Tesi Doctoral

NEW PROTECTING GROUPS FOR THE SYNTHESIS OF COMPLEX PEPTIDES

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INDEX GENERAL

ÍNDEX D'ABREVIATURES

Alloc: aliloxicarbonil

Boc: *tert*-butiloxicarbonil.

Bn: benzyl.

^{t}Bu: *tert*-butil.

EDOT: 3,4-etilendioxitiofè.

EDOTn: 3,4-etilendioxi-2-tenil.

Fmoc: 9-fluorenilmetoxicarbonil.

Fmoc-OSu: Fmoc-O-succinimidat.

2-MBT: 2-mercaptobenzotiazole.

MIS: 1,2-dimetilindole-3-sulfonil.

MIM: 1-metili-3-indolilmetil.

Pbf: 2,2,4,6,7-pentametildihidrobenzofuran-5-sulfonil.

Pmc: 2,2,5,7,8-pentametilcroman-6-sulfonil.

*p*NB: *p*-nitrobenzil.

*p*NMA: àcid *p*-nitromandèlic.

*p*NZ: *p*-nitrobenziloxicarbonil.

SPPS: Síntesi de Pèptids en Fase Sòlida (Solid Phase Peptide Synthesis)

TFA: àcid trifluoroacètic.

RESUM DE LA MEMÒRIA

El **primer capítol** de la Tesi és un article de revisió sobre els grups protectors usats en química de pèptids tan en solució com en fase sòlida.

En el **segon capítol** s'estudia la utilitat del Fmoc-2-MBT com a nova eina per incorporar el grup Fmoc que evita les reaccions secundàries dels reactius més usats en l'actualitat, Fmoc-Cl i Fmoc-OSu.

El **tercer capítol** és un estudi del grups protectors derivats del *p*-nitrobenzil i està dividit en diversos treballs:

- Al primer es descriuen les utilitats del grup *p*NZ com a protector del grup α-amino per evitar reaccions secundàries associades a l'ús del grup Fmoc.
- Al segon es demostra la utilitat del grup *p*NZ com a protector de les cadenes laterals de la Lys i la Orn i el seu ús en combinació amb l'éster *p*-nitrobenzílic per la preparació de pèptids cíclics.
- Els treballs tercer i quart mostren aplicacions pràctiques del grup *p*NZ a diferents estratègies de síntesi de derivats del pèptid antitumoral Kahalalide F.
- En el darrer treball d'aquest capítol s'explica el desenvolupament d'un nou espaïador bifuncional, el *p*-nitromandèlic, pensat per la síntesi de derivats peptídics làbils a base.

Al **quart capítol**, que també es divideix en varis treballs, s'estudia la utilitat dels derivats del 3,4-etilendioxitiofè i de l'1-metilindole com a nous grups protectors làbils a medi àcid.

- En el primer treball es demostra la utilitat del 3,4-etilendioxitenil i del 1-metil-3-indolilmetil com a protectors d'amides de l'esquelet peptídic.
- Al segon treball es parla de la síntesi i utilitats de nous derivats fenílics del 3,4-etilendioxitenil com a protectors d'àcids carboxílics làbils a concentracions molt baixes de TFA (0.01-0.5%)
- En el tercer treball, basant-nos en els resultats del treball precedent, es demostra la utilitat de l'alcohol 5(4-hidroxifenil)-3,4-etilendioxitenílic per a síntesi de pèptids sensibles a medi àcid.
- Per últim, en el darrer treball, s'ha desenvolupat un nou grup protector per la cadena lateral de l'Arginina, l'1,2-dimetilindole-3-sulfonil (MIS), que és més làbil a medi àcid que els protectors més usats en l'actualitat (Pbf i Pmc).

INTRODUCCIÓ
I
OBJECTIUS

INTRODUCCIÓ

L'elevat interès biològic dels pèptids fa necessari el desenvolupament de noves metodologies de síntesi que permetin l'accés a estructures peptídiques complexes de manera fàcil, ràpida, econòmica i respectuosa amb el medi ambient.

La present Tesi Doctoral se centra en el desenvolupament de grups protectors que poden ser molècules orgàniques de baix pes molecular o unides a un suport polimèric. En aquest darrer cas s'anomenen espaïadors bifuncionals.[1] Les principals raons per al desenvolupament de nous grups protectors són les següents:

- Ortogonalitat: s'entén que un conjunt de grups protectors és ortogonal quan es pot eliminar qualsevol d'ells deixant la resta inalterats. En la síntesi de pèptids complexos, ja sigui ramificats i/o cíclics és necessari l'ús d'esquemes de protecció ortogonals per tal de funcionalitzar la molècula en la posició desitjada.
- Síntesi de pèptids sensibles: quan l'objectiu es sintetitzar un pèptid que conté grups funcionals làbils és necessari l'ús de grups protectors que puguin ser eliminats en condicions suaus.
- Síntesi de seqüències peptídiques anomenades "difícils": les seqüències "difícils" estan definides a la literatura com aquelles que, tot i tenir una mida assequible, són inesperadament difícils de sintetitzar degut a acoblaments i/o desproteccions incompletes. L'ús de determinats grups protectors pot fer accessibles aquestes seqüències.
- Síntesi de seqüències peptídiques amb molta tendència a donar reaccions secundàries com la formació de dicetopiperazines o aspartimides. Per a la seva obtenció calen grups protectors que minimitzin aquestes reaccions secundàries.

L'impuls decisiu per la química de pèptids va ser el desenvolupament de la metodologia de fase sòlida per part de Merrifield als anys 60 i que es basava en la unió del pèptid per l'extrem *C*-terminal a un suport polimèric i en el

[1] En anglès "linkers" o "handles".

creixement d'aquest en la direcció *C-N*.[2] En l'estratègia original de Merrifield, anomenada Boc/Bn (*tert*-butiloxicarbonil/benzil), el grup α-amino dels aminoàcids està protegit amb el grup Boc, làbil a àcid trifluoroacètic (TFA) i que actua com a protector temporal ja que s'elimina vàries vegades durant la síntesi. Mentre que les cadenes laterals dels aminoàcids estan en general protegides amb protectors de tipus benzílic que actuen com a protectors permanents ja que són estables al TFA i no s'eliminen fins al final de la síntesi quan s'escindeix el pèptid del suport polimèric mitjançant tractament amb àcids més forts com l'HF. En l'actualitat però l'estratègia més usada és la Fmoc/^{t}Bu (9-fluorenilmetoxicarbonil/*tert*-butil) , en la qual el grup α-amino es protegeix amb el grup Fmoc, làbil a bases, i les cadenes laterals amb grups de tipus *tert*-butil (làbils a TFA). Aquesta estratègia té com a principals avantatges respecte a la Boc/Bn que és realment ortogonal ja que els grups protectors de l' α-amino i de les cadenes laterals s'eliminen mitjançant mecanismes diferents i a part s'evita el tractament final amb HF.

[2] R.B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149; R.B. Merrifield, *Angew. Chem.* Int. Ed. **1985**, *24*, 799; R.B. Merrifield. *Science* **1986**, *232*, 341.

OJECTIUS DE LA TESI

L'objectiu general de la present Tesi és realitzar una aportació en el camp dels grups protectors en síntesi de pèptids i derivats, ja sigui en fase sòlida, principalment mitjançant l'estratègia Fmoc/^{t}Bu però també la Boc/Bn, o en solució. Els objectius concrets són:

- Dissenyar i sintetitzar diferents grups protectors ja siguin estàndar o de tipus espaïador bifuncional que solucionin problemes reals en química de pèptids i derivats, demostrant-ne la seva eficàcia mitjançant la síntesi de pèptids model.
- Descobrir noves aplicacions i optimitzar l'ús de grups protectors ja existents.
- Aplicar els grups protectors desenvolupats a la síntesi de pèptids amb interès biològic.

1 Grups Protectors per a Aminoàcids

Amino Acid-Protecting Groups

Grups Protectors per a Aminoàcids

Albert Isidro-Llobet, Mercedes Álvarez, Fernando Albericio

Chemical Reviews (En preparació)

Resum

Aquest capítol és un article de revisió i està pensat com una introducció al tema dels grups protectors per posar la Tesi en el seu context.
En ell s'ha fet un resum dels principals grups protectors estàndar (no units a suport polimèric) per síntesi de pèptids tan en solució com en fase sòlida, fent especial incís en els protectors més usats en els darrers temps i en els desenvolupats a partir de l'any 2000. L'article està dividit en seccions segons el tipus de grup funcional a protegir. Dins de cadascuna d'aquestes seccions s'explica quines reaccions secundàries pot donar aquesta funcionalitat, quins són els principals grups que s'usen per protegir-la (ordenats per mecanisme d'eliminació), les seves principals característiques i com s'introdueixen i s'eliminen.

Albert Isidro-Llobet, Mercedes Álvarez, Fernando Albericio. Amino Acid-Protecting Groups. *Chemical Reviews* **2009** (submitted).

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Ús del Fmoc-2-MBT, per a la introducció del grup Fmoc sense reaccions secundàries

Fmoc-2-Mercaptobenzothiazole, for the Introduction of the Fmoc Moiety Free of Side-Reactions

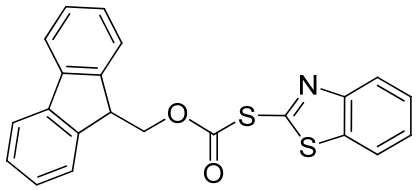
Fmoc-2-Mercaptobenzotiazole, per la Introducció del grup Fmoc sense Reaccions Secundàries

Albert Isidro-Llobet, Xavier Just-Baringo, Ariel Ewenson, Mercedes Álvarez, Fernando Albericio

Biopolymers 88 (2007), 733-737

Resum

A part de tenir unes característiques d'eliminació adequades és important que un grup protector es pugui incorporar d'una manera ràpida, efectiva i sense reaccions secundàries. El treball publicat en el següent article té com a objectiu l'estudi del Fmoc-2-mercaptobenzothiazole com a nou reactiu per la incorporació del grup Fmoc que eviti les reaccions secundàries que es donen en aquest procés quan s'usen els reactius clàssics (Fmoc-Cl i Fmoc-OSu). Aquestes reaccions secundàries són la formació de Fmoc-dipèptids, que es dóna tan amb Fmoc-Cl com amb Fmoc-OSu, i la formació de derivats de Fmoc-ß-Ala-OH, que es dóna quan s'usa Fmoc-OSu. Dels experiments realitzats s'ha pogut concloure que la menor reactivitat del Fmoc-2-mercaptobenzothiazole deguda a la menor acidesa del 2-MBT respecte a l'HCl i la HOSu evita tant la formació de Fmoc-dipèptids com de derivats de Fmoc-ß-Ala-OH.



Fmoc-2-Mercaptobenzotiazole (Fmoc-MBT)

Fmoc-2-Mercaptobenzothiazole, for the Introduction of the Fmoc Moiety Free of Side-Reactions

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ABSTRACT:

A double side-reaction, consisting in the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH, during the preparation of Fmoc protected amino acids (Fmoc-AA-OH) with Fmoc-OSu is discussed. Furthermore, the new Fmoc-2-MBT reagent is proposed for avoiding these side-reactions as well as the formation of the Fmoc-dipeptides (Fmoc-AA-AA-OH) and even tripeptides, which is another important side-reaction when chloroformates such as Fmoc-Cl is used for the protection of the α-amino function of the amino acids. © 2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88: 733–737, 2007.

Keywords: Alloc; Fmoc-dipeptides; Fmoc-OSu, p-NZ; protecting group; side-reaction

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INTRODUCTION

Carbamates such as Boc,[1,2] Fmoc,[3] Z,[4] and to a lesser extent Alloc,[5,6] pNZ,[7] and Troc[8] are the most efficient way to mask the nucleophilicity of the amino function during peptide synthesis.[9,10] The introduction of the alkoxycarbonyl moiety was first carried out under weakly basic conditions via the Schotten–Baumann reaction, by using the corresponding chloroformates (also known as chlorides).[3] However, our group[11] and others[12–15] described in the early eighties that during the preparation of Fmoc-amino acids from Fmoc-Cl, most amino acids become contaminated with significant levels (1–20%) of corresponding Fmoc-dipeptides and even tripeptides. As an example, even when the relatively hindered Alloc-Val-OH was prepared in a laboratory scale, 14% of the corresponding dipeptide was obtained.[9] This high incidence of protected dipeptide by-products would lead to the insertion of an unwanted extra amino acid in the final peptide synthesis, an event which cannot be tolerated in the preparation of an API. This side-reaction can be avoided by carrying out an in situ bis-trimethylsilylation protection of the amino acid, followed by reaction with the chloride.[14,16] The fact that the temporal protection of the carboxylic function avoids the side-reaction reinforces that the mechanism goes through an *O*-acylcarbonate (see Figure 1).

As an alternative to the chloride and since this side-reaction is associated with the quality of the leaving group, we have proposed the use of the less reactive azide derivative, which can be readily prepared and isolated from the chloroformate[3,11] or prepared in situ before reacting with the amino acid.[17] Several approaches, based on the use of other less reactive species such as the 1,2,2,2-tetrachloroethyl,[18,19]

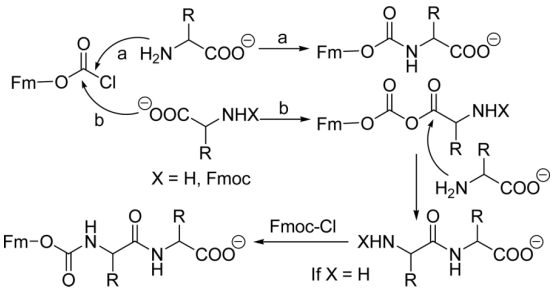

FIGURE 1 Mechanism for the formation of protected dipeptides during the protection of amino acids.

the 5-norbornene-2,3-dicarboximido,[20] the pentafluorophenyl,[21] and the symmetrical pyrocarbonates[22] have been proposed, but the hydroxysuccinimido (Su)[12,13,15,23,24] ester has been considered the reagent of choice for the introduction of the protecting moiety.

However, lately the use of Fmoc-OSu has been questioned, because it has been shown that commercial Fmoc-amino acids prepared with Fmoc-OSu were found to contain Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH as contaminants (0.1–0.4%).[25] These levels of impurity become highly problematic when manufacturing drug substances.

Herein, a discussion regarding the formation of this double side-reaction is carried out as well as an alternative for the preparation of protected amino acids free of side reactions.

EXPERIMENTAL SECTION

General

2-MBT was provided by Luxembourg Industries from (Tel-Aviv, Israel). HPLC was performed on a reversed-phase C_{18} (4.6 × 150 mm^2, 5 μm) with a linear gradient of 0.045% aqueous TFA and 0.036% TFA in CH_3CN at 1.0 mL/min flow rate, from 1:0 to 0:1 over 30 min, with UV detection at 220 nm.

Fmoc-2-MBT

MBT · DCHA Salt. To a solution of 2-MBT (5 g, 29.9 mmol) in 250 mL of EtOAc, DCHA was added and the resulting suspension was stirred overnight. The precipitate obtained was isolated by filtration washed with ethyl acetate and dried in vacuo yielding a white solid (9.44 g, 91% of yield).

Fmoc-2-MBT. To a solution of Fmoc-Cl (7.01 g, 27.1 mmol) in 70 mL of $CHCl_3$, MBT · DCHA salt (9.44 g, 27.1 mmol) was slowly added and stirred overnight. The suspension was filtered and the solid washed with $CHCl_3$ (2 × 10 mL). The filtrate was washed with 10% of aq. citric acid (2 × 30 mL), 10% of aq. $NaHCO_3$ (2 × 30 mL), H_2O (2 × 30 mL), dried over $MgSO_4$ and evaporated to dryness to yield the title compound as a white solid (8.53 g, 81.9% of yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.01 (d, 1H, J = 8.2 Hz), 7.85 (d, 1H, J = 8.0 Hz), 7.72 (d, 2H Fmoc, J = 7.5 Hz), 7.53 (d, 2H Fmoc, J = 7.5 Hz), 7.47 (dd, 1H, J = 8.0, 7.4 Hz), 7.39 (m, 2H Fmoc and 1H MBT), 7.28 (t, 2H Fmoc, J = 7.5 Hz), 4.60 (d, 2H, J = 7.3 Hz), 4.27 (t, 1H, J = 7.3 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.5, 157.5, 152.4, 143.0, 141.5, 137.0, 128.3, 127.5, 126.7, 126.0, 125.3, 123.5, 121.5, 120.4, 71.0, 46.8.

Fmoc-Amino Acids Using Fmoc-2-MBT

Fmoc-Glycine. H-Gly-OH (318 mg, 4.24 mmol) was suspended in a mixture of 1% aqueous Na_2CO_3-dioxane (1:1, v/v) (24 mL) and cooled with and ice bath. A mixture of Fmoc-MBT (1.5 g, 3.85 mmol) in dioxane (4 mL) was slowly added keeping the pH at 9.5 with 10% aq. Na_2CO_3. The ice bath was removed and the suspension stirred at room temperature keeping the pH at 9.5. The course of the reaction was followed by TLC. After 24 h of stirring H_2O (50 mL) were added to the reaction mixture, the pH was adjusted to 8 with 1*N* HCl and washings with MTBE (3 × 30 mL) were carried out. The aq. phase was acidified to pH 1 with HCl-H_2O (1:2) and extracted with EtOAc (3 × 40 mL). The organic fractions were dried with $MgSO_4$, evaporated to dryness and the resulting yellow solid was washed with DCM to give Fmoc-Gly (627 mg, 55% of yield, yield not optimized) as a white solid. The product was characterized by HPLC and ESMS.

RESULTS AND DISCUSSION

A possible explanation for the double side-reaction leading to the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH is depicted in Figure 2. One of the cornerstones of this process involves the presence of a nucleophile and two nucleophiles are present: ($^-$OSu and $^-$OH) are present in the reaction environment.

The β-Ala structure (1) can be formed through a Lossen rearrangement after the attack of a nucleophile, probably $^-$OSu, on one of the carbonyls of the HOSu moiety present in Fmoc-OSu. A similar pathway for this first part of the mechanism was initially reported by Gross and Bilk, during the reaction of HOSu with DCC,[26] and then by Wilcheck and Miron.[27] Furthermore, similar β-Ala impurities have been found by Zalipsky during the use PEG-OSu as pegylation reagent.[28]

The formation of the free amine of β-alanine in the carbamic acid (Nu_2=OH, pathways b and c) or from the rather unstable *ON*-succinimide carbamate (Nu_2=OSu, pathways a and d). Furthermore, the formation of the Su ester of β-Ala,[2] which is susceptible to reacting with the free amino acid to give the dipeptide, is formed when Nu_1=OSu (pathways a and b). This mechanism may also help to explain the larger content found by Hlebowicz et al.[25] of β-Ala by amino acid analysis than that found by HPLC (Fmoc-β-Ala-Arg-OH + Fmoc-β-Ala-OH). This higher amount of β-Ala can be inter-

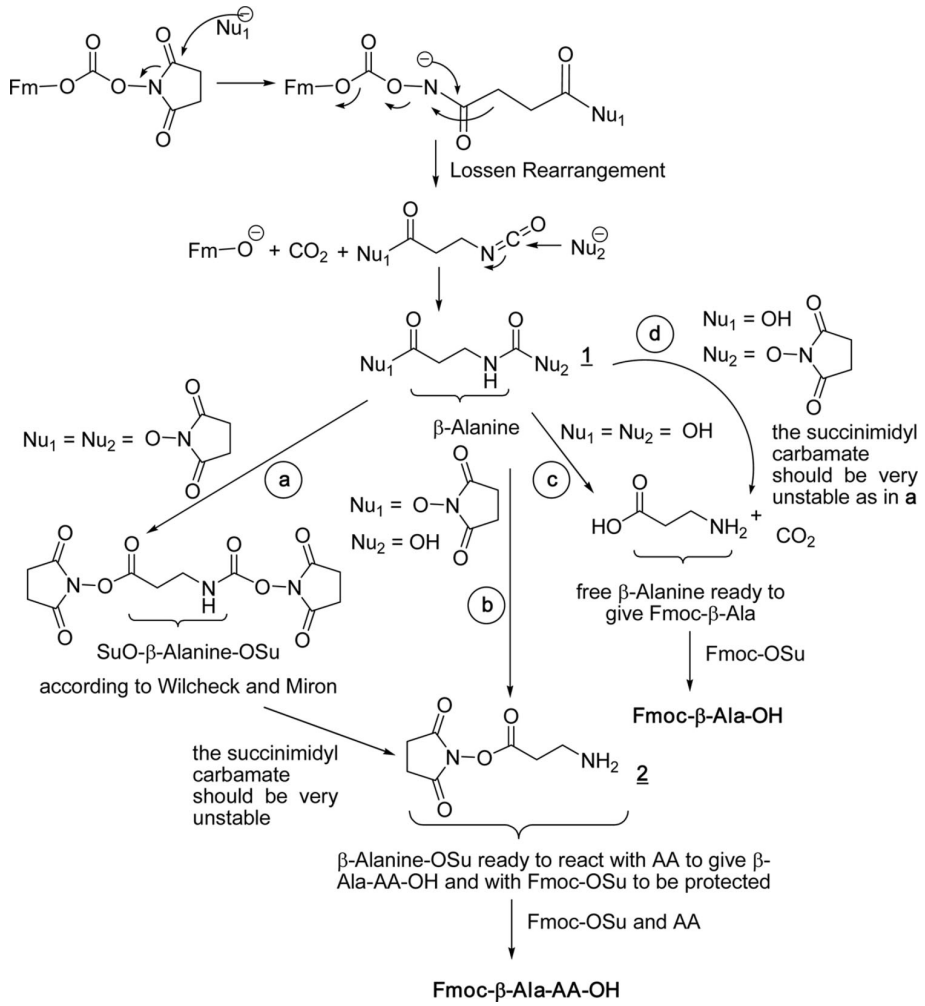

FIGURE 2 Mechanism for the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH during the protection of amino acids.

preted from **2**, which can undergo polymerization and therefore the corresponding peaks can be more difficult to be identified. Furthermore, this scheme agrees with the fact that no formation of Fmoc-AA-β-Ala-OH takes place, as pointed out by Hlebowicz et al.[25]

Formation of H-β-Ala-OH or H-β-Ala-OSu can take place either during the preparation of Fmoc-OSu or during the preparation of the protected amino acids. However, the HPLC analysis of commercial Fmoc-OSu has revealed the absence of Fmoc-β-Ala-OH and therefore indicated that the side-reaction does probably take place during the protection of the amino acids.

Thus, if a substitute to Fmoc-OSu is to be found, this should be of a reactivity similar to OSu esters and therefore lower that other more active species such as the chloroformate or the OBt carbonate, which leads to the formation of protected peptides or tripeptides.[12] In this regard, the 2-MBT (Figure 3), recently proposed by Evans as an additive in carbodiimides based coupling schemes, substituting HOBt, drew our attention.[29] Thus, p*K*a calculations carried out with ChemAxon software showed that the p*K*a of both tautomers of 2-MBT (*pK*a's: NH tautomer, 10.90; SH tautomer, 7.49) was higher than that of HOSu (*pK*a HOSu, 7.19) making the 2-MBT derivatives potentially less reactive than those bearing OSu as the leaving group.

An undeniable advantage of 2-MBT is its availability at low cost (see Ref. 28), since it is a commodity used as a rub-

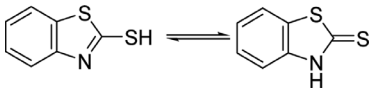

FIGURE 3 Structure of 2-MBT.

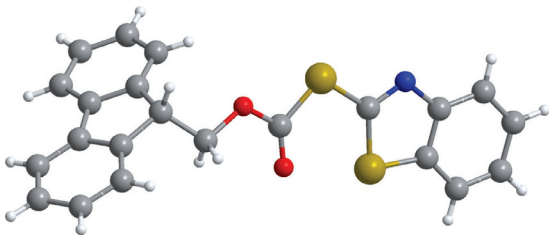

FIGURE 4 Structure of the Fmoc-2-MBT obtained by X-ray difraction.

ber vulcanization accelerator or in the preparation of fungicides.[30]

Preparation of Fmoc-2-MBT was readily achieved by reaction of 2-MBT with Fmoc-Cl following a well reported method described for other reagents.[23] Although, in the literature it is described that 2-MBT active species are presented as a mixture of S- and N-regioisomers, a structure obtained by X-ray diffraction of a Fmoc-2-MBT crystal, showed it to consist solely of the S-regioisomer (see Figure 4).

Fmoc-2-MBT reacts smoothly with amino acids to yield the corresponding Fmoc-AA-OH. It is worth indicating that the acylation reaction is slower when it is carried out with Fmoc-2-MBt than when Fmoc-OSu is used (24 h *vs* 0.5 h to accomplish a total conversion). This rather slow rate indicates than 2-MBT active species is less reactive than those based on OSu. Therefore the N-acylation may take place without the formation of Fmoc-dipeptides. The reactivity modulation afforded by the 2-MBT moiety enhances the differentiation between a stronger nucleophile (the α-amine of the amino acid substrate) and a weaker one (the carboxylate of the latter), thus significantly lessening the possibility of mixed anhydride formation and consequently that of the unwanted di- and tripeptides.

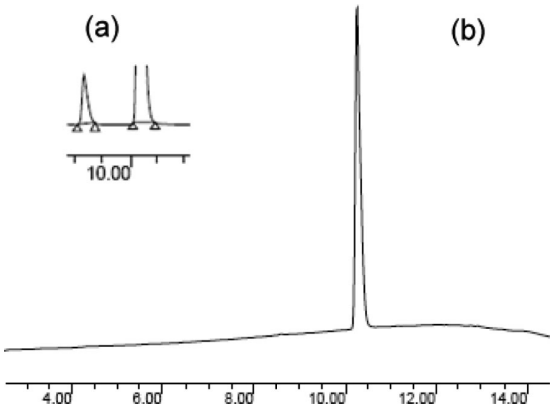

FIGURE 5 HPLC analysis: (a) coinjection of Fmoc-Gly-OH obtained and Fmoc-Gly-Gly-OH, (b) Fmoc-Gly-OH obtained.

The 2-MBT liberated during the reaction can be removed by washing the final product with DCM. HPLC analysis of the crude Fmoc-amino acids obtained following this method showed that no dipeptide formation took place and therefore that the products are free of side-products (Figure 5), confirming the first hypothesis about the reactivity of the 2-MBT derivatives.

CONCLUSIONS

The use of the novel Fmoc-2-MBT reagent allows the preparation of Fmoc-amino acids, free of the three problematic side-products: (a) Fmoc-dipeptides associated to the quality of the leaving group, which takes place when Fmoc-Cl is used; (b) Fmoc-β-Ala-OH; and (c) Fmoc-β-Ala-AA-OH. These two latter side-products being produced when Fmoc-OSu is used as the acylating reagent. A mechanism for the presence of the side-products has been also proposed.

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REFERENCES

1. Carpino, L. A. J Am Chem Soc 1957, 79, 4427–4431.
2. Anderson, G. W.; McGregor, A. C. J Am Chem Soc 1957, 79, 6180–6183.
3. Carpino, L. A.; Han, G. Y. J Org Chem 1972, 37, 3404–3405.
4. Bergmann, M.; Zervas, L. Ber Dtsch Chem Ges 1932, 65, 1192–1201.
5. Loffet, A.; Zhang, H. X. Int J Pept Protein Res 1993, 42, 346–351.
6. Kates, S. A.; Sole, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. Tetrahedron Lett 1993, 34, 1549–1552.
7. Isidro-Llobet, A.; Guasch-Camell, J.; Álvarez, M.; Albericio, F. Eur J Org Chem 2005, 3031–3039.
8. Windholz, T. B.; Johnston, D. B. R. Tetrahedron Lett 1967, 8, 2555–2557.
9. Albericio, F. Biopolymers (Pept Sci) 2000, 55, 123–139.
10. Wade, J. D. In Solid-Phase Synthesis. A Practical Guide; Kates, S. A.; Albericio, F., Eds.; Marcel Dekker: New York, 2000; pp 103–128.
11. Tessier, M.; Albericio, F.; Pedroso, E.; Grandas, A.; Eritja, R.; Giralt, E.; Granier, C.; Van-Rietschoten, J. Int J Pept Protein Res 1983, 22, 125–128.
12. Sigler, G. F.; Fuller, W. D.; Chaturvedi, N. C.; Goodman, M.; Verlander, M. Biopolymers 1983, 22, 2157–2162.
13. Lapatsanis, L., Milias, G., Froussios, K.; Kolovos, M. Synthesis 1983, 671–673.
14. Bolin, D. R.; Sytwu, I. I.; Humiec, F.; Meienhofer, J. Int J Pept Protein Res 1989, 33, 353–359.
15. Ten Kortenaar, P. B. W.; Van Dijk, B. G.; Peeters, J. M.; Raaben, B. J.; Adams, P. J.; Hans, M.; Tesser, G. I. Int J Pept Protein Res 1986, 27, 398–400.
16. Barlos, K.; Papaioannou, D.; Theodoropoulos, D. J Org Chem 1982, 47, 1324–1326.

17. Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. Org Proc Res Dev 2004, 8, 920–924.
18. Barcelo, G.; Senet, J.-P.; Sennyey, G. J Org Chem 1985, 50, 3951–3953.
19. Barcelo, G.; Senet, J.-P.; Sennyey, G.; Bensoam, J.; Loffet, A. Synthesis 1986, 627–632.
20. Henklein, P.; Heyne, H.-V.; Halatsch, W. R.; Niedrich, H. Synthesis 1987, 166–167.
21. Schön, I.; Kisfaludy, L. Synthesis 1986, 303–305.
22. Sennyey, G.; Barcelo, G.; Senet, J. P. Tetrahedron Lett 1986, 27, 5375–5376.
23. Paquet, A. Can J Chem 1982, 60, 976–980.
24. Milton, R. C.; Becker, E.; Milton, S. C.; Baxter, J. E. J.; Elsworth, J. F. Int J Pept Protein Res 1987, 30, 431–432.
25. Hlebowicz, E.; Andersen, A. J.; Andersson, L.; Moss, B. A. J Peptide Res 2005, 65, 90–97.
26. Gross, H.; Bilk, L. Tetrahedron, 1968, 24, 6935–6939.
27. Wilchek, M.; Miron, T. Biochemistry 1987, 26, 2155–2161.
28. Zalipsky, S. Chem Commun 1998, 69–70.
29. Evans, D. J. Speciality Chem Mag 2005, 25, 50–52.
30. The Merck Index, 13th ed.; Merck & Co.: Whitehouse Station, NJ, USA, 2001. Monograph Nr. 5893.

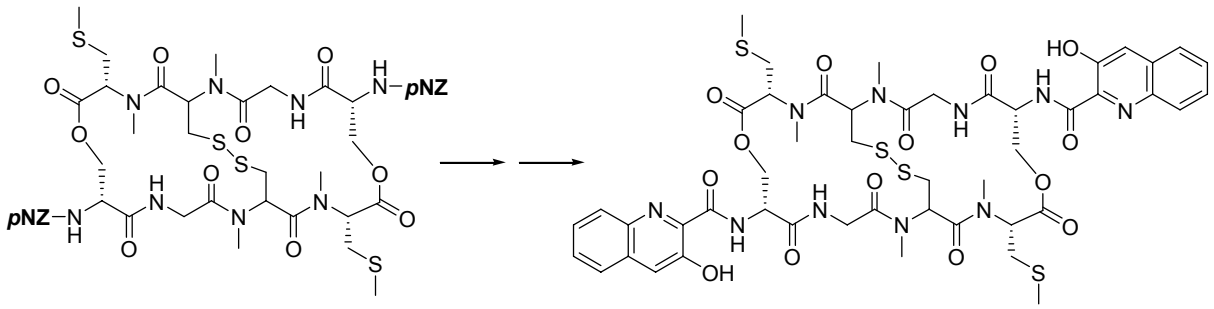
3 Derivats del *p*-Nitrobenzil com a Grups Protectors

Els derivats del *p*-nitrobenzil fa anys que es van introduir com a grups protectors però no han estat massa utilitzats en síntesi de pèptids.

Els objectius generals del següent capítol són:

- El descobriment de noves aplicacions del grup *p*-nitrobenziloxicarbonil (*p*NZ) (parts 1 i 2)
- L'aplicació del grup *p*NZ en diferents estratègies de síntesi del pèptid antitumoral Kahalalide F i derivats (parts 3 i 4)
- El desenvolupament de nous derivats del *p*-nitrobenzil (part 5).

A més, l'estudi del grup *p*NZ realitzat en aquest capítol, ha permès la seva utilització recent en la síntesi de l'Oxathiocoraline, un potent pèptid antitumoral.[1]



Oxathiocoraline

[1] Tulla-Puche, J.; Bayó-Puxan, N.; Moreno, J.A.; Francesch, A.M.; Cuevas, C.; Álvarez, M.; Albericio, F. *J. Am. Chem. Soc.* **2007**, *129*, 5322-5323.

p-Nitrobenzyloxycarbonyl (*p*NZ) as a Temporary N^{α} Protecting Group in Orthogonal Solid-Phase Peptide Synthesis – Avoiding Diketopiperazines and Aspartimide Formation

p-Nitrobenziloxicarbonil (pNZ) com a Protector Temporal del Grup N^{α} en Síntesi Ortogonal de Pèptids en Fase Sòlida - Evitant la Formació de Dicetopiperazines i Aspartimides

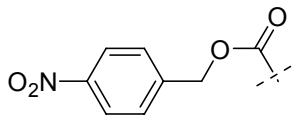
Albert Isidro-Llobet, Judit Guasch-Camell, Mercedes Álvarez and Fernando Albericio

European Journal of Organic Chemistry 2007, 3031-3039

Resum

Les característiques avantatjoses del grup Fmoc el fan molt difícil de substituir com a protector temporal del grup α-amino per a síntesi de pèptids en fase sòlida. De tota manera hi ha casos particulars en què el seu ús provoca reaccions secundàries derivades de la utilització de base en la seva eliminació i que poden reduir el rendiment de les síntesis o fins i tot fer impossible la obtenció del producte final.

En aquest article, es demostra la utilitat del grup *p*NZ com a protector temporal de funcionalitats α-amino en síntesi de pèptids en fase sòlida. Els corresponents *p*NZ aminoàcids són sòlids fàcils de sintetitzar i amb bones propietats per l'ús en SPPS. El grup *p*NZ és ortogonal als grups protectors més comuns en química de pèptids i s'elimina mitjançant reducció amb $SnCl_2$ en condicions pràcticament neutres en presència de quantitats catalítiques d'àcid. La combinació dels *p*NZ i Fmoc aminoàcids evita les principals reaccions secundàries de l'estratègia Fmoc/^{t}Bu associades a l'ús de piperidina, com per exemple la formació de dicetopiperazines i aspartimides.



Grup *p*-nitrobenziloxicarbonil (*p*NZ)

p-Nitrobenzyloxycarbonyl (pNZ) as a Temporary N^{α}-Protecting Group in Orthogonal Solid-Phase Peptide Synthesis – Avoiding Diketopiperazine and Aspartimide Formation

Albert Isidro-Llobet,[a] Judit Guasch-Camell,[a] Mercedes Álvarez,[a,b] and Fernando Albericio*[a,c]

Dedicated to Professor Josep Castells on the occasion of his 80th birthday

Keywords: Amino protecting group / Nitro reduction / $SnCl_2$ reduction / PNZ group in SPPS / Combinatorial chemistry / Side reactions

p-Nitrobenzyloxycarbonyl (pNZ) was used as a temporary protecting group for α-amino functionalities in solid-phase peptide synthesis. The corresponding derivatives are readily synthesized solids that perform well on solid phase. The pNZ moiety is orthogonal with the most common protecting groups used in peptide chemistry, and is removed under neutral conditions in the presence of catalytic amounts of acid. The use of pNZ derivatives in conjunction with Fmoc chemistry circumvents typical side reactions associated with the use of piperidine, such as DKP and aspartimide formation. The flexibility of pNZ can be exploited for the preparation of libraries of small organic molecules.
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Introduction

The solid-phase methodology for the preparation of peptides developed by Bruce R. Merrifield[1] in the late 1950s and early 1960s was crucial for elucidating the utility of peptides as important biochemical tools and, more importantly, their roles in several therapeutic areas.[2] Currently, more than 40 peptide-based drugs are on the market, with four more in registration, 200 in clinical trials, and more than 400 in advanced preclinical development.[3] Solid-phase peptide synthesis (SPPS) strategies are characterized by the lability of both *temporary* (for the N^{α}-amine) and *permanent* (for side chains and for anchoring the *C*-terminal to the solid support through the handle) protecting groups used.[4] The seminal SPPS strategy proposed by Merrifield and fine-tuned over time is based on the graduated acid lability of *tert*-butyloxycarbonyl (Boc), as a *temporary* group, and benzyl-type *permanent* protecting groups. The former is ultimately removed by trifluoroacetic acid (TFA), usually 25–50% in CH_2Cl_2, whereas the latter are removed by strong acids, such as anhydrous HF or trifluoromethanesulfonic acid.[4] Although the Boc-based method has proven successful for the preparation of large numbers of peptides, exposure of the peptide chain to TFA during removal of the Boc group can also cause premature removal of the benzyl protecting group. Furthermore, certain peptides containing fragile residues do not survive the relatively harsh acidic conditions. Finally, HF can be considered a dangerous gas as it must be stored and used in a special Teflon reactor. The aforementioned factors have fueled the development of milder deprotection strategies. Thus, the majority of peptides now produced on solid phase are prepared using the fluorenylmethoxycarbonyl (Fmoc)–*tert*-butyl (*t*Bu) orthogonal protection strategy.[4–6] In this scheme the Fmoc group is normally removed with 20% piperidine in DMF, while the *permanent* protecting groups are removed by TFA in the presence of scavengers. Thus, selective deprotection is governed by alternative cleavage mechanisms rather than by reaction rates. Although this has become the strategy of choice for the preparation of simple peptides, the synthesis of more complex molecules such as cyclic or branched systems may require the use of other protecting groups.[7] Furthermore, the conditions used to remove the Fmoc group are too harsh for certain cases and may be incompatible with several sequences. The main drawbacks associated with the use of Fmoc are that both piperidine, which is a

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base and a rather good nucleophile, and the resulting free amine can provoke side reactions. Thus, an optimal alternative to Fmoc for the protection of N^{α}-amines would be removed under neutral conditions and would leave the resulting amine masked to prevent side reactions. In addition, the protecting group should be orthogonal with *tert*-butyl- and allyl-based protecting groups as well as Fmoc, among others, if it is to be used in the synthesis of complex peptides or other organic molecules.

Herein, the use of the *p*-nitrobenzyloxycarbonyl (pNZ) (Figure 1) group for the *temporary* protection of α-amines in an SPPS strategy is reported.

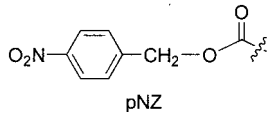

Figure 1. Structure of pNZ protecting group.

The pNZ group, which is a carbamate-type protecting group, was first described by Carpenter and Gish as an alternative to benzyloxycarbonyl (Z).[8] Furthermore, it has been used for the protection of the ε-amino group of Lys.[9] The related *p*-nitrobenzyl (pNB) group has been used to protect the *C*-terminal carboxylic group in side-chain anchoring strategies for the SPS of head-to-tail cyclic peptides,[10] as well as for various functional groups in traditional organic synthesis.[11]

Results and Discussion

Preparation of pNZ-Amino Acids

The classical method for the preparation of N^{α}-carbamate-protected amino acids is via the corresponding chloroformates using Schotten–Baumann conditions, which can generate protected dipeptides as side products.[12] Substantial quantities of protected dipeptide can lead to the insertion of an extra amino acid in the final peptide, which is unacceptable for compounds with therapeutic applications. Several approaches based on the use of activated formates have been proposed to minimize this problematic side reaction.[13] An efficient, competitive and inexpensive procedure is the azide method.[12,14] For the case at hand, an optimized version of a recently described *one-pot* protocol based on the azide was used.[13] Thus, pNZ–Cl was allowed to react with NaN_3, and the resulting pNZ–N_3 was directly treated with free amino acids to afford pNZ–amino acids. These derivatives, which were obtained in relatively high yields (71–94%) and purity, were characterized by HPLC, IR, $^1H/^{13}C$ NMR, and HRMS.

Solubility of pNZ- and Fmoc-Amino Acids

Protected amino acids should be highly soluble, especially for running reactions at high concentration and if automated equipment is to be used to dispense the reagents, a technique often employed for SPPS. In this regard, pNZ-amino acids demonstrate superior solubility in DMF to their corresponding Fmoc derivatives (Table 1).

Table 1. Solubility comparison of pNZ-aa-OH with Fmoc derivatives.

Amino acid	Solubility in DMF[a] [g/mL]
pNZ-L-Phe-OH	0.80
Fmoc-L-Phe-OH	0.31
pNZ-L-Gly-OH	1.33
Fmoc-L-Gly-OH	0.80
pNZ-L-Asp(O*t*Bu)-OH	1.00
Fmoc-L-Asp(O*t*Bu)-OH	0.67

[a] All derivatives except pNZ-L-Asp(O*t*Bu)-OH show negligible solubility in CH_2Cl_2.

Removal of the pNZ Group

The pNZ group can be removed by catalytic hydrogenation as well as other nitro-reducing methods. For the case at hand, the first step was reduction of the nitro group to give the *p*-aminobenzyloxycarbonyl derivative, which suffers spontaneous collapse by 1,6-electron pair shift to afford the quinonimine methide and the carbamic acid. Finally, the carbamic acid decomposes to the corresponding free amine (Figure 2).[15] If this process is carried out in the presence of catalytic amounts of acid, the final product is obtained as an ammonium salt.

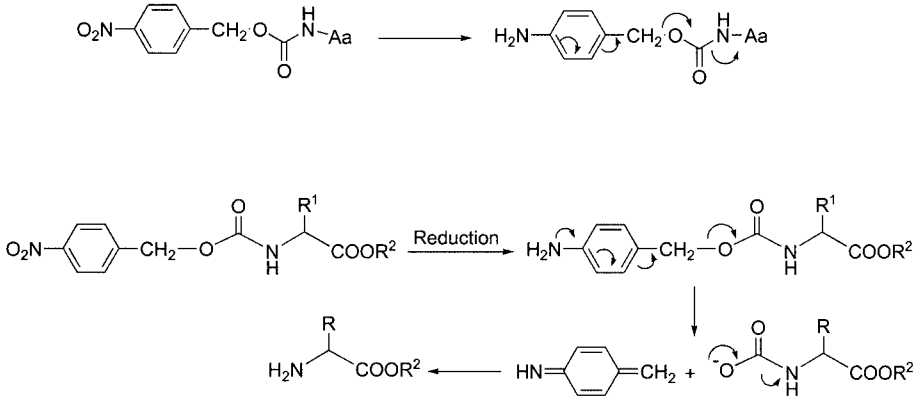

Figure 2. Mechanism of pNZ group removal.

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As shown in Figure 2, the key step in the pNZ removal process is the reduction of the nitro group to the corresponding amine. Although the most common method for this transformation is catalytic hydrogention,[16] it is not useful for SPPS. Other common methods are the use of metals such as Zn,[11b] Fe,[17] or Sn[17] in acidic solutions. However, the difficulty of working with metals on solid phase, as well as the highly acidic conditions required for this chemistry, make it problematic for the case at hand. Thus, two reducing reagents envisaged to be compatible with SPS, $Na_2S_2O_4$,[18] and $SnCl_2$,[8b,19,20] were explored.

$Na_2S_2O_4$

Although the effective nitro-reducing agent $Na_2S_2O_4$ is typically used under basic conditions,[21] there are several reports of its application in neutral or nearly neutral media.[18]

The main drawback associated with the use of $Na_2S_2O_4$ on solid phase is its solubility. It is highly insoluble in DMF and other resin-swelling solvents. Furthermore, the reduction of nitro groups by $Na_2S_2O_4$ requires H_2O, which is not a good solvent for most solid supports. In an effort to overcome the water use, 15-crown-5 was used to solubilize $Na_2S_2O_4$. Several attempts at removing the pNZ group of pNZ-Orn(Boc)-OH in solution using DMF as a solvent were first carried out using varying amounts of water and 15-crown-5. It was found by TLC (1% HOAc in EtOAc) that the rate of cleavage increases with the concentration of H_2O and 15-crown-5.

$SnCl_2$

$SnCl_2$ is a good nitro-reducing agent in the presence of catalytic amounts of acid. It was initially probed with HOAc, but non-carboxylic acids were used in latter probes to prevent acetylation on solid phase.

pNZ-Phe-Gly-Gly-Leu-NH-Rink-polystyrene resin was used as a model. Removal of the pNZ group was carried out under different conditions using both $Na_2S_2O_4$ and $SnCl_2$ as reducing agents. The resulting crude peptides were analyzed by HPLC (Table 2).

The data outlined in Table 2 illustrate that $SnCl_2$ is a superior reducing agent than $Na_2S_2O_4$ for the model case. A 6 M $SnCl_2$ solution is more convenient to use than an 8 M solution, as the latter is supersaturated and some solid $SnCl_2$ may precipitate. HCl (Entry 13) was slightly superior to Tos-OH (Entry 12) and HOAc (Entry 6), while the performance of the alcohols with a relatively acidic hydrogen [HFIP (Entry 10) and TFE (Entry 8)] was inferior.

HCl was thus determined to be the most effective acid. Increasing the concentration of HCl from 1.6 mM (Entry 14) to 64 mM (Entries 15, 16, and 17) did not improve the rate of deprotection. The presence of phenol does not increase the rate of deprotection or the purity of the final product. As might be expected, deprotection occurs faster at high temperature (50 °C) than at room temp. (Entries 18 and 19).

Orthogonality of pNZ-Amino Acids with Fmoc, Boc, and Alloc Groups

The preparation of complex targets such as cyclic or branched peptides, as well as those containing chemicaly fragile moieties, often requires the use of orthogonal protecting groups.[6,7] To demonstrate the orthogonality of pNZ-amino acids with common protecting groups, samples of pNZ-Phe-OH were dissolved in piperidine/DMF (1:4) or TFA (9:1). TLC (1% AcOH in EtOAc) indicated that the pNZ-amino acids are totally stable to both deprotection reagents after 24 h. Likewise, pNZ-Phe-OH was also stable to $Pd(PPh_3)_4$ and phenylsilane in DCM. Furthermore, Boc-

Table 2. Removal of pNZ group from pNZ-Phe-Gly-Gly-Leu-NH-Rink-polystyrene resin.

Entry	Removal conditions[a]	T [°C]	t [min]	Yield [%][b]
1	1 M $Na_2S_2O_4$ in H_2O/AcCN/EtOH (1:1:1)	r.t.	360	0
2	1 M $Na_2S_2O_4$ and 15-Crown-5 in DMF/H_2O (9:1)	r.t.	60	11
3	1 M $Na_2S_2O_4$ and 15-Crown-5 in DMF/H_2O (9:1)	r.t.	420	35
4	1 M $Na_2S_2O_4$, 15-Crown-5, and DIEA in DMF/H_2O (9:1)	r.t.	60	12
5	1 M $Na_2S_2O_4$, 15-Crown-5, and DIEA in DMF/H_2O (9:1)	r.t.	420	13
6	8 M $SnCl_2$, 1.6 mM HOAc, 0.04 M phenol in DMF	r.t.	60	91
7	8 M $SnCl_2$, 1.6 mM HOAc, 0.04 M phenol in DMF	r.t.	300	100
8	6 M $SnCl_2$, 0.04 M phenol in DMF/TFE (19:1)	r.t.	60	68
9	6 M $SnCl_2$, 0.04 M phenol in DMF/TFE (19:1)	r.t.	300	100
10	6 M $SnCl_2$, 0.04 M phenol in DMF/HFIP (19:1)	r.t.	60	76
11	6 M $SnCl_2$, 0.04 M phenol in DMF/HFIP (19:1)	r.t.	300	100
12	6 M $SnCl_2$,1.6 mM TosOH, 0.04 M phenol in DMF	r.t.	60	86
13	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M phenol in DMF	r.t.	60	93
14	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M phenol in DMF	r.t.	2×30	98
15	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	r.t.	2×10	58
16	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	r.t.	2×20	85
17	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	r.t.	2×30	97
18	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M of phenol in DMF	50 °C	2×10	97
19	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M of phenol in DMF	50 °C	2×20	100

[a] Experiments were carried out with 10 mg of resin and 0.5 mL of solvent. [b] Yield was calculated by comparing the areas of the HPLC peaks corresponding to the protected and the deprotected peptides. As the ε of the unprotected peptide should be lower than that of the protected peptide, the actual yields are higher than those reported.

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Phe-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Orn(Alloc) were stable to a mixture of 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF for 2 h. Minimal degradation was only observed for Boc-Phe-OH after 24 h under these conditions. The aforementioned data hence confirm that the pNZ group is orthogonal with Boc, Fmoc, and Alloc protecting groups.

Solid-Phase Peptide Synthesis Using a pNZ/*tert*-Butyl Strategy

Using pNZ-amino acids with *tert*-butyl side chain protecting groups, Leu-enkephalinamide and human phospholipase A2 (18–23) were synthesized on a Rink-resin.

Two parallel syntheses of H-Tyr-Phe-Gly-Gly-Leu-NH_2 (Leu-enkephalinamide) were carried out. Removal of the pNZ group accomplished with 6 M $SnCl_2$, 1.6 mM HCl/dioxane in DMF (2 × 30 min) at room temperature and at 50 °C, followed by extensive washings with DMF (3 × 30 s), DMF/H_2O (3 × 30 s), THF/H_2O (3 × 30 s), DMF (3 × 30 s), and DCM (3 × 30 s) to remove excess $SnCl_2$ as well as any side products from the protecting group. Before coupling the subsequent pNZ-amino acid, the resin was neutralized with diisopropylethylamine (DIEA)/CH_2Cl_2 (1:9).[22] Couplings were performed using an *N*,*N*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) mediated method. After removal of the last pNZ group, free peptides were released from the resin by acidolytic cleavage [TFA/H_2O/DCM (90:5:5)] and worked up. Both crude products were obtained in good purity as characterized by HPLC (Figure 3a) and HPLC-MS.

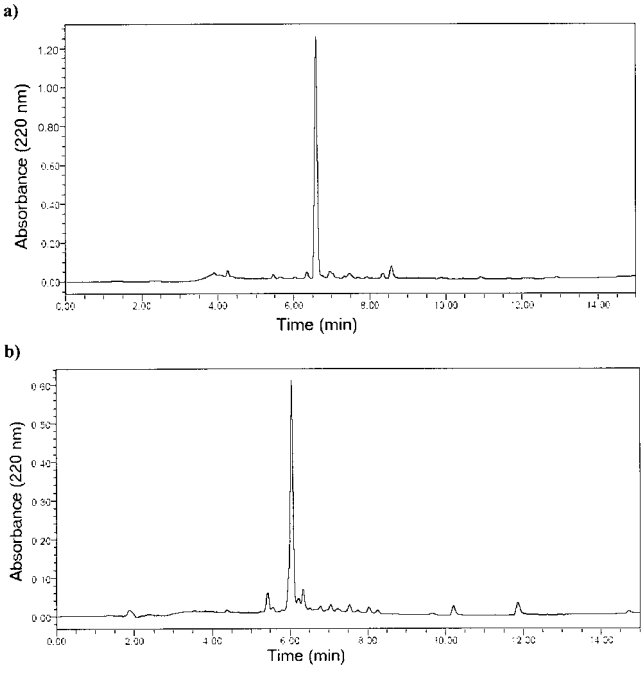

Figure 3. HPLC of (a) Leu-enkephaline and (b) the human phospholipase A2 (18–23) (see Exp. Sect. for chromatographic conditions).

