** vs. **18a**, **4**). In both cases, COMU (**18c**) was superior to HATU (**4**), the most potent of the currently commercially available coupling reagents. This superiority was more remarkable when only 1 equiv of base was used (Table 3 and Table 4), thereby reaffirming the hydrogen bond acceptor role of the oxygen in the morpholino moiety.

Table 3. Levels of coupling of Fmoc-Val-Val-NH_2 with use of several different coupling reagents and a range of equivalents of DIEA in DMF as a solvent.[a]

time [min]	HATU (**4**) yield [%]		HDMA (**7**) yield [%]		COMU (**18c**) yield [%]		HOTU (**18a**) yield [%]	
	2 equiv	1 equiv	2 equiv	1 equiv	2 equiv	1 equiv	2 equiv	1 equiv
5	83.0	70.0	94.8	80.0	95.1	82.0	85.0	71.0
10	87.6	76.0	95.0	85.0	96.0	86.0	89.0	78.0
20	90.5	80.0	96.4	90.0	98.0	90.1	91.0	83.0
30	92.5	82.0	98.0	93.5	98.5	94.5	93.0	86.0
60	93.0	82.0	99.0	95.5	100.0	96.0	94.0	87.0
120	94.0	83.0	99.0	96.0	100.0	98.0	96.0	88.0

[a] HPLC conditions: linear gradient of 10 to 90% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm. Flow rate = 1 mL min^{-1} Column: Waters C_{18}, 5 μm, 4.6 × 150 mm (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler). t_R (Fmoc-Val-OH) = 20.89 min, t_R (Fmoc-Val-Val-NH_2) = 19.88 min.

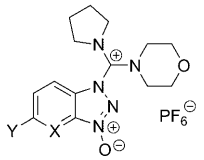
Table 4. Levels of coupling of Z-Aib-Val-OMe with use of several different coupling reagents and a range of equivalents of DIEA in DMF as a solvent.[a]

time [min]	HATU (**4**) yield [%]	HBTU (**5**) yield [%]	HDMA (**7**) yield [%]		COMU (**18c**) yield [%]		HOTU (**18a**) yield [%]	
	2 equiv	2 equiv	2 equiv	1 equiv	2 equiv	1 equiv	2 equiv	1 equiv
2	80.0	70.0	89.0	80.5	89.0	85.0	85.0	69.0
5	85.0	76.0	91.0	86.5	92.0	89.0	88.0	74.0
10	87.0	78.0	93.0	88.0	93.0	90.0	89.0	77.0
20	89.0	80.0	94.0	89.5	95.0	91.0	90.0	82.0
30	90.0	81.0	96.0	90.0	97.0	93.0	91.0	86.0
60	91.0	83.0	97.0	91.0	98.0	96.0	91.3	86.0
120	92.0	83.0	98.0	91.0	99.0	97.0	92.0	86.0

[a] HPLC conditions: linear gradient of 10 to 90% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm, flow rate = 1 mL min^{-1}, Column: Waters C_{18}, 5 μm, 4.6 × 150 mm (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler). t_R (Z-Aib-OH) = 18.25 min, t_R (Z-Aib-Val-OMe) = 20.90 min. t_R (Z-Aib-OAt) = 22.30 min, t_R (Z-Aib-OBt) = 22.80 min, t_R (Z-Aib-Oxyma) = 23.90 min.

Once these encouraging results with the Oxyma-based COMU (**18c**) had been obtained, a deeper study using several oxime derivatives were carried out. Two model peptides, Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2, were used to study the retention of configuration achieved with the new coupling reagents.[7]

The novel uronium coupling reagents were tested and compared with classical immonium salts (including the benzotrizole derivatives **19**, **20**, and **21**, containing pyrrolidino-morpholino systems, Figure 6) with the aid of these models, which involve stepwise and also [2+1] segment coupling (Tables 5 and 6). For the stepwise coupling of Z-Phg-OH to H-Pro-NH_2 to produce Z-Phg-Pro-NH_2, the oxime-based COMU (**18c**), HOTU (**18a**), HTODC (**18e**), and HDMODC (**18g**) gave better conservation of chirality than the benzotriazole-based HATU (**4**), HBTU (**5**), HDMA (**7**), HDMB (**8**), and 6-HDMCB (**9**) (Table 5).[7] The dimethyl-morpholino derivative COMU (**18c**) induced less racemization than other Oxyma derivatives containing different iminium moieties (**18a**, **18b**, **18d**) and than other uronium salts containing different oxime substituents (**18g**, **18l**). In the oxime series, the worst results were obtained



HMPyA (**19**): X = N, Y = H
HMPyB (**20**): X = CH, Y = H
HMPyC (**21**): X = CH, Y = Cl

Figure 6. General structures of benzotriazole-based immonium salts containing pyrrolidino-morpholino systems.

Table 5. Yields and racemization for the formation of Z-Phg-Pro-NH_2 in DMF (solution-phase synthesis).[a]

Coupling reagent	Base (equiv)	Yield [%]	D,L [%]
HATU (**4**)	DIEA (2)	78.4	3.1
	TMP (2)	77.9	2.1
	DIEA (1)	74.8	2.4
HBTU (**5**)	DIEA (2)	80.2	8.2
	TMP (2)	81.2	6.4
	DIEA (1)	75.0	5.3
HDMA (**7**)	DIEA (2)	81.2	1.6
	TMP (2)	80.3	3.9
	DIEA (1)	82.3	1.6
HDMB (**8**)	DIEA (2)	80.8	3.8
	TMP (2)	79.9	7.8
	DIEA (1)	82.3	3.1
6-HDMCB (**9**)	DIEA (2)	84.5	1.5
HMPyA (**19**)	DIEA (2)	89.9	2.6
HMPyB (**20**)	DIEA (2)	88.9	4.9
HMPyC (**21**)	DIEA (2)	90.1	2.1
HOTU (**18a**)	DIEA (2)	78.9	0.17
	TMP (2)	90.0	1.20
	TMP (1)	80.3	0.70
COMU (**18c**)	DIEA (2)	88.2	0.12
	TMP (2)	91.0	0.90
	TMP (1)	93.0	0.40
HDmPyOC (**18b**)	DIEA (2)	89.3	0.31
HMPyOC (**18d**)	DIEA (2)	89.8	0.32
HTODC (**18e**)	DIEA (2)	89.3	0.39
HDMODC (**18g**)	DIEA (2)	90.1	0.40
HDmPyODC (**18f**)	DIEA (2)	88.7	0.44
HMPyODC (**18h**)	DIEA (2)	90.2	0.35
HTOPC (**18k**)	DIEA (2)	85.3	28.9
HDMOPC (**18l**)	DIEA (2)	86.0	13.6

[a] LL and DL forms of the test dipeptide are described elsewhere.[7] The t_R values for LL and DL were identified by co-injection with authentic and pure samples of LL. HPLC system: linear gradient of 20 to 50% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm, $t_{R\,LL}$ = 26.01 min., $t_{R\,DL}$ = 27.40 min.

Table 6. Yields and racemization for the formation of Z-Phe-Val-Pro-NH_2 (2+1) in DMF (solution-phase synthesis).[a]

Coupling reagent	Base (equiv)	Yield [%]	LDL [%]
HATU (**4**)	DIEA (2)	85.8	13.9
	DIEA (1)	83.2	11.0
	TMP (2)	78.0	5.3
	TMP (1)	76.1	4.9
HBTU (**5**)	DIEA (2)	89.7	27.7
	DIEA (1)	78.6	16.3
	TMP (2)	81.2	14.2
HDMA (**7**)	DIEA (2)	89.3	10.5
	DIEA (1)	87.4	5.1
	TMP (2)	86.2	3.7
	TMP (1)	84.1	3.8
HDMB (**8**)	DIEA (2)	88.7	20.3
	DIEA (1)	86.3	11.5
	TMP (2)	87.1	13.3
	TMP (1)	80.1	10.5
6-HDMCB (**9**)	DIEA (2)	79.9	13.9
	TMP (2)	80.8	6.5
HMPyA (**19**)	TMP (2)	90.1	4.1
HMPyB (**20**)	TMP (2)	86.8	15.3
HMPyC (**21**)	TMP (2)	89.9	8.7
HOTU (**18a**)	DIEA (2)	91.2	23.6
	TMP (2)	88.7	7.4
	TMP (1)	80.3	7.5
COMU (**18c**)	DIEA (2)	91.3	19.3
	TMP (2)	89.8	7.0
	TMP (1)	90.3	3.5
HDmPyOC (**18b**)	DIEA (2)	92.0	20.7
	TMP (2)	88.0	7.9
HMPyOC (**18d**)	DIEA (2)	92.3	26.3
	TMP (2)	91.0	10.2
HTODC (**18e**)	TMP (2)	89.1	17.7
	TMP (1)[b]	82.3	16.3
HDMODC (**18g**)	DIEA (2)	88.0	17.9
	TMP (1)[b]	90.0	13.2
HDmPyODC (**18f**)	TMP (2)	87.2	22.1
HMPyODC (**18h**)	TMP (2)	89.4	23.2
HTOPC (**18k**)	TMP (2)	88.3	43.1
	TMP (1)	79.8	40.7
HDMOPC (**18l**)	TMP (2)	90.2	43.6
	TMP (1)	87.2	40.2

[a] LLL and LDL forms of the test tripeptide are described elsewhere.[7] Samples were co-injected with authentic and pure samples of LLL. HPLC system: linear gradient of 20% to 80% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm, Waters C_{18} 5 μm 4.6× 150 mm column, $t_{R\,LLL}$ = 19.98 min., $t_{R\,LDL}$ = 21.05 min. [b] Extra peak related to the starting material (Z-Phe-Val-OH) was observed in 3–5% ratio.

with the cyano-pyridinyl system (**18k**, **18l**) because of the higher acidity of the corresponding oxime.

For the same model but with the reaction carried out on solid-phase, COMU (**18c**) gave the best coupling yield, together with levels of racemization similar to those seen with the HOBt derivatives (Table 7).

To check the effectiveness of the new reagents, the demanding Leu-enkephalin derivative H-Tyr-Aib-Aib-Phe-Leu-NH_2[6f] was manually assembled on Fmoc-RinkAmide-AM-resin with the use of amino acid/activator (3 equiv), DIEA (6 or 3 equiv) and 30 min coupling times, except in the case of Aib-Aib, for which 1 h double coupling was used. Percentages of incorporation for the coupling of

Table 7. Yields and racemization for the formation of Z-Phe-Val-Pro-NH_2 (2+1) in DMF (solid-phase synthesis).[a]

Coupling reagent	Base (equiv)	Yield [%]	LDL [%]
HATU (**4**)	TMP (2)	90.0	13.0
HBTU (**5**)	TMP (2)	89.0	27.0
HDMA (**7**)	TMP (2)	90.0	12.0
HDMB (**8**)	TMP (2)	92.0	23.0
6-HDMCB (**9**)	TMP (2)	92.0	20.0
HOTU (**18a**)	TMP (2)	91.0	27.1
	TMP (1)	85.0	25.0
COMU (**18c**)	TMP (2)	94.0	23.0
	TMP (1)	92.0	21.0
HTODC (**18e**)	TMP (2)	92.0	38.0
	TMP (1)	86.0	28.9
HDMODC (**18g**)	TMP (2)	94.0	25.7
	TMP (1)	88.0	23.3

[a] The coupling was performed with Fmoc-Pro-Rink amide-PS-resin and 3 equiv of Z-Phe-Val-OH, 3 equiv of coupling reagent, 6 equiv of base (TMP), and preactivation for 10–30 s in DMF at RT. The peptide was recovered after deblocking with water in TFA (10%) for 1 h at RT. The solvent was removed under vacuum and then washed with hexane. The crude peptide was injected into the HPLC system by using a previously reported method.[7] Extra peaks related to the starting material (Z-Phe-Val-OH) were observed in 3–5% percentages. LLL and LDL forms of the test tripeptide are described elsewhere.[7] Samples were co-injected with authentic and pure samples of LLL.

Fmoc-Aib-OH onto the Aib-containing resin were determined by reversed-phase HPLC analysis, after cleavage of the peptide from the resin (Table 8). The best results were obtained with the Oxyma-based COMU (**18c**) and HOTU (**18a**), with higher percentages of target pentapeptide being

Table 8. The percentages of des-Aib (H-Tyr-Aib-Phe-Leu-NH_2) obtained during solid-phase assembly of the pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2).[a]

Coupling reagent	Base (equiv)	Pentapeptide [%]	des-Aib [%]
HATU (**4**)	DIEA (2)	83.0	17
	DIEA (1)	68.0	32
HBTU (**5**)	DIEA (2)	47.0	53
	DIEA (1)	33.0	67
HDMA (**7**)	DIEA (2)	98.0	<1
	DIEA (1)	90.0	10
HDMB (**8**)	DIEA (2)	89.0	10
	DIEA (1)	64.0	36
6-HDMCB (**9**)	DIEA (2)	98.7	1.3
	DIEA (1)	39.0	62.0
HOTU (**18a**)	DIEA (2)	99.0	1.0
	DIEA (1)[b]	0.3	99.7
	DIEA (2)[c]	87.5	12.5
COMU (**18c**)	DIEA (2)	99.7	0.26
	DIEA (1)[b]	29.3	70.7
	DIEA (2)[c]	99.86	0.14
HTODC (**18e**)	DIEA (2)	85.5	13.5
HDMODC (**18g**)	DIEA (2)	95.3	4.7
HTOPC (**18k**)	DIEA (2)	27.4	72.9
HDMOPC (**18 L**)	DIEA (2)	41.3	58.3

[a] Pentapeptide and deletion tetrapeptide des-Aib were confirmed by peak overlap in the presence of authentic samples. HPLC-MS showed the correct mass for the pentapeptide at 612.0. [b] 1 h standard single coupling was performed for Aib-Aib with only 1 equiv of base. [c] 30 min standard single coupling was performed for Aib-Aib with 2 equiv of base.

obtained than with HDMA (**7**) or HATU (**4**). Synthesis with COMU (**18c**) led to only a 0.26% yield of des-Aib when 2 equiv of DIEA were used, whereas its tetramethyl derivative **18a** gave a 1% yield under the same conditions. For a 30 min coupling, **18c** gave a 0.14% yield of des-Aib whereas **18a** gave a 12.5% yield. This observation indicates that the morpholino moiety increases the reactivities of uronium salts relative to tetramethyl derivatives. These results are consistent with what is discussed above.

In view of the superiority showed by the morpholino-containing uronium salts over their tetramethyl counterparts, the effect of the leaving group was further tested by comparing the benzotriazole-based HDMA (**7**) and HDMB (**8**) and the Oxyma-based COMU (**18c**) in the manual solid-phase assembly of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH_2 on Fmoc-RinkAmide-AM-PS-resin. The strategy followed for the assay began with the elongation of resin-bound tripeptide H-MeLeu-Phe-Leu-resin by use of DIC/Oxyma (**10**) in 30 min couplings. Quantitative yields were verified by means of the Kaiser test for primary amines. After this preliminary step, comparisons were made for the stepwise incorporation of the two last residues, with use of the corresponding immonium/uronium salt and Fmoc-amino acid. Samples were preactivated for 20–30 s with DIEA (2 or 1 equiv relative to uronium salt/Fmoc-amino acid) in order to avoid guanidylation of the growing peptide chain. The coupling times were shortened to 5 min, so that significant differences in the reactivities of the coupling reagents could arise. After cleavage from the resin with 90% TFA/10% H_2O and lyophilization, relative performances were checked in terms of percentages of pentapeptide and deletion peptides, as determined by reversed-phase HPLC analysis (Table 9).

The experiments were carried out either in standard (99.8% purity, as determined by GC) or in treated (anhydrous, dried over molecular sieves and bubbled with N_2 to remove Et_2NH) DMF, as in the rest of experiments, in order to examine the effect of the solvent's purity (entries 1–6 vs. 7–12). The assay with standard DMF reflected the huge difference in reactivity between HOAt- and HOBt-derived uronium salts, with an impressive—for such demanding conditions—91% yield of pentapeptide being obtained with HDMA (**7**), whereas HDMB (**8**) only afforded a poor 7% (entries 1, 2). The Oxyma-based COMU (**18c**) gave a much higher purity than HDMB but, unlike in the previous synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2, it was far from that afforded by HDMA (43%, entry 3). The experiment was repeated, but with preactivation with only 1 equiv of base, a second equiv being added once the coupling mixture had been added onto the resin, in order to examine whether the base had any effect on the stability of the active ester during preactivation. The results showed no significant variation, except in the case of COMU (**18c**), for which the yield rose from 43% to 55% (due to the higher rate of coupling for the MeLeu residue), suggesting a high reactivity of the Oxyma-derived active ester (Table 9, entries 4, 5, and 6). The percentage of pentapeptide also increased to the same extent with COMU (**18c**) when the experiment with 2 equiv DIEA was carried out with treated, instead of standard, DMF, confirming the positive effect of the higher purity of the solvent, although this was not noticeable in the experiments with HDMA and HDMB (Table 9, entries 7, 8, and 9). Finally, an experiment was conducted with only 1 equiv DIEA, displaying the same tendency as observed previously: the performance of COMU (**18c**) was superior to that of the HOBt derivative but not as potent as that of the HOAt one (Table 9, entries 10, 11, and 12).

The effectiveness of COMU (**18c**) was compared with that of HOTU (**18a**) in the synthesis of the common decapeptide model ACP (65–74) on solid-phase (Table 10).[6f]

Table 9. Percentages of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH_2 and related deletion peptides obtained in solid-phase assembly in 5 min with use of various dimethyl-morpholino immonium/uronium salts.[a]

Entry	Coupling conditions		Coup. reagent	Penta [%]	des-MeLeu [%]	des-Tyr [%]	Trip [%]
1	standard DMF	2 equiv DIEA	HDMA	91.4	4.5	3.8	0.3
2			HDMB	7.0	42.2	9.7	41.1
3			COMU	42.9	48.5	4.4	4.2
4		2 equiv DIEA[b]	HDMA	91.5	5.0	3.1	0.4
5			HDMB	6.9	42.4	9.4	41.3
6			COMU	55.5	39.5	3.0	2.0
7	treated DMF	2 equiv DIEA	HDMA	89.4	6.7	3.6	0.3
8			HDMB	6.8	43.0	9.2	41.0
9			COMU	56.0	38.5	3.3	2.2
10		1 equiv DIEA	HDMA	73.7	18.5	7.4	0.4
11			HDMB	5.7	37.8	8.9	47.6
12			COMU	33.6	51.9	6.3	8.2

[a] HPLC-MS showed the correct mass for the pentapeptide at 695.5. Fmoc-amino acids were preactivated for 20–30 s. [b] Fmoc-amino acids were preactivated with only 1 equiv DIEA, with addition of another 1 equiv onto the resin after the first addition.

Table 10. Preparation of ACP (65–74) (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2) with use of the different coupling reagents (2 equiv) and 2 min coupling times.[a]

Coupling reagent	ACP [%]	Des-Asn [%]	Des-Val [%]	Des-Ile^{69} [%]	Des-Ile^{72} [%]
HOTU (**18a**)	66.0	0.27	2.72	21.7	7.56
COMU (**1cc**)	79.4	0.74	–	18.0	0.98

[a] The HPLC-MS showed the correct mass for the ACP at 1065.0.

The peptide was manually elongated on a Fmoc-Rink Amide-AM-resin (0.7 mmol g^{-1}). Coupling times of 2 min were used and excesses of reagents were 2 equiv for coupling reagents and amino acids and 4 equiv for DIEA. Incorporation was detected for Ile^{72} onto Asn and for Ile^{69} onto Asp. Peptide purity was determined by reversed-phase HPLC analysis, after cleavage of the peptide from the resin by treatment with TFA/H_2O (9:1) for 2 h at RT. HPLC analysis again showed a better performance of the morpholino-containing derivative than for the tetramethyl analogue: some deletion peptides, such as des-Val, were not observed with COMU (**18c**) and the percentage of pentapeptide ob-

tained was higher than that obtained with HOTU (**18a**) (Table 10, entry 1 vs 2).

The safety profile of COMU (**18c**), the most efficient of the uronium salts tested, was also considered and compared to those of HDMA (**7**) and HDMB (**8**). This issue was highly relevant in view of the explosive properties of benzotriazole-based additives and derived stand-alone coupling reagents, which limit their transportation. Although explosivity has never been reported for HDMA (**7**) or HDMB (**8**), the fact that they contain HOBt/HOAt and that other immonium salts, such as TBTU, have also shown explosive properties[10] means that a certain safety risk is assumed. Our main interest was focused on checking whether the novel coupling reagent COMU (**18c**) would display the common pattern observed in explosive compounds: high release of pressure associated with rapid decomposition.[14]

The thermal risk was assessed through a combination of two calorimetry assays: Differential Scanning Calorimetry (DSC) and Accelerating Rate Calorimetry (ARC). A preliminary assessment of the risk associated with a given decomposition can be determined by means of a dynamic DSC experiment. The heat released during this assay is measured by comparing it with a reference that has undergone the same thermal process. In addition, the relative decomposition kinetics (which are linked to explosivity) are highlighted.[15] In this assay, samples are heated in a closed crucible under a flow of N_2 from 30 to 300 °C at a constant heating rate of 10 °C min^{-1}. Diagrams displaying the heat flow as a function of time and temperature showed a distinct difference in decomposition behavior between benzotriazole-based HDMA (**7**) and HDMB (**8**) and the novel COMU (Figure 7). During the decomposition of HDMA (**7**) and

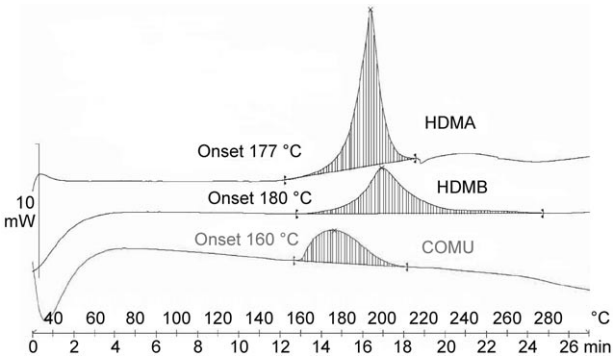


Figure 7. Thermograms showing heat flow versus temperature and time in the DSC experiments with HDMA (**7**), HDMB (**8**), and COMU (**18c**).

HDMB (**8**) the release of heat was slow at the beginning and increased very sharply, reaching a maximum and finally decreasing. This decomposition profile as observed for HDMA (**7**) and HDMB (**8**) resembles that of an autocatalytic reaction, in which the product also acts as a catalyst, the rate of the reaction increasing at the same time as the conversion.[16] These self-accelerating reactions warrant special attention because of their great unpredictability, resulting from the starting induction period with no thermal signal and their unexpected steep initiation. Therefore, a temperature alarm in an industrial process is not effective with compounds that show this kinetic behavior. Nevertheless, a dynamic DSC experiment can provide only indications of the autocatalytic nature of decomposition.

In contrast, COMU decomposed in a more constant manner that did not resemble this autocatalytic pattern. The normalized exothermic ΔH values should also be noted: 209 kJ mol^{-1} for HDMB, 245 kJ mol^{-1} for HDMA, and 183 kJ mol^{-1} for COMU. These observations indicate that in the event of an explosion, COMU would have less thermal severity. The ΔT_{ad} value (adiabatic temperature rise), calculated from experimentally ascertained exothermic ΔH values, also shows this relative severity: 248 °C for HDMB (**8**), 290 °C for HDMA (**7**), and 214 °C for COMU (**18c**). To conclude with the information that can be extracted from the DSC assay, the onset temperature at which decomposition began was lower in the case of the experiment with COMU (**18c**) (160 °C) than with HDMA (**7**) or HDMB (**8**) (177 °C and 180 °C, respectively).

A further evaluation of the risks associated with these compounds was carried out under adiabatic conditions by the ARC technique.[17] By this approach, the pressure released and the ΔT_{ad} can be directly determined. The assay begins with the application of the "heat-wait-seek" method, and when self-heating of the sample at a rate higher than 0.02 °C min^{-1} is detected, the experiment is changed to adiabatic mode. When decomposition occurs, the temperature and the pressure rise, and once the temperature reaches values above 300 °C the assay is stopped manually. In all cases the pressure rises detected were relatively low, in comparison with those of related additives.[11] In particular, the released pressure measured in the COMU (**18c**) experiment was similar to that seen with HDMA (**7**) (53 vs. 55 bar) and slightly higher than that seen with HDMB (**8**) (24 bar) (Figure 8).

Additionally, the increase in temperature during decomposition (ΔT_{ad}) was considerably lower with COMU (**18c**) (64 °C) than with HDMA (**7**) or HDMB (**8**) (164 °C and 121 °C, respectively), thereby confirming the lower thermal

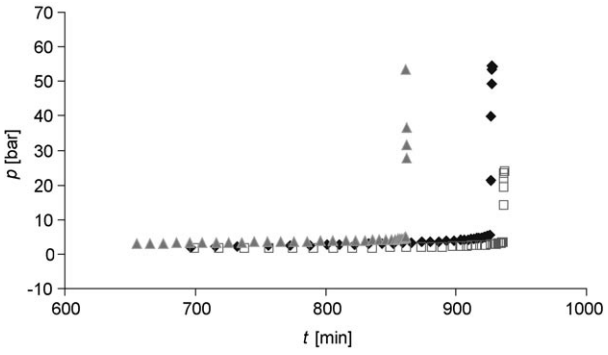


Figure 8. Decomposition profiles observed during ARC experiments with HDMB (**8**, □), HDMA (**7**, ◆), and COMU (**18c**, ▲) showing released pressure (bar) as a function of time (min).

severity of COMU observed in the DSC assay (Figure 9). Also consistent with the previous assay were the distinct kinetic profiles of COMU (**18c**) and the benzotriazole-based

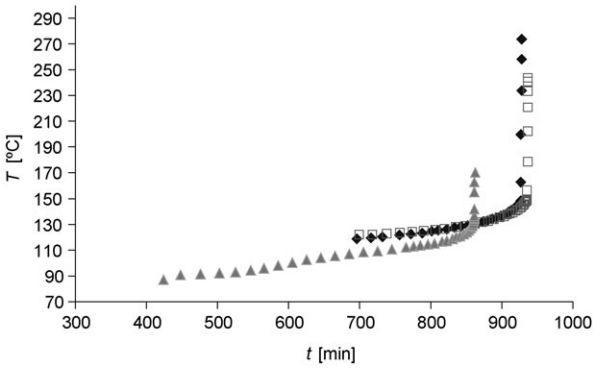


Figure 9. Decomposition profiles observed during ARC experiments with HDMB (**8**, □), HDMA (**7**, ◆), and COMU (**18c**, ▲) showing temperature (°C) as a function of time (min).

immonium salts. Whereas the temperatures in the HDMA (**7**) and HDMB (**8**) experiments increased slowly at the beginning and rose suddenly to their maxima (similarly to the DSC results, suggesting autocatylic kinetics), in the COMU (**18c**) experiment the temperature reached approximately one third of the total ΔT_{ad} over a period of time longer than had been required for the whole decomposition of HDMA (**7**) and HDMB (**8**). These slower kinetics could enable pressure originating from decomposition to be released into the environment.

With regard to the onset temperatures, ARC allows more accurate determination than DSC, which is known to suffer from uncertainty due to the smaller scale. For COMU (**18c**), decomposition began at a lower temperature than for HDMA (**7**) and HDMB(**8**) (91 °C vs. 119 °C and 122 °C). For safe working, it is recommended that the temperature of a given compound be maintained at values at which the time to maximum rate under adiabatic conditions is longer than 24 h.[18] This temperature can commonly be estimated after running an ARC assay, by subtraction of 50 K from the observed onset.[19] This safety value is at a lower temperature with COMU (**18c**) than with HDMA (**7**) and HDMB (**8**) (41 °C vs. 69 °C and 72 °C). Although peptide chemistry is usually performed at room temperature, and therefore below this safety threshold value, these results show that COMU has less thermal stability than benzotriazole-based immonium salts that also contain the morpholino moiety. The experimentally determined and calculated values determined from calorimetric studies and discussed above are shown in Table 11.

To study further the autocatalytic natures of the decompositions of HDMA (**7**) and HDMB (**8**), as suggested by the results obtained from the dynamic DSC and ARC experiments, in contrast with the results obtained for COMU (**18c**), we performed isothermal DSC assays. This technique is the most reliable way to detect whether decompositions

Table 11. Experimentally determined and calculated values obtained from dynamic DSC and ARC assays with the different stand-alone coupling reagents.

Coupling reagent	DSC			ARC		
	Onset [°C]	ΔH [kJ mol^{-1}]	calcd ΔT_{ad} [°C] [a]	Onset [°C]	Δp [bar]	exptl ΔT_{ad} [°C]
HDMA (**7**)	177	245	290	119	55	164
HDMB (**8**)	180	209	248	122	24	121
COMU (**18c**)	160	183	214	91	53	64

[a] Calculated ΔT_{ad} (°C) was obtained from the general formula ΔT_{ad} (°C) = ΔH (J mol^{-1})/[MW (g mol^{-1})·c_p (kJ kg^{-1} °C^{-1})], with estimated Cp = 2 kJ kg^{-1} °C^{-1}.

follow autocatalytic or non-autocatalytic kinetics. Temperatures were set at 10 °C below the onset observed in the corresponding dynamic DSC assay, and remained constant for 480 min. As a result, we obtained thermograms showing heat flow versus time (Figure 10). As would be expected for

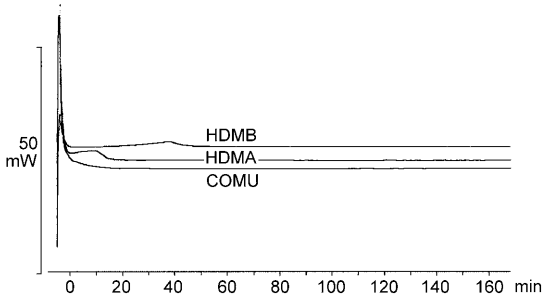


Figure 10. Thermograms showing heat flow versus time for isothermal DSC experiments set at 10 °C below the onset temperatures of HDMA (**7**, 167 °C), HDMB (**8**, 170 °C), and COMU (**18c**, 150 °C).

an autocatalytic reaction, HDMA (**7**) and HDMB (**8**) each defined a bell-shaped heat release curve, reported for this type of kinetics, in which the reaction accelerated, passing through a maximum of heat release and then decreasing. In contrast, COMU (**18c**) displayed a typical non-autocatalytic, *n*th-order kinetic profile, in which the rate of heat release decreased uniformly with time.[16] The former distinct kinetic profile strongly suggests the risk of a thermal runaway, which may lead to a sudden explosion, and consequently the safety measures that need to be taken.

Conclusions

In conclusion, we describe a new class of *O*-form uronium-type coupling reagents that differ in their immonium moieties and also in the leaving groups. The presence of the morpholino group has a marked influence on the polarity of the carbon skeleton, which affects the solubility, stability, and the reactivity of the reagent. As would be expected, HOAt derivatives were in all cases confirmed to be superior to HOBt ones in terms both of coupling yields and of retention

of configuration. Remarkably, Oxyma derivatives often gave results similar to or even better than those obtained with the aza derivatives, and also performed extremely well in the presence of only 1 equiv of base, thereby confirming the effect of the oxygen as a hydrogen bond acceptor in the reaction. The excellent performances of Oxyma derivatives, despite their acidities being similar to that of HOBt and higher than that of HOAt, can be explained by the fact that the *O*-form is the only form present during the coupling. In the benzotriazole derivatives, the *N*-form is the predominant one.

By means of calorimetry techniques, the safety profile of COMU (**18c**) was compared with those of benzotriazole-based HDMA (**7**) and HDMB (**8**). Although the pressure rises with the three coupling reagents were similar, COMU (**18c**) decomposed in a much slower and more controlled manner, with less thermal severity but greater predictability.[20] Moreover, the decomposition of HDMA (**7**) and HDMB (**8**), unlike that of COMU (**18c**), displayed autocatalytic kinetics, being highly unpredictable and difficult to detect before ending up in thermal runaway.

Experimental Section

General: TLC was performed on silica plates (8×4 cm, Albet) in suitable solvent systems with visualization with a Spectroline Model CM-10 UV lamp (254 nm). Melting points were measured in open capillary tubes with a Buchi B-540 melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded on a Thermo Nicolet series Fourier Transformer instrument as KBr pellets. The absorption bands (ν_{max}) are given in wavenumbers (cm^{-1}). A Shimadzu UV-250/PC instrument was used as a UV/Vis spectrophotometer. NMR spectra were recorded on a Varian mercury 400 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra, with chemical shifts reported as ppm relative to TMS. HPLC analyses were carried out with a Waters Symmetry Column C18, 5 μm, 4.6×150 mm with dual λ absorbance detector. HPLC-MS analyses were carried out with a Waters Symmetry Column C18, 5 μm, 4.6×150 mm with dual detector. All solvents used for recrystallization, extraction, column chromatography, and TLC were of commercial grade, distilled before use, and stored under dry conditions.

***N,N*-Dimethylmorpholine-4-carboxamide (DMU, 14a)**:[7] The urea derivative was distilled and collected at 127–129 °C as a colorless oil in a yield of 92.4 % (73 g from 0.5 mol reaction). ^{1}H NMR ($CDCl_3$): δ = 2.84 (s, 6H; $2CH_3$), 3.22–3.2 (m, 4H; $2CH_2$), 3.68–3.70 ppm (m, 4H; $2CH_2$); ^{13}C NMR ($CDCl_3$): δ = 38.62, 47.51, 66.89, 164.96 ppm.

***N,N*-Dimethylpiperidine-1-carboxamide (DmPyU, 14b)**:[24,25] The pure urea was obtained as a colorless oil at 98–100 °C in a yield of 85.5 %. ^{1}H NMR ($CDCl_3$): δ = 1.81–2.10 (m, 4H; $2CH_2$), 2.81(s, 6H; $2CH_3$), 3.15–3.18 ppm (m, 4H; $2CH_2$).

Morpholino(pyrrolidin-1-yl)methanone (MPyU, 14c): The urea derivative was distilled and collected at 127–129 °C as a colorless oil in a yield of 92.4 % (73 g from 0.5 mol reaction). ^{1}H NMR ($CDCl_3$): δ = 2.84 (s, 6H; $2CH_3$), 3.22–3.2(m, 4H; $2CH_2$), 3.68–3.70 ppm (m, 4H; $2CH_2$); ^{13}C NMR ($CDCl_3$): δ = 38.62, 47.51, 66.89, 164.96 ppm.

General Procedure for the synthesis of chlorouronium salts:[26] Oxalyl chloride (100 mmol) in CH_2Cl_2 (100 mL) was added dropwise at room temperature over 5 min to a solution of urea derivative (100 mmol) in dry CH_2Cl_2 (200 mL). The reaction mixture was stirred under reflux for 3 h, and the solvent was removed under vacuum. The residue was washed with anhydrous ether (2×100 mL) and was then bubbled with N_2 to remove the excess of the ether. The obtained residue was very hygroscopic, so it was dissolved directly in CH_2Cl_2, and saturated aqueous potassium hexafluorophosphate (100 mmol in 50 mL water, KPF_6) was added at room temperature with vigorous stirring for 10–15 min. The organic layer was collected, washed once with water (100 mL), dried over anhydrous $MgSO_4$, and filtered. The solvent was removed under reduced pressure to give a white solid that was recrystallized from CH_2Cl_2/ether or acetonitrile/ether to give white crystals.

4-[(Dimethylamino)chloromethylene]morpholin-4-iminium hexafluorophosphate (DMCH, 15a):[7] The salt was obtained as white crystals in a yield of 89.6 % (28.9 g). M.p. 94– 95 °C; ^{1}H NMR (CD_3COCD_3): δ = 3.39 (s, 6H; $2CH_3$), 3.75 (t, 4H; $2CH_2$), 3.86 ppm (t, 4H; $2CH_2$); ^{13}C NMR (CD_3COCD_3): δ = 44.36, 52.82, 65.99, 162.79 ppm.

***N*-[Chloro(pyrrolidin-1-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate (DmPyCH, 15b)**:[23] The product was obtained as a white solid in a yield of 89.0 %. M.p. 93–95 °C; ^{1}H NMR (CD_3COCD_3): δ = 2.00–2.13 (m, 4H; $2CH_2$), 3.49 (s, 6H; $2CH_3$), 3.90–4.02 ppm (m, 4H; $2CH_2$).

1-[Chloro(morpholino)methylene]pyrrolidinium hexafluorophosphate (MPyCH, 15c): The chloro salt was obtained by the method described above. The product was obtained as a white solid in a yield of 83.9 %. M.p. 99–100 °C; ^{1}H NMR ([D_6]acetone): δ = 2.10–2.14(m, 4H; $2CH_2$), 3.87 (t, 4H; $2CH_2$), 4.00 (t, 4H; $2CH_2$), 4.04–4.06 ppm (m, 4H; $2CH_2$); ^{13}C NMR ($CDCl_3$): δ = 25.80, 51.75, 55.97, 65.97, 154.85 ppm; elemental analysis (%) calculated for $C_9H_{16}ClF_6N_2OP$ (348.65): C 31.00, H 32.69, N 8.03; found: C 31.00, H 32.69, N 8.31.

General Procedure for preparation of uranium-type coupling reagents: The chloro salt (20 mmol) was added at 0 °C to a solution of an oxime potassium salt or a benzotriazole derivative (20 mmol) in acetonitrile (50 mL). The reaction mixture was stirred at this temperature for 30 min and was then allowed to come to room temperature with stirring over 6 h. The crude product was filtered and washed with acetonitrile. The solvent was concentrated to a small volume (1/4) under reduced pressure, and dry ether was then added to afford the product as a white solid in a pure state.

Synthesis of the potassium salt of hydroxycarbonimidoyl dicyanide (17a):[21] Sodium nitrite (14.2 g, 206 mmol) was slowly added at 0 °C (20–30 min addition) to a solution of malononitrile (9.06 g, 138 mmol) in acetic acid (20 mL) and water (50 mL). This mixture was then stirred at the same temperature for 45 min. After quenching of the reaction with HCl (2 N, 100 mL), the compound was extracted three times with ether (3×100 mL). The extracts were dried over anhydrous Na_2SO_4, and the ether was removed under vacuum to give an oily residue. The oily product was added slowly to a cold solution of KOH (8.0 g) in MeOH (100 mL), and then the reaction mixture was stirred for 20 min. Excess ether was added to afford the potassium salt as yellow crystals in a yield of 14.1 g (77.5 %). ^{13}C NMR (DMSO): δ = 107,40, 113.57, 119.78 ppm.

Synthesis of the potassium salt of diethyl 2-(hydroxyimino)malonate (17c):[22] A solution of diethyl malonate (16 g, 0.1 mol) in glacial acetic acid (17.5 mL, 0.3 mol) was stirred vigorously at 0–5 °C while a solution of $NaNO_2$ (20.7 g, 0.3 mol) in water (250 mL) was added dropwise over 3–4 h. The ice bath was removed and the mixture was stirred vigorously for a further 20 h. The nitrosation was carried out in three-necked flasks with appropriate fittings and a small vent to allow nitric oxide to escape. The reaction mixture was extracted with CH_2Cl_2 (400 mL and then 3× 100 mL portions). The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4. The CH_2Cl_2 was removed under vacuum and the resulting oily product was dissolved in CH_2Cl_2 (400 mL) and then stirred with anhydrous K_2CO_3 (32 g) for 15 min. After filtration, the CH_2Cl_2 was removed until 200 mL was reached, ether was added until the solution became cloudy, and the mixture was then kept in the refrigerator overnight to afford off-white crystals in 63.4 % yield. M.p. 116–118 °C; ^{1}H NMR ($CDCl_3$): δ = 1.24–1.29 (q, 6H; $2CH_3$), 4.20–4.29 ppm (m, 4H; $2CH_2$).

Synthesis of 2-pyridylhydroxyiminoacetonitrile (17d):[23] A solution of sodium nitrite (4.5 g, 0.065 mol in 5 mL water) was added slowly, with cooling, to a solution of 2-pyridylacetonitrile (2.2 g, 0.019 mol) in glacial acetic acid (4.5 mL). After 12 h standing, the precipitate was filtered off, washed with water, dried, and then recrystallized from ethanol to afford

the product in 65% yield. M.p. 220–222°C (lit. mp. 219–222°C, 68%); ^{1}H NMR ([D$_6$]DMSO: δ = 7.45–7.52 (1H), 7.87–7.90 (2H), 8.67 (d, 1H), 14.12 ppm (OH).

General Procedure for the preparation of urea derivatives:[24] The *N,N*-dialkylcarbamoyl chloride (0.6 mol) was added dropwise at 0°C to a stirring mixture of secondary amine (0.5 mol) and triethylamine (TEA, 0.5 mol) in CH_2Cl_2 (400 mL). When the addition was complete, the mixture was stirred for 3–4 h at room temperature. The reaction mixture was basified with NaOH (10%), and the organic layer was then collected and the aqueous layer was washed with CH_2Cl_2 (100 mL). The combined CH_2Cl_2 solution was washed with H_2O (2×100 mL) and saturated solution of NaCl (2×100 mL). Finally, the organic solvent was dried over anhydrous $MgSO_4$ and filtered, and the solvent was removed under reduced pressure. The oily residue obtained was purified by vacuum distillation.

***O*-[Cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTU, 18a)**: The product was obtained as a white solid in a yield of 6.32 g (82.1%). M.p. 135–137°C (dec). The triethylamine/HOXt approach gave a lower yield (68.7%) than the potassium salt strategy, maybe due to the washing with water. ^{1}H NMR ([D$_6$]acetone): δ = 1.37 (1, 3H; CH_3), 3.37 (s, 12H; $4CH_3$), 4.82 ppm (q, 2H; CH_2); ^{13}C NMR ([D$_6$]acetone): δ = 13.46, 40.71, 64.56, 106.78, 135.09, 156.11, 161.43 ppm; elemental analysis (%) calculated for $C_{10}H_{17}F_6N_4O_3P$ (386.23): C 31.10, H 4.44, N 14.51; found: C 31.34, H 4.35, N 14.75.

1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-pyrrolidinomethylene)]methanaminium hexafluorophosphate (HDmPyOC, 18b): The product was obtained as a white solid, in a yield of 6.8 g (82.7%). M.p. 126–127°C (dec); ^{1}HNMR ([D$_6$]acetone): δ = 1.37 (t, 3H; CH_3), 2.10, 2.13 (m, 4H; $2CH_2$), 3.36 (s, 6H; $2CH_3$), 3.95–3.99(m, 4H; $2CH_2$), 4.48 ppm (q, 2H; CH_2); ^{13}C NMR ([D$_6$]acetone): δ = 13.47, 25.09, 40.40, 51.58, 64.52, 106.74, 134.82, 156.12, 158.66 ppm; elemental analysis (%) calculated for $C_{12}H_{19}F_6N_4O_3P$ (412.27): C 34.96, H 4.65, N 13.59; found: C 35.07, H 4.79, N 13.72.

1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate (COMU, 18c): The product was obtained as white crystals in a yield of 7.6 g (88.8%), and decomposes without melting at 159.90°C according to dynamic DSC (Figure 3). ^{1}H NMR ([D$_6$]acetone): δ = 1.38 (t, 3H; CH_3), 3.41 (s, 6H; $2CH_3$), 3.82 (t, 4H; $2CH_2$), 3.89 (t, 4H; $2CH_2$), 4.48 ppm (q, 2H; CH_2); ^{13}C NMR ([D$_6$]acetone): δ = 13.48, 40.70, 49.94, 64.59, 66.04, 106.76, 135.03, 156.14, 160.61 ppm; elemental analysis (%) calcd for $C_{12}H_{19}F_6N_4O_4P$ (428.27): C 33.65, H 4.47, N 13.08; found: C 33.79, H 4.59, N 13.30.

1-[(1-Cyano-2-ethoxy-2-oxoethylideneaminooxy)(morpholino)methylene]pyrrolidinium hexafluorophosphate (HMPyOC, 18d): The product was obtained as white solid in a yield of 8.2 g (90.3%). M.p. 171–172°C; ^{1}H NMR ([D$_6$]acetone): δ = 1.26 (t, 3H; CH_3), 1.98–2.02 (m, 4H; $2CH_2$), 3.65–3.68 (m, 4H; $2CH_2$), 3.74–3.76 (m, 4H; $2CH_2$), 3.84–3.87 (m, 4H; $2CH_2$), 4.37–4.40 ppm (q, 2H; CH_2); ^{13}C NMR ([D$_6$]acetone): δ = 13.49, 25.09, 49.20, 51.57, 55.98, 51.76, 64.54, 66.06, 106.69, 134.63, 156.11, 157.88 ppm; elemental analysis (%) calcd for $C_{14}H_{21}F_6N_4O_4P$ (454.31): C 37.01, H 4.66, N 12.33; found: C 37.25, H 4.78, N 12.50.

***O*-[(Dicyanomethylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate (HTODC, 18e)**: The product was obtained as a white solid in a yield of 5.0 g (74.0%). M.p. 180–181°C (dec); ^{1}H NMR ([D$_6$]acetone): δ = 3.27 (s, 12H; $4CH_3$) ppm; ^{13}C NMR ([D$_6$]acetone): δ = 40.80, 105.10, 108.21, 119.65, 160.67 ppm; elemental analysis (%) calcd for $C_8H_{12}F_6N_5OP$ (339.18): C 28.33, H 3.57, N 20.65; found: C 28.52, H 3.65, N 20.88.

1-{[1-(Dicyanomethylideneaminooxy)-dimethylamino-pyrrolodinomethylene]}methanaminium hexafluorophosphate (HDmPyODC, 18f): The product was obtained as a light yellow solid, in a yield of 5.4 g (74%). M.p. 146–147°C (dec). ^{1}H NMR ([D$_6$]acetone): δ = 1.97–2.00 (m, 4H; $2CH_2$), 3.28 (s, 6H; $2CH_3$), 3.96–4.04 (m, 4H; $2CH_2$) ppm. ^{13}C NMR ([D$_6$]acetone): δ = 25.07, 40.41, 51.77, 105.06, 108.23, 119.30, 157.86 ppm; elemental analysis (%) calculated for $C_{10}H_{14}F_6N_5OP$ (365.22): C 32.89, H 3.86, N 19.18; found: C 33.12, H 3.99, N 19.51.