The synthesis of H-Ala-Leu-Ser-Tyr-Gly-Phe-NH_2 [human phospholipase A2 (18–23)] was carried out similarly to the procedure outlined above. The pNZ was removed at room temperature, and the couplings employed an *N*-[(1*H*-3-oxy-4-azabenzotriazol-1-yl)(dimethylamino)methylene]-*N*-dimethyliminium hexafluorophosphate (HATU)/DIEA mediated method. In this case the neutralization step was omitted. After final acidolytic cleavage and workup the crude peptide was obtained in good purity as characterized by HPLC (Figure 3b) and HPLC-MS.

Use of the pNZ Group to Circumvent Problems Associated with Fmoc Chemistry

As mentioned above, the main drawback associated with the Fmoc strategy is related to the use of piperidine, which is an excellent nucleophile and a medium-strength base. Furthermore, after removal of the Fmoc group, the amino functionality remains a free amine. Both, the piperidine and the free amine can cause side reactions such as the formation of diketopiperazines (DKP) and, in the case of Asp-containing peptides, aspartimides.

DKP Formation

The free amino group of a resin-bound dipeptide can attack the peptide-resin anchorage intramolecularly to form cyclic dipeptides or DKPs.[4a,23,24] Thus, the solid-phase synthesis of *C*-terminal peptide acids sometimes requires special protocols for the incorporation of the second and third amino acids (Figure 4).

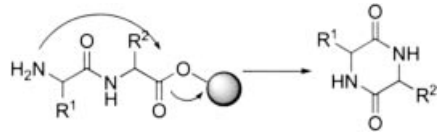

Figure 4. Mechanism of DKP formation.

Although DKP formation is governed by various factors, the side reaction is more severe in Fmoc-based syntheses than in Boc-based syntheses.[25] First of all, piperidine catalyzes the side reaction,[26] secondly, removal of the Boc group is carried out in acidic medium, hence the amine remains protonated and incorporation of the third Boc-amino acid can be done with in situ neutralization, which minimizes DKP formation.[27] DKP formation in Fmoc chemistry can be minimized by various approaches, including the use of sterically hindered resins such as those based on trityl (Trt) or *tert*-butyl (*t*Bu) groups.[28] Likewise, incorporation of the second residue with Trt protection, followed by Trt removal in acidic medium and subsequent incorporation of the third residue with in situ neutralization, can also be employed.[29] Under mild conditions, DKP can be also minimized by incorporation of the second amino acid protected with the Alloc group in conjunction with a tandem deprotection–coupling reaction, which is carried out with

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the corresponding fluoride derivative and is favored over DKP formation.[30]

Owing to its facile removal, it was thought that the pNZ group would be a good candidate for the protection of the second amino acid of a growing peptide on solid phase. To demonstrate this methodology, the tripeptide H-Phe-D-Val-Pro-OH was chosen as a model.[31] Thus, pNZ-D-Val-Pro-AB-Leu-aminomethylresin and Fmoc-D-Val-Pro-AB-Leu-aminomethylresin were synthesized with Fmoc-Pro-OH and pNZ/Fmoc-D-Val-OH, using a Leu residue as internal reference amino acid (IRAA) to calculate the yield {AB linker = 3-[4-(hydroxymethyl)phenoxy]propionic acid}. While 100% of DKP formation was detected after removal of the Fmoc group of the D-Val residue [piperidine/DMF (2:8)] and incorporation of the Fmoc-Phe-OH, no DKP was observed in the case of the pNZ peptide resin. In this case, the pNZ was removed with 6 M $SnCl_2$, 1.6 mM HCl/dioxane in DMF (2 × 30 min) at room temperature and after extensive washings, the Fmoc-Phe-OH (5 equiv.) was incorporated in the presence of (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (5 equiv.) and DIEA (10 equiv.) using in situ neutralization.[32]

Aspartimide Formation

The cyclization of Asp residues to form aspartimides is a common side reaction in peptide synthesis.[4a,33] Although this side reaction is favored under various conditions (strong acids, excess coupling reagents or basic medium) and is sequence-dependent, it is most relevant to Fmoc-based syntheses due to the repetitive use of piperidine (Figure 5). Hence, Fmoc strategies not only yield the aspartimide-containing peptide but also generate the corresponding α- and β-peptides (by hydrolysis of α-imides) and α- and β-piperidides (via attack of the aspartimide by piperidine).

The side reaction is more severe when the β-carboxyl group of the Asp is protected with the allyl group, since it forms a better leaving group than *tert*-butyl.[34] Although the side reaction can be minimized to some extent through the use of hindered protecting groups[35] for the Asp and by protecting the preceeding amide,[36] a more convenient and general method is needed.

A possible solution is a hybrid strategy in which Fmoc is used to protect all of the residues preceding an Asp, at which point pNZ is used to protect the Asp and remaining residues. The use of pNZ here would be advantageous as its removal does not require the repetitive use of a base, and the final cleavage and deprotection of the peptide would be carried out with TFA, which is less prone to generate aspartimide than the HF normally used in a Boc/Bzl chemistry strategy.[4a]

The hybrid strategy was studied using the peptide H-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 model since it contains the sequence Asp-Gly-Tyr-Ile, which is considered to generate a large amount of aspartimides.[33b] Two parallel syntheses were carried out on a Rink-resin. In the first synthesis, Fmoc protection was used for Ile, Tyr, and Gly, and pNZ for Asp(O*t*Bu), Orn(Boc), and Ala, whereas the second synthesis used exclusively Fmoc-amino acids. Furthermore, the peptide H-Ala-Orn-Asp(Gly-Tyr-Ile-NH_2)-OH[37] was synthesized (using Fmoc chemistry) because it is a possible side product of the aspartimide corresponding to the model peptide. Fmoc and pNZ groups were removed with 4 h treatments of 20% piperidine/DMF or 6 M $SnCl_2$/DMF, respectively. While both piperidides were detected in the synthesis using exclusively Fmoc-protection, neither aspartimide nor β-peptide were observed in the synthesis using the Fmoc-pNZ hybrid strategy.

Conclusions

This work has demonstrated the benefits of using pNZ as a temporary protecting group for the α-amines in SPPS. The corresponding derivatives are readily synthesized and, in contrast to other α-amino protecting groups such as Trt and Alloc, they are solids. pNZ is removed under simple, neutral conditions in the presence of catalytic amounts of acid. pNZ is orthogonal to the most common SPPS protecting groups such as *t*Bu/Boc, Fmoc, and Alloc. In addition to its utility for the total elongation of a peptide chain, the pNZ group can be used in conjuction with Fmoc chemistry to overcome side reactions associated with the use of piperidine, such as DKP and aspartimide formation. It is also predicted that the use of pNZ for the preparation of *C*-terminal Cys peptides would circumvent the formation

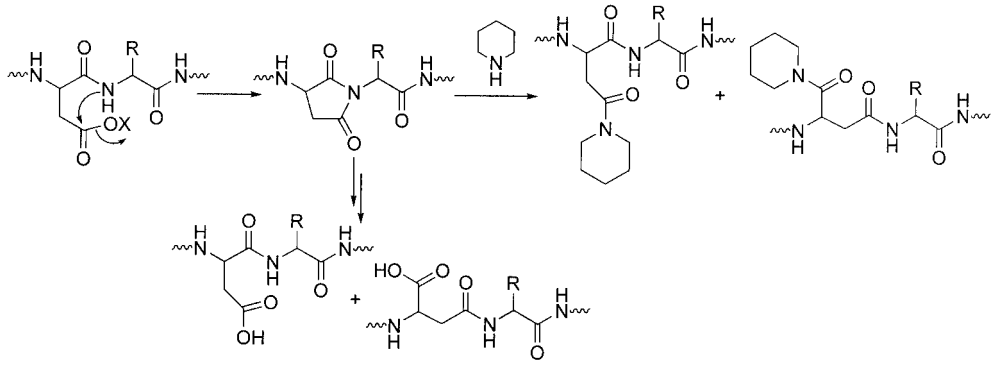

Figure 5. Mechanism of aspartimide formation with typical side products shown.

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of *N*-piperidylalanine, a frequent side reaction when Fmoc protection is used.[38] The flexibility of pNZ can be exploited for the preparation of libraries of small organic molecules.

Experimental Section

General Procedures: Commercial-grade reagents and solvents were used without further purification. Resins, linkers and amino acid derivatives, HOBt, DIPCDI, PyAOP, HATU, *p*-nitrobenzyl chloroformate and sodium azide were obtained from NovaBiochem (Läufelfingen, Switzerland), Bachem (Bubendorf, Switzerland), Iris Biotech (Marktredwitz, Germany), Aldrich (Milwaukee, WI), Acros (Geel, Belgium), Neosystem (Strasbourg, France), and Luxembourg Industries (Tel Aviv, Israel). Analytical HPLC was carried out with a Waters instrument, comprising two solvent-delivery pumps and automatic injector (Waters Separations Module 2695) and a variable-wavelength detector (model Waters 996 Photodiode Array). UV detection was performed at 220 nm, and linear gradients of CH_3CN (0.036% TFA) into H_2O (0.045% TFA) were run at a flow rate of 1.0 mL/min. MS-HPLC was carried out with a Waters instrument, comprising two solvent-delivery pumps and automatic injector (Waters Separations Module 2795), a dual-wavelength detector (Waters 2487, Dual λ Absorbance Detector), and an electrospray detector (Waters micromass ZQ). NMR spectra were acquired with a Mercury-400 (400 MHz) spectrometer (High Field NMR Unit, Barcelona Science Park), data are given on the δ scale referenced to TMS (see Supporting Information for ^1H and ^{13}C NMR spectra). Amino acid analyses were performed using a Beckman System 6300 High Performance Analyzer. Resins were treated with a mixture of HCl and propionic acid (1:1) at 160 °C for 1 h, and after evaporating the acid under reduced pressure, they were suspended in amino acid analysis buffer and filtered.

pNZ-Amino Acid Synthesis

Method 1: *p*-Nitrobenzyl chloroformate (1.73 g, 8 mmol) was dissolved in 1,4-dioxane (3.5 mL) and a solution of sodium azide (0.624 g, 9.6 mmol) in H_2O (2.5 mL) was added. The resulting emulsion was stirred for 2 h and the formation of the azide was monitored by TLC (CH_2Cl_2). Gly (0.600 g, 8 mmol), dissolved in 1,4-dioxane/2% aqueous Na_2CO_3 (1:1) (10 mL), was then added dropwise, and the resulting white suspension was stirred for 24 h, keeping the pH between 9 and 10 by adding 10% aqueous Na_2CO_3. At this point, TLC (CH_2Cl_2) showed that there was no remaining azide, H_2O (75 mL) was added and the suspension was washed with methyl *tert*-butyl ether (MTBE) (3×40 mL). The aqueous portion was acidified to pH = 2–3 with 12 N HCl/H_2O (3:1) and a white precipitate appeared, which was filtered off and dried to yield 1.44 g (71%) of the title compound as a white solid.

Method 2: The synthesis was performed as in Method 1 but the acidic suspension was extracted with EtOAc. The organic layers were dried with $MgSO_4$, filtered, and the solvent was removed under reduced pressure to yield an oil, which solidified as white solid by washing with diethyl ether or suspended in acetonitrile (AcCN)/H_2O and lyophilized (H-D-Val-OH and H-L-Ala-OH). The pNZ-amino acids were recrystalized in H_2O or in diethyl ether/hexane.

All protected amino acids were characterized by HPLC [Gradient A: from H_2O to AcCN; Gradient B: from AcCN/H_2O (3:7) to AcCN, where H_2O contained 0.045% of TFA and AcCN contained 0.036% of TFA], HPLC-MS, ^1H and ^{13}C NMR, HR-MS, and IR; for used method and yields see Table 3.

pNZ-Ser(*t*Bu)-OH: M.p. 92.8–93.8 °C. HPLC: R_t = 11.28 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3364, 3170, 2980, 1741, 1697, 1525, 1347 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.6 Hz, 2 H), 7.61 (d, J = 8.6 Hz, 2 H), 7.45 (d, J = 8.3 Hz, NH), 5.18 (s, 2 H), 4.10 (m, 1 H), 3,55 (m, 2 H), 1.09 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 172.44 (C), 156.42 (C), 147.60 (C), 145.71 (C), 128.78 (CH), 124.15 (CH), 73.50 (C), 64.93 (CH_2), 62.03 (CH_2), 55.61 (CH), 27.84 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{15}H_{21}N_2O_7$ [M + H^+] 341.1350, found 341.1359.

pNZ-D-Val-OH: M.p. 125.5–127.5 °C.[8a] HPLC: R_t = 10.5 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3322, 2970, 1693, 1540, 1351 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.6 Hz, 2 H), 7.63 (d, J = unresolved, NH), 7.61 (d, J = 8.6 Hz, 2 H), 5.17 (s, 2 H), 3.86 (dd, J = 8.5, 5.9 Hz, 1 H), 2.48 (m, 1 H), 0.89 (d, J unresolved, 3 H), 0,87 (d, J unresolved, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.82 (C), 156.81 (C), 147.61 (C), 145,75 (C), 128.77 (CH Ar), 124.18 (CH Ar), 64.91 (CH_2), 60.30 (CH), 30.23 (CH), 19.83 (CH_3), 18.68 (CH_3) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{13}H_{16}N_2O_6Na$ [M + Na^+] 319.0906, found 319.0889.

pNZ-Gly-OH: M.p. 121.5–122.5 °C (ref.[8a] 122.5–124 °C). HPLC: R_t = 4.50 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3383, 3117, 2941, 1751, 1705, 1522, 1350 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.70 (t, J = 6.1 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.18 (s, 2 H), 3.67 (d, J = 6.1 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 172.12 (C), 156.9 (C), 147.62 (C), 145.7 (C), 128.77 (CH), 124.20 (CH), 64.95 (CH_2), 42.84 (CH_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{10}H_{11}N_2O_6$ [M + H^+] 255.0618, found 255.0611.

pNZ-Leu-OH: M.p. 81.0–85.5 °C (ref.[8a] 60–61 °C). HPLC: R_t = 7.90 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3421.3, 2961, 1693, 1524, 1345 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.8 Hz, 2 H), 7.70 (d, J = 8.2 Hz, NH), 7.59 (d, J = 8.8 Hz, 2 H), 5.16 (d, J = 1.96 Hz, 2 H), 3.95 (m, 1 H), 1.63 (m, 1 H), 1.50 (m, 2 H), 0.87 (d, J = 6.6 Hz, 3 H), 0.83 (d, J = 6.5 Hz, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 174.93 (C), 156.55 (C), 147.61 (C), 145.73 (C), 128.75 (CH), 124.19(CH), 64.87 (CH_2), 52.94 (CH), 40.27 (CH_2), 25.00 (CH), 23.54 (CH_3), 21.80 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{14}H_{19}N_2O_6$ [M + H^+] 311.1244, found 311.1252.

pNZ-Ile-OH: M.p. 79–83 °C (ref.[8a] 77.5–80 °C). HPLC: R_t = 11.21 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3321, 2967, 1727, 1659, 1538, 1349 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.7 Hz, 2 H), 7.64 (d, J = 8.5 Hz, NH), 7.61 (d, J = 8.7 Hz, 2 H), 5.17 (s, 2 H), 3.90 (dd, J = 8.5, 6.1 Hz, 1 H), 2.48 (m, 1 H), 1.77 (m, 1 H), 1.38 (m, 1 H), 1.20 (m, 1 H), 0.86 (d, J = 6.8 Hz, 3 H), 0.82 (t, J = 7.4 Hz, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.84 (C), 156.72 (C), 147.62 (C), 145.76 (C), 128.78 (CH), 124.19 (CH), 64.92 (CH_2), 59.32 (CH), 36.76 (CH), 25.35 (CH_2), 16.29 (CH_3), 11.95 (CH_3) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{14}H_{18}N_2O_6Na$ [M + Na^+] 333.1063 found 333.1063.

pNZ-Tyr(*t*Bu)-OH: M.p. 130–136.5 °C. HPLC: R_t = 9.61 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3351, 2980, 1703, 1522, 1352 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.18 (d, J = 8.7 Hz, 2 H), 7.69 (d, J = 8.6 Hz, NH), 7.45 (d, J = 8.7 Hz, 2 H), 7.13 (d, J = 8.4 Hz, 2 H), 6.84 (J = 8.4 Hz, 2 H), 5.09 (dd, J = 24,1 Hz, 14,2 Hz, 2 H), 4.15 (m, 1 H), 3.04 (dd, J_{gem} = 13.8, J = 4.2 Hz, 1 H), 2.77 (dd, J_{gem} = 13.8, J = 10.7 Hz, 1 H), 1.24 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.99 (C), 156.28 (C), 154.15 (C), 147.51 (C), 145.86 (C), 133.22 (C), 130.35 (CH), 128.46 (CH), 124.11 (CH), 123.95 (CH), 78.27 (C), 64.62 (CH_2), 56.47 (CH), 36.70 (CH_2), 29.21 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{21}H_{25}N_2O_7$ [M + H^+] 417.1663, found 417.1665.

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Table 3. Method used and yield for the preparation of pNZ-amino acids.

Amino acid	Leu	Ile	D-Val	Phe	Gly	Ser(*t*Bu)	Orn(Boc)	Asp(O*t*Bu)	Ala	Tyr(*t*Bu)
Method	2	2	2	1	1	2	2	2	2	1
Yield [%]	77	94	83	84	71	85	83	89	81	82

pNZ-Phe-OH: M.p. 129.0–135.1 °C (ref.[8b] 134.5–136.5 °C). HPLC: R_t = 7.90 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3329, 1702, 1516, 1346 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.17 (d, J = 8.7 Hz, 2 H), 7.76 (d, J = 8.5 Hz, NH), 7.48 (d, J = 8.7 Hz, 2 H), 7.25 (m, 5 H), 5.10 (s, 2 H), 4.16 (m, 1 H), 3.08 (dd, J_{gem} = 13.8, J = 4.3 Hz, 1 H), 2.82 (dd, J_{gem} = 13.8, J = 10.6 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.88 (C), 156.34 (C), 147.53 (C), 145.76 (C), 138.61 (C), 129.80 (CH), 128.83 (CH), 128.57 (CH), 127.01 (CH), 124.12 (CH), 64.74 (CH_2), 56.33 (CH), 37.20 (CH_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{17}H_{17}N_2O_6$ [M + H^+] 345.1087, found 345.1079.

pNZ-Ala-OH: Amorphous solid (ref.[8b] 132.5–134 °C). HPLC: R_t = 9.28 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3348, 1697, 1530, 1351 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.74 (d, J = 7.6 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.16 (s, 2 H), 3.99 (m, 1 H), 1.26 (d, J = 7.4 Hz, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 174.94 (C), 156.23 (C), 147.61 (C), 145.71 (C), 128.79 (CH), 124.18 (CH), 64.84 (CH_2), 49.97 (CH), 17.70 (CH_3) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{11}H_{12}N_2O_6Na$ [M + Na^+] 291.0593, found 291.0599.

pNZ-Orn(Boc)-OH: Amorphous solid. HPLC: R_t = 7.51 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3405, 2978, 1717, 1523, 1348 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.6 Hz, 2 H), 7.68 (d, J = 8.0 Hz, NH), 7.60 (d, J = 8.6 Hz, 2 H), 6.77 (t, J = 4.8 Hz, NH), 5.17 (s, 2 H), 3.90 (m, 1 H), 2.91 (m, 2 H), 1.58 (m, 1 H), 1.53 (m, 1 H), 1.42 (m, 2 H), 1.35 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 174.40 (C), 156.50 (C), 147.60 (C), 145.77 (C), 128.73 (CH), 124.19 (CH), 78.07 (C), 64.84 (CH_2), 54.45 (CH), 40.09 (CH_2), 28.94 (CH_3), 28.88 (CH_2), 26.87 (CH_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{18}H_{26}N_3O_8$ [M + H^+] 412.1721, found 412.1719.

pNZ-Asp(O*t*Bu)-OH: M.p. 79.5–82.6 °C. HPLC: R_t = 11.37 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3342, 3157, 1723, 1699, 1521, 1349 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.76 (d, J = 8.6 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.17 (s, 2 H), 4.33 (m, 1 H), 2.68 (dd, J_{gem} = 16.0, J = 5.7 Hz, 1 H), 2.52 (dd, J_{gem} = 16.0, J = 8.4 Hz, 1 H), 1.35 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.03 (C), 169.87 (C), 156.21 (C), 147.62 (C), 145.62 (C), 128.82 (CH), 124.16 (CH), 80.98 (C), 64.97 (CH_2), 51.32 (CH), 37.88 (CH_2), 28.31 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{16}H_{21}N_2O_8$ [M + H^+] 369.1299, found 369.1309.

Solid-Phase Synthesis: Solid-phase syntheses were carried out in polypropylene syringes (2–10 mL) fitted with a porous polyethylene disk. Solvents and soluble reagents were removed by suction.

Fmoc: Deprotection was accomplished with piperidine/DMF (2:8) (2 × 3 min, 1 × 4 min, and 1 × 5 min). Washings between deprotection, coupling, and final deprotecion steps were carried out in DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL solvent/g resin for each wash.

pNZ: Unless otherwise indicated, deprotection was accomplished with 6 M $SnCl_2$ and 1.6 mM HCl/dioxane, in DMF (2 × 30 min). The resin was then washed with DMF (3 × 30 s), DMF/H_2O (3 × 30 s), THF/H_2O (3 × 30 s), DMF (3 × 30 s), and DCM (3 × 30 s).

Coupling of Fmoc-Amino Acids and Handles: This was performed by adding the carboxylic acid reagent (4 equiv.) and the coupling reagent [DIPCDI (4 equiv.) and HOBt (4 equiv.), or HATU (3.8 equiv.) and DIEA (12 equiv.)] in DMF (0.5 mL) to the resin and stirring the mixture for 60–90 min. For all cases, reaction progress was monitored by the ninhydrin test[39] and couplings were repeated if a positive test result was obtained. Peptide synthesis transformations and washes were performed at 25 °C unless otherwise indicated.

Final Cleavage and Deprotection: The acidolytic cleavage was carried out with TFA/H_2O/DCM (90:5:5) (10 mL/g of resin). The TFA was removed by evaporation and the residue was taken up in HOAc/H_2O (7:3), washed three times with $CHCl_3$, and the aqueous phase lyophilized.

Synthesis of H-Tyr-Phe-Gly-Gly-Leu-NH_2

Synthesis 1: Rink amide resin (50 mg, 0.66 mmol/g). Couplings DIPCDI/HOBt method. Then, the pNZ was removed and the resin was neutralized with DIEA/CH_2Cl_2 (1:9), washed with DCM (5 × 1 min), and DMF (5 × 1 min) before performing the next coupling. After final acydolitic cleavage and workup, the crude product was analyzed by HPLC [R_t = 6.37 min; gradient: from H_2O (0.045% of TFA) to AcCN (0.036% of TFA)], and HPLC-MS.

Synthesis 2: The same synthetic process as outline above was repeated but carrying out the removal of the pNZ at 50 °C.

Synthesis of H-Ala-Leu-Ser-Tyr-Gly- Phe-NH_2: Rink amide resin (50 mg, 0.66 mmol/g). Couplings: pNZ-amino acids (4 equiv.), HATU (3.8 equiv.) and DIEA (12 equiv.) in DMF (0.5 mL) were added to the resin and the reaction mixture was stirred for 1 h. Then, the pNZ group was removed and the resin was washed before performing the next coupling without previous neutralization. After final acydolitic cleavage and workup, the crude product was analyzed by HPLC [R_t = 6.05 min; gradient: from H_2O/AcCN (95:5) to H_2O/AcCN (5:95) where H_2O contained 0.045% of TFA and AcCN contained 0.036% of TFA] and HPLC-MS.

Tests of DKP Formation: Aminomethyl resin (100 mg, f = 1.2 mmol/g) was used as the base resin. Fmoc-L-Pro-OH (4 equiv.), DMAP (0.4 equiv.) and DIPCDI (4 equiv.) in DMF (1 mL) were added to the HO-AB-Leu-aminomethyl resin, which is a Wang-type resin,[40] and the reaction mixture was stirred for 1 h. After washing, the TosCl-PNBP test[41] confirmed the correct coupling of the amino acid. Then the Fmoc group was removed, the resin was divided into two parts and the peptides were synthesized.

Fmoc Synthesis: The Fmoc-D-Val-OH was incorporated using DIPCDI/HOBt-mediated coupling, the Fmoc was removed; after washings, the Fmoc-Phe-OH was incorporated using the same protocol, the Fmoc was removed and the amino acid hydrolysis of a peptide resin aliquot indicated that the extension of side reactions has been of 100%.

pNZ Synthesis: pNZ-D-Val-OH was incorporated using DIPCDI/HOBt-mediated coupling, the pNZ was removed; after washings, the Fmoc-Phe-OH (4 equiv.), PyAOP (4 equiv.),[42] and DIEA (8 equiv.) in DMF (0.5 mL) were added and the reaction mixture was stirred for 1 h. After washing as indicated above, the ninhydrin test was negative. The resin was then washed and the amino acid hydrolysis of a peptide resin aliquot indicated that side reactions did not occur.

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Tests of Aspartimide Formation: Rink amide resin (200 mg, 0.66 mmol/g) was used and the synthesis was carried out as outlined above using DIPCDI/HOBt-mediated couplings. After removal of the Fmoc group of the Gly, the resin was divided into two parts for the Fmoc and the pNZ synthesis. After acidolytic cleavage and workup, the crude products were analyzed by HPLC [R_t = 9.04 min; gradient: from H_2O to H_2O/AcCN (7:3), where H_2O contained 0.045% of TFA and AcCN contained 0.036% of TFA] and HPLC-MS.

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[1] a) R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149; b) R. B. Merrifield, *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 799; c) R. B. Merrifield, *Science* **1986**, *232*, 341.
[2] S. Lien, H. B. Lowman, *Trends Biotechnol.* **2003**, *21*, 556.
[3] a) A. Loffet, *J. Pept. Sci. J. Peptide Sci.* **2002**, *8*, 1; b) T. Bruckdorfer, O. Marder, F. Albericio, *Current Pharm. Biotech.* **2004**, *5*, 29.
[4] a) P. Lloyd-Williams; F. Albericio; E. Giralt, *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC, Boca Raton, FL, USA, **1997**; b) T. S. Yokum, G. Barany, in: *Solid-phase Synthesis. A Practical Guide*, Marcel Dekker Inc., New York, USA, **2000**, pp. 79–102; c) G. B. Fields, J. L. Lauer-Fields, R.-q. Liu, G. Barany, in: *Synthetic Peptides: A User's Guide* (Eds.: G. A. Grant), 2nd ed., W. H. Freeman & Co., New York, **2001**, pp. 93–219; d) M. Goodman, A. Felix, L. A. Moroder, C. Toniolo (Eds.), *Houben-Weyl*, vol. E22a–e ("Synthesis of Peptides and Peptidomimetics"), Georg Thieme Verlag, Stuttgart, Germany, **2002**.
[5] W. C. Chan; P. D. White (Eds.), *Fmoc Solid Phase Peptide Synthesis*, Oxford University Press, Oxford, UK, **2000**.
[6] The *orthogonal* concept is based on the use of independent classes of protecting groups, removed by different mechanisms so that they may be removed in any order and in the presence of all other types of groups. a) G. Barany, R. B. Merrifield, *J. Am. Chem. Soc.* **1977**, *99*, 7363; b) G. Barany, F. Albericio, *J. Am. Chem. Soc.* **1985**, *107*, 4936.
[7] F. Albericio, *Biopolymers* **2000**, *55*, 123.
[8] a) F. H. Carpenter, D. T. Gish, *J. Am. Chem. Soc.* **1952**, *74*, 3818; b) D. T. Gish, F. H. Carpenter, *J. Am. Chem. Soc.* **1953**, *75*, 950.
[9] a) J. E. Shields, F. H. Carpenter, *J. Am. Chem. Soc.* **1961**, *83*, 3066; b) M. D. Hocker, C. G. Caldwell, R. W. Macsata, M. H. Lyttle, *Pept. Res.* **1995**, *8*, 310; c) S. Peluso, P. Dumy, C. Nkubana, Y. Yokokawa, M. Mutter, *J. Org. Chem.* **1999**, *64*, 7114.
[10] a) P. Romanovskis, A. F. Spatola, *J. Pept. Res.* **1998**, *52*, 356; b) M. Royo, J. Farrera-Sinfreu, L. Solé, F. Albericio, *Tetrahedron Lett.* **2002**, *43*, 2029.
[11] a) M. D. Bachi, J. Ross-Peterson, *J. Chem. Soc., Chem. Commun.* **1974**, 12; b) R. R. Chauvette, P. A. Pennington, *J. Med. Chem.* **1975**, *18*, 403; c) S. R. Lammert, A. I. Ellis, R. R. Chauvette, S. Kukolja, *J. Org. Chem.* **1978**, *43*, 1243; d) K. Fukase, H. Tanaka, S. Torii, S. Kusumoto, *Tetrahedron Lett.* **1982**, *23*, 885; e) R. Balasuriya, S. J. Chandler, M. J. Cook, *Tetrahedron Lett.* **1983**, *24*, 1385; f) S. Hashiguchi, H. Natsugari, M. Ochiai, *J. Chem. Soc., Perkin Trans. 1* **1988**, 2345; g) M. Namikoshi, B. Kundu, K. L. Rinehart, *J. Org. Chem.* **1991**, *56*, 5464.
[12] M. Tessier, F. Albericio, E. Pedroso, A. Grandas, R. Eritja, E. Giralt, C. Granier, J. Van-Rietschoten, *Int. J. Pept. Protein Res.* **1983**, *22*, 125.
[13] For a broad discussion of this subject, see: L. J. Cruz, N. G. Beteta, A. Ewenson, F. Albericio, *Org. Process Res. Dev.* **2004**, *8*, 920.
[14] Although the existence of this side reaction was not mentioned, Fmoc-N3 was already proposed as an alternative to the chloroformate in the seminal papers of Carpino and Han: L. A. Carpino, G. Y. Han, *J. Am. Chem. Soc.* **1970**, *92*, 5748 and L. A. Carpino, G. Y. Han, *J. Org. Chem.* **1972**, *37*, 3404.
[15] In the literature, the *p*-azidobenzyloxycarbonyl group, which behaves similarly to pNZ, is also described. The reduction of the azide with dithiothreitol (DTT) or $SnCl_2$ should lead to the *p*-aminobenzyloxycarbonyl derivative, which liberates the free amine by the same mechanism as pNZ. However, in our hands this azide derivative gave substantially worse results than pNZ. a) B. Loubinoux, P. Gerardin, *Tetrahedron Lett.* **1991**, *32*, 351; b) R. J. Griffin, E. Evers, R. Davison, A. E. Gibson, D. Layton, W. J. Irwin, *J. Chem. Soc., Perkin Trans. 1* **1996**, 1205.
[16] J.-F. Wen, W. Hong, K. Yuan, T. C. W. Mak, H. N. C. Wong, *J. Org. Chem.* **2003**, *68*, 8918, and references therein.
[17] J. T. Manka, F. Guo, J. Huang, H. Yin, J. M. Farrar, M. Sienkowska, V. Benin, P. Kaszynski, *J. Org. Chem.* **2003**, *68*, 9574, and references therein.
[18] a) W. Liao, C. F. Piskorz, R. D. Locke, K. L. Matta, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 793; b) X. Qian, O. Hindsgaul, *Chem. Commun.* **1997**, 1059, and references therein.
[19] a) A. A. Pletnev, Q. Tian, R. C. Larock, *J. Org. Chem.* **2002**; *67*, 9276; b) M. Schlitzer, M. Böhm, I. Sattler, H.-M. Dahse, *Bioorg. Med. Chem.* **2000**, *8*, 1991.
[20] $SnCl_2$ has also been used to remove the pNB ester on solid phase, see ref.[10]
[21] a) E. Guibé-Jampel, M. Wakselman, *Synth. Commun.* **1982**, *12*, 219; b) R. A. Scheurerman, D. Tumelty, *Tetrahedron Lett.* **2000**, *41*, 6531.
[22] If aminium/uronium salt coupling methods are used, this neutralization step can be omitted. See synthesis of phospholipase A2 (18–23).
[23] B. F. Gisin, R. B. Merrifield, *J. Am. Chem. Soc.* **1972**, *94*, 3102.
[24] M. C. Khosla, R. R. Smeby, F. M. Bumpus, *J. Am. Chem. Soc.* **1972**, *94*, 3102.
[25] For instance, the peptide-resin anchorage: DKP formations are favored when the peptide is bound to the resin as a benzyl ester; the nature of the first and second amino acids of the peptide: the presence of Pro, Gly or of *N*-alkylamino acids can lead to a substantial extent of DKP formation because all of them favor the *cis* configuration of the amide bond, which is the optimal configuration to form DKPs. Another unfavorable combination is to have one D- and one L-amino acid in the dipeptide because it decreases the steric hindrance during the cyclization. See ref.[4a]
[26] E. Pedroso, A. Grandas, X. de las Heras, R. Eritja, E. Giralt, *Tetrahedron Lett.* **1986**, *27*, 743.
[27] a) K. Suzuki, K. Nitta, N. Endo, *Chem. Pharm. Bull.* **1975**, *23*, 222; b) M. Gairi, P. Lloyd-Williams, F. Albericio, E. Giralt, *Tetahedron Lett.* **1990**, *31*, 7363.
[28] a) K. Akaji, Y. Kiso, L. A. Carpino, *J. Chem. Soc., Chem. Commun.* **1990**, 584; b) K. Barlos, D. Gatos, W. Schaefer, *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 590; c) C. Chiva, M. Vilaseca, E. Giralt, F. Albericio, *J. Pept. Sci.* **1999**, *5*, 131.
[29] J. Alsina, E. Giralt, F. Albericio, *Tetrahedron Lett.* **1996**, *37*, 4195.
[30] N. Thieriet, J. Alsina, E. Giralt, F. Guibé, F. Albericio, *Tetrahedron Lett.* **1997**, *38*, 7275.
[31] The sequence D-Val-Pro is prone for DKP formation because it contains the combination of one D- and one L-amino acid with a Pro residue. On the other hand, the Val is a β-branched residue and therefore its acylation is not trivial. See refs.[4a,26,27b,29]
[32] For couplings without preactivation of the protected amino acid, phosphonium salts such as PyAOP are preferred to aminium/uronium salts, because the latter can give guanidinium for-

mation. F. Albericio, J. M. Bofill, A. El-Faham, S. A. Kates, *J. Org. Chem.* **1998**, *63*, 9678.
[33] a) J. P. Tam, M. W. Riemen, R. B. Merrifield, *Pept. Res.* **1988**, *1*, 6; b) E. Nicolás, E. Pedroso, E. Giralt, *Tetrahedron Lett.* **1989**, *30*, 497; c) Y. Yang, W. V. Sweeney, K. Schneider, S. Thornqvist, B. T. Hait, J. P. Tam, *Tetrahedron Lett.* **1994**, *35*, 9689; d) R. Doelling, M. Beyermann, J. Haenel, F. Kernchen, E. Krause, P. Franke, M. Brudel, M. Bienert, *J. Chem. Soc., Chem. Commun.* **1994**, 853; e) J. L. Lauer, C. G. Fields, G. B. Fields, *Lett. Pept. Sci.* **1995**, *1*, 197; f) J. Cebrian, V. Domingo, F. Reig, *J. Pept. Res.* **2003**, *62*, 238.
[34] a) D. Delforge, M. Dieu, E. Delaive, M. Art, B. Gillon, B. Devreese, M. Raes, J. Van Beeumen, J. Remacle, *Lett. Pept. Sci.* **1996**, *3*, 89; b) S. C. Vigil-Cruz, J. V. Aldrich, *Lett. Pept. Sci.* **1999**, *6*, 71.
[35] a) A. Karlstroem, A. E. Unden, *Tetrahedron Lett.* **1995**, *36*, 3909; b) A. Karlstroem, A. Unden, *Int. J. Pept. Protein Res.* **1996**, *48*, 305; c) M. Mergler, F. Dick, B. Sax, P. Weiler, T. Vorherr, *J. Pept. Sci.* **2003**, *9*, 36; d) M. Mergler, F. Dick, B. Sax, C. Staehelin, T. Vorherr, *J. Pept. Sci.* **2003**, *9*, 518.
[36] a) M. Quibell, D. Owen, L. C. Peckman, T. Johnson, *J. Chem. Soc., Chem. Commun.* **1994**, *20*, 2343; b) L. Urge, L. Otvos, Jr., *Lett. Pept. Sci.* **1995**, *1*, 207; c) L. C. Packman, *Tetrahedron Lett.* **1995**, *36*, 7523.
[37] The peptide backbone is through the β-carboxyl group of the Asp residue.
[38] J. Lukszo, D. Patterson, F. Albericio, S. A. Kates, *Lett. Pept. Sci.* **1996**, *3*, 157.
[39] E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970**, *34*, 595.
[40] a) F. Albericio, G. Barany, *Int. J. Pept. Protein Res.* **1984**, *23*, 342; b) F. Albericio, G. Barany, *Int. J. Pept. Protein Res.* **1984**, *26*, 92.
[41] O. Kuisle, M. Lolo, E. Quiñoa, R. Riguera, *Tetrahedron* **1999**, *55*, 14807.
[42] (Benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) can also be used instead of PyAOP.
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Semipermanent *p*-Nitrobenzyloxycarbonyl (*p*NZ) Protection of Orn and Lys Side Chains: Prevention of Undesired α-Fmoc Removal and Application to the Synthesis of Cyclic Peptides

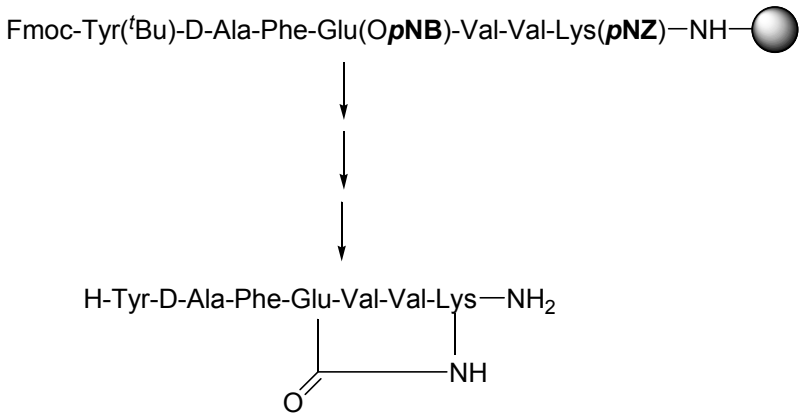
Protecció de les Cadenes Laterals d'Orn i Lys amb el Grup *p*-Nitrobenziloxicarbonil (*p*NZ) per Evitar l'Eliminació no Desitjada del Grup α-Fmoc i Aplicació a la Síntesi de Pèptids Cíclics

Albert Isidro-Llobet, Mercedes Álvarez and Fernando Albericio

Tetrahedron Letters 46 (2005), 7733-7736

Resum

Un cop estudiades les aplicacions del grup *p*NZ com a protector temporal del grup α-amino, s'han investigat les seves propietats com a protector semipermanent de les cadenes laterals de Orn i Lys en química Fmoc/^{t}Bu. En aquest sentit, s'ha comprovat que després d'eliminar el grup *p*NZ, no es produeix l'eliminació no desitjada del grup α-Fmoc que si que succeeix quan s'usen grups protectors com l'Alloc. També s'ha demostrat la utilitat del grup *p*NZ en combinació amb l'ester *p*-nitrobenzílic (*p*NB) per a la síntesi de pèptids cíclics cadena lateral-cadena lateral.



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Tetrahedron Letters

Semipermanent *p*-nitrobenzyloxycarbonyl (*p*NZ) protection of Orn and Lys side chains: prevention of undesired α-Fmoc removal and application to the synthesis of cyclic peptides

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Abstract—Semipermanent side-chain protection of Orn and Lys with *p*-nitrobenzyloxycarbonyl (*p*NZ) for Fmoc/^{t}Bu chemistry does not result in the unwanted removal of α-Fmoc that occurs when groups such as Alloc are used for the same application. Furthermore, *p*NZ can be used in conjuction with *p*-nitrobenzyl ester (*p*NB) to prepare cyclic peptides.
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1. Introduction

Solid phase has become the preferred mode for peptide synthesis.[1] The success of these syntheses depends to a great extent on the coupling reagents used[1,2] as well as the temporary and permanent protecting strategies employed.[1,3] Among the most useful temporary protection groups for amino acid side chains is fluorenylmethoxycarbonyl (Fmoc), which is removed after the incorporation of each protected amino acid. In contrast, the *tert*-butyl (^{t}Bu), which is removed at the end of the synthesis, is the permanent group of choice for most peptides.[4]

In the case of cyclic[5] or branched[6] peptides, semipermanent protecting groups must also be used. These groups must be stable to the conditions used to remove the temporary protecting group (i.e., piperidine for Fmoc) and should be removed without affecting any permanent protecting groups. Currently, the semipermanent protecting groups most widely used in tandem with the Fmoc/^{t}Bu strategy are allyl derivatives.[7] However, the palladium complex used in their removal is expensive. Furthermore, deprotection of allyloxycarbonyl (Alloc) from the side chains of Orn and Lys generates a highly basic free amine that causes premature removal of Fmoc groups.[8] This side reaction can have disastrous effects for a synthesis, cleaving up to 20% of Fmoc groups present.

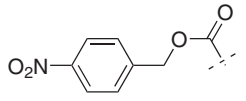

Figure 1. Structure of the *p*NZ protecting group.

Herein reported is the use of *p*-nitrobenzyloxycarbonyl (*p*NZ) as an alternative to Alloc for semipermanent side-chain protection of Orn and Lys. The application of *p*NZ to avoid undesired α-Fmoc removal and in the synthesis of cyclic peptides is also discussed (Fig. 1).

2. Results and discussion

The *p*NZ group was first described by Carpenter and Gish as an alternative to benzyloxycarbonyl (Z).[9] It has also been used for the protection of the ε-amino group of Lys.[10] Very recently, the *p*NZ has been described as a temporary protecting group for the α-functionalities in solid-phase peptide synthesis.[11] *p*NZ is orthogonal to the most common solid-phase peptide synthesis protecting groups such as ^{t}Bu/*tert*-butyloxycarbonyl (Boc), Fmoc, and Alloc, and is removed under

Keywords: Combinatorial chemistry; Orthogonal protecting group; Side reactions.
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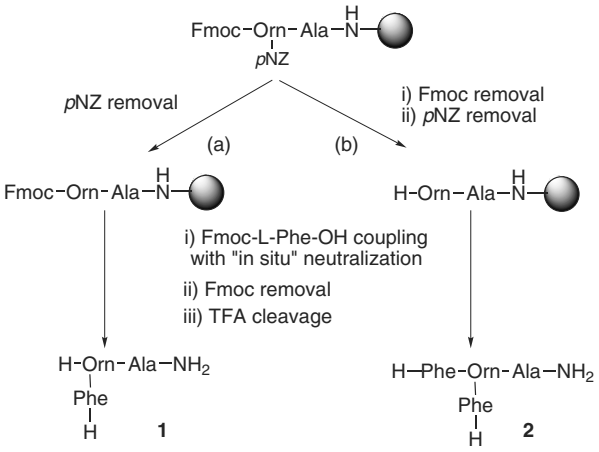
reductive and nearly neutral conditions with $SnCl_2$ in the presence of low concentrations of acid (1.6 mM HCl/dioxane).[11] Furthermore, its use circumvents typical side reactions associated with piperidine, such as the formation of aspartiimides or diketopiperazines (DKP).[11]

2.1. Avoiding undesired α-Fmoc removal

The basis for using *p*NZ to avoid DKP formation is that its removal implies catalytic amounts of acid. Thus, the deprotected amino acid is obtained as an ammonium salt, leaving the basicity and nucleophilicity of the amine function masked.[11] The same principle was thus applied to circumvent the unwanted removal of Fmoc caused by the primary amines of Lys or Orn side chains upon deprotection during solid-phase synthesis.

To demonstrate the utility of *p*NZ as a temporary protecting group for the side chains of Orn and Lys, the peptide {[H-Orn(&)-Ala-NH_2][H-Phe&]}[12] (**1**) was prepared from Fmoc-Orn(*p*NZ)-OH (Scheme 1, pathway **a**).[13] After *p*NZ removal in mildly acidic conditions, Fmoc-Phe-OH was directly coupled without a prior neutralization step. Instead, an in situ neutralization method similar to that used to avoid DKP formation was used.[11,14] If the α-Fmoc of Orn had been prematurely removed, then the byproduct {[H-Phe-Orn(&)-Ala-NH_2][H-Phe&]} (**2**) would have been generated, as was observed upon following pathway **b** of Scheme 1. As expected, peptide **2** was not detected by HPLC (Fig. 2) in crude **1**, indicating that premature Fmoc removal had been avoided by using *p*NZ.



Scheme 1. Strategy used to demonstrate that premature α-Fmoc elimination does not occur when *p*NZ is used to protect amino acid side chains.

2.2. Use of *p*NZ/*p*NB protection for the solid-phase synthesis of cyclic peptides

Similarly to the pair Alloc/Allyl used for the side-chain protection of Lys or Orn and Glu or Asp, *p*NZ can be used in conjunction with the related *p*-nitrobenzyl (*p*NB) for the synthesis of side chain to side-chain cyclic peptides.[15] This was demonstrated for the synthesis of the cyclic peptide H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2, a conformationally restricted analog of deltorphin.[16]

The peptide was synthesized on a Rink amide resin using Fmoc-Lys(*p*NZ)-OH and Fmoc-Glu(O*p*NB)-OH[17] together with other Fmoc/*t*Bu amino acids as shown in Scheme 2. The elongation of the peptide chain proceeded smoothly using *N*,*N*-diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt) as coupling agents. The semipermanent *p*NZ and *p*NB groups were removed with $SnCl_2$ and catalytic HCl. The cyclization was then carried out using benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP)/(*N*,*N*-*d*iisopropylethyl amine (DIEA)). Finally, cleavage of the peptidyl resin with trifluoroacetic acid (TFA) rendered the target peptide with a yield of 14.1% after HPLC purification (Fig. 3).

3. Conclusions

The use of *p*NZ as a temporary protecting group of Orn and Lys avoids premature α-Fmoc removal after side-chain deprotection of the ω-amino function. Furthermore, *p*NZ has been used in combination with its derivative *p*NB to synthesize a cyclic peptide via side-chain to side-chain cyclization. The *p*NZ/*p*NB combination has thus been validated as a new member

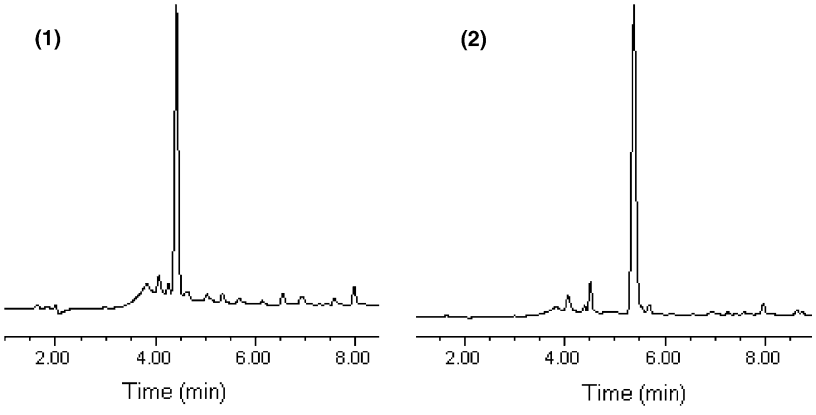
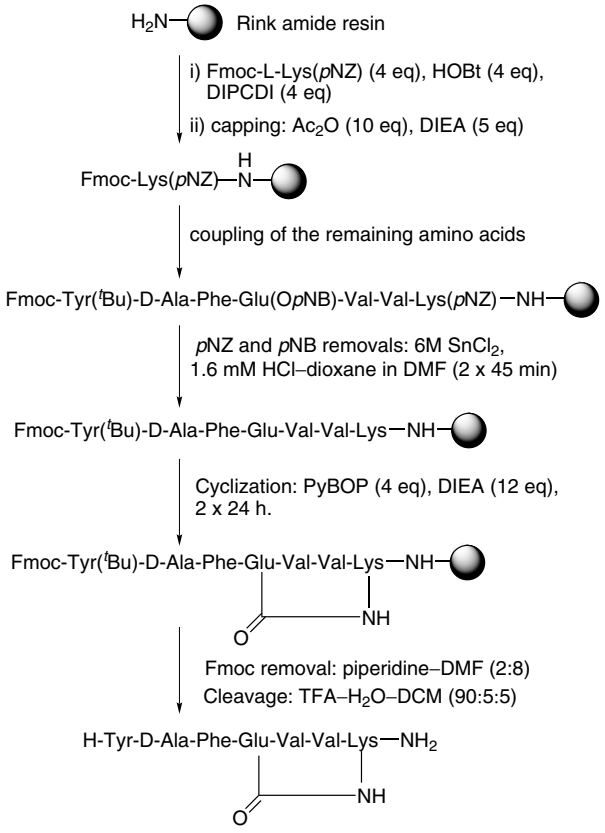


Figure 2. HPLC chromatograms of H-Orn(Phe)-Ala-NH_2 (**1**) and H-Phe-Orn(Phe)-Ala-NH_2 (**2**).



Scheme 2. Solid-phase synthesis of the deltorphin cyclic analog, H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2.

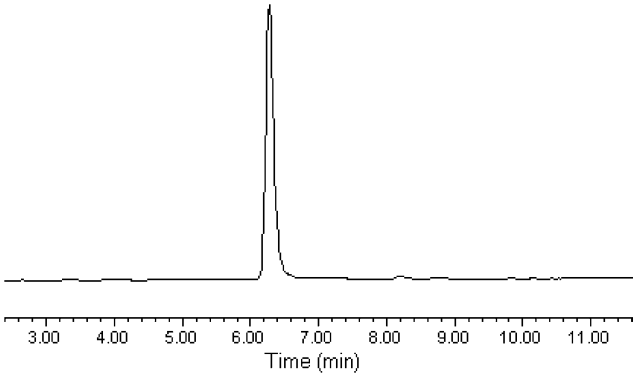


Figure 3. HPLC chromatogram of the cyclic deltorphin analog H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2.

of the family of orthogonal protecting group pairs[18] used in the synthesis of cyclic peptides as well as other complex peptides and organic molecules. Using the *p*NZ/*p*NB pair in conjunction with the Alloc/Allyl pair could enable regioselective synthesis of bicyclic peptides.

4. Experimental section

Analytical HPLC was carried out in a Waters instrument comprising two solvent-delivery pumps and automatic injector (Waters Separations Module 2695) and a variable-wavelength detector (model Waters 996 Photodiode Array). UV detection was performed at 220 nm, and linear gradients of CH_3CN (0.036% TFA) into H_2O (0.045% TFA) were run at a flow rate of 1.0 mL/min (Gradient A: from H_2O to CH_3CN in 15 min; Gradient B: from CH_3CN–H_2O (80:20) CH_3CN–H_2O (65:35) in 15 min).

4.1. Avoiding undesired α-Fmoc removal

To a Rink amide resin ($f = 0.66$ mmol/g, 100 mg), placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disk, was added Fmoc-Ala-OH (4 equiv) in the presence of equimolar amounts of DIPCDI and HOBt in *N*,*N*-dimethylformamide (DMF). The mixture was stirred for 1 h, at which point the Fmoc group was removed with piperidine–DMF (1:4). Fmoc-Orn(*p*NZ)-OH (4 equiv) was then coupled as described above. The resin was dissolved in DMF and divided into two equal portions (**a**,**b**).

The *p*NZ group of resin **a** was removed using 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF (2×30 min), and Fmoc-Phe-OH was subsequently coupled with PyBOP (5 equiv) and DIEA (8 equiv) in DMF. The Fmoc groups were removed, the peptidyl resin was cleaved with TFA/DCM/H_2O (90:5:5), and crude **1** was concentrated by evaporation, then characterized by HPLC ($t_R = 5.39$ min, gradient A) and ESMS (calcd for $C_{26}H_{36}N_6O_4$, 496.3, found *m*/*z*, 497.5 $[M+H]^+$).

The Fmoc and *p*NZ groups of **b** were removed as previously described and Fmoc-Phe-OH was coupled as above. Then, Fmoc group was removed and the peptide submitted to the same protocol as above to give **2**, which was dissolved in CH_3CN–H_2O and characterized by HPLC ($t_R = 4.42$ min, gradient A) and ESMS (calcd for $C_{17}H_{27}N_5O_3$, 349.2, found *m*/*z*, 350.4 $[M+H]^+$).

4.2. Preparation of the cyclic deltorphin analog H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2

Fmoc-Lys(*p*NZ)-OH (0.9 equiv), HOBt (0.9 equiv), and DIPCDI (0.9 equiv) in DMF were added to Rink amide resin (0.2 g, $f = 0.68$ mmol/g) and stirred for 1 h. The resin was then treated with Ac_2O (10 equiv) and DIEA (5 equiv) in DMF for 30 min in order to acetylate the free amino groups. The new loading of the resin was calculated by UV titration of the Fmoc group ($f = 0.49$ mmol/g). The remaining amino acids were coupled using 4 equiv each of Fmoc-Aaa-OH/DIPCDI/HOBt in DMF. Once the linear peptide was assembled, the Fmoc group of the last residue (Tyr) was left on, and the *p*NZ and *p*NB groups were removed by treating the resin with 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF (2×45 min). An aliquot of the resin was then treated with piperidine/DMF (1:4) to remove the Fmoc group, treated with TFA/H_2O/DCM (90:5:5) to cleave the peptide from the resin, and analyzed by HPLC ($t_R = 6.08$ min, gradient A) and ESMS (calcd for $C_{42}H_{63}N_9O_{10}$, 853.5, found *m*/*z*, 854.2 $[M+H]^+$).

The remaining resin was treated with PyBOP (4 equiv) and DIEA (12 equiv) in DMF for 24 h to cyclize the

peptide. An aliquot of the resin was then treated with piperidine/DMF (1:4), TFA/H_2O/DCM (90:5:5), and analyzed by HPLC and HPLC–MS, which indicated that the crude product comprised 65% linear peptide and 35% cyclic peptide. The remaining resin was re-treated with the same reagents and after 24 h an aliquot of the resin was analyzed as described above, indicating 50% linear peptide and 50% cyclic peptide. The cyclization was completed by stirring the resin with PyBOP (4 equiv), DIEA (12 equiv), and HOAt (8 equiv) in DMF for 72 h. The Fmoc group was then removed and the peptide was cleaved from the resin with TFA/H_2O/DCM (90:5:5). The TFA was removed by evaporation and the crude was dissolved in CH_3CN/H_2O (7:3) and washed three times with $CHCl_3$. H_2O was added to the aqueous layer, which was lyophilized and found to contain a 1:1 mixture of the linear and cyclic peptides among other impurities. The crude product was purified by semipreparative HPLC to afford 1.8 mg of the cyclic peptide, which was characterized by HPLC (t_R = 6.26 min, 99% of purity, gradient B) and ESMS (calcd for $C_{42}H_{63}N_9O_{10}$, 835.5, found m/z, 836.7 $[M+H]^+$).

Acknowledgments

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References and notes

1. (a) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; (b) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC: Boca Raton, FL, USA, 1997; (c) *Houben-Weyl, Synthesis of Peptides and Peptidomimetics*; Goodman, M., Felix, A., Moroder, L. A., Toniolo, C., Eds.; Georg Thieme Verlag: Stuttgart, Germany, 2002; Vol. E 22a-e; (d) Bruckdorfer, T.; Marder, O.; Albericio, F. *Curr. Pharm. Biotechnol.* **2004**, *5*, 29–43.
2. (a) Kates, S. A.; Albericio, F. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker: New York, NY, USA, 2000; pp 275–330; (b) Albericio, F.; Chinchilla, R.; Dodsworth, D.; Nájera, C. *Org. Prep. Proc. Int.* **2001**, *33*, 203–303.
3. (a) Wade, J. D. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker: New York, NY, USA, 2000; pp 103–128; (b) Doherty-Kirby, A. L.; Lajoie, G. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker: New York, NY, USA, 2000; pp 129–195.
4. (a) *Fmoc Solid Phase Peptide Synthesis*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford (UK), 2000; (b) Fields, G. B.; Lauer-Fields, J. L.; Liu, R.-q.; Barany, G. In *Synthetic Peptides: A User's Guide*, 2nd ed.; Grant, G. A., Ed.; W. H. Freeman & Co.: New York, 2001; pp 93–219.
5. Rovero, P. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker: New York, NY, USA, 2000; pp 331–364.
6. Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303–310.
7. Guibé, F. *Tetrahedron* **1997**, *53*, 13509–13556, and **1998**, *54*, 2967–3042.
8. (a) Farrera-Sinfreu, J.; Royo, M.; Albericio, F. *Tetrahedron Lett.* **2002**, *43*, 7813–7815; (b) Vig, B. S.; Murray, T. F.; Aldrich, J. V. *Biopolymers* **2003**, *71*, 620–637.
9. (a) Carpenter, F. H.; Gish, D. T. *J. Am. Chem. Soc.* **1952**, *74*, 3818–3821; (b) Gish, D. T.; Carpenter, F. H. *J. Am. Chem. Soc.* **1953**, *75*, 950–952; (c) Shields, J. E.; Carpenter, F. H. *J. Am. Chem. Soc.* **1961**, *83*, 3066–3070.
10. (a) Hocker, M. D.; Caldwell, C. G.; Macsata, R. W.; Lyttle, M. H. *Pept. Res.* **1995**, *8*, 310–315; (b) Peluso, S.; Dumy, P.; Nkubana, C.; Yokokawa, Y.; Mutter, M. *J. Org. Chem.* **1999**, *64*, 7114–7120.
11. Isidro-Llobet, A.; Guasch-Camell, J.; Álvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, *14*, 3031–3039.
12. The nomenclature used for branched and cyclic peptides is described in: Spengler, J.; Jiménez, J. C.; Giralt, E.; Albericio, F. *J. Pept. Res.* **2005**, *65*, 550–555.
13. Fmoc-Orn(*p*NZ)-OH and Fmoc-Lys(*p*NZ)-OH were synthesized from Fmoc-Aaa(Boc)-OH and *p*-nitrobenzyl chloroformate as starting materials, by the azide method: Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. *Org. Proc. Res. Dev.* **2004**, *8*, 920–924.
14. (a) Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1990**, *31*, 7363–7366; (b) Alsina, J.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1996**, *37*, 4195–4198.
15. The *p*NB group has been used to protect the *C*-terminal carboxylic group in side-chain or backbone anchoring strategies for the solid-phase synthesis of head-to-tail cyclic peptides. Romanovskis, P.; Spatola, A. F. *J. Pept. Res.* **1998**, *52*, 356–374; Royo, M.; Farrera-Sinfreu, J.; Solé, L.; Albericio, F. *Tetrahedron Lett.* **2002**, *43*, 2029–2032.
16. Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. *J. Med. Chem.* **1992**, *35*, 3956–3961.
17. Synthesis of Fmoc-L-Glu(O *p*NB)-OH: Fmoc-L-Glu-O′Bu (3.25 g, 7.63 mmol, 1.1 equiv) and *p*-nitrobenzyl alcohol (1.06 g, 6.94 mmol, 1 equiv) were dissolved in a minimum amount of DCM (7.5 mL). DIPCDI (2.16 g, 6.94 mmol, 1 equiv) and DMAP (0.085 g, 0.69 mmol, 0.1 mmol) were added. The reaction was followed by TLC (DCM) and after 2 h, EtOAc (40 mL) was added, and the solution was washed with 10% aqueous Na_2CO_3 (3× 25 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under vacuum to afford a yellow oil (3.42 g, 88% yield).
Fmoc-L-Glu(ONB)-O′Bu (1.68 g, 3 mmol) was dissolved in TFA/DCM (1:1) (20 mL) and stirred for 1 h. The solvent was then eliminated in vacuo and diethyl ether was added and evaporated four times in order to ensure that the TFA was removed. After drying the product in vacuo, a white solid was obtained (1.51 g, 3 mmol, nearly quantitative yield).
18. Albericio, F. *Biopolym. (Pept. Sci.)* **2000**, *55*, 123–139.

Use of *p*-Nitrobenzyloxycarbonyl (*p*NZ) as a Permanent Protecting Group in the Synthesis of Kahalalide F Analogs

Ús del Grup *p*-Nitrobenziloxicarbonil (*p*NZ) com a Protector Permanent per a la Síntesi d'Anàlegs de la Kahalalide F

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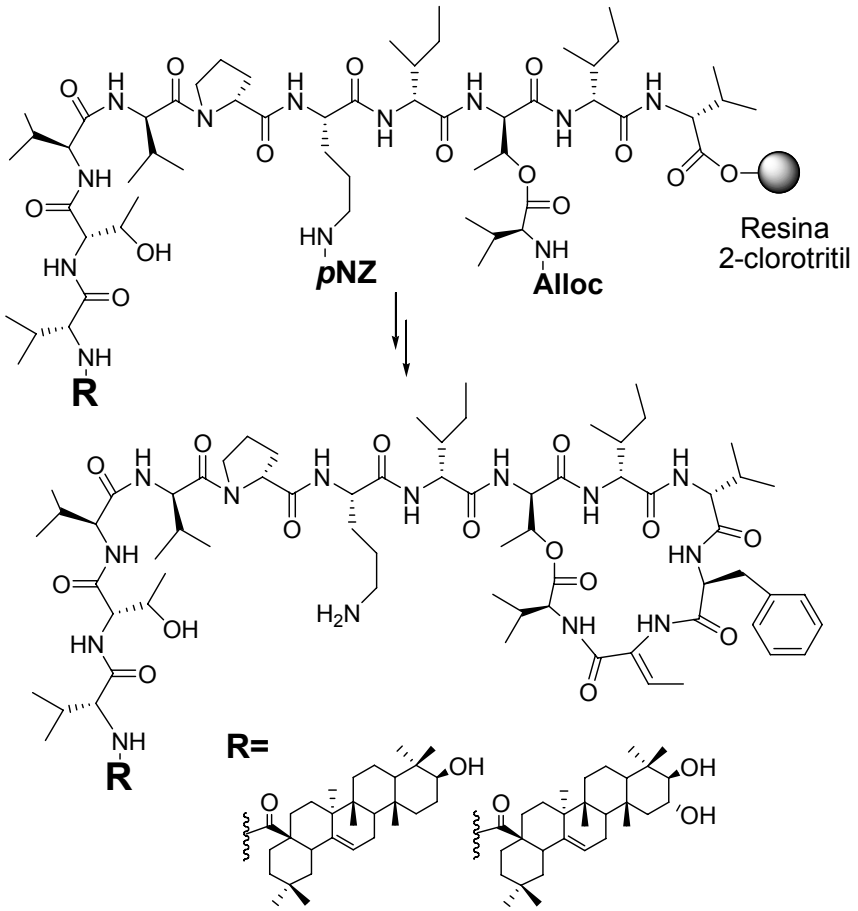
Tetrahedron Letters 46 (2005), 7737-7741

Resum

Aquest article mostra una primera aplicació del grup *p*NZ a un cas real: la síntesi de derivats del ciclodepsipèptid antitumoral Kahalalide F amb substituents làbils a medi àcid com els àcids grassos oleanolic i maslínic. En la síntesi es combina el grup *p*NZ amb la resina 2-clorotritil i el grup Alloc. Les condicions suaus d'eliminació del grup *p*NZ no afecten als àcids oleanòlic i maslínic i permeten l'obtenció del producte final.



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Use of *p*-nitrobenzyloxycarbonyl (*p*NZ) as a permanent protecting group in the synthesis of Kahalalide F analogs

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Abstract—*p*-Nitrobenzyloxycarbonyl (*p*NZ) is used for the permanent protection of ornithine in the synthesis of derivatives of the anti-tumor cyclodepsipeptide Kahalalide F that contain acid labile residues.
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1. Introduction

Solid-phase fluorenylmethoxycarbonyl (Fmoc)-*tert*-butyl (^{t}Bu) chemistry is the strategy of choice for the preparation of peptides in basic research and commercial applications.[1,2] In this strategy, the Fmoc group is normally removed with piperidine in DMF, while *permanent* protecting groups are removed by trifluoroacetic acid (TFA) in the presence of scavengers during the cleavage of the peptide from the resin.[1,2] However, certain peptides can be degraded by the treatment with high concentrations of TFA. For these cases, ^{t}Bu-type protecting groups must be substituted with protecting groups that can be removed in neutral or nearly neutral conditions.

Herein, the synthesis of analogs of the anti-tumor cyclodepsipeptide Kahalalide F that contain acid labile substituents (oleanolic and maslinic acids) using *p*-nitrobenzyloxycarbonyl (*p*NZ)[3] as a permanent protecting group for the side chain of Orn is reported (Fig. 1).

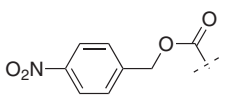

Figure 1. Structure of the *p*NZ protecting group.

2. Results and discussion

Kahalalide F (**1a**), the only member of the kahalalide peptide family to be isolated from the sacoglossan mollusc *Elysya rufescens* and the green alga *Bryopsis* sp.,[4] is in clinical trials for several types of cancers.[4,5] Structurally, Kahalalide F is a head to side-chain cyclic depsipeptide that terminates in an aliphatic acid. The establishment of an efficient solid-phase synthesis of Kahalalide F[6] by our group spawned an in-house program dedicated to creating analogs for SAR.[7]

The original solid-phase synthesis,[6] similar to that outlined in Figure 2, is based on an orthogonal protecting group scheme using a chlorotrityl chloride (ClTrt-Cl, Barlos) resin together with Fmoc and allyloxycarbonyl (Alloc) as temporary protecting groups, and ^{t}Bu and Boc for the side-chain protection of Thr and Orn, respectively.

We envisioned Kahalalide F analogs in which the N-terminal aliphatic acid of the parent compound is substituted with oleanolic acid (3β-hydroxy-12-oleanen-28-oic) or maslinic acid (2α,3β-dihydroxy-12-oleanen-28-oic), pentacyclic triterpenoids widely found in nature.[8]

Keywords: Combinatorial chemistry; Cyclic pepides; Orthogonal protecting group.
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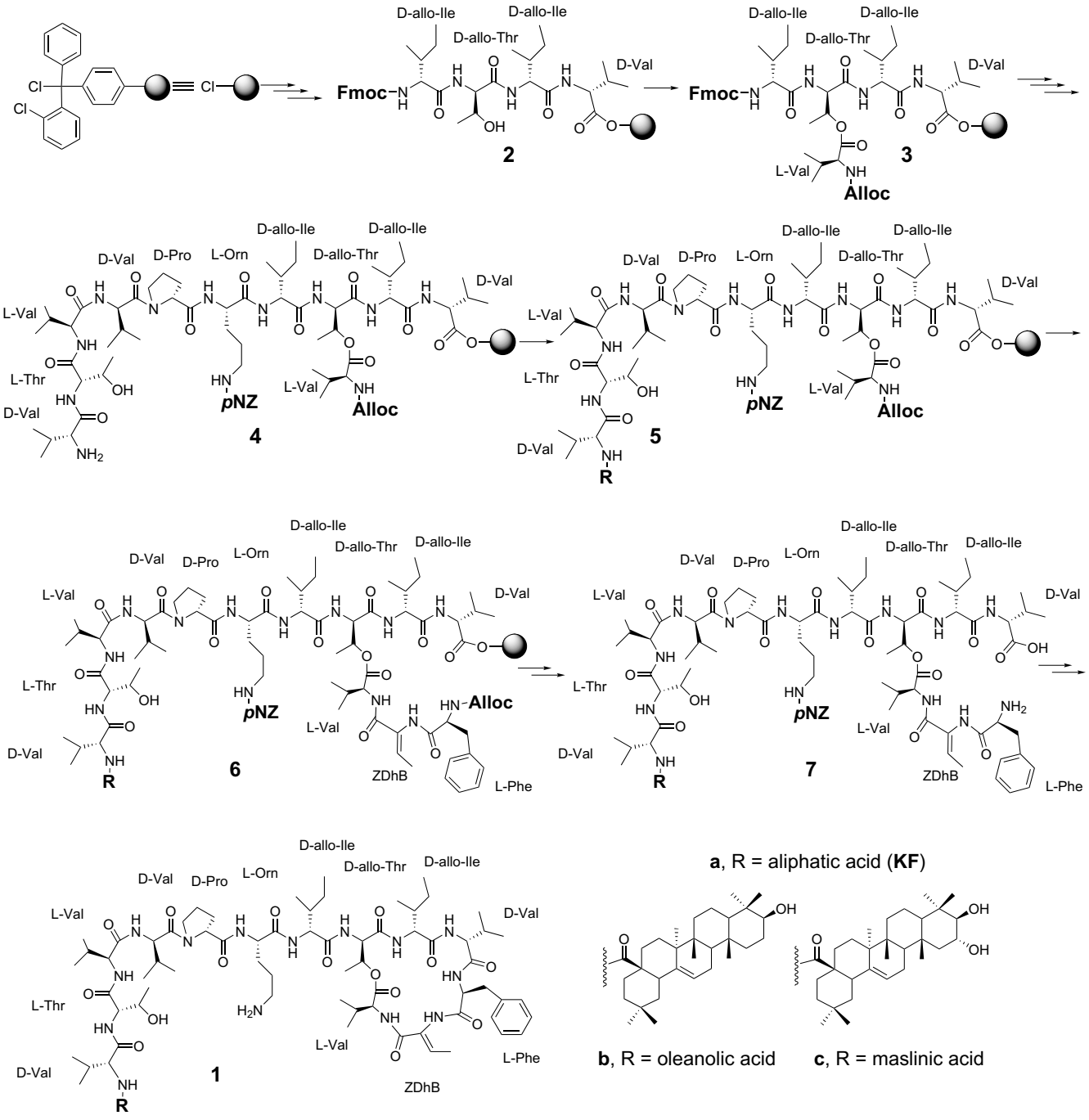

Figure 2. Synthetic strategy developed for the synthesis of KF analogs.

These compounds are isolated in high yields from olive-pressing residues following an extraction process.[9] Both acids, as well as structural analogues, have interesting pharmacological profiles,[10] including in vitro anti-HIV activity[11] and antioxidant properties.[12]

The first attempt at synthesizing the aforementioned analogs progressed smoothly until the last step. Final treatment of the peptidyl-resin with TFA, which should have removed the ^{t}Bu and Boc groups, led to a complex mixture in which the target compound could not be identified by HPLC-MS. It was later established that oleanolic and maslinic acids are not stable to high concentrations of TFA.

We thus sought a new protecting group for the δ-amino function of Orn that would be orthogonal to the Fmoc, Alloc, and Cl-Trt protecting groups.[13] The Thr near the N-terminal can be incorporated with its secondary alcohol function unprotected due to its low nucleophilicity and the few coupling cycles implied in the synthesis.

Specifically, the group had to be stable to piperidine (used for Fmoc removal), Pd(0) in the presence of scav-

engers (used for Alloc removal), and 1% TFA–CH_2Cl_2 (used to cleave the protected peptide from the Cl-Trt resin before cyclization). The *p*NZ group, recently described for the temporary protection of α-functionalities in solid-phase peptide synthesis meets the aforementioned conditions.[3f] The *p*NZ group, removed with $SnCl_2$ in the presence of catalytic amounts of acid, also does not induce side reactions that commonly result from other temporary protecting groups.[3f]

A new synthesis was thus initiated with limited incorporation of Fmoc-D-Val-OH to ClTrt-resin,[14] followed by the stepwise coupling of the next three N^{α}-protected amino acids, with the side chain of D-allo-Thr unprotected. Subsequent incorporation of Alloc-Val-OH using DIPCDI/DMAP allowed formation of the ester bond.[15] At this point, the peptide chain was completed by first introducing Fmoc-Orn(*p*NZ)-OH, followed by the rest of protected amino acids and terminated by either oleanolic or maslinic acid. The incorporation of these two acids required strong coupling conditions, such as benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP)/1-hydroxy-7-azabenzotriazole (HOAt)/*N*,*N*-diisopropylethylamine (DIEA). The dipeptide Alloc-L-Phe-*Z*Dhb-OH was introduced and the completed linear peptide was cleaved from the resin with TFA–CH_2Cl_2 (1:99), cyclized with *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt), and deprotected using the *p*NZ removal conditions described above. The target KF analog was obtained with excellent purity and was characterized by MS.[16]

3. Conclusions

This work demonstrates the utility of the *p*NZ group as a permanent protecting group in solid-phase peptide synthesis. *p*NZ can be used with Cl-Trt-resin to prepare peptides having acid sensitive residues, for which ^{t}Bu-type protection is not possible. The *p*NZ group is removed in solution or on-resin with $SnCl_2$ and a catalytic amount of acid.

4. Experimental section

4.1. Fmoc-L-Orn-OH

Fmoc-L-Orn(Boc)-OH (2 g, 4.4 mmol) was dissolved in TFA–DCM (3:7) and stirred for 30 min. After evaporating off the solvent, diethyl ether was added to, and evaporated from, the crude residue five times in order to ensure that all of the TFA had been removed. The final product (1.31 g, 84% yield) was characterized by analytical HPLC (<98% purity) and ES-MS (calcd for $C_{20}H_{22}N_2O_4$: 354.15, found *m*/*z* 355.1 $(M+1)^+$).

4.2. Fmoc-L-Orn(pNZ)-OH

Protection of the side chain of Orn was carried out using the azide method.[17] *p*-Nitrobenzyl chloroformate (0.797 g, 3.7 mmol) was dissolved in dioxane (1.7 mL) and a solution of sodium azide (0.289 g, 4.44 mmol) in H_2O (1.1 mL) was added. The resulting emulsion was stirred for 2 h and the formation of the azide was followed by TLC (CH_2Cl_2). This solution was added dropwise to a suspension of Fmoc-L-Orn-OH (1.31 g, 3.7 mmol) in 6 mL of dioxane–2% aqueous Na_2CO_3 (1:1) and the resulting white suspension was stirred for 24 h with the pH kept between 9 and 10 by adding 10% aqueous Na_2CO_3. At this point, TLC (CH_2Cl_2) results indicated that there was no azide left, H_2O (50 mL) was then added and the suspension was washed with *tert*-butyl methyl ether (MTBE) (3 × 40 mL). The aqueous portion was acidified to pH 1 with 9 N HCl, affording a white precipitate that was filtered off and dried to yield 1.67 g (85% of yield) of the title compound. The white solid was characterized by HPLC (>98% purity), ESMS (calcd for $C_{28}H_{27}N_3O_8$: 533.2, found *m*/*z* 534.4 $(M+1)^+$), ^{1}H and ^{13}C NMR.[18]

4.3. KF analogs

To chlorotrityl resin (500 mg, $f = 1.27$ mmol/g) placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk, was added Fmoc-D-Val-OH (0.7 equiv), DIEA (7 equiv) and dichloromethane, and the resulting slurry was stirred for 1 h. The reaction was then terminated by adding piperidine–DMF (1:4) and the loading was calculated by UV ($f = 0.56$ mmol/g). The chain was elongated by sequentially coupling Fmoc-D-allo-Ile-OH, Fmoc-D-allo-Thr-OH and Fmoc-D-allo-Ile-OH (4 equiv), using DIPCDI (4 equiv) and HOBt (4 equiv) in DMF for 90 min. The completion of all couplings was confirmed with a negative ninhydrin test result. All Fmoc groups were removed as indicated above except the last group, which was not removed. Alloc-Val-OH (5 equiv) was then coupled to the peptide with DIPCDI (5 equiv) and DIEA (1 equiv) in the presence of DMAP (0.5 equiv). This coupling was repeated twice using the same conditions. An aliquot of the resin was then treated with TFA–H_2O (1:99) for 1 min and, after evaporation, the crude product corresponding to the peptide resin **3** was characterized by HPLC (>95% purity) and ESMS (calcd for $C_{30}H_{53}N_5O_9$, 627.4 Found: *m*/*z* 628.2 $[M+H]^+$). Then, the Fmoc group of Fmoc-D-allo-Ile-OH was removed and Fmoc-Orn(*p*NZ)-OH (4 eq), Fmoc-D-Pro-OH (5 equiv), Fmoc-D-Val-OH (4 equiv, a recoupling was needed), Fmoc-L-Val-OH (4 equiv), Fmoc-Thr-OH (5 equiv) and Fmoc-D-Val-OH (4 equiv) were sequentially added to the peptide resin and stirred for 90 min in the presence of equimolar amounts of DIPCDI and HOBt in DMF.

After removing the last Fmoc group, an aliquot of the peptide resin was cleaved as indicated above and the crude product obtained (corresponding to peptide resin **4**) characterized by HPLC (>98% of purity) and MALDI-TOF (calcd for $C_{67}H_{109}N_{13}O_{20}$: 1416.7, found: *m*/*z* 1417.1 $[M+H]^+$, 1439.1 $[M+Na]^+$, 1455.0 $[M+K]^+$). The remaining resin was suspended in DMF and divided into two portions. Oleanolic acid (3 equiv) was coupled to one of the portions, and maslinic acid (3 equiv) was

coupled to the other using PyBOP (3 equiv), HOAt (3 equiv) and DIEA (9 equiv) in DMF. An aliquot of each peptide resins was cleaved and characterized by HPLC (**5b**: >78% of purity, **5c**: >80% of purity) and ESMS (**5b**: *m/z* calcd for $C_{97}H_{155}N_{13}O_{22}$: 1854.1, found *m/z* 1855.6 $[M+H]^+$; **5c**: *m/z* calcd for $C_{97}H_{155}N_{13}O_{23}$: 1870.1, found *m/z* 1871.6 $[M+H]^+$).

The Alloc group of both resins was removed using $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (10 equiv) in DMF (three treatments of 15 min). The resin was then washed with DCM and DMF, followed by 0.02 M sodium diethyldithiocarbamate in DMF (3×15 min). Alloc-Phe-*Z*Dhb-OH (2.5 equiv), HOAt (2.5 equiv) and DIPCDI (2.5 equiv) in DMF were added to each resin, and the mixture was stirred overnight to yield the peptidyl resins **6b** and **6c**. The removal of Alloc group and subsequent washings were carried out as described above.

Both protected peptides were cleaved from the resin with TFA–DCM (1:99) (5×30 s) and successive washings with DCM. Filtrates were collected in H_2O and the solvent was partially removed in vacuo. Acetonitrile (MeCN) was added to dissolve the solid that appeared during H_2O removal, the solutions were lyophilized and the products characterized by HPLC (**7b**: >91% of purity, **7c**: >73% of purity) and ESMS (**7b**: calcd for $C_{106}H_{165}N_{15}O_{22}$, 2001.5, found *m/z* 2002.0 $[M+H]^+$; **7c**: calcd for $C_{106}H_{165}N_{15}O_{23}$, 2017.5, found *m/z* 2018.5 $[M+H]^+$).

The resulting crudes were dissolved in DCM and cyclized in the presence of DIPCDI (3 equiv), HOBt (4 equiv), and DIEA (3 equiv). After 1 h of stirring, no starting materials remained as indicated by HPLC, so the solvent was removed.

Removal of the *p*NZ groups was carried out by stirring the peptides with 1 M $SnCl_2$ and 1.6 mM HCl–dioxane in DMF for 1 h. The solvent was removed in vacuo, and the crudes were purified by HPLC (Kromasil C_8 5 μm, 205×50 mm) then characterized by HPLC (**1b**: 32 mg, >98% of purity, **1c**: 19 mg, >97% of purity) and MALDI-TOF-MS (1b: calcd for $C_{98}H_{157}N_{14}O_{17}$, 1803.8. Found: *m/z* 1804.8 $[M+H]^+$, 1825.8 $[M+Na]^+$, 1841.8 $[M+K]^+$; **1c**: calcd for $C_{98}H_{158}N_{14}O_{18}$, 1819.2. Found: *m/z* 1820.0 $[M+H]^+$, 1842.9 $[M+Na]^+$, 1857.9 $[M+K]^+$).

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References and notes

1. (a) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; (b) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC: Boca Raton, FL (USA), 1997; (c) *Houben-Weyl, Synthesis of Peptides and Peptidomimetics*; Goodman, M., Felix, A., Moroder, L. A., Toniolo, C., Eds.; Georg Thieme: Stuttgart (Germany), 2002; Vol. E 22a-e; (d) Bruckdorfer, T.; Marder, O.; Albericio, F. *Current Pharm. Biotech.* **2004**, *5*, 29–43.
2. (a) *Fmoc Solid Phase Peptide Synthesis*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford (UK), 2000; (b) Fields, G. B.; Lauer-Fields, J. L.; Liu, R.-q.; Barany, G. In *Synthetic Peptides: A User's Guide*, 2nd ed.; Grant, G. A., Ed., WH. Freeman & Co.: New York, 2001; pp 93–219.
3. (a) Carpenter, F. H.; Gish, D. T. *J. Am. Chem. Soc.* **1952**, *74*, 3818–3821; (b) Gish, D. T.; Carpenter, F. H. *J. Am. Chem. Soc.* **1953**, *75*, 950–952; (c) Shields, J. E.; Carpenter, F. H. *J. Am. Chem. Soc.* **1961**, *83*, 3066–3070; (d) Hocker, M. D.; Caldwell, C. G.; Macsata, R. W.; Lyttle, M. H. *Pept. Res.* **1995**, *8*, 310–315; (e) Peluso, S.; Dumy, P.; Nkubana, C.; Yokokawa, Y.; Mutter, M. *J. Org. Chem.* **1999**, *64*, 7114–7120; (f) Isidro-Llobet, A.; Guasch-Camell, J.; Álvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, 3031–3039.
4. Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825–5826.
5. (a) Jimeno, J.; Lopez-Martin, J. A.; Ruiz-Casado, A.; Izquierdo, M. A.; Scheuer, P. J.; Rinehart, K. *Anti-Cancer Drugs* **2004**, *15*, 321–329; (b) Hamann, M. T. *Curr. Op. Molecular. Therap.* **2004**, *6*, 657–665; (c) 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*, 1854–1862.
6. Lopez-Macia, A.; Jimenez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, 11398–11401.
7. Albericio, F.; Fernandez-Donis, A.; Giralt, E.; Gracia, C.; Lopez-Rodriguez, P.; Varon, S.; Cuevas, C.; Lopez-Macia, A.; Francesch, A.; Jiménez-Garcia, J. C.; Royo, M. PCT Int. Appl., 2005, WO 2005023846.
8. (a) *Dictionary of Natural Products on CD-ROM*, ISSN 0966-2146 ver. 5:1 Chapman & Hall: New York, 1996; oleanolic acid, CAS [508-02-1]; maslinic acid, CAS [4373-41-5]; (b) Phytochemical and Ethnobotanical DB. http://www.ars-grin.gov/duke/plants.html.
9. García-Granados, A.; Martínez, A.; Parra, A.; Rivas F. PCT Int. Appl. W0 98 04331, 1998. *Chem. Abstr.* **1998**, *128*, 179706.
10. (a) Liu, J. *Ethnopharmacology* **1995**, *49*, 57; (b) Assefa, H.; Nimrod, A.; Walker, L.; Sindelar, R. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 619; (c) Honda, T.; Rounds, B. V.; Bore, L.; Finlay, H. J.; Favaloro, F. G., Jr.; Suh, N.; Wang, Y.; Sporn, M. B.; Gribble, G. W. *J. Med. Chem.* **2000**, *43*, 4233.
11. (a) Kashiwada, Y.; Ikeshiro, Y.; Nagao, T.; Okabe, H.; Cosentino, L. M.; Lee, K. H. *J. Nat. Prod.* **2000**, *63*, 1619; (b) Mengoni, F.; Lichtner, M.; Battinelli, L.; Marzi, M.; Mastronianni, C. M.; Vullo, V.; Mazzanti, G. *Planta Med.* **2002**, *68*, 111.
12. Montilla, M. P.; Agil, A.; Navarro, C.; Jimenez, M. I.; Garcia-Granados, A.; Parra, A.; Cabo, M. M. *Planta Med.* **2003**, *69*, 472–474.
13. (a) Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942; (b) Albericio, F. *Biopolymers (Pept. Sci.)* **2000**, *55*, 123–139.

14. The use of high loading (F >0.7 mmol/g) ClTrt-Cl resins for the preparation of hydrophobic peptides such as Kahalalide F usually leads to impurities Chiva, C.; Vilaseca, M.; Giralt, E.; Albericio, F. *J. Pept. Sci.* **1999**, *5*, 131–140.
15. When this Val was incorporated after the peptide chain had been completed, the yields were low. This is presumably due to the hydrophobicity of the peptide chain, which favors interchain aggregation. See Ref. 6.
16. Tin metal content in the purified KF analogue was showed to be nil by atomic absorption.
17. Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. *Org. Proc. Res. Develop.* **2004**, *8*, 920.
18. ^{1}H NMR (400 MHz, DMSO-d_6): 8.2 (2H, d, $J = 8.4$ Hz, H3′ and H5′); 7.9 (2H, d, $J = 7.2$ Hz, H1 and H8); 7.7 (2H, d, $J = 7.2$ Hz, H4 and H5); 7.6 (2H, d, $J = 8.8$ Hz, H2′ and H6′); 7.4 (2H, dd, $J_1 = J_2 = 7.2$ Hz, H3 and H6); 7.3 (2H, dd, $J_1 = J_2 = 7.2$ Hz, H2 and H7); 5.1 (2H, s, –O–CH_2–4-nitrophenyl); 4.2 (3H, m, H9 and fluorene–CH_2–O–); 3.8 (1H, m, α̃CH Orn); 3.0 (2H, m, α̃CH_2 Orn); 1.7 (1H, m, α̃CH_2 Orn); and 1.6 (1H, m, α̃CH_2 Orn); 1.4 (2H, m, α̃CH_2 Orn). ^{13}C NMR (100 MHz, DMSO-d_6): 174.5 (–*C*O–OH); 156.6 and 156.5 (–N–*C*O–O–) 147.6 (C4′); 146.1 (C1′); 144.6 and 144.5 (C12 and C13); 141.4 (C10 and C11); 128.7 (C2′ and C6′); 128.3 (C2 and C7); 127.8 (C3 and C6); 126.0 (C4 and C5); 124.2 (C3′ and C5′); 120.8 (C1 and C8); 66.2 (fluorene–*C*H_2O–); 64.6 (–O–*C*H_2–4-nitrophenyl); 54.8 (α̃CH Orn); 47.36 (C9); 40.8 (α̃CH_2 Orn); 29.4 (α̃CH_2 Orn); 26.7 (α̃CH_2 Orn).

Convergent Approaches for the Synthesis of the Antitumoral Peptide, Kahalalide F. Study of Orthogonal Protecting Groups

Estratègies Convergents per la Síntesi del Pèptid Antitumoral, Kahalalide F. Estudi de Grups Protectors Ortogonals

Carolina Gracia, Albert Isidro-Llobet, Luis J. Cruz, Gerardo A. Acosta, Mercedes Álvarez, Carmen Cuevas, Ernest Giralt and Fernando Albericio

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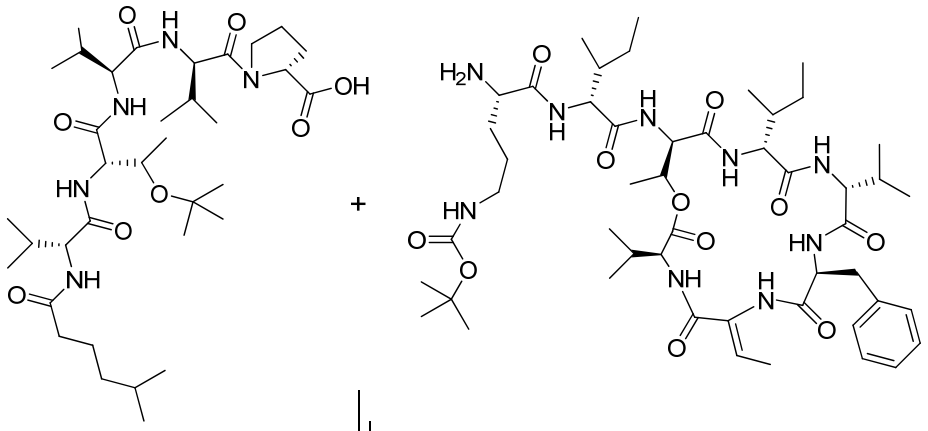
Resum

A continuació es mostra una segona aplicació del grup *p*NZ a un cas real: una nova estratègia convergent de síntesi de la Kahalalide F.

Els compostos de la família Kahalalide són pèptids aïllats del mol·lusc herbívor Hawaià *Elysia rufescens*, i la seva dieta, l'alga verda *Bryopsis* sp. La Kahalalide F i els seus anàlegs sintètics són els compostos més prometedors de la família Kahalalide degut a la seva activitat antitumoral. En treballs previs s'havien desenvolupat estratègies lineals de síntesi en fase sòlida de la Kahalalide F. En aquest article es descriuen vàries estratègies convergents de síntesi on s'usen diferents esquemes ortogonals de protecció, un d'ells implicant l'ús del grup *p*NZ. Aquestes estratègies permeten un millor control i caracterització dels intermedis ja que es realitzen més reaccions en solució.



JOC*Article*

Convergent Approaches for the Synthesis of the Antitumoral Peptide, Kahalalide F. Study of Orthogonal Protecting Groups

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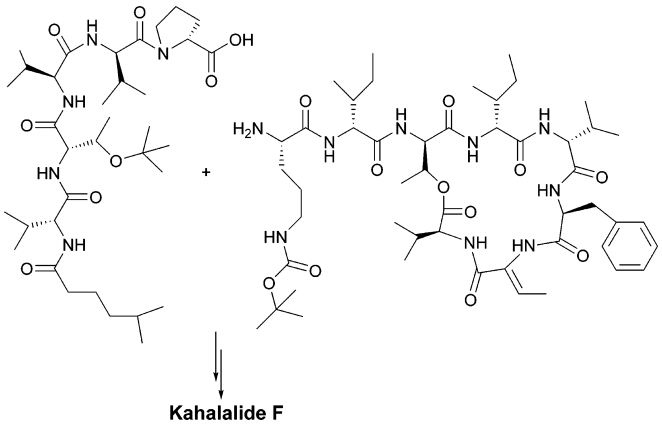

Kahalalide compounds are peptides that are isolated from a Hawaiian herbivorous marine species of mollusc, *Elysia rufescens*, and its diet, the green alga *Bryopsis* sp. Kahalalide F and its synthetic analogues are the most promising compounds of the Kahalalide family because they show antitumoral activity. Linear solid-phase syntheses of Kahalalide F have been reported. Here we describe several new improved synthetic routes based on convergent approaches with distinct orthogonal protection schemes for the preparation of Kahaladide analogues. These strategies allow a better control and characterization of the intermediates because more reactions are performed in solution.

Introduction

Kahalalide compounds are peptides that are isolated from a Hawaiian herbivorous marine species of mollusc, *Elysia rufescens*, and its diet, the green alga *Bryopsis* sp. Kahalalide F[1] and analogues are the most promising compounds of the Kahalalide family because of their antitumoral activities.[2] Kahalalide F alters the function of the lysosomal membrane, a characteristic that distinguishes it from all other known antitumor agents. This compound also inhibits TGF-α expression, blocks intracellular signaling pathways downstream of the EGF and ErbB2 receptor family, and induces non-p53-mediated apoptosis.[3] Recent studies demonstrate that Kahalalide F induces cell necrosis in vivo (oncosis) and shows selectivity for tumor cells compared with healthy cells in vitro.[4] Its activity is independent of multidrug resistance (MDR) expression.[5]

Kahalalide F is currently undergoing Phase II clinical trials in various solid tumors: melanoma, non-small-cell lung cancer, and hepatocellular carcinoma. A Phase II trial for the treatment of patients with severe psoriasis is also ongoing.[6]

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(1) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.
(2) Hamann, M. T. *Curr. Opin. Mol. Ther.* **2004**, *6*, 657−665.
(3) Janmaat, M. L.; Rodriguez, J. A.; Jimeno, J.; Kruyt, F. A. E. *Mol. Pharmacol.* **2005**, *68*, 502−510.

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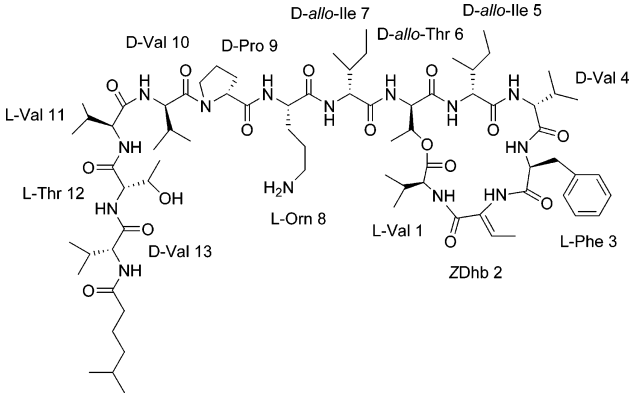
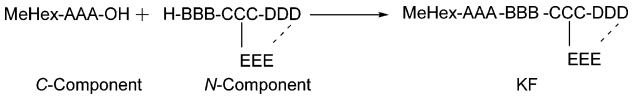


FIGURE 1. Structure of Kahalalide F.

Members of the Kahalalide F family, which are head-to-side chain cyclodepsipeptides, have a complex structure, comprising six amino acids, among these (*Z*)-didehydro-α-aminobutyric acid (ZDhb) (formed from dehydratation of a Thr residue), as a cyclic part between the carboxylic acid of a L-Val 1 and the secondary alcohol of the D-*allo*-Thr 6, and an exocyclic chain of seven amino acids with a terminal aliphatic/fatty acid group (R) (Figure 1).[1,7−9] Compounds in which R is a methylhexanoic acid are of greatest interest.[10]

Linear solid-phase syntheses of Kahalalides involving cyclization and final deprotection performed in solution have been described.[8,11] However, synthetic routes for Kahalalide compounds are still required. Here we report several new convergent strategies for the synthesis of Kahalalide F derivatives. Convergent strategies are defined as those in which peptide fragments are coupled to give the desired target molecule.[12−15] The condensation of peptide fragments should lead to fewer problems in the isolation and purification of intermediates. The difference between the condensation product desired and the segments themselves, in terms of molecular size and chemical nature, should be sufficiently pronounced so as to permit their separation relatively easily. Several orthogonal protecting schemes have been used in these strategies.[16]

SCHEME 1. Convergent Strategy for the Synthesis of Kahalalide F Analogues; AAA, BBB, CCC, DDD, and EEE Are Amino Acid Residues



Although solid-phase strategies are crucial for peptide synthesis,[12b,17−20] the method of choice for a convergent strategy involves a combination of solid-phase and solution chemistries. Thus, protected peptides are prepared in solid phase and then combined in solution.[21,22a] Paradigm of this strategy is the large-scale commercial production of enfuvitide (T-20 or Fuzeon), a 36-amino acid peptide.[23]

Results and Discussion

To render Kahalalide F, a 14-building-block peptide [13 amino acids and the methylhexanoic acid], the most convenient synthetic strategy involves dividing the peptide in two fragments, the *N*-terminal and the fragment containing the cycle Scheme 1). Fragment composition can be modified in order to optimize condensation yields and minimize side reactions, such as epimerization.

The preparation of the *N*-Component requires cyclization in solution and therefore the *N*-protection should also be kept during the cleavage of the lineal fragment from the resin.

Solid-phase synthesis of protected peptides requires a resin that facilitates the release of the peptide without removing the side-chain protecting groups, which are removed as the last step of the synthesis.[12a,13,14] In this regard, the *t*Bu-based protecting groups are highly convenient because they are removed with high concentrations of trifluoroacetic acid (TFA) in the presence of scavengers and are stable to piperidine, used to remove the α-amino protecting group, and to low concentrations of TFA. The super-acid-labile chlorotrityl chloride polystyrene (CTC, Barlos, Cl-●) resin allows the release of peptides with 1−2% of TFA in CH_2Cl_2 or even with trifluoroethanol or hexafluoroacetone solutions.[24] An additional advantage of the CTC resin is that its hindered structure minimizes the formation of the diketopiperazines (DKP) during removal of the temporal protecting group of the second amino acid.[25,26]

General Strategies. Three sets of fragments were prepared. Schemes 2 and 3 show the *C*-Components and their complementary *N*-Components synthesized.

(4) Suarez, Y.; Gonzalez, L.; Cuadrado, A.; Berciano, M.; Lafarga, M.; Muñoz, A. *Mol. Cancer Ther.* **2003**, *2*, 863−872.
(5) Jimeno, J.; Faircloth, G.; Fernandez Souse-Faro, J. M.; Scheuer, P.; Rinehart, K. *Mar. Drugs* **2004**, *2*, 14−29.
(6) Izquierdo Delso, M. A. PCT Int. Appl. (2004) WO 2004075910, A1 20040910, CAN 141:236712, AN 2004:740175.
(7) Goetz, G.; Yoshida, W. Y.; Scheuer, P. J. *Tetrahedron* **1999**, *55*, 7739−7746 and 11957.
(8) Lopez-Macia, A.; Jimenez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, 11398−11401.
(9) Bonnard, I.; Manzanares, I.; Rinehart, K. L. *J. Nat. Prod.* **2003**, *66*, 1466−1470.
(10) Albericio, F.; Fernandez, A.; Giralt, E.; Gracia, C.; Lopez, P.; Varon, S.; Cuevas, C.; Lopez-Macia, A.; Francesch, A.; Jimenez, J. C.; Royo, M. PCT Int. Appl. (2005) WO 2005023846, A1 20050317, CAN 142:298335, AN 2005:239012.
(11) Lopez, P. E.; Isidro-Llobet, A.; Gracia, C.; Cruz, L. J.; Garcia-Granados, A.; Parra, A.; Alvarez, M.; Albericio, F. *Tetrahedron Lett.* **2005**, *46*, 7737−7741.
(12) (a) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49*, 11065−11133. (b) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997.
(13) Benz, H. *Synthesis* **1994**, *4*, 337−358.
(14) (a) Barlos, K.; Gatos, D. *Biopolymers* **1999**, *51*, 266−278. (b) Barlos, K.; Gatos, D. In *Fmoc Solid-Phase Peptide Synthesis*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford, UK, 2000; pp 215−228.
(15) Sakakibara, S. *Biopolymers* **1999**, *51*, 279−296.
(16) An orthogonal protecting scheme is defined as one based on completely different classes of protecting groups such that each class of groups can be removed in any order and in the presence of all other classes of protecting groups. Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936−4942.
(17) Fields, G. B. *Methods in Enzymology, Solid-Phase Peptide Synthesis*; Academic Press: Orlando, Florida, 1997; Vol. 289.
(18) Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C. *Houben-Weyl. Methods of Organic Chemistry. Synthesis of Peptides and Peptidomimetics*; Georg Thieme Verlag: Stuttgart, Germany, 2001; Vol. E 22.
(19) Bruckdorfer, T.; Marder, O.; Albericio, F. *Curr. Pharm. Biotechnol.* **2004**, *5*, 29−43.
(20) Albericio, F. *Curr. Opin. Chem. Biol.* **2004**, *8*, 211−221.
(21) Nisiuchi, Y.; Inui, T.; Nishio, H.; Bodi, J.; Kimura, T.; Tsuji, F. T.; Sakakibara, S. P *Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13549−13554.
(22) Chiva, C.; Barthe, P.; Codina, A.; Gairi, M.; Molina, F.; Granier, C.; Pugniere, M.; Inui, T.; Nishio, H.; Nishiuchi, Y.; Kimura, T.i; Sakakibara, S.; Albericio, F.; Giralt, E. *J. Am. Chem. Soc.* **2003**, *125*, 1508−1517.
(23) Bray, Brian L. *Natl. Rev. Drug Discovery* **2003**, *2*, 587−593.
(24) (a) Barlos, K.; Gatos, D.; Schäfer, W. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 590−593. (b) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513−520.

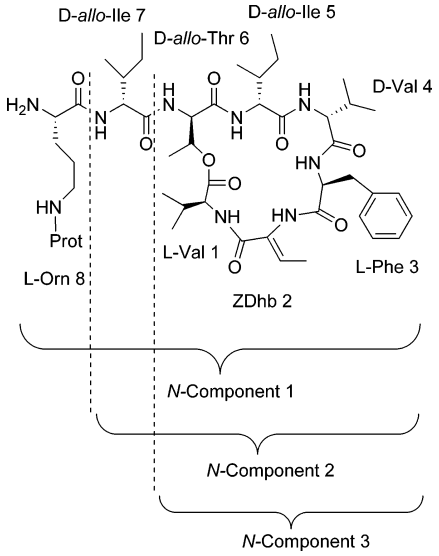
SCHEME 2. *C*-Components Synthesized

C-Component 1: MeHex–D-Val–L-Thr(*t*Bu)–L-Val–D-Val–D-Pro–OH (13 12 11 10 9)

C-Component 2: MeHex–D-Val–L-Thr(*t*Bu)–L-Val–D-Val–D-Pro–L-Orn(Boc)–OH (13 12 11 10 9 8)

C-Component 3: MeHex–D-Val–L-Thr(*t*Bu)–L-Val–D-Val–D-Pro–L-Orn(Boc)–D-*allo*-Ile–OH (13 12 11 10 9 8 7)

SCHEME 3. *N*-Components Synthesized



A priori, the main advantage of strategy 1 is that the hardly racemizable Pro is the *C*-terminal amino acid for the *C*-Component. In contrast, the advantage of strategies 2 and 3 is that the *N*-Component does not contain any side-chain protecting group and therefore a level of protecting groups can be avoided for these fragments.

All fragments were synthesized on the CTC resin, starting with a limited incorporation of the first 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid in the presence of *N*,*N*-diisopropylethylamine (DIEA).[27] The remaining resin chloride functions were capped with methanol (MeOH) to prevent the formation of tertiary amines after treatment with the piperidine used to remove the Fmoc group.[28] The Fmoc-protecting group was removed with piperidine−*N*,*N*-dimethylformamide (DMF) (1:4) and the peptide chain was elongated with *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. Finally, the peptides were cleaved from the resin with TFA−CH_2Cl_2 (1:99), characterized by HPLC and ESMS, and purified by semipreparative HPLC when necessary.

Synthesis of the *N*-Components. The ZDhb moiety was incorporated through the dipeptide allyloxycarbonyl (Alloc)-Phe-ZDhb-OH, wich is much faster than incorporating sequentially Thr and Phe and performing the dehydratation of Thr to ZDhb on solid phase.[8,11]

Four synthetic strategies were used to prepare the three distinct *N*-Components (Scheme 4). All strategies are based on the solid-phase synthesis of a branched peptide using a tri- or tetra-orthogonal protecting scheme and subsequent cyclization and deprotection of the *N*-terminal function in solution. Strategy 1, which produces the *N*-Component 1, follows the procedure previously described[8,11] and starts with the incorporation of Fmoc-D-Val-OH on the CTC resin, followed by elongation until the D-*allo*-Ile,[7] esterification of the β-hydroxyl of the D-*allo*-Thr with Alloc-Val-OH. The incorporation of protected Orn-(Boc) residue on the main chain is then followed by the incorporation of the Alloc-Phe-ZDhb-OH on the branched one. Strategy 3, which leads to the *N*-Component 2, is similar to 1, but elongation is stopped after incorporation of D-*allo*-Ile.[7] In Strategies 2 and 4, which yield *N*-Component 1 and *N*-Component 3, respectively, synthesis starts with the incorporation of the Alloc-Phe-ZDhb-OH onto the CTC resin (see below for the discussion of the strategy).