1-{[1-(Dicyanomethyleneaminooxy)-dimethylamino-morpholinomethylene]}methanaminium hexafluorophosphate (HDMODC, 18g): The product was obtained as white solid in a yield of 5.7 g (74.8%). M.p. 118–119°C; ^{1}H NMR ([D$_6$]acetone): δ = 3.42 (s, 6H; $2CH_3$), 3.80–3.88 ppm (m, 8H; $4CH_2$); ^{13}C NMR ([D$_6$]acetone): δ = 40.97, 49.93, 65.92, 105.13, 108.15, 119.84, 159.77 ppm; elemental analysis (%) calcd for $C_{10}H_{14}F_6N_5O_2P$ (381.21): C 31.51, H 3.70, N 18.37; found: C 31.68, H 3.84, N 18.61.

1-[(Dicyanomethyleneaminooxy)(morpholino)methylene]pyrrolidinium hexafluorophosphate (HMPyODC, 18h): The product was obtained as light yellow solid in a yield of 6.4 g (78.6%). M.p. 158–159°C; ^{1}H NMR ([D$_6$]acetone): δ = 2.11–2.14 (m, 4H; $2CH_2$), 3.79–3.85 (m, 4H; $2CH_2$), 3.86–3.88 (m, 4H; $2CH_2$), 3.98–4.01 ppm (m, 4H; $2CH_2$); ^{13}C NMR ([D$_6$]acetone): δ = 25.28, 49.15, 65.95, 105.07, 108.17, 119.48, 157.00 ppm; elemental analysis (%) calcd for $C_{12}H_{16}F_6N_5O_2P$ (407.25): C 35.39, H 3.96, N 17.20; found: C 35.60, H 4.06, N 17.56.

***O*-[(Diethoxycarbonylmethylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate (HTODeC, 18i)**: The product was obtained as a pale yellow oil in a yield of 84.5%. ^{1}H NMR ($CDCl_3$): δ = 1.35 (t, 6H; $2CH_3$), 3.18 (s, 12H; $4CH_3$), 4.39–4.48 ppm (q, 4H; $2CH_2$); elemental analysis (%) calcd for $C_{12}H_{22}F_6N_3O_5P$ (433.28): C 33.26, H 5.12, N 9.70; found: C 33.44, H 5.25, N 9.98.

1-[(1,3-Diethoxy-1,3-dioxoprop-2-ylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate (HDMODeC, 18j): The product was obtained as pale yellow oil in a yield of 84.3%. ^{1}H NMR ($CDCl_3$): δ = 1.35 (t, 6H; $2CH_3$), 3.21(s, 6H; $2CH_3$), 3.35 (t, 2H; CH_2), 3.61 (t, 2H; CH_2), 3.78 (t, 2H; CH_2), 3.87 (t, 2H; CH_2), 4.37–4.50 ppm (q, 4H; $2CH_2$); elemental analysis (%) calcd for $C_{14}H_{24}F_6N_3O_6P$ (475.32): C 35.38, H 5.09, N 8.84; found: C 35.53, H 5.28, N 9.11.

***N*-{[Cyano(pyridin-2-yl)methyleneaminooxy](dimethylamino)methylene}-*N*-methylmethanaminium hexafluorophosphate (HTOPC, 18k)**: The product was obtained as a light reddish brown solid in a yield of 6.2 g (82.7%). M.p. 169–171°C; ^{1}H NMR ([D$_6$]acetone): δ = 3.30 (s, 12H; $4CH_3$), 7.57–7.61 (m, 1H), 7.94 (td, 1H), 8.10 (dd, 1H), 8.70 ppm (dd, 1H); ^{13}C NMR ([D$_6$]acetone): δ = 40.48, 107.56, 122.52, 127.87, 138.18, 142.46, 146.67, 150.68, 162.03 ppm; elemental analysis (%) calcd for $C_{12}H_{16}F_6N_5OP$ (391.25): C 36.84, H 4.12, N 17.90; found: C 37.00, H 4.21, N 18.11.

***N*-{[Cyano(pyridin-2-yl)methyleneaminooxy](dimethylamino)methylene}-*N*-morpholinomethanaminium hexafluorophosphate (HDMOPC, 18l)**: The product was obtained as a light brown solid in a yield of 7.74 g (89.4%). M.p. 154–155°C; ^{1}H NMR ([D$_6$]acetone): δ = 3.34 (s, 12H; $4CH_3$), 3.79–3.83 (m, 8H; $4CH_2$), 7.58–7.62 (m, 1H), 7.96 (td, 1H), 8.12 (dd, 1H), 8.71 ppm (dd, 1H); ^{13}C NMR ([D$_6$]acetone): δ = 40.76, 49.79, 66.13, 107.60, 122.53, 127.92, 138.23, 142.74, 146.63, 150.69, 161.06 ppm; elemental analysis (%) calcd for $C_{14}H_{18}F_6N_5O_2P$ (433.29): C 38.81, H 4.19, N 16.16; found: C 39.04, H 4.33, N 16.47.

1-[Morpholino(pyrrolidinium-1-ylidene)methyl]-1*H*-[1.2.3]triazolo[4,5-I]pyridine 3-oxide hexafluorophosphate (HMPyA, 19): The product was obtained as a white solid in a yield of 7.3 g (81.3%). M.p. 206–208°C (dec); ^{1}H NMR ([D$_6$]acetone): δ = 2.11–2.15 (m, 2H; CH_2), 2.18–2.30 (m, 2H; CH_2), 3.48–3.63 (m, 2H; CH_2), 3.79–4.16 (m, 10H; $5CH_2$), 8.02 (dd, 1H), 8.53 (dd, 1H), 8.85 ppm (dd, 1H); ^{13}C NMR ([D$_6$]acetone): δ = 25.09, 41.74, 42.35, 50.82, 51.58, 66.19, 66.42, 124.37, 127.84, 149.65 ppm; elemental analysis (%) calcd for $C_{14}H_{19}F_6N_6O_2P$ (448.30): C 37.51, H 4.27, N 18.75; found: C 37.75, H 4.42, N 19.02.

1-[Morpholino(pyrrolidinium-1-ylidene)methyl]-1*H*-benzo[*d*]-[1.2.3]triazole 3-oxide hexafluorophosphate (HMPyB, 20): The product was obtained as a white solid in a yield of 7.4 g (82.8%). M.p. 203–204°C (dec); ^{1}H NMR ([D$_6$]acetone): δ = 2.12–2.15 (m, 2H; CH_2), 2.16–2.29 (m, 2H; CH_2), 3.42–3.58 (m, 2H; CH_2), 3.80–4.26 (m, 10H; $5CH_2$), 7.75 (td, 1H), 7.97–8.02 (m, 2H), 8.07 ppm (d, 1H); ^{13}C NMR ([D$_6$]acetone): δ = 25.09, 41.76, 42.23, 50.81, 51.51, 66.19, 66.41, 109.99, 114.56, 127.52, 143.54 ppm. elemental analysis (%) calcd for $C_{15}H_{20}F_6N_5O_2P$ (447.32): C 40.28, H 4.51, N 15.66; found: C 40.45, H 4.66, N 15.89.

5-Chloro-1-[morpholino(pyrrolidinium-1-ylidene)methyl]-1*H*-benzo[*d*]-[1.2.3]triazole 3-oxide hexafluorophosphate (HMPyC, 21): The product was obtained as a white solid in a yield of 7.96 g (82.7%). M.p. 217–218°C (dec); ^{1}H NMR ([D$_6$]acetone): δ = 2.10–2.15 (m, 2H; CH_2), 2.19–2.29 (m, 2H; CH_2), 3.45–3.65 (m, 2H; CH_2), 3.81–4.09 (m, 10H; 5CH_2), 7.99 (d, 1H), 8.02 (d, 1H), 8.12 ppm (dd, 1H); ^{13}C NMR ([D$_6$]acetone): δ = 25.93, 52.89, 53.36, 66.09, 66.42, 115.79, 115.84, 132.57, 132.62, 134.06, 134.12, 147.38 ppm; elemental analysis (%) calculated for $C_{15}H_{19}ClF_6N_5O_2P$ (481.76): C 37.40, H 3.98, N 14.54; found: C 37.65, H 4.13, N 14.79.

Fmoc-Val-Val-NH$_2$ in solution phase: An authentic sample was prepared as follows: Tetramethylfluoroformamidiniun hexafluorophosphate (TFFH, 1 mmol) was added at 0°C to a mixture of Fmoc-Val-OH (1 mmol), and DIEA (1 mmol) in DMF (5 mL) and the reaction mixture was activated for 5 min, followed by the addition of H-Val-NH$_2$ (1 mmol) and DIEA (1 mmol). The reaction mixture was stirred at 0°C for 1 h and at RT for 3 h. Water (50 mL) was added and the precipitate was collected and washed with sat. Na_2CO_3 and HCl (1 N), and was then dried in air and recrystallized from ethyl acetate/hexane to give a white solid in a yield of 87.0%. M.p. 226–228°C. ^{1}H NMR ([D$_6$]DMSO): δ = 0.95 (t, 12H; 4CH_3), 1.80–2.00 (m, 2H; 2CH), 3.87 (t, 1H; CH), 4.10 (t, 1H; CH), 4.18–4.20 (m, 3H; CH, CH_2), 7.02 (t, 2H; NH), 7.28–7.85 ppm (m, 12, ar, NH).

This sample was used as an authentic sample and injected into the HPLC (Waters 2487) under the following conditions: linear gradient of 10 to 90% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm. Flow rate = 1 mL min^{-1}. Column: Waters C_{18}, 5 μm, 4.6×150 mm (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler). t_R = 20.89 min (Fmoc-Val-OH), t_R = 19.88 min (Fmoc-Val-Val-NH$_2$). This reaction was repeated under several sets of coupling conditions and samples (10 μL) were taken at different intervals. In addition, the reaction was quenched by addition of acetonitrile/water (1:1, 2 mL), and a sample of the solution (10 μL) was then injected into the HPLC system under the same conditions as described above.

Z-Aib-Val-OMe in solution phase: An authentic sample was prepared as follows: TFFH (0.264 g, 1 mmol) was added at 0°C to a mixture of Z-Aib-OH (0.237 g, 1 mmol), and DIEA (0.174 mL, 1 mmol) in DMF (5 mL) and the reaction mixture was activated for 8 min, followed by addition of H-ValO-Me·HCl (0.169 g, 1 mmol) and DIEA (2 mmol). The reaction mixture was stirred at 0°C for 1 h and at RT for 3 h. Water (50 mL) was added, and the precipitate was collected and washed with sat. Na_2CO_3 and HCl (1 N), and was then dried in air and recrystallized from dichloromethane/hexane (white needles after two days at room temperature) to give a white solid in 87.0% yield. M.p. 76–77°C; ^{1}H NMR ([D$_6$]DMSO): δ = 0.85 (t, 6H; 2CH_3), 1.56 (d, 6H; 2CH_3), 2.13–2.18 (m, 1H; CH), 3.72 (s, 3H; OCH_3), 4.49–4.53 (m, 1H; CH), 5.09 (s, 2H; CH_2), 5.36 (brs, 1H; NH), 7.28–7.37 (m, 6H; ar, NH) ppm. This sample was used as an authentic sample and injected into the HPLC (Waters 2487) under the following conditions: linear gradient of 10 to 90% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min, detection at 200 nm. Flow rate = 1 mL min^{-1}. Column: Waters C_{18}, 5 μm, 4.6×150 mm. (Waters Dual Wavelength Detector and Waters 717 Plus auto sampler). t_R (Z-Aib-OH) = 18.25 min, t_R (Z-Aib-Val-OMe) = 20.90 min, t_R (Z-Aib-OAt) = 22.30 min, t_R (Z-Aib-OBt) = 22.80 min, t_R (Z-Aib-Oxyma) = 23.90 min.

Model segment coupling reaction: Test couplings were carried out as previously described for Z-Phg-Pro-NH$_2$[7] and Z-Phe-Val-Pro-NH$_2$.[7]

Synthesis of Tyr-Aib-Aib-Phe-Leu-NH$_2$ on solid phase:[6d] Fmoc-Rink Amide-PS (100 mg, 0.7 mmol g^{-1}) was deblocked with piperidine in DMF (20%, 10 mL) for 10 min and washed with DMF (2×10 mL), CH_2Cl_2 (2×10 mL), and then DMF (2×10 mL). Fmoc-Leu-OH (3 equiv), coupling reagent (3 equiv), and DIEA (6 equiv) were mixed in DMF (0.5 mL) for activation (1–2 min) and then added to the resin. The reaction mixture was then stirred slowly for 1 min and allowed to couple for 30 min (1 h, double coupling only for the Aib-Aib). The resin was washed with DMF, and then deblocked with piperidine in DMF (20%) for 7 min. It was then again washed with DMF, CH_2Cl_2, and DMF and coupled with the next amino acid. Coupling and deblocking steps were repeated to provide the pentapeptide. The crude product was analyzed by HPLC with a linear gradient of 20 to 80% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 30 min on a Symmetry Waters C_{18} column (4.6×150 mm, 4 μm), with detection at 220 nm, flow rate 1.0 mL min^{-1}, t_R (penta) = 8.82 min, t_R (des-Aib) = 9.10 min.

Synthesis of H-Tyr-MeLeu-MeLeu-Phe-Leu-NH$_2$ on solid phase: Tripeptide H-MeLeu-Phe-Leu-NH$_2$ was manually assembled on Fmoc-RinkAmide-Aminomethyl-PS-resin (0.63 mmol g^{-1}), after Fmoc removal with piperidine in DMF (20%, 2×5 min). The resin was washed with DMF (×10), CH_2Cl_2 (×10), and DMF (×10). Residues were introduced after 30 min coupling, with preactivation of Fmoc-amino acids (3 equiv) with Oxyma (3 equiv) and DIC (3 equiv) in DMF for 1.5 min. Quantitative incorporation was checked at each step by use of the Kaiser test for primary amines. Sample cleavage (10 mg) with TFA/H_2O (9:1) confirmed the tripeptide in >99.5% purity, as analyzed by reversed-phase HPLC and ESI-MS ([M+H]$^+$ = 405.32). The two last residues, Tyr and MeLeu, were introduced by preparation of 0.3 M solutions of coupling reagent (3 equiv) and Fmoc-amino acid (3 equiv) in standard or treated DMF, and preactivation of the mixture with DIEA (3 or 6 equiv) for 20–30 s. The peptide chain was cleaved from the resin with TFA/H_2O (9:1) over 2 h at room temperature. The solution was filtered and the resin was washed with CH_2Cl_2 (1 mL×2), which was removed together with TFA under nitrogen flow. The crude peptide was purified with cold Et_2O (2 mL×3) and after lyophilization, purity was checked on reversed-phase HPLC, with use of a Waters SunFire C18 Column (3.5 μm, 4.6×100 mm), with a 20% to 50% linear gradient of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min, with detection at 220 nm. The t_R of the pentapeptide was 6.5 min, whereas the t_R values of des-MeLeu, des-Tyr, and tripeptide H-MeLeu-Phe-Leu-NH$_2$ were 5.0, 5.7, and 3.0 min respectively.

Synthesis of ACP (65–74) (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH$_2$):[6d] The peptide was elongated manually on an Fmoc-Rink Amide-AM-resin (0.7 mmol g^{-1}). Coupling times of 2 min were used, and excesses of reagents were 2 equiv for Fmoc-amino acids and coupling reagents and 4 equiv for DIEA. Incorporation was detected for Ile^{72} onto Asn and for Ile^{69} onto Asp. Peptide purity was determined by reversed-phase HPLC analysis (Symmetry Waters C_{18} (4.6×150 mm, 4 μm), linear gradient over 30 min of 10 to 90% CH_3CN in H_2O/0.1% TFA, flow rate 1.0 mL min^{-1}, t_R decapeptide = 10.43 min, t_R des-Asn = 10.8 min, t_R des-Ile^{72} = 7.5 min, t_R des-Ile^{69} = 9.1 min, t_R des-Val = 8.43 min), after cleavage of the peptide from the resin by treatment with TFA/H_2O (9:1) for 2 h at room temperature.

General Procedure for dynamic differential scanning calorimetry assays: The thermal behavior of HDMA (**7b**), HDMB (**8**), and COMU (**18c**) was tested. Samples (1 mg) were heated from 30°C to 300°C at a rate of 10°C min^{-1} in a closed high-pressure crucible with N_2 flow in a Mettler–Toledo DSC-30 differential scanning calorimeter. Diagrams showing heat flow as a function of temperature and time were obtained.

General Procedure for isothermal differential scanning calorimetry assays: The autocatalytic natures of HDMA (**7b**), HDMB (**8**), and COMU (**18c**) was tested. Samples (1 mg) were heated to 10°C below the onset temperatures detected in the dynamic DSC [HDMA (**7**): 167°C, HDMB (**8**): 170°C, and COMU (**17c**): 150°C] for 480 min in a closed high-pressure crucible with N_2 flow in a Mettler–Toledo DSC-30 differential scanning calorimeter. Diagrams showing heat flow as a function of time were obtained.

General Procedure for ARC experiments: Assays were carried out on an Accelerating Rate Calorimeter (ARC) from Thermal Hazard Technology, with use of ARCTC-HC-MCQ (Hastelloy) test cells. Samples [1.837 g of HDMB (**8**), 1.605 of HDMA (**7**), and 2.350 g of COMU (**18c**)] were introduced into the calorimetric test cell at room temperature, without stirring. The cell was heated at the initial temperature (30°C) and the "heat-wait-seek" method was applied. This procedure consisted of heating the sample by 5°C and, after 15 min of equilibrium, measuring whether self-heating was occurring at a rate higher than 0.02°C min^{-1} (default sensitivity threshold). When self-heating was detected, the system was changed to adiabatic mode. After decomposition, the assay

was stopped when temperature rose above 300 °C. The phi factors[27] were: 2.02371 (HDMA), 2.76093 (HDMB), and 2.40644 (COMU).

Acknowledgements

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[1] Abbreviations not defined in text: Aib, α-aminoisobutyric acid; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; HOAt, 7-aza-1-hydroxybenzotriazole; N-HATU, N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene)-*N*-methylmethanaminium hexafluorophosphate N-oxide; N-HBTU, N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]N-methylmethanaminium hexafluorophosphate N-oxide; DMCH,4-((dimethyamino)chloromethylene)morpholin-4-Iminium hexaflurophosphate HDMA, 1-((dimethylimino)morpholino > methyl)3-*H*-[1,2,3]triazolo[4,5-*b*]pyridine-1–3-olate hexafluorophosphate; HDMB, 1-((dimethylimino)morpholino > methyl)3-*H*-benzo-[1,2,3]triazolo-1-ium-3-olate hexafluorophosphate; 6-HDMCB, 1-((dimethylimino)morpholino > methyl)3-*H*-6-chlorobenzo[1,2,3] triazolo-1-ium-3-olate hexafluorophosphate; DMU, *N,N*-dimethylmorpholine-4-carboxamide; DmPyU, *N,N*-Dimethylpiperidine-1-carboxamide; MPyU, Morpholino(pyrrolidin-1-yl)methanone; *N*-DmPyCH, (Chloro(pyrrolidin-1-yl)methylene)-*N*-methylmethanaminium hexafluorophosphate; MPyCH, 1-(chloro(morpholino)methylene)pyrrolidinium Hexafluorophosphate; HOTU, O-[Cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate; HdmPyOC, 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate; COMU, 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate; HMPyOC, 1-((1-cyano-2-ethoxy-2-oxoethylideneaminooxy)(morpholino)methylene) pyrrolidinium Hexafluorophosphate; HTODC, O-[(Dicyanomethylidene)-amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate; HDmPyODC, 1-[(1-(dicyanomethylideneaminoxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate; HDMODC, 1-[(1-(dicyanomethyleneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate HMPyODC, 1-((dicyanomethyleneaminooxy)morpholinomethylene)pyrrolidinium Hexafluorophosphoate; HTODeC, O-[(diethoxycarbonylmethylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate; HDMODeC, 1-[(1,3-diethyoxy-1,3-dioxopropan-2-ylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate; HTOPC, *N*-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino)methylene)-*N*-Methylmethanaminium Hexafluorophosphate; HDMOPC, *N*-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino)methylene)-*N*-morpholinomethanaminium Hexafluorophosphate; HMPyA, 1-(morpholino-(pyrrolidinium-1-ylidene)methyl)-*1H*-[1,2,3]triazolo[4,5-I]pyridine 3-oxide Hexafluorophospahte; HMPyB, 1-(morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-benzo[*d*][1,2,3]triazole 3-oxide Hexafluorophosphate; HMPyC, 5-chloro-1-(morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-benzo[*d*][1,2,3]triazole 3-oxide hexafluorophosphate; Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.* **247**, 977 (1972)].

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COMU, a Safer and More Effective Replacement for Benzotriazole-based Uronium Coupling Reagents

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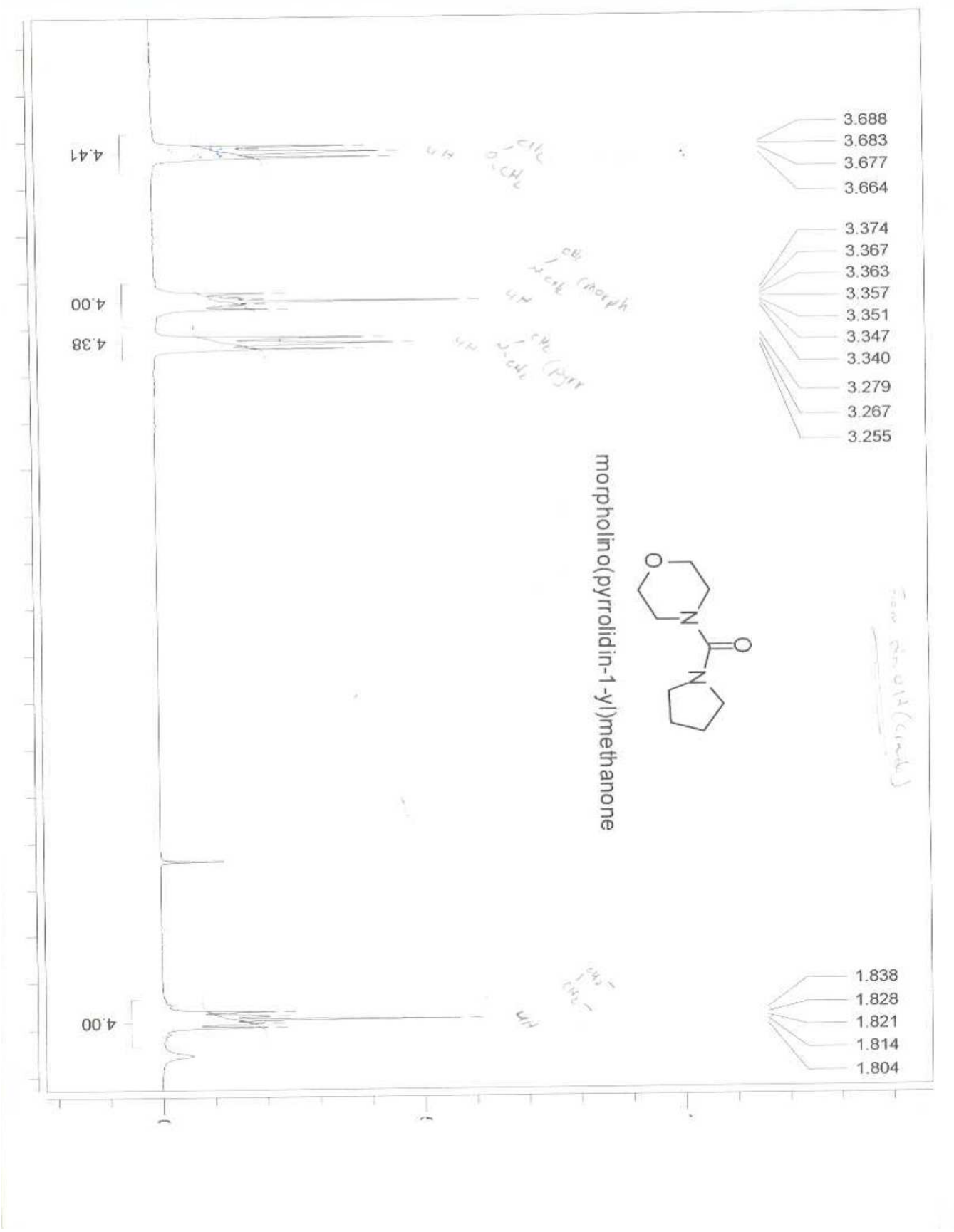
Morpholino(pyrrolidin-1-yl)methanone (MPyU, 14c, ^{1}H-NMR)



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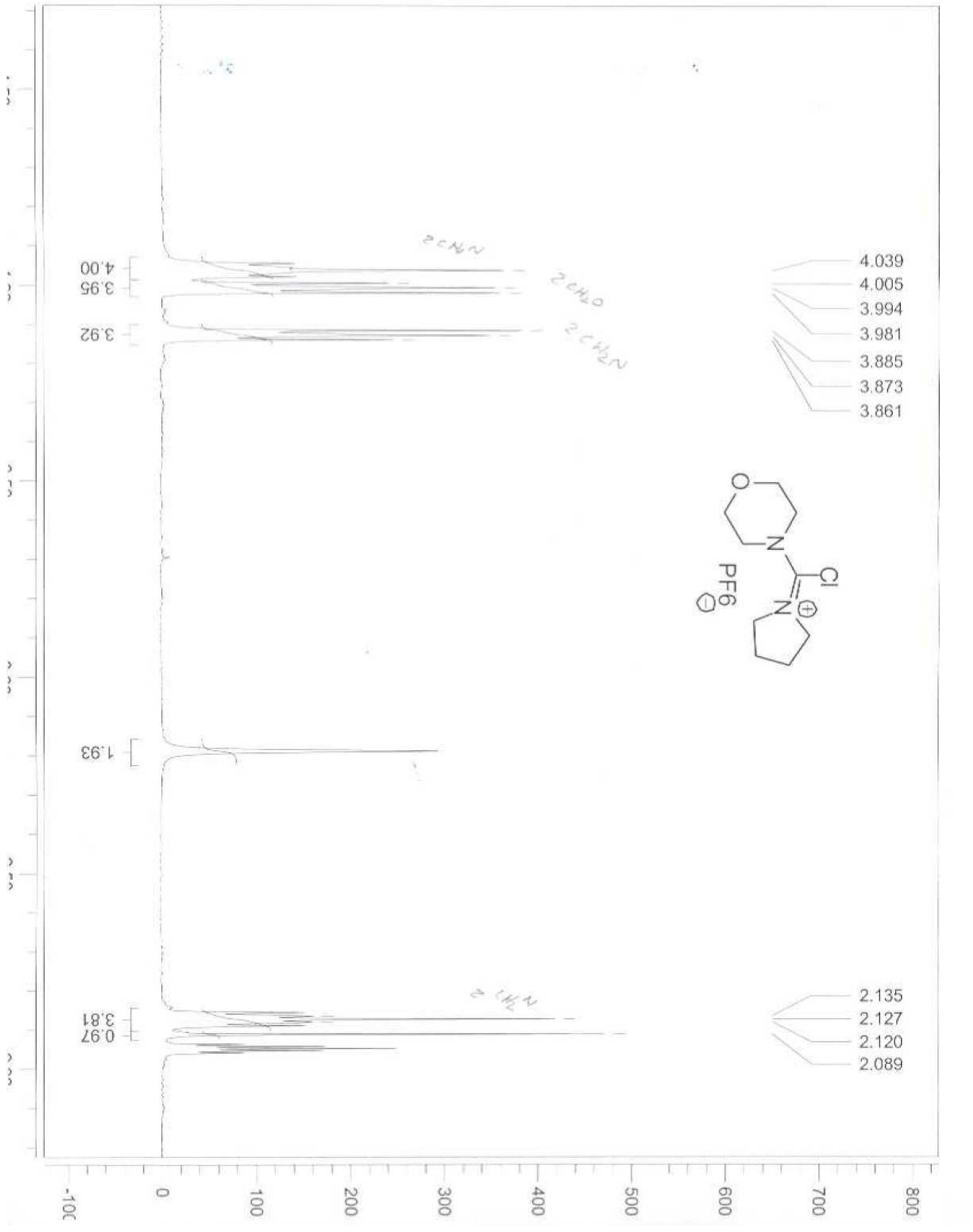

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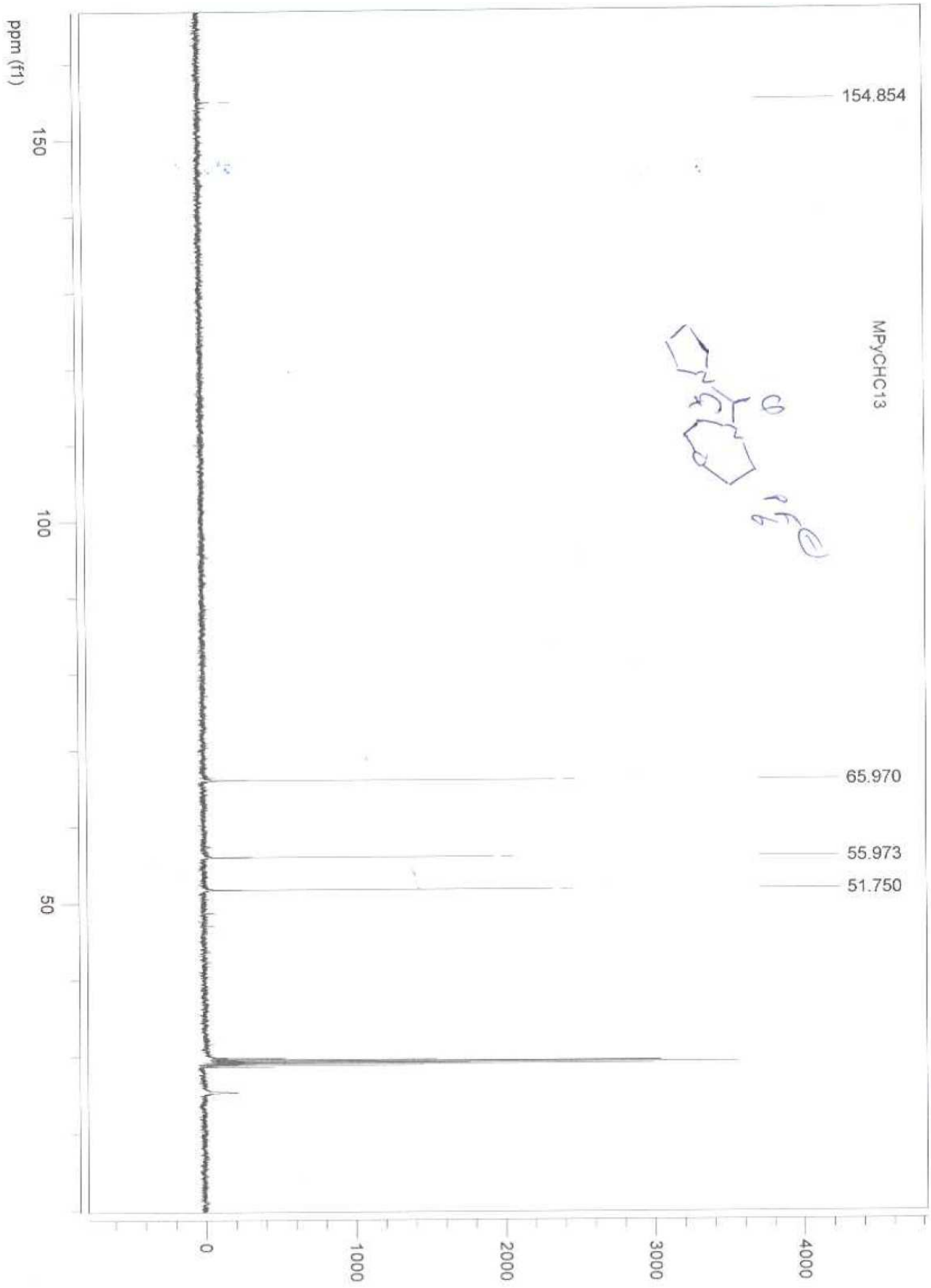
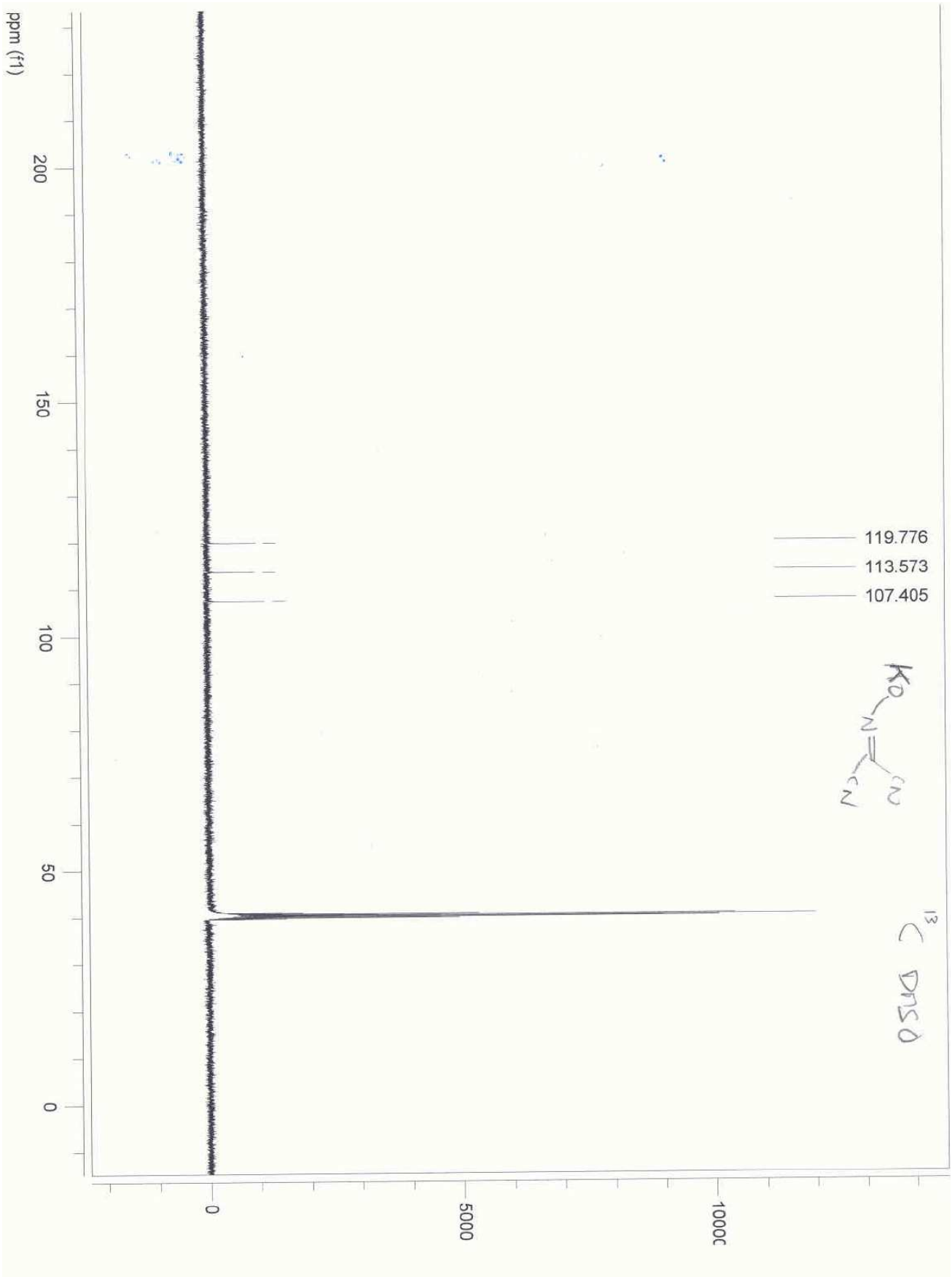
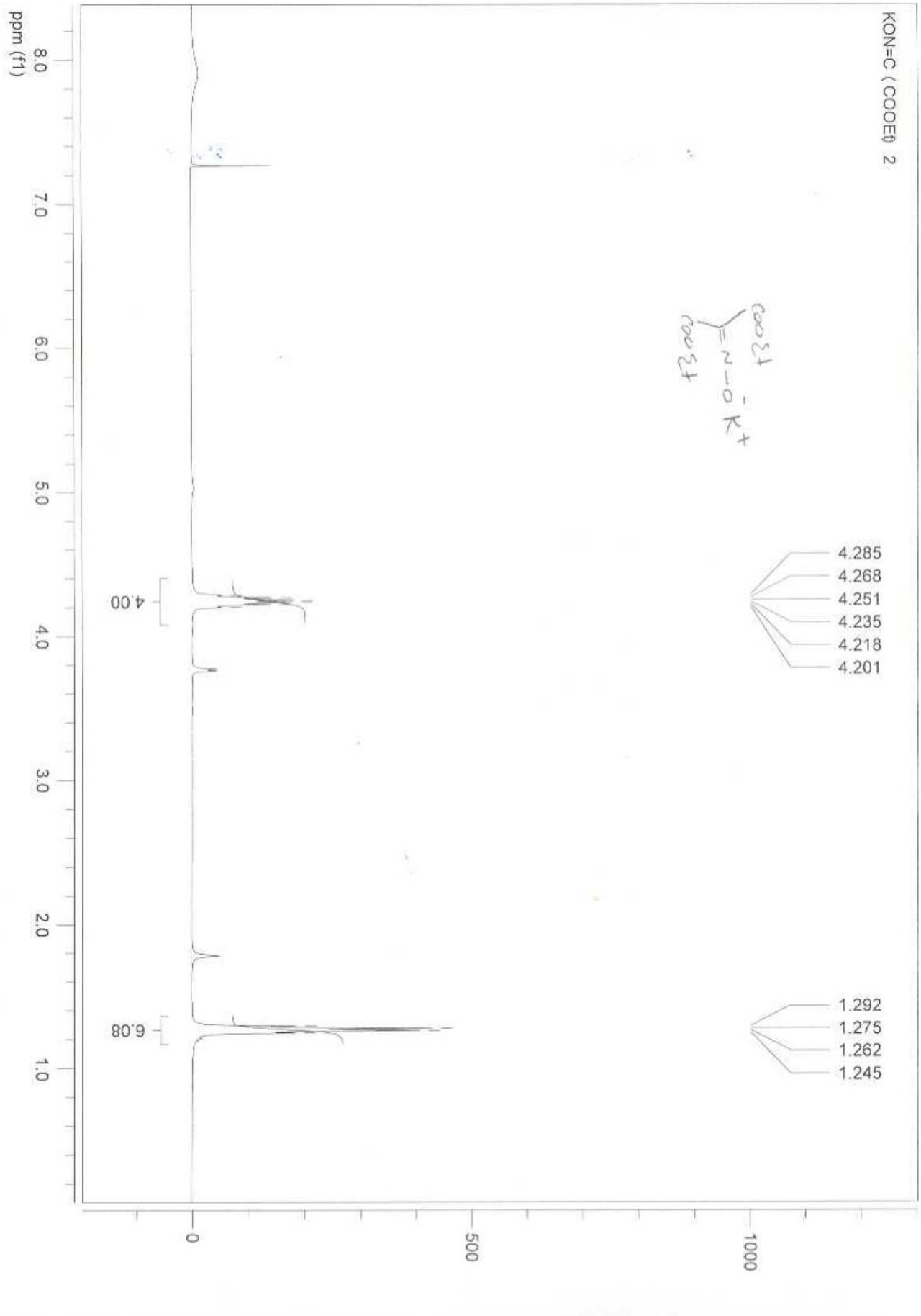


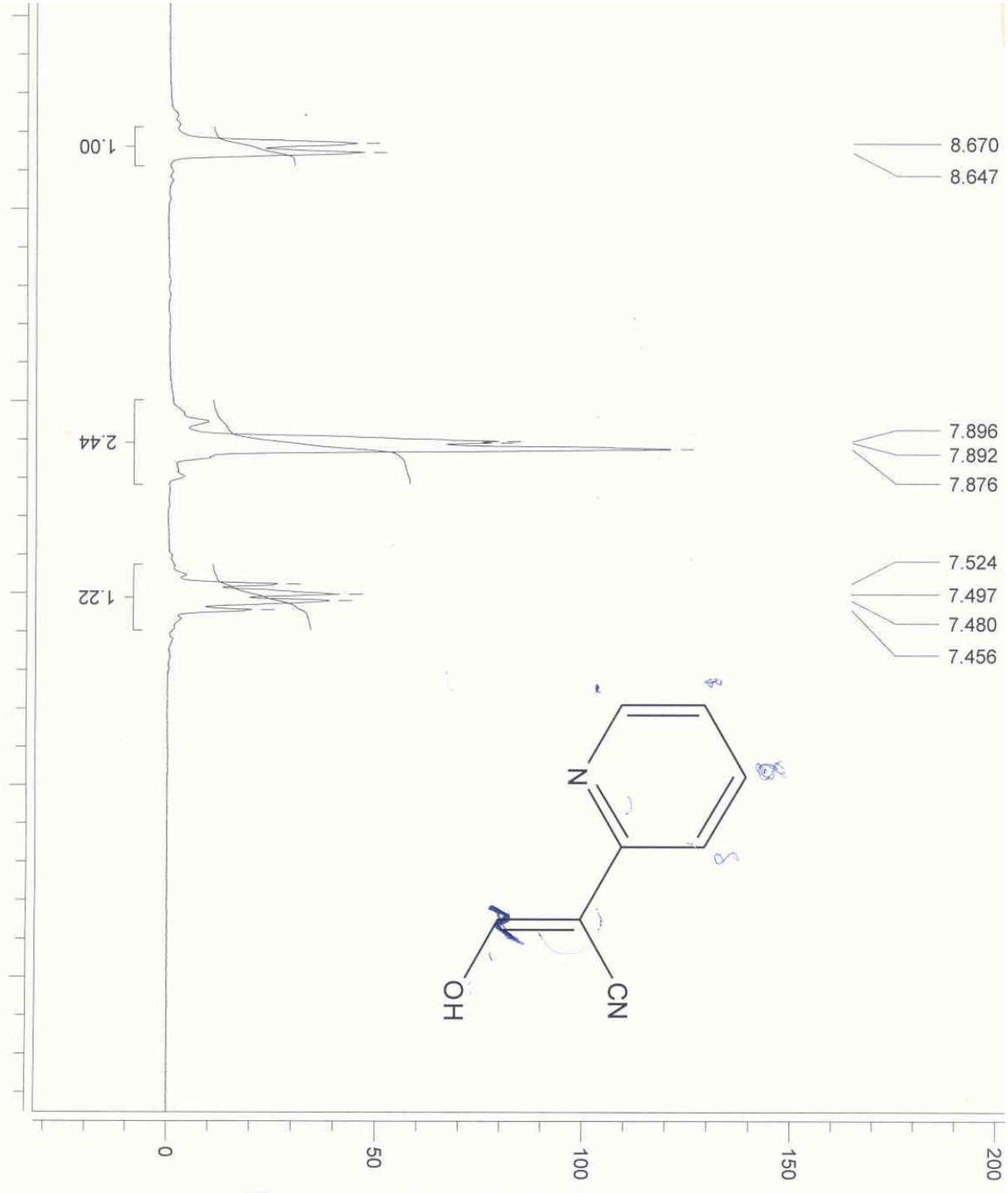
Potassium Salt of Hydroxycarbonimidoyl dicyanide (17a, ^{13}C-NMR)



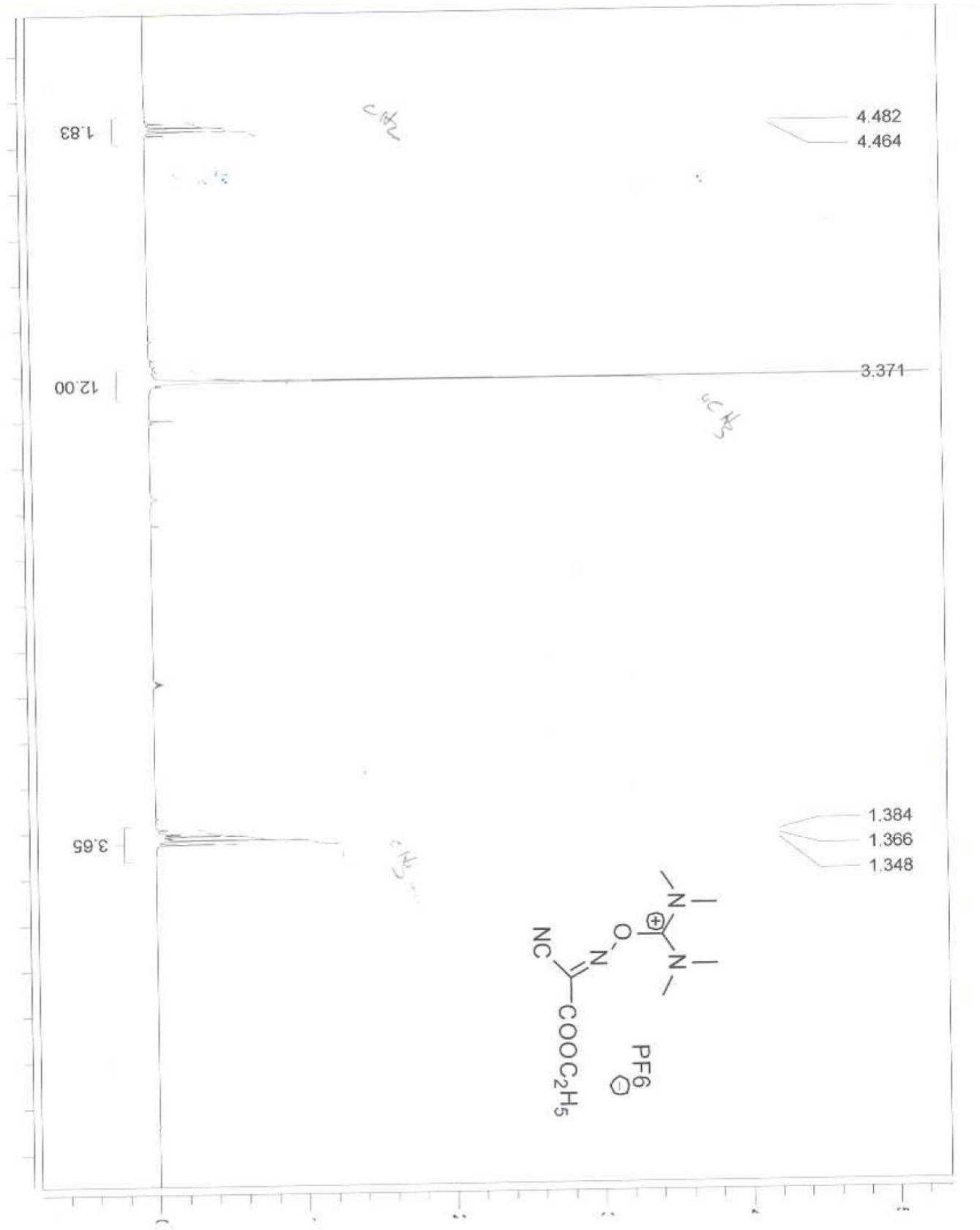
Potassium Salt of Diethyl 2-(hydroxyimino)malonate (17c, ^{1}H-NMR)