This study addressed (i) the most convenient strategy in terms of coupling yield and racemization of the *C*-terminal amino acid of the *C*-Component (Strategies 1 or 2 vs 3 and 4); (ii) for Strategies 1 and 2 (*N*-Component 1), the best semi-permanent protecting group [Fmoc, *p*-nitrobenzyloxycarbonyl (*p*NZ)[11,29,30] in Strategy 1 and Alloc in Strategy 2][31] for the N^{α}-amino of Orn, taking into account that the *tert*-butoxycarbonyl (Boc) group is reserved as permanent protecting group for the N^{ϵ}-amino of the same Orn; and (iii) whether the dipeptide Alloc-Phe-ZDhb-OH is a good starting point for the elongation of the peptidic chain (Strategies 2 and 4) when compared with the traditional Fmoc-D-Val-OH.[4]

As protecting group for the N^{α}-amino of Orn, Fmoc, Alloc, and the recently described *p*NZ[11,29,30] were examined (Scheme 4, Strategies 1 and 2) as well as Boc (Scheme 4, Strategies 3 and 4). Alloc was removed by Pd(0) in rather neutral conditions in the presence of a scavenger of the allyl carbocations.[32] *p*NZ is orthogonal to the most common solid-phase peptide synthesis (SPPS) protecting groups such as *tert*-butyl (*t*Bu)/Boc, Fmoc, and Alloc. It was removed under simple and practical neutral conditions by $SnCl_2$ in the presence of catalytic amounts of acid.[29] When the Fmoc group (Strategy 1) was removed under the classical conditions piperidine−DMF (1:4), the major product was the linear peptide, which formed by aminolysis of the ester bond.[33] However, the use of diethylamine (DEA) (30 equiv of DEA for 90 min at 25 °C) minimized the undesired side reaction. In this case, although no opening of the cycle was detected, some starting product (around 5%) remained in

(25) Rovero, P.; Vigano, S.; Pegoraro, S.; Quartara, L. *Lett. Pept. Sci.* **1996**, *2*, 319−323.

(26) Chiva, C.; Vilaseca, M.; Giralt, E,; Albericio, F. *J. Pept. Sci.* **1999**, *5*, 131−140.

(27) If the loading is greater than 1 mmol/g, as most of the commercial available resins of this kind are, the analysis by HPLC-ESMS of the crude product shows additional peaks, corresponding to terminated sequences (see ref 26).

(28) Garcia-Martin, F.; Bayo, N.; Cruz, L. J.; Tulla, J.; Bohling, J.; Albericio, F. Manuscript in preparation.

(29) Isidro-Llobet, A.; Guasch-Camell, J.; Alvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, 3031−3039.

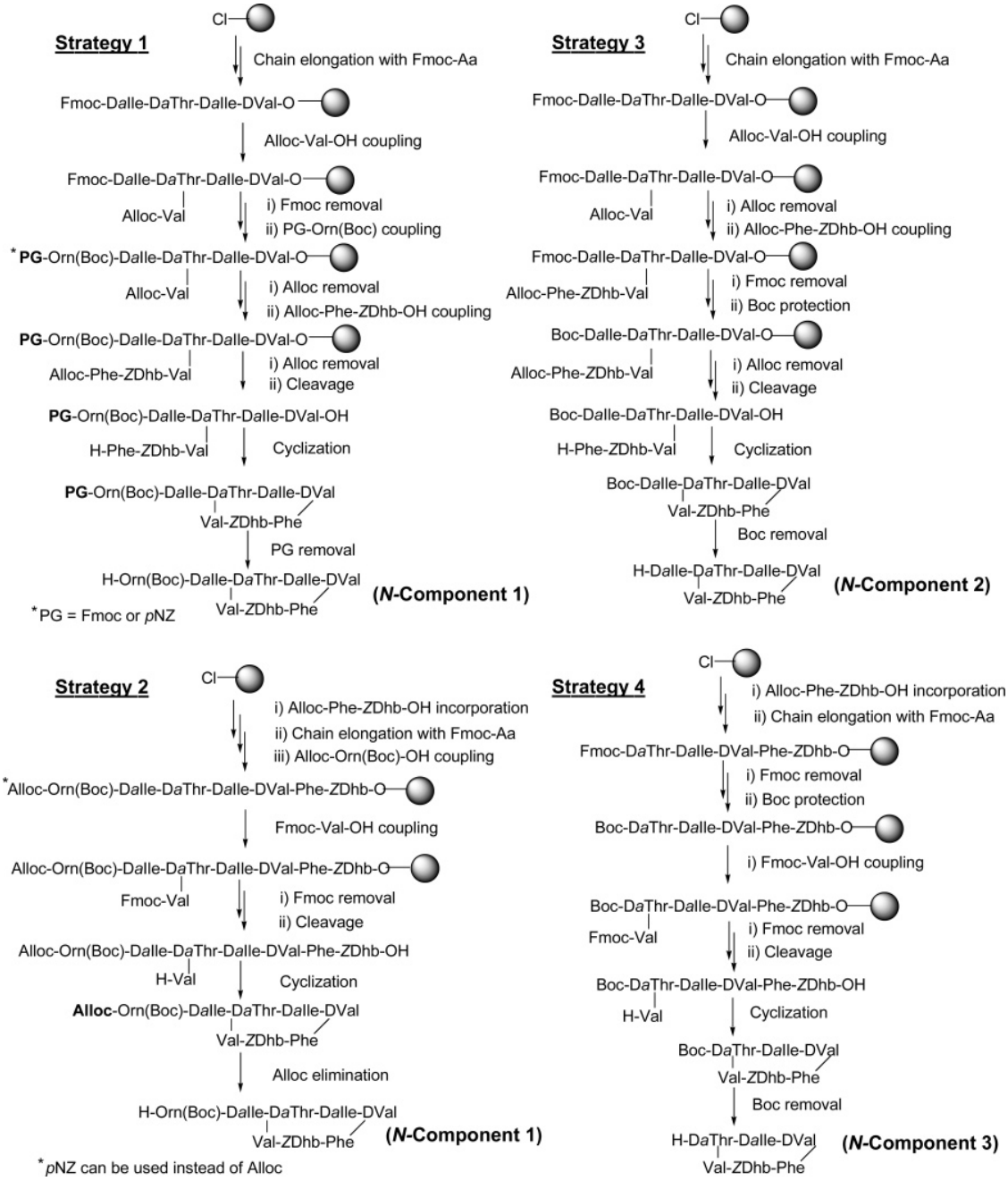
(30) Isidro-Llobet, A.; Alvarez, M.; Albericio, F. *Tetrahedron Lett.* **2005**, *46*, 7733−7736.

(31) The synthesis of peptides by convergent approaches and/or the cyclic peptides require the use of semi-permanent protecting groups, which must be stable to the conditions used to remove the temporal protecting group, which are those that are removed after each synthetic cycle and should be removed without affecting any permanent protecting groups, which removed in the final step of the synthetic process: Albericio, F. *Biopolymers* **2000**, *55*, 123−139.

(32) Guibe, F. *Tetrahedron* **1997**, *53* (40), 13509−13556; **1998**, *54*, 2967−3042.

(33) It is interesting to point out that the ester bond between D-*allo*-Thr[6] and L-Val[1] is perfectly stable to the repetitive piperidine treatments during the stepwise elongation of the peptidic chain where the cycle is still not formed, the same ester bond is unstable to just one piperidine treatment once the cycle is already formed. This is a illustrative example of that the rigidity conferred by a cycle can change the physchem properties of a molecule.

SCHEME 4. Strategies for the Syntheses of the Cyclic Fragment



the reaction crude. (The starting peptide contains a Fmoc group, which makes a difference in the ϵ of both compounds and therefore makes the yield calculation difficult.) In the same strategy, the use of *p*NZ-Orn(Boc)-OH instead of Fmoc-Orn-(Boc)-OH was examined. The ester bond was stable to the $SnCl_2$ treatment used to remove *p*NZ.

Furthermore, the same stability was achieved when the Alloc group (Scheme 4, Strategy 2) was removed with Pd(0) in the presence of triethylsilane (TES). To use Alloc as a semi-permanent protecting group, the classical strategy 1 used for the synthesis of Kahalalide F could not be followed because it would have been necessary to remove Alloc as N^{α}-protecting group of the Phe in the presence of the semi-permanent protecting group of the Orn (Scheme 4, Strategy 1). Thus, a new strategy to circumvent this problem was assayed. This strategy began with the incorporation of Alloc-Phe-ZDhb-OH as the first building block to the CTC resin using the same conditions required for the introduction of protected amino acids (Scheme 4, Strategy 2). The peptide chain was elongated using Fmoc for the α-amino protection of D-Val, D-*allo*-Ile, D-*allo*-Thr, and D-*allo*-Ile, and Alloc for the Orn(Boc). (In this strategy the Orn(Boc) could be also introduced as *p*NZ-Orn(Boc)-OH.) This new strategy worked well in terms of cyclization yield and in addition showed two key advantages. Due to the defect of the first protected building block required for the incorporation onto the CTC resin, a smaller amount of the precious Alloc-Phe-ZDhb-OH was required compared with Strategy 1, in which the dipeptide was introduced in excess. Second and more importantly, ZDhb does not show chirality and therefore no

TABLE 1. Screening of *C*- and *N*-Components Coupling Methods

conditions	% of final product in the HPLC
DIPCDI/HOAt (1.5 equiv, 1.5 equiv)	47% (t = 48 h)
EDC/HOBt/DMAP (4 equiv, 4 equiv, 0.4 equiv)	16% (t = 6 h); 23% (t = 20 h)
PyAOP/DIEA (1 equiv, 3 equiv) + 1 equiv PyAOP at t = h	35% (t = 30 min); 54% (t = overnight)
HATU/DIEA (1 equiv, 3 equiv) + 1 equiv PyAOP at t = 3 h	43% (t = 30 min); 49% (t =overnight)
EDC/HOBt (3 equiv, 3 equiv)	5.8% (t = 30 min); 53% (t = overnight)

epimerization occurred during the cyclization step, which is the most prone for inducing racemization.

In Strategies 3 and 4, and as the *N*-Components 2 and 3 did not contain any side-chain protecting group, the Boc group was used as N^{α}-amino protecting group of the *N*-terminal residue (D-*allo*-Ile[7] in Strategy 3 and D-*allo*-Thr in Strategy 4). In addition to the full stability of the lactone bond to the conditions (TFA) used to remove that protecting group, the main advantages associated with the use of the Boc group were that the reagent to remove this group as well as the side products formed were volatile and therefore could be removed by simple evaporation.

Condensation of Linear and Cyclic Fragments. First of all, and using *C*- and *N*-Components as models, we screened the coupling methods [DIPCDI/7-aza-1-hydroxybenzotriazole (HOAt) (1.5 equiv each); 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/HOBt (3 equiv each); EDC/HOBt/*N*,*N*-(dimethylamino)pyridine (DMAP) (4 equiv, 4 equiv, 0.4 equiv); (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/DIEA (1 equiv, 3 equiv) + PyAOP (1 equiv after 1 h), and 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU)/DIEA (1 equiv, 3 equiv)]. On the basis of this screening, we concluded that the method based on PyAOP gave the best results in terms of yields and the absence of side products in the HPLC chromatograms (Table 1). Phosphonium derivatives such as PyAOP are more convenient for slow couplings compared with aminium/uronium reagents such as HATU, because the latter can terminate the peptide chain through a guanidination reaction.[34] Furthermore, PyAOP contains HOAt, which is the most reactive benzotriazole.[35]

Each linear *C*-Component was then condensed with its corresponding *N*-Component in solution phase using PyAOP and DIEA. The three protected peptides obtained were treated with TFA−H_2O (9:1) to remove the Boc and *t*Bu groups and yielded Kahalalide F.

A range of results were obtained depending on which *C*- and *N*-terminal amino acids of the *C*- and *N*-Components, respectively, were involved. From the point of view of the purity of the final product, the strategies that involved the concourse of *C*- and *N*-Components 1 and 2 were superior to that corresponding to fragments 3. This can be explained because the latter involves coupling between two hindered β-branched amino acids (D-*allo*-Ile and D-*allo*-Thr). Furthermore, D-*allo*-Thr is part of the cycle, which may also impair the coupling of the protected peptide. The *C*- and *N*-Component 1 strategy also gave slightly superior results.

Furthermore, when the *C*-terminal amino acids of the *C*-Component were Orn [*C*-Component 2 and *N*-Component 2 (Strategy 3, Scheme 4)] or D-*allo*-Ile [*C*-Component 3 and

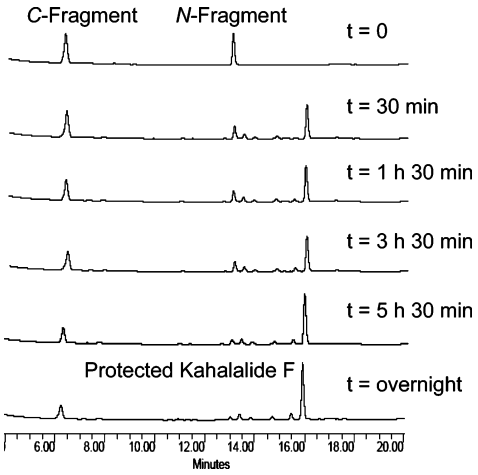


FIGURE 2. HPLC analysis of the solution coupling between *C*- and *N*-Components 1 over time. Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

N-Component 3 (Strategy 4, Scheme 4)], epimerization of these amino acids was observed [4% for the case of Orn (Figure 3) and more than 10% for D-*allo*-Ile (data no shown)].[36] However, when the *C*-terminal amino acid of the linear fragment was D-Pro [*C*- and *N*-Components 1 (Strategy 1 or 2, Scheme 4)], epimerisation was not observed (Figure 4). These results were obtained by comparing the HPLC crudes of the Kahalalide F compounds synthesized by the strategies and the three epimers, which were also synthesized by a stepwise strategy (substitution of D-Pro for L-Pro, of L-Orn for D-Orn, and of D-*allo*-Ile for Ile). Our observations confirm that, as a result of its cyclic secondary amino function, Pro is less prone to racemize during fragment coupling.

Conclusions

Here we developed several convergent strategies for the synthesis of the analogues of the antitumor peptide Kahalalide F. The best approaches were those in which the *C*-terminal amino acid of the *C*-Component is D-Pro because it avoids epimerization during the coupling of the fragment in solution. The choice of the semi-permanent protecting group for the N^{α}-amino of Orn-protecting groups requires additional discussion. Thus, the use of the less common groups, *p*NZ, and Alloc, which ensure total stability of the lactone, implies the concourse of metallic cations or metals, which may demand a purification step before the final fragment coupling. Furthermore, the Fmoc group requires fine-tuning of the experimental process, which can be detrimental for the development of a large-scale process in which conditions may be more difficult to control. The use of the *N*-Component prepared by Strategy 2 (Scheme 4) shows

(34) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678−9683.

(35) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, 201−203.

(36) These epimers did not show any remarkable biological activity in the typical cell lines used for evaluation the antitumorals activity of Kahalalide F and its analogues.

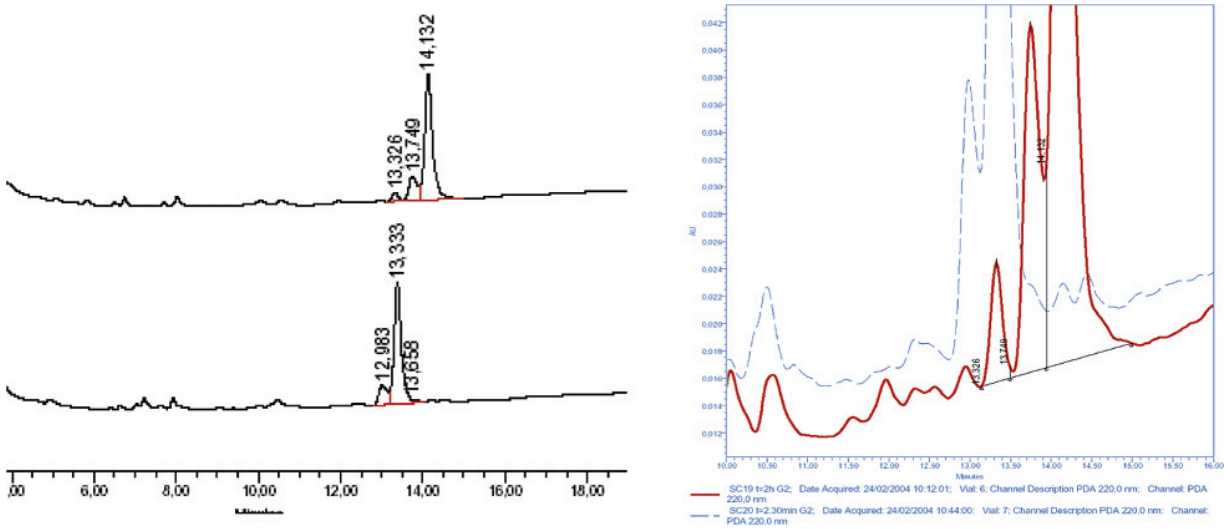


FIGURE 3. HPLC analysis of the Boc-protected Kahalalide F prepared from *C*- and *N*-Components 2 (up and red) compared with the (DOrn^{8})-Kahalalide F (down and blue). Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

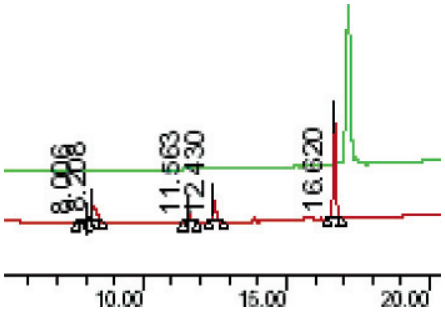


FIGURE 4. HPLC analysis of the Boc-protected Kahalalide F prepared from *C*- and *N*-Components 1 (green) compared with the (LPro^{9})-Kahalalide F (red). Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

several advantages because it allows the preparation of Kahalalide F without risk of epimerization during cyclization and uses lower amounts of the precious Alloc-Phe-*Z*Dhb-OH.

Experimental Section

General Procedures. Cl-TrtCl-resin, protected Fmoc-amino acid derivatives, HOBt, and HOAt were purchased from different sources as well as DIPEA, DIPCDI, EDC·HCl, piperidine, TFA, DMF, MeCN (HPLC grade), and CH_2Cl_2. All commercial reagents and solvents were used as received with the exception of DCM, which was passed through an alumina column to remove acidic contaminants.

*p*NZ-Orn(Boc)-OH was prepared as described previously[29] and Alloc-amino acids as essentially described by Dangles et al. and Cruz et al.[37,38] Alloc-*Z*Dhb-Phe-OH was prepared as before.[39]

Solid-phase syntheses were performed in polypropylene syringes (10−50 mL), each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine−DMF (2:8, v/v) (1 × 2 min, 2 × 10 min). Washings between deprotection, coupling, and again, deprotection steps were performed with DMF (5 × 0.5 min) and CH_2Cl_2 (5 × 0.5 min) using 10 mL solvent/g resin each time. Peptide synthesis transformations and washings were done at 25 °C. Syntheses carried out on solid phase were controlled by HPLC of the intermediates obtained after cleaving an aliquot (approximately 2 mg) of the peptidyl-resin with TFA−H_2O (1:99) for 1 min. HPLC reversed-phase columns Symmetry C_{18} 4.6 mm × 150 mm, 5 μm (column A) and Symmetry300 C_{18} 4.6 mm × 50 mm, 5 μm (column B) were used. Analytical HPLC was performed on an instrument comprising two solvent delivery pumps, automatic injector dual wavelength detector, and system controller (Breeze V3.20) and on an instrument comprising two solvent delivery pumps, automatic injector, and a variable wavelength detector (photodiode array). UV detection was at 215 or 220 nm, using linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

MALDI-TOF and ES-MS analysis of peptide samples were performed using ACH matrix. Peptide-resin samples for amino acid analysis were hydrolyzed in 12 N aqueous HCl−propionic acid (1:1), at 155 °C for 1−3 h, and peptide-free samples were hydrolyzed in 6 N aqueous HCl at 155 °C for 1 h.

The "&" symbol is used in the nomenclature for cyclic peptides and precursors.[40] The appearance of "&" in a given position of the one-line formula indicates the location of one end of a chemical bond and the second "&" the point to which this bond is attached. Thus, "&" represents the start or the end of a chemical bond, which is "cut" with the aim to facilitate the view of a complex formula. In this way, two "&" symbols indicate one chemical bond.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from Fmoc-Orn(Boc)-OH.**

***H*-D-Val-*O*-TrtCl-resin (1).** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), and a solution of Fmoc-D-Val-OH (238 mg, 0.7 mmol, 0.43 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added. The mixture was then stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was stopped by adding MeOH (800 μL) and stirring for 10 min. The Fmoc-D-Val-O-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), piperidine as indicated in General

(37) Dangles, O.; Guibe, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J. Org. Chem.* **1987**, *52*, 4984−4993.

(38) Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. *Org. Process Res. Dev.* **2004**, *8*, 920−924.

(39) Albericio, F.; Giralt, E.; Jimenez, J. C.; Lopez, A.; Manzanares, I.; Rodrigues, I.; Royo, M. PCT Int. Appl. (2001) WO 2001058934, A2 20010816, CAN 135:167039, AN 2001:598019.

(40) Spengler, J.; Jimenez, J. C.; Burger, K.; Giralt, E.; Albericio, F. *J. Pept. Res.* **2005**, *65*, 550−555.

Procedures, and DMF (5 × 0.5 min). The loading was 0.50 mmol/g, as calculated by Fmoc determination.

[Fmoc-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (2). Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Thr-OH (free hydroxy group) (683 mg, 2 *mmol*, 4 equiv), and Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv) were added sequentially to the above obtained *H*-D-Val-O-TrtCl-resin (**1**) using DIPCDI (310 μL, 2 mmol, 4 equiv) and HOBt (307 mg, 2 mmol, 4 equiv) in DMF (2.5 mL). In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures. Alloc-Val-OH (502 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 mg, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated twice in the same conditions. An aliquot of the peptidyl-resin was treated with TFA and the HPLC (R_t 14.2 min, column A) of the crude obtained after evaporation showed a purity of >98%. ESMS calcd for $C_{45}H_{63}N_5O_{11}$ 849.45; found *m*/*z* 850.1 $[M + H]^+$, 871.9 $[M + Na]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (3). The Fmoc group of the peptide resin (**2**) was removed, and Fmoc-Orn(Boc)-OH (912 mg, 2 mmol, 4 equiv) was added using DIPCDI (310 μL, for 2.0 mmol and 4 equiv; and 388 μL, for 2.5 mmol and 5 equiv) and HOBt (307 mg, for 2.0 mmol and 4 equiv; and 395 mg, for 2.5 mmol and 5 equiv) for 90 min. Ninhydrin test after the incorporation was negative. An aliquot of the peptidyl-resin was treated with TFA and the HPLC (R_t 12.8 min, column A) of the crude obtained after evaporation showed a purity of 90%. ESMS calcd for $C_{56}H_{81}N_7O_{14}$ 1,063.58; found *m*/*z* 1,086.77 $[M + Na^+]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Phe-ZDhb-Val&] (4). The Alloc group of the peptide resin (**3**) was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), followed by washings with diethyldithiocarbamate 0.02 M (3 × 15 min). Alloc-Phe-*Z*Dhb-OH (666 mg, 2 mmol, 4 equiv) and HOAt (273 mg, 2 mmol, 4 equiv) were dissolved in DMF (1.25 mL) and added to peptidyl-resin. DIPCDI (310 μL, 2 mmol, 4 equiv) was then added, and the mixture was stirred for 5 h. The ninhydrin test was negative. After washings with DMF and CH_2Cl_2, an aliquot of the peptidyl-resin was treated with TFA−H_2O (1:99) for 1 min, and the product was characterized by MALDI-TOF-MS: calcd for $C_{68}H_{95}N_9O_{16}$ 1,293.69; found *m*/*z* 1,294.35 $[M + H]^+$, 1,316.39 $[M + Na]^+$, 1,333.34 $[M + K]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-OH][*H*-Phe-ZDhb-Val&] (5). The Alloc group of the peptide resin (**4**) was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), the resin was washed with sodium diethyldithiocarbamate in DMF 0.02 M (3 × 15 min), and the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). The filtrate was collected on H_2O (4 mL) and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve solid that formed during the removal of H_2O, and the solution was lyophilized to give 700 mg of title compound (578 μmol, 99% yield) of the title compound with a purity of > 91% as checked by HPLC (Column A, R_t 8.59 min)], which was used without further purification. MALDI-TOF-MS calcd for $C_{64}H_{91}N_9O_{14}$ 1,209.67; found *m*/*z* 1,210.45 $[M + H]^+$, 1,232.51 $[M + Na]^+$, 1,248.45 $[M + K]^+$.

Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (6). The protected peptide (**5**) was dissolved in CH_2Cl_2 (580 mL, 1 mM), and HOBt (137 mg, 2.3 mmol) dissolved in the minimum volume of DMF to dissolve HOBt, DIPEA (302 μL, 1.73 mmol, 3 equiv), and DIPCDI (356 μL, 2.3 mmol, 4 equiv) were added. The mixture was stirred for 1 h, and the course of the cyclization step was then checked by HPLC (column A, R_t 12.4 min). The solvent was removed by evaporation under reduced pressure and the product was used without further purification. MALDI-TOF-MS calcd for $C_{64}H_{89}N_9O_{13}$ 1,191.66; found *m*/*z* 1,092.17 $[M + H]^+$, 1,214.14 $[M + Na]^+$, 1,230.10 $[M + K]^+$.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** The protected peptide (**6**) (50 mg, 42 μmol) was dissolved in DMF (5 mL), then DEA (130 μL, 30 equiv) was added and the mixture was stirred for 90 min. The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 × 100 mm), linear gradient of MeCN (30% to 75% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm. The product was characterized by HPLC (R_t 8.7 min, Condition A) and MALDI-TOF-MS calcd for $C_{49}H_{79}N_9O_{11}$ 969.59; found *m*/*z* 970.87 $[M + H]^+$, 870.78 $[M - Boc]^+$.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from pNZ-Orn(Boc)-OH.**

pNZ-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (7). The synthesis was performed in the same way as for compound **6**, but Fmoc-Orn(Boc)-OH was replaced by *p*NZ-Orn(Boc)-OH.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** The protected peptide (14.7 mg, 12.8 μmol) was dissolved in 1.6 mM HCl in DMF (10 mL), $SnCl_2$ (3.8 g, 20 mmol) was then added, and the mixture was stirred until HPLC (Column A) showed the completion of the reaction (1 h). The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), gradient of MeCN (30% to 75% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (4.8 mg, 4.9 μmol, 40% yield). The product was characterized by HPLC (R_t 8.2 min, Column A) and by MALDI-TOF-MS: calcd for $C_{49}H_{79}N_9O_{11}$ 969.59; found *m*/*z* 992.35 $[M + Na]^+$, 870.34 $[M - Boc]^+$, 892.34 $[M + Na - Boc]^+$.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from Alloc-Orn(Boc)-OH**

***H*-Phe-ZDhb-*O*-TrtCl-resin (8).** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), and a solution of Alloc-Phe-*Z*Dhb-OH (232 mg, 0.7 mmol, 0.42 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added. This mixture was then stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was arrested by adding MeOH (800 μL), after stirring for 10 min. The Alloc-Phe-*Z*Dhb-*O*-TrtCl-resin was subjected to washings with CH_2Cl_2 (3 × 0.5 min) and DMF (3 × 0.5 min), and the Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv) in CH_2Cl_2. The resin was washed as described in General Procedures. The loading was 0.68 mmol/g, as calculated by Fmoc determination.

[Alloc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-OH][*H*-Val&] (9). Fmoc-D-Val-OH (678 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Thr-OH (free hydroxy group) (683 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), and Alloc-Orn-(Boc)-OH (630 mg, 2 mmol, 4 equiv) were added sequentially to the above obtained *H*-Phe-*Z*Dhb-*O*-TrtCl-resin (**8**) using DIPCDI (310 μL, 2 mmol, 4 equiv) and HOBt (307 mg, 2 mmol, 4 equiv) in DMF (2.5 mL). In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures. Fmoc-Val-OH (848.2 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 mg, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated twice in the same conditions. After removal of the Fmoc group as described in General Procedures, the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL), and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that formed during H_2O removal,

and the solution was lyophilized to give 650 mg of target compound (606 μmol, 90% yield) of the title compound with a purity of >75% as checked by HPLC (Column A, R_t 9.93 min). ESMS calcd for $C_{53}H_{85}N_9O_{14}$ 1072.29; found *m*/*z* 1074.4 $[M + H]^+$.

Alloc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (10). Peptide **9** (250 mg, 233 μmol) was dissolved in CH_2Cl_2 (240 mL, 1 mM), and HOAt (126 mg, 9.325 mmol, 4 equiv) in the minimum volume of DMF, and DIPCDI (143 μL, 9,325 mmol, 4 equiv) were added. The mixture was stirred for 24 h, and the course of the cyclization step was then checked by HPLC (column A, R_t 12.82 min). The solvent was removed by evaporation under reduced pressure, and the product was used without further purification. MALDI-TOF-MS calcd for $C_{53}H_{83}N_9O_{13}$ 1,054.28; found *m*/*z* 1,056.4 $[M + H]^+$.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** Peptide **10** was dissolved in 10 mL of CH_2Cl_2, then $Pd(PPh_3)_4$ (8 mg, 6,94 μmol, 0.03 equiv) in the presence of $PhSiH_3$ (94 μL, 763,6 μmol, 3.3 equiv) was added, and the mixture was stirred for 90 min. The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), linear gradient of MeCN (20% to 80% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm. The product was characterized by HPLC (R_t 9.19 min, Condition A) and by MALDI-TOF-MS: calcd for $C_{49}H_{79}N_9O_{11}$ 970.21; found *m*/*z* 972.1 $[M + H]^+$.

***C*-Component 1: MeHex-D-Val-Thr(tBu)-Val-D-Val-D-Pro-OH.** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), a solution of Fmoc-D-Pro-OH (237 mg, 0.7 mmol, 0.43 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added, and the mixture was stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was arrested by stirring the resin in MeOH (800 μL) for 10 min. The Fmoc-D-Pro-*O*-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), piperidine as indicated in General Procedures, and DMF (5 × 0.5 min). The loading was 0.27 mmol/g, as calculated by Fmoc determination.

Fmoc-D-Val-OH (458 mg, 1.32 mmol, 5 equiv), Fmoc-Val-OH (360 mg, 1.06 mmol, 4 equiv), Fmoc-Thr(*t*Bu)-OH (527 mg, 1.32 mmol, 5 equiv), Fmoc-D-Val-OH (360 mg, 1.06 mmol, 4 equiv), and MeHex-OH (138 mg, 1.06 mmol, 4 equiv) were added sequentially to the above peptide resin using DIPCDI (165 μL, for 1.06 mmol and 4 equiv; and 205 μL, for 1.32 mmol and 5 equiv) and HOBt (162 mg, for 1.06 mmol and 4 equiv; and 203 mg, for 1.32 mmol and 5 equiv) for 90 min. In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures.

The partially protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL) and H_2O was partially removed in a rotavapor. MeCN was then added to dissolve the solid that formed during H_2O removal, and the solution was lyophilized to give 154.4 mg (226 μmol, 86% yield) of the title compound with a purity of >94% as checked by HPLC (Column A, R_t 12.13 min). The crude obtained after evaporation showed a purity of >94%. The product was characterized by ES-MS: calcd for $C_{35}H_{63}N_5O_8$, 681.9; found *m*/*z* 682.15.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from *C*- and *N*-Components 1. *N*-Component 1 (8.25 mg, 8.5 μmol) and *C*-Component 1 (7 mg, 10.2 μmol, 1.2 equiv) were dissolved in DMF (10 mL), and PyAOP (5.32 mg, 10.2 μmol, 1.2 equiv) and DIPEA (5.3 μL, 30.6 μmol, 3.6 equiv) were added at room temperature. The mixture was stirred for 1 h, when extra PyAOP (5.32 mg, 10.2 μmol, 1.2 equiv) was added. The mixture was allowed to react for 2 h at room temperature until HPLC (Column A) showed completion of the reaction. HPLC showed that the crude obtained after evaporation had a purity of >75%.

The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), linear gradient of MeCN (+0.05% TFA) in water (+0.05% TFA) (30% to 100% in 15 min), 20 mL/h, detection at 220 nm, to give the protected Kahalalide F (6.9 mg, 4.2 μmol, 49% yield). MALDI-TOF-MS calcd for $C_{84}H_{140}N_{14}O_{18}$ 1,633.05; found *m*/*z* 1,534.33 $[M - Boc]^+$,1,556.26 $[M - Boc + Na]^+$ 1,656.33 $[M + Na]^+$.

Protected Kahalalide F was dissolved in TFA−H_2O (19:1, 700 μL), and the mixture was stirred for 1 h. The solvent was removed by evaporation under reduced pressure, and dioxane was added (245 μL). Again, the solvent was removed by evaporation under reduced pressure (the process was repeated three times), H_2O (1 mL) was then added, and the solution was lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), isocratic 44% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (5 mg, 3.4 μmol, 80% yield, 93.3%).

The HPLC of the crude product did not show the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-*Pro*-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& (Epimer 1). This observation indicates racemization during the coupling step between the two protected peptides. MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$ 1,476.93; found *m*/*z* 1,478.17 $[M + H]^+$ 1,500.14 $[M + Na]^+$, 1,516.12 $[M + K]^+$.

Epimer 1: MeHex-D-Val-Thr-Val-D-Val-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&. Experimental procedures were as described for the stepwise synthesis of Kahalalide F, except that Fmoc-D-Pro-OH was replaced by Fmoc-Pro-OH.[8] The product was characterized by HPLC (R_t 11.23 min, Column A) and by MALDI-TOF-MS: calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,500.23 $[M + Na]^+$, 1,515.97 $[M + K]^+$.

***N*-Component 2: *H*-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&.** Starting with [Fmoc-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (**2**), the Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), and the resin was washed with sodium diethyldithiocarbamate in DMF 0.02 M (3 × 15 min). Alloc-Phe-*Z*Dhb-OH (666 mg, 2 mmol, 4 equiv) and HOAt (273 mg, 2 mmol, 4 equiv) were dissolved in DMF (1.25 mL) and added to peptidyl-resin. DIPCDI (310 μL, 2 mmol, 4 equiv) was then added, and the mixture was stirred for 5 h; at which point the HPLC showed completion of reaction (R_t 7.09 min, Column A).

The Fmoc group was removed and after extensive DMF washings, Boc_2O (546 mg, 5 equiv) and DIPEA (0.87 mL, 10 equiv) in DMF were added and left to react for 2 h. The ninhydrin test was negative at this time point. (Alternatively, Boc-D-*allo*-Ile-OH can be introduced instead of the Fmoc derivative.) After DMF washings, the Alloc group was removed, and the protected peptide was cleaved from the resin with TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL) and H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that formed during H_2O removal, and the solution was then lyophilized.

Cyclization was performed as for compound **6**, and Boc was then removed with TFA−H_2O (19:1) (1 h). The solvent was removed under reduced pressure, and dioxane was added (245 μL). Again, the solvent was removed by evaporation under reduced pressure (the process was repeated three times), and then H_2O (1 mL) was added and lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 × 100 mm), isocratic 44% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (207 mg, 273 μmol, 55% yield, 93.3%). The product was characterized by HPLC (R_t 7.27 min, Column A) and by MALDI-TOF-MS: calcd $C_{39}H_{61}N_7O_8$, 755.46; found *m*/*z* 756.56 $[M + H]^+$, 778.55 $[M + Na]^+$, 794.53 $[M + K]^+$.

***C*-Component 2: MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-OH.** Experimental procedures as for *C*-Component 1, except that the peptide synthesis was initiated by incorporation of Fmoc-Orn(Boc)-OH to the Cl-TrtCl-resin. The product was characterized by HPLC (R_t 13.27 min, Column A) and by Electrospray: calcd $C_{45}H_{81}N_7O_{11}$, 895,60; found *m*/*z* 895,10.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from *C*- and *N*-Components 2. The synthesis was performed as described above except that the condensed fragments were *N*-Component 2 and *C*-Component 2. The HPLC of the crude final product showed the presence of the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-D-Pro-D-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (4.1%) (Epimer 2), which indicates racemization during the coupling step between both protected peptides. The product was characterized by HPLC (R_t 10.5 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,477.99 $[M + H]^+$ 1,499.97 $[M + Na]^+$, 1,515.93 $[M + K]^+$.

Epimer 2: MeHex-D-Val-Thr-Val-D-Val-D-Pro-D-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&. The synthesis was carried out as described for the stepwise synthesis of Kahalide except that Fmoc-Orn(Boc)-OH is replaced by Fmoc-D-Orn(Boc)-OH.[8] The product was characterized by HPLC (R_t 9.89 min, Column A). MALDI-TOF-MS calcd $C_{75}H_{124}N_{14}O_{16}$ 1,476.93; found *m*/*z* 1,478.06 $[M + H]^+$ 1,500.15 $[M + Na]^+$, 1,516.04 $[M + K]^+$.

***N*-Component 3: *H*-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&.** Starting with Fmoc-D-*allo*-Thr-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-*O*-TrtCl-resin, the Fmoc group was removed, and after extensive DMF washings, Boc_2O (546 mg, 5 equiv) and DIPEA (0.87 mL, 10 equiv) in DMF were added and left to react for 2 h, after which the ninhydrin test was negative. After DMF washings, Fmoc-Val-OH (848.2 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 μL, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated in the same conditions twice. After removal of the Fmoc group as described in General Procedures, the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL), and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that appeared during the H_2O removal, and the solution was lyophilized, to give 57 mg (75 μmol, 90% yield) of the title compound with a purity of >95%, as checked by HPLC (Column A, R_t 7.95 min). ESMS calcd for $C_{38}H_{60}N_6O_{10}$ 760.44; found *m*/*z* 762.3 $[M + H]^+$.

Cyclization was carried out as for compound **6**, and the Boc was then removed with TFA−H_2O (19:1) (1 h). The solvent was removed under reduced pressure, and dioxane was added (245 μL). The solvent was removed by evaporation under reduced pressure (the process was repeated three times), and then H_2O (1 mL) was added and lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), isocratic 30% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (50.2 mg, 67.6 μmol, 90% yield).

The product was characterized by HPLC (R_t 5.32 min, Column A) and by ES-MS: calcd $C_{33}H_{61}N_6O_9$ 642.37; found *m*/*z* 642.035.

***C*-Component 3: MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-OH.** Experimental procedures as for the obtention of *C*-Component 1, except that the peptide synthesis was initiated by incorporation of Fmoc-D-*allo*-Ile-OH to the Cl-TrtCl-resin. The product was characterized by HPLC (R_t 10.25 min, Column A) and by MALDI-TOF-MS: calcd for $C_{51}H_{92}N_8O_{12}$ 1,008.68; found *m*/*z* 1,009.8.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from *C*- and *N*-Components 3. Experimental procedures were as described for the other syntheses of Kahalalide F. The HPLC of the final product showed the presence of the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-Ile-D-*allo*-Thr(&)-D-*allo*Ile-D-Val-Phe-*Z*Dhb-Val& (Epimer 3), which indicates racemization during the coupling step between the two protected peptides. The product was characterized by HPLC (R_t 7.92 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,478.5 $[M + H]^+$ 1,501.4 $[M + Na]^+$, 1,517.6 $[M + K]^+$.

Epimer 3: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&. Experimental procedures were as described for the stepwise synthesis of Kahalaide F, except that Fmoc-Ile-OH was used instead of Fmoc-D-*allo*-Ile-OH.[8] MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-Ile-OH: (R_t 10.25 min, Column A and MALDI-TOF-MS calcd for $C_{51}H_{92}N_8O_{12}$ 1,008.68; found *m*/*z* 1,009.5). The final product was characterized by HPLC (R_t 8.02 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,478.2 $[M + H]^+$ 1,501.1 $[M + Na]^+$, 1,517.3 $[M + K]^+$.

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Supporting Information Available: HR-MS and ^{1}H NMR characterization of *C*- and *N*-Components 1, 2, and 3, as well as ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Convergent Approaches for the Synthesis of the Anti-tumoral Peptide, Kahalalide F. Study of Orthogonal Protecting Groups

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Convergent Synthesis of Kahalalide F

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Contents:

***C*-Component 1:** ^{1}H NMR (400 MHz): 8.18 (d, *J*= 8.7 Hz, NH), 7.91 (d, *J*= 8.5 Hz, NH), 7.64 (d, *J*= 8.9 Hz, NH), 7.60 (d, *J*= 8.1 Hz, NH), 4.29 (m, 4H, αCH), 4.16 (m, 1H, αCH), 3.91 (m, 1H, βCH from Thr), 3.82 (m, 1H, δCH_2 from D-Pro), 3.60 (m, 1H, δCH_2 from D-Pro), 2.15 (t, *J*= 7.2 Hz, 2H, αCH_2 from MeHex), 2.08 (m, 1H), 1.95 (m, 4H), 1.83 (m, 2H), 1.52 (m, 1H, CH from MeHex), 1.26 (m, 3H, γCH_3 from Thr),1.08 (s, 9H from ^{t}Bu), 0.95 (d, *J*= 6.3 Hz, 3H), 0.89 (d, *J*= 6.7 Hz, 3H), 0.81 (m, 22H). HRMS (ESI) m/z for $C_{35}H_{64}N_5O_8$ ($M+H^+$) calcd.: 682.4750, found: 682.4749.

***C*-Component 2:** ^{1}H NMR (400 MHz): 8.19 (d, *J*= 8.7 Hz, NH), 7.95 (d, *J*= 8.3 Hz, NH), 7.88 (d, *J*= 8.6 Hz, NH), 7.67 (d, *J*= 8.9 Hz, NH), 7.57 (d, *J*= 8.1 Hz, NH), 6.76 (t, *J*=5.5 Hz, δNH from Orn), 4.26 (m, 6H, αCH), 3.91 (m, 1H, βCH from Thr), 3.76 (m, 1H, δCH_2 from D-Pro), 3.57 (m, 1H, δCH_2 from D-Pro), 2.85 (m, 2H, δCH_2 from Orn), 2.12 (m, 2H, CH_2 from MeHex), 1.34 (s, 9H from Boc), 1.08 (s, 9H from ^{t}Bu), 0.95 (d, *J*= 6.3 Hz, 3H), 0.88 (d, *J*= 6.7 Hz, 3H), 2.05-0.79 (m, 37H). HRMS (ESI) m/z for $C_{45}H_{82}N_7O_{11}$ ($M+H^+$) calcd.: 895.6067, found: 896.6067.

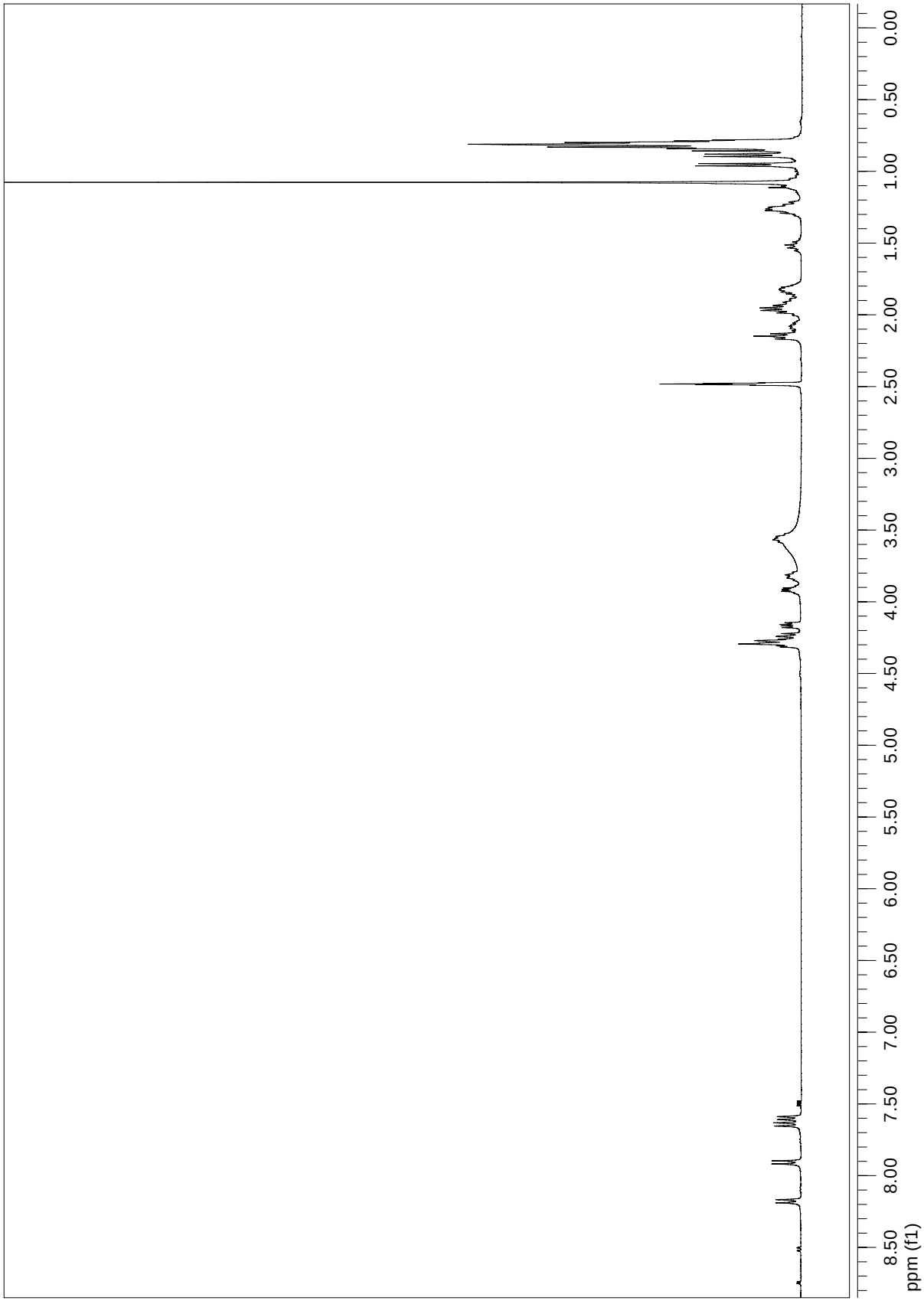
***C*-Component 3:** ^{1}H NMR (400 MHz): 8.22 (d, *J*= 8.7 Hz, NH), 7.93 (d, *J*= 8.7 Hz, NH), 7.90 (d, *J*= 8.7 Hz, NH), 7.84 (d, *J*= 9 Hz, NH), 7.69 (d, *J*= 9 Hz, NH), 7.54 (d, *J*= 8.2 Hz, NH), 6.74 (t, *J*=5.7 Hz, δNH from Orn), 4.32 (m, 7H, αCH), 3.90 (m, 1H, βCH from Thr), 3.83 (m, 1H, δCH_2 from D-Pro), 3.55 (m, 1H, δCH_2 from D-Pro), 2.84 (m, 2H, δCH_2 from Orn), 2.15 (t, *J*= 7.4 Hz, 2H, αCH_2 from MeHex), 1.34 (s, 9H from Boc), 1.08 (s, 9H from ^{t}Bu), 0.95 (d, *J*= 6.3 Hz, 3H), 0.87 (d, *J*= 6.6 Hz, 3H), 2.05-0.79 (m, 46H). HRMS (ESI) m/z for $C_{51}H_{93}N_8O_{12}$ ($M+H^+$) calcd.: 1009.6908, found: 1009.6907.

***N*-Component 1:** ^{1}H NMR (400 MHz): 9.62 (s, NH from ZDhb), 8.78 (m, 2H, NH), 8.66 (d, *J*= 8.9 Hz, NH), 8.37 (d, *J*= 8.8 Hz, NH), 8.06 (s, NH_2), 7.63 (NH, *J*= 8.4 Hz from D-Val 4), 7.26 (m, 4H Ar from Phe), 7.20 (m, 1H Ar from Phe), 6.9 (t, *J*= 5.7 Hz, δNH from Orn), 6.73 (d, 6.9 (t, *J*= 8.9 Hz, NH from L-Val 1), 6.33 (q, *J*= 7.1 Hz, CH from ZDhb), 4.93 (m, 1H, βCH from D-aThr), 4,54 (dd, *J*= 10.2 Hz, J'= 9.3 Hz, 1H, αCH), 4.44 (m, 3H, αCH), 4.30 (dd, *J*= 10.4 Hz, *J'*= 5.8 Hz, 1H, αCH), 3.80 (m, 2H, αCH), 2.90 (m, 4H, βCH_2 from Phe and δCH_2 from Orn), 2.16 (m, βCH from D-Val 4), 1.73 (m, 2H, βCH from D-aIle 5 and 7), 1.67 (m, 4H, βCH from D-aIle 5 and 7, γCH_2 from Orn), 1.35 (s, 9H from Boc), 1.30 (m, 4H), 1.28 (d, *J*= 6.2 Hz, 3H), 1.06 (d, *J*= 6.2 Hz, 3H), 1.01 (m, 1H), 0.80-0.60 (m, 24H). HRMS (ESI) m/z for $C_{49}H_{80}N_9O_{11}$ ($M+H^+$) calcd.: 970.5972, found: 970.5972.

***N*-Component 2:** ^{1}H NMR (400 MHz): 9.63 (s, NH from ZDhb), 8.99 (d, *J*= 9.0 Hz,NH), 8.89 (d, *J*= 10.5 Hz,NH), 8.79 (d, *J*= 5.5 Hz, NH), 8.10 (s, NH_2), 7.58 (d, , *J*= 8.3 Hz, NH from D-Val 4), 7.26 (m, 4H Ar from Phe), 7.20 (m, 1H Ar from Phe), 6.75 (d, , *J*= 8.9 Hz, NH from L-Val1), 6.34 (q, *J*= 7.1 Hz, CH from ZDhb), 4.92 (m, 1H, βCH from D-aThr), 4,59 (dd, *J*= 9.7 Hz, J'= 9.7 Hz, 1H, αCH), 4.42 (m, 2H, αCH), 4.32 (dd, *J*= 10.4 Hz, *J'*= 5.5 Hz, 1H, αCH), 3.86 (dd, *J*= 10.3 Hz, *J'*= 9.1 Hz, 1H, αCH), 3.66 (d, *J*= 5.3, 1H, αCH), 2.93 (m, βCH_2 from Phe), 2.16 (m, βCH from D-Val 4), 1.73 (m, 2H, βCH from D-aIle 5 and 7), 1.39 (m, 2H), 1.27 (m, 5H), 1.12 (d, *J*= 6.3 Hz, 3H), 1.07 (m, 1H), 0.80-0.60 (m, 24H). HRMS (ESI) m/z for $C_{39}H_{62}N_7O_8$ ($M+H^+$) calcd.: 756.4655, found: 756.4654.

***N*-Component 3:** ^{1}H NMR (400 MHz): 9.50 (s, NH from ZDhb), 8.91 (d, *J*= 9.7 Hz, NH), 8.78 (d, *J*= 5.9 Hz, NH), 8.65 (s, NH_2), 7.48 (d, *J*= 7.4, NH from D-Val 4), 7.25 (m, 4H Ar from Phe), 7.18 (m, 1H Ar from Phe) 6.98 (d, *J*= 9 Hz, NH from L-Val 1), 6.37 (q, *J*= 7.0 Hz, CH from ZDhb), 5.03 (m, 1H, βCH from D-aThr), 4.5-4.3 (m, 3H, αCH), 4.05-3.95 (m, 2H, αCH), 2.94 (m, βCH_2 from Phe), 2.04 (m, βCH from D-Val 4), 1.84 (m, βCH from D-aIle), 1.52 (m, 1H, βCH from L-Val 1), 1.32 (m, 1H, γCH_2 from D-aIle), 1.28 (m, 6H), 1.12 (m, 1H, γCH_2 from D-aIle), 0.86-0.62 (m, 18H). HRMS (ESI) m/z for $C_{33}H_{51}N_6O_7$ ($M+H^+$) calcd.: 643.3814, found: 643.3814.

C-Component 1



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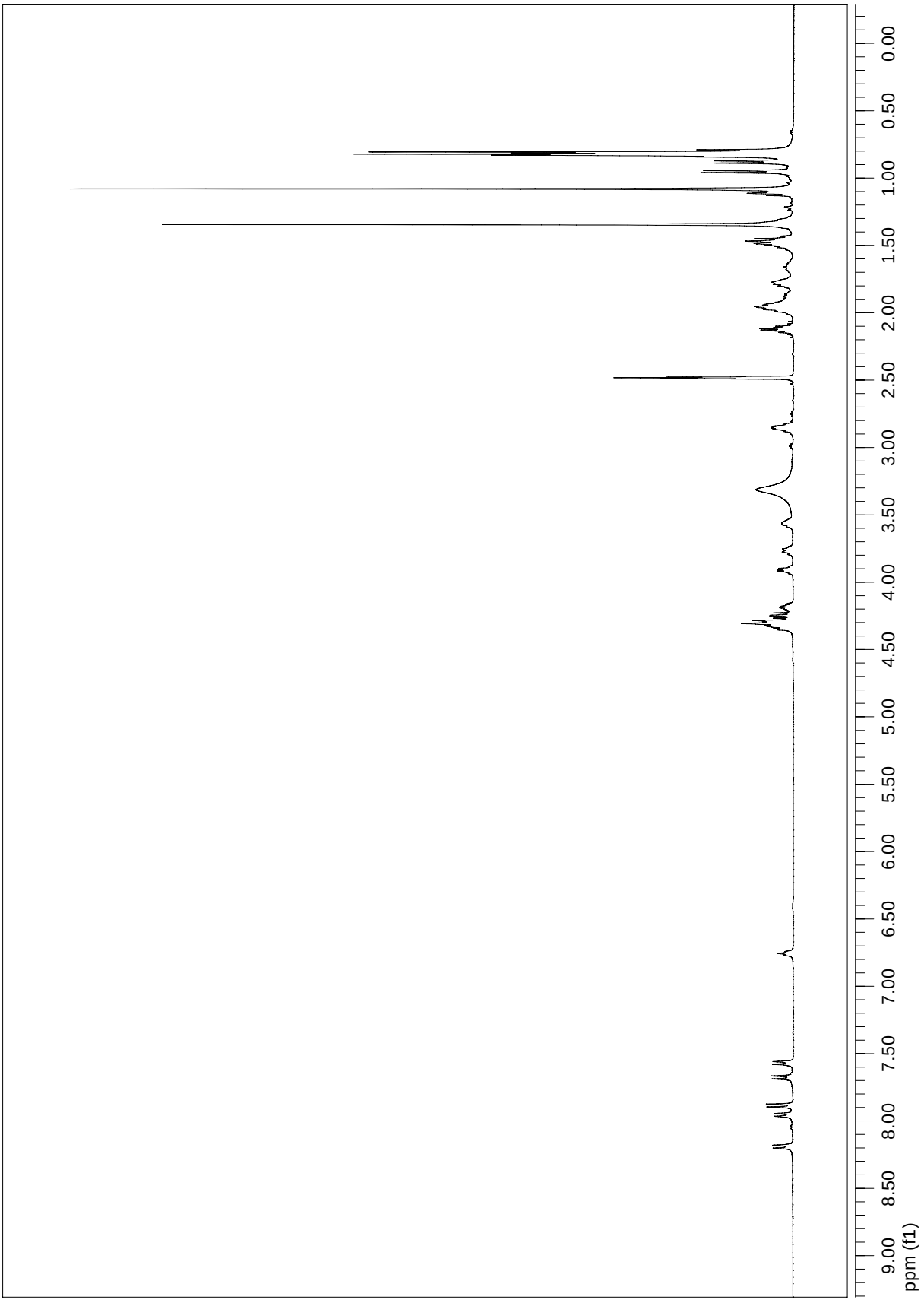

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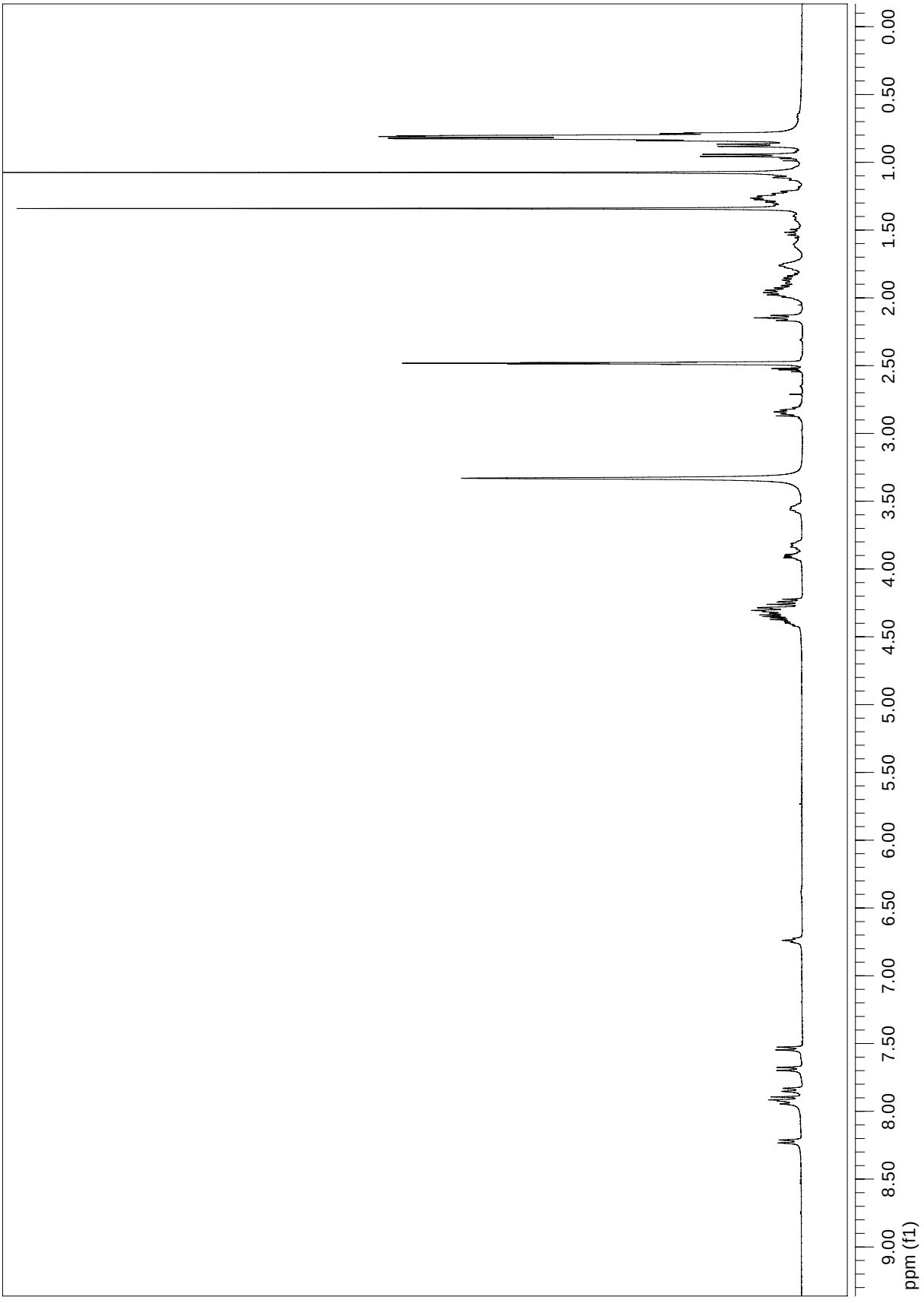

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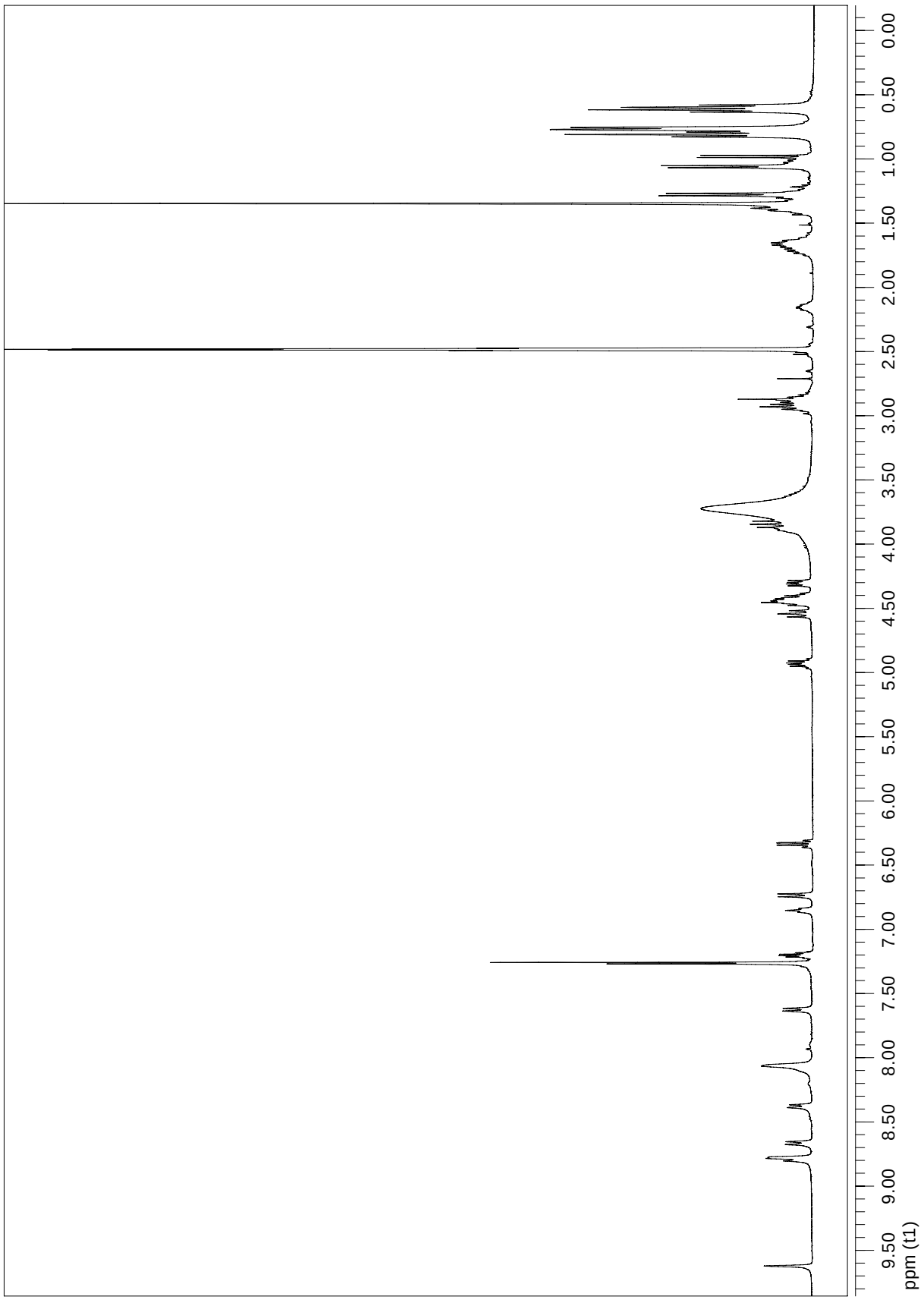

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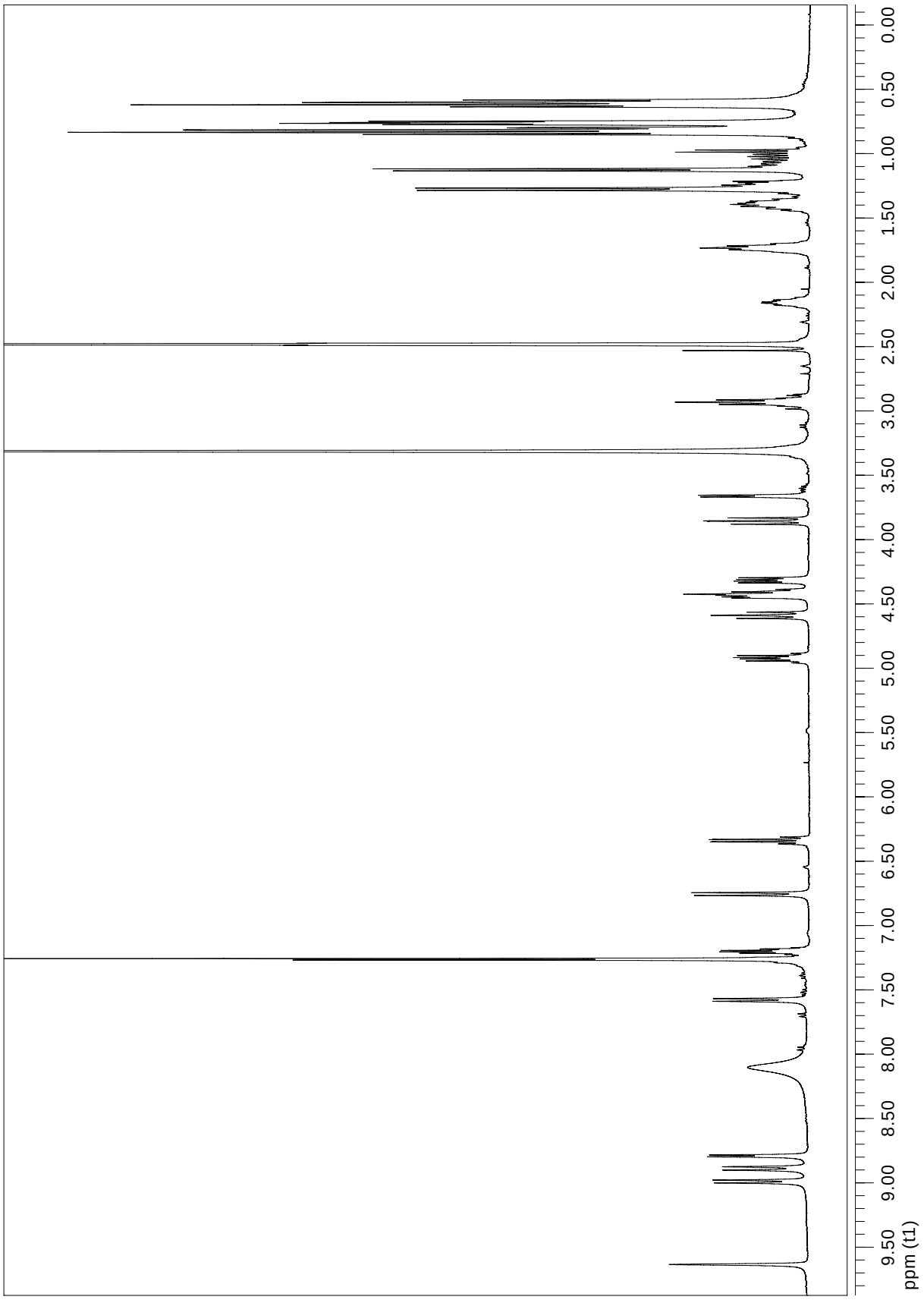

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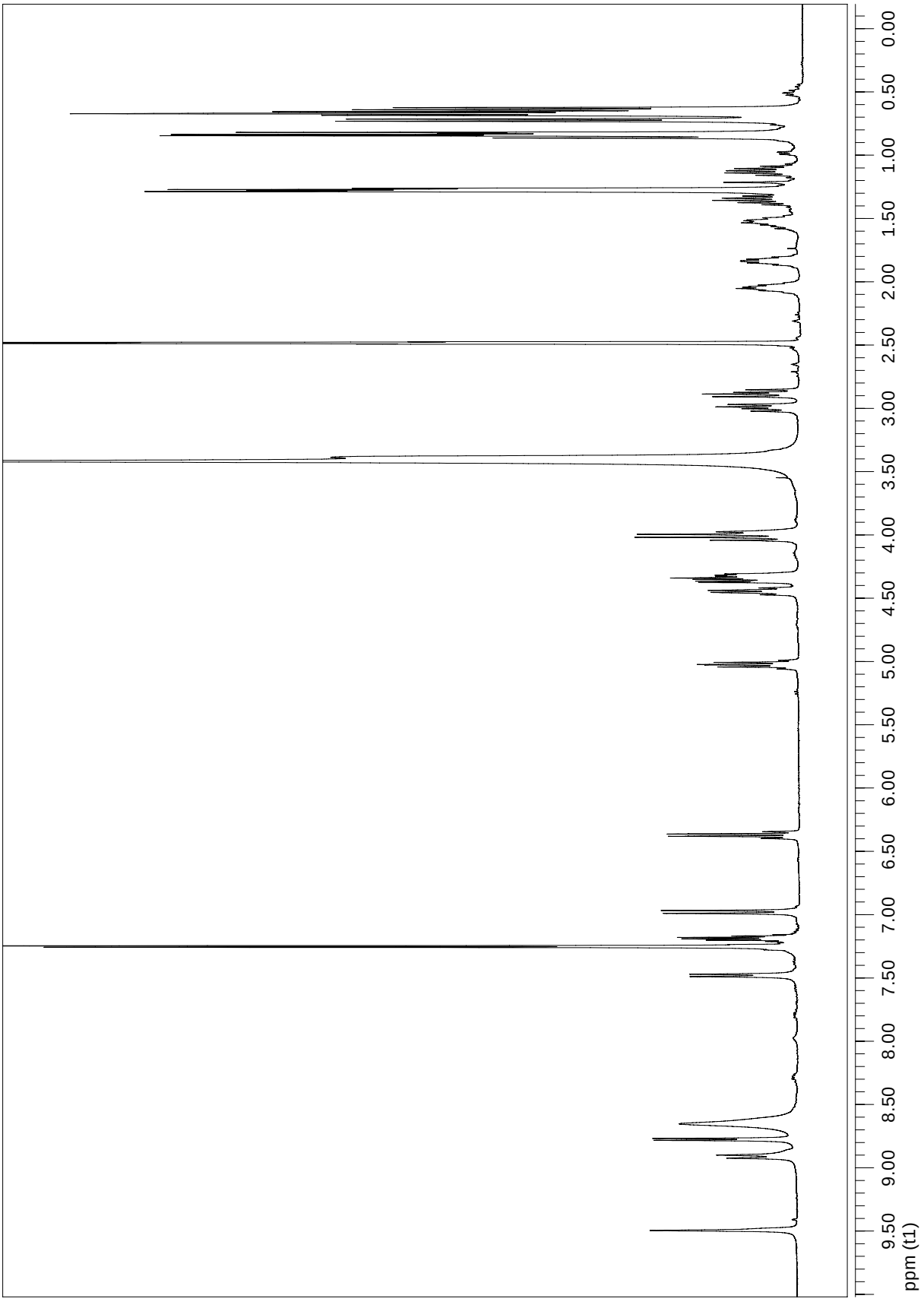


p-Nitromandelic Acid as a Highly Acid-Stable Safety-Catch Linker for Solid-Phase Synthesis of Peptide and Depsipeptide Acids

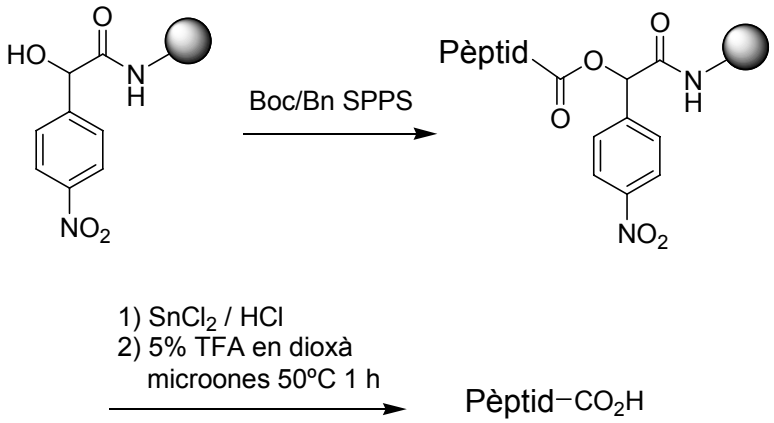
L'Àcid *p*-Nitromandelic com a Espaïador Bifuncional de Tipus "Safety-Catch" Molt Estable a Medi Àcid per a la Síntesi de Pèptids i Depsipèptids Àcids Carboxílic

Albert Isidro-Llobet, Mercedes Álvarez, Klaus Burger, Jan Spengler and Fernando Albericio

Organic Letters 9 (2007), 1429-1432

Resum

En el següent treball es presenta un nou derivat del *p*-nitrobenzil, l'àcid *p*-nitromandèlic (*p*NMA), com a espaïador bifuncional o "linker" per a la síntesi de compostos làbils a base, ja siguin pèptids o depsipèptids, mitjançant l'estratègia Boc/Bn. El "linker" *p*NMA és del tipus "safety catch" ja que per escindir el producte desitjat de la resina calen dues etapes: una primera en la qual es converteix l'enllaç "linker"-compost en més làbil i una segona d'escissió pròpiament dita. En el cas concret del *p*NMA, en primer lloc es redueix el grup nitro amb $SnCl_2$ i després de rentar la resina per eliminar l'agent reductor s'obtenen els (depsi)pèptids amb les cadenes laterals lliures (si la resina es tracta amb TFMSA-tioanisol abans de l'escissió del pèptid) o protegides mitjançant irradiació de la resina amb microones a 50ºC en TFA-dioxà (5:95).



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p-Nitromandelic Acid as a Highly Acid-Stable Safety-Catch Linker for Solid-Phase Synthesis of Peptide and Depsipeptide Acids

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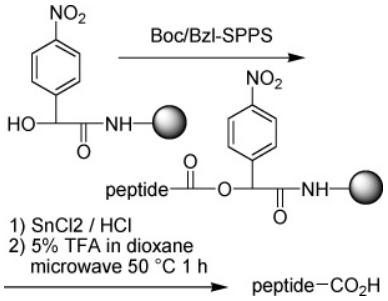
ABSTRACT



***p*-Nitromandelic acid as a safety-catch linker for Boc/Bzl-SPPS of base-labile compounds like peptides and depsipeptides is described. This linker permits acidic removal of side-chain protection groups from the resin. For cleavage from the solid support, the *p*-nitro group was reduced with tin(II) chloride. After washing off the reducing agents, the (depsi)peptide acids with or without the side-chain protection schemes were obtained by microwave irradiation at 50 °C with 5% TFA in dioxane.**

Depsipeptides are naturally occurring compounds that show a broad range of biological activities.[1] They are often cyclic compounds of relatively small ring sizes (5−12-membered). As for cyclic peptides, synthesis of the linear chain on solid support and cyclization after cleavage is a common strategy for their preparation in small-scale runs. However, the choice between the two main SPPS strategies, the Fmoc/^{t}Bu and Boc/Bzl routes, respectively, requires a number of considerations. Ester bonds are base-sensitive and consequently show limited stability toward Fmoc-removal conditions. Thus, there is a risk of continuous loss of ester product if consecutive coupling cycles involving Fmoc-removal are performed after ester bond formation. However, ester bonds are stable toward the acidic conditions of the Boc/Bzl strategy. Consequently, for the synthesis of long linear depsipeptides, the Boc/Bzl route is clearly favored.[2] After cleavage from the solid support with anhydrous HF, the product can be efficiently separated by precipitation from the cleavage cocktail, which normally consists of low-volatile

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(1) Hamada, Y.; Shioiri, T. *Chem. Rev.* **2005**, *105*, 4441−4482.

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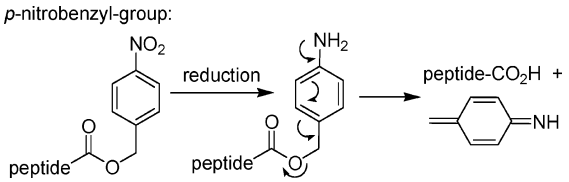
scavengers (anisole, thioanisole, etc.) or, in case of using trifluoromethane sulfonic acid (TFMSA), also nonvolatile strong acid. Unfortunately, precipitation of small (depsi)-peptides may be incomplete and therefore isolation then requires tedious purification procedures. Moreover, the use of conventional Boc/Bzl-protected amino acid building blocks rules out the preparation of the fully side-chain protected peptides required for convergent synthesis or head-to-tail cyclization.

With the aim to overcome the difficulties associated with the synthesis of small depsipeptides, we searched for a linker compatible with the Boc route, which also allows prior removal of the side-chain protection schemes from the resin or cleavage of the fully protected segments, respectively. Safety-catch linkers can meet these challenges. The safety-catch principle involves the conversion of a relatively stable form of a linker into a labile, isolable and cleavable one.[3] However, safety-catch linkers are designed for the Fmoc/^{t}-Bu-route are not sufficiently acid-stable. The requirement of nucleophiles or basic conditions for final cleavage after switching on the activity of the linker-bond carbonyl group is shared by a broad range of safety-catch linkers that can be classified as "hidden active esters". We considered this strategy to be less suitable for our purpose because the ester bonds of depsipeptides are sensitive to bases and nucleophiles.

The recently described safety-catch linkers based on the substructure 4-mercapto benzylalcohol are, in their oxidized (sulfinyl) form, stable toward acids and bases. Final cleavage of the peptide is performed by reductive acidolysis.[4a,b] However, under these conditions, benzyl-type protecting groups used in the Boc/Bzl-SPPS are also cleaved.[4c] It is particulary difficult to remove Bzl-type side-chain protecting groups while the peptide is anchored on the resin. *S*-Alkylation occurred in the presence of the scavengers anisole and *p*-cresol and prevents future cleavage from the resin. In contrast, the scavengers thioanisole and thiophenol reduced the linker and caused premature cleavage.[4b]

We studied the potential of *p*-nitromandelic acid (Pnm) as a safety-catch linker. This linker is structurally related to the *p*-nitrobenzyloxycarbonyl (*p*NZ) and *p*-nitrobenzyl groups.[5] This protecting group was, e.g., used for *C*-terminal protection in side-chain anchoring techniques for the SPPS of head-to-tail cyclic peptides.[6] Reduction of the *p*-nitro group of *p*NZ can be performed orthogonally to the Fmoc, Boc, and Alloc protection on the solid phase and causes spontaneous

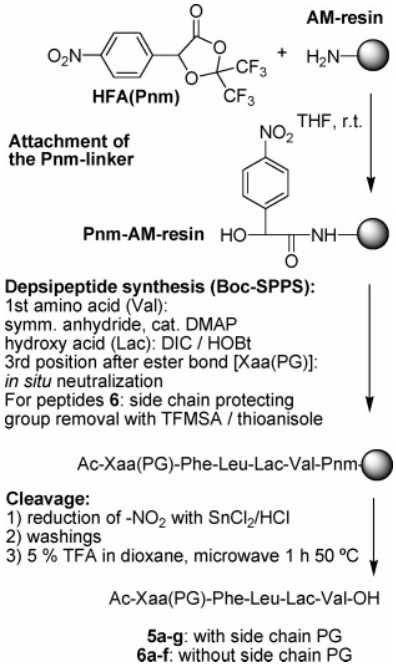
Scheme 1



deprotection of amino and hydroxy groups via 1,6-electron pair shift (Scheme 1).[7] *p*-Nitromandelic acid bears an additional carboxylic group, which allows anchoring on a resin. The (depsi)peptide chain can then be constructed on the α-OH-function. Reduction of the *p*-nitro group would give a *p*-aminomandelic (Pam)-bound peptide, which should be cleaved analogously from the quinonimine methide generated by a 1,6-electron pair shift.

Racemic Pnm was prepared from *p*-nitrobenzaldehyde[8] and protected with hexafluoroacetone (HFA).[9] HFA(Pnm) was then coupled to aminomethylated polystyrene (AM-resin, 1.1 mmol/g) (Scheme 2). The filtrate with the excess of HFA-

Scheme 2



(Pnm) can be reused for further couplings.[9b] Fmoc-Leu-OH was attached as symmetric anhydride in the presence of cat. DMAP to the free hydroxy-function of HO-Pnm-AM to give

(2) (a) Beligere, G. S.; Dawson, P. E. *J. Am. Chem. Soc.* **2000**, *122*, 12079−12082. (b) Silinski, P.; Fitzgerald, M. C. *Biochemistry* **2003**, *42*, 6620−6630. (c) Blankenship, J. W.; Balambika, R.; Dawson, P. E. *Biochemistry* **2002**, *41*, 15676−15684.

(3) (a) Patek, M.; Lebl, M. *Biopolymers* **1998**, *47*, 353−363. (b) James, I. W. *Tetrahedron* **1999**, *55*, 4855−4946.

(4) (a) Kiso, Y.; Fukui, T.; Tanaka, S.; Kimura, T.; Akaji, K. *Tetrahedron Lett.* **1994**, *35*, 3571−3574. (b) Erlandsson, M.; Unden, A. *Tetrahedron Lett.* **2006**, *47*, 5829−5832. (c) Yajima, H.; Fujii, N.; Funakoshi, S.; Watanabe, T.; Murayama, E.; Otaka, A. *Tetrahedron* **1988**, *44*, 805−819.

(5) Carpenter, F. H.; Gish, D. T. *J. Am. Chem. Soc.* **1952**, *74*, 3818−3821.

(6) (a) Romanovskis, P.; Spatola, A. F. *J. Pept. Res.* **1998**, *52*, 356−374. (b) Royo, M.; Farrera-Sinfreu, J.; Sole, L.; Albericio, F. *Tetrahedron Lett.* **2002**, *43*, 2029−2032.

(7) Isidro-Llobet, A.; Guasch-Camell, J.; Alvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, 3031−3039.

(8) Fosdick, L. S.; Wessinger, G. D. *J. Am. Chem. Soc.* **1938**, *60*, 1465−1466.

(9) (a) Spengler, J.; Böttcher, C.; Albericio, F.; Burger, K. *Chem. Rev.* **2006**, *106*, 4728−4746. (b) Albericio, F.; Burger, K.; Ruíz-Rodríguez, J.; Spengler, J. *Org. Lett.* **2005**, *7*, 597−600.

Fmoc-Leu-Pnm-AM-resin **1**. For the synthesis of Fmoc-Ile-Phe-Leu-Pnm-AM-resin **2**, Boc-Leu-OH was attached first and the following amino acids were coupled with DIC/HOBt activation. The drastic decrease in resin loading to 0.11 mmol/g demonstrates the high propensity to form diketopiperazine, which was circumvented in further peptide synthesis by the in situ neutralization method for couplings of amino acid in the third position after the ester bond.[10]

The *p*-nitro function of Pnm in **1** and **2** was then reduced using the same conditions applied for reduction of the *p*NZ-group (6 M $SnCl_2$/1.6 mM HCl/dioxane-solution in DMF for 1 h).[7] Two intensive IR-signals associated with aryl-bound nitro-groups at 1524 and 1347 cm^{-1} disappeared. In comparison with the rapid cleavage of the *p*NZ group, which proceeds spontaneously after reduction, the resin-bound depsipeptides **3** and **4** with *C*-terminal *p*-aminomandelic acid (Pam) were relatively stable. The loss of peptide or amino acid during reduction and subsequent washings of the resin was in the range of 1.3−23.4%, with an average of 12.2% (10 experiments).[11] This stability enabled the removal of most of the tin salt excess.[12]

Next, we tested several conditions to cleave the amino acid or peptide from Pam-AM resin. Short treatment with 10% DIEA in DCM showed only a small effect (Table 1, entry 1) and prolonged treatment caused, as expected, an unselective ester bond rupture (Table 1, entry 2 vs 3). The resin-bound peptide was also relatively stable in organic solvents at room temperature (entry 4), but the amount of cleaved product increased by heating, which was performed in a controlled manner by microwave irradiation (entries 5 and 6). Better results were achieved with 90% TFA (entries 7 and 8). However, prolonged treatment up to 72 h did not lead to complete cleavage of product (entry 9). Combining acidic media (5% TFA in dioxane) with 1 h heating to 50 °C gave the best results (entry 10) and was found to be selective for the reduced form of the linker, because from the unreduced resin **2** practically no product was cleaved (Table 1, entry 11).

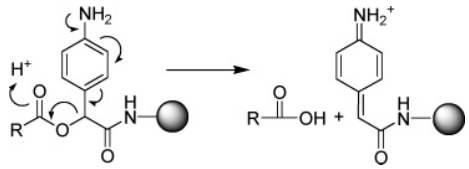
Table 1. Cleavage Conditions

entry	resin[a]	conditions	% cleavage (% remaining on resin)[b]
1	**3**	3 × 2 min, 10% DIEA in DCM	5 (77)
2	**4**	10% DIEA in DCM, overnight	50 (44)
3	**2**	10% DIEA in DCM, overnight	27
4	**3**	DMF, overnight	33
5	**4**	DMF, MW 50 °C, 1 h	75 (9)
6	**4**	THF, MW 50 °C, 1 h	65 (22)
7	**3**	TFA/DCM/H_2O (90:5:5), overnight	58 (19)
8	**4**	TFA/DCM/ H_20 (90:5:5), overnight	56
9	**3**	TFA/DCM/ H_20 (90:5:5), 72 h	66 (22)
10	**4**	TFA/dioxane (5:95), MW 50 °C, 1 h	85 (12)
11	**2**	TFA/dioxane (5:95), MW 50 °C, 1 h	99[c]

[a] Unreduced form of the linker: **1**, Fmoc-Leu-*Pnm*-AM; **2**, Fmoc-Ile-Phe-Leu-*Pnm*-AM. Reduced form of the linker: **3**, Fmoc-Leu-*Pam*-AM; **4**, Fmoc-Ile-Phe-Leu-*Pam*-AM. [b] Quantified by Fmoc determination measuring UV absorbance at 290 nm. [c] Colored solution.

The quinonimine methide generated from the 1,6-electron shift can also react with nucleophiles, which explains why TFA cleaved the Pam-bound depsipeptides more efficiently than DIEA. This principle was recently used in the design of activity-based fluorescent probes that target proteases.[13] We propose that, in our case, the quinonimine methide and the *C*-terminal carboxylic anion of the peptide recombined quickly under neutral conditions or in the presence of DIEA. However, under acidic conditions, the carboxylic acid, which acts as leaving group, is formed. This would explain the better cleavage rates in acidic medium (Scheme 3).

Scheme 3



By means of Boc/Bzl-SPPS standard procedures, we then prepared a number of 5-membered depsipeptides containing at least one amino acid with an additional functionality bearing the common benzylic side-chain protecting group (Scheme 2). To avoid diketopiperazine formation, the second position after the ester bond was a hydroxy acid (lactic acid, Lac) and an amino acid was introduced on fifth position by in situ neutralization.[10] To remove the side chain protecting groups, a part of each resin was treated with TFMSA in the presence of thioanisole as scavenger. These reagents were removed from the resin by filtration and washing. After reduction of the *p*-nitro group, the depsipeptide was cleaved under the conditions described above (5% TFA in dioxane at 50 °C, microwave heating). The solutions were evaporated, and the residues redissolved in water/acetonitrile and lyophilized. The main peaks in the HPLC spectra were the desired products (HPLC-MS, ESI). Table 2 shows the yield and the purity of the protected (**5a**−**g**) and unprotected (**6a-f**) depsipeptides obtained. For side-chain unprotected Cys-depsipeptide **6e** dimerization decreased the yield. A lower

(10) Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1990**, *31*, 7363−7366.

(11) Calculated as the difference between inital loading and the sum of product remaining after reduction on the resin and cleaved product (for conditions, see Table 1) because direct determination of UV absorbance of tin-containing solutions is not credible due to yellow color and precipitates.

(12) The amount of tin was found to be 20 ppm for a product washed with water in a Diaion (Supelco) column as determined by ICP (inductively-coupled plasma).

(13) A peptide recognition sequence was attached to the *p*-amino group of Pam, a fluorescence reporter was linked to the carboxy group, and the 2-hydroxy group was exchanged for fluorine as leaving group. After cleavage of the peptide by the protease, the fluorine is released and the quinonimine methide reacts with a nucleophilic site present in the enzyme to form a covalent bond with the fluorescence-reporter system. Srinivasan, R.; Huang, X.; Ng, S. L.; Yao, S. Q. *ChemBioChem* **2006**, *7*, 32−36.

Table 2. Yields and Purities of Cleaved Products

product (Xaa)[a]	amount in mg (from mg of resin)	% HPLC purity (retention time in min)
5a [Glu(OBzl)	26 (80)	76 (7.40[b])
6a (Glu)	9.5 (39)	82 (5.90[c])
5b [Lys(2ClZ)]	25 (90)	78 (8.03[b])
6b (Lys)	8 (40)	75 (7.95[d])
5c [Thr(Bzl)]	19.5 (90)	77 (7.79[b])
6c (Thr)	9.5 (40)	66 (6.02[c])
5d [Tyr(Bzl)]	22.5 (90)	72 (9.07[b])
6d (Tyr)	10 (40)	77 (7.23[c])
5e [Cys(pMeBzl)]	21 (85)	73 (8.45[b]); 5 (oxidized product) (6.19[b])
6e (Cys)	4 (35)	18 (10.45[d]); 32 dimer (11.91,[d] 1186.3)
5f [Arg(Tos)]	10.5 (50)	72 (5.46[b])
6f (Arg)	4.5 (20)	72 (6.91[d])
5g [Asn(Xan)]	7 (80)	53 (without Xan) (10.49[e])

[a] See Scheme 2. [b] Gradient 40−100% ACN, 15 min. [c] Gradient 30−100% ACN, 15 min. [d] Gradient 20−100% ACN, 15 min. [e] Gradient 0−100% ACN, 15 min.

yield was also observed for Asn **5g**. However, the other depsipeptides were obtained in reasonable purity and yields.

In summary, we have demonstrated that *p*-nitromandelic acid can be used as a highly acid-stable safety-catch linker, compatible with the Boc/Bzl strategy for the synthesis of peptides and depsipeptides. Cleavage of the final products can be performed without base. Therefore, on the basis of these observations, we propose that this linker is suitable for future applications especially for the synthesis of base-labile compounds on the solid phase.

Acknowledgment. This study was partially supported by CICYT (CTQ2006-03794/BQU), the Generalitat de Catalunya (2005SGR 00662), and the Barcelona Science Park. A.I.-L. thanks the DURSI, Generalitat de Catalunya, and European Social Funds for a predoctoral fellowship.

Supporting Information Available: Synthesis and NMR-spectroscopic data of HFA-*p*-nitromandelic acid, protocols used for peptide synthesis, details of HPLC-measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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p-Nitromandelic Acid (Pnm) as a Highly Acid-Stable Safety-Catch Linker for Solid Phase Synthesis of Peptide and Depsipeptide Acids

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Supporting Information

General

Commercial compounds

Commercial grade reagents and solvents were used without further purification. AM-resin (aminomethylated polystyrene, 100-200 mesh, f = 1.1 mmol/g), PyBOP for in situ neutralization and HOBt were purchased from NovaBiochem, lactic acid, leucic acid and diisopropylcarbodiimide (DIC) from Aldrich, Boc-amino acids and Fmoc-Leu-OH from Iris Biotech and DIEA from Merck.

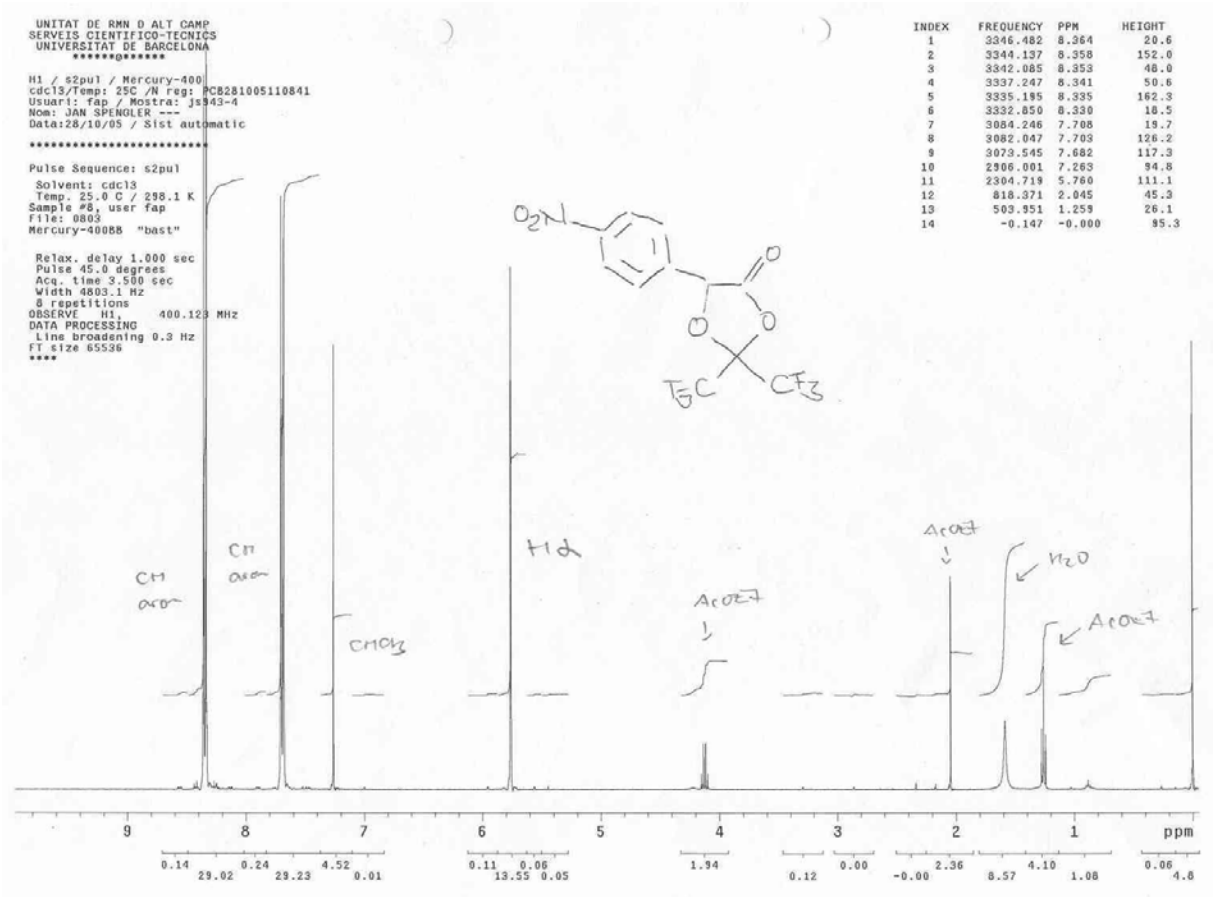
Apparatus

Analytical HPLC was carried out with a Waters instrument on a C8-column. Linear gradients (given in the table) of CH_3CN (0.036% TFA) into H_2O (0.045% TFA) were run at a flow rate of 1.0 mL/min. UV detection was performed at 220 nm. The mass signals in the HPLC-MS spectra were obtained with an electrospray detector (Waters micromass ZQ). NMR spectra were acquired with a Mercury-400 spectrometer (^1H at 400.125 MHz, ^{13}C at 100.625 MHz with TMS as internal reference) (High-field NMR Unit, Barcelona Science Park).
The following abbreviations are used to indicate multiplicity: s, singlet; d, doublet, dd, double doublet; t, triplet; dt, double triplet; m, multiplet, br s, broad signal. Microwave experiments were performed with a CEM Discover apparatus. Temperature was measured with a non-contact infrared sensor.

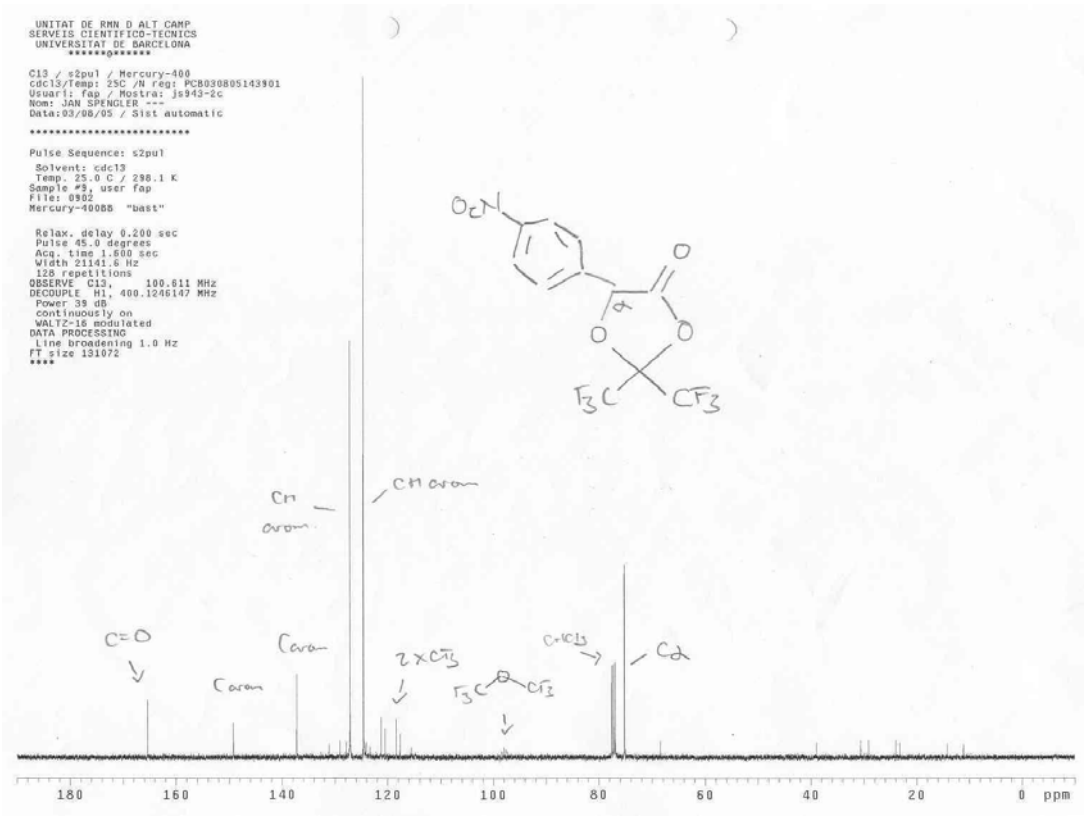
2,2-Bis(trifluoromethyl)-1,3-dioxolan-5-(4-nitrophenyl)-4-one (HFA-Pnm)

p-Nitromandelic acid was synthesized from *p*-nitrobenzaldehyde (32.5 mmol, 4.9 g) and potassium cyanide. The crude nitrile was transformed in the methyl ester as described earlier.[1] The crude methyl ester (3.8 g) was then saponified by refluxing with glacial HOAc / conc. HCl mixture (5 : 1), until no starting material was detected by TLC. Note: saponification with LiOH gave no desired product. After evaporation, a mixture of products was obtained, which was dissolved in DMSO and directly subjected to reaction with HFA. The extracted product (1.7 g) was purified by flash chromatography (hexane / ethyl acetate 4 : 1, $R_f = 0.6$) 2,2-bis(trifluoromethyl)-1,3-dioxolan-5-(4-nitrophenyl)-4-one [HFA(Pnm), 0.54 g, 5% overall yield from *p*-nitrobenzaldehyde]. It spectroscopical data are in agreement with those already described for such compounds.[2] ^{1}H NMR ($CDCl_3$): δ (ppm) = 5.75 (s, 1H), 7.69 (d, J = 8.67 Hz, 2H), 8.34 (d, J = 8.91 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 74.9, 97.8 (m), 118.8 (q, J = 285 Hz), 119.5 (q, J = 288 Hz), 124.3, 126.9, 137.0, 148.9, 165.1. ^{19}F NMR ($CDCl_3$): δ (ppm) = -81.0 (q, J = 7.75 Hz), -80.45 (q, J = 7.75 Hz). IR (film): ν = 1855, 1531, 1352, 1238, 1134 cm^{-1}.