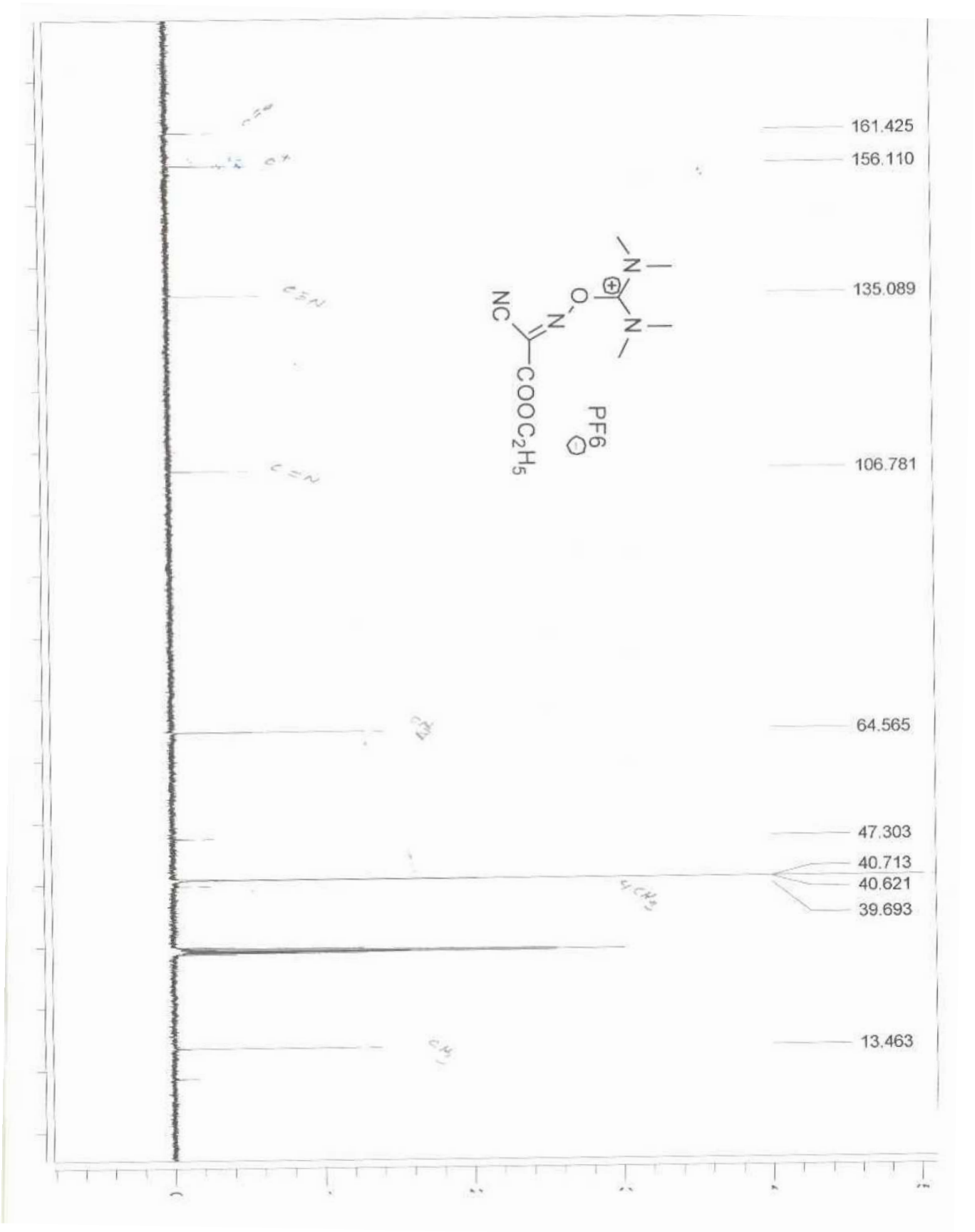
2-Pyridylhydroxyiminoacetonitrile (17d, ^{1}H-NMR)



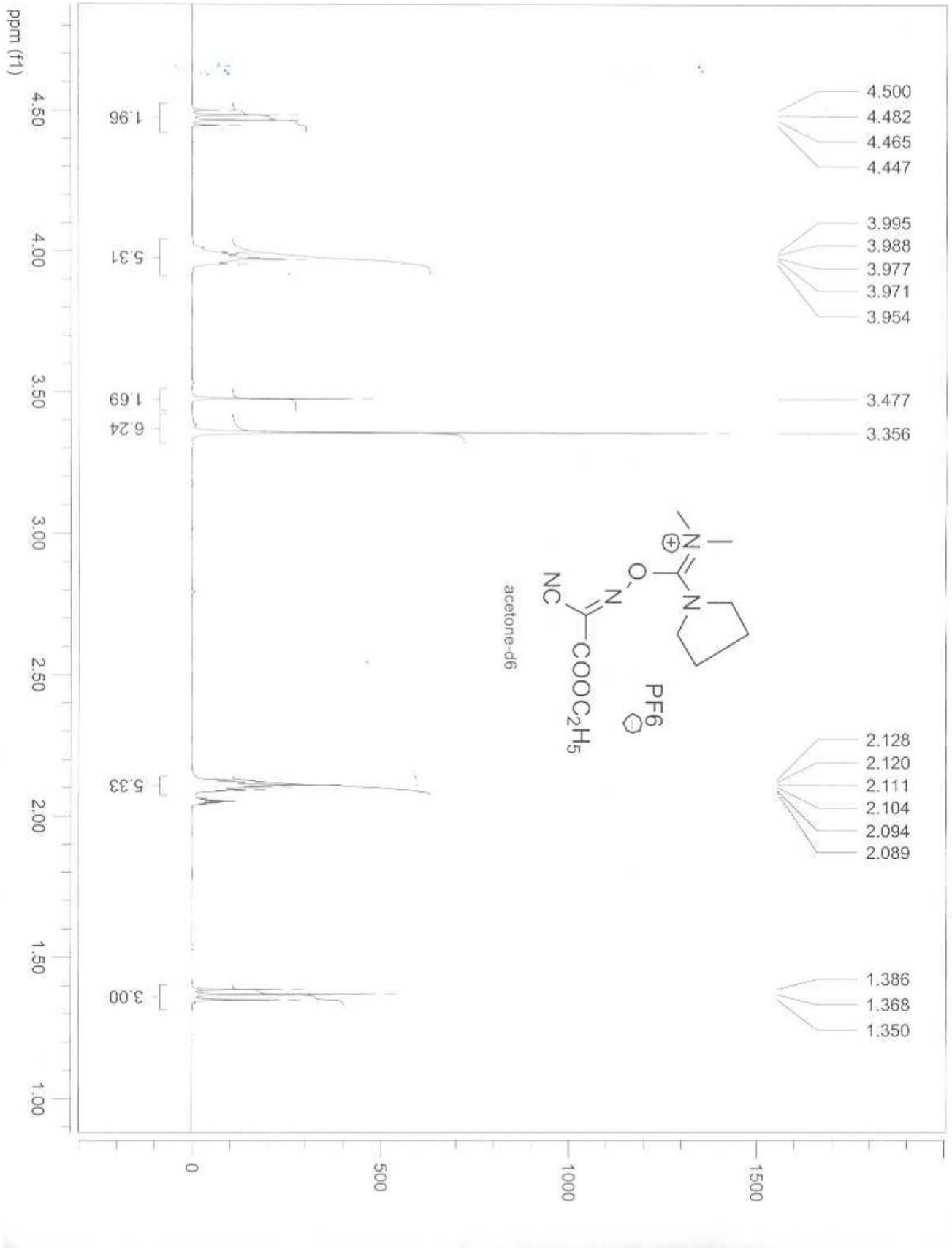
O-[Cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate (HOTU, 18a, ^{1}H-NMR)



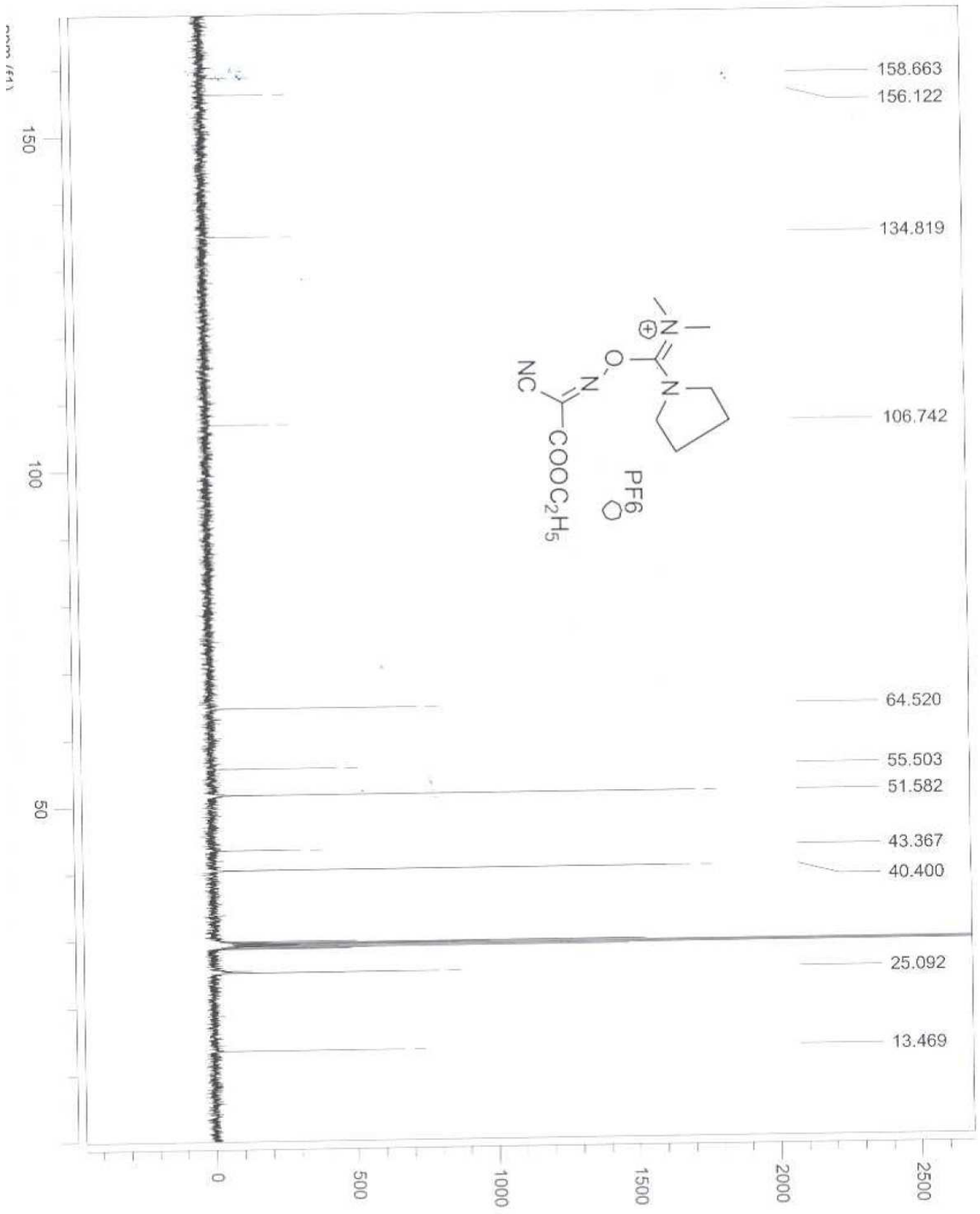
O-[Cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate (HOTU, 18a, ^{13}C-NMR)



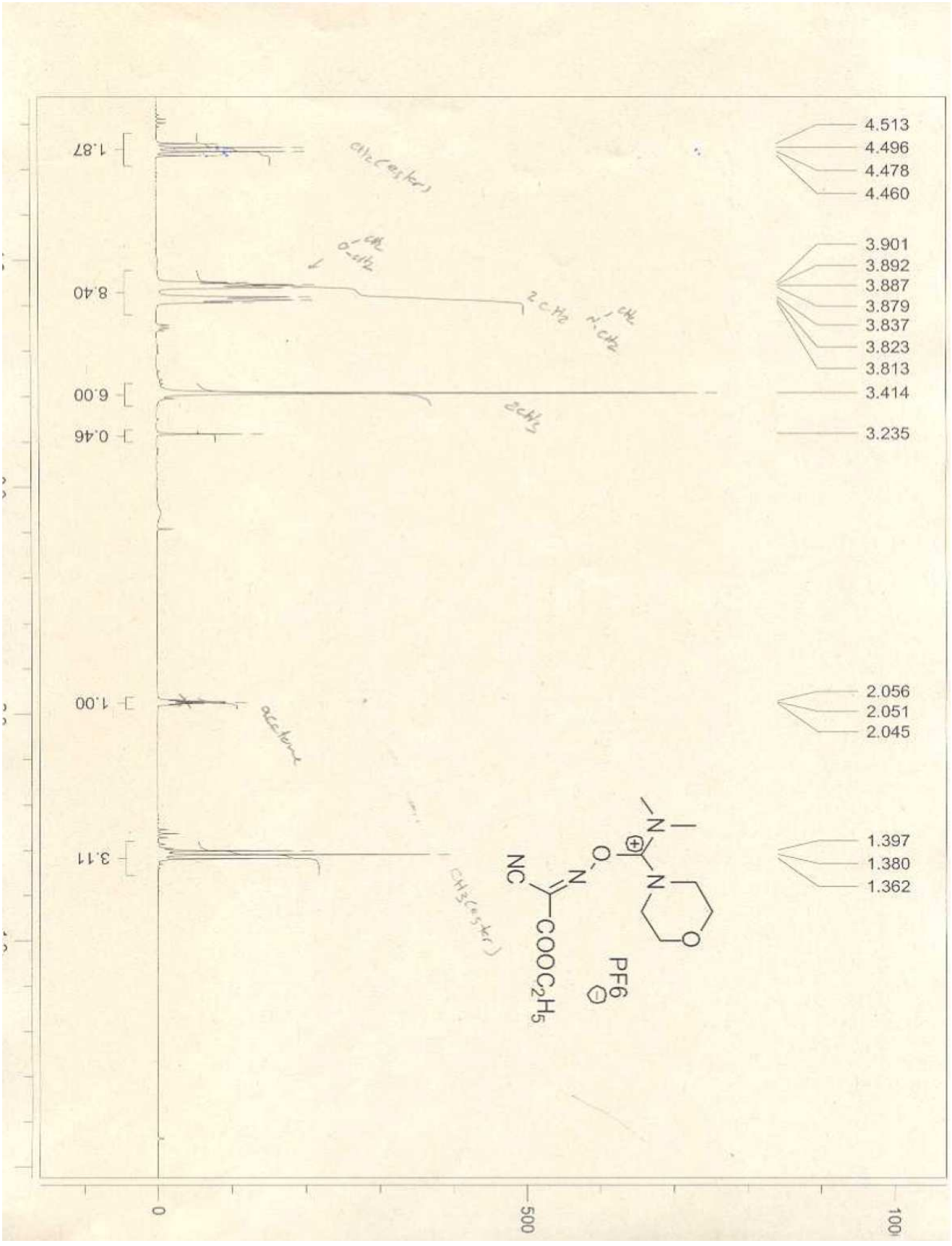
1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate (HDmPyOC, 18b, ^{1}H-NMR)



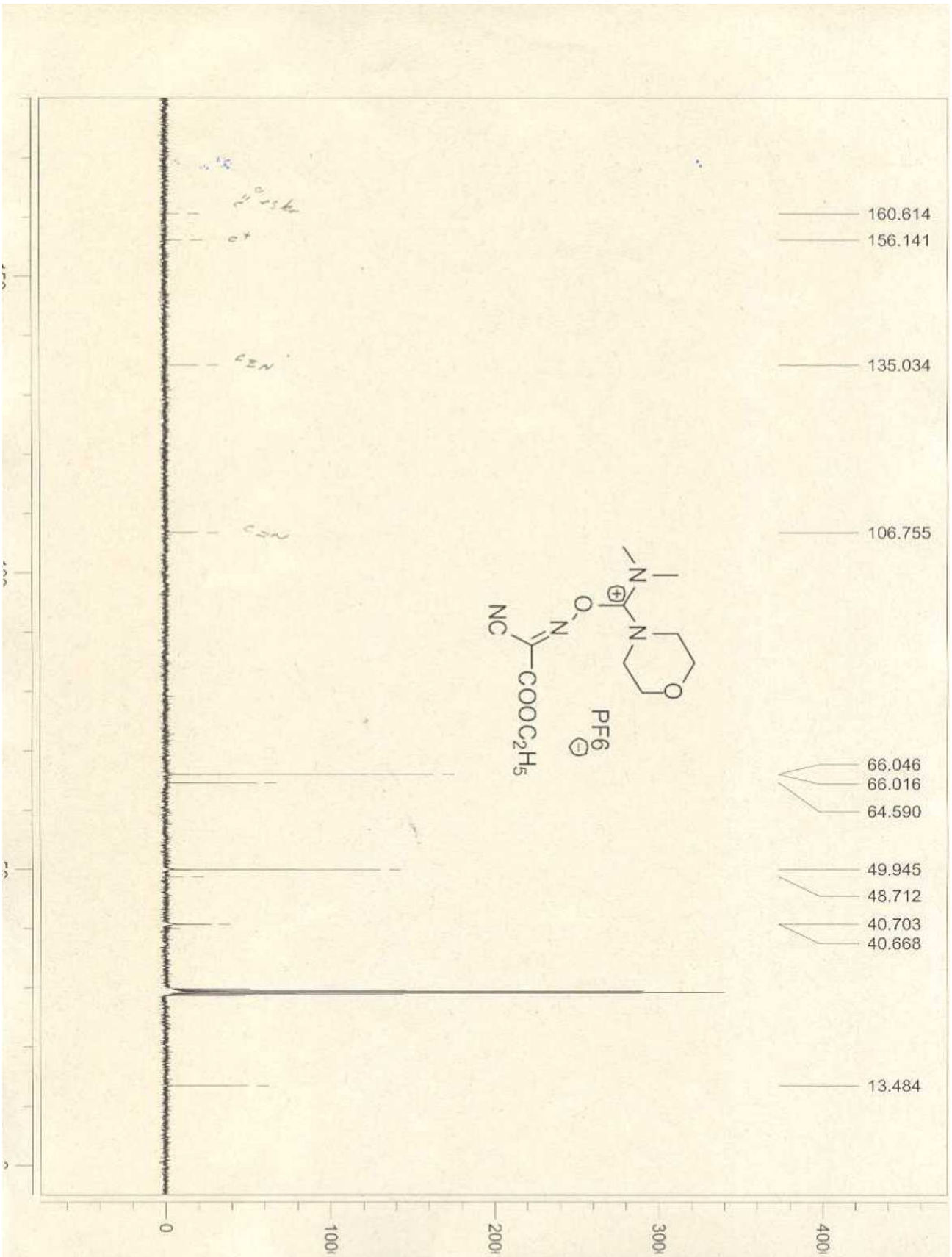
1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate (HDmPyOC, 18b, ^{13}C-NMR)



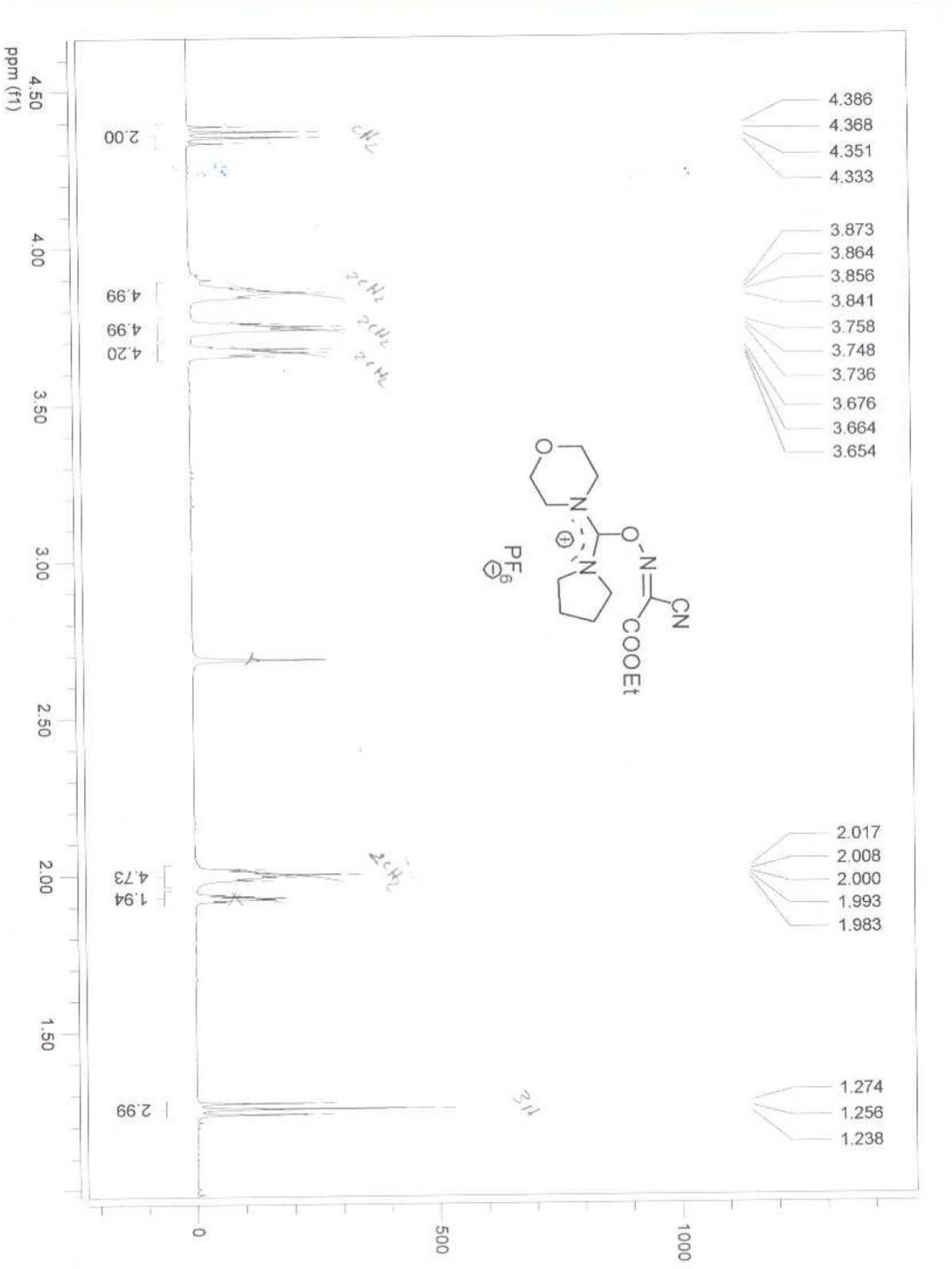
-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate (COMU, 18c, ^{1}H-NMR)



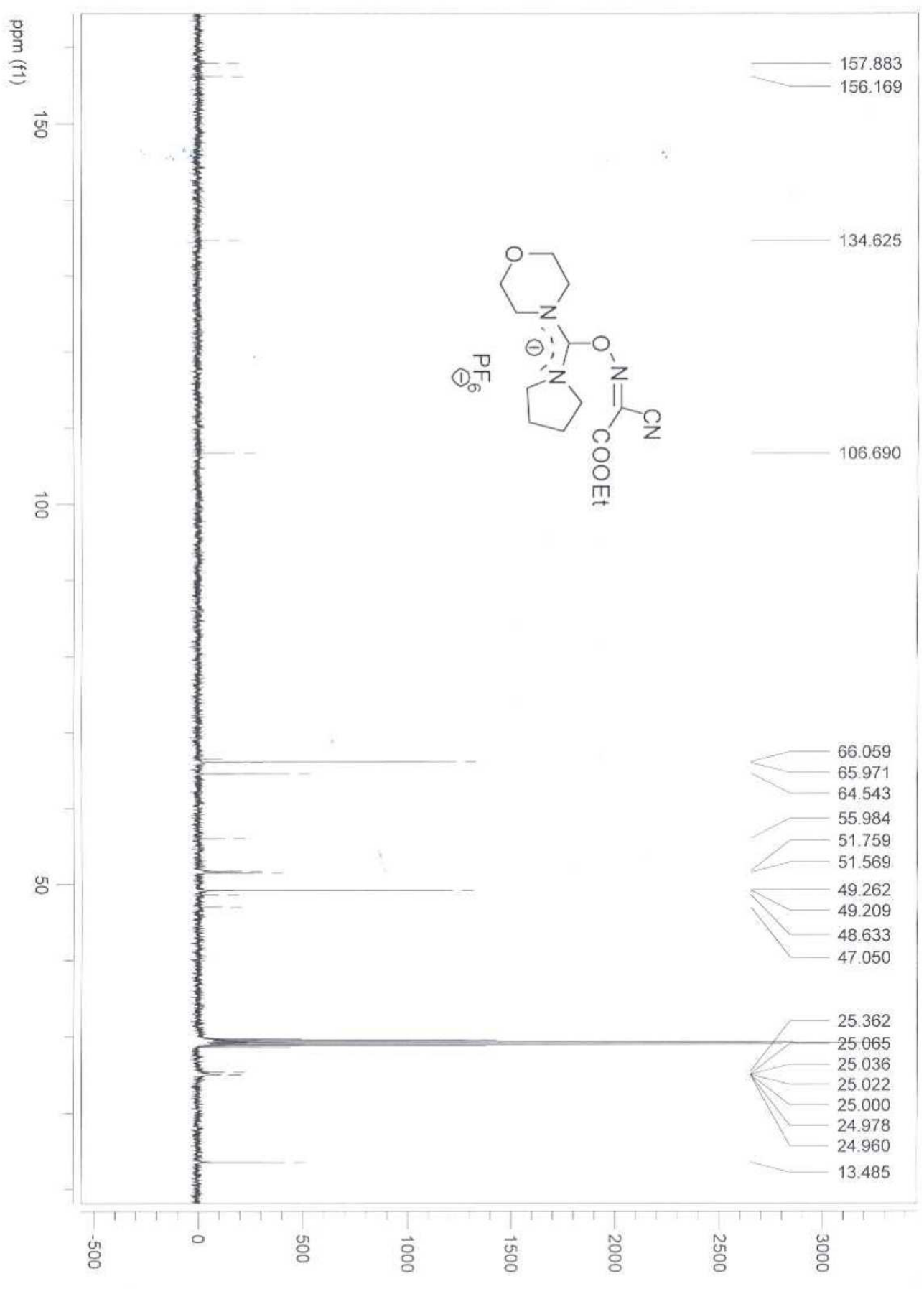
1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate (COMU, 18c, ^{13}C-NMR)



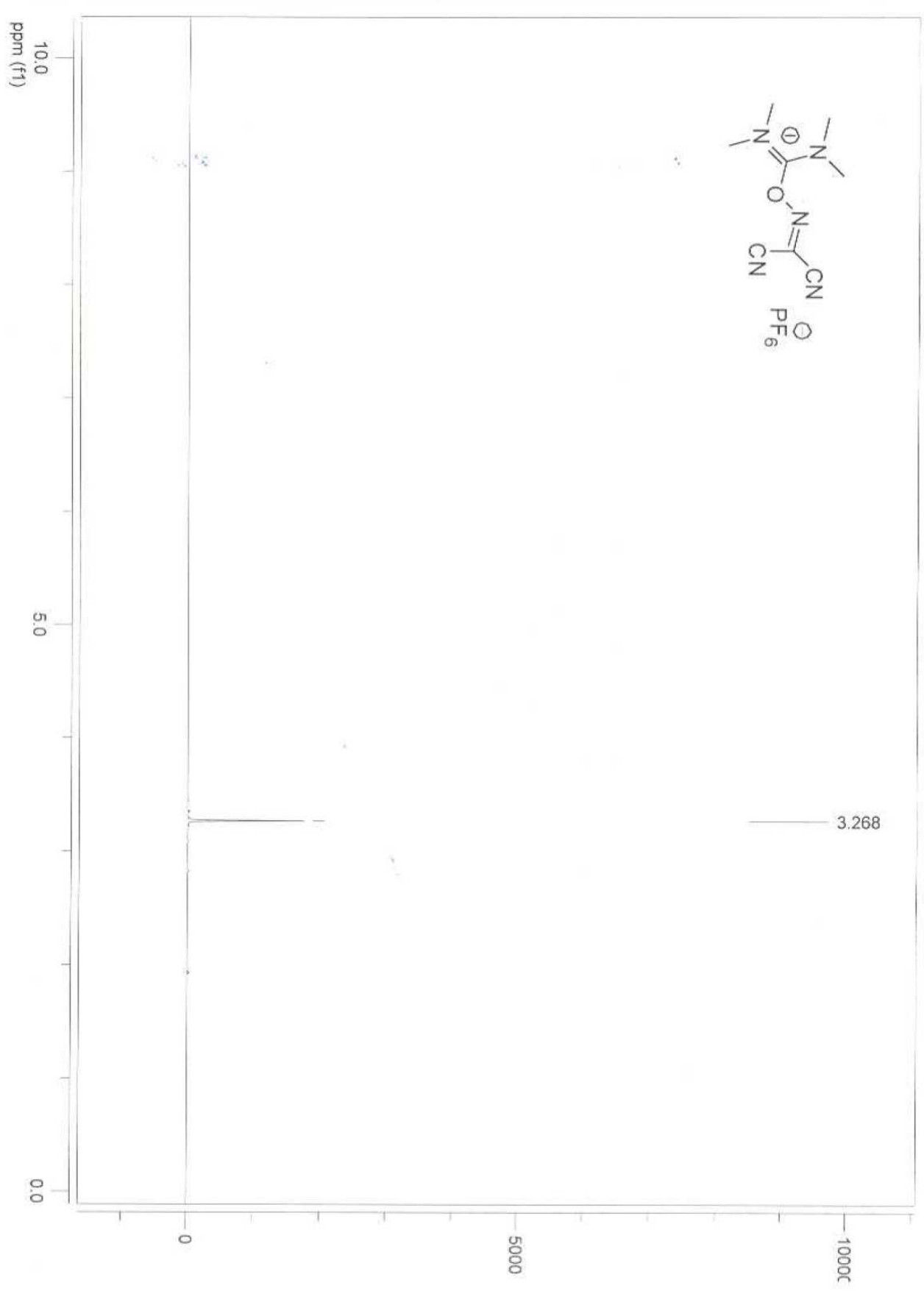
1-((1-Cyano-2-ethoxy-2-oxoethylideneaminooxy)(morpholino)methylene) pyrrolidinium Hexafluorophosphate (HMPyOC, 18d, ^{1}H-NMR)



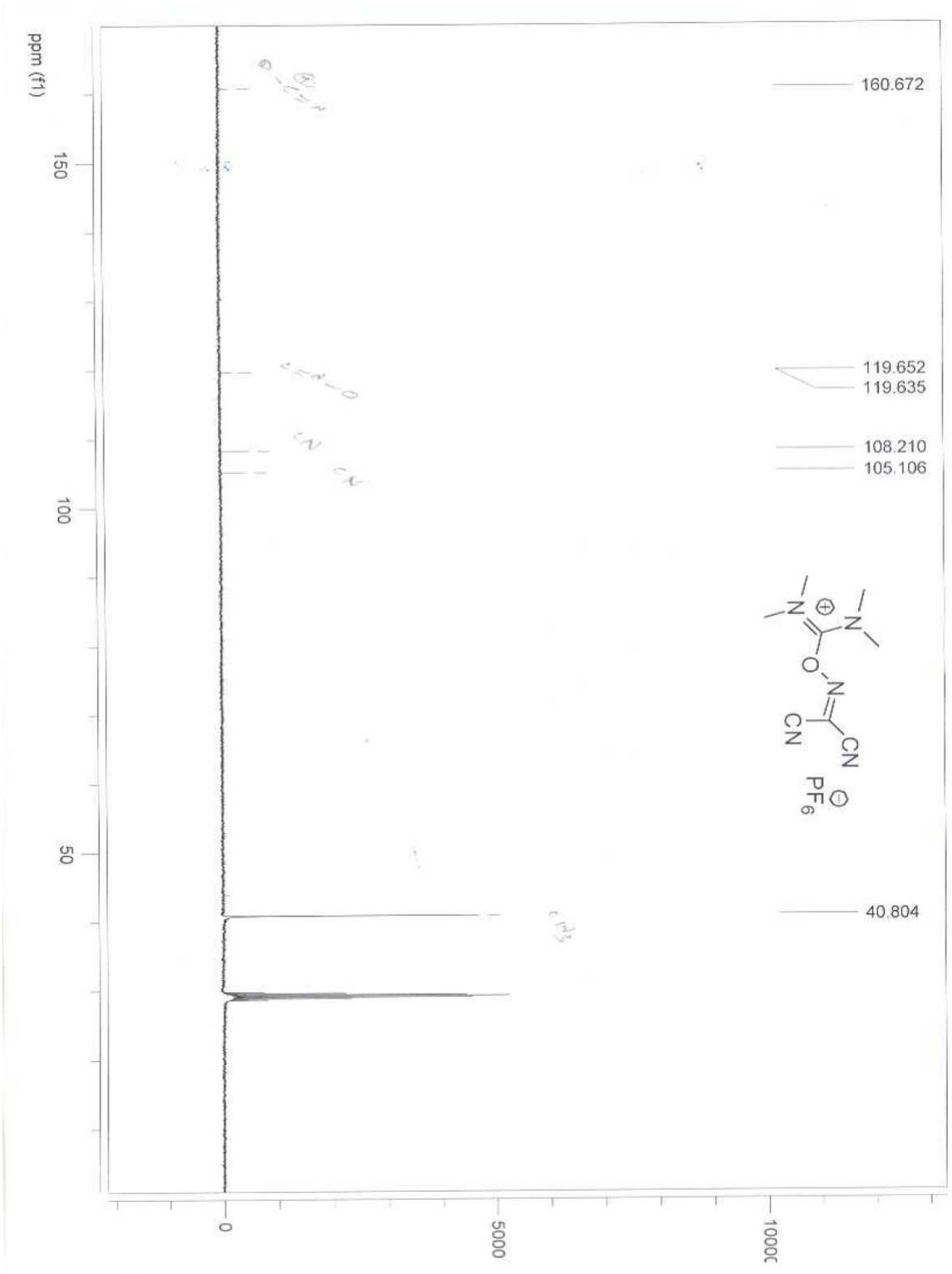
1-((1-Cyano-2-ethoxy-2-oxoethylideneaminooxy)(morpholino)methylene) pyrrolidinium Hexafluorophosphate (HMPyOC, 18d, ^{13}C-NMR)



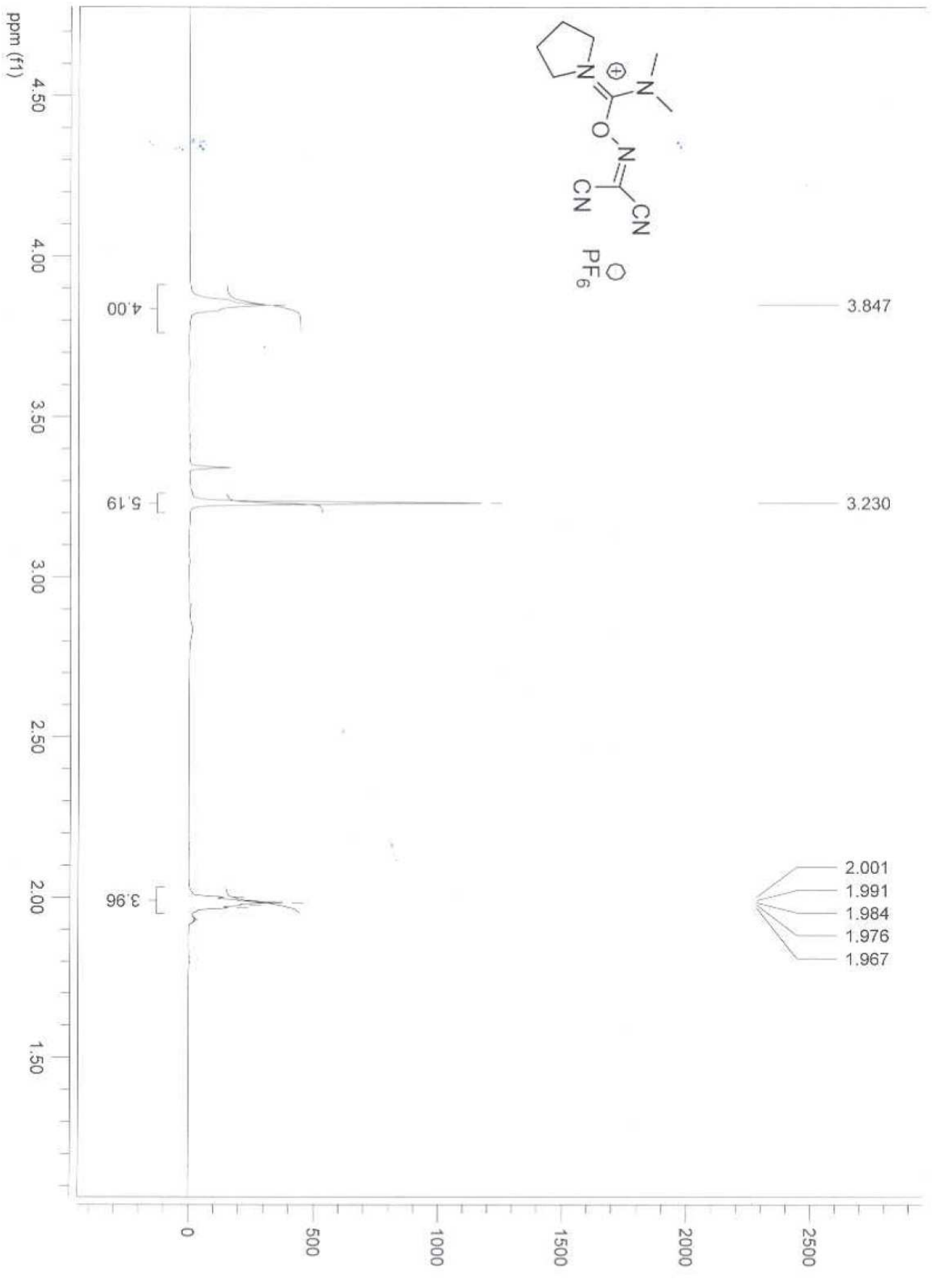
_O_-[(Dicyanomethylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate (HTODC, 18e, ^{1}H-NMR)



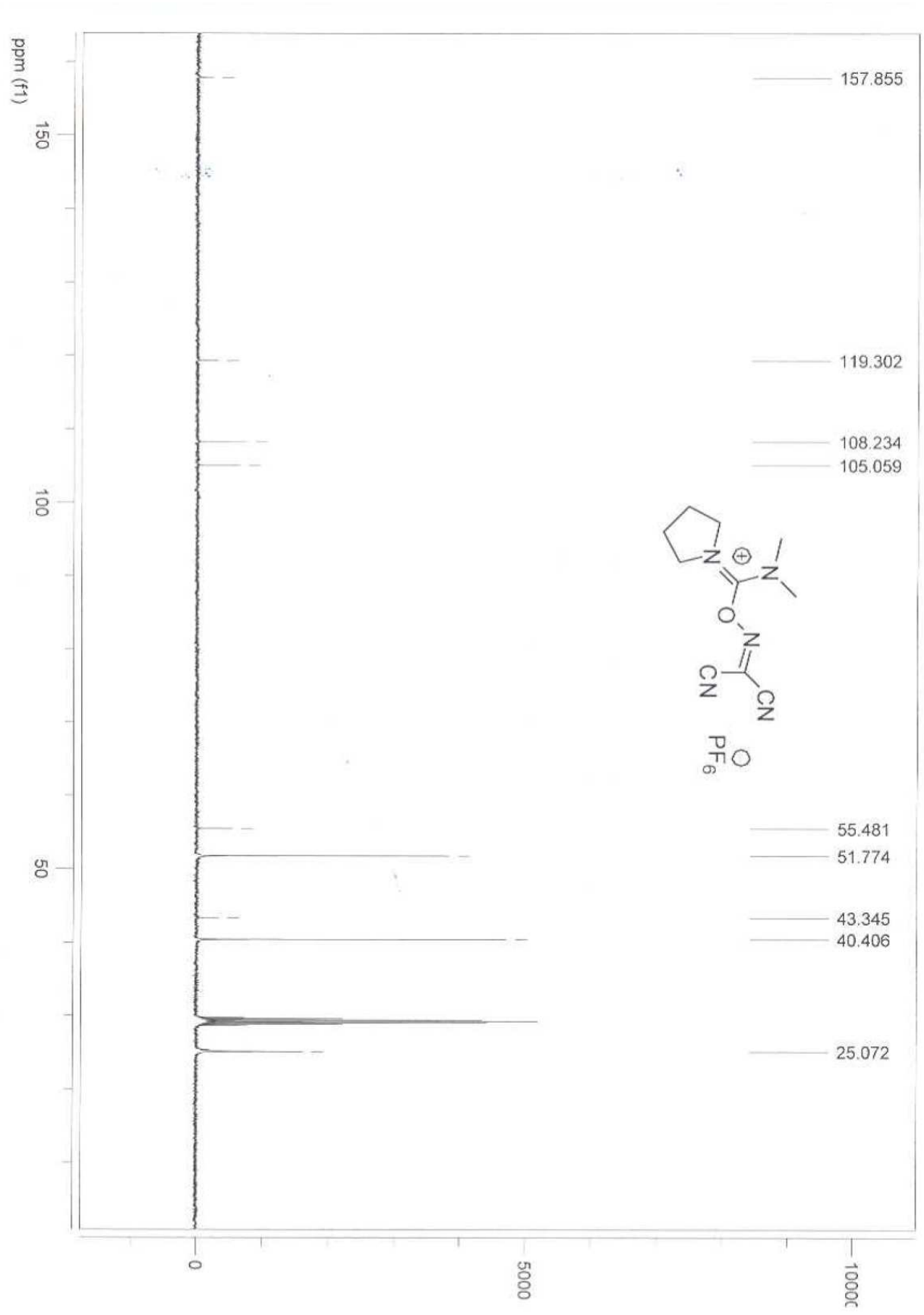
_O_-[(Dicyanomethylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate (HTODC, 18e, ^{13}C-NMR)



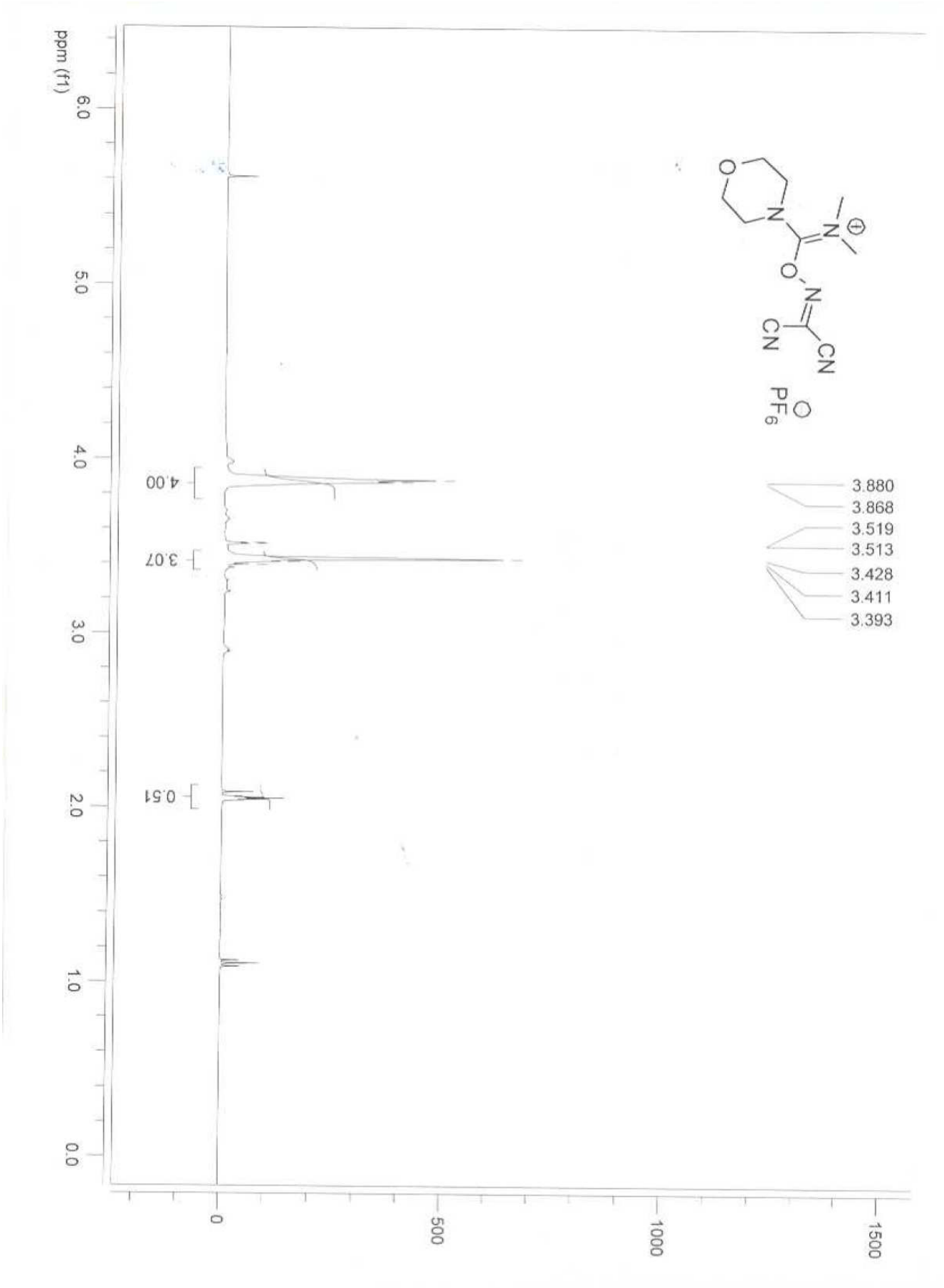
1-[(1-(dicyanomethylideneaminooxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate (HDmPyODC, 18f, ^{1}H-NMR)



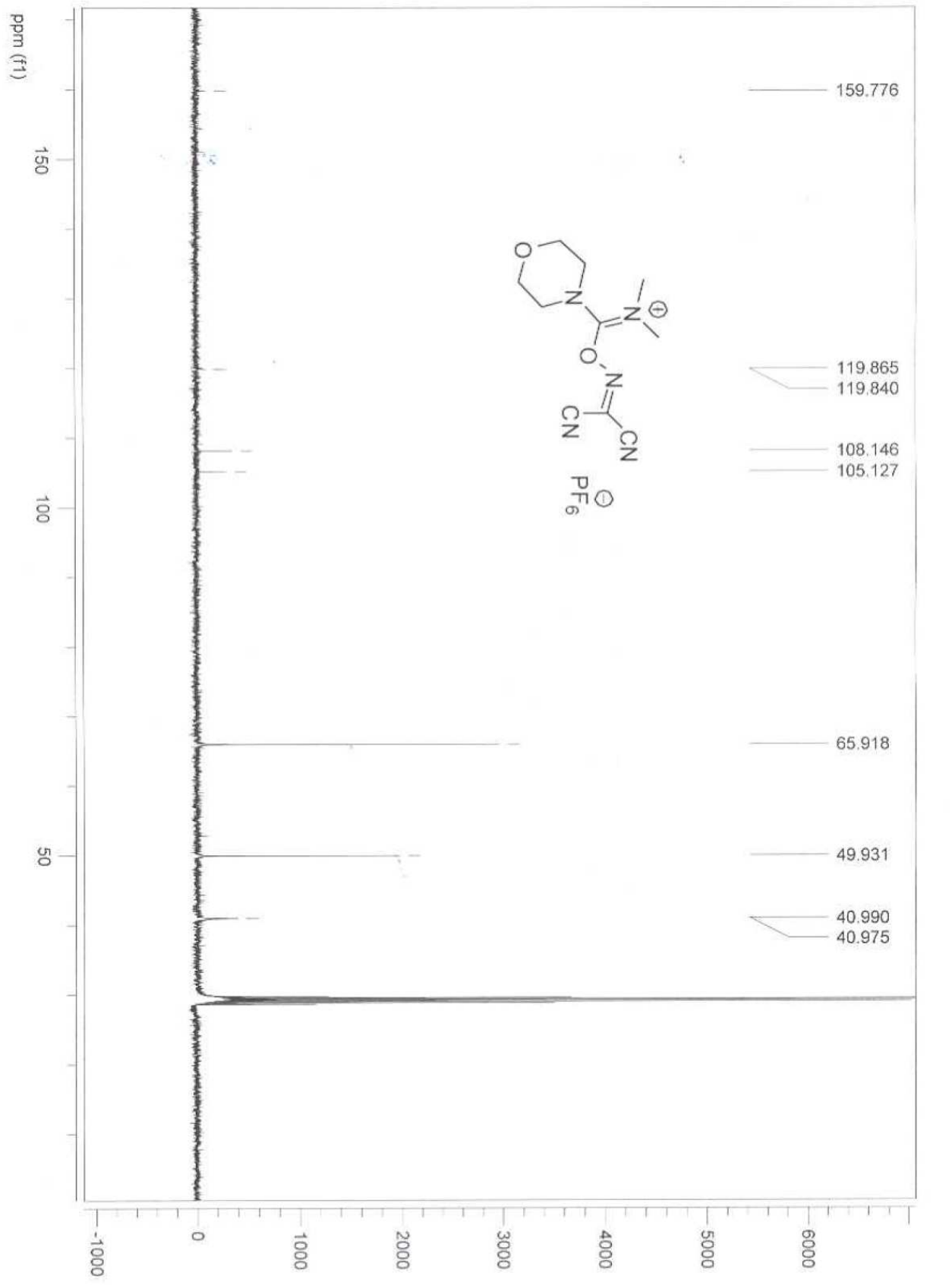
1-[(1-(dicyanomethylideneaminooxy)-dimethylamino-pyrrolodinomethylene)] methanaminium Hexafluorophosphate (HDmPyODC, 18f, ^{13}C-NMR)



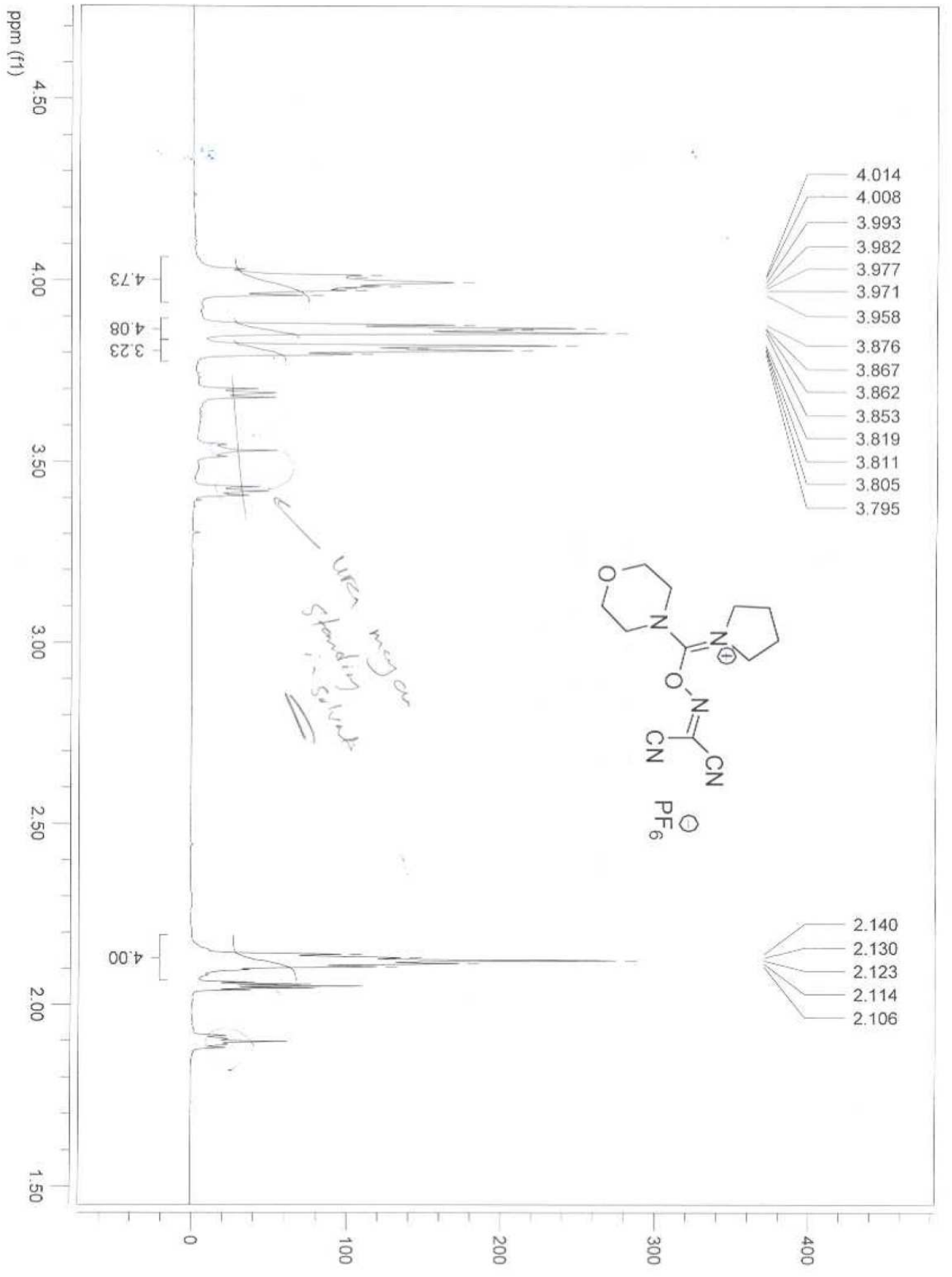
1-[(1-(dicyanomethyleneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate (HDMODC, 18g, ^{1}H-NMR)



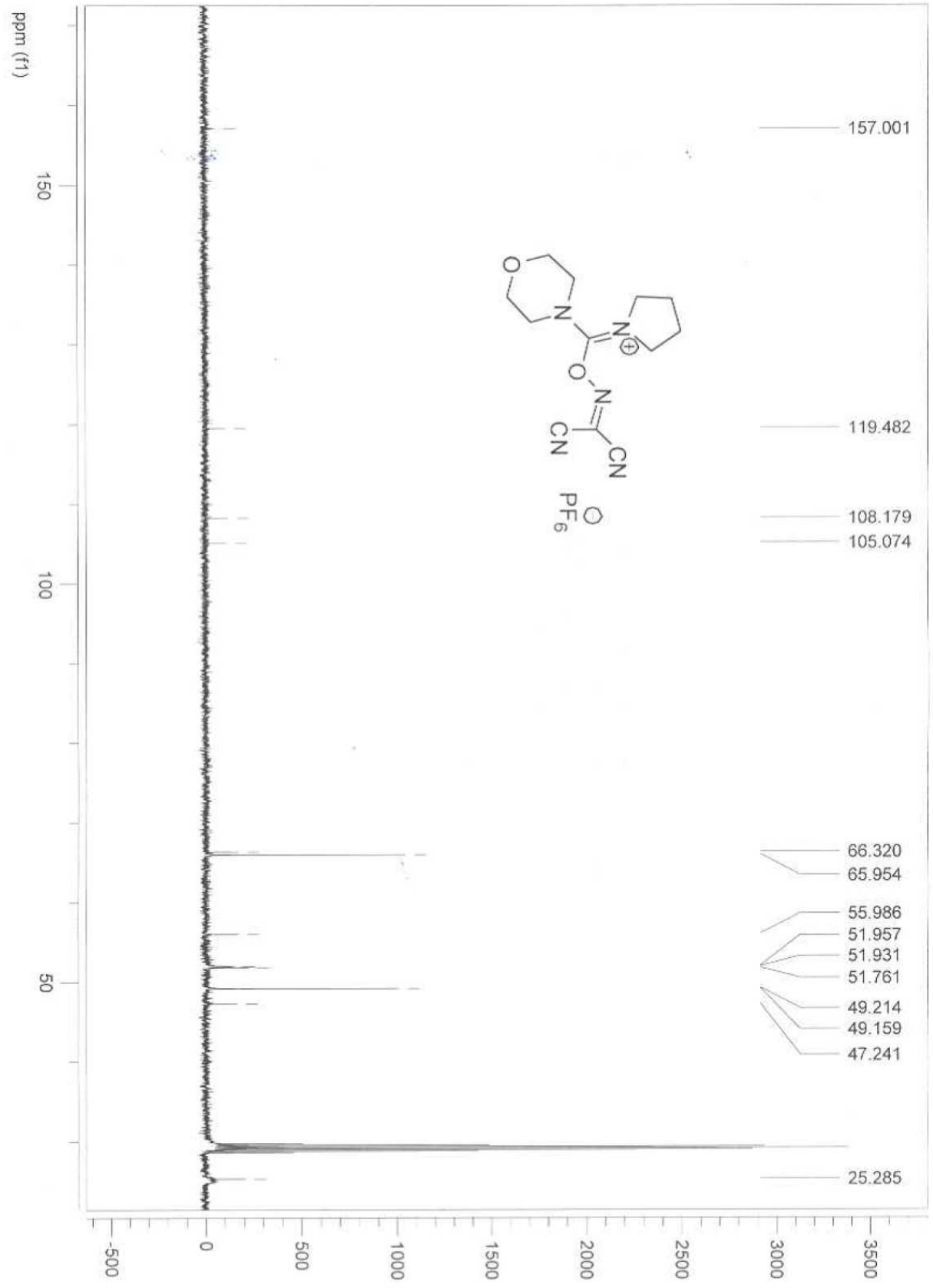
1-[(1-(dicyanomethyleneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate (HDMODC, 18g, ^{13}C-NMR)



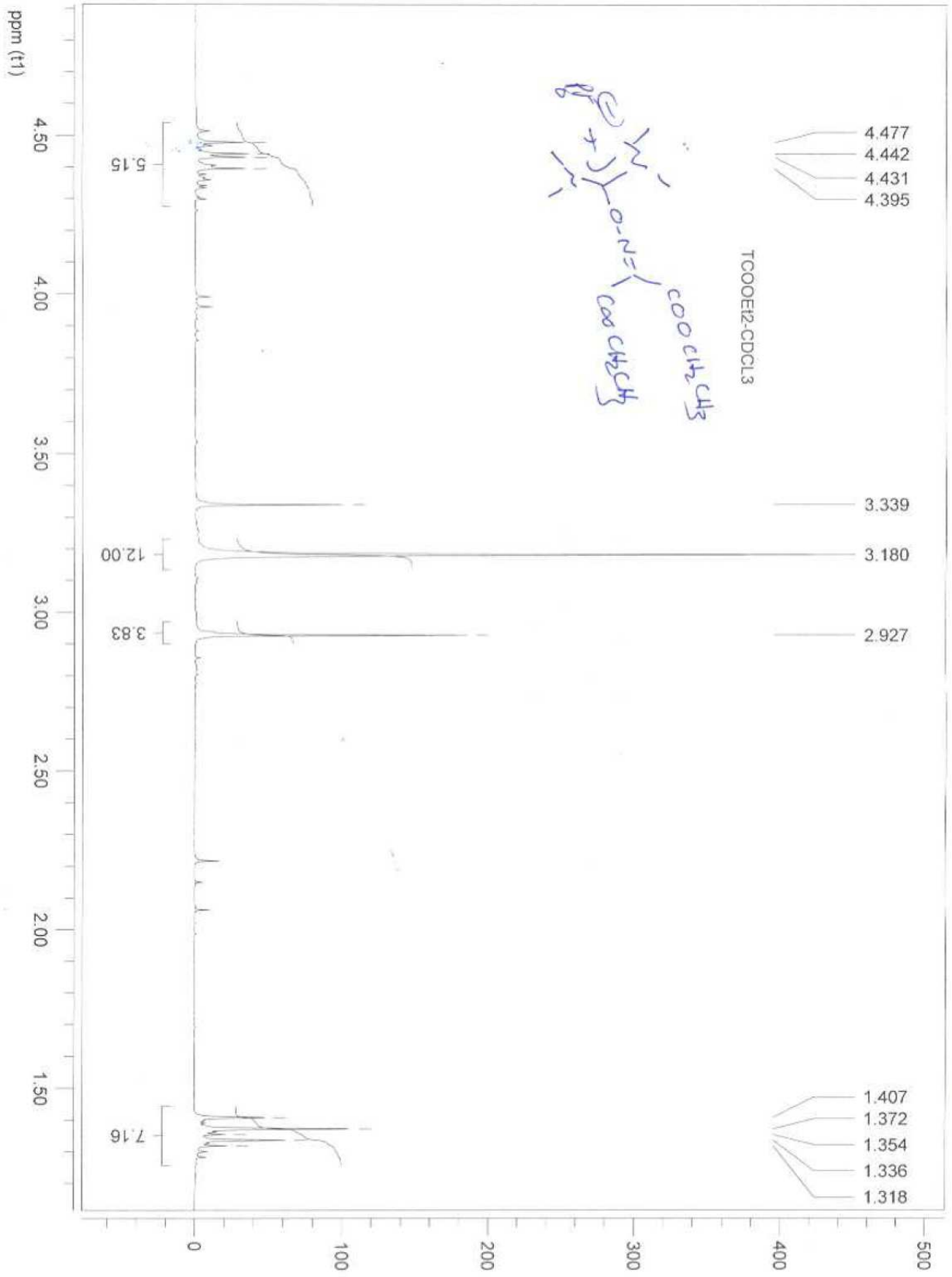
1-((Dicyanomethyleneaminooxy)(morpholino)methylene)pyrrolidinium Hexafluorophosphoate (HMPyODC, 18h, ^{1}H-NMR)



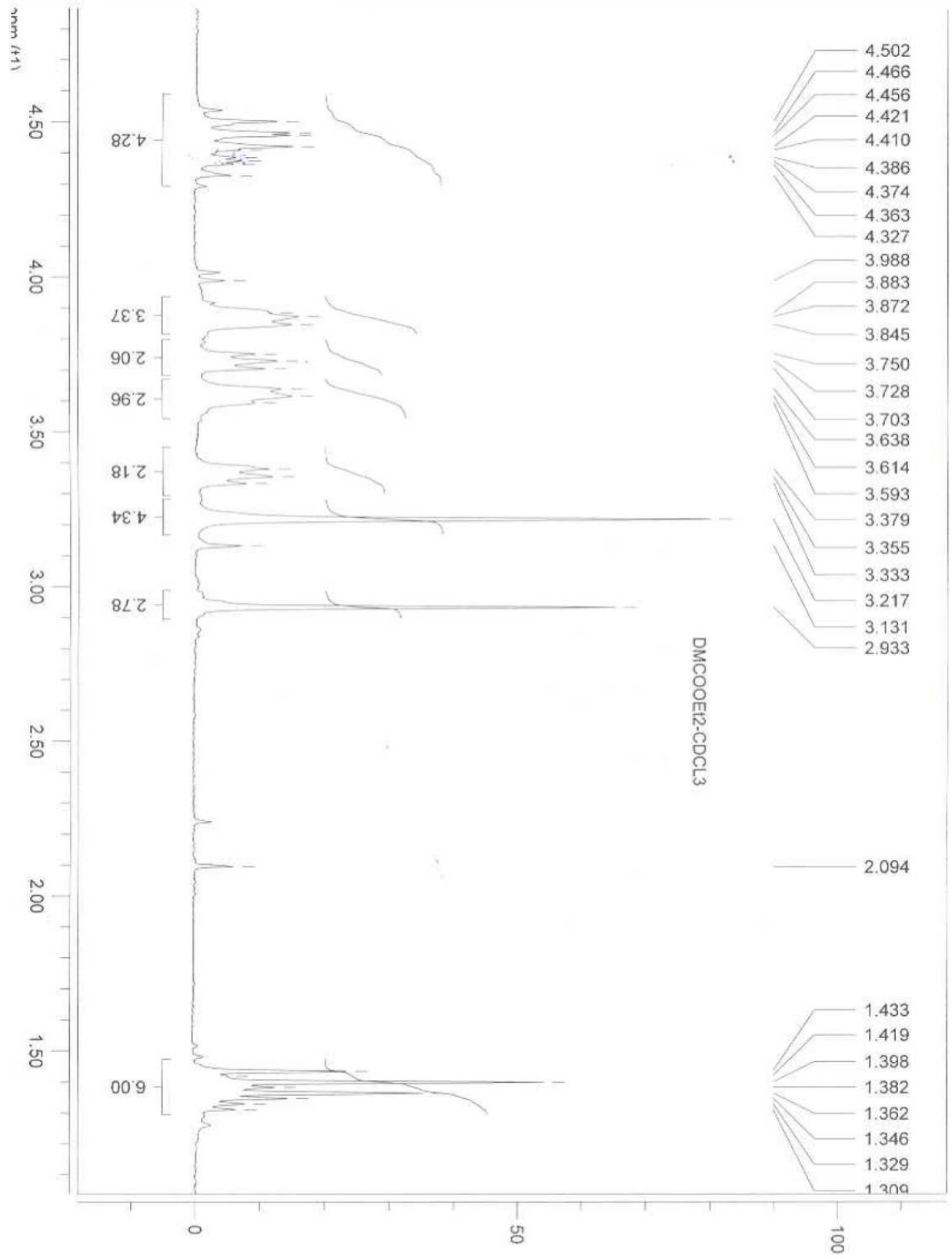
1-((Dicyanomethyleneaminooxy)(morpholino)methylene)pyrrolidinium Hexafluorophosphoate (HMPyODC, 18h, ^{13}C-NMR)



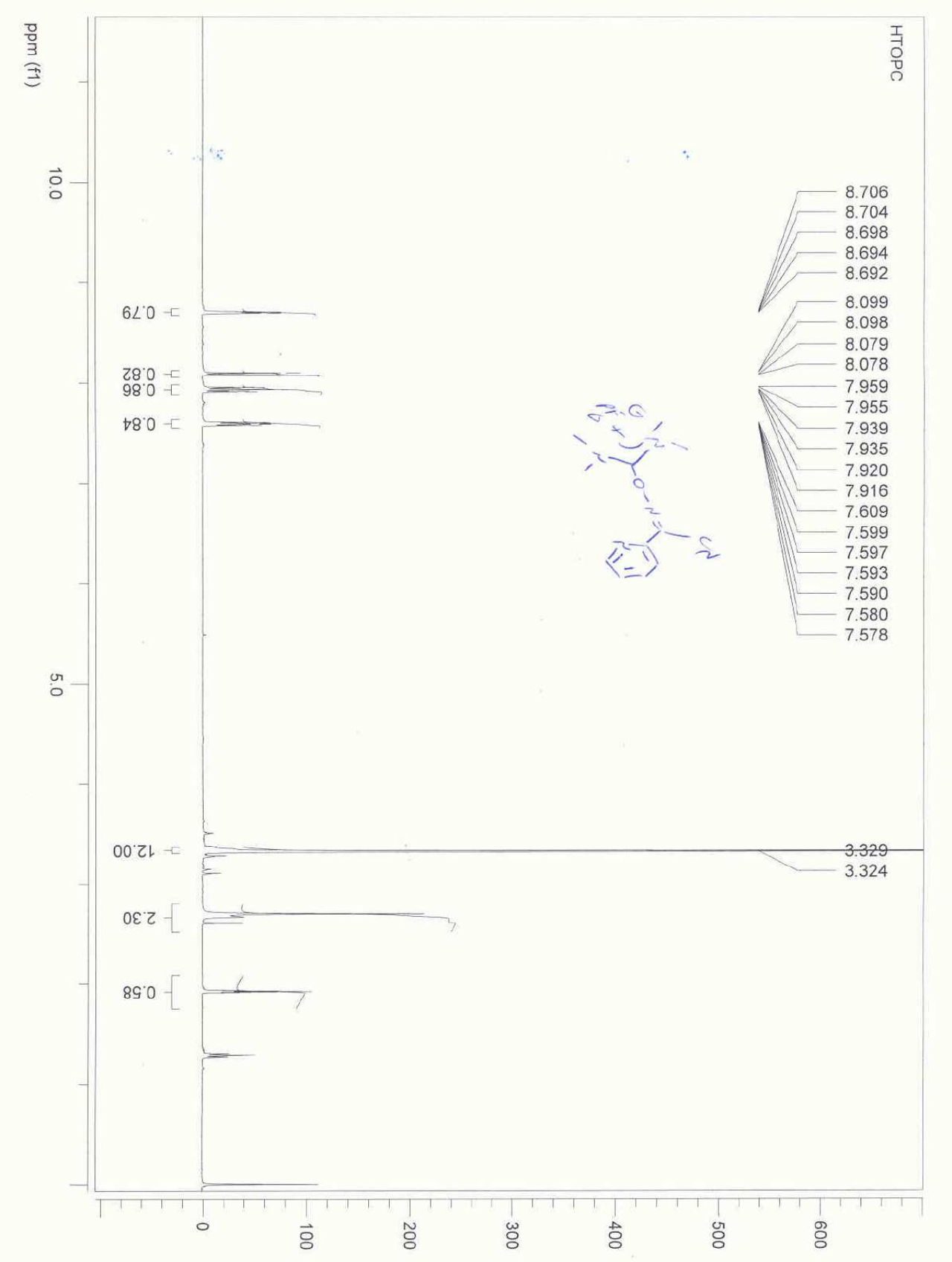
_O_-[(Diethoxycarbonylmethylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate (HTODeC, 18i, ^{1}H-NMR)



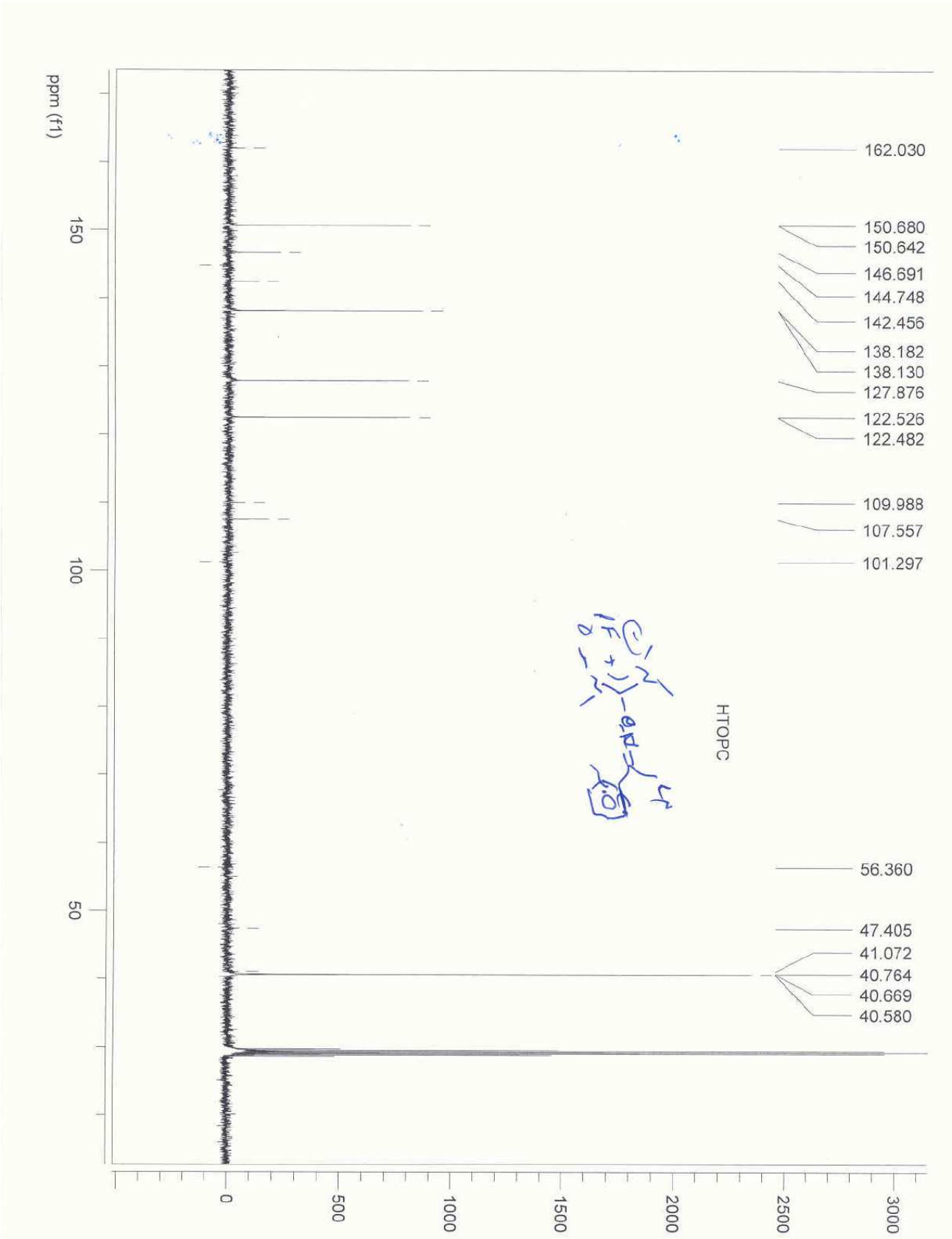
1-[(1,3-diethyoxy-1,3-dioxopropan-2-ylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium Hexafluorophosphate (HDMODeC, 18j, ^{1}H-NMR)



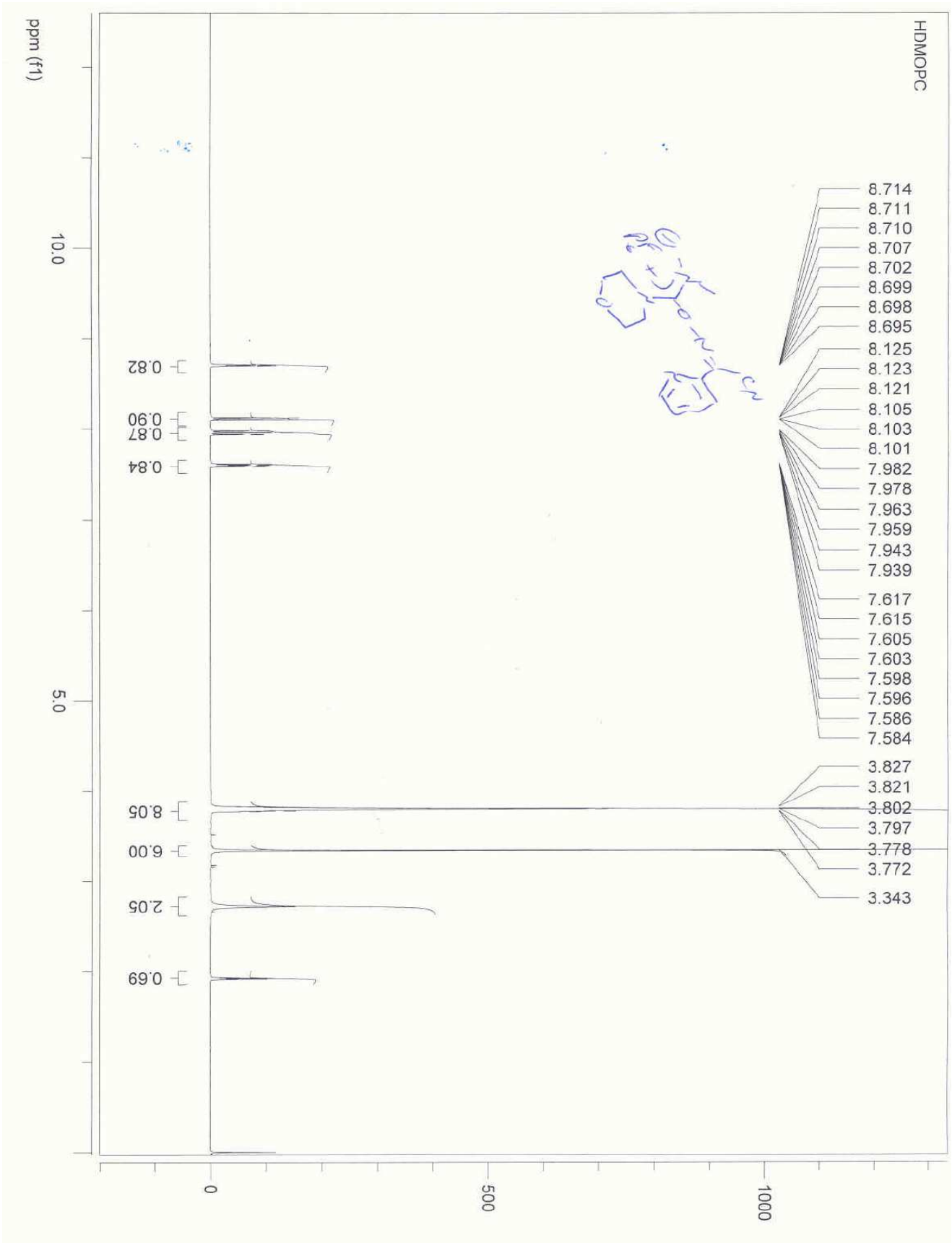
_N_-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino) methylene)-N-Methylmethanaminium Hexafluorophosphate (HTOPC, 18k, ^{1}H-NMR)



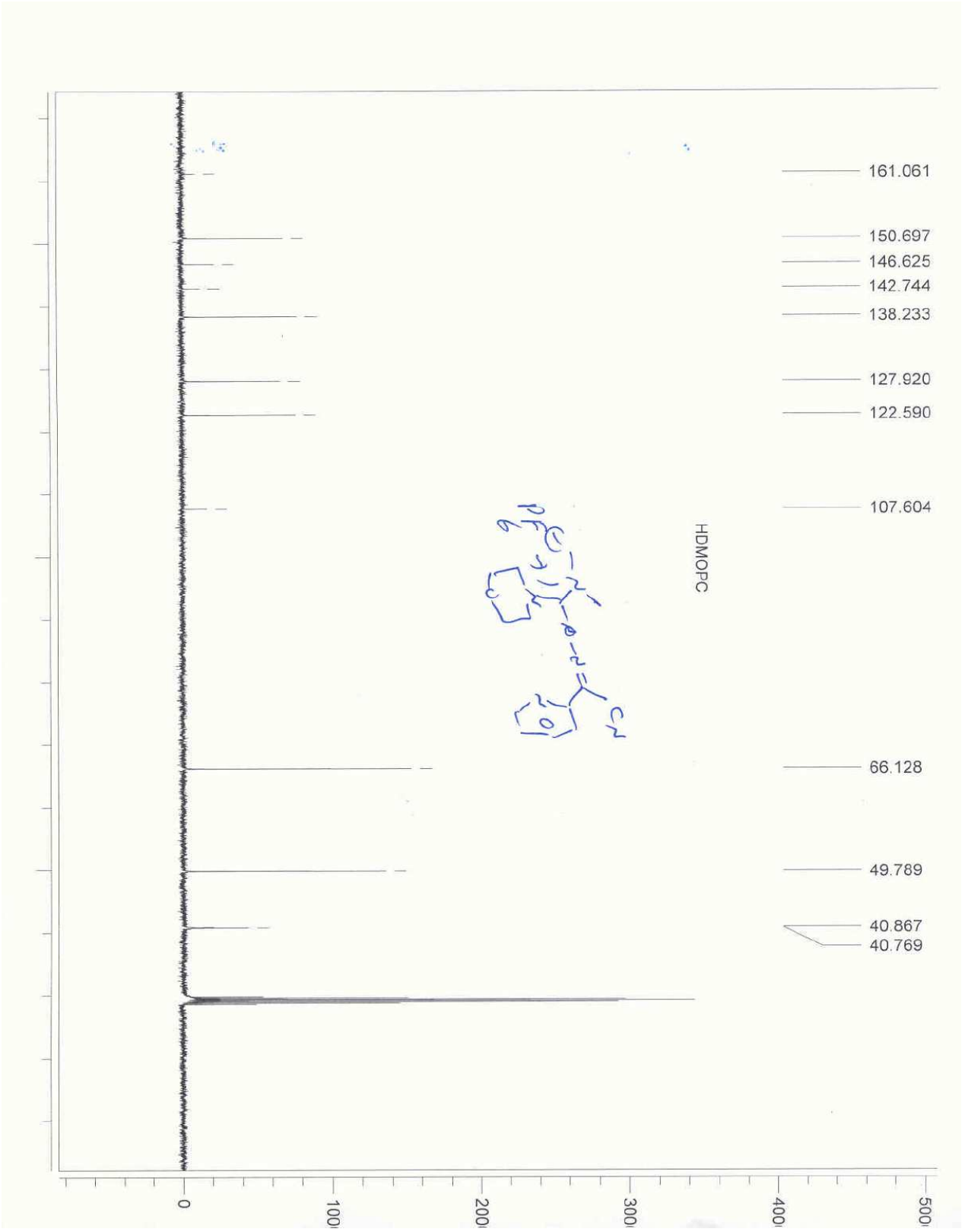
_N_-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino) methylene)-N-Methylmethanaminium Hexafluorophosphate (HTOPC, 18k, ^{13}C-NMR)



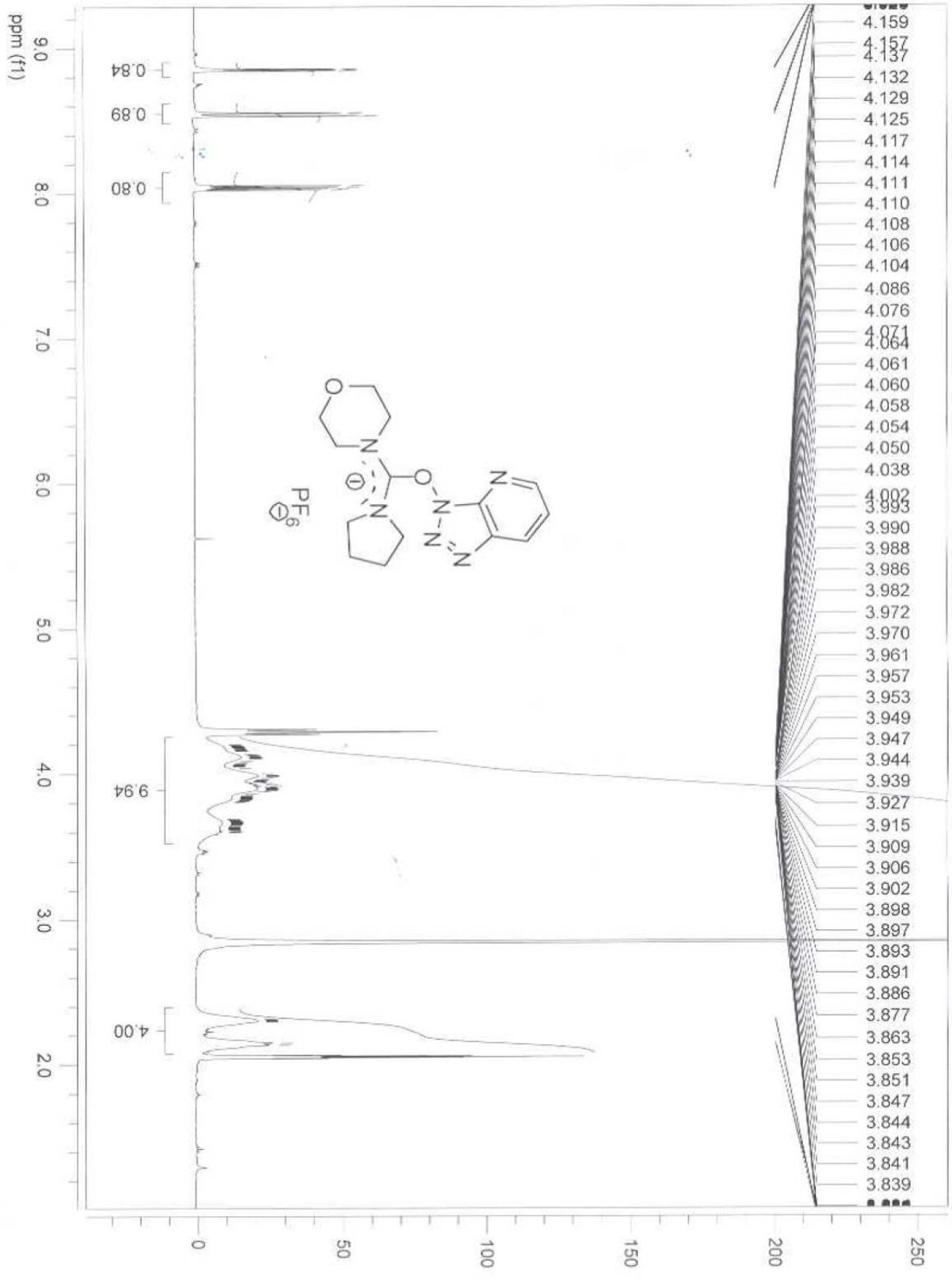
N-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino) methylene)-N-morpholinomethanaminium Hexafluorophosphate (HDMOPC, 18l, ^{1}H-NMR)



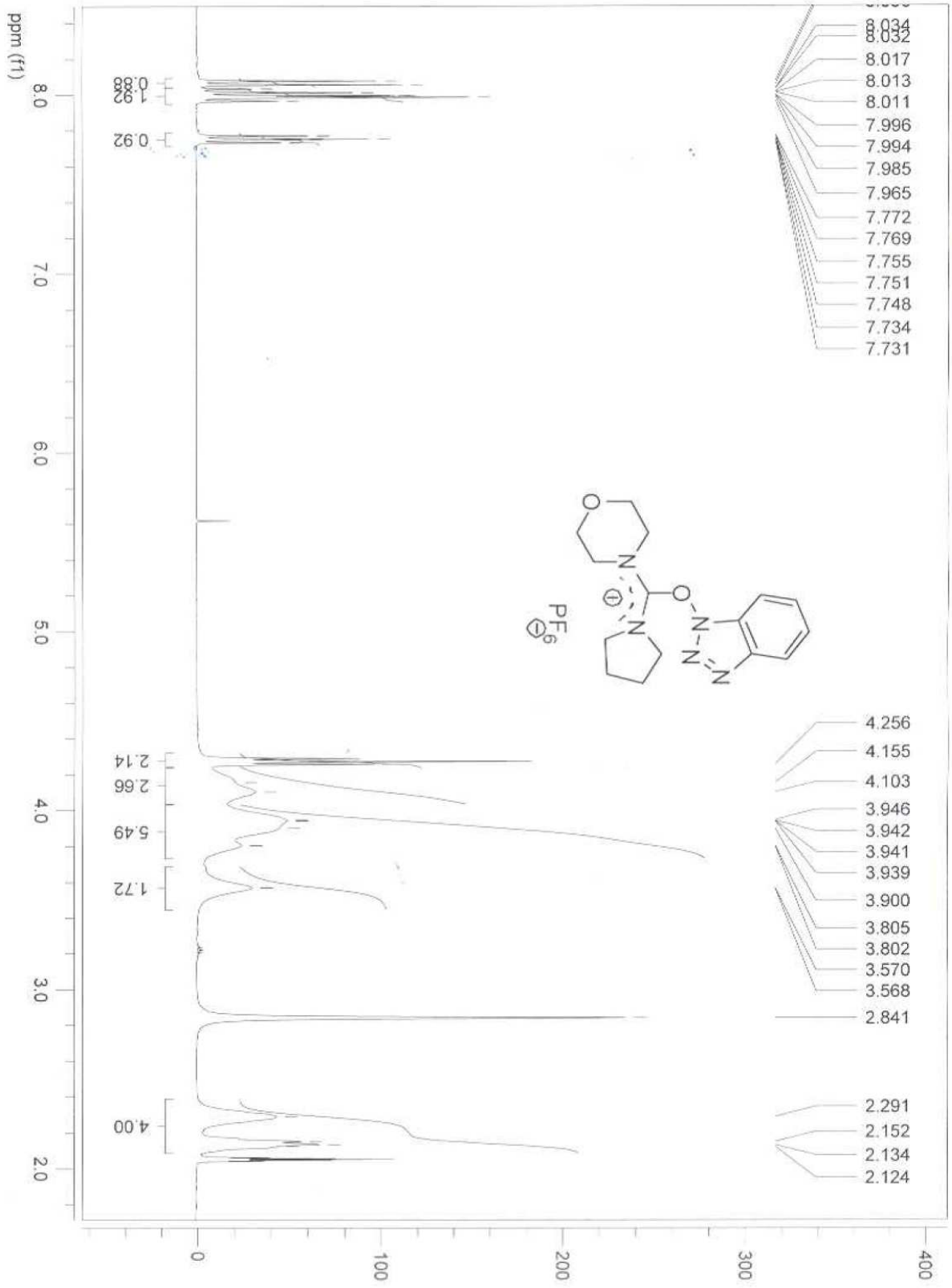
N-[(cyano(pyridine-2-yl)methyleneaminooxy)(dimethylamino) methylene)-N-morpholinomethanaminium Hexafluorophosphate (HDMOPC, 18l, ^{13}C-NMR)



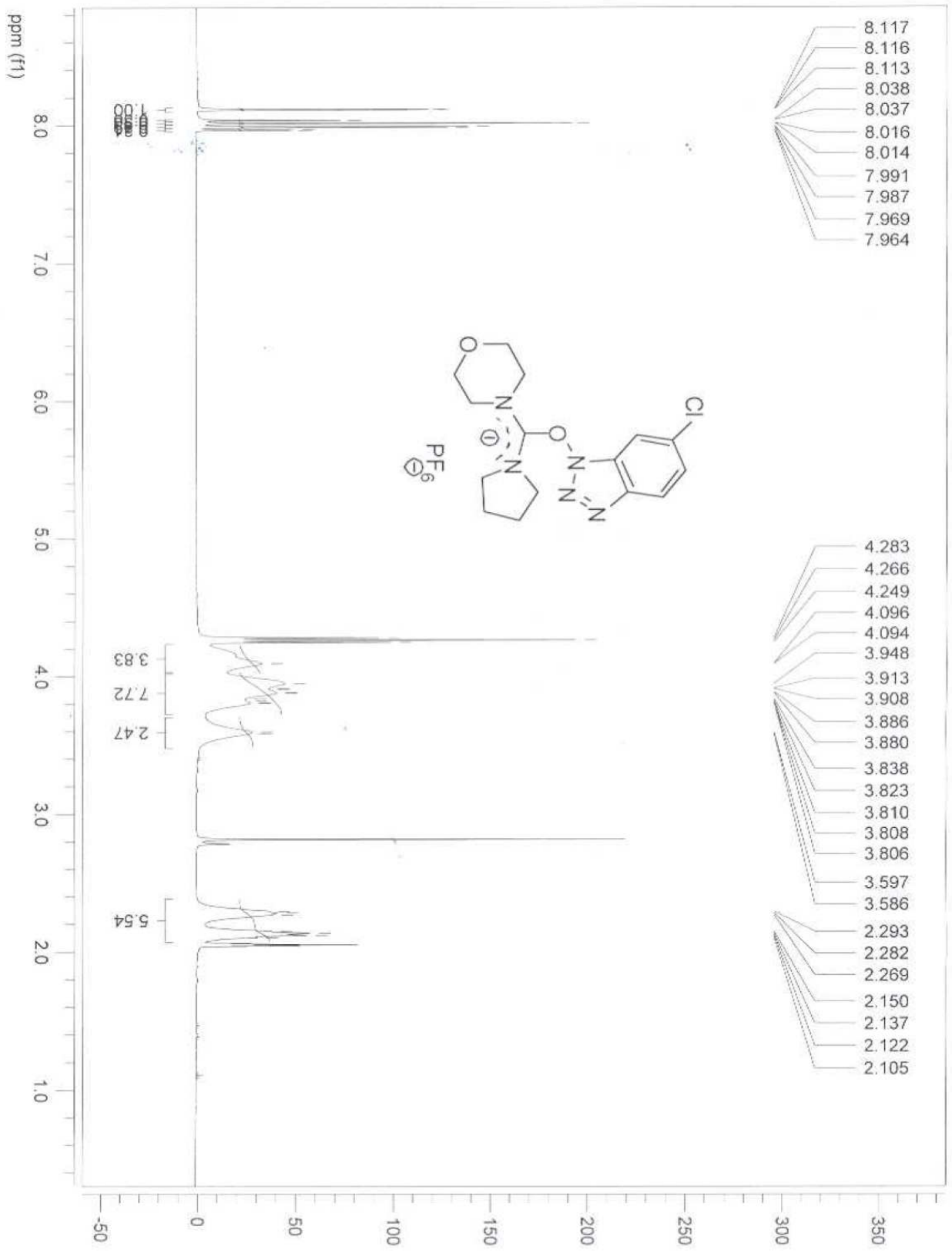
1-(Morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-[1,2,3]triazolo[4,5-I]pyridine 3-oxide Hexafluorophospahte (HMPyA, 19, ^{1}H-NMR)



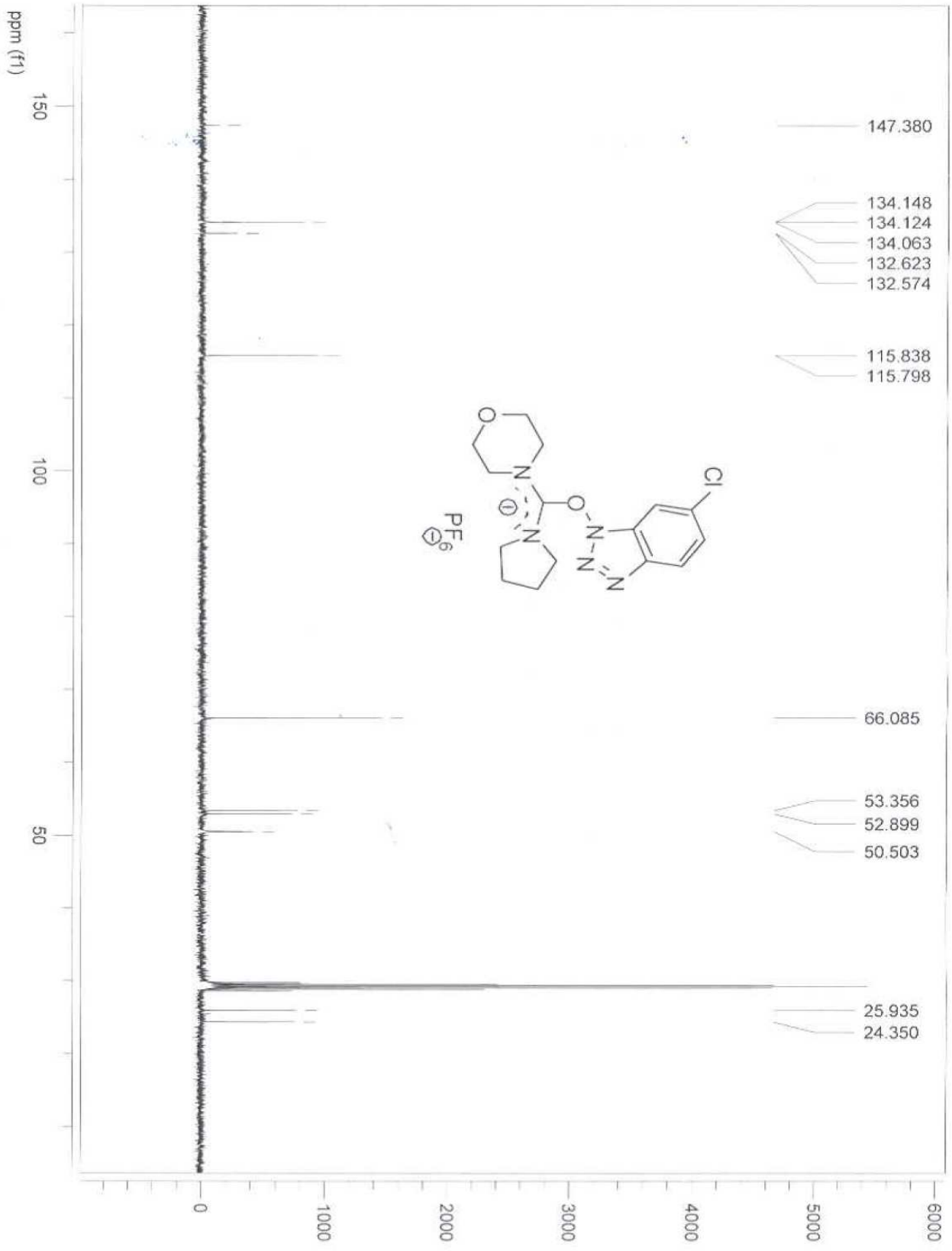
1-(Morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-benzo[*d*][1,2,3]triazole 3-oxide Hexafluorophosphate (HMPyB, 20, ^{1}H-NMR)



5-Chloro-1-(morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-benzo[*d*][1,2,3]triazole 3-oxide Hexafluorophosphate (HMPyC, 21, ^{1}H-NMR)



5-Chloro-1-(morpholino(pyrrolidinium-1-ylidene)methyl)-*1H*-benzo[*d*][1,2,3]triazole 3-oxide Hexafluorophosphate (HMPyC, 21, ^{13}C-NMR)



Publicació VII

Microwave irradiation and COMU: a potent combination for solid-phase peptide synthesis †

Irradiació per microones i COMU: una combinació potent per síntesi de pèptids en fase sòlida

Ramon Subirós Funosas[a,b], Gerardo A. Acosta[a,b], Ayman El-Faham[a,c,d*], Fernando Albericio[a,b,e*]

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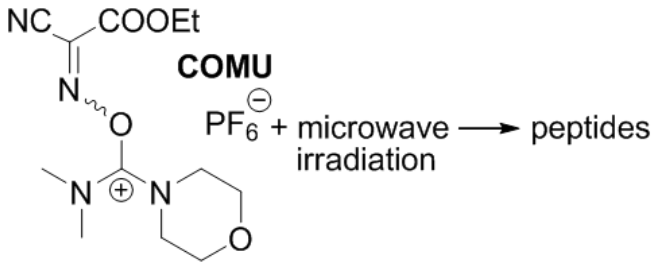
† Ramon Subirós Funosas va dur a terme els assajos d'eficiència dels diferents reactius d'acoblament al sintetitzador assistit per microones i la comprovació de l'absència de subproductes de guanidilació, així com l'elaboració del manuscrit.