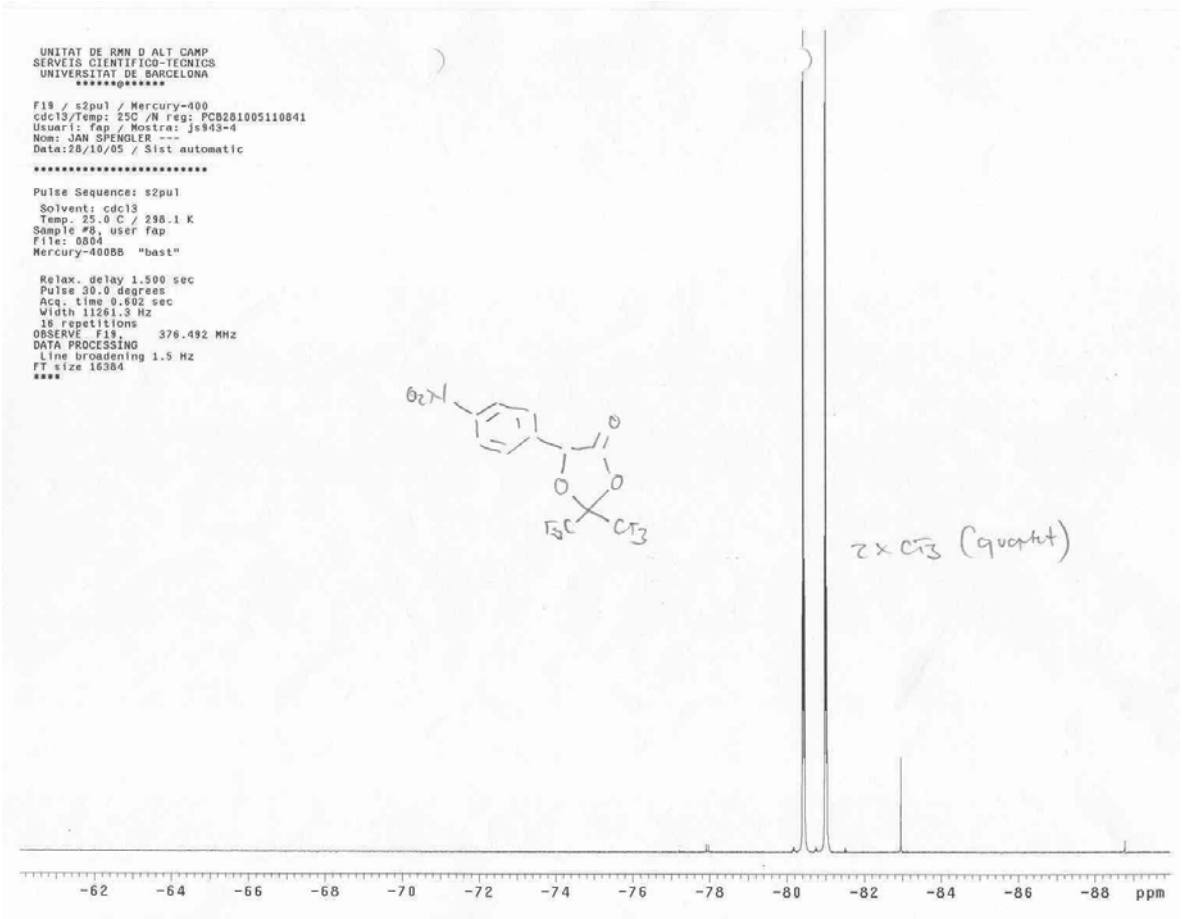
^{1}H NMR:



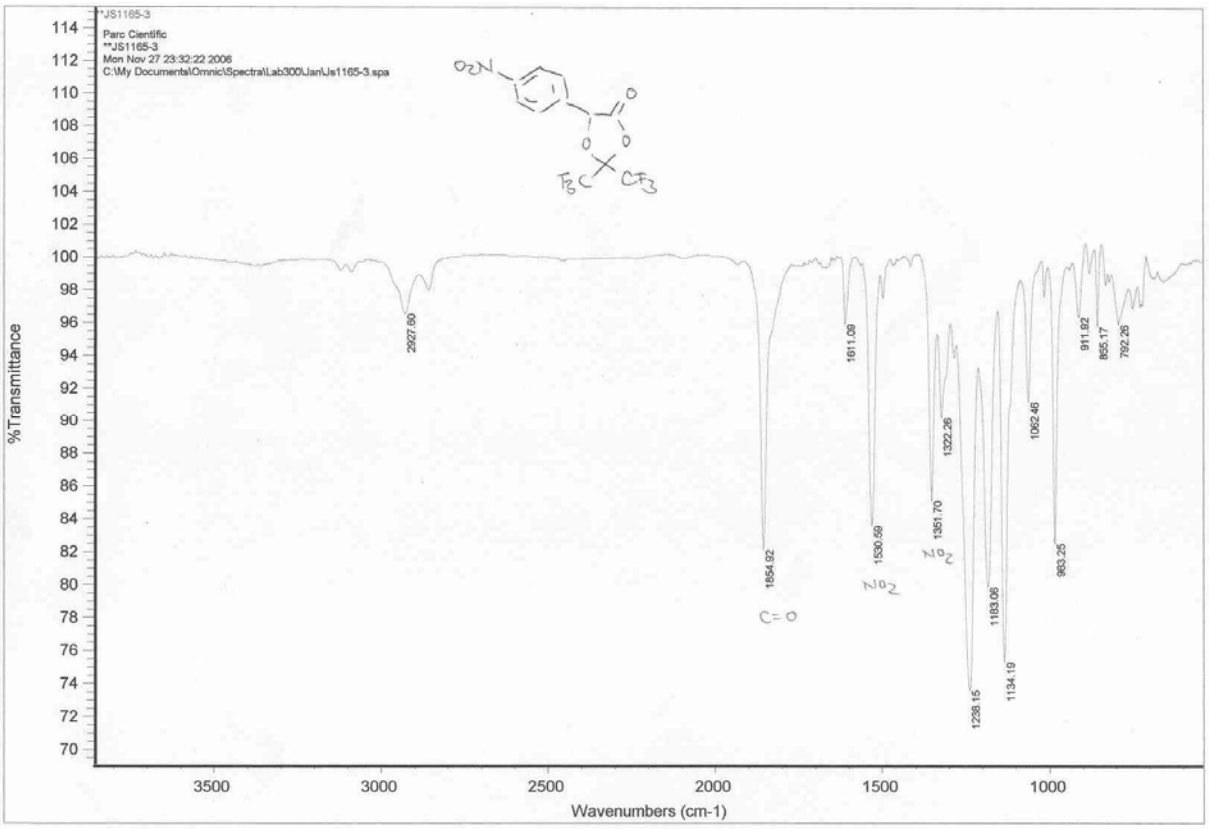
^{13}C NMR:



^{19}F NMR:



IR (film):



Preparation of H-Pnm-AM-resin and depsipeptide synthesis

4 eq of HFA-Pnm were dissolved in THF and added to aminomethylated polystyrene (which turned blue) and the mixture was left to shake until the ninhydrin-test was negative. The filtrate and washing solutions with the excess of HFA-Pnm were then evaporated and stored for future reuse. The depsipeptides were then synthesized by standard protocols. Attachment of the first amino acid: symmetric anhydride generated with DIC, cat. amounts of DMAP. Third position after an ester bond: in-situ neutralization using the PyBOP-reagent.[3] Lactic and leucic acid were coupled without α-OH protection schemes with DIC/HOBt.

For side-chain deprotection:

The resins were treated with 150 µL of thioanisol at 0 ºC followed by addition of 1 mL of TFA and 10 min of stirring. 100 µL of Trifluoromethanesulfonic acid (TFMSA) were then added and the resins were stirred 2 h at rt. Finally, the following washings were carried out: TFA (3 x 1 min), DCM (5 x 1 min), isopropanol (5 x 1 min) and DMF (5 x 1min).

For final cleavage, the resins were treated with:

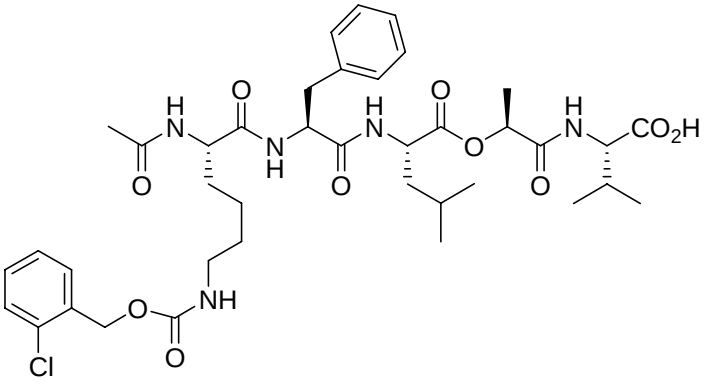
i) 6 M $SnCl_2$, 1.6 mM HCl/dioxane in DMF, 1 h at rt.

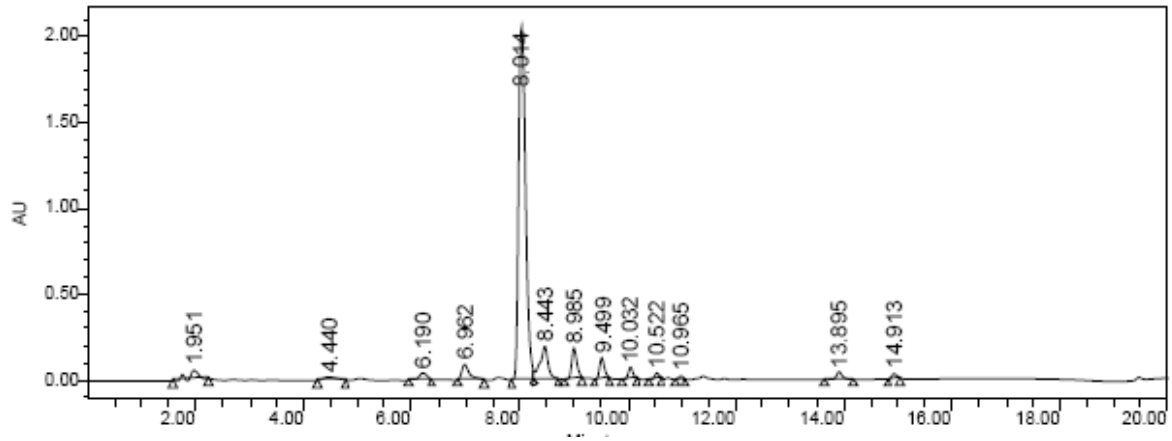
Washings: 5 x 1 min. with DMF and 5 x 30 seconds with dioxane.

ii) 5% TFA in dioxane 1h at 50 ºC (Microwave heating).

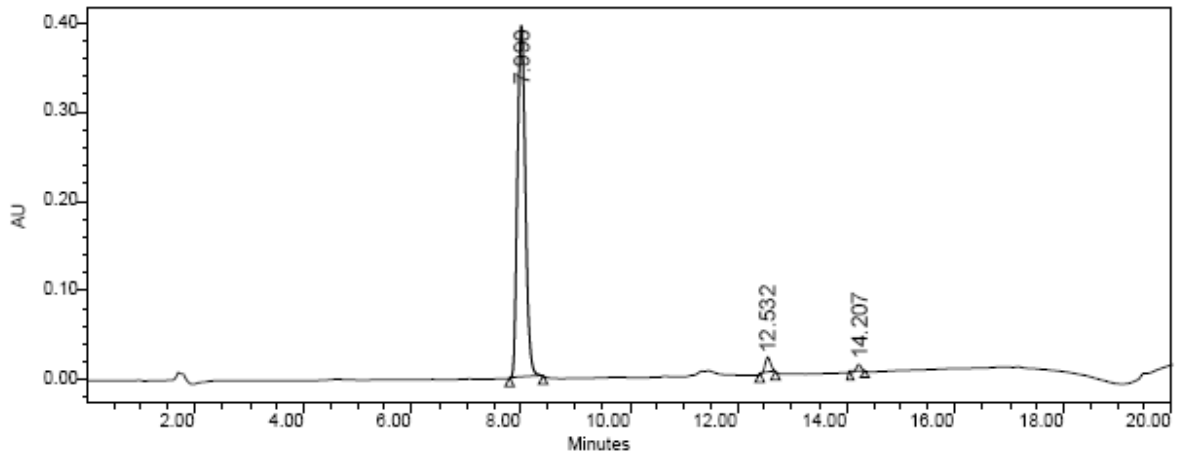
Ac-Lys(2ClZ)-Phe-Leu-Lac-Val-OH (5b) :

HRMS (ESI-MS, ES^+): $C_{39}H_{53}ClN_5O_{10}$ ($M+H^+$) calcd.: 789.34721, found: 789.36602.



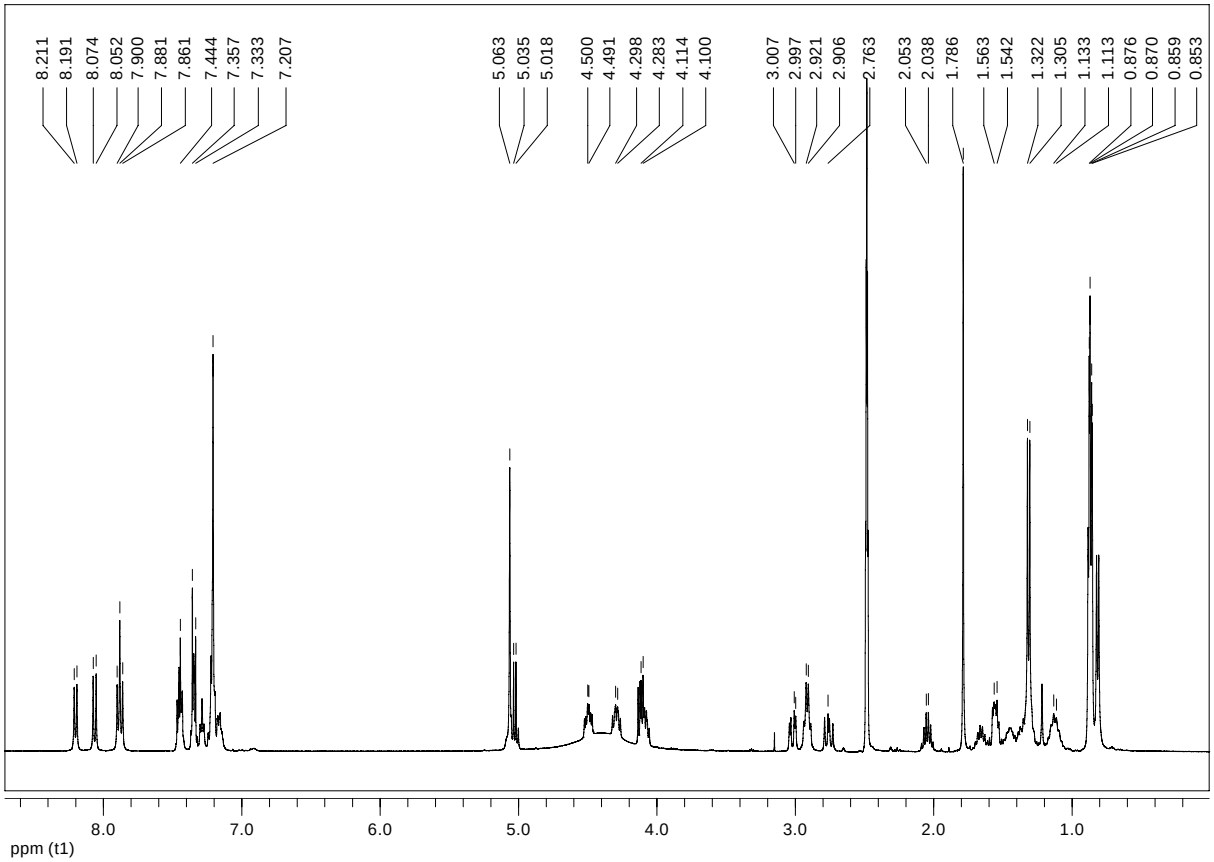


The depsipeptide **5b** (Ac-Lys(2ClZ)-Phe-Leu-Lac-Val-OH) (25 mg) was purified by semipreparative HPLC



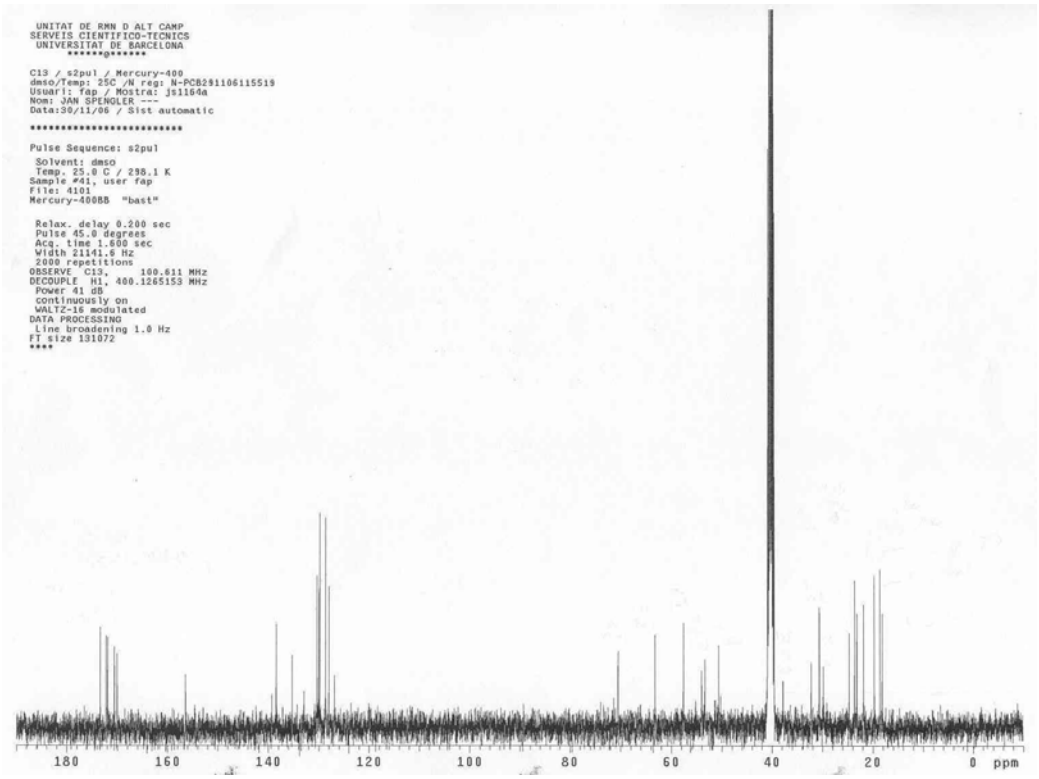
^{1}H NMR of **5b** (400 MHz, DMSO):

8.21 (d, *J*= 7.79 Hz, NH from Leu), 8.06 (d, *J*= 8.62 Hz, NH from Val), 7.88 (m, 2H, NH from Phe and Lys), 7.45 (dd, *J*= 9.2 Hz, *J'*= 5.6 Hz, 2 CH ar.), 7.35 (m, 2 CH ar.), 7.28 (t, *J*= 5.5 Hz, εNH from Lys), 7.2 (m, 4 CH ar.), 7.16 (m, CH ar.), 5.06 (s, CH_2 from 2-Cl-Z), 5.03 (q, *J*= 6.8 Hz, CH from lactic acid), 4.50 (m, αCH from Phe), 4.28 (m, αCH from Leu), 4.10 (m, αCH from Val and Lys), 3.02 (dd, *J*= 14.0 Hz, *J*= 4.0 Hz, 1H, CH_2 from Phe), 2.91 (m, εCH_2 from Lys), 2.76 (dd, *J*= 14.0 Hz, *J*= 9.8 Hz, 1H, CH_2 from Phe), 2.05 (m, βCH_2 from Val), 1.79 (s, acetyl CH_3), 1.66 (m, βCH Leu), 1.56 (m, CH_2 from Leu), 1.45 (m, 1H, βCH_2 from Lys), 1.36 (m, 1H, βCH_2 from Lys), 1.31 (d, *J*= 6.8 Hz, CH_3 from lactic acid), 1.31 (m, δCH_2 from Lys), 1.12 (m, γCH_2 from Lys), 0.87 (m, 9H, 2CH_3 from Val, CH_3 from Leu), 0.81 (d, *J*= 6.3 Hz, CH_3 from Leu).

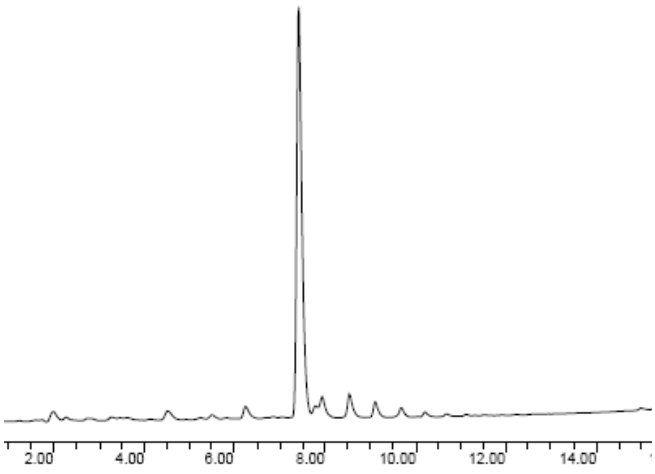


^{13}C-NMR of **5b** (100 MHz):

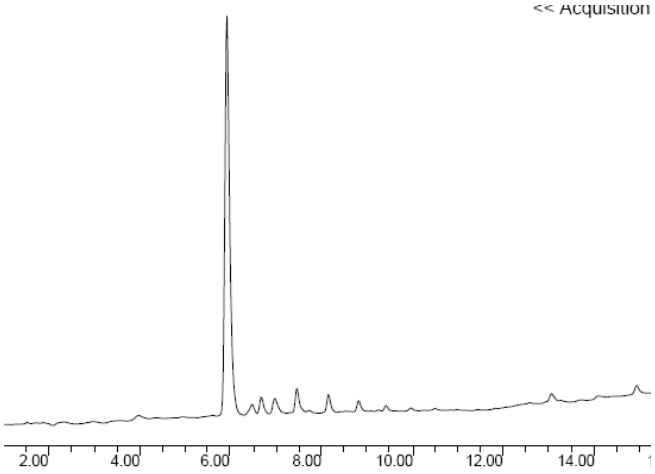
173.39, 172.27, 172.25, 171.94, 170.59, 170.05 , 156.42, 138.44 , 135.30, 130.35, 129.93, 129.83, 128.66, 127.98, 126.89, 70.49, 63.21, 57.59, 54.03, 53.39, 50.66, 37.81, 32.22, 30.62, 29.85, 24.69, 23.62, 23.20, 23.15, 21.84, 19.72, 18.58, 18.13.



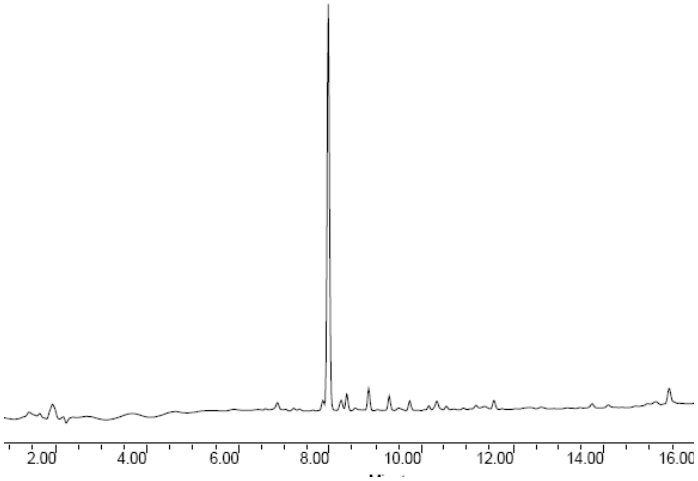
Ac-Glu(OBzl)-Phe-Leu-Lac-Val-OH (5a): HRMS (ESI-MS, ES^+): $C_{37}H_{50}N_4O_{10}$ ($M+H^+$) calcd.: 711.35997, found: 711.35844.



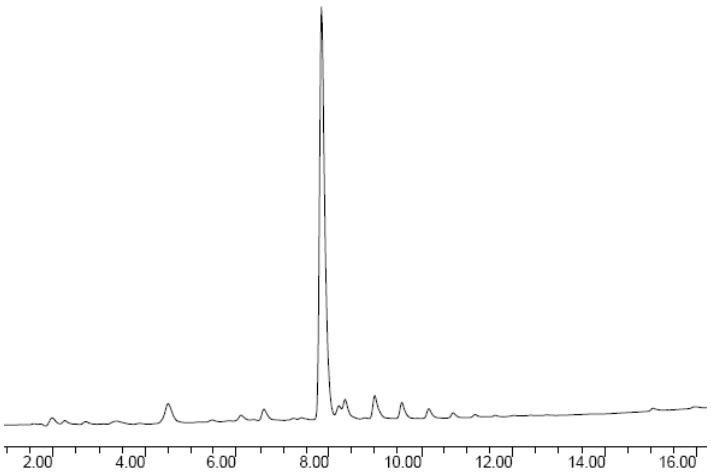
Ac-Glu-Phe-Leu-Lac-Val-OH (6a): HRMS (ESI-MS, ES^+): $C_{30}H_{44}N_4O_{10}$ ($M+H^+$) calcd.: 621.31302, found: 621.31208.



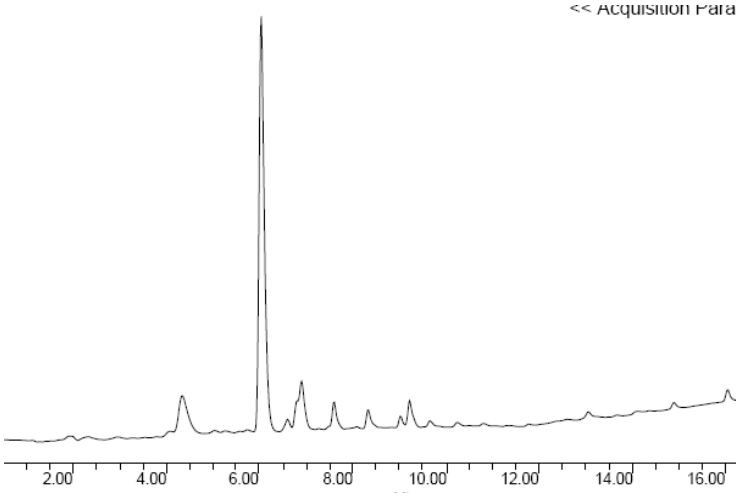
Ac-Lys-Phe-Leu-Lac-Val-OH (6b): HRMS (ESI-MS, ES^+): $C_{31}H_{49}N5O_8$ ($M+H^+$) calcd.: 621.34941, found: 621.36843.



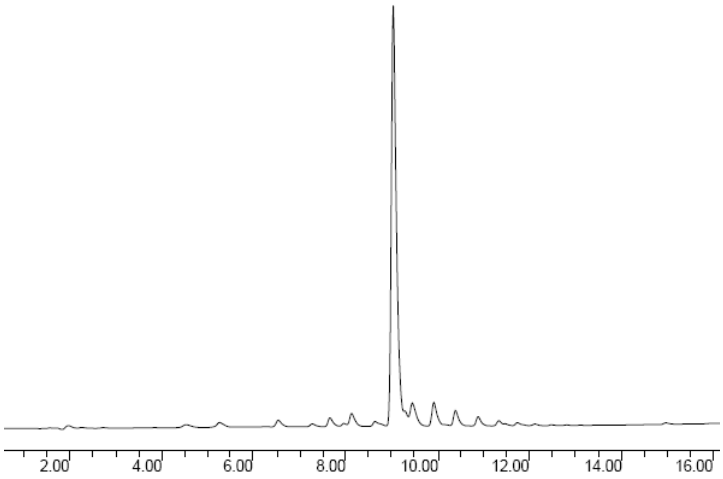
Ac-Thr(Bzl)-Phe-Leu-Lac-Val-OH (5c): HRMS (ESI-MS, ES^+): $C_{36}H_{50}N_4O_9$ ($M+H^+$) calcd.: 683.36506, found: 683.36511.



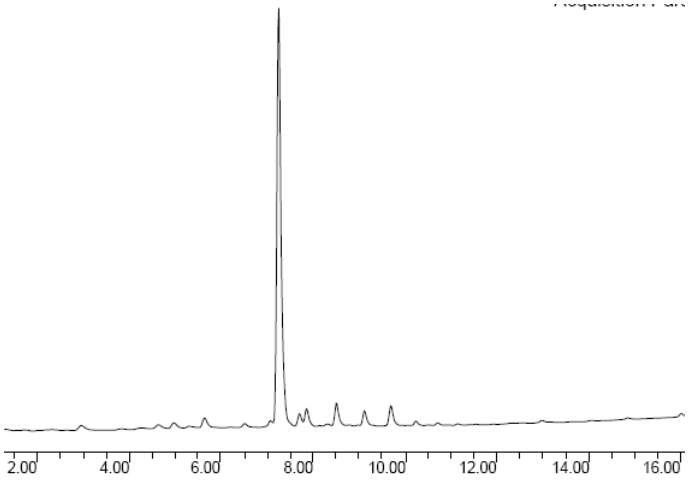
Ac-Thr-Phe-Leu-Lac-Val-OH (6c): HRMS (ESI-MS, ES^+): $C_{29}H_{44}N_4O_9$ ($M+H^+$) calcd.: 593.31811, found: 593.31692.



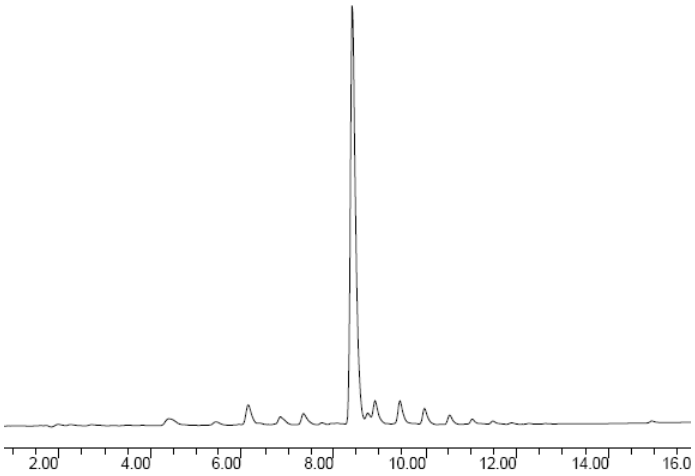
Ac-Tyr(Bzl)-Phe-Leu-Lac-Val-OH (5d): HRMS (ESI-MS, ES^+): $C_{41}H_{52}N_4O_9$ ($M+H^+$) calcd.: 745.38071, found: 745.38043.



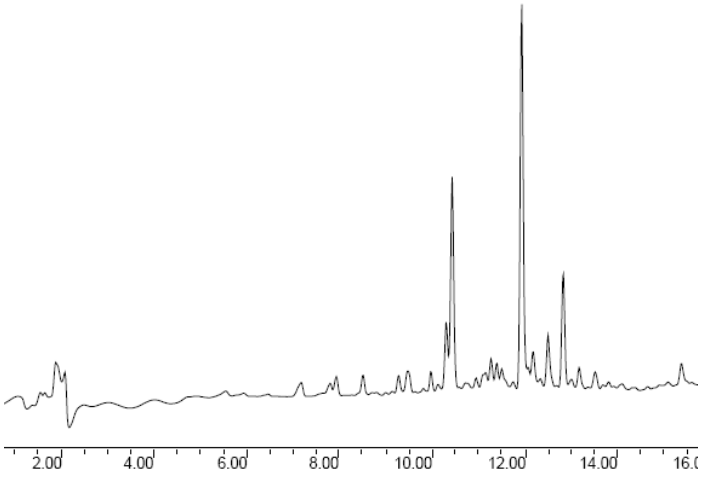
Ac-Tyr-Phe-Leu-Lac-Val-OH (6d): HRMS (ESI-MS, ES^+): $C_{34}H_{46}N_4O_9$ ($M+H^+$) calcd.: 655.33376, found: 655.33270.



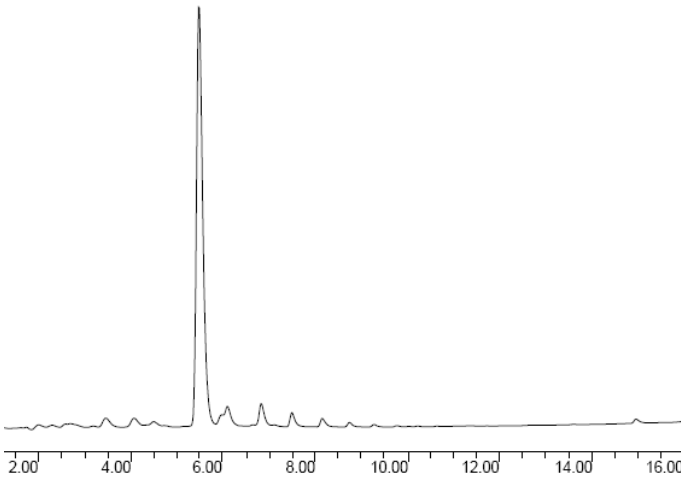
Ac-Cys(pMeBzl)-Phe-Leu-Lac-Val-OH (5e): HRMS (ESI-MS, ES^+): $C_{36}H_{50}N_4O_8S$ ($M+H^+$) calcd.: 699.34221, found: 699.34108.



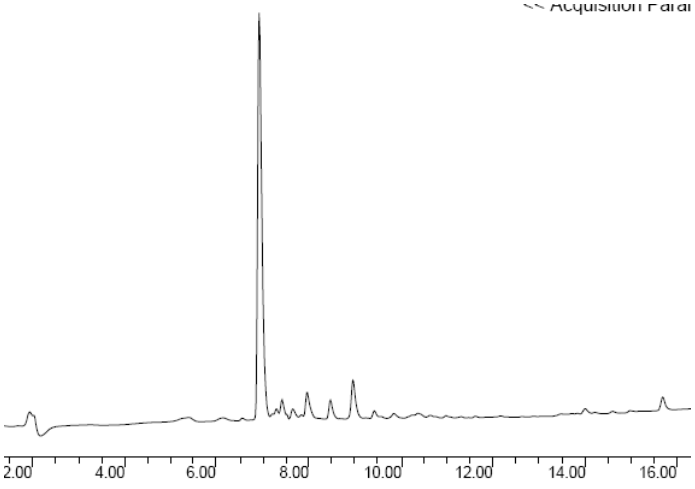
Ac-Cys-Phe-Leu-Lac-Val-OH (6e): HRMS (ESI-MS, ES^+): $C_{28}H_{42}N_4O_8S$ ($M+H^+$) calcd.: 595.27961, found: 595.27728.



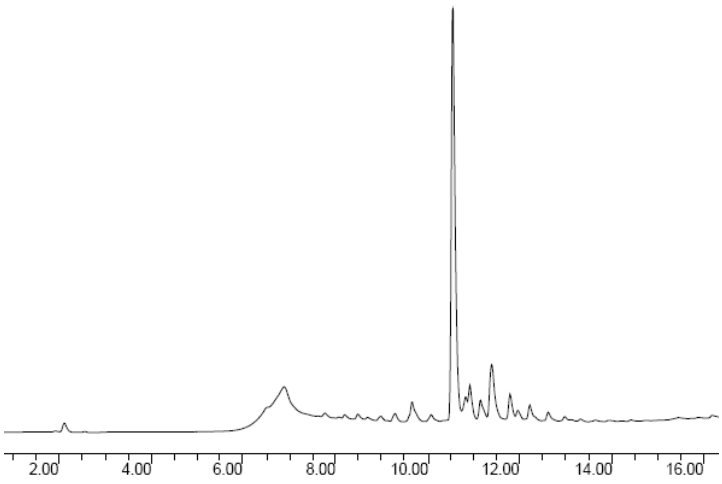
Ac-Arg(Tos)-Phe-Leu-Lac-Val-OH 5f : HRMS (ESI-MS, ES^+): $C_{38}H_{55}N_7O_{10}S$ ($M+H^+$) calcd.: 802.38039, found: 802.38051.



Ac-Arg-Phe-Leu-Lac-Val-OH 6f : HRMS (ESI-MS, ES^+): $C_{31}H_{49}N_7O_8$ ($M+H^+$) calcd.: 648.37154, found: 648.37143.



Ac-Asn(Xan)-Phe-Leu-Lac-Val-OH 5g : HRMS (ESI-MS, ES^+): $C_{29}H_{43}N_5O_9$ ($M+H^+$) calcd.: 606.31335, found: 606.31338.



Quantification of resin functionalization

The Fmoc-group was cleaved with 20% piperidine in DMF (3 x 5 min). The solutions were diluted with DMF and UV-absorbance at 290 nm was measured.

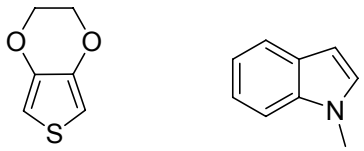
[1] Fosdick, L. S.; Wessinger, G. D. *J. Am. Chem. Soc.* **1938**, *60*, 1465-1466.
[2] Pumpor, K.; Windeisen, E.; Burger K. *J. Heterocyclic Chem.* **2003**, 40, 435-442.
[3] Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1990**, *31*, 7363-7366.

4

Derivats del 3,4- Etilendioxitiofè i l'1-Metilindole

Els anells heteroaromàtics del 3,4-etilendioxitiofè (EDOT) i el 1-metilindole són molt rics en electrons degut a que són sistemes π-excedents amb substituents donadors d'electrons units a ells. Això fa que els seus derivats siguin bons candidats a grups protectors làbils a medi àcid ja que els cations resultants de la seva eliminació seran molt estables.

L'objectiu del següent capítol és demostrar la utilitat de grups protectors derivats del 3,4-etilendioxitiofè (EDOT) i l'1-metilindole com a grups protectors làbils a medi àcid. Les funcionalitats que s'han protegit han estat àcids carboxílics, amides i el grup guanidini de l'arginina.



3,4-Etilendioxitiofè i 1-metilindole

EDOTn and MIM, New Peptide Backbone Protecting Groups

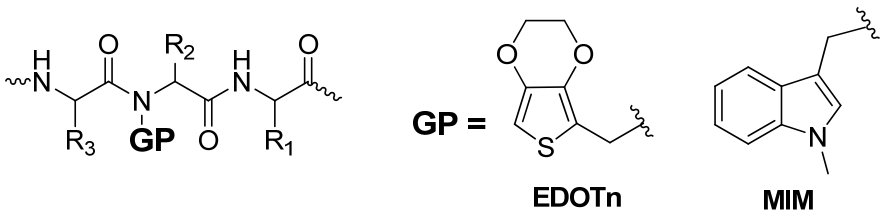
EDOTn i MIM, Nous Protectors d'Amides de l'Esquelet Peptídic

Albert Isidro-Llobet, Xavier Just-Baringo, Mercedes Álvarez and Fernando Albericio

Biopolymers 90 (2008), 444-449

Resum

El següent treball, tracta del desenvolupament de nous protectors per amides de l'esquelet peptídic ("backbone protection"). Recentment la protecció de part de les amides de l'esquelet peptídic ha permès la síntesi de pèptids complexos d'elevat interès evitant l'agregació de les cadenes peptídiques i reaccions secundàries com la formació d'aspartimides. Els nous protectors presentats: EDOTn (3,4-etilendioxi-2-tenil) i MIM (1-metil-3-indolilmetil) solucionen alguns dels inconvenients associats als protectors actuals com la seva difícil eliminació i el seu impediment estèric.



EDOTn and MIM, New Peptide Backbone Protecting Groups

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ABSTRACT:

In recent years, backbone protection has allowed the synthesis of complex peptidic sequences of high interest by preventing chain aggregation and aspartimides formation. Nevertheless, the backbone protectors currently used have some drawbacks: they are difficult to remove and show high steric hindrance. The new backbone protectors presented in this study (EDOTn and MIM) represent an improvement in both aspects. © *2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 444–449, 2008.*

Keywords: aspartimides; backbone protection; "difficult" peptide sequences; diketopiperazines; EDOT; EDOTn; MIM; side-reactions; solid phase

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One of the authors (F.A.) recalls a short and informal talk with Bruce in Braga (Portugal), after attending the Josef Rudinger Memorial Lecture delivered by Bob Sheppard, in which he introduced Hmb backbone protection. Bruce mentioned that backbone protection could be one of the solutions to make SPS applicable for all kinds of peptides. Hopefully, EDOTn and MIM are a step in this direction.

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Contract grant number: 2005SGR 00662
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INTRODUCTION

Peptide synthesis in both solid-phase and solution strategies are hallmarked by controlled formation of the peptide bond[1] and an appropriate combination of protecting groups.[2] With regard to the latter, intense research effort has been devoted to the development of C-terminal-protecting groups, mainly polymeric ones for solid-phase strategies,[3] or α-amino[2b] and side-chain-protecting groups.[2c] In contrast, little attention has been dedicated to the protection of the amide backbone,[4] in the belief that the NH was already protected. However, this is not strictly true because at least three interactions involving the NH backbone have been described. First of all, growing synthetic peptides can aggregate as a result of intra- and interchain interaction, thereby hindering the synthetic process.[5] Second, the NH of the amino acid (usually Gly, Ser or Thr) before the Asp residue can react with the β-carboxyl group of the Asp to render aspartimides, which, in addition to the corresponding β-peptide, can produce more side-reactions (Figure 1).[6] Although this reaction is more severe with the Fmoc/^{t}Bu strategy and the sequence Asp-Gly, it can occur in many other cases.

Finally, and although less frequent, internal diketopiperazine formation involving the NH and the activated carboxylic acid of the previous amino acid has been reported (Figure 2).[7]

Protection of this amide bond can result in the suppression or at least minimization of these undesired interactions. Thus, it has been described that solid-phase synthesis (SPS) of long peptides with sequences prone to aggregation can be improved by protecting some of the amides of the peptide.[8] This backbone protection prevents the formation of hydrogen bonds between peptide chains and aggregation due to steric hindrance, thereby leading to faster and more predictable coupling and deprotection reactions.

The most used backbone protectors are pseudoprolines (for Thr and Ser),[9] Hmb (2-hydroxy-4-methoxybenzyl),[10] and

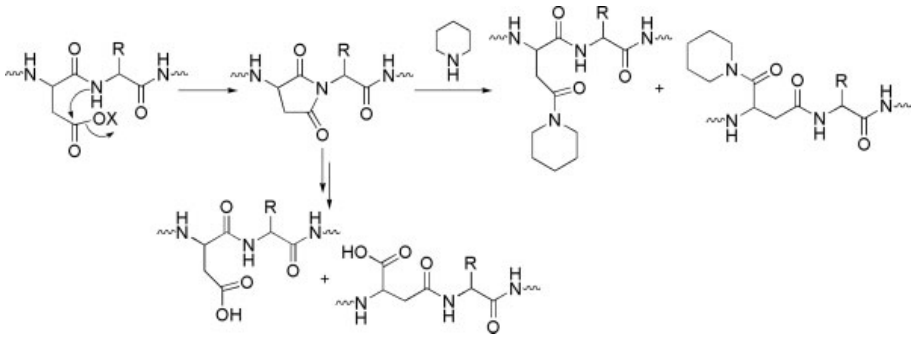

FIGURE 1 Mechanism of aspartimide formation with typical side products shown.

Dmb (2-4-dimethoxybenzyl, used basically for Gly).[11] Several examples of their use for the synthesis of "difficult" peptides have been described.[12] Furthermore, building blocks protected with Hmb and Dmb (single amino acids or dipeptides) have also been used to prevent aspartimide formation.[13]

Nevertheless, the backbone-protecting groups available at present have some drawbacks. Thus, pseudoprolines, which are commercially available, are limited to sequences containing Ser and Thr. Hmb can hinder depsipeptide synthesis and postsynthetic phosphorylation of the free phenolic function,[11] whereas Dmb is sterically hindered and several authors report that Dmb removal can sometimes be problematic as prolonged treatments with TFA are required.[14]

Here we present two new backbone-protecting groups with the aim to solve some of the above-mentioned problems. The 1-methyl-3-indolylmethyl (MIM) and 3,4-ethylenedioxy-2-thenyl (EDOTn) were inspired by linkers for the SPS of C-terminal-modified peptides.[15,16]

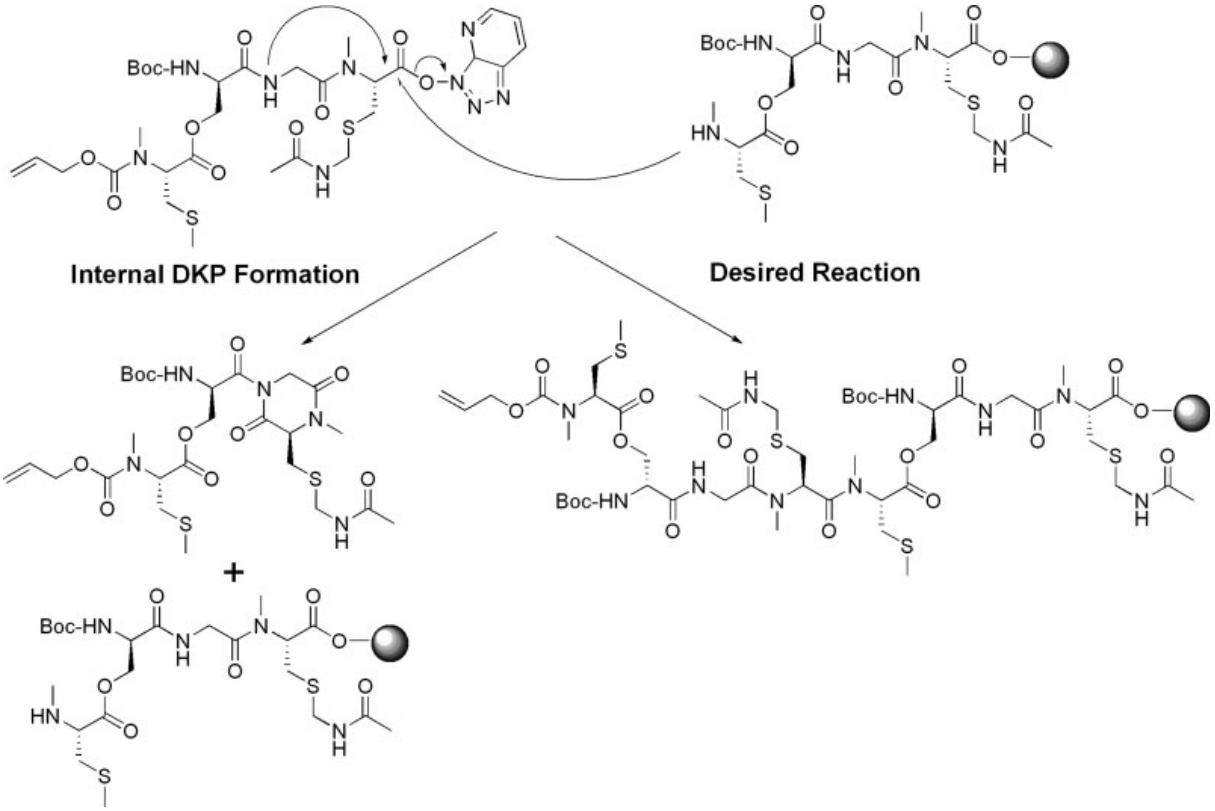

FIGURE 2 Internal DKP formation.[7]

EXPERIMENTAL SECTION

General

The 3,4-ethylenedioxythiophene and 1-methylindole-3-carbaldehyde were provided by Sigma-Aldrich. Rinkamide MBHA-resin, protected Fmoc-amino acid derivatives, HOBt, DIPEA, DIC, piperidine, TFA, DMF, MeCN (HPLC grade), and CH_2Cl_2 were purchased from several sources. All commercial reagents and solvents were used as received. Solid-phase syntheses were performed in polypropylene syringes (2–5 mL), each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine–DMF (2:8, v/v) (1 × 2 min, 2 × 10 min). Washings between deprotection, coupling, and deprotection steps were performed with DMF (5 × 0.5 min) and CH_2Cl_2 (5 × 0.5 min) using 10 mL solvent/g resin each time. Peptide synthesis transformations and washings were done at 25°C. A HPLC reversed-phase column Symmetry C18, 4.6 mm × 150 mm, 5 μm was used. Analytical HPLC was performed on an instrument comprising two solvent delivery pumps, an automatic injector dual wavelength detector, and a system controller (Breeze V3.20), and on an instrument with two solvent delivery pumps, an automatic injector, and a variable wavelength detector (photodiode array). UV detection was at 220 nm, using linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA).

3,4-Ethylenedioxythiophene-2-Carbaldehyde

The 3,4-ethylenedioxythiophene (5g, 35 mmol) was dissolved in 65 mL of dry THF under Argon. The solution was cooled to −78°C and *n*-butyl lithium (21.5 mL, 35 mmol) was slowly added. The temperature was slowly raised to 0°C and the resulting suspension was stirred for 1 h. The solution was then cooled to −78°C, dry DMF was added, and the mixture was stirred for 90 min at room temperature. The mixture was then poured onto crushed ice containing HCl and the white precipitate of aldehyde was filtered, washed with cold H_2O, and dried, yielding 5.3 g of a white solid (89% of yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.91 (d, 1H, CHO, J = 1.1 Hz), 6.80 (d, 1H, J = 1.1 Hz), 4.37 (m, 2H, CH_2), 4.27 (m, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.4 and 180.3 (CHO), 148.7 (C), 142.0 (C), 118.7 (C), 111.0 (CH), 65.5 (CH_2), 64.6 (CH_2).

Fmoc-(EDOTn)-Gly-OH

$NaBH_3CN$ (310 mg, 4.9 mmol) was added to a suspension of 3,4-ethylenedioxythiophene-2-carbaldehyde (700 mg, 4.1 mmol) and H-Gly-OH (247 mg, 3.3 mmol) in H_2O-dioxane (1:1) (10 mL), the pH was adjusted to 5–6 with 1*N* HCl and the resulting suspension was stirred overnight.

The pH was acidified to 3–4 and the solution was evaporated to dryness, yielding 1.21 g of a brown solid, which was used without further purification. This solid was suspended in 10% aqueous Na_2CO_3 (18 mL) at 0°C, and Fmoc-Cl (759 mg, 2.93 mmol) in 1,4-dioxane (15 mL) was added. The solution was warmed to room temperature and the pH was kept between 8 and 10 by adding 10% aqueous Na_2CO_3 H_2O (10 mL) and dioxane (7 mL) were added to make the stirring easier. The mixture was then left to stir overnight. The mixture was poured into H_2O (120 mL) and washed with MTBE (3 × 75 mL). The aqueous phase was acidified to pH 3 with HCl—H_2O (1:2) and extracted with EtOAc (3 × 75 mL). The organic phases were pooled, dried over $MgSO_4$, and evaporated to dryness. MeOH was added and the solution was evaporated again, to yield 1.4 g of a brownish oil, which, after column chromatography, rendered the final product as a solid (404 mg, 27% of yield). The product was characterized by HPLC, ESMS, and NMR.

^{1}H NMR (400 MHz, DMSO): δ = 7.87 (m, 2H Fmoc), 7.63 and 7.59 (2d, 2H Fmoc, J = 7.4 Hz), 7.40 (m, 2H Fmoc), 7.29 (m, 2H Fmoc), 6.49 and 6.47 (2s, 1H thiophene), 4.42 and 4.38 (2s, 2H, CH_2), 4.36–4.22 (m, 3H, CH and CH_2 from Fmoc), 4.16 (m, 4H, $2CH_2O$), 3.88 and 3.84 (2s, 2H, CH_2 from Gly). ^{13}C NMR (100 MHz, DMSO): δ = 171.3 and 171.1 (COOH), 154.6 (CO from Fmoc), 144.4 and 144.3 (C), 141.7 and 141.7 (C), 141.5 and 141.3 (C), 140.5 and 140.2 (C), 128.4 (CH), 127.8 (CH), 125.8 (CH), 120.8 (CH), 112.4 and 112.2 (C), 99.1 and 99.0 (CH), 68.0 and 67.9 (CH_2), 65.3 and 65.0 (CH_2), 48.4 (CH_2), 47.9 (CH_2), 47.2 (CH), 42.6 and 42.4 (CH_2).

Fmoc-(MIM)-Gly-OH

$NaBH_3CN$ (948 mg, 14.1 mmol) was added to a suspension of 1-methylindole-3-carbaldehyde (2g, 12.6 mmol) and H—Gly—OH (755 mg, 10.1 mmol) in H_2O-dioxane (1:1) (30 mL), the pH was adjusted to 5–6 with 1*N* HCl and the resulting suspension was stirred overnight. The pH was acidified to 3–4 and the solution was evaporated to dryness. MeOH at 50°C (40 mL) was added to the residue and the mixture was stirred for 5 min and filtered, yielding a white solid (489 mg). The liquors were evaporated to dryness, a minimum amount of MeOH was added and the white solid obtained was filtered, washed with cold MeOH, and dried (588 mg). Both solids were identical by HPLC and had the mass of MIM-Gly-OH.

Fmoc-Cl (1.33 g, 5.15 mmol) in dioxane (15 mL) was slowly added to a solution of MIM-Gly-OH (900 mg, 4.12 mmol) in 10% aqueous Na_2CO_3 (15 mL) at 0°C. The solution was warmed to room temperature and the pH was kept between 8 and 10 by adding 10% aqeuous Na_2CO_3, and was then stirred overnight. After that, TLC (EtOAc–MeOH, 9:1) showed that all MIM-Gly-OH had reacted. H_2O (75 mL) was added to the reaction mixture, which was washed with MTBE (3 × 75 mL). The aqueous phase was acidified to pH 3 with HCl—H_2O (1:2) and extracted with EtOAc (3 × 75 mL). The organic phases were pooled, dried with $MgSO_4$, and evaporated to dryness to yield 2.1 g of a yellow oil, which, after column chromatography, (DCM–MeOH, 99:1) yielded the final product as a white solid (795 mg, 43% of yield). The product was characterized by HPLC, ESMS, and NMR.

^{1}H NMR (400 MHz, DMSO): δ = 7.86 (d, 2H Fmoc), 7.65 and 7.60 (2d, 2H Fmoc, J = 7.5 Hz), 7.53–6.92 (m, 9H), 4.60–4.21 (m, 5H), 3.79 (s, 1H), 3.74 and 3.68 (2s, 2H), 3.71 (s, 1H). ^{13}C NMR (100 MHz, DMSO): δ = 171.6 and 171.3 (COOH), 156.2 (CO from Fmoc), 144,6 and 144.4 (C), 141.5 and 141.3 (C), 137.6 and 137.4 (C), 130.3 and 129.7 (CH), 128.4 and 128.3 (CH), 127.8 (CH), 127.6 and 127.4 (C), 125.8 and 125.6 (CH), 122.1 and 122.0 (CH), 120.8 and 120.8 (CH), 119.7 and 119.6 (CH), 119.6 and 119.1 (CH), 110.4 (CH), 109.7 (C), 67.8 and 67.3 (CH_2), 47.5 and 46.8 (CH_2), 47.4 (CH), 42.8 and 42.3 (CH_2), 33.1 (CH_3).