Gerardo A. Acosta va col·laborar en la escissió dels pèptids de la resina i la posada a punt de les condicions més idònies per realitzar la comparació dels reactius al sintetitzador Liberty CEM.

Resum

El treball que es presenta a continuació tracta sobre la compatbilitat de la sal d'uroni novell basada en Oxyma de tipus dimetilmorfolino, COMU, amb sintetitzadors de pèptids automàtics assistits per irradiació de microones.

De manera consistent amb anteriors estudis realitzats en la sal d'uroni COMU, aquest demostra una eficiència major que les sals d'amini basades en benzotriazol HATU i HBTU, en la elongació del pentapèptid Aib-encefalina, el qual inclou acoblaments directes Aib-Aib, considerats d'alta dificultat sintètica pel caràcter α,α-disubstituït del resiu en qüestió. A més, com s'ha demostrat en experiments similars amb Oxyma, no s'observa cap subproducte d'addició nucleòfila al carboxil de l'èster que podrien bloquejar el grup amino *N*-terminal. En comparació amb la síntesi del mateix model peptídic en mode manual, aquesta tècnica, que combina dos metodologies potents, es capaç de donar lloc a percentatges de pèptid desitjat superiors al 90%, en temps totals de síntesi comparativament molt més curts.



La combinació de COMU i microones resulta en una estratègia potent per la síntesi de pèptids, sobretot si estan implicats elements de dificultat estèrica.

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Microwave irradiation and COMU: a potent combination for solid-phase peptide synthesis

Ramon Subiros-Funosas [a,b], Gerardo A. Acosta [a,b], Ayman El-Faham [a,c,d], Fernando Albericio [a,b,e,*]

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ABSTRACT

Here we demonstrate the compatibility of Oxyma-based uronium-type coupling reagent COMU with microwave-assisted peptide synthesizers. Consistent with previous reports, COMU displayed higher efficiency than benzotriazole classical immonium salts HATU and HBTU in the demanding synthesis of the Aib derivative of Leu-Enkephalin pentapeptide and did not yield Oxyma-based byproducts. Thus, the combination of microwave irradiation and COMU resulted in a similar performance in considerably shorter time to that achieved by manual synthesis.

Uronium-type coupling reagent 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate, **1** (COMU, Fig. 1) is a more efficient alternative to classical benzotriazole immonium salts in terms of racemization suppression, coupling effectiveness, stability, and solubility.[1] In addition to the morpholino moiety, this superiority is attributed to the introduction of ethyl 2-cyano-2-(hydroxyimino)acetate, **2** (Oxyma, Fig. 1) in its structure. Oxyma displays high control of optical purity and extension of coupling in demanding sequences.[2]

Both Oxyma and its related uronium salt can be handled with a considerably lower risk than its explosive benzotriazole counterparts, as determined by calorimetry techniques. However, the thermal stability of Oxyma is relatively low.[1,2] Nonetheless, no incident has been reported during extreme coupling stability assays, carried out using microwave irradiation at 80 °C.[2]

Previous studies examined the stability of Oxyma toward the nucleophilic N-terminus of the growing peptide chain in extreme experiments. To enhance the likelihood of this side reaction, these experiments used a high excess of the additive in the absence of carbodiimide and Fmoc-AA-OH.[2] In the assay conducted at room temperature overnight, no Oxyma-based byproduct was found. In contrast, a similar experiment with microwave irradiation gave rise to some of these undesired byproducts (Fig. 2). In that demanding experiment, the main byproduct resulted from the formylation of the amino function (**3**, 47.9%), attack of the amino group on the carboxylate (**4**) and the carbonyl of the oxime (**5**), which was also hydrolyzed (**6**). In all the cases, impurities were detected in the range 2–4%.[2]

In order to unequivocally determine the compatibility of the Oxyma-derived coupling reagents with microwave-assisted solid-phase peptide synthesis (SPPS), we compared the performance of COMU with that of classical immonium salts HBTU **7** and HATU **8** in the synthesis of a true peptide model conducted in an automated peptide synthesizer with microwave irradiation (Fig. 3).[3]

Since its first appearance in 1986, microwave irradiation has been established as a highly useful tool for solid-phase organic

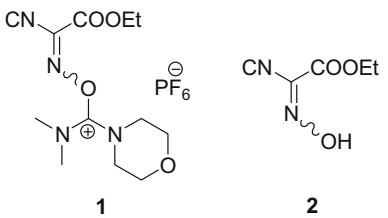

Figure 1. Structure of COMU and Oxyma.

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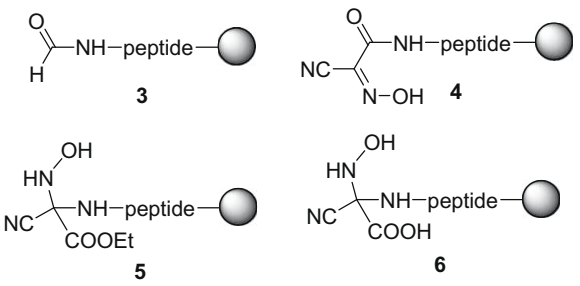

Figure 2. Side products obtained by reaction of Oxyma with a peptide in the absence of carbodiimide and Fmoc-AA-OH.

synthesis, both in academy and in industry, by increasing product yield and purity and reducing reaction times, in combination with thermal effects.[4–6] However, few studies have reported the application of this approach in the field of peptide synthesis since the early report of Wang and co-workers in 1991.[7] This low general acceptance could be partly attributed to the widespread belief that the acceleration rate of coupling and deprotection steps also leads to instability of coupling reagents and an increase in typical side reactions, such as racemization and aspartimide formation. Nevertheless, recent studies have confirmed that these drawbacks can be controlled with an adequate choice of reaction conditions, and also that resin loading is critical for optimal microwave-assisted peptide synthesis.[8,9] The availability of peptide synthesizers equipped with microwave irradiation has undoubtedly contributed to the sudden increasing popularity of this tool in SPPS, which has proved compatible with both Fmoc and Boc approaches in the assembly of difficult sequences including phospho and glycopeptides, cymantrene-peptide bioconjugates, and cyclotides.[10–16]

One of the key advantages offered by microwave irradiation (which cannot be explained by thermal effects) derives from the dipolar polarization mechanism, which provides extra energy for the rotation of the molecules with a dipolar moment, such as peptides.[8,12] This effect is highly relevant during the elongation of long and hydrophobic peptides, since the polar peptide backbone constantly aligns with the electric field, thereby disrupting chain aggregation. Thus, peptides of up to 30-mer can be prepared easily overnight.[8]

To further demonstrate the strong performance of microwave-assisted SPPS in challenging sequences, here we report the assembly of the highly demanding Aib-analog of Leu-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) by means of a Liberty CEM Microwave Peptide Synthesizer. The Aib-Aib sterically hindered coupling is an excellent scenario to compare the performance of COMU **1** and benzotriazole-based HBTU **7** and HATU **8**,[17] and also to study the formation of the side reactions associated with the presence of Oxyma during the coupling.

The synthesis was carried out in a 0.1 mmol scale of H-RinkAmide-ChemMatrix resin (loading = 0.42 mmol/g), a totally PEG-based solid support that shows good swelling properties in both polar and non-polar solvents.[18–21] A conditioning protocol was followed to remove any remaining base from the commercial resin before its transfer with DMF into a suitable-sized polypropylene vial. Solutions of reagents for coupling and deprotection steps were placed in appropriate vessels (Table 1).

Short single couplings were applied to introduce the five residues (6 min at 80 °C), using 1 mmol of Fmoc-aminoacid (0.6 mmol for Aib, in order to enhance relative differences), 1 equiv of coupling reagent, and 2 equiv of base, relative to amino acid.[22] Deprotection steps were conducted with a solution of piperidine in DMF (Table 1) at 75 °C (3 min × 2). Once automated synthesis of the pentapeptide was completed (proceeding safely with COMU **1** under the abovementioned conditions), the final cleavage from the resin was accomplished with a 2-h treatment with 90% TFA/10% H_2O at room temperature. After precipitation with cold Et_2O and lyophilization, the samples were analyzed by reverse-phase HPLC-PDA and HPLC–MS. An example of the crude mixtures obtained is shown for the synthesis with COMU **1** (Fig. 4).

Two main compounds were observed in the crude mixtures: the desired pentapeptides (H-Tyr-Aib-Aib-Phe-Leu-NH_2) and des-Aib (H-Tyr-Aib-Phe-Leu-NH_2). Only traces of other possible deletion peptides such as des-Tyr (H-Aib-Aib-Phe-Leu-NH_2) and des-Aib,Tyr (H-Aib-Phe-Leu-NH_2) were detected. Some minor peaks were observed and these were not related to the coupling reagents or deletion peptides. The relative percentages of pentapeptide/des-Aib for each coupling reagent are shown in Table 2. The highest percentage of pentapeptide was obtained with COMU **1** (92%). This performance was better than that observed for HATU **8** (79%) or HBTU **7**, (23%). The manual synthesis with COMU at room temperature afforded a higher percentage of the Aib-enkephalin pentapeptide (99.7%);[1] however, coupling times were much longer (60 min, double coupling) than those in the abovementioned microwave synthesis (6 min). These results therefore highlight the enhanced efficiency of the method presented.

In our experiment with COMU, carried out in more realistic conditions than those abovementioned, no byproducts (**4–6**) related to

Table 1
Reagents and solvents used for microwave-assisted Fmoc-SPPS

	Reagent	Concentration	Solvent
Amino acid	Fmoc-AA-OH	0.2 M	DMF
Activator	HBTU/HATU/COMU	0.5 M	DMF
Base	DIEA	2 M	DMF
Deprotection	Piperidine	20% v/v	DMF

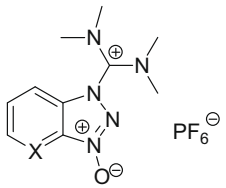

Figure 3. Structure of benzotriazole-based immonium salts HBTU and HATU.

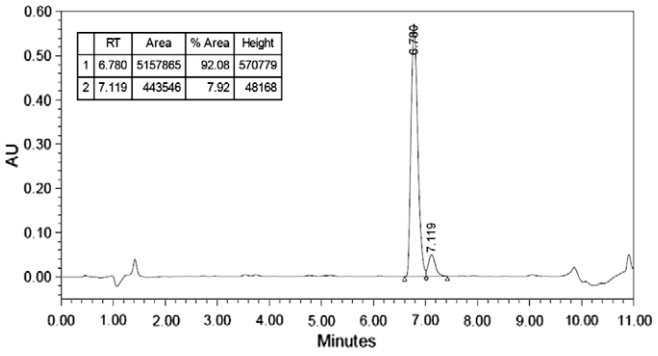

Figure 4. HPLC analysis of the crude obtained from the experiment using COMU. HPLC conditions: Waters SunFire C18 column (3.5 μm, 4.6 × 100 mm); linear gradient 20–30% of 0.036% TFA in CH_3CN/0.045% TFA in H_2O over 8 min; flow = 1.0 ml/min; detection at 220 nm; t_R (penta) = 6.780 min, $[M+H]^+$ = 611.35; t_R (des-Aib) = 7.119 min, $[M+H]^+$ = 526.30.

Table 2
Relative percentages of pentapeptide/des-Aib with various coupling reagents

Coupling reagent	Pentapeptide (%)	Des-Aib (%)	Yield (%)
COMU (**1**)	92.1	7.9	88.5
HBTU (**7**)	23.1	76.9	20.5
HATU (**8**)	79.5	20.5	76.0

the Oxyma moiety contained in the coupling reagent were detected. However, traces of guanidylation were found at the dipeptide stage[23] with each coupling reagent, during the introduction of the first Aib residue.

In summary, here we demonstrate the compatibility of Oxyma-based COMU **1** with microwave-assisted SPPS, during the automated synthesis of Aib-enkephalin pentapeptide. The Oxyma moiety contained in COMU also displayed high nucleophilic stability to the N-terminus of the growing peptide chain, as shown by the absence of Oxyma-based byproducts. Consistent with the previous reports, COMU showed better performance than classical immonium salts HATU and HBTU, which yielded lower percentages of the desired pentapeptide. On the basis of our findings, we conclude that the combination of COMU and microwave irradiation is an efficient approach for SPPS, as it yielded more than 90% of Aib-enkephalin, a highly demanding pentapeptide, in considerably less time than the conventional manual synthesis.

Acknowledgments

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Publicació VIII

PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts †

PyOxP i PyOxB: la família novell de sals de fosfoni basades en Oxyma

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Org. Biomol. Chem., ***2010***, *8 (16), 3665-3673*

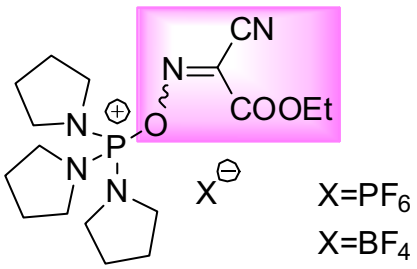
† Ramon Subirós Funosas va realitzar la síntesi dels reactius de fosfoni, així com els experiments d'eficiència en dímers de Aib-encefalina i ACP. Així mateix, el doctorand va realitzar l'escriptura del manuscrit.

Ayman El-Faham va dur a terme els experiments de solubilitat, estabilitat, minimització d'epimerització i la resta d'assajos d'eficiència, inclòs el model cíclic.

Resum

Anteriors estudis han posat de manifest el gran impacte i acollida que ha rebut la nova família de reactius d'acoblament autosuficients basats en el 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma), establint-se en poc temps com un mètode eficient i segur per la preparació de pèptids.

En el treball descrit en aquest apartat es presenta la síntesi i evaluació de les sals de fosfoni derivades d'Oxyma, PyOxP [hexafluorofosfat de *O*-[(1-ciano-2-etoxi-2-oxoetiliden)amino]-oxitris(pirrolidin-1-il)fosfoni] i PyOxB [tetrafluoroborat de *O*-[(1-ciano-2-etoxi-2-oxoetiliden)amino]-oxitris(pirrolidin-1-il)fosfoni]. Ambdues sals d'oni exhibeixen una supressió de l'epimerització del carboni-α en diversos models peptídics i una solubilitat en DMF i DCM, superior a la mostrada pels anàlegs de benzotriazol, fins i to d'HOAt. D'entre aquestes, la sal d'hexafluorofosfat (PyOxP) combina una extraordinària estabilitat amb una gran eficiència en l'elongació de seqüències lineals impedides que inclouen residus Aib consecutius. Els models de ciclació realitzats mostren les avantatges de PyOxP, ja que dóna lloc a una puresa de material cíclic major que la d'altres potents sals de fosfoni, com ara PyClock o PyAOP.



Estructura de les sals de fosfoni que contenen Oxyma: PyOxP i PyOxB.

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PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts†

Ramon Subirós-Funosas,[a,b] Ayman El-Faham*[a,c,d] and Fernando Albericio*[a,b,e]

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Recent studies described the great impact of a non-benzotriazolic family of coupling reagents based on ethyl 2-cyano-2-(hydroxyimino)acetate, Oxyma, as a powerful coupling methodology for peptide synthesis. Here we present the synthesis and evaluation of the derived phosphonium salts *O*-[(1-cyano-2-ethoxy-2-oxoethylidene)amino]-oxytri(pyrrolidin-1-yl) phosphonium hexafluorophosphate (PyOxP) and tetrafluoroborate (PyOxB). Both coupling reagents exhibited higher capacity to suppress racemization in various peptide models and enhanced solubility in DMF and DCM than benzotriazole-based reagents. In addition, the hexafluorophosphate analog PyOxP, combined excellent stability with outstanding efficiency in the assembly of demanding penta and decapeptides that include consecutive Aib residues. Cyclization models revealed the advantages of PyOxP, which rendered a higher percentage of cyclic material than other known potent phosphonium salts.

Introduction

Success in peptide synthesis is highly dependent on the coupling strategy used to form the peptide bond.[1,2] Traditionally, carbodiimides were considered an efficient approach to assemble low to moderate sequences, in consumption with additives (HOBt **1**, HOAt **2** and 6-Cl–HOBt **3**; Fig. 1 left), which lower the likelihood of racemization and other side reactions.[3–5] However, the need for stronger activating reagents led to the introduction of stand-alone onium salts (mainly aminium/uronium and phosphonium ones), which have become the preferred coupling choice in solution- and solid-phase peptide synthesis.[6]

The most powerful onium salts are the aminium/uronium ones. Their structure comprises a carbocation skeleton and an additive, usually benzotriazole-based (HATU **4**, HBTU **5**/TBTU **6**, HCTU **7**/TCTU **8**; Fig. 1 center).[7–9] The tetramethylamino group included in the carbocation skeleton of these classical coupling reagents has recently evolved to a dimethylmorpholino moiety (HDMA **9**, HDMB **10**, 6-Cl–HDMB **11**; Fig. 1 right),[10] thereby resulting in improved stability and solubility in common solvents used in peptide synthesis, as well as an increase in optical purity and reactivity. The high reactivity of both types of aminium/uronium salts may also lead to the appearance of side reactions, usually during slow couplings, such as cyclizations or in the introduction of hindered residues. In these situations, an excess of such a coupling reagent usually causes the attachment of a guanidine moiety to the free N-terminus of the growing peptide chain, thereby blocking any further reaction.[11] Moreover, these reagents are costly for peptide synthesis purposes. Phosphonium salts differ from the former in the nature of the electrophilic core as they have a positively charged phosphorus center and they do not undergo the above mentioned termination of the peptide chain when added in excess. Thus phosphonium salts show similar potency to their aminium/uronium counterparts.[12] These types of onium salts were first introduced by Kenner and coworkers in 1969, although they did not gain general acceptance until the 1990s, when many became commercially available.[13] The most predominant phosphonium salts display a tris(pyrrolidino)phosphonium-type electrophilic core (PyAOP **12**, PyBOP **13**, PyClock, **14**; Fig. 2)[14–16] while their tris(dimethylamino) analogs, of which BOP is the most representative, are slightly less reactive and also originate HMPA as a byproduct, which is a carcinogenic and toxic phosphoramide.[17–19]

In both classes of onium salts, aminium/uronium and phosphonium, the same trend is observed: HOAt (**2**) analogs showed superiority to HOBt (**1**) and 6-Cl–HOBt (**3**) ones in activation and coupling steps and also in reducing the loss of optical purity.[12] In addition, derivatives of 6-Cl–HOBt (**3**) are generally less hazardous and more reactive than HOBt (**1**) counterparts.[9] This relative performance is connected to the acidity of the additive, which is linked to the reactivity of the corresponding active ester. Therefore, PyAOP (**12**) is considered the most reactive phosphonium salt (pK_a of HOAt=3.28), whereas PyClock (**14**) is a good alternative to PyBOP (**13**), because the presence of Cl at position 6 of the benzotriazole ring results in a better leaving group (pK_a of 6-Cl–HOBt=3.35 *vs.* pK_a of HOBt=4.60).[20] However, additional factors contribute to the great difference in reactivity. Thus, it has been proposed that the nitrogen at the 7-position of HOAt creates a neighboring group effect, which results in enhanced reactivity and solubility of its analogs.

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† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C-NMR spectra for PyOxP and PyOxB, and HPLC characterization of racemization and coupling efficiency experiments. See DOI: 10.1039/c003719b

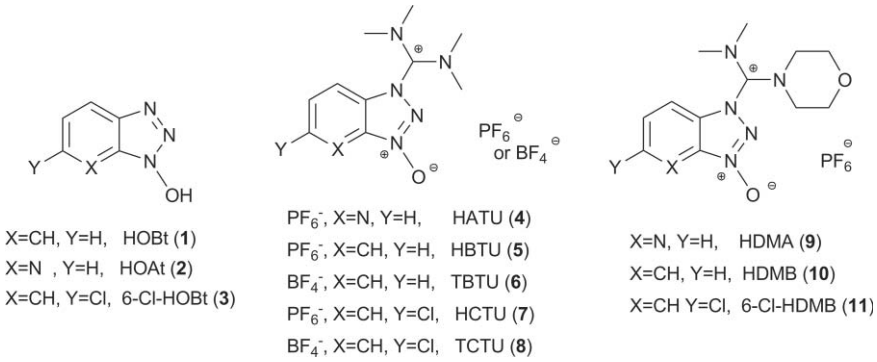


Fig. 1 Benzotriazole-based additives (left), tetramethyl (center) and dimethylmorpholino (right) aminium salts.

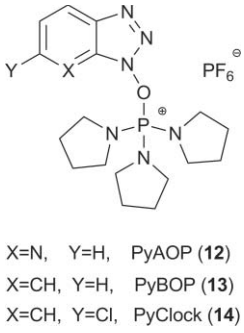


Fig. 2 Benzotriazole-based phosphonium salts.

Recently, we reported ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma **15**, Fig. 3) and its derived uronium salt 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate (COMU **16**, Fig. 3) as efficient additive and coupling reagent for peptide synthesis.[21,22] The active esters formed by these reagents are extremely reactive due to the low hindrance and acidity of Oxyma (pK_a = 4.60), which results in an excellent leaving group.[23] Oxyma (**15**) and COMU (**16**) show superiority to HOBt (**1**) derivatives and a similar or even higher potency than HOAt (**2**) analogs in suppressing racemization and assembling demanding peptide models. In terms of safety, **15** and **16** also have a considerably lower risk of explosion than benzotriazole-based reagents and are compatible with microwave irradiation.[24,25]

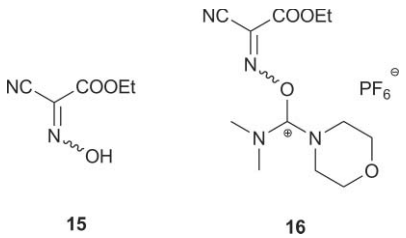


Fig. 3 Oxyma and its derived uronium salt, COMU.

In view of these outstanding results, the next step was to include Oxyma (**15**) in a tris(pyrrolidino)-type phosphonium salt, in both hexafluorophosphate and tetrafluoroborate forms. The synthesis and structure determination of the hexafluorophosphate version was accomplished by Hoffmann *et al.* in 2003, although its suitability as a coupling reagent was not tested.[26] In the present study, we compared the capacity of *O*-[(cyano-(ethoxycarbonyl)methyliden-amino]-yloxytripyrrolidinophosphonium hexafluorophosphate and tetrafluoroborate (PyOxP **17** and PyOxB **18**, Fig. 4) to lower racemization and enhance coupling extension in linear and cyclic peptide models with the abovementioned benzotriazole-based phosphonium salts PyAOP (**12**), PyBOP (**13**) and PyClock (**14**).

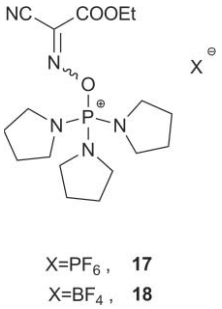
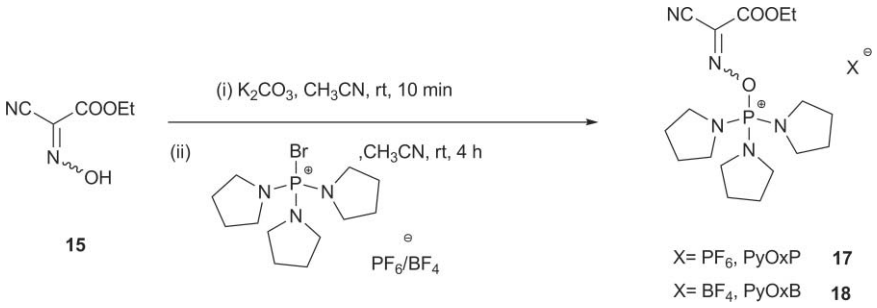


Fig. 4 Structure of the hexafluorophosphate and tetrafluoroborate tris(pyrrolidino)phosphonium salts derived from Oxyma.

Results and discussion

The Oxyma-based phosphonium salts PyOxP (**17**) and PyOxB (**18**) were successfully synthesized in one-pot fashion from Oxyma (**15**) (Scheme 1). The first step consisted of the rapid formation of the intermediate potassium salt of the oxime with potassium carbonate (K_2CO_3) after 10 min of gently stirring. Subsequent addition of PyBroP or the analogous tetrafluoroborate salt to the reaction mixture afforded, after 4 h, the desired phosphonium salts **17** and **18**, in 76% yield after recrystallization from DCM–hexane. Previous formation of the potassium salt is preferred over the addition of a tertiary base to Oxyma (**15**), like Et_3N, to generate the anion, since this method yields $Et_3N^+Br^-$, which is difficult to separate from the desired phosphonium salt.

The stability of phosphonium salts is a basic feature for their optimal performance in peptide synthesis, since they are predominantly used in slow-rate couplings, specially cyclizations, and therefore a rapid hydrolysis of the reagent dramatically decreases their efficiency. In order to check and compare the stability of the proposed oxime-based phosphonium salts with their benzotriazole counterparts, we conducted hydrolysis experiments in an NMR tube (3 days in acetone) and in an open vial (5 days in DMF)



Scheme 1

Table 1 Hydrolytic stability (%) of phosphonium salts during NMR and HPLC assays

Entry	Coupling Reagent	Method of analysis	
		NMR, 3 days (%)[a]	HPLC, 5 h (%)[b]
1	PyAOP (**12**)	10	29
2	PyBOP (**13**)	20	39
3	PyClock (**14**)	50	35
4	PyOxP (**17**)	60	40
5	PyOxB (**18**)	30	25

[a] The samples were dissolved in acetone-d_6 and placed in an NMR tube (closed vial). [b] A solution of the phosphonium salt in DMF was kept at rt in an open vial.

(Table 1). As envisaged, stability in the closed vial was higher than in the open vessel. The hexafluorophosphonium derivative **17** showed slower hydrolysis than its tetrafluoroborate analog **18** in both conditions (entries 4 *vs.* 5). The lower stability of PyOxB (**18**) was evident during the NMR assays, when a freshly prepared solution rapidly changed color (from colorless to yellow in DMF, from yellow to red in DCM), showing 6–7% hydrolysis in NMR. These observations were consistent with the melting points observed for the two Oxyma-based phosphonium salts (131–132 °C for PyOxB *vs.* 185–186 °C for PyOxP). Not only did PyOxP (**17**) show greater stability than PyOxB (**18**), but it was also the most stable phosphonium salt tested, including the benzotriazole analogs PyAOP (**12**), PyBOP (**13**) and PyClock (**14**) (entry 4 *vs.* 1, 2, 3).[12] Amongst these classical reagents, **14** exhibited slower hydrolysis than **12** and a similar rate to **13**, as previously reported (entry 3 *vs.* 1, 2).[16] Finally, in the DMF/DIEA mixture PyOxB (**18**) hydrolyzed the most quickly.

Solubility is also a key feature in determining the suitability of a coupling reagent for peptide synthesis. The solubility of the various phosphonium salts in DMF (generally the solvent of choice in peptide bond formation) and DCM was evaluated (Table 2). All reagents exhibited higher solubility in the more polar solvent (DMF), as expected. The Oxyma derivatives **17** and **18** were considerably more soluble than the benzotriazole ones (**12**, **13**, **14**) in both solvents (entries 4, 5 *vs.* 1, 2, 3). Although displaying lower stability than PyOxP (**17**), PyOxB (**18**) was more soluble, affording up to 3.24 M solutions in DMF (entry 5 *vs.* 4).

Efficient peptide assembly is highly dependent on extension of coupling and yield, but also on the control of optical purity. In order to compare the retention of configuration induced by PyOxP (**17**) and PyOxB (**18**) with that achieved by the benzotriazole-based derivatives **12**, **13** and **14**, three previously studied peptide

Table 2 Solubility of phosphonium salts in DMF and DCM

Entry	Coupling Reagent	DMF		DCM	
		Wt/g mL^{-1}	Molarity	Wt/g mL^{-1}	Molarity
1	PyAOP (**12**)	1.04	1.99	0.92	1.79
2	PyBOP (**13**)	0.80	1.54	0.64	1.23
3	PyClock (**14**)	0.80	1.44	0.40	0.72
4	PyOxP (**17**)	1.36	2.58	1.04	1.97
5	PyOxB (**18**)	1.52	3.24	1.32	2.82

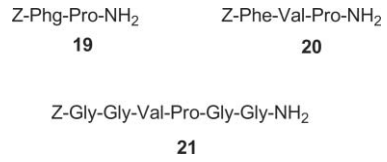


Fig. 5

models were used, which involve stepwise and segment couplings (Fig. 5).[27–29]

The [1+1] stepwise coupling of *Z*-Phg-OH onto H-Pro-NH_2 to afford *Z*-Phg-Pro-NH_2 **19** was performed in the presence of DIEA, in solution and solid phase (Table 3). The Oxyma-based phosphonium salts showed better performance than the benzotriazole-based derivatives in both approaches. The superiority of these

Table 3 Racemization during the [1+1] formation of *Z*-Phg-Pro-NH_2 in DMF (solution and solid-phase synthesis)[a,b,c]

Entry	Coupling approach	Coupling Reagent	DL (%)
1	Solution-phase	PyAOP (**12**)	2.2
2		PyBOP (**13**)	5.8
3		PyClock (**14**)	1.6
4		PyOxP (**17**)	0.3
5		PyOxB (**18**)	0.6
6		PyAOP (**12**)/HOAt (**2**)	2.6
7		PyBOP (**13**)/HOBt (**1**)	5.7
8		PyClock (**14**)/6-Cl-HOBt (**3**)	1.4
9		PyOxP (**17**)/Oxyma (**15**)	0.6
10		PyOxB (**18**)/Oxyma (**15**)	0.6
11	Solid-Phase	PyAOP (**12**)	41.4[d]
12		PyBOP (**13**)	44.2[d]
13		PyClock (**14**)	50.2[d]
14		PyOxP (**17**)	7.4[d]
15		PyOxB (**18**)	10.8[d]

[a] DIEA (2 equiv.) was used as base (for detailed procedure and analysis, see Experimental section).[27] [b] LL and DL forms of the model dipeptide were identified after co-injection with a pure sample of LL. [c] Yields obtained were in the range 88–92%. [d] The percentage in the table is the average of two runs.

Table 4 Racemization during the [2+1] formation of *Z*-Phe-Val-Pro-NH_2 in DMF (solution and solid-phase synthesis) [a,b,c]

Entry	Coupling approach	Coupling Reagent	LDL (%)
1	Solution-phase	PyAOP (**12**)	3.0
2		PyBOP (**13**)	12.5
3		PyClock (**14**)	8.6
4		PyOxP (**17**)	5.7
5		PyOxB (**18**)	5.3
6		PyAOP (**12**)/HOAt (**2**)	1.0
7		PyBOP (**13**)/HOBt (**1**)	8.8
8		PyClock (**14**)/6-Cl-HOBt (**3**)	7.9
9		PyOxP (**17**)/Oxyma (**15**)	3.4
10	Solid-Phase	PyAOP (**12**)	17.3
11		PyBOP (**13**)	29.9
12		PyClock (**14**)	28.6
13		PyOxP (**17**)	23.0
14		PyOxB (**18**)	27.8
15		PyAOP (**12**)/HOAt (**2**)	15.0
16		PyBOP (**13**)/HOBt (**1**)	29.5
17		PyClock (**14**)/6-Cl-HOBt (**3**)	25.2
18		PyOxP (**17**)/Oxyma (**15**)	22.2

[a] TMP (2 equiv.) was used as base (for detailed procedure and analysis, see Experimental section).[27,29] [b] LLL and LDL forms of the model tripeptide were identified after co-injection with a pure sample of LLL. [c] Yields obtained were in the range 88–92%.

Table 5 Yield and racemization during the [3+3] formation of *Z*-Gly-Gly-Val-Pro-Gly-Gly-NH_2 in DMF (solid-phase synthesis)[a,b,c]

Entry	Coupling Reagent	Base (equiv.)	DL (%)
1	PyAOP (**12**)	DIEA (2)	33.7
2	PyBOP (**13**)		51.0
3	PyClock (**14**)		43.1
4	PyOxP (**17**)		22.2
5	PyOxB (**18**)		22.2
6	PyAOP (**12**)	TMP (2)	4.4
7	PyBOP (**13**)		10.2
8	PyClock (**14**)		11.5
9	PyOxP (**17**)		4.1
10	PyOxB (**18**)		6.9

[a] For detailed procedure and analysis see Experimental section. [b] LL and DL forms of the model hexapeptide were identified after co-injection with pure samples. [c] Yields obtained were in the range 88–92%.

salts was greater in the solid-phase synthesis, where racemization with PyOxP (**17**) and PyOxB (**18**) was 4–5 times lower than with PyAOP (**12**), PyBOP (**13**) and PyClock (**14**) (entries 14, 15 *vs.* 11, 12, 13). Among these novel phosphonium salts, **17** afforded the best results regarding optical purity (entries 4, 14 *vs.* 5, 15). In solution, **14** gave lower racemization than **12**, the most potent phosphonium salt known to date (entry 3 *vs.* 1).[16] Addition of the corresponding additive to the onium salt did not have a siginificant effect on the percentage of racemization (entries 6–10 *vs.* 1–5). The performance of the phosphonium salts in stepwise synthesis was also tested in the *Z*-Phe-Ser-Pro-NH_2 model, although this system was not sensitive enough to allow a comparative study (racemization was < 0.1% in all experiments).

The loss of optical purity during the [2+1] segment coupling of dipeptide *Z*-Phe-Val-OH onto H-Pro-NH_2 to yield *Z*-Phe-Val-Pro-NH_2 **20** was higher than in the previous stepwise model. This result is attributed to the greater sensitivity of peptide fragments than single amino acids (Table 4). Given that TMP reduces racemization in segment couplings more efficiently than other bases, its use is recommended in these experiments.[29] Oxyma-based PyOxP (**17**) and PyOxB (**18**) showed a similar performance, giving lower racemizations than HOBt and 6-Cl–HOBt analogs (entries 4, 5 *vs.* 2, 3 and entries 13, 14 *vs.* 11, 12). The addition of additives in peptide model **20** produced a clear increase in the control of optical purity, in contrast to the less sensitive segment coupling **19** (entries 6–9 *vs.* 1–5 and 15–18 *vs.* 10–14).

The retention of configuration in fragment couplings was further tested in the [3+3] formation of the model hexapeptide *Z*-Gly-Gly-Val-Pro-Gly-Gly-NH_2 **21**, a modified version of the well-known system *Z*-Gly-Gly-Val-Ala-Gly-Gly-NH_2, in solid-phase fashion (Table 5).[29] The results indicate the suitability of using the highly hindered base TMP instead of DIEA for segment couplings. Again, Oxyma-based phosphonium salts showed an outstanding performance, regardless of the base used. Of particular note was the percentage of racemization detected with PyOxP (**17**), which was even lower than that recorded for the HOAt analog **12** (entry 4, 9 *vs.* 1, 6).

After addressing loss of optical purity, the coupling efficiency of PyOxP (**17**), the most stable and strongest racemization-suppressing analog of the Oxyma-based phosphonium salts, was tested in the assembly of complex peptide models, based on Leu-enkephalin pentapeptide (H-Tyr-Gly-Gly-Phe-Leu-NH_2), its linear dimer (H-Tyr-Gly-Gly-Phe-Leu-Tyr-Gly-Gly-Phe-Leu-NH_2) and ACP (65–74) decapeptide (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2). The design of the analogs consisted of introducing the hindered α,α-disubstituted amino acid Aib to replace consecutive Gly (Leu-enkephalin and dimer derivatives **22**, **23** and **24**) or Ala (ACP derivative **25**) residues, in order to reduce coupling extension, thereby improving our capacity to distinguish differences between coupling reagents (Fig. 6).

H-Tyr-Aib-Aib-Phe-Leu-NH_2
22

H-Tyr-Aib-Aib-Phe-Leu-Tyr-Gly-Gly-Phe-Leu-NH_2
23

H-Tyr-Gly-Gly-Phe-Leu-Tyr-Aib-Aib-Phe-Leu-NH_2
24

H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2
25

Fig. 6

Coupling efficiency was first evaluated in the synthesis of the highly demanding Aib-enkephalin analog **22** (H-Tyr-Aib-Aib-Phe-Leu-NH_2).[7a] Coupling times of 30 min, except for the Aib-Aib assembly (30 min double coupling), were applied during solid-phase elongation. The pentapeptide/deletion tetrapeptide ratios obtained with the reagents are given in Table 6. Following the trend observed in the previous experiments, PyOxP (**17**) provided higher yield and extent of coupling than PyAOP (**12**), which was the benzotriazole derivative that best performed, followed by PyClock (**14**) (entry 4 *vs.* 1, 2, 3). The desired pentapeptide was obtained with PyBOP (**13**) in a poor 48.6% yield, in contrast to its Oxyma analog **17**, which afforded near quantitative

Table 6 Percentage of Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) and des-Aib deletion tetrapeptide (H-Tyr-Aib-Phe-Leu-NH_2) during solid-phase assembly [a,b]

Entry	Coupling Reagent	Yield (%)	Penta (%)	des-Aib(%)
1	PyAOP (**12**)	89.0	84.8	14.2
2	PyBOP (**13**)	80.2	48.6	47.3
3	PyClock (**14**)	85.0	76.7	23.3
4	PyOxP (**17**)	89.2	98.5	1.5

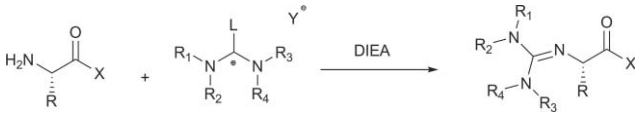
[a] Couplings were performed with 2 equiv. of DIEA [b] Tetrapeptide (des-Aib) was confirmed by peak overlap in the presence of a true sample (for detailed procedure and analysis, see Experimental section).[7a]

Aib-enkephaline **22**. Thus, **17** showed better performance than its benzotriazole counterparts.

The phosphonium salts were further examined in the elongation of the sterically hindered 8,9- and 3,4-Aib analogs of Leu-enkephaline dimer (**23** and **24**), which displays difficult Leu and Tyr couplings.[30] After short couplings, many nonapeptides (des-Phe, des-Leu, des-Tyr, des-Aib and des-Gly) and octapeptides (des-Aib,Leu, des-Aib,Tyr, des-Leu,Tyr, des-Gly,Tyr and des-Gly,Aib) were produced. The introduction of Aib and the adjacent Tyr was the most demanding coupling (Table 7). The experiments with PyAOP (**12**) resulted in an excellent degree of coupling, although PyOxP (**17**) performed better, affording the target decapeptides in >95% yield (entries 4, 8 *vs.* 1, 5) in solid-phase syntheses. In the benzotriazole series, PyClock (**14**) and PyBOP (**13**) obtained similar percentages of the 8,9-analog **23** (entry 3 *vs.* 2); however, in the synthesis of the 3,4-derivative **24**, PyClock (**14**) showed higher potency than its HOBt counterpart **13** (entry 7 *vs.* 6).

To conclude the coupling efficiency assays, the Aib[67]-Aib[68] modified ACP (64–75) decapeptide (H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2) **25** was assembled by means of 5 min couplings, except in the formation of Aib-Ile and Aib-Aib peptide bonds (30 min and 30 min double coupling respectively).[31] Misincorporation of one residue (des-Aib, des-Asn, des-Gln and des- Ile[69]) or two (des-Ile[69],Ile[72]) occurred (Table 8). PyOxP (**17**) allowed the introduction of Aib, Asn and Ile[69] to a greater extent than PyAOP (**12**) and PyBOP (**13**), thereby affording 81% of the Aib-decapeptide **25**. In contrast, the benzotriazole counterparts provided only 64% and 48% yield (entry 3 *vs.* 1,2). Higher yield was also obtained with the Oxyma-based phosphonium salt **17** (83.2%).

Finally, the suitability of PyOxP (**17**) and PyOxB (**18**) as coupling reagents in cyclization steps was tested in the synthesis of cyclic Ala-pentapeptide (H-Ala-Ala-*N*MeAla-Ala-Ala-OH).[32,33] The main risk associated with this step is the capping of the N-terminus amino group. This effect has been reported when aminium/uronium salts are used and it leads to guanidylation and termination of the peptide chain (Scheme 2).



Scheme 2

Once the linear pentapeptide was successfully assembled in solid-phase and cleaved from the resin, a 0.01M solution in DMF was mixed with the corresponding coupling reagent and DIEA. After 24 h, samples were taken and their content was analyzed by reverse-phase HPLC-PDA and HPLC-MS (Table 9).