H-Tyr-Gly-Xaa-Phe-Leu-NH_2 (Xaa = MIM-Gly-OH, EDOTn-Gly-OH or 2,4-dimethoxybenzyl-Gly-OH)

Rinkamide MBHA resin (300 mg, 0.67 mmol/g) was placed in a polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed. Fmoc-L-Leu-OH (284.2 mg, 0.805 mmol, 4 eq) and Fmoc-L-Phe-OH (311.5 mg, 0.804 mmol, 4 eq) were coupled sequentially using DIC (124.5 μL, 0.805 mmol, 4 eq) and HOBt (108.6 mg, 0.805 mmol, 4 eq) in DMF, $t = 1$ h. The last Fmoc group was removed and the resin was divided into three equal parts.

Fmoc-(EDOTn)-Gly-OH (121 mg, 0.268 mmol, 4 eq), Fmoc-(MIM)-Gly-OH (118.1 mg, 0.268 mmol, 4 eq), and Fmoc-(Dmb)-Gly-OH (119.9 mg, 0.268 mmol, 4 eq) were coupled to each aliquot of the resins using DIC (41.5 μL, 0.268 mmol, 4 eq) and HOBt (36.2 mg, 0.536 mmol, 4 eq) in DMF, for 1 h. Fmoc group was removed and after washing with DMF and DCM, each resin was then divided into five equal parts in order to perform coupling assays.

Coupling Assays

An aliquot of each resin was treated with Fmoc-Gly-OH (39.8 mg, 0.134 mmol, 10 eq), DIC (10.4 μL, 0.067 mmol, 5 eq), and DMAP (2 mg, 0.016 mmol, 1.25 eq) in DCM–DMF for 3 h. The resin was divided into two equal aliquots. The Fmoc group of the first aliquot was removed and Fmoc-Tyr(^{t}Bu) (12.3 mg, 0.027 mmol, 4 eq) was coupled using DIC (4.15 μL, 0.027 mmol, 4 eq) and HOBt (3.6 mg, 0.027 mmol, 4 eq). The coupling was repeated in the same conditions, the Fmoc group was removed and the peptide was cleaved from the resin with TFA-DCM-H_2O (95:2.5:2.5), for 1 h. TFA was evaporated, washings (2) with diethyl ether were performed, H_2O was added, and the solution was lyophilized.

Fmoc-Gly (15.9 mg, 0.054 mmol, 8 eq) was recoupled in the second aliquot using HATU (19.4 mg, 0.051 mmol, 7.6 eq) and DIPEA (27.5 μL, 0.161 mmol, 24 eq) in DMF for 3 h, then the same procedure as for the first aliquot was followed. The crude products from the two aliquots of resin were analyzed by HPLC and ESMS.

H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2 from EDOTn-Gly-OH and MIM-Gly-OH

Rinkamide MBHA resin (200 mg, 0.67 mmol/g) was placed in a polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed. Fmoc-L-Ile-OH (189.4 mg, 0.536 mmol, 4 eq) and Fmoc-L-Tyr(^{t}Bu)-OH (245.7 mg, 0.536 mmol, 4 eq) were coupled sequentially using DIC (83 μL, 0.536 mmol, 4 eq) and HOBt (72.4 mg, 0.536 mmol, 4 eq) in DMF, for 1 h. The last Fmoc group was removed and the resin was divided into two equal aliquots.

Fmoc-(EDOTn)-Gly (121 mg, 0.268 mmol, 4 eq) and Fmoc-(MIM)-Gly-OH (118.1 mg, 0.268 mmol, 4 eq) were coupled to the two resins using DIC (41.5 μL, 0.268 mmol, 4 eq) and HOBt (36.2 mg, 0.268 mmol, 4 eq) in DMF, for 1 h. The Fmoc group was removed and Fmoc-L-Asp(O^{t}Bu)-OH (275.7 mg, 0.67 mmol, 10 eq) was coupled to the resin through its symmetric anhydride formed with DIC (51.9 μL, 0.335 mmol, 5 eq), in the presence of DMAP (12.3 mg, 0.101 mmol, 1.5 eq) in DCM–DMF, for 3 h. A recoupling was performed using Fmoc-L-Asp(O^{t}Bu)-OH (110.3 mg, 0.268 mmol, 4 eq), HATU (96.8 mg, 0.255 mmol, 3.8 eq), and DIPEA (135.6 μL, 0.804 mmol, 12 eq). Fmoc group was removed and Fmoc-L-Lys(Boc) (125.6 mg, 0.268 mmol, 4 eq) and Fmo-L-Val (91.0 mg, 0.268 mmol, 4 eq) were coupled sequentially using DIC (83 μL, 0.536 mmol, 4 eq) and HOBt (72.4 mg, 0.536 mmol, 4 eq) in DMF, for 1 h. (both couplings were performed twice).

After removal of the last Fmoc group, the resins were treated for 1 h with TFA-DCM-H_2O (95:2.5:2.5). The TFA was evaporated, Et_2O washings were performed, H_2O was added and the solutions were lyophilized to render 13.0 mg and 15.4 mg of EDOTn and MIM peptides, respectively, which were analyzed by HPLC and ESMS.

RESULTS AND DISCUSSION

General

A suitable new backbone protector should be easy to synthesize, should not be sterically hindered, and should be easily removed from amides during the final TFA cleavage treatment. MIM and the EDOTn, which are electron-rich systems, have been shown to be acid-labile as amide linkers.[15,16]

The backbone-protected derivatives of Gly were easily prepared via reductive amination with the commercially available 1-methylindole-3-carbaldehyde and the 3,4-ethylenedioxythiophene-2-carbaldehyde, which was easily prepared from the heterocycle.[17,18] Finally, Fmoc introduction rendered the appropriate building block for the synthesis (Figure 3). All reactions gave acceptable yields and excellent purities.

Leu-enkephalinamide (H-Tyr-Gly-Gly-Phe-Leu-NH_2) was taken as a model to assay the acylation of the ^{3}Gly with the two backbone-protecting groups and with Dmb, which was used as a reference.

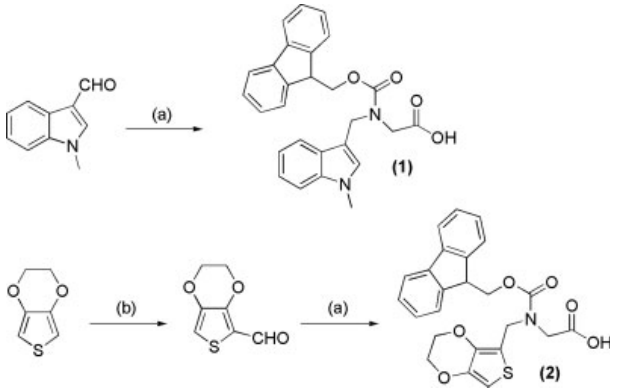

FIGURE 3 (a) (i) H-Gly-OH (0.8 eq), $NaBH_3CN$ (1.2 eq) in H_2O-dioxane (1:1), pH 5–6, room temperature, overnight. (ii) Fmoc-Cl, $Na_2CO_{3\ (aq)}$-dioxane, pH 8–10, room temperature, overnight; 43% overall yield for Fmoc-(MIM)-Gly-OH (1) and 27% overall yield for Fmoc-(EDOTn)-Gly-OH (2). (b) BuLi (1 eq), DMF in dry THF; 89% yield.

Table I Incorporation of Fmoc-Gly-OH on PG-Gly-Phe-Leu-NH-Rinkamide Resin[a]

Dmb (%)	MIM (%)	EDOTn (%)
65.3[b] + 12.2[c]	51.6%[b,d]	94.2%[b,d]
67.6[b] + 28.1[c]	81.6%[b,d]	97.6%[b,d]

[a] Yields were calculated by HPLC of the crude product, H-Tyr-Gly-Gly-Phe-Leu-NH_2 vs. H-Tyr-Gly-Phe-Leu-NH_2; Coupling conditions: Fmoc-Gly-OH (10 eq), DIC (5 eq) DMAP (0.5 eq), 3 h, room temperature in DCM–DMF; Fmoc-Gly-OH (8 eq), HATU (7.6 eq), DIPEA (16 eq), 3h, room temperature in DMF.
[b] Free pentapeptide.
[c] Dmb-protected pentapeptide.
[d] No protected pentapeptide was detected.

EDOTn was less sterically hindered than Dmb, whereas MIM was more sterically hindered but moderate coupling yields were achieved with the two coupling cycles performed (Table I).

In addition, we detected considerable amounts of backbone-protected Leu-enkephalinamide when Dmb was used. In contrast, no protected Leu-enkephalinamide was found where EDOTn and MIM were used (Table I).

Use of the Backbone Protection to Prevent Aspartimide Formation

Here we used two new backbone-protecting derivatives of Gly to synthesize the peptide H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2, a sequence prone to aspartimide formation.[6b]

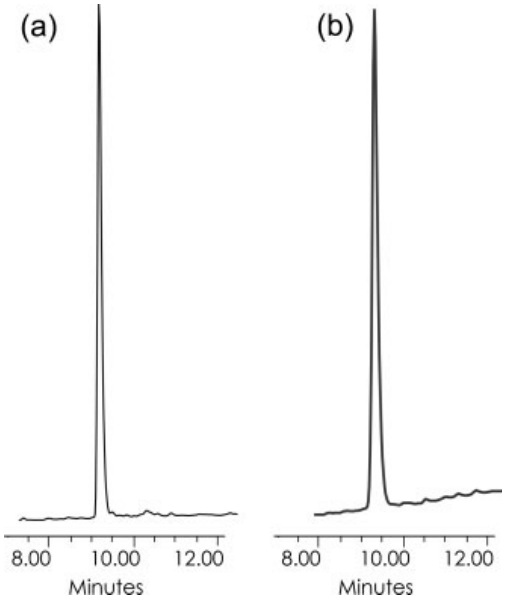

FIGURE 4 HPLC of H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2: (a) using Fmoc-(EDOTn)-Gly-OH, (b) using Fmoc-(MIM)-Gly-OH.

The peptide was synthesized on a Rinkamide resin using Fmoc amino acids and DIC, and HOBt as coupling reagents. DIC/DMAP and HATU/DIPEA were used to incorporate the Asp residue on the derivatizated Gly-containing resin. After final TFA cleavage from the resin, the final peptides were obtained with good purities in both cases (Figure 4). HPLC-MS did not show the presence of the corresponding piperidides, which would indicate aspartimide formation.

CONCLUSIONS

The two backbone protectors proposed, MIM and EDOTn, are more easily removed in acidic conditions than the Dmb group. In addition, 1-methylindole-3-carbaldehyde is commercially available and inexpensive, and 3,4-ethylenedioxythiophene-2-carbaldehyde is easily synthesized from the commercially available 3,4-ethylenedioxythiophene.

The backbone-protecting group derived from 3,4-ethylenedioxythiophene-2-carbaldehyde is less sterically hindered than Dmb, thereby allowing better coupling yields.

The two backbone-protecting groups can be useful to mask the NH of the peptide bond and therefore to prevent side interactions caused by its presence. The preparation of dipeptides containing these backbone-protecting groups will open a broader application of these derivatives.

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REFERENCES

1. (a) Albericio, F.; Carpino, L. A. In Methods in Enzymology, Solid-Phase Peptide Synthesis; Fields, G. B., Ed.; Academic Press: Orlando, 1997; Vol. 289, pp 104–126; (b) Albericio, F.; Chinchilla, R.; Dodsworth, D.; Nájera, C. Org Prep Proc Int 2001, 33, 203–303; (c) So-Yeop, H.; Young-Ah, K. Tetrahedron 2004, 60, 2447–2467.
2. (a) Albericio F. Biopolymers (Pept Sci) 2000, 55, 123–139; (b) Wade, J. D. In Solid-Phase Synthesis; Kates, S. A.; Albericio, F., Eds., Marcel Dekker: New York, 2000; pp 103–128; (c) Doherty-Kirby, A. L.; Lajoie, G. A. In Solid-Phase Synthesis; Kates, S. A.; Albericio, F., Eds.; Marcel Dekker: New York, 2000; pp 129–195.
3. (a) Guillier, F.; Orain, D.; Bradley, M. Chem Rev (Washington, DC) 2000, 100, 2091–2157; (b) Alsina, J.; Albericio, F. Biopolymers (Pept Sci) 2003, 71, 454–477.
4. Johnson, T.; Owen, D.; Quibell, M.; Sheppard, R. C. Peptides 1992, In Proceedings to the 22nd European Peptide Symposium; Schneider, C. H.; Eberle, A. N., Eds.; ESCOM Science: Leiden, 1993; pp 23–24.
5. Meister, S. M.; Kent, S. B. H. In Peptides: Structure, Function, Proceedings to the 8th American Peptide Symposium; Hruby, V. J.; Rich, D. H., Eds.; Pierce Chemical Company, Rockford, Ill, 1983; pp 103–106.

6. (a) Tam, J. P.; Riemen, M. W.; Merrifield, R. B. Pept Res 1988, 1, 6–18; (b) Nicolás, E.; Pedroso, E.; Giralt, E.; Tetrahedron Lett 1989, 30, 497–451; (c) Yang, Y.; Sweeney, W. V.; Schneider, K.; Thornqvist, S.; Chait, B. T.; Tam, J. P. Tetrahedron Lett 1994, 35, 9689–9692; (d) Doelling, R.; Beyermann, M.; Haenel, J.; Kernchen, F.; Krause, E.; Franke, P.; Brudel, M.; Bienert, M. J Chem Soc Chem Commun 1994, 853–854; (e) Lauer, J. L.; Fields, C. G.; Fields, G. B. Lett Pept Sci 1995, 1, 197–205; (f) Cebrian, J.; Domingo, V.; Reig, F. J Pept Res 2003, 62, 238–244; (g) Zahariev, S.; Guarnaccia, C.; Pongor, C. I.; Quaroni, L.; Cemazar, M.; Pongor, S. Tetrahedron Lett 2006, 47, 4121–4124.
7. Tulla-Puche, J.; Bayó-Puxan, N.; Moreno, J. A.; Francesch, A. M.; Cuevas, C.; Álvarez, M.; Albericio, F. J Am Chem Soc 2007, 129, 5322–5323.
8. (a) Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sheppard, R. C. Int J Pept Protein Res 1992, 40, 300–307; (b) Hyde, C.; Johnson, T.; Owen, D.; Quibell, M.; Sheppard, R. C. Int J Pept Protein Res 1994, 43, 431–440.
9. (a) Mutter, M.; Nefzi, A.; Sato, T.; Sun, X.; Wahl, F.; Wuhr, T. Pept Res 1995, 8, 145–153; (b) Haack, T.; Muter, M. Tetrahedron Lett 1992, 33, 1589–1592.
10. Johnson, T.; Quibell, M.; Owen, D.; Shepard, R. C. J Chem Soc Chem Commun 1993, 369–372.
11. Johnson, T. Packman, L. C.; Hyde, C. B.; Owen, D.; Quibell, M. J Chem Soc Perkin Trans 1 1996, 719–728.
12. (a) Use of pseudoprolines: White, P. In Peptides 1998, Proceedings to the 25th European Peptide Symposium; Bajusz, S.; Hudecz, F., Eds.; Akadémiai Kiadó: Budapest, 1999; pp 120–121; (b) Use of Hmb: Ede, N. J.; Ang, K. H.; James, I. W.; Bray, A. M. Tetrahedron Lett 1996, 37, 9097–9100; (c) Use of Dmb:Zahariev, S.; Guarnaccia, C.; Zanuttin, F.; Pintar, A.; Esposito, G.; Maravic, G.; Krust, B.; Hovanessian, A. G.; Pongor, S. J Pept Sci 2005, 11, 17–28; (d)Comparative study: Sapson, W.; Patsiouras, H.; Ede, N. J. J Pept Sci 1999, 5, 403–409.
13. (a) Quibell, M.; Owen, D.; Packman, L. C.; Johnson, T. J Chem Soc Chem Commun 1994, 20, 2343–2344; (b) Mergler, M.; Dick, F.; Sax, B.; Weiler, P.; Vorherr, T. J Pept Sci 2003, 9, 36–46.
14. (a) Weygand, F.; Steglich, W.; Bjarnason, J.; Akhtar, R.; Khan, M. Tetrahedron Lett 1966, 29, 3483–3487; (b) Thamm, P.; Kolbeck, W.; Musiol, H.-J.; Moroder, L. In Synthesis of Peptides and Peptidomimetics; Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C., Eds.; Houben-Weyl, Methods of Organic Chemistry, Georg Thieme Verlag: Stuttgart, Vol. E 22a, 2002; pp 260–275.
15. Jessing, M.; Brandt, M.; Jensen, K. J.; Christensen, J. B.; Boas, U. J Org Chem 2006, 71, 6734–6741.
16. Estep, K. G.; Nelpp, C. E.; Stephens Stramiello, L. M.; Adam, M. D.; Allen, M. P.; Robinson, S.; Roskamp, E. J Org Chem 1998, 63, 5300–5301.
17. Zhang, Z.; Chaltin, P.; Van Aerschot, A.; Lacey, J.; Rozenski, J.; Busson R.; Herdewijn, P. Bioorg Med Chem 2002, 10, 3401–3413.
18. Mohankrishnan, A. K.; Hucke, A.; Lyon, M. A.; Lakshmikantham, M. V.Cava, M. P. Tetrahedron 1999, 55, 11745–11754.

Phenyl-EDOTn Derivatives as Super Acid Labile Carboxylic Acid Protecting Groups for Peptide Synthesis

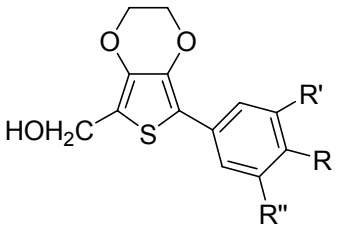
Derivats del Fenil-EDOTn com a Grups Protectors d'Àcids Carboxílics Molt Làbils a Medi Àcid per Síntesi de Pèptids

Albert Isidro-Llobet, Mercedes Álvarez and
Fernando Albericio

Tetrahedron Letters 49 (2008), 3304-3307

Resum

Aquest article té com a objectiu presentar nous grups protectors per a àcids carboxílics làbils a molt baixes concentracions de TFA. Aquests protectors es basen en l'alcohol 3,4-etilendioxitenílic (EDOTn-OH) sobre el qual s'han incorporat en posició 5 anells benzènics rics en electrons per tal d'incrementar la seva labilitat a àcids per efecte ressonant. S'ha realitzat un estudi comparatiu d'aquests derivats i s'ha justificat la seva diferent labilitat a àcids amb raonaments teòrics basats en la bibliografia existent que posteriorment s'han demostrat experimentalment. Tots els derivats estudiats han resultat ser làbils a molt baixes concentracions de TFA (0.01-0.5 %).



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Phenyl-EDOTn derivatives as super acid labile carboxylic acid protecting groups for peptide synthesis

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Abstract

A series of new 3,4-ethylenedioxy-2-thenyl (EDOTn) derived alcohols have been synthesized and evaluated as super acid labile carboxylic acid protecting groups. All the derivatives are labile to very low concentrations of TFA (0.01–0.5%).
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Keywords: Peptide synthesis; EDOTn; EDOT; Acid labile protecting groups; Carboxylic acid protection

1. Introduction

Nowadays, most peptide syntheses are carried out on solid phase in the C to N direction with the α-carboxylate of the C-terminal amino acid attached to the solid support.[1] Nevertheless, the synthesis of several important peptides involves side chain or backbone attachment to the resin or synthesis in solution.[2–4] In all these strategies, a suitable protecting group for the C-terminal α-carboxylate is mandatory. In addition to that, carboxylic acid protection is also necessary for the side chains of Asp and Glu and broadly for other carboxylic groups.

In the case of solid phase peptide synthesis, the most widely used strategy is the Fmoc/^{t}Bu strategy, which involves α-amino temporary protection by the base labile Fmoc group, side chain protection with trifluoroacetic acid (TFA) labile protecting groups, usually ^{t}Bu type ones; and cleavage from the resin also with TFA. Therefore, a suitable carboxyl protecting group for solid phase peptide synthesis should be resistant to the base treatments used to remove the Fmoc group and, for most of the applications, the conditions for its removal should leave ^{t}Bu type groups unaltered, allowing the obtention of ^{t}Bu protected peptides as well as other labile moieties present in the molecule such as sugars.

3,4-Ethylenedioxythiophene (EDOT) is a highly electron-rich compound. Thus, protecting groups based on 3,4-ethylenedioxy-2-thenyl (EDOTn) should be very acid labile due to the stabilization of the resulting thenyl carbocation. EDOTn derivatives have recently been described as very acid labile BAL-type linkers[5] as well as amide backbone protectors,[6] which can be removed by TFA–DCM (1:99) and (95:5), respectively.

New EDOTn-derived compounds (**1a**–**e**) as very acid labile carboxylic acid protecting groups are herein described (Fig. 1).

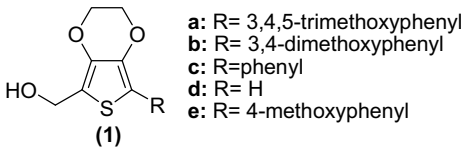

Fig. 1. EDOTn-derived alcohols prepared.

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In the previous works, the acid lability of EDOTn was slightly increased by adding electron-donating substituents via an inductive effect such as alkyl or alkylsulfide.[5]

In the present work, EDOTn has been functionalized with electron-rich phenyl rings in order to increase the acid lability by resonance effect. A series of esters of different phenyl-EDOTn derivatives have been synthesized and their lability to TFA evaluated, investigating also the effect of adding electron-donating substituents to the phenyl ring.

2. Synthesis of alcohols based on electron-rich EDOTn

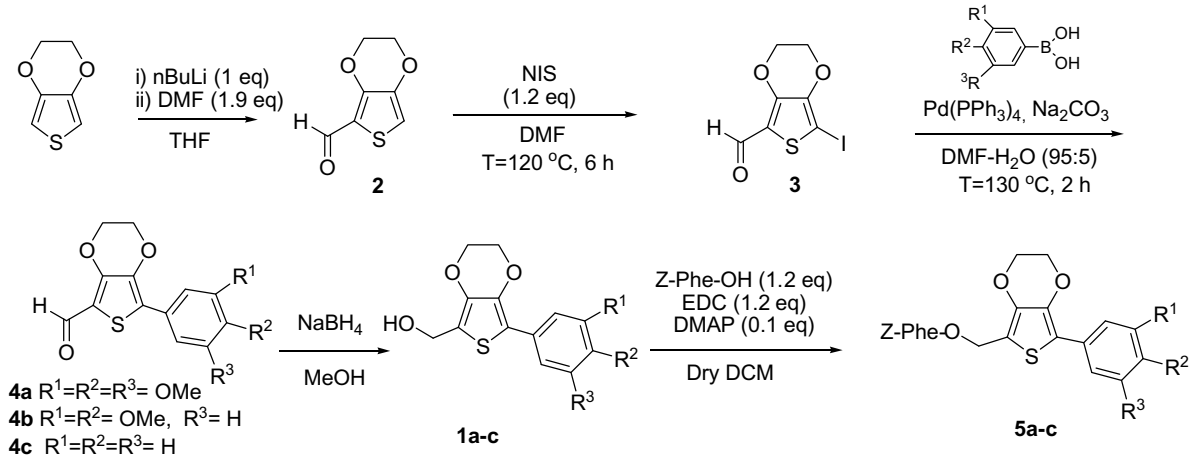
Two different synthetic methods were tested. In Method A (Scheme 1), the commercially available EDOT was formylated in high yield using *n*-BuLi and DMF as described in the previous works of the group[6] yielding **2**. This latter was iodinated by reaction with *N*-iodosuccinimide (NIS) in DMF to afford **3**. Suzuki–Miyaura reaction[7] of the iodinated aldehydes with different phenylboronic acids yielded furnished **4a–c**, which were reduced with $NaBH_4$ to the corresponding alcohols **1a–c**. In Method B, compound **2** was reduced with $NaBH_4$ to alcohol **1d**. Then, it was protected as acetate and iodinated with NIS in DMF. The iodide was converted to **1e** by reaction with 4-methoxyphenyl boronic acid (Scheme 2).

From the two methods, A is the best because although the iodination of **2** requires harsher conditions than the iodination of **1d**, the yields of the Suzuki reactions on compound **3** are much higher than those on compound **6**.[8]

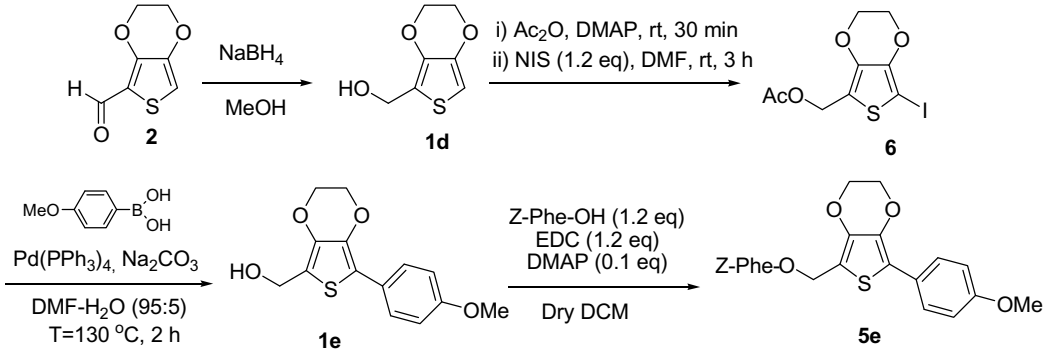
Finally, for evaluation purposes alcohols **1a–e** were esterified with *N*-benzyloxycarbonylphenylalanine (Z–Phe–OH) affording **5a–e** esters in high yields.[9]

3. Removal assays[10]

All the EDOTn derivatives prepared are labile to very low concentrations of TFA (0.01–0.5% TFA in DCM) (Table 1). Thus, they can be removed in the presence of ^{t}Bu type protecting groups. **1a–c** and **1e** are more acid labile than **1d** confirming that the conjugation provided by the aromatic ring stabilizes the resulting carbocation. Among the phenyl-EDOTn derivatives **1a–c** and **1e**, **1b** is more labile than **1e** and the latter more labile than **1c**. Interestingly, **1a** exhibits very similar lability to **1c**. A probable explanation is that the steric hindrance between the three methoxy substituents makes them go out of the aromatic ring plane and consequently their electron-donating effect decreases dramatically. Similar results have been found in a protecting group for Arg.[11] To corroborate this hypothesis, UV spectra of compounds **4a–c** were registered



Scheme 1. The synthesis of *N*-(benzyloxycarbonyl)phenylalanine (Z–Phe–OH) esters of EDOTn derivatives, Method A.



Scheme 2. The synthesis of *N*-(benzyloxycarbonyl)phenylalanine (Z–Phe–OH) esters of EDOTn derivatives, Method B.

Table 1
Acid labilities of the different EDOTn derivatives

	1a (%)	**1b** (%)	**1e** (%)	**1c** (%)	**1d** (%)
0.01% TFA, $t = 10$ min	5	35	20	3	0
0.01% TFA, $t = 1$ h	—	87	68	—	—
0.5% TFA, $t = 5$ min	100	100	100	96	59
0.5% TFA, $t = 1$ h	—	—	—	—	100

showing the following λ_{max}: 362.0, 373.8 and 339.4 nm, respectively. The decrease in λ_{max} (hypsochromic shift) of compound **4a** compared to compound **4b** is probably due to the fact that in compound **4a** the lone pairs of the methoxy groups are less delocalized into the aromatic nucleus.

4. Orthogonality to the Fmoc group

Nowadays, most peptides synthesized on solid phase are prepared using the Fmoc/^{t}Bu strategy. Therefore, it is interesting to check how resistant alcohols **1a–e** are used to the piperidine-mediated removal of the Fmoc group. 3,4-Ethylenedioxythenyl *N*-(benzyloxycarbonyl)phenylalaninate (**5d**) was chosen as a model because it contains the least electron-rich system and can, therefore, be the most base labile. **5d** was treated with piperidine-DMF (2:8) for 48 h. HPLC analysis revealed that only 5% of EDOTn removal took place, confirming that the EDOTn derivatives described are compatible and orthogonal with the Fmoc group.

5. Conclusions

In conclusion, the new PhEDOTn carboxyl protecting groups can be removed using very low concentrations of TFA, being rather stable to the standard protocols to remove the Fmoc group. These groups can be useful alternatives for the preparation of complex peptides. Furthermore, the unexpected stability of **1c** should be useful for the design of new protecting groups.

Acknowledgments

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References and notes

1. Tulla-Puche, J.; Bayó-Puxan, N.; Moreno, J. A.; Francesch, A. M.; Cuevas, C.; Álvarez, M.; Albericio, F. *J. Am. Chem. Soc.* **2007**, *129*, 5322–5323.
2. Kates, S. A.; Solé, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 1549–1552.
3. Jensen, K. J.; Alsina, J.; Songster, M. F.; Vagner, J.; Albericio, F.; Barany, G. *J. Am. Chem. Soc.* **1998**, *120*, 5441–5452.
4. Jou, G.; González, I.; Albericio, F.; Lloyd-Williams, P.; Giralt, E. *J. Org. Chem.* **1997**, *62*, 354–366.
5. Jessing, M.; Brandt, M.; Jensen, K. J.; Cristensen, J. B.; Boas, U. *J. Org. Chem.* **2006**, *71*, 6734–6741.
6. Isidro-Llobet, A.; Just-Baringo, X.; Álvarez, M.; Albericio, F. *Biopolymers.* **2008**, doi:10.1002/bip.20823.
7. *Topics in Current Chemistry*; Miyaura, N., Ed.; Springer Verlag: Berlin Heidelbert, 2002.
8. *Method A*: *5-Iodo-3,4-ethylenedioxythiophene-2-carbaldehyde* (**3**). Compound **2** (510 mg, 3 mmol) and *N*-iodosuccinimide (NIS) (810 mg, 3.6 mmol) were dissolved in dry DMF (5 mL) and stirred at 120 °C until no starting material was detected by HPLC (usually 6–8 h). The reaction mixture was cooled to room temperature. Diethylether (60 mL) was added and the resulting solution was washed with H_2O (3 × 50 mL). The organic portion was dried over $MgSO_4$ and filtered; the solution was then stored at −20 °C and used within 24 h maximum. (The dry product is very unstable even at low temperatures). A small aliquot was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.78$ (s, 1H), 4.36 (s, 4H) HPLC.
5-(3,4,5-Trimethoxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde (**4a**): DMF (25 mL) was added to the above obtained solution of **3** in diethyl ether (60 mL). The diethyl ether was evaporated and more DMF was added to reach a total volume of 85 mL, then 3,4,5-trimethoxyphenylboronic acid (859 mg, 4.05 mmol), $Pd(PPh_3)_4$ (212 mg, 0.184 mmol), and 2 M aqueous Na_2CO_3 (5.4 mL) were also added and the mixture was stirred at 135 °C for 4 h. The course of the reaction was followed by TLC (EtOAc–hexane; 1:1). The reaction mixture was evaporated to dryness, DCM (100 mL) was added and the solution was washed with H_2O (3 × 100 mL). The organic phase was dried over $MgSO_4$ and evaporated to dryness. The crude obtained was purified by silica gel chromatography (hexane, EtOAc) to render 620 mg of **4a** (62% yield). Mp 146.3–149.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.93$ (s, 1H), 7.02 (s, 2H), 4.42 (m, 4H), 3.9 (s, 6H), 3.88 (s, 3H).
5-(3,4-Dimethoxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde (**4b**): 630 mg (69% yield, 90% purity). Mp 185.4–190.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.92$ (s, 1H), 7.40 (dd, 1H, $J = 8.4$ and 2.1 Hz), 7.31 (d, 1H, $J = 2.1$ Hz), 6.90 (d, 1H, $J = 8.4$ Hz), 4.40 (m, 4H), 3.93 (s, 3H), 3.92 (s, 3H).
5-Phenyl-3,4-ethylenedioxythiophene-2-carbaldehyde (**4c**): 474.1 mg (64% yield, 90% purity) mp 135.2–139.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.94$ (s, 1H), 7.80 (dd, 2H, $J = 7.2$ and 1.4 Hz), 7.37 (m, 3H), 4.41 (m, 4H).
5-(3,4,5-Trimethoxyphenyl)-3,4-ethylenedioxythenyl alcohol (**1a**): Compound **4a** (150 mg, 0.45 mmol) was dissolved in MeOH (5 mL) and the resulting suspension was cooled in an ice bath. $NaBH_4$ (135.1 mg, 3.57 mmol) was added. The evolution of H_2 was observed and the initial suspension became a solution which was stirred at room temperature for 1 h. After that, H_2O (20 mL) was added and the pH adjusted to 8 by adding NH_4Cl; DCM extractions were then carried out (3 × 20 mL). The organic extracts were dried with $MgSO_4$, evaporated to dryness, and dried in the vacuum desiccator to get rid of all the MeOH. 133.6 mg of a solid was obtained (89% yield, 90% purity).
^{1}H NMR (400 MHz, DMSO): $\delta = 6.86$ (s, 2H), 5.29 (t, 1H, $J = 5.6$ Hz), 4.46 (d, 2H, $J = 5.6$ Hz), 4.26 (m, 4H), 3.78 (s, 6H), 3.65 (s, 3H).
5-(3,4-Dimethoxyphenyl)-3,4-ethylenedioxythenyl alcohol (**1b**): 129.7 mg (86% yield, 95% purity) ^{1}H NMR (400 MHz, DMSO): $\delta = 7.16$ (d, 1H, $J = 2.1$ Hz), 7.13 (dd, 1H, $J = 8.4$ Hz, $J = 2.1$ Hz), 6.95 (d, 1H, $J = 8.4$ Hz), 5.25 (t, 1H, $J = 5.6$ Hz), 4.45 (d, 2H, $J = 5.6$ Hz), 4.25 (m, 4H), 3.76 (s, 3H), 3.75 (s, 3H).
5-Phenyl-3,4-ethylenedioxythenyl alcohol (**1c**): 92.5 mg (84% yield, 85% purity) ^{1}H NMR (400 MHz, DMSO): $\delta = 7.61$ (dd, 2H, $J = 8.4$ Hz and 1.1 Hz), 7.36 (dd, 2H, $J = 7.4$ and 7.4 Hz), 7.20 (dd, 1H, $J = 7.8$ and 7.8 Hz), 5.30 (t, 1H, $J = 5.6$ Hz), 4.47 (d, 2H, $J = 5.6$ Hz), 4.27 (m, 4H).

3,4-Ethylenedioxythenyl alcohol (**1d**): 2 g, (99% yield, 99% purity). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.29$ (s, 1H), 4.66 (s, 2H), 4.21 (m, 4H).
Method B: 5-Iodo-3,4-ethylenedioxythenyl acetate (**6**) *3,4-Ethylenedioxythenyl acetate:* **1d** (700 mg, 4.07 mmol) was dissolved in Ac_2O (3 mL) and DMAP (49 mg, 0.41 mmol) was added. The reaction mixture was stirred for 30 min at room temperature, poured into saturated aqueous Na_2CO_3 (25 mL), and extracted with EtOAc (20 mL). The organic layer was washed with saturated aqueous Na_2CO_3 (6 × 15 mL) and 0.1% HCl in H_2O (3 × 20 mL), dried with $MgSO_4$, and then evaporated to dryness to yield 760 mg of an oil (87% yield).
^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.35$ (s, 1H), 5.11 (s, 2H), 4.22 (m, 4H), 2.08 (s, 3H).
5-Iodo-3,4-ethylenedioxythenyl acetate (**6**) 3,4-Ethylenedioxythenyl acetate (760 mg, 3.56 mmol) was dissolved in dry DMF(5.5 mL), and NIS (958 mg, 4.27 mmol) was added. After 3 h of stirring at room temperature, Et_2O (75 mL) was added and the resulting solution was washed with H_2O (3 × 100 mL). The organic layer was dried with $MgSO_4$, filtered and a small aliquot was taken for ^{1}H NMR. DMF (10 mL) was added to the remaining solution before removing Et_2O (the dry product is unstable) and this solution was immediately used for the next reaction.
^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.07$ (s, 2H), 4.25 (4H), 2.07 (s, 3H).
5-(4-Methoxyphenyl)-3,4-ethylenedioxythenyl alcohol (**1e**): DMF (90 mL) was added to the above-mentioned DMF solution of **6**. 4-methoxyphenylboronic acid (810 mg, 5.33 mmol), $Pd(PPh_3)_4$ (278 mg, 0.241 mmol), and 3.5 M aqueous Na_2CO_3 (8 mL) were successively added and the mixture was stirred at 135 °C for 5 h. The course of the reaction was followed by TLC (AcOEt–hexane, 1:1). The reaction mixture was evaporated to dryness. Et_2O (100 mL) was added and the solution was washed with H_2O (3 × 100 mL). Et_2O (40 mL) was added to the organic phase and it was washed with brine (3 × 100 mL). Then it was dried with $MgSO_4$ and evaporated to dryness. The crude obtained was purified by column chromatography (hexane, AcOEt) and 97 mg of an orange solid was obtained (10% yield).
^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.61$ (d, 2H, $J = 8.9$ Hz), 6.90 (d, 2H, $J = 8.9$ Hz), 4.37 (d, 2H, $J = 4.4$ Hz), 4.27 (m, 4H), 3.82 (s, 3H).

9. *5-(3,4,5-Trimethoxyphenyl)-3,4-ethylenedioxythenyl-N-(benzyloxycarbonyl)phenylalaninate* (**5a**): Compound **1a** (130 mg, 0.39 mmol), Z–Phe–OH (140 mg, 0.47 mmol), EDC (145 mg, 0.47 mmol), and DMAP (4.8 mg, 0.04 mmol) were dissolved in dry DCM (1.5 mL). The reaction mixture was stirred for 90 min and checked by TLC (DCM-MeOH, 95:5). After that, EtOAc (25 mL) was added, and the organic layer was washed with saturated aqueous Na_2CO_3 (3 × 25 mL) and H_2O (6 × 25 mL). The organic phase was dried with $MgSO_4$ and evaporated to dryness to yield 245.1 mg of an oil (98% yield, 80% purity).
^{1}H NMR (400 MHz, DMSO): $\delta = 7.83$ (d, 1H, $J = 8.2$ Hz), 7.25 (m, 10H), 6.89 (s, 2H), 5.09 (s, 2H), 4.96 (m, 3H), 4.30 (m, 4H), 3.78 (s, 6H), 3.66 (s, 3H), 3.0 (dd, 1H, $J = 13$ and 5.0 Hz) 2.86 (dd, 1H, $J = 13.9$ Hz and 10.2 Hz).
5-(3,4-Dimethoxyphenyl)-3,4-ethylenedioxythenyl-N-(benzyloxycarbonyl)phenylalaninate (**5b**): 150.6 mg, (78% yield, 70% purity) ^{1}H NMR (400 MHz, DMSO): $\delta = 7.82$ (d, 1H, $J = 8.1$ Hz), 7.21 (m, 12H), 6.89 (d, 1H, $J = 8.1$ Hz), 5.09 (s, 2H), 4.96 (m, 3H), 4.29 (m, 4H), 3.76 (s, 3H, CH_3), 3.75 (s, 3H), 3.01 (dd, 1H, $J = 13.8$ and 5.2 Hz) 2.85 (dd, 1H, $J = 13.8$ Hz and 10.1 Hz).
5-Phenyl-3,4-ethylenedioxythenyl-N-(benzyloxycarbonyl)phenylalaninate (**5c**): 163.9 mg, (91% yield, 75% purity). ^{1}H NMR (400 MHz, DMSO): $\delta = 7.83$ (d, 1H, $J = 8.2$ Hz), 7.60 (m, 2H), 7.28 (m, 13H), 5.01 (m, 5 H), 4.29 (m, 4 H), 3.01 (dd, 1H, $J = 13.8$ Hz and 5.2 Hz), 2.85 (dd, 1H, $J = 13.8$ and 10.1 Hz).
3,4-Ethylenedioxythenyl-N-(benzyloxycarbonyl)phenylalaninate (**5d**): 592 mg, (87% yield, 90% purity) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.27 (m, 8H), 7.03 (m, 2H), 6.37 (s, 1H, CH), 5.12 (m, 3H), 4.68 (m, 1H), 4.19 (m, 4H), 3.11 (m, 2H).
5-(4-Methoxyphenyl)-3,4-ethylenedioxythenyl-N-(benzyloxycarbonyl)-phenylalaninate (**5e**): 67.4 mg, (82% yield, 80% purity). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.62$ (d, 2H, $J = 8.9$ Hz), 7.33 (m, 6H), 7.19 (m, 3H), 7.05 (m, 2H), 6.91 (d, 2H, $J = 8.9$ Hz), 5.25–5.06 (m, 5H), 4.70 (m, 1H), 4.28 (m, 4H), 3.83 (s, 3H), 3.12 (m, 2H).

10. For each removal test, 1 mg of amino acid was treated with a solution of the corresponding concentration of TFA with 2% of triethylsilane as a scavenger. In all the solutions, a minimum of 2 equiv of TFA was used. The reactions were stopped by adding 2 equiv of pyridine per equivalent of TFA, evaporated, dissolved in H_2O–AcCN, and analyzed by HPLC. The removal percentage was calculated by comparing the areas at $\lambda = 220$ nm of the peaks corresponding to the free and protected amino acid.

11. Fujino, M.; Wakimasu, M.; Kitada, C. *Chem. Pharmacol. Bull.* **1981**, *29*, 2825–2831.

Hydroxyphenyl-EDOTn-OH, a Sterically Unhindered Linker for the Solid-Phase Synthesis of Acid Sensitive Protected Peptide Acids

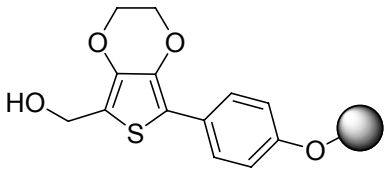
Hidroxifenil-EDOTn-OH: Un Espaïdor Bifuncional no Impedit Estèricament per a la Síntesi en Fase Sòlida de Pèptdis Protegits Sensibles a Medi Àcid

Albert Isidro-Llobet, Ulrik Boas, Knud J. Jensen, Mercedes Álvarez and Fernando Albericio

(Journal of Organic Chemistry, Submitted)

Resum

Un cop demostrada l'elevada labilitat a àcids dels derivats fenílics del 3,4-etilendioxitiofè (veure article anterior) s'ha decidit desenvolupar un espaïador bifuncional relacionat, l'alcohol 5-(4-hidroxifenil)-3,4-etilendioxitenílic, que permeti escindir pèptids de resines usant molt baixes concentracions de TFA. L'espaïador bifuncional sintetitzat té com a principal avantatge el seu baix impediment estèric ja que és un alcohol primari. Per demostrar-ne la utilitat s'ha usat per sintetitzar pèptids model lliures i amb les cadenes laterals protegides amb *tert*-butil, obtenint-se els millors resultats en el darrer cas.



Hydroxyphenyl-EDOTn-OH, a Sterically Unhindered Linker for the Solid-Phase Synthesis of Acid Sensitive Protected Peptide Acids

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albericio@irbbarcelona.org; *mercedes.alvarez@irbbarcelon.org;*

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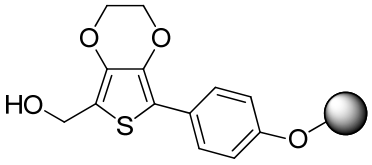


The 5-(4-hydroxyphenyl)-3,4-ethylenedioxythienyl alcohol is described as a new linker for the solid phase synthesis of peptide carboxylic acids. It is based on the electron rich 3,4-ethylenedioxythenyl (EDOTn) moiety and allows the obtention of free and *tert*-butyl protected peptides by cleavage with 90% and 0.5% of TFA respectively. This very high acid-lability makes it useful for the synthesis of sensitive peptides. Free and *tert*-butyl protected Leu-enkephalinamide have been synthesized as models to demonstrate the utility of the linker.

At present most solid phase peptide syntheses are performed using the Fmoc/^{t}Bu orthogonal strategy. [1 2 3 4 5] The obtention of biologically interesting sensitive or complex peptides requires the use of mild reagents for deprotection and cleavage operations. In this aspect, super acid-labile resins are of vital importance because they allow the cleavage of very acid sensitive peptidic moieties or *tert*-butyl protected peptide fragments, which will be further modified after the cleavage. The 2-chlorotrityl resin (2-CTC)[6] has been the resin of choice for most of these cases and although it has given very good results in a large number of complicated synthesis, [7 8] it has the drawback of its steric hindrance due to the bulkiness of the 2-chlorotrityl moiety.

In previous work we demonstrated the utility of EDOTn derivatives as super acid labile protecting groups. [9,10,11] In the present work we propose 5-(4-hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol (Figure 1) as a sterically non-hindered super acid-labile linker for the solid phase synthesis of peptide carboxylic acids, in particular as the C-terminal acid.

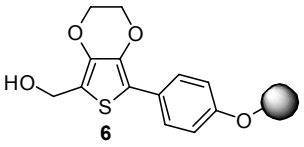


Figure 1. 5-(4-Hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol resin.

5-(4-Hydroxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde was prepared by formylation of 3,4-ethylenedioxythiophene, followed by iodination and a Suzuki coupling with *p*-hydroxyphenylboronic acid (Scheme 1) and it was coupled to a conventional hydroxymethyl Merrifield polystyrene resin. From the different coupling methods and resins tried the best approach was the coupling via formation of a trichloroacetimidate on the hydroxymethyl polystyrene resin. After the coupling of the phenol the resin was treated with DMF-H_2O to hydrolyze the remaining trichloroacetimidate. Then, it was acetylated with Ac_2O and DMAP and the aldehyde was reduced using $NaBH_4$. (Scheme 2). Fmoc-Leu was coupled via formation of the symmetric anhydride with DIC and DMAP. The loading of the resin was 0.29 mmol/g (calculated by Fmoc UV titration at λ=290 nm),.

Acidolytic release studies

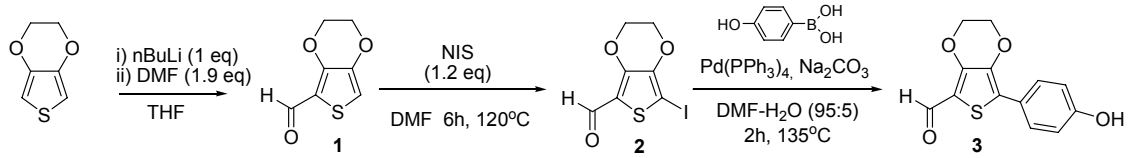
In order to test the acid lability of the new linker, the tripeptide Fmoc-Gly-Phe-Leu-OH was prepared on the resin. Aliquots of the peptide bonded to the resin were treated with solutions containing 0.1 % TFA, 0.5 % TFA and 90 % TFA.

It has been observed that phenyl-EDOTn derivatives are completely removed using concentrations of 90 % TFA. [9] The percentage of cleavage was calculated by HPLC analysis of the crude cleavage mixtures using *p*-nitrobenzyloxycarbonyl-Ala-OH (*p*NZ-Ala) as a standard. To determine the cleavage rates, the same amount of a 1 mg/mL standard solution of pNZ-Ala was added to the cleavage crude of the three aliquots. Then, they were analyzed by HPLC and the ratio of the areas from the peaks corresponding to pNZ-Ala and Fmoc-Gly-Phe-Leu-OH of the 0.1 % and 0.5 % TFA samples were compared with the 90 % of TFA sample to calculate the percetage of cleavage in each case (Table 1).

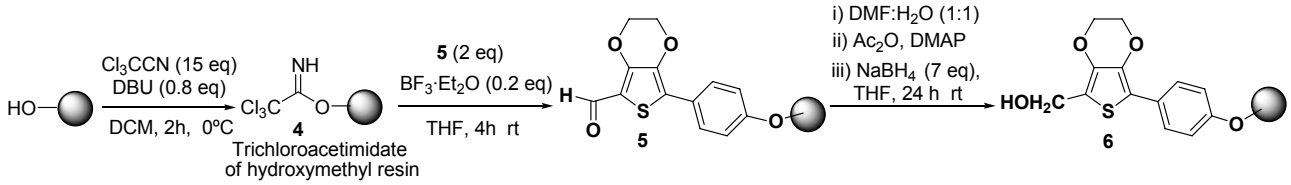
	% of Cleavage
TFA-TES-CH_2Cl_2 (0.1:2:97.9), 10 min.	80 %
TFA-TES-CH_2Cl_2 (0.5:2:97.5), 10 min.	100 %

Table 1. Percentages of cleavage of Fmoc-Gly-Phe-Leu-OH

Scheme 1. Synthesis of 5-(4-hydroxyphenyl)-3,4-ethylenedioxythiophene carbaldehyde.



Scheme 2. Obtention of the 5-(4-hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol based resin



Synthesis of unprotected and protected Leu-enkephalin: H-Tyr-Gly-Gly-Phe-Leu-OH using the 5-(4-hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol linker

The Leu-enkephalinamide was chosen as a model peptide to demonstrate the utility of the new derivatized resin. The coupling of Fmoc-Leu was performed as indicated above. All the remaining couplings were performed using DIC and HOBt as coupling reagents. After the last Fmoc removal, the peptide was cleaved from the resin using two different conditions: 0.5 % TFA for 10 min and 90 % TFA for 1h in order to obtain the *tert*-butyl protected and free peptide respectively. In the former case the crude showed an excellent purity by HPLC (Figure 2, left), whereas in the case of the latter the purity was slightly inferior (Figure 2, right).. This lower purity at high concentrations of TFA was also observed for the case of the 2-CTC resin and suggests that a best method for obtaining free peptides using the 5-(4-hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol resin is performing the cleavage of the protected peptide with low concentrations of TFA, and after filtering the resin, a global deprotection by increasing the TFA concentration to remove the *tert*-butyl type protecting groups.

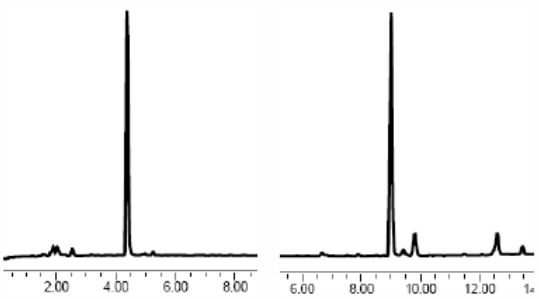


Figure 2. HPLC (λ=220 nm) of H-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-OH and H-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-OH respectively.

Conclusions

The present 5-(4-hydroxyphenyl)-3,4-ethylenedioxythenyl alcohol based resin allows release of peptide acids at low concentrations of TFA (0.5 % TFA within 10 min). Thus *tert*-butyl protected peptides and possibly very acid-sensitive peptidic structures can easily be obtained. In addition, cleavage with high concentrations of TFA provides unprotected peptides with acceptable purities. As the new resin is based on a primary alcohol, it is likely to cause little steric hindrance.