Table 7 Percentage of 8,9-(H-Tyr-Aib-Aib-Phe-Leu -Tyr-Gly-Gly-Phe-Leu-NH_2) and 3,4-(H-Tyr-Gly-Gly-Phe-Leu-Tyr-Aib-Aib-Phe-Leu-NH_2) Aib-enkephalin decapeptides and deletion peptides during solid-phase assembly[a,b]

Analog	Entry	Coupling Reagent	deca (%)	des-Phe (%)	des-Aib-Leu (%)	des-Aib, Tyr (%)	des-Leu (%)	des- Leu, Tyr (%)	des-Gly, Tyr (%)	des Tyr (%)	des-Aib (%)	des-Gly (%)	des-Gly, Aib (%)
8,9-Aib **23**	1	PyAOP (**12**)	93.3	0.1	—[c]	0.2	0.4	0.2	—[c]	0.9	3.6	1.3	—[c]
	2	PyBOP (**13**)	56.1	0.1	0.3	0.7	0.3	0.2	—[c]	3.4	37.7	1.2	—[c]
	3	PyClock (**14**)	56.2	0.1	0.4	0.1	0.2	0.2	—[c]	2.4	39.1	1.3	—[c]
	4	PyOxP (**17**)	96.5	0.1	—[c]	0.5	0.4	0.2	—[c]	0.1	0.5	1.7	—[c]
3,4-Aib **24**	5	PyAOP (**12**)	85.5	0.3	0.1	0.1	0.3	0.3	0.1	1.2	9.9	1.6	0.6
	6	PyBOP (**13**)	51.5	0.1	0.2	0.3	0.1	0.7	0.1	0.5	44.8	1.2	0.6
	7	PyClock (**14**)	71.5	1.0	0.1	0.1	0.1	0.5	0.1	1.0	24.6	0.8	0.3
	8	PyOxP (**17**)	95.4	0.1	0.1	0.1	0.3	0.7	0.1	1.1	0.5	1.5	0.1

[a] Couplings were performed with 2 equiv. of DIEA (for detailed procedure and analysis, see Experimental section). [b] Apart from the detailed deletion peptides, unknown peaks were observed in all cases at 5.2 and 5.8 min in the 8,9 analog and at 4.6 and 6.6 min in the 3,4-analog (in the range 0.5–3.0%). [c] No misincorporation was detected.

Table 8 Percentage of Aib[67]-Aib[68] analog of ACP (64–75) decapeptide (H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2) and deletion peptides during solid-phase assembly [a,b]

Entry	Coupling Reagent	Yield (%)	Aib-deca (%)	des-Aib (%)	des-Asn (%)	des-Gln (%)	des-Ile[69], Ile[72] (%)	des-Ile[69] (%)
1	PyAOP (**12**)	81.0	64.0	12.0	3.8	1.0	1.0	10.4
2	PyBOP (**13**)	78.0	48.0	32.0	2.0	0.8	5.0	12.0
3	PyOxP (**17**)	83.2	81.0	2.2	2.2	1.0	1.7	10.1

[a] Fmoc-amino acids were preactivated with coupling reagents in the presence of 1 equiv. of DIEA and then, after addition to the resin, a second equiv. of DIEA was added (for detailed procedure and analysis, see Experimental section). [b] Other unknown peaks were observed, but these were not related to the peptide.

Table 9 Cyclization of H-Ala-Ala-*N*MeAla-Ala-Ala-OH in DMF after 24 h (10^{-2} M).[a,b]

Entry	Coupling Reagent	Cyclic penta (%)	Linear penta (%)	Linear dimer (%)
1	PyAOP (**12**)	54	10	36
2	PyBOP (**13**)	43	28	29
3	PyClock (**14**)	61	15	24
4	PyOxP (**17**)	70	10	20
5	PyOxB (**18**)	47	15	38

[a] The linear pentapeptide was elongated on a 2-chlorotrityl resin by means of DIC/HOAt (**2**)-mediated couplings. [b] 4 equiv. excess of DIEA was used (for detailed procedure and analysis, see Experimental section). [c] In analogous cyclizations performed with HDMA (**9**) and COMU (**16**) in DCM, a high amount of guanidylated material was found (90% and 60% respectively), thus confirming the unsuitability of uronium salts in cyclization steps.

In all experiments, the cyclic product was detected along with the starting linear pentapeptide and the linear dimer. PyOxP (**17**) afforded the highest percentage of cyclized material, closely followed by PyClock (**14**), which showed stronger performance than its HOAt derivative PyAOP (**12**) (entry 4 *vs.* 1, 3). PyBOP (**13**) and PyOxB (**18**) were the least efficient in providing the cyclic pentapeptide, thus confirming the superiority of the hexafluorophosphate version of the Oxyma-based phosphonium salt over the tetrafluoroborate counterpart (entry 4 *vs.* 5).

Conclusions

In summary, PyOxP (**17**) and PyOxB (**18**), the hexaflurophosphate and tetrafluoroborate phosphonium salts derived from Oxyma (**15**), have been successfully obtained in a one-pot procedure from ethyl 2-cyano-2-(hydroxyimino)acetate. This was achieved through reaction of the intermediate potassium salt and PyBroP or its analogous tetrafluoroborate salt to afford the reagents in good yields and high purities. The presence of Oxyma (**15**) conferred the reagents higher solubility in standard solvents for peptide synthesis than PyAOP (**12**), PyBOP (**13**) and PyClock (**14**). Consistently, the analysis of racemization models, involving either [1+1] stepwise or [2+1] and [3+3] segment couplings, showed a higher control of optical purity of PyOxP (**17**) and PyOxB (**18**) over their HOBt and 6-Cl–HOBt counterparts, and similar performance to that of the HOAt derivative.

Although PyOxP (**17**) and PyOxB (**18**) allow the preparation of more concentrated solutions, the use of the former is preferred in view of its higher stability and coupling efficiency, both in the assembly of linear and cyclic peptide models. Apart from being more stable in solution than PyAOP (**12**), PyBOP (**13**) and PyClock (**14**), PyOxP (**17**) also showed greater coupling performance during the elongation of the sterically demanding Leu-enkephalin and ACP (64–75) derivatives that present consecutive Aib residues. With respect to the cyclization of H-Ala-Ala-NMeAla-Ala-Ala-OH, PyOxP (**17**) afforded the cyclized product in the highest percentage, superior to that achieved in the synthesis with PyAOP and with a lower percentage of the linear by-products.

In conclusion, PyOxP (**17**) showed superiority not only over PyBOP (**13**) and PyClock (**14**), but also over PyAOP (**12**), the most potent phosphonium salt known to date. Moreover, given its high stability in open and closed vials, the use of PyOxP is advantageous and highly recommended in automated peptide synthesizers.

Experimental

Materials

The solvents used were of HPLC reagent grade. Oxyma Pure was obtained from Luxembourg Biotechnologies. Melting points were measured in open capillary tubes with a Buchi B-540 melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR magnetic resonance spectra were recorded on a Varian Mercury 400 MHz spectrometer at rt with chemical shift values reported in δ units (ppm) relative to tetramethylsilane (TMS) as internal standard. For analytical separation and characterization, a reverse-phase Waters 2695 HPLC separation module, coupled to a Waters 2998 PDA UV detector, was used. Chromatograms were processed with Empower software. Peptide mass was detected by means of an HPLC-PDA system as described above, coupled to a Waters Micromass ZQ mass detector, using the MassLynx 4.1 software. Elemental analyses were performed on a Perkin-Elmer 2400 elemental analyzer, and the values found were within ±0.3% of the theoretical values. Follow-up of the synthesis of the phosphonium salts was done by TLC on silica gel-protected aluminium sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for a few seconds. The compounds were named using ChemBioDraw Ultra version 12.0, Cambridge soft Corporation. Samples were introduced into a Waters Synapt HDMS mass spectrometer by direct infusion and exact masses were determined (ESI positive polarity, W analyzer, 3000 V capillary voltage, 150 °C and 100 °C desolvation and source temperature, 40 V sample cone, 100–1500 m/z).

One-pot synthesis of PyOxP (17) and PyOxB (18)

Oxyma (20 mmol; 2.84 g) was suspended in ACN (50 mL) and potassium carbonate (10 mmol; 1.38 g) was added. The reaction mixture was stirred at rt for 10 min and then 20 mmol of the bromophosphonium salt (PyBroP or its tetrafluoroborate analog) was added under nitrogen atmosphere. The reaction mixture was stirred at rt for 4 h, filtered from the precipitate and washed with ACN (10 mL). The filtrate was concentrated under vacuum and diethylether was added with stirring to provide the phosphonium salts in 76% yield (7.9 g), after recrystallization from DCM–hexane. PyOxP: mp = 185–186 °C; ^{1}H NMR ($CDCl_3$): δ 1.41 (t, 3H, CH_3), 2.01–2.04 (m, 12H, 6 C-CH_2), 3.37–3.41 (m, 12H, 6 N-CH_2), 4.47 (q, 2H, CH_2) ppm; ^{13}C NMR ($CDCl_3$):δ 14.00, 26.39, 48.29, 65.07, 106.31, 135.80, 135.95, 155.87 ppm.; elemental analysis (%) calculated for $C_{17}H_{29}F_6N_5O_3P_2$ (527.38): C, 38.72; H, 5.54; F, 21.61; N, 13.28. Found: C, 38.59; H, 5.45; N, 13.38. PyOxB: mp = 131–132 °C; H^1 NMR (acetone-d_6): δ 1.36 (t, 3H, CH_3), 2.04–2.05 (m, 12H, 6 C-CH_2), 3.51–3.53 (m, 12H, 6 N-CH_2), 4.45 (q, 2H, CH_2) ppm,^{13}C NMR (acetone-d_6):δ 13.47, 26.21, 47.97, 64.28, 106.97, 136.69, 136.84, 156.36 ppm. A sample for exact mass determination was prepared, dissolving in CH_3CN and diluting 1 to 100 in H_2O–CH_3CN (1 : 1): $[M]^+$=382.2009. PyOxP and PyOxB were also characterized by HPLC, using a Sun fire C_{18} (3.5 μm, 4.6 × 100 mm) column, linear gradient 5 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (PyOxP)= 5.19 min, t_R (PyOxB) = 5.17 min.

Racemization tests using model peptides in solution and solid phase[27,29]

Solution-phase tests were conducted adding the coupling reagent (0.125 mmol, 1 equiv.) to a solution of the amino acids (1 equiv. each) in DMF (1 mL) in the presence of base (2 equiv.) at 0 °C. The resulting mixture was stirred for 3–4 h in an ice bath. The work-up of the reaction mixture was as previously described for *Z*-Phg-Pro-NH_2 and *Z*-Phe-Val-Pro-NH_2.[27] For solid-phase assays, the coupling was performed with H-Pro-RinkAmide-PS-resin or H-Pro-Gly-Gly-RinkAmide-PS-resin, 3 equiv. of the corresponding acid (*Z*-Phg-OH, *Z*-Phe-Val-OH or *Z*-Gly-Gly-Val-OH) and 3 equiv. of the corresponding coupling reagent in DMF at rt, with preactivation of 10–30 s with 6 equiv. of base (DIEA or TMP).[22] The reaction mixture was left 3–4 h at rt. The peptide was recovered after cleavage from the resin with 50% TFA in DCM for 1 h at rt. TFA and DCM were removed *in vacuo* and the crude peptide was precipitated with ether or dissolved in ACN and injected directly into the HPLC apparatus. Crude products from the solution- and solid-phase tests were analyzed by reverse-phase HPLC-PDA, using a Sun fire C_{18} (3.5 μm, 4.6 × 100 mm) column, linear gradient of 20 to 50% (*Z*-Phg-Pro-NH_2) over 20 min, linear gradient of 20 to 70% (*Z*-Phe-Val-Pro-NH_2) over 8 min or linear gradient of 10% to 60% (*Z*-Gly-Gly-Val-Pro-Gly-Gly-NH_2) CH_3CN/0.036% TFA in H_2O/0.045% TFA over 8 min; flow = 1.0 mL min^{-1} detection at 220 nm. t_R (*Z*-L-Phg-Pro-NH_2) = 12.51 min, t_R (*Z*-D-Phg-Pro-NH_2) = 13.07 min, t_R (*Z*-Phe-L-Val-Pro-NH_2) = 6.95 min, t_R (*Z*-Phe-D-Val-Pro-NH_2) = 7.43 min, t_R (*Z*-Gly-Gly-L-Val-Pro-Gly-Gly-NH_2) = 5.53 min, t_R (*Z*-Gly-Gly-D-Val-Pro-Gly-Gly-NH_2) = 5.91 min.

Solid-phase synthesis of Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2)[7a]

The synthesis was carried out in a plastic syringe attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-AM-PS (loading = 0.63 mmol g^{-1}, 100 mg) resin was swelled in DCM and conditioned in DMF (2 × 10 mL each). The resin was deblocked by treatment with 20% piperidine in DMF (10 mL) for 10 min. The resin was washed with DMF, DCM and DMF (2 × 10 mL each) and then acylated with a solution of Fmoc-Leu-OH (3 equiv.) and coupling reagent (3 equiv.) in 0.4 mL of DMF, previously preactivated with DIEA (6 equiv.) for 1–2 min before addition to the resin. The reaction mixture was stirred slowly for 1 min and allowed to couple for 30 min (30 min double coupling for Aib-Aib peptide bond formation). The resin was washed with DMF, and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM and DMF (2 × 10 mL each) and then coupled with the next amino acid to obtain the pentapeptide. The peptide was cleaved from the resin after treatment with TFA–H_2O (9 : 1) at rt for 2 h. TFA was removed *in vacuo* and the crude peptide was precipitated with diethyl ether. The weight of the crude product was recorded and purity was determined by reverse-phase HPLC-PDA, using a Sun fire C_{18} (3.5 μm, 4.6 × 100 mm) column, linear gradient 0 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 15 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (penta) = 6.68 min, $[M+H]^+$=611.0; t_R (des-Aib) = 6.78 min, $[M+H]^+$=526.4. Neither des-Tyr (448.3) nor tripeptide (363.5) were observed.

Solid-Phase Synthesis of Aib-ACP (65–74) decapeptide (H-Val-Gln-Aib[67]-Aib[68]-Ile[69]-Asp-Tyr-Ile[72]-Asn-Gly-NH_2)[31]

The synthesis was conducted in a plastic syringe attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-MBHA-PS resin (loading = 0.45 mmol g^{-1}, 100 mg) was swelled in DCM and conditioned in DMF (2 × 10 mL each). The Fmoc-protecting group was removed by treatment with 20% piperidine in DMF (10 mL) for 10 min, and the resin was washed with DMF, DCM and DMF (2 × 10 mL each). Acylation took place by mixing the resin with a solution of Fmoc-Gly-OH (3 equiv.), and coupling reagent (2 equiv.) in 0.4 mL DMF, previously preactivated with DIEA (2 equiv.) for 1–2 min, followed by addition of extra 2 equiv. of DIEA. The reaction mixture was stirred slowly for 1 min and allowed to couple for 5 min (30 min coupling for Aib-Ile and 30 min double coupling in case of Aib-Aib). The resin was washed with DMF, and then deblocked with a solution of 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, DMF (2 × 10 mL each) and then coupled with the next amino acid to obtain the pentapeptide. The peptide was cleaved from the resin by treatment with TFA–H_2O (9 : 1) at rt for 2 h. TFA was removed *in vacuo* and the crude peptide was precipitated with ether. The precipitate was then redissolved in H_2O and lyophilized. The weight of the crude product was recorded and the purity was determined by HPLC analysis, using a Sun fire C_{18} (3.5 μm, 4.6 × 100 mm) column, linear gradient 10 to 35% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (Aib-ACP) = 6.41 min, $[M+H]^+$=1091.2; t_R (des-Aib) = 6.57 min, $[M+H]^+$ = 1005.1; t_R (des-Asn) = 6.67 min, $[M+H]^+$ = 997.2; t_R (des-Gln) = 6.95 min, $[M+H]^+$ = 963.4; t_R (des-Ile[69], Ile[72]) = 5.12 min, $[M+H]^+$= 863.9; t_R (des-Ile[69]) = 6.22 min, $[M+H]^+$= 976.5.

Solid-phase synthesis of 8,9-Aib enkephalin decapeptide (H-Tyr-Aib-Aib-Phe-Leu-Tyr-Gly-Gly-Phe-Leu-NH_2)[30]

The synthesis was carried out in a plastic syringe attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The resin Fmoc-Rink-amide-AM-PS (loading = 0.63 mmol g^{-1}, 100 mg) resin was swelled in DCM and conditioned in DMF (2 × 10 mL each). The resin was deblocked by treatment with 20% piperidine in DMF (10 mL) for 10 min. The resin was washed with DMF, DCM and DMF (2 × 10 mL each) and then acylated with a solution of Fmoc-Leu-OH (3 equiv.) and coupling reagent (3 equiv in 0.4 mL of DMF, previously preactivated with DIEA (6 equiv.) for 1–2 min before addition to the resin. The reaction mixture was stirred slowly for 1 min and allowed to couple for 30 min (30 min double coupling for Aib-Aib coupling). The resin was washed with DMF and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM and DMF (2 × 10 mL each) and then coupled with the next amino acid to obtain the pentapeptide. The peptide was cleaved from the resin after treatment with TFA–H_2O (9 : 1) at rt for 2 h. TFA was removed *in vacuo* and the crude peptide was precipitated with diethyl ether. The weight of the crude product was recorded and purity was determined by reverse-phase HPLC-PDA, using a XBridge C_{18} (2.5 μm, 4.6 × 75 mm) column, linear gradient 30 to 37% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O

over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (des-Phe) = 3.31 min, $[M+H]^+$ = 1001.5; t_R (des-Aib,Tyr) = 3.65 min, $[M+H]^+$ = 900.6; t_R (des-Leu) = 3.74 min, $[M+H]^+$ = 1035.5; t_R (des-Leu,Tyr) = 4.26 min, $[M+H]^+$ = 872.5; t_R (des-Aib,Leu) = 4.40 min, $[M+H]^+$ = 950.5; t_R (des-Tyr) = 4.82 min, $[M+H]^+$ = 985.7; t_R (decapeptide) = 6.17 min, $[M+H]^+$ = 1148.7; t_R (des-Aib) = 6.73 min, $[M+H]^+$ = 1063.6; t_R (des-Gly) = 8.11 min, $[M+H]^+$ = 1091.6.

Solid-phase synthesis of 3,4-Aib enkephalin decapeptide (H-Tyr-Gly-Gly-Phe-Leu-Tyr-Aib-Aib-Phe-Leu-NH_2)[30]

The synthesis was carried out in a plastic syringe attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The resin Fmoc-Rink-amide-AM-PS (loading = 0.63 mmol g^{-1}, 100 mg) resin was swelled in DCM and conditioned in DMF (2 × 10 mL each). The resin was deblocked by treatment with 20% piperidine in DMF (10 mL) for 10 min. The resin was washed with DMF, DCM and DMF (2 × 10 mL each) and then acylated with a solution of Fmoc-Leu-OH (3 equiv.) and coupling reagent (3 equiv.) in 0.4 mL of DMF, previously preactivated with DIEA (6 equiv.) for 1–2 min before addition to the resin. The reaction mixture was stirred slowly for 1 min and allowed to couple for 30 min (only 10 min for the last five residues). The resin was washed with DMF and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, DMF (2 × 10 mL each) and the coupling and deblocking cycles were repeated to obtain the decapeptide. The peptide was cleaved from the resin after treatment with TFA–H_2O (9 : 1) at rt for 2 h. TFA was removed *in vacuo* and the crude peptide was precipitated with diethyl ether. The weight of the crude product was recorded and purity was determined by reverse-phase HPLC-PDA, using a XBridge C_{18} (2.5 μm, 4.6 × 75 mm) column, linear gradient 32 to 35% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (des-Phe) = 3.43 min, $[M+H]^+$ = 1001.4; t_R (des-Aib,Leu) = 3.71 min, $[M+H]^+$ = 950.6; t_R (des-Leu) = 4.27 min, $[M+H]^+$ = 1035.5; t_R (des-Leu,Tyr) = 4.81 min, $[M+H]^+$ = 872.5; t_R (des-Gly,Tyr) = 5.69 min, $[M+H]^+$ = 928.7; t_R (des-Tyr) = 5.90 min, $[M+H]^+$ = 985.8; t_R (des-Aib,Tyr) = 6.24 min, $[M+H]^+$ = 950.5; t_R (decapeptide) = 7.54 min, $[M+H]^+$ = 1148.7; t_R (des-Aib) = 8.21 min, $[M+H]^+$ = 1063.7; t_R (des-Gly) = 8.53 min, $[M+H]^+$ = 1091.8; t_R (des-Gly,Aib) = 9.04 min, $[M+H]^+$ = 1006.8

Synthesis and cyclization of H-Ala-Ala-NMeAla-Ala-Ala-OH.[32,33]

A: Solid-phase synthesis.[32]. The synthesis was conducted in a plastic syringe attached to a vacuum manifold in order to effect rapid removal of reagents and solvent. The 2-chlorotritylchloride resin (loading = 1.55 mmol g^{-1}; 1 g) was swelled in DCM (3 × 50 mL), and then acylated with Fmoc-Ala-OH (2 mmol) and DIEA (14 mmol) in 10 mL of DCM. The reaction mixture was stirred slowly for 1 min and allowed to couple for 15 min. Then, an extra 6 mmol of DIEA was added and the resin was left to stand with stirring from time to time for 45 min. 1 mL of MeOH was added to the resin, stirred for 5 min and allowed to stand for an additional 5 min. The resin was filtered and then washed with DCM and DMF (2 × 50 mL each). Deblocking of the N-terminus was accomplished with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF (2 × 50 mL each) and then coupled with the next Fmoc-Ala-OH (3 mmol) by means of DIC (3 mmol), and HOAt (3 mmol) for 1 h (1 h double coupling for the coupling of Fmoc-Ala-OH with H-NMeAla-Ala-Ala-Resin). The coupling and deblocking cycles were repeated to obtain the pentapeptide. The peptide was cleaved from the resin by treatment with 50 mL of TFA/DCM (2%) at rt for 5 min. The resin was filtered and then washed with an extra solution of 2% in TFA/DCM (20 mL). TFA and DCM were removed *in vacuo* and the crude peptide was precipitated with cold diethyl ether. The precipitate was then collected and washed two extra times. After drying the peptide under vacuum (1.0 g, 66.5% yield), purity (99.0%) was determined by reverse-phase HPLC analysis using a Sun fire C_{18} (3.5 μm, 4.6 × 100 mm) column, linear gradient 5 to 25% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (penta Ala) = 2.99 min, $[M+H]^+$ = 388.2.

B: Cyclization. The cyclization step was performed as reported in a previous study.[32] The linear pentapeptide (0.1 mmol) was cyclized by treatment with the corresponding coupling reagent (0.11 mmol) and 0.44 mmol of DIEA in DMF (10 mL, 0.01 M). After removing DMF under reduced pressure, the crude product was extracted with methanol and precipitated with ether. The sample was dissolved with 100 μl of 1%TFA/H_2O, completing the volume to 1 ml with ACN. 10 μl of the resulting solution was injected into a reverse-phase HPLC apparatus using a Sun fire C18 (3.5 μm, 4.6 × 100 mm) column, linear gradient 5 to 55% of 0.036% TFA in CH_3CN/0.045%TFA in H2O over 8 min; flow rate = 1.0 ml min^{-1}; detection at 220 nm; t_R (linear pentapeptide) = 2.65 min, $[M+H]^+$ = 388.2; t_R (cyclic pentapeptide)= 7.12 min, $[M+H]^+$ = 370.7; t_R (linear dimer) = 8.67 min, $[M+H]^+$ = 739.5.

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Notes and references

1 Abbreviations not defined in text: Aib, α-aminoisobutyric acid; ACN, CH_3CN, ACP, acyl carrier protein decapeptide (65–74); AM-PS, aminomethyl-polystyrene resin; DIC, *N*,*N*′-diisopropylcarbodiimide; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; COMU, 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate DCM, dicloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; HOAt, 7-aza-1-hydroxybenzotriazole; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HCTU, *N*-[(6-chloro-1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HDMA, 1-((dimethylimino)(morpholino)-methyl)3-*H*-[1,2,3]triazolo[4,5-*b*]pyridine-1-3-olate hexafluorophosphate; HDMB, 1-((dimethylimino)(morpholino)methyl)3-*H*-benzo-[1,2,3]triazolo-1-ium-3-olate hexafluorophosphate; 6-Cl-HDMB,

1-((dimethylimino)(morpholino)methyl)3-*H*-6-chlorobenzo[1,2,3]triazolo-1-ium-3-olate hexafluorophosphate; MBHA-PS, 4-methylbenzhydrylamine resin; Oxyma, ethyl 2-cyano-2-(hydroxyimino) acetate; Phg, α-phenylglycine; PyAOP, azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyClock, 6-chloro-benzotriazol-1-yl-*N*-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyOxB, *O*-[(1-cyano-2-ethoxy-2-oxoethylidene)amino]-oxytri(pyrrolidin-1-yl) phosphonium tetrafluoroborate; PyOxP, *O*-[(1-cyano-2-ethoxy-2-oxoethylidene)amino]-oxytri(pyrrolidin-1-yl) phosphonium hexafluorophosphate TBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TCTU, *N*-[(6-chloro-1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]*N*-methylmethanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; TMP, 2,4,6-trimethylpyridine (collidine); Z, benzyloxycarbonyl; Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.*, 1972, **247**(4), 977–983].

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Suplementary Information

PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts

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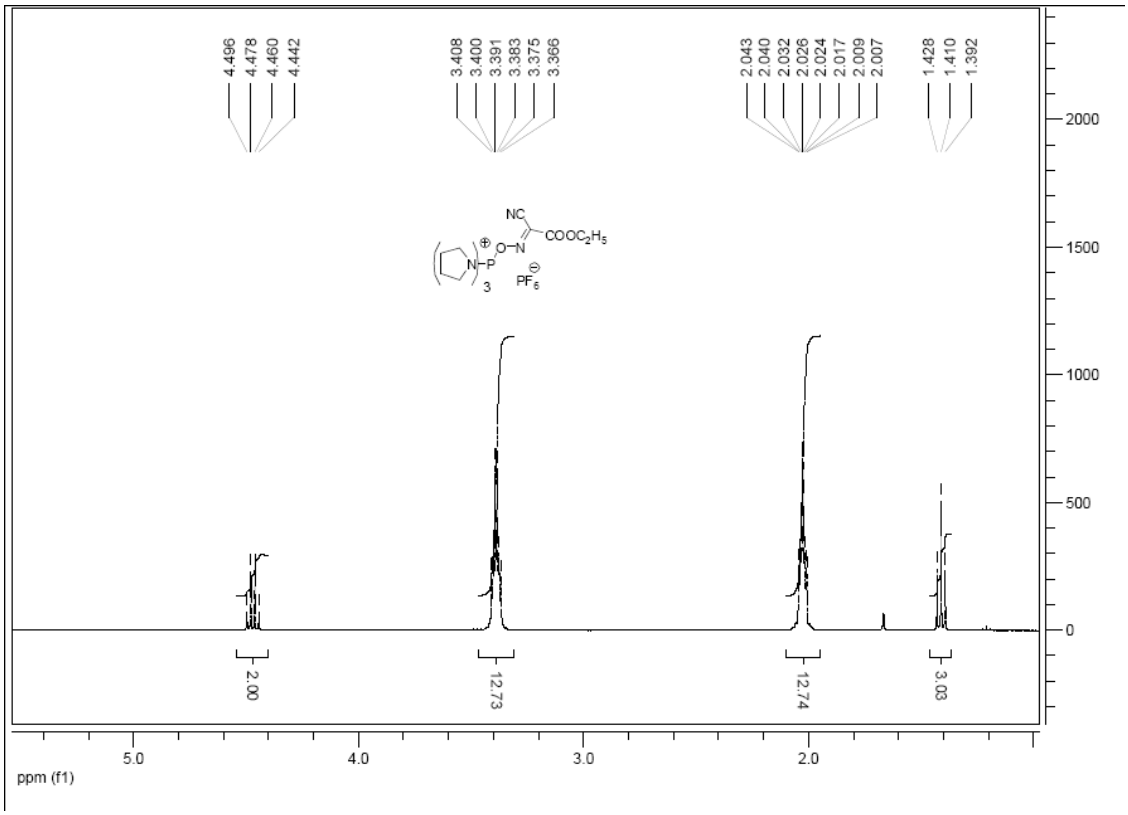

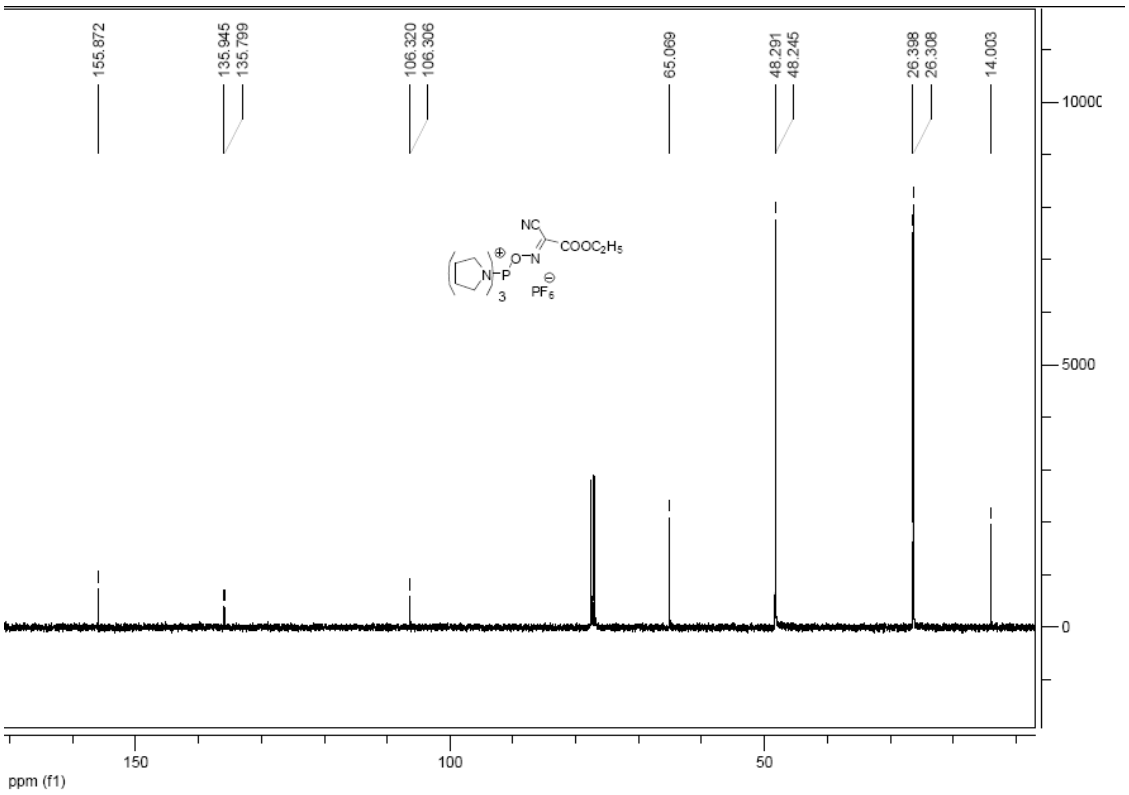
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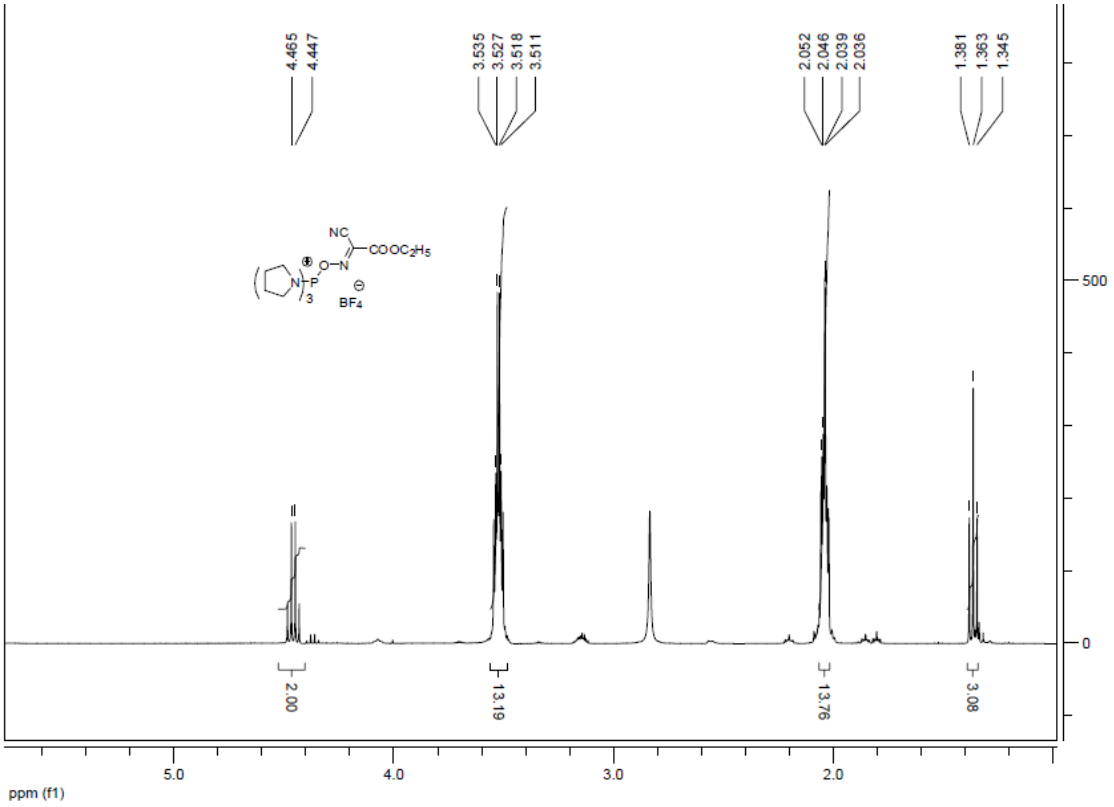
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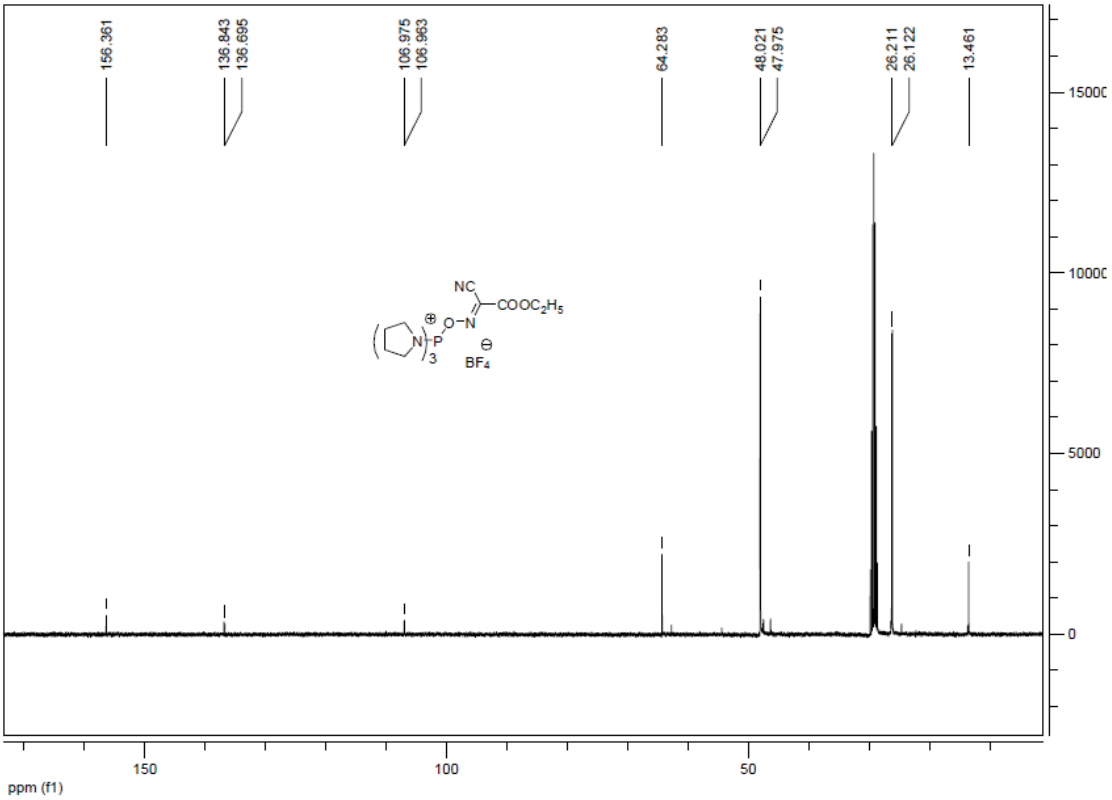
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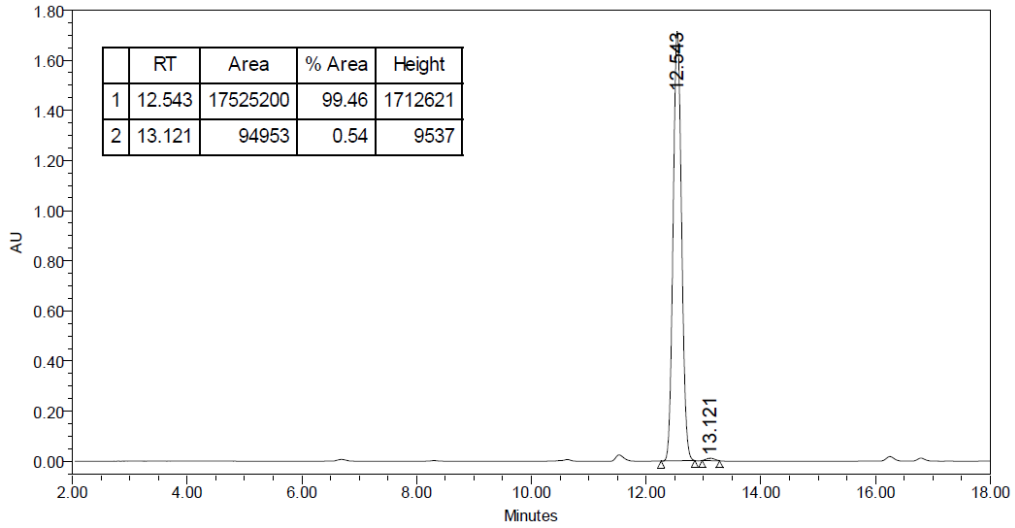
P
3
BF4

- **Z-Phg-Pro-NH_2 (Solution phase,PyOxP)**



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1	12.543	17525200	99.46	1712621
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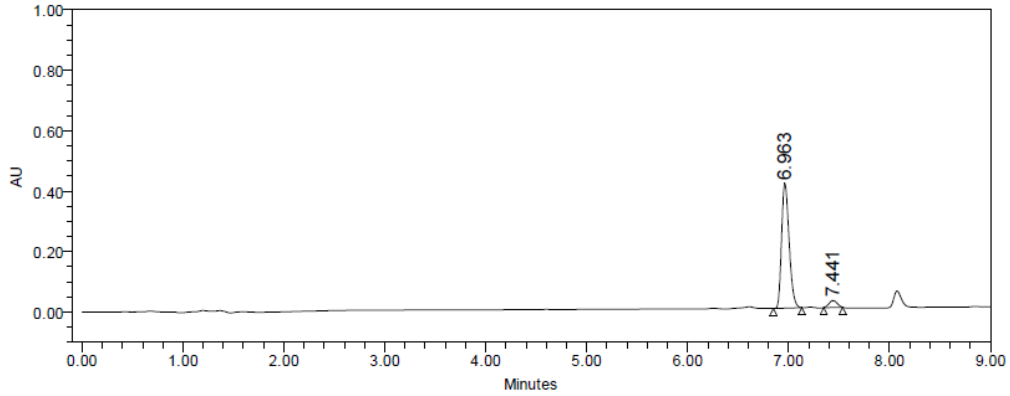
- **Z-Phe-Val-Pro-NH_2 (Solution phase, PyOxP)**



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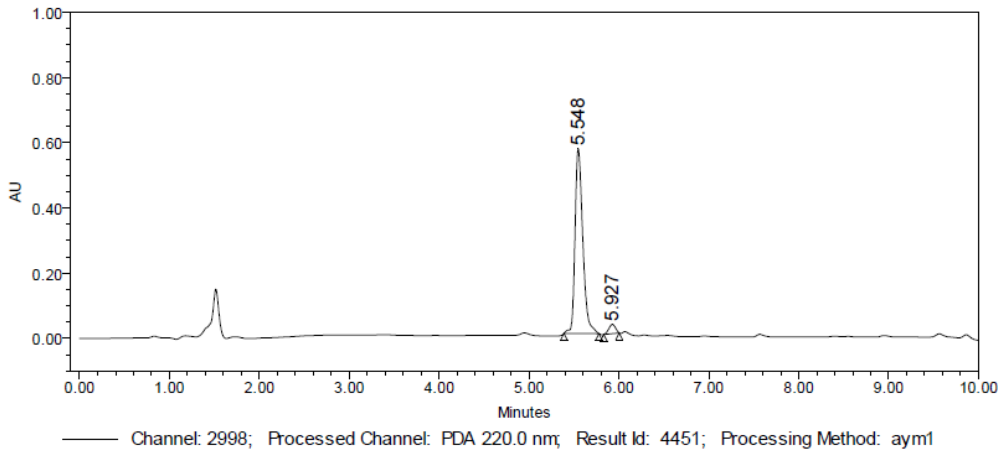
1	6.963	2146215	94.26	412808
2	7.441	130789	5.74	23430

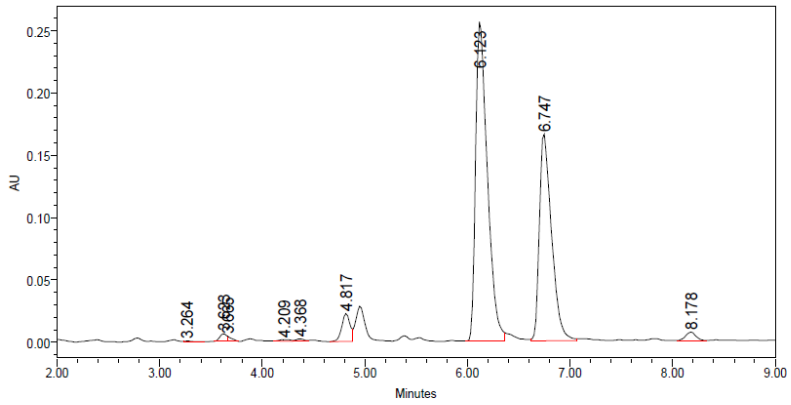
- **Z-Gly-Gly-Val-Pro-Gly-Gly-NH_2 (TMP, PyOxP)**



Processed Channel Descr.: PDA 220.0 nm

	Processed Channel Descr.	RT	Area	% Area
1	PDA 220.0 nm	5.548	3406698	95.86
2	PDA 220.0 nm	5.927	147132	4.14

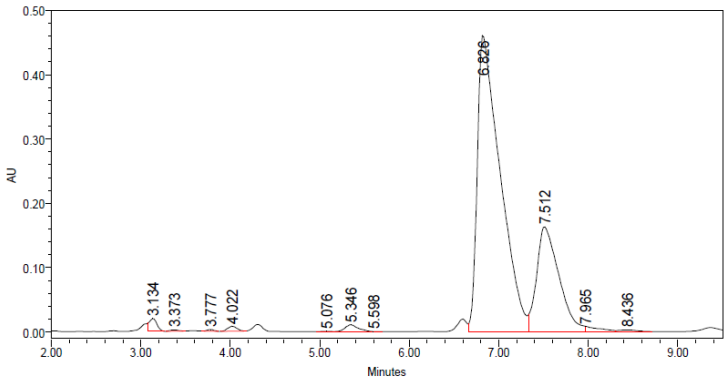
- **8,9-Aib-enkephalin decapeptide (PyBOP)**



Peak Results

	RT	Area	Height	Total Area	% Area
1	3.264	3132	575	3642306	0.09
2	3.623	27849	6079	3642306	0.76
3	3.665	9821	3591	3642306	0.27
4	4.209	7593	971	3642306	0.21
5	4.368	10408	1783	3642306	0.29
6	4.817	122722	21997	3642306	3.37
7	6.123	2043712	258129	3642306	56.11
8	6.747	1371699	165531	3642306	37.66
9	8.178	45370	6754	3642306	1.25

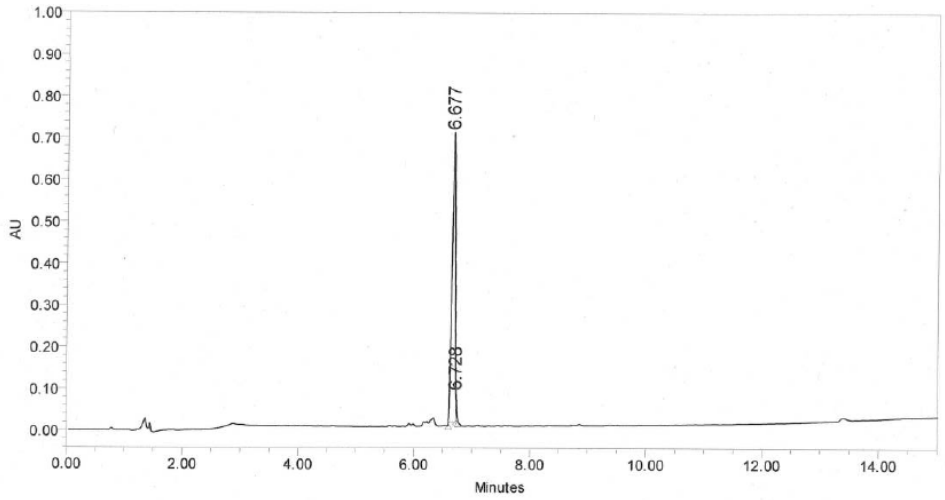
- **3,4-Aib-enkephalin decapeptide (PyClock)**



Peak Results

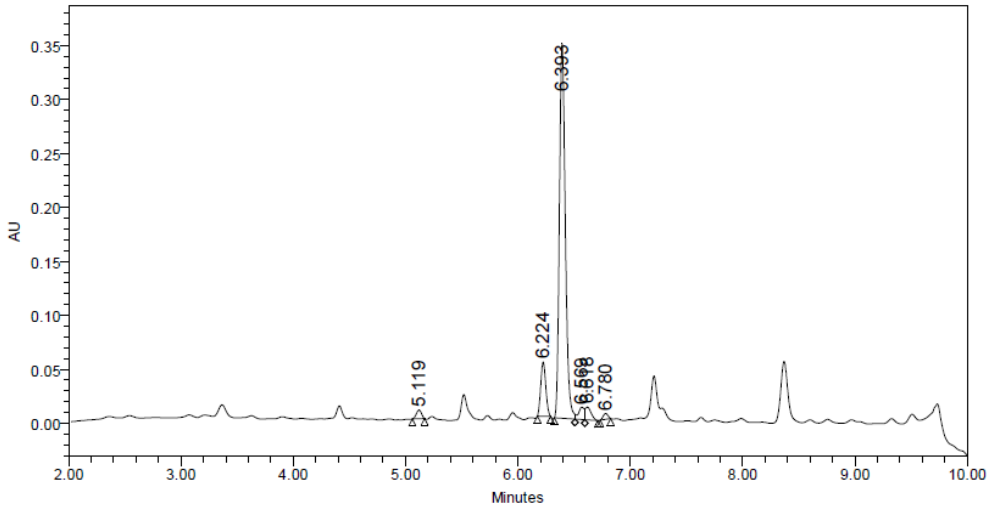
	RT	Area	Height	Total Area	% Area
1	3.134	115458	19562	11421075	1.01
2	3.373	12728	1990	11421075	0.11
3	3.777	14301	2600	11421075	0.13
4	4.022	53650	7427	11421075	0.47
5	5.076	6530	873	11421075	0.06
6	5.346	116747	10894	11421075	1.02
7	5.598	760	235	11421075	0.01
8	6.826	8169101	464691	11421075	71.53
9	7.512	2804617	163023	11421075	24.56
10	7.965	89971	8235	11421075	0.79
11	8.436	37211	2692	11421075	0.33

- **Aib-enkephalin pentapeptide (PyOxP)**



	RT	Area	% Area	Height
1	6.677	2762192	98.55	693691
2	6.728	40643	1.45	67262

- **Aib-ACP decapeptide (PyOxP)**



	RT	Area	% Area	Height
1	5.119	25692	1.66	8086
2	6.224	156714	10.12	50323
3	6.393	1265508	81.75	347229
4	6.569	41255	2.66	11538
5	6.618	42908	2.77	11883
6	6.780	15982	1.03	5421

Discussió Global

Discussió global

En capítols anteriors s'han descrit els estudis realitzats en la present Tesi Doctoral en referència a la utilització d'Oxyma i reactius derivats, d'aplicació en metodologia de síntesi peptídica. És la voluntat d'aquest apartat aprofundir en el comportament de Oxyma i les espècies reactives anàlogues, així com debatre de forma global els avantatges que comporta el seu ús vers altres compostos presents al mercat.

❖ *Reactivitat d'Oxyma i sals d'oni derivades*:

L'acidesa del grup *N*-hidroxilamino és clau per explicar el comportament i l'eficiència tant de les *N*-hidroxilamines usades com a additius per carbodiimides com la dels reactius d'acoblament "autosuficients" que se'n deriven. Un cop format l'èster actiu corresponent, independentment del mètode d'activació utilitzat, l'habilitat de la *N*-hidroxilamina com a grup sortint és clau en la rapidesa i extensió d'acilació, el qual està intimament lligat a aquest acidesa. En la sèrie benzotriazòlica, per exemple, la reactivitat relativa observada per HOBt (pK_a=4.60), 6-Cl-HOBt (pK_a=3.35) i HOAt (pK_a=3.28) és coherent amb l'escala d'acidesa que presenten.[1] La relació acidesa/reactivitat es manté en comparar diferents famílies. Així, la benzotriazina HODhbt (pK_a=3.97), d'acidesa intermitja entre HOBt i HOAt, mostra una reactivitat del mateix ordre.[2] Un altre exemple el trobem amb la imida cíclica HOSu (pK_a=6.0), els ésters actius de la qual presenten una baixa reactivitat.[3]

Tenint en compte l'acidesa d'Oxyma (pK_a=4.60), es podria esperar una reactivitat comparable al HOBt, que presenta la mateixa constant de dissociació.[4] No obstant, els experiments d'eficàcia en acoblaments peptídics realitzats amb aquests dos compostos i les sals d'oni anàlogues han mostrat un escenari totalment oposat (Publicacions II, VI, VII, VIII). Contràriament al que es podia preveure, Oxyma, COMU, HOTU i PyOxP (comercialment com a PyOxim) mostren una extensió d'acoblament superior a HOBt, HBTU, HDMB i PyBOP en tots els sistemes assajats, sense excepció. Generalment, aquesta diferència en reactivitat creix al mateix temps que ho fa la dificultat de l'acoblament, amb la introducció d'amino àcids *N*-metilats o α,α-disubstituïs en posicions veïnals. L'exemple més representatiu el trobem en el pentapèptid Aib-encefalina (H-Tyr-Aib-Aib-Phe-Leu-NH_2), on Oxyma és molt superior en eficacia a HOBt (56% vs. 10% en 1 hora). La mateixa tendència s'observa en els altres derivats d'aquest model peptídic.

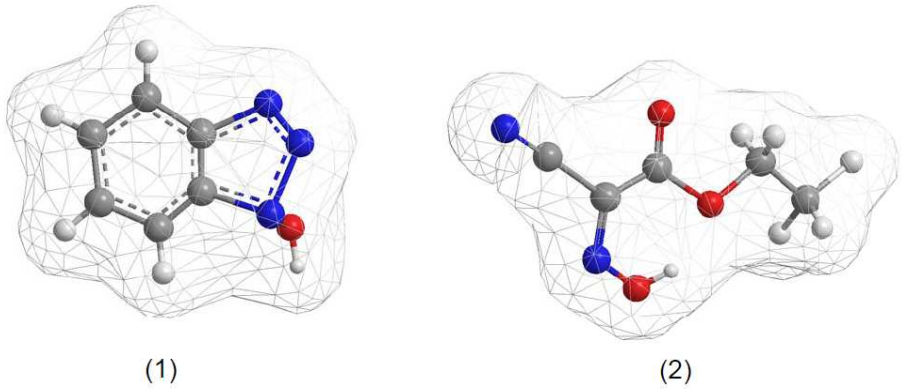


Figura 1. Representació del volum espaial ocupat per HOBt (**1**) i Oxyma (**2**)†

[1] Subiros-Funosas, R.; El-Faham, A.; Albericio, F. "*N*-hydroxylamines for Peptide Synthesis**",** *Patai's Chemistry of Functional Groups, Ed. John Wiley & Sons***, 2011,** *2 (2)*, 623-730.
[2] a) Konig, W.; Geiger, R. *Chem. Ber.,* **1970**, *103*,2034; b) Carpino, L. A. *J. Am. Chem. Soc*., **1993**, *115*, 4397.
[3] El-Faham, A.; Albericio, F. *J. Org. Chem.*, **2008**, *73*, 2731.
[4] Itoh, M. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2219.

† Per tal de representar el volum espaial ocupat per les mol·lècules, es va utilitzar el programa *ChemBio3D Ultra 11.0* de Cambridge Soft Corporation, tot calculant la superficie mol·lecular i mostrant-la com a *wire mesh.*

Encara més sorprenent en termes de potència d'acilació és la comparació entre els reactius de HOAt i Oxyma. L'acidesa del 7-aza-derivat de HOBt és notòriament superior a la d'Oxyma (1.4 unitats de pK_a), el qual condueix a priori a l'estimació d'una reactivitat també força major. Els resultats obtinguts, però, mostren una evident dependència de l'impediment estèric del sistema d'acoblament en la reactivitat relativa, més clara fins i tot que en l'anterior comparació amb HOBt ja que en aquest cas, en unions peptídiques on la dificultat es podria qualificar de mitjana, els reactius d'Oxyma són inferiors als de HOAt. És el cas dels sistemes H-Tyr-*N*MeGly-*N*MeGly-Phe-Leu-NH_2 i ACP (65-74) en la comparació Oxyma/HOAt. Seguint amb la comparació entre aquests additius, un cas fronterís el representaria el pentapèptid H-Tyr-*N*MeAla-*N*MeAla-Phe-Leu-NH_2, on Oxyma és lleugerament més eficient que HOAt (79% vs. 74%), tot i que tan sols s'ha introduït un grup metil respecte l'anàleg *N*MeGly. En el cas de sistemes on es veuen implicats residus Aib o Val, la reactivitat d'Oxyma s'accentúa encara més en detriment de HOAt. Per exemple, en el sistema H-Tyr-Aib-Aib-Phe-Leu-NH_2, HOAt aconsegueix 33% de pentapèptid en 1 hora per 55% d'Oxyma. La mateixa tendència es segueix en el model Aib-encefalina amb COMU i PyOxP (Publicacions VI, VII i VIII), Fmoc-Val-Val-NH_2 i Z-Aib-Val-OMe amb HOTU i COMU (Publicació VI), 8.9- i 3,4-Aib decapèptids encefalina i Aib^{67}-Aib^{68} ACP (65-74) amb PyOxP (Publicació VIII). En el darrer model, queda palès la contribució estèrica en la reactivitat dels èsters actius formats *in situ*, ja que en l'anàleg més senzill (Ala^{67}-Ala^{68}) Oxyma es va situar a nivell intermig entre HOBt i HOAt. Una excepció a la tendència observada el representa el pentapèptid *N*MeLeu-encefalina, que tot i implicar una dificultat estèrica considerable, va donar lloc a percentatges d'acoblaments majors amb HDMA que amb COMU (Publicació VI).

Una explicació raonable per la sorprenent reactivitat relativa entre Oxyma i HOBt/HOAt, desafiant les regles d'acidesa anteriorment establertes, es podria basar en el volum mol·lecular de les *N*-hidroxilamines implicades. Un simple càlcul del volum espaial aproximat d'Oxyma i HOBt (com a representant dels benzotriazols) mostra com HOBt ocupa un volum més concentrat a una regió de l'espai, pròxima al grup *N*-hidroxilamino, mentre que el volum d'Oxyma és més dispers i distribuït espaialment (**Fig. 1**). Encara més important és la rigidesa de l'anell de benzotriazol, que fa que aquest volum estigui pràcticament fixat a l'espai, en contraposició amb la flexibilitat de l'estructura d'Oxyma, el qual explicaria la diferència d'eficàcia entre Oxyma i HOBt/HOAt depenent de la dificultat estèrica dels amino àcids que s'uneixen per mitjà d'enllaç peptídic. Tot i que l'acidesa del grup *N*-hidroxilamino és sens dubte un factor bàsic en el comportament com a grup de sortida, en casos en que l'acidesa és igual o de l'ordre de pKa=1.0-1.5, el factor estèric i la rigidesa del sistema poden marcar la diferència de reactivitat.

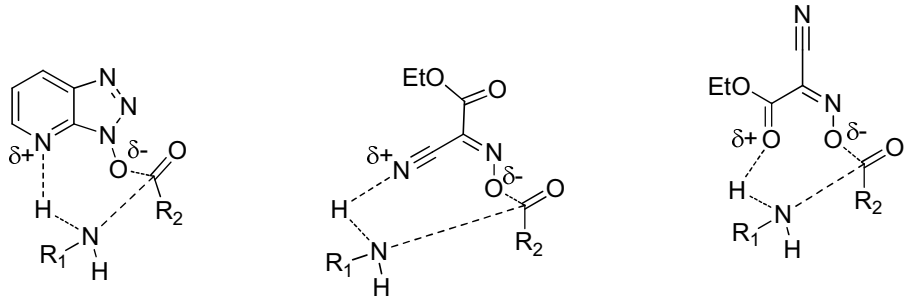


Figura 2. Estats de transició proposats durant l'acilació de grups amino amb èsters actius de HOAt (**3**), (*E*)-2-ciano-2-hidroxiiminoacetat d'etil (**4**) i (*Z*)-2-ciano-2-hidroxiiminoacetat d'etil (**5**), mostrant la participació veïnal d'àtoms de nitrogen o oxigen.

Tot i considerant la importància del volum espaial ocupat per aquestes mol·lècules en la reactivitat dels èsters actius derivats, la superioritat d'Oxyma vers HOAt és veritablement impactant. En conseqüència, cal preguntar-se si altres factors no termodinàmics (entenent com a tal acidesa/pK_a) podrien influïr en l'elevada reactivitat d'Oxyma. La contribució de tals efectes no és cap novetat, tenint en compte que precisament pel cas dels èsters de HOAt s'ha postulat l'existència d'un efecte d'assistència veïnal del nitrogen en posició 7, actuant com a catalitzador general bàsic i potenciant la reacció d'acilació (**3**, **Fig. 2**).[2b] En base a aquest efecte veïnal, s'explica la diferència substancial de reactivitat entre HOAt i 6-Cl-HOBt (l'acidesa dels quals dista tan sols 0.07 unitats de pK_a) i entre HOAt i el 4-aza-isomer, el qual tot i ser més àcid (pK_a=3.02) no pot donar lloc a aquesta

catàlisi i és menys eficient.[1,2b,5] Considerant aquests precedents, seria possible un efecte d'assistència veïnal anàleg en el cas d'Oxyma, que afegís una contribució addicional a la inesperada reactivitat? Per respondre, cal valorar l'existència de dos estereoisòmers possibles en quant a la configuració del doble enllaç de tipus imino. Pel que fa a l'èster d'Oxyma de configuració (*E*), podria existir un estat de transició en el qual el nitrogen del grup ciano assisteix com a catalitzador general bàsic de l'amino entrant. No obstant, donada la geometría lineal del grup ciano el centre nucleòfil quedaria massa allunyat del carbonil reactiu (**4**, **Fig. 2**). En canvi, l'èster d'Oxyma de configuració (*Z*) si permetria un estat de transició on l'oxigen carbonílic d'Oxyma (o el grup alcoxi, amb menor força) assisteix de forma veïnal, posicionant el grup amino nucleòfil prop del centre electròfil (**5**, **Fig. 2**). En aquest cas, l'assistència veïnal seria menys intensa que en el cas de HOAt i implicaria un estat de transició de 8 baules (per un de 7 amb HOAt). En conseqüència aquest efecte veïnal de l'estereoisòmer (*Z*) podria explicar en part la major reactivitat dels èsters d'Oxyma en comparació amb els de HOBt però no la superioritat d'acilació en comparació amb els de HOAt.

Una altra qüestió es planteja: quin estereoisòmer d'Oxyma és el que està present en els experiments realitzats? Les fonts comercials de les que s'ha obtingut el reactiu no n'especifiquen la configuració del doble enllaç, ni les estratègies sintètiques esmentades a la literatura (nitrosació del cianoacetat d'etil) són de caràcter estereoselectiu.[1] En estat sòlid, molts autors, utilitzant entre d'altres tècniques ^{13}C-RMN en un interval de temperatura 20-110ºC, apunten a la presència de l'estereoisòmer (*Z*) de forma exclusiva.[1,6,7,8] A més, aquest estereoisòmer estaria estabilitzat per pont d'hidrogen intramolecular, cosa que no pot oferir l'isomer (*E*) per la geometría lineal del grup ciano, que s'allunya espaialment del grup hidroxil. Dos conformacions són possibles per a l'estereoisòmer (*Z)*, en base a la rotació de l'enllaç entre el carboni de tipus imino i el de tipus carboxil: *cis* (**6**) i *trans* (**7**), en referència a la geometría del doble enllaç imino (**Fig. 3**). Ambdòs isomers conformacionals s'interconverteixen a temperatures pròximes a temperatura ambient, i ambdòs poden participar del pont d'hidrogen intramol·lecular.[9] L'isòmer *cis* (**6**), però, que situa el carbonil com a acceptor, formaria un pont d'hidrogen més fort pel caràcter gairebé aniònic de l'oxigen i s'espera que tingui més presència. De fet, l'espectre de IR d'Oxyma en estat sòlid s'apropa més a l'isòmer *cis* (**6**) que al *trans* (**7**), prenent com a referència l'estereoisòmer (*E*) (veure Apèndix II).[9]

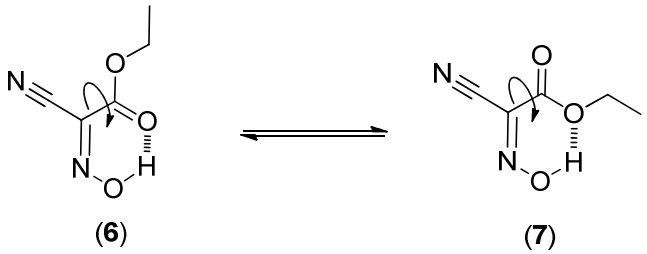


Figura 3. Equilibri conformacional entre els dos rotàmers *cis* (**6**) i *trans* (**7**) possibles del (*Z*)-2-ciano-2-hidroxiiminoacetat d'etil, estabilitzats per pont d'hidrogen intramolecular.

Per tant, l'isòmer (*Z*) *cis* (**6**) és presumiblement el majoritari en estat sòlid, el qual potenciaria més l'efecte veïnal durant l'acilació de l'èster actiu. No obstant, sembla arriscat assumir que aquesta proporció d'estereoisòmers i rotàmers és extrapol·lable al que succeeix en solució, encara més considerant que la forma aniònica és molt important, el qual elimina la formació de pont d'hidrogen intramolecular. Una situació similar la podriem trobar en els reactius d'acoblament basats en Oxyma, on l'absència del protó àcid es tradueix en estereoisomeria (*E*) en el cas de COMU, com

[5] a) Carpino, L. A.; Imazumi, H.; Foxman, B. M.; Vela, M. J.; Henklein, P.; El-Faham, A.; Klose, J.; Bienert, M. *Org. Lett*., **2000**, *2*, 2253; b) Xu, Y.; Miller, M. J. *J. Org. Chem*., **1988**, *63*, 4314.
[6] Conrad, M.; Schulze, A. *Ber. Dtsch. Chem. Ges*. **1909**, *42*, 735.
[7] Jayachitra, G.; Yasmeen, N.; Srinivasa Rao, K.; Ralte,S. L.; Srinivasan, R.; Singh, A. K. *Synth. Commun*. **2003,** *33,* 1532.
[8] Eddings, D.; Barnes, C.; Gerasimchuk, N.; Durham, P.; Domasevich, K. *Inorg. Chem*. **2004**, *43*,3894.
[9] Rachwalska, M.; Urbanek, Z. H. *Z. Phys. Chem*. **2008**, *222*, 1625

sembla indicar l'estructura obtinguda per raigs X (Publicació VI). Sense la possibilitat de formar pont d'hidrogen, i si pogués haver efecte paraigües, l'esteroisòmer (*E*) és estèricament més favorable ja que el grup ciano s'allunya per la seva geometria lineal de la oxima. La estereoisomeria de l'èster actiu d'Oxyma per tant, que tampoc presenta la possibilitat de pont d'hidrogen intramol·lecular com COMU, no és fàcilment assignable, si bé podria estar més a prop del (*E*), que no pot participar d'efecte veïnal amb grups amino entrant.

Per últim pel que fa a aquest apartat, hi ha un tret estructural de les sals d'oni derivades d'0xyma que es podria relacionar amb la major eficiència observada vers els derivats de HOAt en molts models impedits. Contràriament al que succeeix amb els anàlegs de benzotriazol, no s'obtenen sals de amini, sino d'uroni, quan el grup sortint de l'agent d'acoblament és Oxyma (tal com s'ha comprovat en el cas de COMU, per raigs X). Fa pocs anys, Carpino i col·laboradors van demostrar que les sals d'uroni són més reactives que les d'amini, degut a que l'additiu està connectat per l'oxigen, i no pel nitrogen, a l'esquelet carbocatiònic.[10] Es pot esperar doncs, que l'estructura de COMU (i HOTU/TOTU) sigui responsable en part dels resultats observats en relació a HDMA/HATU.

❖ ***Evaluació del risc tèrmic inherent als compostos que contenen Oxyma***:

Un aspecte fonamental a tractar en l'estudi d'Oxyma i COMU és el risc tèrmic que implica la seva descomposició i la possibilitat de que s'origini un descontrol tèrmic que acabi derivant en explosió. En aquest sentit, i donat que no es disposava d'eines per realitzar experiments d'explosivitat (on es determina inequívocament el caràcter explosiu d'una substància), es va decidir valorar de forma indirecta la seguretat tèrmica que ofereixen tant els derivats d'Oxyma com els de HOBt i HOAt, mitjançant tècniques calorimètriques. Per tal d'assolir-ho, en primera instància cal considerar que una explosió sol ser provocada per una descomposició tèrmica ràpida, tot alliberant simultàniament molta pressió.[11] Els assajos calorimètrics DSC dinàmic i ARC permeten investigar aquests dos factors claus.

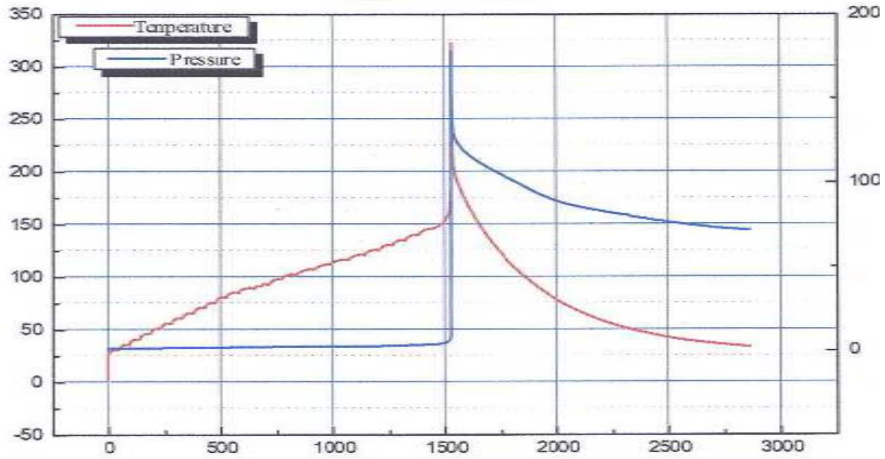


Figura 4. Evolució de la temperatura i pressió amb el temps durant assajos ARC, mostrant la temperatura a l'eix vertical esquerra (ºC), la pressió a l'eix vertical dret (bars) i el temps (min) a l'eix horitzantal.

Per un costat, l'assaig DSC ofereix vàries possibilitats: detectar canvis de fase en compostos cristal·lins, determinar acuradament el punt de fusió d'un sòlid i realitzar valoracions preliminars de seguretat tèrmica.[12] En aquest darrer apartat, la mostra s'escalfa en un calorímetre provist de cilindre i termoparell interior, sota pressió de nitrogen a velocitat constant, entre dues temperatures establertes, permetent observar la cinètica de descomposició, factor clau en la valoració del risc d'explosió. D'altra banda, les característiques en que es du a terme l'assaig ARC

[10] Carpino, L. A.; Imazumi, H.; El-Faham, A.; Ferrer, F. J.; Zhang, C.; Lee, Y.; Foxman, B.M.; Henklein, P.; Hanay, C.; Mügge, C.; Wenschuh, H.; Klose, J.; Beyermann, M.; Bienert, M. *Angew. Chem. Int. Ed.* **2002**, *41 (3)*, 441.
[11] King, R.; Hirst, R. *Safety in the Process Industries*, 2nd ed., Elsevier: Oxford, **2002**.
[12] Steinbach, J. *Safety Assessment for Chemical Processes*, Wiley-VCH: Weinheim, **1999**, 29.

(condicions adiabàtiques que simulen el pitjor escenari possible: acumulació de calor a la massa de reacció) permeten una mesura directa de l'augment de pressió del sistema, gràcies al transductor de pressió de la cambra tancada on es situa el bloc hermètic amb la mostra.[13] En aquest assaig, s'aplica el mètode "heat-wait-seek", pel qual s'escalfa la mostra 5ºC, s'espera 15 min i es comprova si la mostra s'autoescalfa a un ritme superior a 0.02ºC/min (linia vermella en forma d'escales petites a la **Fig. 4**). En el moment en que es detecta aquest autoescalfament, s'atura la calefacció i la mostra descomposa alliberant pressió (linia blava a la **Fig. 4**).

Els resultats exposats a les Publicacions II i VI, emprant una combinació de DSC dinàmic i ARC, mostren clarament que el perfil de descomposició tant de HOBt i HOAt, com de HDMB i HDMA, és consistent amb la d'una substància explosiva, fet que paral·lelament confirma la validesa dels assajos calorimètrics emprats amb aquesta finalitat. En canvi, Oxyma no segueix el patró marcat pels benzotriazols, sinó que descomposa de manera més progressiva i constant, provocant 1/3 de l'augment de la pressió observada amb aquests. D'altra banda, el comportament relatiu de COMU, HDMA i HDMB en els experiments calorimètrics descrits segueix la línia marcada pels additius corresponents. Així doncs, la descomposició de COMU té lloc de manera molt més lenta que els derivats de benzotriazol, la corba dels quals permet especular amb una cinètica autocatalítica. Aquest comportament es caracteritza per la catàlisi que efectuen els productes de descomposició sobre la mateixa, augmentant la velocitat del procés amb la conversió.[14] En conseqüència, s'introdueixen elements d'imprevisibilitat en la descomposició, el qual es tradueix en perillositat i la inutilització d'alarmes de seguretat. El caràcter autocatalític de HDMA i HDMB es va confirmar amb assajos DSC isotèrmics tot escalfant la mostra 10ºC sota la temperatura d'inici de la descomposició en DSC dinàmics durant 480 min. Mentre COMU posseeix una cinètica d'ordre *n*, disminuint l'alliberament de calor amb el temps, en el cas dels benzotriazols s'assoleix un màxim i decreix ràpidament. Tot i no haver assajat aquest comportament amb els corresponents additius, existeix una alta probabilitat de que tant HOBt com HOAt mostrin també cinètiques d'aquest tipus. Per últim, la pressió mesurada fou similar amb totes les sals d'oni, destacant la reducció global de la pressió en comparació amb les *N*-hidroxilamines lliures. En anteriors estudis, s'havia demostrat el menor risc que impliquen aquests compostos quan s'inclouen en agents d'acoblament "autosuficients".[15] La sal de fosfoni d'Oxyma no s'han evaluat en no tenir constància de la perillositat dels anàlegs de benzotriazol.

Taula 1. Valors experimentals i calculats obtinguts en assajos DSC dinàmic i ARC utilitzant additius i sals d'uroni/amini basats en *N*-hidroxilamines.

Reactiu Acoblament	**DSC dinàmic**			**ARC**		
	Onset (ºC)	**ΔH (kJ/mol)**	**calc ΔT_{ad} (ºC)‡**	**Onset (ºC)**	**ΔP (bar)**	**exp ΔT_{ad} (ºC)**
HOBt	190	234	764	145	178	414
HOAt	218	226	830	178	167	384
Oxyma	131	125	440	124	61	307
HDMA	177	245	290	119	55	164
HDMB	180	209	248	122	24	121
COMU	160	183	214	91	53	64

[13] Townsend, I. *J. Thermal Anal.* **1991**, *37*, 2031.
[14] Stoessel, F. *Thermal Safety of Chemical Processes*, Wiley-VCH, Weinheim, **2008**, 311.
[15] Wehrstedt, K. D.; Wandrey, P. A.; Heitkamp, D. *J. Hazard. Mater.*, **2005**, *126*, 1.

En referència a la valoració del risc tèrmic d'un procés, aquest es considera una suma de la probabilitat de que succeeixi un descontrol tèrmic i de la severitat del mateix. Pel que fa al primer component de l'equació, s'ha discutit amb anterioritat el perfil de descomposició d'Oxyma i COMU, molt més segur que el dels benzotriazols derivats. En relació a la severitat tèrmica del procés, és a dir la virulència de la possible explosió, aquesta s'evalua en base a la temperatura de descomposició adiabàtica (ΔT_{ad}), que es pot mesurar durant un experiment ARC o es pot estimar fent un càlcul a partir de del calor alliberat en un assaig DSC dinàmic. Els resultats obtinguts als estudis recollits a les Publicacions II i VI mostren com l'estimació d'aquest paramètre s'ajusta a la tendència relativa dels compostos, però no en el valor absolut (**Taula 1**). Tant Oxyma com COMU condueixen a una severitat tèrmica menor que els anàlegs de tipus benzotriazol, que en el cas de la sal d'uroni és de l'ordre de la meitat. Tenint en compte la probabilitat i severitat tèrmica del possible descontrol tèrmic es pot assegurar que el risc tèrmic dels reactius basats en Oxyma és considerablement menor al dels benzotriazols.

Finalment, l'únic aspecte negatiu que es pot extreure dels experiments realitzats consisteix en la baixa temperatura d'inici de la descomposició d'Oxyma i COMU (*onset* en anglès), valor el qual s'obté de forma més acurada a partir de l'asaig ARC (**Taula 1**). Per treballar en condicions segures, estaments de caire calorimètric recomanen mantener la temperatura en valors on el temps fins la velocitat máxima de descomposició adiabática és >24 h.[16,17] Una estimació d'aquesta temperatura barrera la podem obtenir restant 50K de l'onset de l'assaig ARC: 74ºC en el cas d'Oxyma i 41ºC en el cas de COMU.[18,19]Ara bé, existeix risc real en sobrepassar aquests valors? Ni en els experiments d'estabilitat d'Oxyma a addició nucleòfila realitzats en microones, ni en l'evaluació de la compatibilitat de COMU amb sintetitzadors assistits per microones, ambdos a 80ºC, es va observar cap incident. La rigurositat d'aquesta barrera pot ser excessiva i fins i tot innecessària en casos en que el risc tèrmic no és elevat (com el que ens ocupa), ja que fins i tot tenint lloc la descomposició s'ha demostrat que la seguretat no està en entredit. A més, de forma habitual la síntesi peptidica té lloc a temperatura ambient o a alta temperatura en temps curts, on la cinètica de descomposici dels reactius d'Oxyma permetria dissipar al medi la pressió creada.

❖ ***Control de la configuració del caboni-α induïda pels reactius d'Oxyma***:

De manera similar al que es pot observar en la reactivitat de les *N*-hidroxilamines, la reducció de l'epimerització al carboni-α també està relacionada amb l'acidesa de les mateixes. D'aquesta manera, normalment *N*-hidroxilamines més àcides i eficients en l'assistència de la formació de l'enllaç peptídic promouen a la vegada majors retencions de la configuració òptica.[2b] No obstant, en la mateixa línia de la soprenent reactivitat dels èsters d'Oxyma en relació amb HOBt i HOAt, el control de la puresa òptica també és superior en molts casos quan s'utilitzen els reactius basats en Oxyma. Aquest comportament es detecta tant amb Oxyma, com COMU i PyOxP. El fet que la reducció d'epimerització no es correpon totalment amb l'acidesa ja s'havia descrit en anys anteriors.[20]

La minimització de la pèrdua de configuració òptica en relació amb els derivats de HOAt, no obstant, depèn dels sistema d'acoblament escollit: en el sistema sequencial Z-Phg-OH + H-Pro-NH_2 els anàlegs d'Oxyma són clarament més eficients, mentre que en el sistema de tipus fragment [2+1] entre Z-Phe-Val-OH i H-Pro-NH_2 té un nivel intermig entre HOAt i HOBt. Aquesta tèndència és evident en qualsevol reactiu d'Oxyma utilitzat i és independent de si l'acoblament és en solució o fase sòlida (Publicació II, VI, VIII). Una mirada més precisa permet descartar que aquest disminució de l'eficiència relativa vingui donada pel tipus d'acoblament (fragment o sequencial), ja que utilitzant PyOxP (i PyOxB) en el model [3+3] entre Z-Gly-Gly-Val-OH i H-Pro-Gly-Gly-NH_2, de nou es

‡ La variació de temperatura adiabàtica es pot calcular a partir del calor alliberat en assaig DSC per mitjà de la fórmula ΔT_{ad} (ºC) = ΔH (J/mol) / [MW (g/mol) · Cp (KJ/ Kg ºC)], estimant Cp= 2 KJ/ Kg · ºC.

[16] Stoessel, F. *Chem. Eng. Prog.* **1993**, *89(10),* 68.
[17] Stoessel, F.; Fierz, H.; Lerena, P.; Killé, G. *Org. Proc. Res. Dev.* **1997**, *1,* 428.
[18] Hofelich, T. C.; Thomas, R. C. *Int. Symp. Runaway React.* **1989**, 74.
[19] Singh, J.; Simms, C. *Institution of Chemical Engineering Symposium Series*, **2001**, *148* (Hazards XVI), 67.
[20] Izdebski, J. *Pol. J. Chem.* **1979**, *53*, 1049

mostra superioritat ver l'anàleg de HOAt. És possible que en el cas concret del dipèptid Z-Phe-Val-OH s'estableixin interaccions menys favorables que en d'altres sistemes.

D'altra banda també s'ha pogut investigar l'efecte de la base utilitzada en la tendència de formació d'epímers, ja que la presència d'aquesta fomenta l'aparició d'aquesta reacció indesitjada. En aquest sentit, s'han utilitzat amb reactius d'acoblament tant DIEA com TMP (també anomenada Colidina), les quals difereixen sensiblement en basicitat (pK_a=10.1 per DIEA i pK_a=7.4 per TMP).[21] Els resultats mostren que, en substituir DIEA per TMP en sistemes sequencials, provoca canvis tant en direcció positiva com negativa depenent del reactiu. En canvi, s'observa una clara reducció d'epimerització quan TMP s'escolleix com a base en sistemes d'acoblament de fragments peptídics [3+3] o [2+1] com els descrits anteriorment, degut al seu caràcter bàsic menys acusat (Publicació VI i VII). Es poden obtenir beneficis addicionals en afegir una additiu a la mescla de reacció, potenciant en tots els casos assajats la minimització d'aquesta reacció secundària.

❖ *Acidesa i poder enmascarador del caràcter bàsic/nucleofílic*:

La utilització de *N*-hidroxilamines i fenols àcids en la reducció de l'impacte de les aspartimides en química Fmoc és una estratègia ràpida, senzilla, econòmica i eficient que presumptament passa per un mecanisme de competició per la base del medi.[22] Tal i com altres autors van observar en el passat, els resultats obtinguts a la Tesi Doctoral (Publicació V) no permeten relacionar de forma consistent acidesa i capacitat supressora del caràcter bàsic o nucleòfil.[22] De forma similar al cas pentafluorofenol/penaclorofenol, l'eficiència relativa Oxyma/HOBt/HOAt no es correspon a la tendència formulada *a priori* en base a l'acidesa.

Com s'observa a la Taula 1 de la Publicació V, l'augment de la puresa en pèptids-resina que contenen Asp a la seqüència es relaciona directament amb la reducció de la presència de piperidides. Aquesta observació permet pensar que l'efecte positiu de l'addició de *N*-hidroxilamines àcides en desproteccions realitzades amb piperidina passa per l'emmascarament del caràcter nuclèofil de la base. La interacció i força que s'estableix entre la base i la *N*-hidroxilamina es postula com clau en aquest mecanisme. Es podria esperar, doncs, que donat que tant piperidina com prolina (els protagonistes de les reaccions secundàries estudiades) són amines secundàries i per tant impedides, el factor estèric torni a ser el responsable dels resultats observats, com en anteriors apartats. Així doncs, tant HOBt com HOAt tindrien una interacció més feble que Oxyma amb piperidina. De totes maneres, és molt important remarcar que aquesta interacció no impedeix que la base realitzi la seva tasca en el medi reacció, ja que en el cas del sobreacoblament de prolina, després de tractar la resina, l'acoblament es completa com de manera habitual.

❖ *Relació reactivitat/eficiència i efecte del substituent en oximes:*

Sovint s'entén que una major acidesa i reactivitat es sinònim d'una major eficiència, però aquesta correlació no sempre es compleix. Un exemple el tenim en l'estudi de la introducció del grup Fmoc amb diferents carbonats d'oximes (Publicació III). S'ha demostrat que en casos com aquest, una acidesa menor que d'altres anàlegs, però que tot i així s'emmarqui en el rang de les *N*-hidroxilamines utilitzades habitualment, condueix a un percentatge més alt del producte esperat. És el cas de la ciano-2-piridina oxima, que tot i que en d'altres treballs (Publicació VI) els reactius que se'n deriven són molt menys eficients que Oxyma, en aquest cas particular d'introducció de grups carbamat, aquesta menor reactivitat aconsegueix un impacte mínim de dipèptid.

En la família de les oximes que s'empren en metodologia de síntesi de pèptids la naturalesa dels substituents del carboni de tipus imino és fonamental en l'acidesa i reactivitat. Tot jugant amb la constitució d'aquests components es poden aconseguir les propietats més adequades en cada context, com s'ha vist anteriorment. En el grup de substituents marcadament electroatraients trobem el ciano i el carboxilat d'etil, sobretot el primer. Precisament la combinació dels dos dóna

[21] Carpino, L. A.; El-Faham, A. *J. Org. Chem.*, **1994**, *59*, 695.
[22] Martinez, J. ; Bodanszky, M. *Int. J. Pept. Protein Res* **1978**, *12*, 277.

lloc a Oxyma, amb característiques idònies com a additiu per carbodiimides.[4] La presència conjunta de dos grups ciano a la mateixa oxima resulta en un oli inestable, posant de manifesta l'elevat efecte elecroatraient d'aquest grup.[20] El carbonat de Fmoc corresponent va donar lloc a rendiments baixos per aquesta excessiva reactivitat (Publicació III). Tot i que no se'n coneix l'acidesa, s'aproxima un valor més baix que el d'Oxyma. Respecte l'efecte dels substituents de tipus carboxilat, l'èster indueix més acidesa que l'amida (pK_a=4.6 vs. 5.2 en els cianoanàlegs).[4] Substituir el ciano d'Oxyma per un altre grup carboxilat d'etil resulta en una reducció drástica de l'acidesa (pK_a=4.6 vs. 7.1) i de l'eficiència, com s'ha comprovat en les sals d'uroni (Publicació VI).[4] A l'extrem oposat del ciano hi trobem el substituent 2-piridino que és manifestament menys reactiu que les altres oximes descrites en aquests estudis, com es confirma en la investigació dels carbonats actius (Publicació III).[20] En conseqüència, es pot esperar una acidesa més baixa que la de la oxima que conté dos carboxilats d'etil.

Conclusions Globals

Conclusions

La investigació duta a terme en la Tesi Doctoral ha permès esbrinar el potencial de 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma) com a additiu per carbodiimides i com a bloc de construcció en sals d'uroni i fosfoni. Altres aplicacions s'han considerat, com la seva capacitat per reduir l'impacte de reaccions secundàries catalitzades per base o la inclusió en carbonats actius per introduïr grups protectors. Les conclusions que es poden extreure d'aquests estudis són:

- La capacitat d'Oxyma d'assistir la formació de l'enllaç peptídic, en combinació amb carbodiimides, és clarament superior a HOBt en tots els models emprats. En relació a HOAt, l'eficiència d'Oxyma depèn de l'impediment estèric dels aminoàcids que participen en l'acoblament: en sistemes senzills la seva reactivitat és intermitja entre HOBt i HOAt, però a mesura que s'introdueix dificultat estèrica, Oxyma és cada cop més eficaç que HOAt.

- La retenció de configuració al carboni-α quan s'empra Oxyma, en relació a HOAt, depèn del tipus de model escollit. En acoblaments sequencials, Oxyma indueix menor epimerització que HOAt, mentre que en acoblaments [2+1] o de fragments peptídics, Oxyma es situa a nivel lleugerament inferior, però més eficaç que HOBt.

- El risc tèrmic inherent a Oxyma s'ha comparat a HOBt i HOAt per mitjà de tècniques calorimetriques (DSC dinàmic i ARC). La combinació d'aquests dos assajos mostren que el comportament tèrmic d'Oxyma és totalment oposat als benzotriazols, el perfil explosiu dels quals es confirma. Es pot assegurar que la probabilitat de descontrol tèrmic resultant en explosió amb Oxyma és molt menor a HOBt i HOAt.

- Possibles reaccions secundàries d'addició nucleòfila al grup carboxil de Oxyma s'han descartat en les condicions habituals de formació de l'enllaç peptídic, tot i que s'observen en condicions dràstiques amb microones. Experiments duts a terme amb COMU i microones permeten excloure subproductes d'addició en estratègies de formació estàndar de l'enllaç peptídic que usen reactius basats en Oxyma.

- Els carbonats actius de Fmoc amb diferents oximes àcides, incloent Oxyma, s'han sintetitzat en dos passos a partir dels compostos metilens actius amb bons rendiments i puresa. Provats en la protecció de H-Gly-OH, opcions menys reactives com la inclusió de la oxima que presenta substituents ciano-2-piridino poden ser més adients en aquests casos, en proporcionar percentages de dipèptid menors (0.01%) i rendiments similars (92%) als carbonats més reactius, com els d'Oxyma. Fruit d'aquesta excessiva reactivitat, el carbonat de la dicianooxima dóna rendiments molt baixos, probablement per hidròlisi del reactiu.

- El carbonat actiu de Alloc de la ciano-2-piridina oxima es va preparar partint de la sal potàssica, una metodologia més potent, en un 82% de rendiment i puresa superior al 99%. La protecció de H-Gly-OH amb el carbonat actiu de Alloc de la ciano-2-piridina oxima va proporcinar una puresa (99.5%) i percentatge de dipèptid (0.02%) similars a l'anàleg de Fmoc, tot i que els carbonats d'Alloc són més sensibles a aquesta reacció secundària que els de Fmoc per factors estèrics.

- Els beneficis obtinguts a partir del caràcter àcid d'Oxyma s'extenen a la minimització de reaccions secundàries causades per una excessiva basicitat o nucleofília de reactius del medi. L'efecte és igual o superior a HOAt, tot i presentar un pK_a menys àcid, suggerint altres efectes, tal vegada estèrics.

- Concentracions creixents d'additiu augmenten progressivament la puresa del pèptid, principalment per reducció del percentatge de piperidides, el qual suggereix que el mecanisme d'acció passa per l'emmascarament nucleofílic de piperidina. Amb 1M Oxyma la puresa del pèptid es pot incrementar 30%, respecte l'experiment sense additiu.

- S'ha comprovat l'existència del sobreacoblament d'aminoàcids originat per la basicitat de Pro. No obstant, l'impacte d'aquesta reacció secundària és considerablement menor al de les aspartimides, ja que és necessari forçar les condicions per veure aproximadament un 3% de subproductes. L'efecte del tractament de pèptid-resina incloent Pro al *N*-terminal amb Oxyma es pot considerar intermig entre HOBt i HOAt.

- L'acidesa d'Oxyma origina una tendència a la prematura escissió de pèptids de la resina 2-clorotritil comparable a HOBt i inferior a HOAt, a tenir en compte en temps d'acoblament llargs.

- La sal d'uroni dimetilmorfolino d'Oxyma, COMU, exhibeix una gran solubilitat en DMF i una eficiència en molts casos superior a HATU en seqüències impedides, així com una retenció de configuració excel·lent en sistemes sequencials. L'efecte de la inclusió del grup morfolino resulta en un increment de la solubilitat i reactivitat, com s'observa en els resultats obtinguts amb HOTU i COMU. La reactivitat de COMU en combinació amb microones resulta en una eficiència comparable a la que s'obté amb el mateix reactiu en temps molt més llargs.

- La combinació d'assajos calorimètrics (DSC isotèrmic i dinàmic, i ARC) va mostrar un perfil de descomposició marcadament diferent als dels benzotriazols, descomposant COMU de manera constant, en contrast amb HDMA i HDMB, el comportament autocatalític dels quals resulta molt perillós per la seva imprevisibilitat. Hi ha altes probabilitats de que extrapol·lant als corresponents additius, tant HOBt com HOAt mostrin un comportament cinètic similar.

- Les sals de fosfoni d'Oxyma es poden assolir fàcilment en *one-pot* i bons rendiments. Tot i que la presència de l'anió tetrafluoroborat aporta més solubilitat al reactiu, ho fa en detriment d'una estabilitat que si mostra PyOxP, el qual és més eficient que PyAOP en molts sistemes lineals i en ciclacions estudiats.

- En resum, es pot concloure que la família de les oximes àcides constitueix una classe de reactius d'aplicació en vàries branques de metodologia de síntesi peptídica amb alta eficiència i seguretat tèrmica. Variant la naturalesa dels substituents electroatraients s'obtenen propietats adequades per diferents àmbits metodològics.

- En general, tant Oxyma com les sals d'oni derivades representen una clara alternativa als reactius basats en el benzotriazol, amb major solubilitat i gran eficiència, sobretot en augmentar la dificultat i l'impediment de la seqüència, superant en molts casos els derivats de HOAt.

- La severitat tèrmica de Oxyma i COMU es força més baixa que la dels anàlegs benzotriazol, el qual sumat a la menor probabilitat de descontrol tèrmic resulta en un risc tèrmic considerablement més baix. El caràcter excessivament conservador de la regla calorimètrica dels 50K, extreta a partir d'assajos ARC, s'ha demostrat en els experiments d'estabilitat i eficiència duts a terme a temperatures superiors a 80ºC amb microones, on no s'ha registrat cap incident, fet que fa reconsiderar la utilitat d'aquests paràmetres quan el risc tèrmic és manifestament baix.

- En relació als anteriors punts, queda palès que tot i ser una eina fonamental en el disseny de les *N*-hidroxilamines que actúen com a grups de sortida, el pK_a per ell mateix no és concloent respecte a la reactivitat relativa de les corresponents espècies reactives. El tamany dels substituents contigus al grup *N*-hidroxilamina és determinant, així com el de possibles efectes veïnals. En altres paraules, no només l'efecte termodinàmic és influent, sinó també el cinètic.

Apèndixs

Apèndix I: EROS Oxyma

Tot seguit s'adjunta la fitxa del 2-ciano-2-hidroxiiminoacetat d'etil (Oxyma), inclosa recentment a l'Enciclopèdia de Reactius per Síntesi Orgànica (EROS; Enciclopedy of Reagents for Organic Synthesis; 2011). Aquesta línea de publicacions proporciona un accés ràpid a les característiques, propietats, síntesi, caracterització i aplicacions d'un reactiu particular en l'àmbit orgànic sintètic.[†]

En aquest cas es descriuen la síntesi, característiques i aplicacions d'Oxyma, detallant els protocols habituals per a la formació de l'enllaç peptídic en solució i fase sòlida, així com una descripció breu de les prestacions i propietats que ofereix. Es dona una visió global de les avantatges que suposa aquest reactiu sobre els benzotriazols disponibles comercialment.

Ethyl 2-cyano-2-(hydroxiimino)acetate

2-ciano-2-hidroxiiminoacetat d'etil

Fernando Albericio[a], Ramon Subirós Funosas[a]

[a] Institut de Recerca Biomèdica de Barcelona (IRB Barcelona)

Encyclopedy of Reagents for Organic Synthesis (EROS), **2011,** *acceptat, manuscript*
EROS CAS 1075198-30-9

[†] Ramon Subirós Funosas va realitzar la cerca a la literatura i l'elaboració del manuscrit

CONTRIBUTION FOR:

ELECTRONIC ENCYCLOPEDIA OF REAGENTS FOR ORGANIC SYNTHESIS

Ethyl 2-cyano-2-(hydroxyimino)acetate

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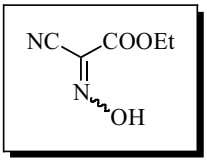
Ethyl 2-cyano-2-(hydroxyimino)acetate



[CAS 3849-21-6] $C_5H_6N_2O_3$ (MW 142.11)

(additive to carbodiimides in peptide bond formation[1] and nucleophilicity/basicity-masking agent in the prevention of base-mediated side reactions[2])

Alternate Name: Oxyma Pure.

Physical Data: mp 128-133ºC.[1] pKa 4.60.[1,3]

Solubility: Sol in DMF, NMP, CH_3CN, DCM and H_2O. Partially sol in toluene.

Form Supplied in: white to off-white crystalline solid, commercially available.

Analysis of Reagent Purity: ^1H and ^{13}C NMR spectra are sufficient to determine purity. Oxyma is identified by a characteristic UV maximum of absorbance at 235-240 nm.[1]

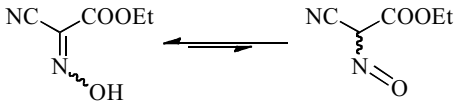
Preparative Methods: Oxyma is easily obtained by nitrosation of ethyl 2-cyanoacetate, in the presence of sodium nitrite and acetic acid.[1,4] Yields are almost quantitative when using phosphoric acid buffered to pH 4.5.[5]

Purification: Recrystallization from EtOH.[6]

Handling, Storage and Precautions: In solid form, it is shelf-stable at room temperature. Solutions of Oxyma in DMF, NMP and MeCN are stable for many weeks. Dynamic DSC calorimetric assay determined decomposition at 135ºC, serving as a safety barrier.[7] A more exact onset for the decomposition (124 ºC) is obtained from an ARC experiment.[7] It is recommended to keep temperature below 74ºC to conserve

calorimetric parameters, although experiments in solution have been safely conducted under microwave irradiation and at temperatures above 80ºC.[7] However, Oxyma demonstrates a decomposition profile opposite to the ones of known explosive *N*-hydroxybenzotriazoles, simultaneously releasing 1/3 of the pressure observed with those heterocyclic compounds.[7]

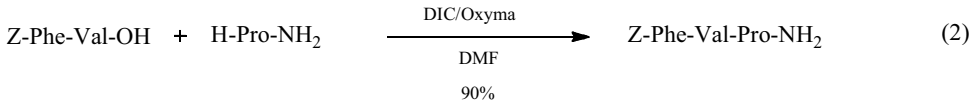
The use of ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) is being employed as an additive to carbodiimides in peptide bond forming processes.[1,7] In contrast to many oximes containing α-hydrogens, Oxyma displays high stability in the solid state as well as in solution.[3,7] The most stable tautomer is the oxime species, but in strong basic media or in the anionic state it is present in the tautomeric nitroso form (eq. 1).[1,8] The Z isomer of the oxime form is thought to be predominant, according to 13C-NMR analysis.[1,9] Intramolecular hydrogen bonding between the ester carbonylic oxygen and the *N*-hydroxy proton could stabilize this isomer.



(1)

A standard solution-phase procedure for amino acid couplings processes using Oxyma consists in adding the carbodiimide (0.11 mmol), typically diisopropylcarbodiimide (DIC) or the water-soluble 1-ethyl-3-(3'-(dimethylamino)propyl)carbodiimide (EDC), to an ice-cooled solution of *N*-protected acid (0.105 mmol), *C*-protected amine (0.1 mmol) and Oxyma (0.11 mmol) in DMF (1 mL).[10] The mixture is left at 0ºC for 1h and then at room temperature overnight, followed by dilution with ethyl acetate, and acidic and basic aqueous washes. Alternatively, a 2 min preactivation can be advantageous, through the addition of the *C*-protected amine to a solution of the remaining components at 0ºC.[7] Using this latter protocol, the epimerization-sensitive tripeptide Z-

Phe-Val-Pro-NH_2 has been efficiently assembled from Z-Phe-Val-OH and H-Pro-NH_2 by means of DIC/Oxyma coupling, with traceless amounts of starting materials (eq.2).[7]



In a similar manner, Oxyma has been tested in solid-phase peptide approaches using an adapted procedure to that of solution-phase.[7] Given the common use of high excess of reagents to ensure quantitative coupling yields in solid-phase strategies, 3 equiv. of Oxyma, *N*-protected acid, and DIC are usually preactivated for 3-5 min prior to the addition to the resin. For the assembly of standard non-hindered residues, no longer than 30 min couplings are necessary for an efficient introduction. Under microwave irradiation at 60ºC, coupling times can be shortened to 20 min. even if sterically encumbered amino acids are employed.[11] The compatibility of Oxyma with automated peptide synthesizers has been demonstrated in the solid-phase elongation of the ACP (65-74) decapeptide, providing a higher percentage of target peptide than 1-hydroxybenzotriazole (HOBt) and performing close to 7-aza-1-hydroxybenzotriazole (HOAt).[7]

The efficiency of Oxyma, relative to HOBt and HOAt, has been evaluated in the solid-phase assembly of Leu-enkephaline (H-Tyr-Gly-Gly-Phe-Leu-NH_2) analogues whereby the central Gly positions were replaced by sterically hindered residues (MeGly, MeAla and Aib). In this study, the percentage of introduction of the last two amino acids of the MeGly and MeAla peptide systems, Oxyma and HOAt were similarly efficient in affording the target pentapeptide, in spite of the pKa difference between them (3.28 vs. 4.60).[7,12] In an opposite corner, HOBt rendered the lowest percentages of desired peptide. This tendency was followed in the elongation of the Aib derivative, tested

using various coupling times (Table 1). The performance of Oxyma clearly superseded that of HOBt and HOAt.

Table 1. *Percentage of target peptide and deletion peptides during stepwise manual introduction of Fmoc-Tyr(tBu)-OH and Fmoc-Aib-OH to quantitatively-assembled tripeptide H-Aib-Phe-Leu-Rinkamide-aminomethyl-PS, using a 3-min preactivation with DIC in DMF.*

entry	coupling reagent	conditions	pentapeptide (%)	des-Aib (%)	other deletion peptides (%)
1	DIC/HOAt	30-min coupling	11.3	86.1	2.6
2	DIC/HOBt	30-min coupling	3.0	91.0	6.0
3	DIC/Oxyma	30-min coupling	28.0	70.5	1.5
4	DIC/HOAt	1-h coupling	28.7	71.3	--
5	DIC/HOBt	1-h coupling	9.8	86.9	3.3
6	DIC/Oxyma	1-h coupling	55.7	44.3	--
7	DIC/HOAt	1-h double coupling	55.0	45.0	--
8	DIC/HOBt	1-h double coupling	18.9	80.6	0.5
9	DIC/Oxyma	1-h double coupling	69.0	31.0	--

The suppression of epimerization induced by Oxyma has been tested in solution and solid-phase in peptide models involving stepwise and segment couplings.[3,4,7] The ability of Oxyma to efficiently avoid epimerization was demonstrated in the coupling of Ac-Ile-OH to H-Gly-OEt, when the addition of this additive caused the percentage of epimerization to decrease from 35 to 1.8%, surpassing HOBt (8.8%) and *N*-hydroxisuccinimide, HOSu (2.7%).[3] Similarly, acidic oximes were designed and tested in the retention of configuration during formation of Bzl-Leu-Gly-OEt in solution.[4] Although hydroxyiminomalonitrile exhibited improved suppression of epimerization, Oxyma gave higher stability and yield. Recently, the reduction of epimerization has been more exhaustively examined in the coupling of Z-Phg-OH and H-Pro-NH_2 Oxyma (1.1% epimerization) induced less epimerization than HOBt (9.3%), *N*-hydroxy-2-pyridinone (HOPO, 26.1%) and even HOAt (3.3%), affording the dipeptide in the highest yield. Comparable percentages of epimerization were obtained in the [2+1] Z-

Phe-Val-Pro-NH_2 peptide model, HOBt and HOPO being the least efficient in the retention of configuration (8.9% and 45.1% respectively).[7] In this segment system, Oxyma induced more epimerization than HOAt (3.8% vs. 2.1%), but gave a higher yield of tripeptide. In solid-phase, the reduction of epimerization in the introduction of Cys(Trt) was examined in the stepwise elongation of H-Gly-Cys(Trt)-Phe-NH_2. Very little epimerization was observed with both Oxyma and HOAt (0.1%).[7]

Several derivatives of Oxyma have been proposed as stand-alone coupling reagents, following its success as safe, efficient and epimerization-suppressant additive.[1] Among aminium/uronium salts, *O*-[cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium tetrafluoroborate (TOTU) was the first Oxyma-based analog to be unveiled.[13] More recently, the hexafluorophosphate version, *O*-[cyano(ethoxycarbonyl)methylidene)amino]-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTU) was designed and tested along with dimethylmorpholino counterparts, such as (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholino-carbenium hexafluorophosphate (COMU), which showed superiority in terms of solubility, stability and reactivity.[14,15] Phosphonium salts including the Oxyma moiety have also been built, presenting either an hexafluorophosphate or tetrafluoroborate counteranion.[16] The former version, *O*-[(1-cyano-2-ethoxy-2-oxoethylidene)amino]-oxytri(pyrrolidin-1-yl) phosphonium hexafluorophosphate (PyOxim), proved to be an attractive alternative to classical benzotriazole analogues, performing better in efficiency and cyclization experiments.[16,17] Oxyma can be added in equimolar amounts to the abovementioned onium salts, in order to reduce the impact of some side reactions, such as epimerization or guanidylation, by accelerating the active ester formation.[16] Apart from onium salts, Oxyma and parent acidic oximes have also been used as building blocks for the

preparation of reactive carbonates, useful in the introduction of Boc, Alloc and Fmoc protecting groups.[18,19] Reactive preformed esters and esterification reagents have also been prepared.[3,20] Oxyma esters have been employed in lipase-catalyzed acyl transfer reactions in secondary alcohols.[21]

The contribution of Oxyma in methodology of peptide synthesis is not restricted, however, in assisting the formation of the peptide bond or the introduction of protecting groups. Its high dissociation constant (pKa=4.60), highly responsible for the unique properties as leaving group, is also beneffitial to prevent base-mediated side reactions in the Fmoc/tBu approach.[1,2] Thus, aspartimide formation and Pro-caused overcoupling can be strongly minimized by addition of a solution of Oxyma. The reduction of undesired aspartic acid intramolecular cyclization by the addition of the acidic HOBt had also been reported in the past.[22] Recently, it has also been showed that the highest the concentration of *N*-hydroxylamine in 20% piperidine/DMF, the least percentage of aspartimides and piperidides are observed.[2] In particular, the addition of 1M Oxyma in DMF results in the most pure crude (from 56 to 86% of unmodified peptide), compared to HOBt and HOAt (79-80%).[2] In a similar manner, the overcoupling observed when Pro is present at the *N*-terminus, coming from premature Fmoc removal from the protected residue being introduced are greatly modified by *N*-hydroxylamines, such as Oxyma. The presence of this oxime, presumably by interacting with the free secondary amino group, causes a reduction of such unwanted side reaction, in a comparable extent to benzotriazoles.[2]

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Apèndix II: *IR d'Oxyma*

L'espectre d'infraroig d'Oxyma es va realitzar en forma de pastilla de KBr en un espectròmetre FT-IR *ThermoNicolet Nexus*, processant l'espectre amb el programa *Omnic vs 6.0*.

Banda	***Freqüència***	***Intensitat Relativa***	***Assignació***
1	3817 cm^{-1}	Alta	O-H stretching associat
2	3127 cm^{-1}	Alta	O-H stretching associat
3	2989 cm^{-1}	Mitjana-alta	CH_3 stretching simètric CH_2 stretching simètric CH_3 stretching assimètric
4	2233 cm^{-1}	Baixa	C≡N triple enllaç stretching
5	1728 cm^{-1}	Alta	C=O stretching
6	1630 cm^{-1}	Baixa	C=N stretching
7	1580 cm^{-1}	Baixa	C_2H_5 bending
8	1472 cm^{-1}	Mitjana	CH_3 bending assimètric CH_2 scissors simètric
9	1433 cm^{-1}	Alta	CH_3 bending assimètric O-H bending
10	1373 cm^{-1}	Mitjana	CH_3 bending simètric
11	1314 cm^{-1}	Alta	CH_2 twisting i wagging C-O stretching assimètric
12	1187 cm-1	Mitjana-baixa	CH_3 rocking CH_2 twisting-rocking
13	1067 cm^{-1}	Alta	C-O stretching simètric
14	1004 cm^{-1}	Mitjana	N-O stretching
15	853 cm^{-1}	Mitjana	C-C stretching
16	767 cm^{-1}	Mitjana	esquelet
17	752 cm^{-1}	Baixa	CH_2 rocking
18	519 cm^{-1}	Baixa	esquelet
19	449 cm^{-1}	Mitjana-baixa	esquelet

Cal destacar:

a) Les assignacions s'han realitzat amb ajuda de la literatura existent[1,2,3,4]

b) No s'observa banda υ(O-H) stretching lliure, suggerint que la totalitat d'hidroxils participen d'associació per pont d'hidrogen, bé inter o intramol·lecular (bandes 1 i 2). En cas de l'isomer *E*, aquestes bandes s'han assignat prèviament com de tipus oligomèric intermol·lecular.[4]

c) Considerant les bandes 5, 6 i 13, en estat sòlid tindria més presència l'isomer conformacional *cis*, (en referència a la disposició relativa dels grups imino i carbonil), prenent com a model l'estereoisomer *E* (veure Figura 3 de Discussió general).[4]

d) La presència de les bandes 1, 2, 6 i 14, unit a l'absència de bandes υ (N=O st) a 1280 cm^{-1} i 1550 cm^{-1} confirma la identitat del tautòmer oxima en detriment del tautòmer nitroso (veure Apèndix I).[1,5]

e) La baixa intensitat de la banda 6 (a T>210K) pot estar correlacionat amb una transferència de càrrega del nitrogen a l'oxigen del grup oxima.[4]

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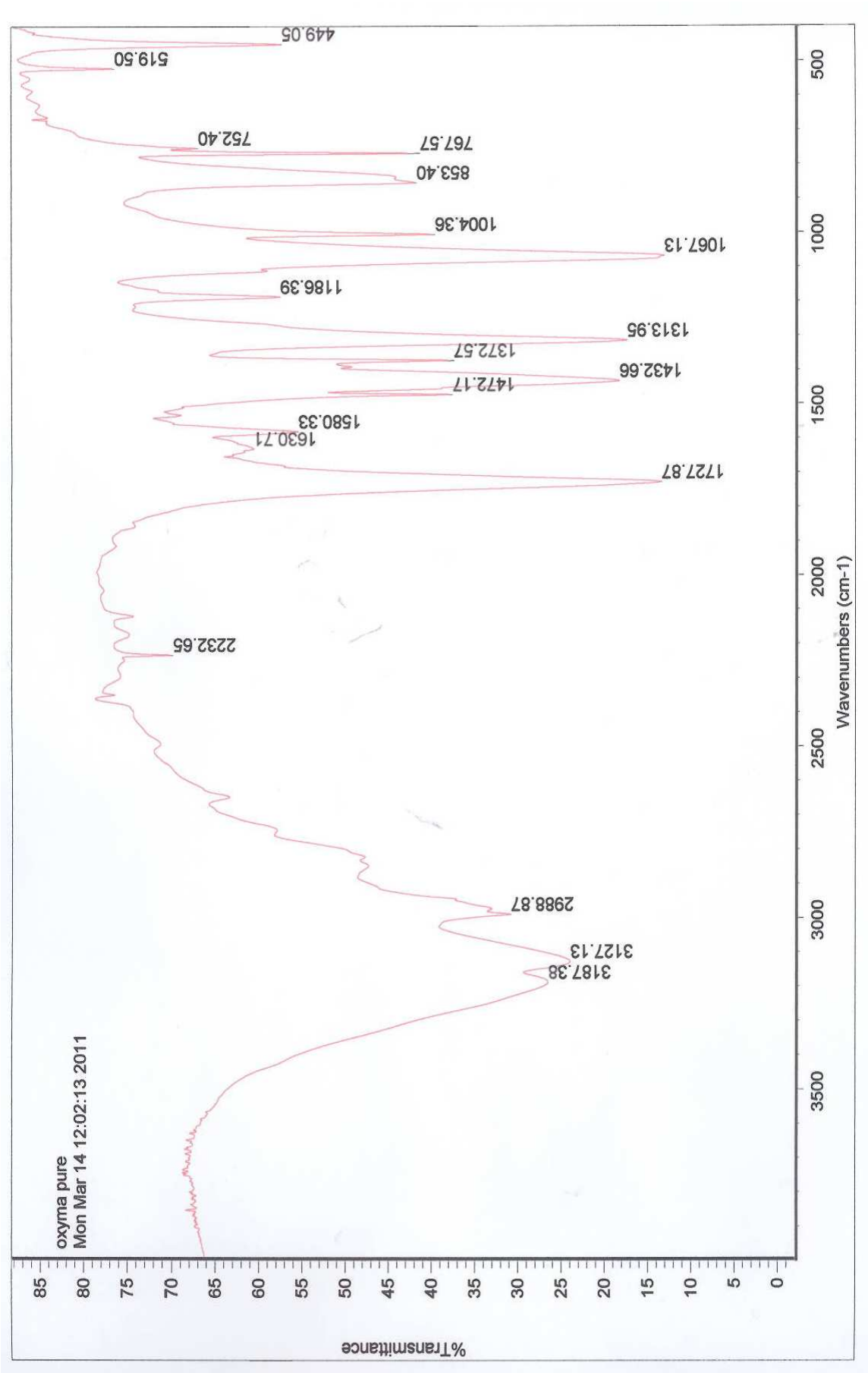
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1372.57
1432.66
1472.17
1580.33
1630.71
1727.87
2232.65
2988.87
3127.13
3187.38

Apèndix III: *Espectres RMN d'Oxyma*

S'adjunten a continuació els espectres de ressonància magnètica nuclear de ^{1}H i ^{13}C d'Oxyma, realitzats en un espectròmetre de RMN Mercury 400MHz. A l'espectre de ^{1}H únicament s'observa el grup de senyals pertanyents al grup etil de l'èster, mentre que el ^{13}C permet distingir a més l'esquelet quaternari de la oxima

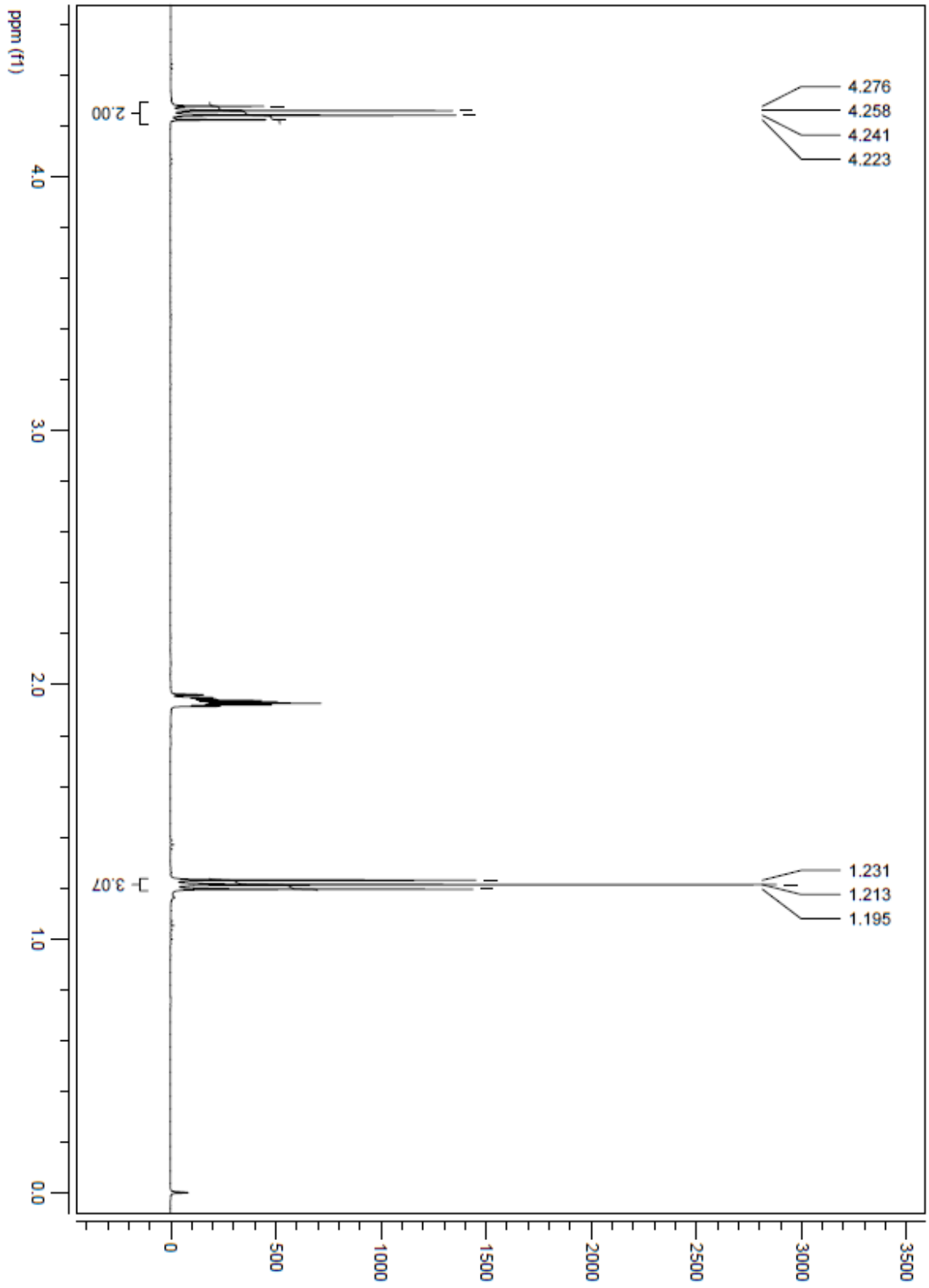
- **^{1}H-RMN** (400MHz, CD_3COCD_3):

  δ= 4.25 (q, 2H, *J*= 7.1 Hz), 1.21 (t, 3H, , *J*= 7.1 Hz)

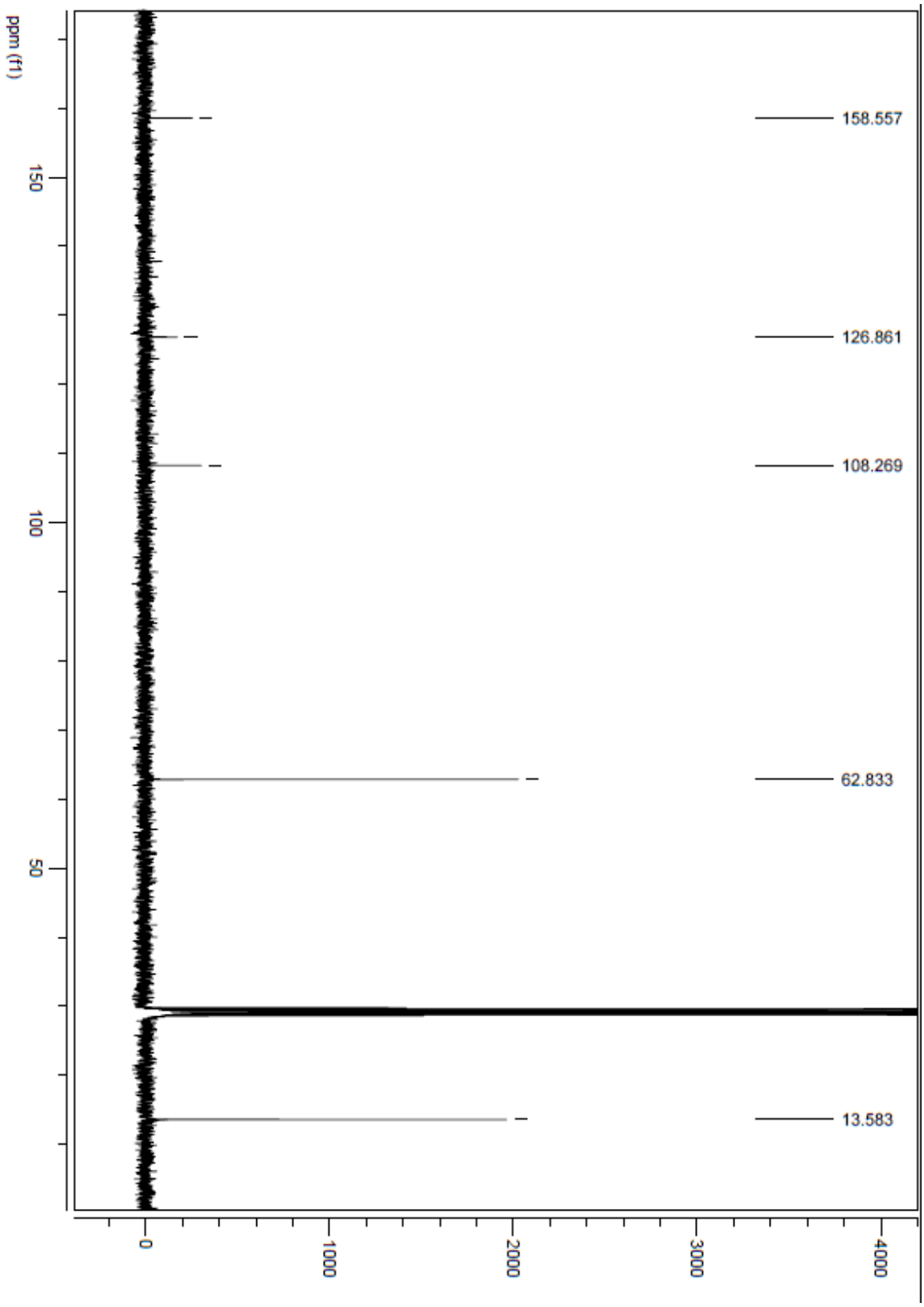
- **^{13}C-RMN** (100MHz, CD_3COCD_3):

  δ= 158.5, 126.9, 108.3, 62.8, 13.6

❖ **^{1}H-RMN** (400MHz, CD_3COCD_3):



❖ **^{13}C-RMN** (400MHz, CD_3COCD_3):



***Apèndix IV*: EROS COMU**

Tot seguit s'adjunta la fitxa de COMU inclosa recentment a l'Enciclopèdia de Reactius per Síntesi Orgànica (EROS; Enciclopedy of Reagents for Organic Synthesis; 2011). Aquesta línia editorial es centra en donar una pincellada de les característiques, propietats, síntesi, caracterització i aplicacions d'un determinat reactiu en l'àmbit orgànic sintètic.[†]

En el cas particular de la sal d'uroni COMU, es detallen les propietats físiques, els protocols més habituals en la formació de l'enllaç peptídic en solució i fase sòlida, així com el seu comportament en la reducció d'epimerització i eficàcia d'acoblament. Es descriu també la utilització d'aquesta sal d'oni en la transformació d'α-aminoàcids en amides de Weinreb.

(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholinocarbenium hexafluorophosphate

Hexafluorofosfat de (1-Ciano-2-etoxi-2-oxoetilidenaminooxi)dimetilaminomorfolinocarbeni

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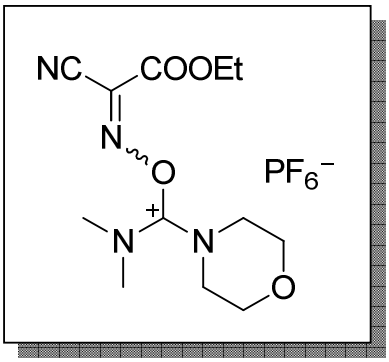
Encyclopedy of Reagents for Organic Synthesis (EROS), **2011,** *acceptat, manuscrit RN01360*

[†] Ramon Subirós Funosas va realitzar la cerca a la literatura i l'elaboració del manuscrit

(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholino-carbenium hexafluorophosphate



[CAS 1075198-30-9] $C_{12}H_{19}F_6N_4O_4P$ (MW 428.27)

(stand-alone coupling reagent for peptide bond formation[1,2] and transformation of α-amino acids into their corresponding Weinreb amides[3])

Alternate Name: COMU.

Physical Data: Sample decomposes without melting at 143-144ºC (color transition to dark brown) in a standard melting point device. A more accurate onset for the decomposition (159.90ºC) is obtained from dynamic DSC (Differential Scanning Calorimetry) assays.[2] COMU contains less than 1% water according to Karl-Fischer studies, using sodium tartrate as reference.

Solubility: sol DMF (1.44 mol L^{-1}), sol NMP/DCM 3:1 (0.5 mol L^{-1}) and in most organic solvents.

Form Supplied in: white to off-white crystalline solid, commercially available.

Analysis of Reagent Purity: 1H-NMR is the most reliable technique for an accurate determination; an approximate purity can be also obtained from HPLC.

Preparative Methods: COMU is easily accessed from (dimethylaminomorpholino)chloroformamidinium hexafluorophosphate, generated in three steps from morpholine and *N,N*-dimethylcarbamoyl chloride, and Oxyma, in the presence of Et_3N in DCM.[2]

Purification: by crystallization or precipitation from acetonitrile.

Handling, Storage and Precautions: Sensitive to hydrolysis, use DMF dried over molecular sieves and bubbled with N_2, to avoid free amines, for long-term storage of its solutions. In solid form is stable at 4ºC several months. In safety terms, COMU displays an opposite decomposition profile to explosive benzotriazole-based analogues. Although conservative calorimetric parameters recommend working at temperatures below 41ºC, the sample can be safely dried several hours at 60ºC and used in microwave syntheses at 80ºC.[2,4] Median lethal dose (LD50) is estimated at 2000 mg/kg. Furthermore, COMU presents low potential as allergenic agent.

(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholino-

carbenium hexafluorophosphate (COMU) is an ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma)-based reagent directed towards the formation of amide bonds, in particular in peptide synthesis, either in solid- or solution-phase.[1,3,5] X-rays have shown that COMU exists as uronium salt (*O*-form) rather than aminium salt (*N*-form), in contrast to benzotriazole counterparts like ***N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate or tetrafluoroborate *N*-oxide**; (HBTU/TBTU), ***N*-[(1*H*-6-chlorobenzotriazol-1-yl)-(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate or tetrafluoroborate *N*-oxide** (HCTU/TCTU) or ***N*-[(dimethylamino)-1H-1,2,3-triazolo**

[4,5-b]pyridin-1-yl-methylene)-N-methylmethanaminium hexafluorophosphate N-oxide (HATU).[2,6,7]Further evidence about the particular connectivity between the *N*-hydroxylamine moiety and the carbocation skeleton is provided by careful analysis of the 13C-NMR chemical shift corresponding to the positively-charged carbon center (156.14 ppm), consistent with *O*-form structural isomer.[2] A typical small-scale solution phase procedure for acylations with COMU implies addition of the coupling reagent (0.125 mmol) to a solution of equimolar amounts (0.125 mmol) of *N*-protected acid and *C*-protected amine, and 0.25 mmol of tertiary base (***Diisopropylethylamine***, DIEA or ***2,4,6-Collidine***) in DMF (1 mL) at 0ºC, followed by stirring at this temperature for 1 h and then at room temperature for extra 2-3h.[8] In example, dipeptide Z-Aib-Val-OMe was efficiently prepared from the hindered amino acids Z-Aib-OH and H-Val-OMe with this methodology in a near quantitative manner in only 2 hours (eq 1).[2] One of the advantages of the presence of the morpholino moiety contained in COMU is that extraordinary coupling extensions can be obtained with only 1 equiv. of base (97% in the same peptide model after 2 hours), as result of the hydrogen-acceptor behavior of the oxygen in the cyclic ring.[2,9,10]

$$\text{Z-Aib-OH} + \text{H-Val-OMe} \xrightarrow[\text{DIEA, DMF}]{\text{COMU}} \underset{99\%}{\text{Z-Aib-Val-OMe}} \qquad (1)$$

The suitability of COMU in solid-phase peptide strategies has also been tested using a similar protocol to that described for solution-phase.[9] Since solid-phase allows the use of excess starting materials, which can be separated from the solid support by filtration, 3 eq. of Fmoc-amino acid and COMU is generally sufficient for successful peptide bond formation. In order to avoid undesired guanidylation of the *N*-terminus of the peptide-

resin, short preactivation (1-2 min) of a solution of the reagents in DMF with 6 eq. of DIEA is highly recommended (longer preactivation times may lead to a significant increase in C_α- racemization).[11,12] Alternatively, addition of 3 eq. of Oxyma accelerates the formation of the active ester, decreasing the risk of guanidylation. However, in couplings where the activation of the carboxylic acid is slow, like cyclizations, this undesired capping inevitably occurs with uronium/aminium salts.[13,14]

With the aim of investigate on the efficiency of COMU and to compare it with other known benzotriazole-based coupling reagents, manual and microwave-assisted automated syntheses of pentapeptide H-Tyr-Aib-Aib-Phe-Leu-NH_2 were conducted (Table 1).[2,4] In both strategies COMU achieved a percentage of target peptide considerably higher than (***1-Hydroxybenzotriazole***) HOBt-based HBTU (entries 3, 4 vs. 7, 8). Surprisingly, the inclusion of Oxyma and morpholino moieties in COMU resulted in superior potency to classical aminium salt HATU, a previously well-established standard of powerful amide bond-assisting reagent (entries 1, 2 vs. 7, 8).[1,2,6] In comparison to analogous dimethylaminomorpholino-based reagents, COMU achieves superior coupling extensions than HOBt-derived 1-((dimethylimino)morpholinomethyl)3-H-benzo-[1,2,3]triazolo-1-ium-3-olate hexafluorophosphate (HDMB), and similar to 1-((dimethylimino)morpholinomethyl)3-H-[1,2,3]triazolo[4,5-b]pyridine-1–3-olate hexafluorophosphate (HDMA) (entries 5, 6 vs. 7).[2,9] However, in the MeLeu analogue of the Aib-enkephaline pentapeptide, COMU performed at an intermediate level between these two aminium salts.[2] The closely related uronium salt *O*-[Cyano(ethoxycarbonyl)

Table 1. Coupling extension during solid-phase assembly of pentapeptide H-Tyr-Aib-Aib-Phe-Leu-NH_2 using 1-2 min. preactivation with 2 eq. DIEA in DMF

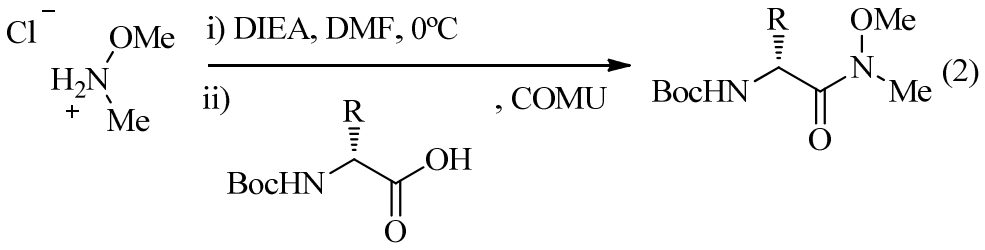
Entry	Coupling Reagent	Conditions	Pentapeptide (%)	des-Aib (%)
1	HATU	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	83.0	17
2	HATU	MW synthesizer, 80ºC, 6 min.	79.5	20.5
3	HBTU	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	47.0	53
4	HBTU	MW synthesizer, 80ºC, 6 min.	23.1	76.9
5	HDMA	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	98.0	<1
6	HDMB	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	89.0	10
7	COMU	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	99.7	0.26
8	COMU	MW synthesizer, 80ºC, 6 min.	92.1	7.9
9	HOTU	Manual, rt, 30 min. (1 h d.c. for Aib-Aib)	99.0	1.0

methylidene)amino]-1,1,3,3-tetramethyluronium Hexafluorophosphate, (HOTU), also containing Oxyma, shows comparable efficiency to COMU in the Aib peptide model with 2 equiv. of base (entry 7 vs. 9), although with only 1 equiv. the morpholino-containing COMU was clearly superior.[2] A similar tendency was observed in the assembly of the ACP (65-74) decapeptide.[2] Interestingly, the microwave-assisted syntheses achieved similar purities with substantial reduction of the coupling time, highlighting the benefits of this revolutionary technique (entries 1, 3, 7 vs. 2, 4, 8).[4,15] The conveniency and compatibility of COMU with automated, microwave-irradiated equipments has been confirmed in the elongation of hindered peptide and protein sequences, such as β-amyloid 1-42 peptide, in which the novel Oxyma-based reagents was the undoubtedly the most efficiency among other HOBt, ***7-aza-1-***

hydroxybenzotriazole (HOAt) and ***1-hydroxysuccinimide*** (HOSu)-derived coupling reagents.[16]

An additional feature of COMU is the extraordinary solubility displayed in the routinely used organic solvents in peptide synthesis.[2,3] Thus, up to 1.44 mol L^{-1} (0.620 g mL^{-1}) solutions can be prepared with COMU, in contrast to the maximum solubility exhibited by HBTU and HATU (0.43-0.46 mol L^{-1}), which translates into higher-rate couplings.[2,4] Furthermore, acidic and basic washes with 1 mol L^{-1} HCl and 1 mol L^{-1} $NaHCO_3$ during solution-phase work ups are greatly facilitated due to the enhanced solubility of COMU and the corresponding urea byproduct in aqueous environments, compared to benzotriazole-based reagents.[1,2,3] In addition, the course of the coupling reaction can be followed due to a clear color change using COMU, depending on the base employed (from pink to colorless with 2,4,6-Collidine, from yellow to orange with DIEA).[2] However, this intense color (UV max at 225 nm) can interfere with the fulvene-piperidine adduct during automonitoring of Fmoc removal in certain synthesizers.

COMU is 100% stable in DMF up to 1 day after the preparation of the sample, and 2 days afterwards decreases to 93%.[2] The oxygen in the morpholine ring within the carbocation skeleton also helps stabilizing the compound because of its solvation character. Nonetheless, impurities coming from the synthetic route of COMU, mainly dimethylmorpholino urea, occasionally catalyse the decomposition of the coupling reagent. The quality of the DMF employed and the content of secondary amines, also influences the stability of COMU.



63-97%

Racemization in the *Cα* is a serious concern in peptide synthesis, since the optically pure peptide is usually difficult to separate from other stereoisomers.[1,7] The retention of configuration induced by COMU has been explored during [1+1] and [2+1] couplings using the Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2 peptide systems.[9] Stepwise couplings with COMU gave rise to a minimum amount of racemization <1%, compared to HATU and other benzotriazole-based aminium salts. In segment condensations, COMU performed similarly to HOBt-based reagents in DIEA but was one of the most efficient in reducing racemization when 2,4,6-Collidine was employed as base. Moreover, since COMU requires only 1 equiv. of base in order to achieve successful couplings, racemization can be further decreased.[2,9] In amino acids very prone to this side reaction, such as Cys or His, addition of Oxyma and preactivation at 0ºC are advantageous. This outstanding control of optical purity has inspired the use of COMU in the transformation of *N*-protected-amino acids into the corresponding Weinreb amides by reaction with *N*-methoxy-*N*-methylamine (eq 2).[3] These intermediates are of particular interest for the reduction of carboxyl components to ketones or aldehydes, by a variety of methodologies.[17] Several amino acid Weinreb amides have been synthesized in high yields and purities, by addition of the corresponding residue and COMU to an ice-cooled solution of *N,O*-dimethylhydroxylamine hydrochloride and DIEA in DMF in less than 3 hours.[3] The abovementioned solubility of COMU byproducts in aqueous workups is a decisive factor in the selection of the appropriate coupling reagent.

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Apèndix V: *Impacte actual dels reactius basats en Oxyma*

Cronològicament, els primers estudis de la Tesi Doctoral que es van publicar en revistes d'àmbit científic van ser l'evaluació d'Oxyma i COMU com a reactius especialitzats en la formació de l'enllaç peptídic (Publicacions II i VI), a l'any 2009. Des de llavors, ambdòs han esdevingut comercialment disponibles, fet que ha promogut la seva acollida en laboratoris de síntesi peptídica. En aquest apèndix es recull breument l'impacte que han tingut Oxyma i COMU en algunes investigacions publicades recentment a la literatura.

Els estudis realitzats sobre la compatibilitat de reactius basats en Oxyma amb irradiació per microones han tingut continuitat en la modificació de micropartícules amino-funcionalitzades mitjançant acoblaments assistits per microones amb Oxyma a 60ºC.[1] Tal com s'apuntava a la Publicació II de la Tesi Doctoral, no es tenen notícies de subproductes d'addició nucleòfila a l'èster d'Oxyma. A més. la elevada eficiència d'aquesta metodologia permet reduïr els temps d'acoblament a 20 min.[1] D'altra banda, la combinació d'Oxyma i reactius d'acoblament de tipus triazina [tetrafluoroborat de 4-(4,6-di[2,2,2-trifluoroetoxi]-1,3,5-triazin-2-il)-4-metilmorfolini] es tradueix en una eficiència major que l'addició de HOAt en models peptídics que impliquen acoblaments Aib-Aib, reforçant les observacions de la present Tesi Doctoral.[2] D'entre les *N*-hidroxilamines afegides al medi de reacció, Oxyma és l'única que augmenta la puresa del pèptid desitjat de 55% a 94%. Tanmateix, s'observen millors resultats si el dissolvent en que es du a terme l'acoblament és una mescla DMF/DCM 1:1, que si aquest és únicament DCM.[2] En la mateixa línia, s'ha investigat l'efecte de l'addició d'Oxyma en acoblaments realitzats amb la sal de formamidini TFFH, mostrant una reducció de l'epimerització en sistemes seqüencials i una eficiència en el model Aib-encefalina superiors a HOAt, reafirmant la tendència relativa descrita a la Publicació II.[3] De la mateixa manera, sistemes d'epimerització de tipus fragment [2+1] no permeten observar una millora respecte HOAt així com l'efecte de la base emprada.[3] Finalment, èsters preformats d'Oxyma s'han utilitzat en reaccions de transferència de grup acil a alcohols secundaris catalitzades per lipases.[4]

Una prova més de la compatibilitat de COMU amb sintetitzadors automàtics irradiats per microones s'ha obtingut durant l'elongació de seqüències considerades difícils, com ara el pèptid *β*-amiloide 1-42. En aquest sistema d'eficàcia, la sal d'uroni de tipus dimetilmorfolino construïda amb Oxyma dóna lloc a percentatges d'acoblament superiors a altres reactius autosuficients basats en HOBt, HOAt i HOSu.[5] L'abilitat de COMU de facilitar la transferencia de grups acil s'ha demostrat fora de l'àmbit peptídic en la transformació d'α-aminoàcids en les amides de Weinreb corresponents, controlant la configuració òptica.[6] Altres avantatges ja apuntades en la Publicació VI, i destacades pels autors són la possibilitat de seguir la reacció d'amidació pel canvi de color de groc a taronja i la solubilitat dels subproductes derivats de COMU, possibilitant una alta puresa.[6] Recentment, també s'ha estudiat el seu comportament en esterificacions amb alcohols primaris, secundaris i terciaris en solució.[7] Tot i que les acilacions amb COMU evolucionen més lentament que TBTU i TATU amb alcohols primaris i secundaris, la sal d'uroni d'Oxyma és l'única que permet formar èsters amb alcohols terciaris, a més de no necessitar temps de preactivació llargs.[7] No obstant, potser l'aportació més interessant d'aquest estudi és un detallat mecanisme d'acilació amb COMU.[7] En una patent recentment publicada es descriu l'ús de COMU per a introduir elements marcadors en biomol·lècules.[8]

Les excel·lents prestacions dels reactius basats en Oxyma en la formació de l'enllaç peptídic ha portat a considerar la implementació d'aquesta oxima àcida en sulfonats actius, anomenats NpsOXY

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i TsOXY, segons el reactiu contingui 2-naftalè o 4-toluè.[9] Com seria d'esperar tenint en compte els resultats presentats a la Tesi Doctoral, els derivats d'Oxyma superen els anàlegs de caire benzotriazòlic en termes de supressió de l'epimerització i eficàcia d'acoblament.[9] La recerca d'oximes àcides que puguin millorar la reactivitat d'Oxyma s'ha iniciat amb la síntesi i evaluació de l'oxima construïda a partir de l'àcid de Meldrum i les sals d'uroni corresponents.[10] Probablement, s'hagi creuat la fina línia de reactivitat que separa un acoblament eficient i una excessiva inestabilitat, tenint en compte que l'oxima esmentada (anomenada HONM) s'addiciona irreversiblement a la carbodiimida, tot inutilitzant-la. Tot i així, la sal d'uroni dimetilmorfolino derivada (HMMU) s'ha posicionat com un reactiu especialment indicat per acilacions d'amines poc reactives, de tipus anilina. Resulta també interessant la investigació de la reactivitat dels èsters actius preformats d'Oxyma, HONM i benzotriazols on, a més d'indicar la major reactivitat de la oxima provinent de l'àcid de Meldrum, es pot observar que la reactivitat de l'èster d'Oxyma es comparable a la de HOBt.[10] Aquests resultats (previsibles abans de la Tesi Doctoral, en base a l'acidesa), reforcen la teoría de que les extraordinàries prestacions d'Oxyma provenen de factors estèrics i/o assistència veïnal.

Les perspectives que s'obren en metodologia de síntesi peptídica després de la implementació d'Oxyma i els reactius derivats sembla anar doncs en dos direccions: per un costat trobar nous reactius de disseny que continguin la mencionada estructura i així aprofitar les avantatges sobre els benzotriazols que es deriven; i d'altra banda trobar noves aplicacions i propietats als reactius ja descrits en la present Tesi Doctoral. Probablement el darrer àmbit ofereix una visió més interessant i àmplia del camp metodològic, ja que habitualment els additius i reactius d'acoblament s'han restringit de forma quasi exclusiva a la formació de l'enllaç peptídic. Els descobriments recents, com la esterificació selectiva de COMU vers altres sals d'uroni, obre les portes de la transferència de grups acil a centres diferents de les tradicionals amines o a la catàlisi de reaccions de caire similar. En qualsevol cas, la comprensió del mecanisme d'acció pel qual Oxyma ofereix uns resultats tan sorprenents tenint en compte l'acidesa, permetria dissenyar de forma racional nous reactius amb l'esperança d'eliminar la barrera sintètica que separa el químic peptídic de les seqüències difícils.

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