Experimental Section:

3,4-Ethylenedioxythiophene-2-carbaldehyde (1): It was synthesized following the procedure described in previous works of the group. 5.3 g of a white solid were obtained (89 % of yield).[9]

(5-Iodo-3,4-ethylenedioxythiophene-2-carbaldehyde (2): 3,4-Ethylenedioxythiophene-2-carbaldehyde (1 g, 5.88 mmol) and *N*-iodosuccinimide (NIS) (1.59 g, 7.05 mmol) were dissolved in dry DMF (10 mL) and stirred at 120ºC until no starting material was detected by HPLC (usually 6-8 hours). The reaction mixture was cooled to room temperature, Et_2O (100 mL) was added and the resulting solution was washed with H_2O (3 x 100 mL). The organic portion was dryed with $MgSO_4$, filtered and the solution was stored at -20ºC and used within 24 hours (The dry product from this procedure was very unstable even at low temperature).[12]

5-(4-Hydroxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde (3): DMF (50 mL) was added to a solution of 5-iodo-3,4-ethylenedioxythiophene-2-carbaldehyde in diethyl ether. The Et_2O was evaporated and more DMF was added to a total volume of 150 mL. 4-Hydroxyphenylboronic acid (1.10 g, 7.94 mmol), $Pd(PPh_3)_4$ (416 mg, 0.360 mmol) and 10 mL of 2M aqueous Na_2CO_3 were added and the mixture was stirred at 135ºC for 2h. The course of the reaction was followed by TLC (CH_2Cl_2). The reaction mixture was evaporated to dryness and an aq. solution of saturated NH_4Cl (100 mL) was added and the mixture was extracted with AcOEt (3 x 125 mL). The organic phase was dried with $MgSO_4$, filtered and evaporated to dryness. MeOH (10 mL) was added to the crude obtained, the mixture was filtered discarding the red liquors and keeping the light brown solid, which was dried under vacuum to yield 666 mg (43% total yield from **1**) of the desired product: mp= 265.1-269.3 ºC. ^{1}H NMR (400 MHz, DMSO): δ= 9.92 (s, 1H, CHO), 9.81 (s, 1H, OH), 7.61 (d, 2H, 2CH arom, *J*= 8.8 Hz), 6.83 (d, 2H, 2CH arom, *J*= 8.8 Hz), 4.42 (m, 4H, CH_2CH_2). ^{13}C NMR (100 MHz, DMSO): δ=

179.49 (CHO), 158.97 (C), 150.52 (C), 137.52 (C), 128.86 (CH), 122.87 (C), 116.58 (CH), 113.88 (C), 65.98 (CH_2), 65.21 (CH_2). HRMS (CI): *m/z* calcd. for $C_{13}H_9O_4S$ $[M - H]^-$ 261.0227, found 261.0225.

Resin preparation:

Trichloroacetimidate of the hydroxymethyl Merrifield resin (4): Hydroxymethylpolystyrene resin (300 mg, 0.98 mmol/g) was placed in a 5 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with dry CH_2Cl_2 (5 x 0.5 min), swollen with CH_2Cl_2 (3 mL) for 20 min and transferred to a microwave tube equiped with a septum and under nitrogen atmosphere. CCl_3CN (427.4 µL, 14.5 eq) was added and the tube was cooled in an ice bath. After 10 min of stirring DBU (35.6 µL, 0.81 eq) was added and the resin was stirred for 2 hours at 0ºC. Then, the resin was transferred to the 5 mL syringe and washed with CH_2Cl_2 (5 x 1 min) and Et_2O (5x 1min). An aliquot of the resin was dried and IR spectrometry was performed showing the formation of the trichloroacetimidate. IR (KBr): ν = 3341, 1663 cm^{-1}.

5-(4-hydroxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde resin (5): The above obtained resin **4** was swelled in dry CH_2Cl_2 for 20 min under nitrogen atmosphere, washed with dry THF (3 x 1 min) and transferred to a microwave tube equiped with a septum and under nitrogen atmosphere. A suspension of 5-(4-hydroxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde, **3**, (154 mg, 2 eq) in dry THF was added and the resin was stirred for 5 min. After that $BF_3{\cdot}Et_2O$ (7.5 µL, 0.2 eq) was added. The resin was gently stirred for 4h at room temperature in a shaker. The green resin obtained was washed with THF, DMF, CH_2Cl_2 and treated with DMF-H_2O (2 x 15 min) in order to hydrolyze the remaining trichloroacetimidate. Then washings with DMF (5 x 1 min), THF anhydrous (5 x 1 min) and Et_2O (5 x 1 min). An aliquot of the resin was dried and IR spectrometry was performed showing the disappearance of the trichloroacetimidate and the formation of the aldehyde. IR (KBr): ν = 1649 cm^{-1}.

The resin was swollen in CH_2Cl_2 (15 min), washed with DMF (5 x 30 s) and acetylated by treatment with Ac_2O (554.8 µL, 20 eq) and DMAP (2 eq) in DMF (2 mL) for 45 min.

5-(4-Hydroxyphenyl)-3,4-ethylenedioxythiophene-2-methylhydroxyl resin (6): Resin **5** was swollen with THF for 15 min in a microwave tube placed in an ice bath. $NaBH_4$ (77.9 mg, 7 eq) was added and the suspension was stirred 15 min in the ice bath and 24 h. at rt. The yellow resin obtained was cooled in an ice bath and saturated aq. NH_4Cl was slowly added, hydrogen evolution was observed. After 10 min, the NH_4Cl was removed, new NH_4Cl was added and the resin was left 5 min. at 0ºC and 15 min. more at rt. The resin was placed to a 5 mL polypropylene syringe fitted with a polyethylene filter disk and washed with H_2O, MeOH, THF-H_2O, THF, MeOH and Et_2O. IR spectrometry was performed showing the disappearance of the aldehyde peak.

General solid-phase peptide synthesis

Fmoc-Leu-OH (10 eq) was coupled to resin **6** by forming the symmetric anhydride with DIC (5 eq) and DMAP (0.1 eq) in CH_2Cl_2 (3 h).The loading of the resin was determined by Fmoc UV titration (Fmoc group was removed using piperidine-DMF (2:8) (1 x 1 min, 2 x 10 min).. The remaining amino acids were coupled as Fmoc derivatives (4eq), using DIC (4 eq) and HOBt (4 eq) as coupling agents in DMF (1h).

H-Tyr(^{t}Bu)GlyGlyPheLeu-OH (peptide 1) *and* H-Tyr-GlyGlyPheLeu-OH (peptide 2) were obtained by treating the resin with TFA-TES-CH_2Cl_2 (0.5:2:97.5) for 10 min and TFA-TES-CH_2Cl_2 (90:2:8) for 1h respectively. Peptide 1 cleavage solution was collected in H_2O, the TFA was evaporated and after addition of CH_3CN, the product was characterized by HPLC (λ= 220 nm) and LC-MS (95 % purity). In the case of peptide 2 cleavage solution, it was filtrated, evaporated to dryness dissolved and characterized by HPLC (λ= 220 nm) and LC-MS. The final product was obtained with 80 % purity.

Cleavage assays (Table 1) Aliquots of resin (10 mg) were taken for cleavage assays. The first aliquot was treated with TFA-TES-CH_2Cl_2 (90:2:8) (0.5 mL) for 1h, the second aliquot was treated with TFA-TES-CH_2Cl_2 (0.5:2:97.5) (1 mL) for 10 min and the third with of TFA-TES-CH_2Cl_2 (0.1:2:97.9) (0.5 mL) for 10 min and third resulting cleavage solutions were separated from the resin by filtration , evaporated to dryness and dissolved in H_2O-CH_3CN. 0.8 mL of a 1mg/mL standard solution of *p*NZ-L-Ala-OH in CH_3CN were added and HPLC of the resulting solution was performed.

Supporting Information Available. ^1H and ^{13}C NMR spectra of the prepared compounds. This material is available free of charge via the Internet at http://pubs.acs.org

Acknowledgements. This work was partially supported by CICYT (CTQ2006-03794/BQU), Instituto de Salud Carlos III (CB06_01_0074), the Generalitat de Catalunya (2005SGR 00662), the Institute for Research in Biomedicine, and the Barcelona Science Park. AI-L thanks the *DURSI*, *Generalitat de Catalunya* and the European Social Funds for a predoctoral fellowship.

References.

[1] Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC, Boca Raton, FL, USA, **1997.** [5] W. C. Chan; P. D. White (Eds.), *Fmoc Solid Phase Peptide Synthesis*, Oxford University Press, Oxford, UK, **2000**.

[2] Yokum, T. S.; Barany, G. in: *Solid phase Synthesis. A Practical Guide*, Marcel Dekker Inc., New York, USA, **2000**, pp. 79–102

[3] Fields, G. B.; Lauer-Fields, J. L.; Liu, R.-q.; Barany, G. in: *Synthetic Peptides: A User's Guide* (Eds.: G. A. Grant), 2nd ed., W. H. Freeman & Co., New York, **2001**, pp. 93–219.

[4] Goodman, M. ; Felix, A.; Moroder, L. A.; Toniolo, C. (Eds.), *Houben-Weyl*, vol. E22a–e ("Synthesis of Peptides and Peptidomimetics"), Georg Thieme Verlag, Stuttgart, Germany, **2002**.

[5] The *orthogonal* concept is based on the use of independent classes of protecting groups, removed by different mechanisms so that they may be removed in any order and in the presence of all other types of groups. a) Barany, G.; Merrifield, R. B. *J. Am. Chem. Soc.* **1977**, *99*, 7363; b) Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936.

[6] Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Yao, W.; Schaefer, W. *Tetrahedron Lett.* **1989**, *30*, 3943-3946.

[7] Han, Yeun-Kwei; Johnston, David A.; Khatri, Hiralal N. Synthesis of T-20 peptides. PCT Int. Appl. (2006), WO 2006069727 A2 20060706 CAN 145:103960 AN 2006:653931

[8] Gracia, C.; Isidro-Llobet, A.; Cruz, L. J.; Acosta, G. A.; Alvarez, M.; Cuevas, C.; Giralt, E.; Albericio, F. *J. Org. Chem.* **2006**, 71, 7196-7204

[9] Isidro-Llobet, A.; Just-Baringo, X.; Álvarez, M.; Albericio, F. *Biopolymers.* **2008** (ASAP).

[10] Jessing, M.; Brandt, M.; Jensen, K. J.; Christensen, J. B.; Boas, U. *J. Org. Chem.* **2006**, *71*, 6734-6741.

[11] Isidro-Llobet, A.; Álvarez, M.; Albericio, F. *Tetrahedron Lett.*. **2008** (Submitted).

[12] Alternatively, compound **2** can be prepared by a more laborious protocol (Ref. 10) which provides a more stable product.

Supporting Information

Hydroxyphenyl-EDOTn-OH, a Sterically Unhindered Linker for the Solid-Phase Synthesis of Acid Sensitive Protected Peptide Acids.

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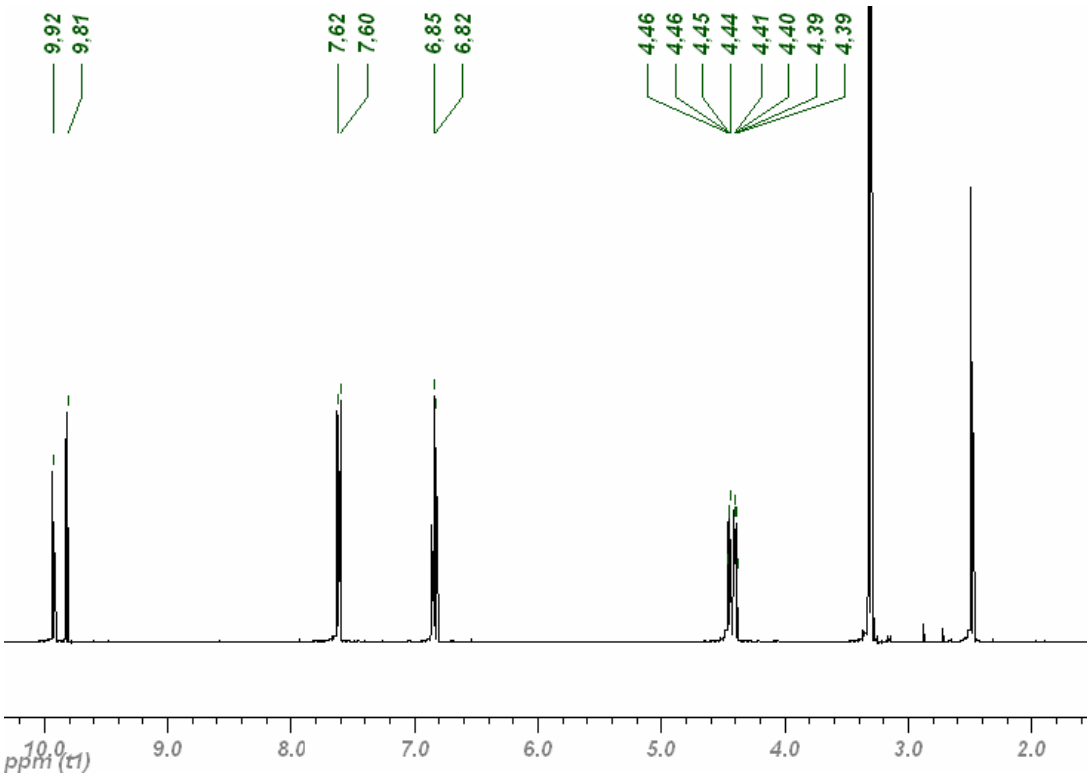
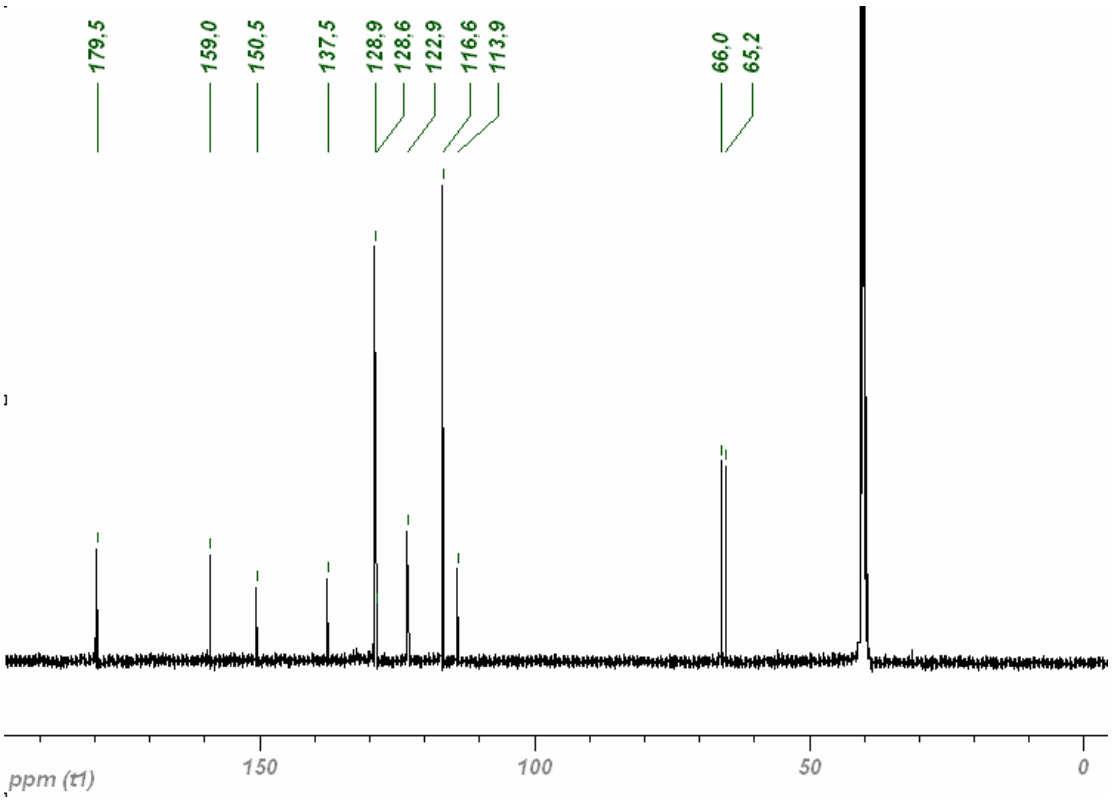
5-(4-Hydroxyphenyl)-3,4-ethylenedioxythiophene-2-carbaldehyde (3)

^{1}H NMR (400 MHz, DMSO)



^{13}C NMR (100 MHz, DMSO)



1,2-Dimethylindole-3-sulfonyl (MIS), the Most Acid-Labile Sulfonyl-Protecting Group for the Side Chain of Arginine

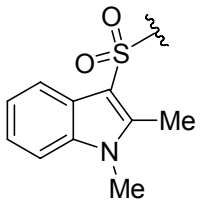
1,2-Dimetilindole-3-sulfonil (MIS), el Grup Protector de Tipus Sulfonil més Làbil a Àcids per la Cadena Lateral de l'Arginina

Albert Isidro-Llobet, Mercedes Álvarez and Fernando Albericio

(Journal of Organic Chemistry, Submitted)

Resum

L'arginina és un aminoàcid present en nombrosos pèptids amb interès terapèutic, en conseqüència molts dels pèptids que es volen sintetitzar contenen arginina. La gran tendència a donar reaccions secundàries del grup guanidini de la cadena lateral de l'arginina, ha fet que la seva protecció hagi estat sempre un tema important en química de pèptids. Actualment, els protectors de tipus sulfonil com el 2,2,5,7,8-pentametilcroman-6-sulfonil (Pmc) i el 2,2,4,6,7-pentametildihidrobenzofuran-5-sulfonil (Pbf) són els més usats. Tot i això la protecció de l'arginina és encara un problema sense resoldre ja que tan el Pmc com el Pbf són encara massa estable als tractaments amb TFA. Aquest problema és particularment greu en seqüències riques en arginina, en pèptids sensibles A medi àcid i en síntesi en gran escala. Per tal d'aconseguir una major labilitat a àcids s'ha dissenyat un nou grup protector, l'1,2-dimetilindole-3-sulfonil (MIS), que es basa en l'1-metilindole, al qual se li ha afegit un metil extra en posició 2 per evitar reaccions secundàries i per augmentar la riquesa en electrons de l'anell indòlic. S'ha demostrat que el nou grup protector és molt més làbil a medi àcid que el Pmc i el Pbf, és compatible amb pèptids que continguin Trp i pot ser una millor opció per protegir la cadena lateral de l'arginina.



1,2-Dimethylindole-3-sulfonyl (MIS)

1,2-Dimethylindole-3-sulfonyl (MIS), the Most Acid-Labile Sulfonyl-Protecting Group for the Side Chain of Arginine

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Abstract The protection of arginine (Arg) side chains is a crucial issue in peptide chemistry because of the propensity of the basic guanidinium group to produce side reactions. Currently, sulfonyl-type protecting groups, such as 2,2,5,7,8-pentamethylchroman (Pmc) and 2,2,4,6,7-pentamethyldihydrobenzofurane (Pbf), are the most widely used for this purpose. Nevertheless, Arg side chain protection remains problematic as a result of the acid stability of these two compounds. This issue is even more relevant in Arg-rich sequences, acid-sensitive peptides and large-scale syntheses. The 1,2-dimethylindole-3-sulfonyl (MIS) group is more acid-labile than Pmc and Pbf and can therefore be a better option for Arg side chain protection. In addition, MIS is compatible with tryptophan-containing peptides.

Keywords. Arginine protection. Peptide synthesis. 1,2-Dimethylindole-3-sulfonyl (MIS). Solid-Phase Synthesis. Guanidinium protection. Tryptophan side reactions.

Introduction.

Most peptides synthesized on solid-phase are prepared using the Fmoc/*tert*-butyl strategy.[1,2] Thus, α-amino temporary protection is achieved with the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group; amino acid side chains are protected by trifluoroacetic acid (TFA)-labile protecting groups, usually ^{t}Bu derivatives; and the *C*-terminal amino acid is anchored to the solid support through a TFA-labile linker/handle.

Nevertheless, *tert*-butyl-type protection of a number of amino acids is not the best option because of factors such as inefficiency at preventing side reactions or inadequate TFA lability. Among these amino acids, protection of the basic guanidinium group of Arginine (Arg) is possibly the most critical case.[3]

Currently, the most frequently used TFA-labile Arg-protecting groups are based on electron-rich benzene sulfonyl moieties. These groups are, by increasing order of acid lability: 4-methoxy-2,6-dimethylbenzenesulphonyl (Mds),[4] 4-methoxy-2,3,6-trimethylsulfonyl (Mtr),[4] 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc),[5] and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf)[6,7] (Figure 1). All of these mask the reactivity of the N^{ω}, are commercially available and have been

extensively used in the Fmoc/^{t}Bu solid-phase strategy.[8] Nevertheless, side chain protection of Arg remains unsolved because even the Pbf group is too stable to TFA and its removal requires high TFA concentrations and long treatment times, which may not be appropriate for acid-sensitive peptides. The situation becomes increasingly more demanding when preparing multiple Arg-containing peptides, which show biological properties of great interest[9]. In addition, the preparation of Pmc and Pbf moieties is expensive.

The design of a new sulfonyl-based Arg-protecting group is not a straightforward process in the sense of simply adding electron-donating groups to an aromatic ring, because the planarity of the system, which is essential for TFA lability, is not easy conserved because of the presence of the sulfonyl group. Thus, trimethoxybenzenesulfonyl (Mtb), which contains more electron-rich substituents (3 MeO) is less acid-labile than Mds (1 MeO, 2 Me) and Mtr (1 MeO, 3 Me).[5] This characteristic is attributed to the loss of planarity caused by the presence of the two methoxy groups near the sulfonyl group. Furthermore, the sulfonyl derivative of the 3,4-ethylenedioxythiophene (EDOT), whose derived compounds are highly labile to TFA as carboxylic acid protectors,[10] is not labile as an Arg side-chain protector, possibly because of the same loss of planarity.[11] Common side-reactions associated with the use of these benzenesulfonyl-based protecting groups are arylation of sensitive residues, such as Trp,[12],or sulfonation of Trp and/or Arg residues themselves.[13] This side reactions are favored by the decomposition of the sulfonyl-protecting group in two moieties, the arylcarbocation and the sulfonyl.[13]

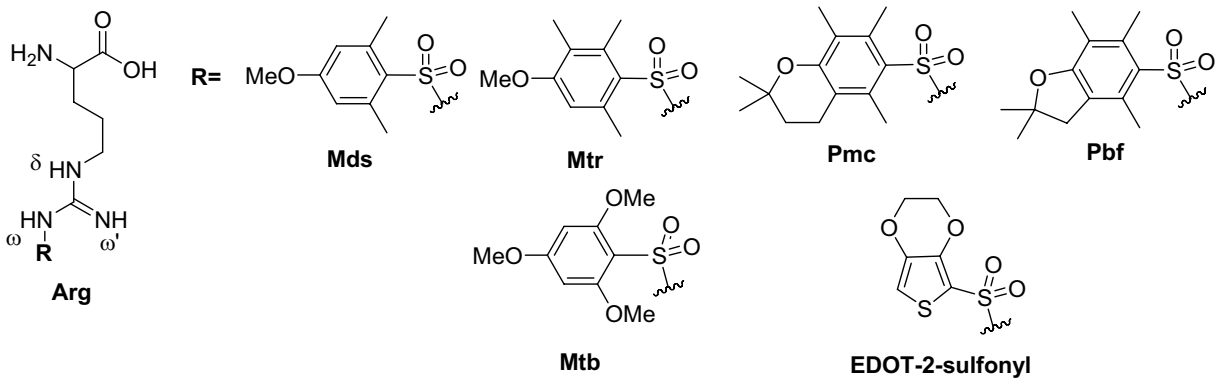


Figure 1. Arg protection

In an attempt to overcome the above mentioned drawbacks, here we describe a new more acid-labile Arg side chain-protecting group based on the indole system.

Results and Discussion.

General

A TFA-labile protecting group should be based on an electron-rich system. In this regard, *N*-alkylindole derivatives have been used as acid-labile amide linkers[14] and amide backbone protectors.[15] Taking this into account, we chose MIS (Figure 2) as guanidinium-protecting group. The extra methyl at position 2 should increase the acid lability of the protecting group and prevent electrophylic aromatic substitution. Furthermore, the 1,2-dimethylindole is commercially available.

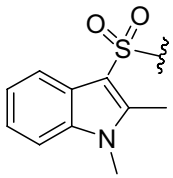


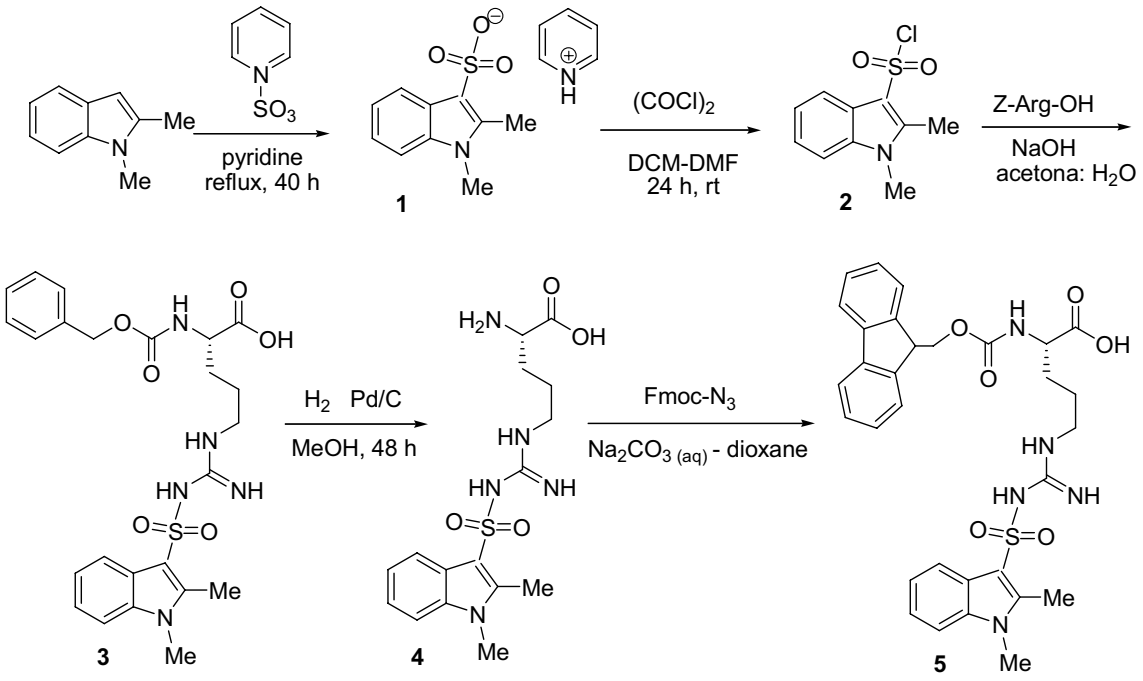
Figure 2. 1,2-Dimethylindole-3-sulfonyl (MIS)

Synthesis of the protecting group and Arg protection

As the 1,2-dimethylindole is prone to polymerize in strong acidic conditions, sulfonation of the indole ring must be carried out in neutral or basic media. Thus, chlorosulfonic acid, which is the reagent of choice for Pmc and Pbf sulfonylation, cannot be used in the case of 1,2-dimethylindole. Nevertheless, the use of sulphur trioxyde pyridine complex yielded the corresponding pyridinium sulfonate in good yield but in our hands longer time than that described in the literature was required.[16] Chlorination under mild conditions by treatment with oxalyl chloride yielded 1,2-dimethylindole-3-sulfonyl chloride (MIS-Cl). These conditions gave similar overall yields to those attained with Pbf and Pmc, with the advantage that 1,2-dimethylindole is commercially available.

We prepared Fmoc-Arg(MIS)-OH (Scheme 1) in a similar way to Pmc/Pbf derivatives[5,6], using Z-Arg-OH as starting material Z-Arg-OH was sulfonylated at the N^{ω} position with MIS-Cl and the Z group was removed via catalytic hydrogenolysis. Final Fmoc protection was achieved via the azide

method because the use of other more active Fmoc derivatives leads to the formation of dipeptides or other side reactions.[17,18]



Scheme 1. Synthesis of Fmoc-L-Arg(MIS)-OH

Synthesis of multiple arginine-containing peptides using MIS and Pbf protection

As Pbf removal is more complicated in multiple Arg-containing peptides, Ac-Phe-Arg-Arg-Arg-Arg-Val-NH_2 was chosen as a model peptide to compare the acid lability of MIS and Pbf. [19] The corresponding Pbf- and MIS-protected peptides were prepared using standard solid-phase peptide synthesis protocols on Sieber amide resin, which allows cleavage from the resin with small amounts of TFA (2%), thereby yielding the MIS- and Pbf-protected peptides respectively with excellent purity.

Removal assays

To compare the acid lability of the Pbf group, which is more acid-labile than the Pmc, with the one of the MIS group, protected peptide-bonded resins were treated with a range of concentrations of TFA in DCM. These assays revealed that the MIS group is considerably more acid-labile than the Pbf one (Table 1).

Also, the MIS derivative generated in the removal process differs from the case of Pbf. For Pbf and Pmc, 2,2,5,7,8-pentamethylchroman and 2,2,4,6,7-pentamethyldihydrobenzofurane, respectively, are formed via a desulfonlylation mechanism[5] , while for MIS, the sulfonic acid (MIS-OH) was stable and was not desulfonated.

	MIS	Pbf
TFA–DCM–H_2O–TIS (**50**:45:2.5:2.5), t= 30 min	100 %	4%
TFA–DCM–H_2O–TIS (**50**:45:2.5:2.5), t= 1 h	100 %	38%

Table 1. Percentages of completely deprotected Ac-FRRRRV-NH_2

Optimization of the scavengers used in the removal:

As MIS-OH is a polar compound, it precipitates during the ether treatment after the cleavage step. Alternative scavengers to H_2O were tested to reduce the amounts of the strongly UV absorbant MIS-OH in order to facilitate purification. Among the scavengers tested, the most optimum were 10% of 3,4-dimethoxyphenol, 1,3,5-trimethoxybenzene (Tmb) or 3,5-dimethoxyphenol. The use of these scavengers reduced the amounts of MIS-OH more than 10 fold (40 times in the case of 1,3,5-trimethoxybenzene), thereby simplifying HPLC purification to yield the final product.

Synthesis of Trp-containing peptides:

To check the compatibility of the MIS group with Trp, we first synthesized the model peptides Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2 and Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 on a Sieber amide resin, which were obtained with an excellent HPLC purity.

Afterwards, both resins were treated with TFA-DCM-trimehtoxybenzene (50:40:10) to compare the purities of Trp-containing peptides after MIS and Pbf removal (Table 2). Trp alkylation or sulfonation was not detected in neither of the cases. The purity of the crude product was greater in the case of MIS and neither the MIS- protected peptides nor MIS-OH were detected by LC-MS. Nevertheless, in the

case of the Pbf experiment, considerable amounts of the Pbf-protected peptide were detected (34% respect to unprotected peptide, HPLC, λ=220 nm).

Conclusions

MIS is the most acid-labile sulfonyl-type protecting group for Arg described to date. This feature makes it highly convenient for the synthesis of multiple Arg-containing peptides or peptides that contain acid-sensitive moieties. Furthermore, MIS is compatible with Trp-containing peptides.

Experimental Section

Synthesis of the protecting group and Arginine protection.

Pyridinium 1,2-dimethylindole-3-sulfonate (1)

1,2-Dimethylindole (19.7 g, 135.9 mmol) and sulphur trioxide pyridine complex (20.4 g, 128.3 mmol) were dissolved in pyridine (100 mL) under Ar atmosphere. The reaction mixture was refluxed for 40 h. It was then cooled to room temperature and H_2O was added (400 mL). The resulting solution was washed with diethyl ether (4 x 250 mL). The aqueous phase was evaporated to dryness and dried in the vacuum dessicator to render a red oil (37.6 g, 96 % yield). ^{1}H NMR (400 MHz, D_2O): δ= 8.44 (d, 2H, 2CH pyr., J= 5.8 Hz), 8.31 (m, 1H, CH pyr.), 7.75 (m, 2H, 2CH pyr), 7.67 (d, 1H, CH arom, J= 7.7 Hz), 7.14 (d, 1H, CH arom, J= 7.4 Hz), 7.05 (m, 2H, 2CH arom), 3.38 (s, 3H, CH_3), 2.41 (s, 3H, CH_3).. ^{13}C NMR (100 MHz, D_2O): δ= 147.0 (CH), 140.9 (CH), 139.2 (C), 135.6 (C), 127.3 (CH), 124.1 (C), 122.0 (CH), 121.0 (CH), 119.2 (CH) 112.8 (C) 109.9 (CH), 29.2 (CH_3), 10.4 (CH_3). HRMS (CI): *m*/*z* calcd. for $C_{10}H_{10}NO_3S$ $[M - H]^+$ 224.0386, found 224.0388.

1,2-Dimethylindole-3-sulfonyl chloride (MIS-Cl) (2)

1 (16.4 g, 53.7 mmol) was suspended in dry DCM (120 mL) under N_2 atmosphere. The solution was cooled in an ice bath and oxalyl chloride (14 mL, 161 mmol) was slowly added. DMF (0.5 mL) was then slowly and carefully added and vigorous effervescence 7the starting material. The reaction mixture

was stirred in an ice bath for a further 30 min until the effervescence ceased and was then stirred at room temperature. After 6 h, the solution was cooled in an ice bath and extra oxalyl chloride (4 mL, 46 mmol) and DMF (0.4 mL) were added and the reaction mixture was stirred at room temperature for further 15 h. A small aliquot (10 μL) was then treated with MeOH for 20 min and injected into the HPLC apparatus, which showed the presence of methyl 1,2-dimethylindole-3-sulfonate (94%) and starting material (6%). Therefore, additional oxalyl chloride (2 mL, 23 mmol) was added and after 4 h more at room temperature the HPLC assay showed that the reaction was completed. The reaction mixture was evaporated to dryness at room temperature, DCM (200 mL) was added, followed by H_2O (100 mL). The mixture was stirred for 5 min with care in order to remove the oxalyl chloride, the phases were separated and the organic phase was washed with H_2O (3x100 mL). The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness to give the target compound as a purple solid (10.2 g, 78% yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.82 (d, 1H, CH, J= 7.8 Hz), 7.36 (d, 1H, NH, J= 8.0 Hz), 7.08 (m, 2H, 2CH), 7.00 (m, 2H, 2CH), 3.63 (s, 3H, CH_3), 2.56 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO): δ= 137.2 (C), 135.9 (C), 125.5 (C), 121.4 (CH), 120.8 (CH), 120.1 (CH), 109.7 (CH), 30.0 (CH_3), 11.3 (CH_3). HRMS (CI): *m*/*z* calcd. for $C_{10}H_{10}NO_2S$ $[M - Cl]^-$ 208.0426, found 208.0427.

Z-L-Arg(MIS)-OH (3)

Z-L-Arg-OH (2 g, 6.5 mmol) was dissolved in acetone (65 mL) and 3 N aqueous NaOH (18 mL, 54 mmol). The reaction was cooled in an ice bath and compound **2** (1.59 g, 6.5 mmol) dissolved in acetone (50 mL) was added over 10 min. The reaction mixture was stirred for 1 h at 0 ºC. Additional **2** (0.95 g, 3.9 mmol) in acetone (20 mL) was then added followed by 90 min of stirring at 0ºC. Finally, a final amount of **2** (0.95 g. 3.9 mmol) in acetone (15 mL) was added and the reaction mixture was stirred for an additional 30 min at 0 ºC and 3 h at room temperature, until no **2** was observed by TLC (hexane–EtOAc 1:1). The pH of the reaction was neutralized with 10% aq. citric acid, the acetone was evaporated *in vacuo*, H_2O (100 mL) was added, the pH was acidified to 3 with 10% aqueous citric acid and the solution was extracted with EtOAc (3 x 100 mL). The organic phases were pooled, washed with H_2O (3 x 75 mL), dried with $MgSO_4$ and evaporated to dryness. The crude product obtained was

purified twice by column chromatogrphy (DCM, MeOH, HOAc). The solvent of the pure fractions was removed *in vacuo* to yield an oil. The minimum amount of a mixture of EtOAc–DCM–MeOH was then added followed by addition of hexane until no further precipitated solid was observed. The solvent was decanted and the solid was washed 4 times with DCM–hexane (enough hexane to precipitate all the product) to remove the HOAc and was dried over $MgSO_4$ to give **3 (**0.61 g, 18 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.85 (d, 1H, CH, J= 7.6 Hz), 7.52 (d, 1H, NH, J= 8.0 Hz), 7.43 (d, 1H, CH, *J*= 8.0 Hz), 7.30 (m, 5H, 5CH Z), 7.10 (m, 2H, 2CH), 5.01 (s, 2H, CH_2), 3.87 (m, 1H, αCH), 3.66 (s, 3H, CH_3), 3.0 (m, 2H, CH_2), 2.60 (s, 3H, CH_3), 1.64 (m, 1H, CH_2), 1.49 (m, 1H, CH_2), 1.41 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO): δ= 174.4 (C), 157.0 (C), 156.8 (C), 139.4 (C), 137.7 (C), 135.9 (C), 129.0 (CH), 128.5 (CH), 128.4 (CH), 125.2 (C), 122.1 (CH), 121.1 (CH), 120.1 (CH), 110.4 (CH), 66.1 (CH_2), 54.3 (CH), 40.0 (CH_2), 30.2 (CH_3), 28.9 (CH_2), 26.4 (CH_2), 11.4 (CH_3). HRMS (CI): *m*/*z* calcd. for $C_{24}H_{30}N_5O_6S$ $[M + H]^+$ 516.1911, found 516.1911.

H-L-Arg(MIS)-OH (4)

A mixture of **3 (**486 mg, 0.94 mmol) and 10 % Pd/C (110 mg) in MeOH (60 mL) was hydrogenated overnight at atmospheric pressure. After this time TLC (DCM–MeOH–HOAc, 90:9:1) still showed some starting material. More 10% Pd/C (100 mg) was added and the reaction was hydrogenated for 24 h more, after which TLC showed the absence of starting material. The reaction mixture was filtered over celite and evaporated to dryness to yield **4** (352 mg, 98 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.83 (d, 1H, CH, J= 7.6 Hz), 7.47 (d, 1H, NH, J= 8.1 Hz), 7.42 (d, 1H, CH, J= 8.1 Hz), 7.11 (m, 2H, 2CH), 3.65 (s, 3H, CH_3), 3.17 (m, 1H, CH), 3.00 (m, 2H, CH_2), 2.60 (s, 3H, CH_3), 1.65 (m, 1H, CH_2), 1.54 (m, 1H, CH_2), 1.42 (m, 2H, CH_2).

Fmoc-Arg(MIS)-OH (5)

Fmoc-Cl (84 mg, 0.32 mmol) was dissolved in 1,4-dioxane (0.5 mL). NaN_3 (25 mg, 0.39 mmol) in H_2O (0.4 mL) was added and the resulting emulsion was stirred for 2 h at room temperature. The emulsion was then slowly added to a solution of **4** (136 mg, 0.36 mmol) in H_2O–dioxane (1:1) at pH 9,

controlled with 10% aqueous Na_2CO_3. The reaction mixture was stirred keeping the pH at 9 and when it was stabilized, it was left to stir overnight. After that, H_2O (30 mL) was added to the reaction mixture and it was washed with *tert*-butyl mehtyl ether (3 x 20 mL).The aqueous phase was acidified to pH 2-3 with 1N HCl and quickly extracted with EtOAc (3 x 30 mL). The organic phases were dried over $MgSO_4$ and evaporated to dryness to yield an oil (115 mg), which was dissolved in the minimum of acetone and aqueous Na_2CO_3 at pH 9 (20 mL) was added. The aqueous solution was washed with *tert*-butyl methyl ether (3 x 30 mL), acidified to pH 2-3 with 1N HCl, and extracted with EtOAc (3 x 20 mL), dried over $MgSO_4$ and evaporated to dryness to yield of the desired product (67.4 mg, 34.3 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.86 (m, 3H, 2CH Fmoc, CH indole), 7.70 (d, 2H, 2CH Fmoc, J= 7.4 Hz), 7.59 (d, 1H, NH, J= 7.9 Hz), 7.42 (d, 1H, CH indole, J= 8.1 Hz), 7.39 (m, 2H, 2CH Fmoc), 7.30 (m, 2H, 2CH Fmoc), 7.10 (m, 2H, 2CH indole), 4.27 (m, 2H, CH_2 Fmoc), 4.20 (m, 1H, CH Fmoc), 3.86 (m, 1H, αCH), 3.66 (s, 3H, CH_3), 3.01 (m, 2H, CH_2), 2.61 (s, 3H, CH_3), 1.65 (m, 1H, CH_2), 1.52 (m, 1H, CH_2), 1.38 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO): δ= 174.4 (C), 157.0 (C), 156.8 (C), 144.5 (C), 141.4 (C), 139.4 (C) , 135.9 (C), 128.3 (CH), 127.8 (CH), 126.0 (CH), 125.2 (C), 122.1 (CH), 121.1 (CH), 120.8 (CH), 120.1 (CH), 110.4 (CH), 66.3 (CH_2), 55.6 (CH), 47.3 (CH), 40.0 (CH_2), 30.2 (CH_3), 28.8 (CH_2), 26.5 (CH_2) 11.4 (CH_3). HRMS (CI): *m/z* calcd. for $C_{31}H_{34}N_5O_6S$ $[M + H]^+$ 604.2224, found 604.2222.

Synthesis of multiple arginine-containing peptides using MIS and Pbf protection:

Ac-Phe-Arg(MIS)-Arg(MIS) -Arg(MIS)-Arg(MIS)-Val-NH_2 (peptide 1)

Sieber amide resin (25 mg, 0.42 mmol/g) was placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed by treatment with piperidine–DMF (2:8) (1 x 1 min, 2 x 10 min). Fmoc-L-Val-OH (14.3 mg, 42.1 μmol) was coupled using HOBt (5.7 mg, 42.1 μmol) and DIC (6.7 μL, 42,1 μmol) in DMF, t = 90 min. The Fmoc group was removed in the usual way and Fmoc-L-Arg(MIS)-OH (15.8 mg, 26.3 μmol) was coupled using PyBOP (13.7 mg, 26.3 μmol) HOAt (3.6 mg, 26.3 μmol)

and DIPEA (13.4 μL, 78.9 μmol) in DMF for 90 min. The resin was acetylated by treatment with Ac_2O (50 eq) and DIPEA (50 eq) in DMF for 25 min. The Fmoc group was removed and the same procedure was repeated three more times, acetylating the resin before each Fmoc removal. After the last Fmoc removal, Fmoc-L-Phe (13.6 mg, 35 μmol) was coupled using PyBOP (18.3 mg, 35 μmol) HOAt (4.8 mg, 35 μmol) and DIPEA (17.9 μL, 105.2 μmol) in DMF for 90 min. The Fmoc group was removed and the resulting free amino group was acetylated as before. The resin was washed with DMF, DCM and diethyl ether, dried *in vacuo*, and divided into five aliquots. One of these was swollen with DCM, and treated with 1.5 mL of TFA–DCM–TIS–H_2O (2:93:2.5:2.5) for 20 min in order to cleave the protected peptide from the resin. The resin was filtered and the collected solution was diluted with DCM and neutralised adding DIPEA (80 μL, 1.2 eq per eq of TFA). The solvent was then removed *in vacuo*, and H_2O and AcCN were added and the solution was frozen and lyophilized. The product obtained was characterised by LC-MS and HRMS (CI): *m*/*z* calcd. for $C_{80}H_{107}N_{23}O_{15}S_4$ $[M + Na]^+$ 1780.7092, found 1780.7152.

Ac-Phe-Arg(Pbf) -Arg(Pbf) -Arg(Pbf)-Arg(Pbf)-Val-NH_2 (peptide 2)

The same procedure as for the synthesis of peptide 1 was used but replacing Fmoc-L-Arg(MIS)-OH by Fmoc-L-Arg(Pbf)-OH (17.1 mg, 26.3 μmol). The product obtained was characterised by LC-MS and HRMS (CI): *m*/*z* calcd. for $C_{92}H_{136}N_{19}O_{19}S_4$ $[M + H]^+$ 1938.9137, found 1938.9202.

Removal assays:

General procedure: the resin (3 mg) was treated with cleavage solution (50 μL). After the cleavage time, the solution was poured into H_2O (4 mL), and TFA and DCM were evaporated. The resulting aqueous solution was washed with DCM (6 x 1 mL), frozen, lyophilized and analyzed by HPLC (λ= 220 nm) and ESMS or MALDI-TOF.

Optimization of the scavengers:

The same procedure as for the removal assays was followed. In all the experiments the resin was treated with TFA-DCM-scavenger (50:40:10) (50 µL) for 1 h. The scavengers tested were 3,4-dimethoxyphenol, 1,3,5-trimethoxybenzene (Tmb) or 3,5-dimethoxyphenol.

Synthesis of Trp-containing peptides:

Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2 and Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 :

Sieber amide resin (70 mg, 0.40 mmol/g) was placed in a 2-mL polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed. Fmoc-L-Gly-OH (33.3 mg, 112 µmol), Fmoc-L-Ala-OH (34.9 mg, 112 µmol) and Fmoc-L-Trp(Boc)-OH (59.0 mg, 112 µmol) were sequentially coupled using PyBOP (58.3 mg, 112 µmol) HOAt (15.2 mg, 112 µmol) and DIPEA (57.4 µL, 336 µmol) in DMF, t= 1.5 h. The resin was divided into two equal parts.

Part 1 (Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2) : Z-Arg(MIS)-OH (28.9 mg, 56 µmol) was coupled using PyBOP (29.2 mg, 56 µmol) HOAt (7.6 mg, 56 µmol) and DIPEA (28.7 µL, 168 µmol) in DMF, t= 1.5 h. The resin was washed with DMF, DCM and diethyl ether, dried *in vacuo* and divided into 4-mg aliquots. One of these was swollen with DCM and treated with 1.5 mL of TFA–DCM–TIS–H_2O (2:93:2.5:2.5) for 20 min in order to cleave the protected peptide from the resin. The resin was filtered and the collected solution was diluted with DCM and neutralised by adding DIPEA (80 µL, 1.2 eq per eq of TFA). The solvent was then removed *in vacuo*, and H_2O and AcCN were added and the solution was frozen and lyophilized. The product obtained was characterised by LC-MS (95 % purity).

Part 2 (Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2) : Fmoc-Arg(Pbf)-OH (36.3 mg, 56 µmol) was coupled using PyBOP (29.2 mg, 56 µmol) HOAt (7.6 mg, 56 µmol) and DIPEA (28.7 µL, 168 µmol) in DMF, t= 1.5 h. The Fmoc group was removed and the free amine was protected with the Z group by treatment with Z-OSu (14.0 mg, 56 µmol) and DIPEA (35.9 µL, 210 µmol). The resin was then washed with

DMF, DCM and diethyl ether, dried *in vacuo*, divided into 4-mg aliquots, one of which was cleaved in the same way as for Part 1. The product obtained was characterised by LC-MS (96 % purity).

Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2: Two aliquots from Part 1 were treated with TFA-DCM-1,3,5-trimehtoxybenzene (50:40:10) and TFA-DCM-H_2O (50:45:5) respectively for 1 h following the General Procedure for the removal assays described above. In the latter case, no DCM washings were performed. The two crude products resulting from these treatments were analyzed by LC-MS. No Trp alkylation or sulfonation nor MIS-protected peptide were observed.

Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2: An aliquot from Part 2 was treated with TFA-DCM-trimehtoxybenzene (50:40:10) for 1 h following the General Procedure for the removal assays described above. The target peptide was analyzed by LC-MS (60% purity). 17% of Pbf-protected peptide was detected and no Trp alkylation or sulfonation was observed.

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Supporting Information Available. ^{1}H and ^{13}C NMR spectra and HPLC chromatograms of the prepared compounds. This material is available free of charge via the Internet at http://pubs.acs.org

References.

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[1] Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins.* Ed. CRC Press, Boca Raton. New York, **1997**.

[2] Cupido, T.; Tulla-Puche, J.; Spengler, J.; Albericio, F. *Curr. Opin. Drug Dis. Develop.* **2007,** *10*, 768-783.

[3] Thamm, P.; Kolbeck, W.; Musiol, H-J.; Moroder, L. Synthesis of Peptides and Peptidomimetics, Houben-Weyl Methods in Organic Chemistry. Eds. Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C. Vol E 22a, 315-333.

[4] Fujino, M.; Wakimasu, M.; Kitada, C. *Chem. Pharm. Bull.* **1981**, *29*, 2825-2831

[5] Ramage, R.; Green, J.; Blake, A. J. *Tetrahedron* **1991**, *47*, 6353-6370.

[6] Carpino, L. A.; Shroff, H.; Triolo, S. A.; Mansour, E. M. E.; Wenschuh, H.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 7829-7832.

[7] Fields, C. G.; Fields, G. B. *Tetrahedron Lett.* **1993**, *42*, 6661-6664.

[8] The NO_2 (Bergmann, M.; Zervas, L.; Rinke, H. *H-S Z. Physiol. Chem...* **1934**, *224*, 40-44), which is also commercially available, has the main drawback of long reaction times, which is an inconvenient in the case of sensitive peptides. For instance in the case of hydrogenolysis partial hydrogenation of Trp or even Phe can occur (Young, G. T.; Schafer, D. J.; Elliott, D. F.; Wade, R., *J. Chem. Soc. C.* **1971**, 46-49). $N^{\omega}N^{\omega'}$,bis-Boc (Verdini, A.S.; Lucietto, P.; Fossati, G.; Giordani, C. *Tetrahedron Lett.* **1992**, *33,* 6541-6542) as well as the 10,11-Dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5-yl [5-dibenzosuberyl] (Sub) (Noda, M.; Kiffe, M. *J. Pept. Res.* **1997**, *50,* 329-335), have been also proposed but no really used.

[9] Futaki, S.; Nakase, I.; Tadokoro, A.; Takeuchi, T.; Jones, A. T. *Biochem. Soc. Trans.* **2007**, *35*, 784-787.

[10] Isidro-Llobet, A.; Álvarez, M.; Albericio, F. *Tetrahedron Lett.* **2008** (submitted).

[11]Isidro-Llobet, A.; Álvarez, M.; Albericio, F. Unpublished results.

[12] Sieber, P. *Tetrahedron Lett.* **1987**, *28,* 1637-1640.

[13] Beck-Sickinger, A.; Schnorrenberg, G.; Metzger, J.; Jung, G. *Int. J. Pept. Prot. Res.* **1991**, *38*, 25-31.

[14] Estep, K. G.; Neipp, C. E.; Stramiello, L. M.; Stephens; A.; Mavis D.; Allen, M. P.; Robinson, S.; Roskamp, E. J. *J. Org. Chem.* **1998**, *63*, 5300-5301.

[15] Isidro-Llobet, A.; Just-Baringo, X.; Álvarez, M.; Albericio, F. *Biopolymers* **2008** (ASAP).

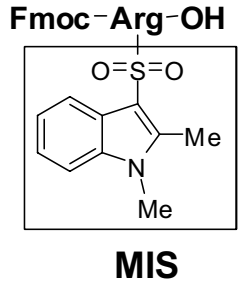
[16] Smith, G. F.; Taylor, D. A. *Tetrahedron* **1973**, *29*, 669-672.

[17] Although the existence of this side reaction was not mentioned, Fmoc-N_3 was already proposed as an alternative to the chloroformate in the seminal papers of Carpino and Han: Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* **1970**, *92*, 5748-5749 and Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404-3409.

[18] For a broad discussion of this subject, see: L. J. Cruz, N. G. Beteta, A. Ewenson, F. Albericio, *Org. Process Res. Dev.* **2004**, *8*, 920-924.

[19] Riniker, B.; Hartman, A. *Pept.: Chem., Struct. Biol., Proc. Am. Pept. Symp., 11th* (Rivier, J.; Marshall, G.R. eds.), Escom, Sci. Pub. (Leiden, The Netherlands) **1990**, pp. 950-952.

TOC



MIS

Supporting Information

1,2-Dimethylindole-3-sulfonyl (MIS), the Most Acid Labile Sulfonyl Protecting Group for the Side Chain of Arginine.

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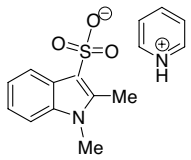
Table of contents:

1. NMR spectra:

1,2-Pyridinium methylindole-3-sulfonate (1)



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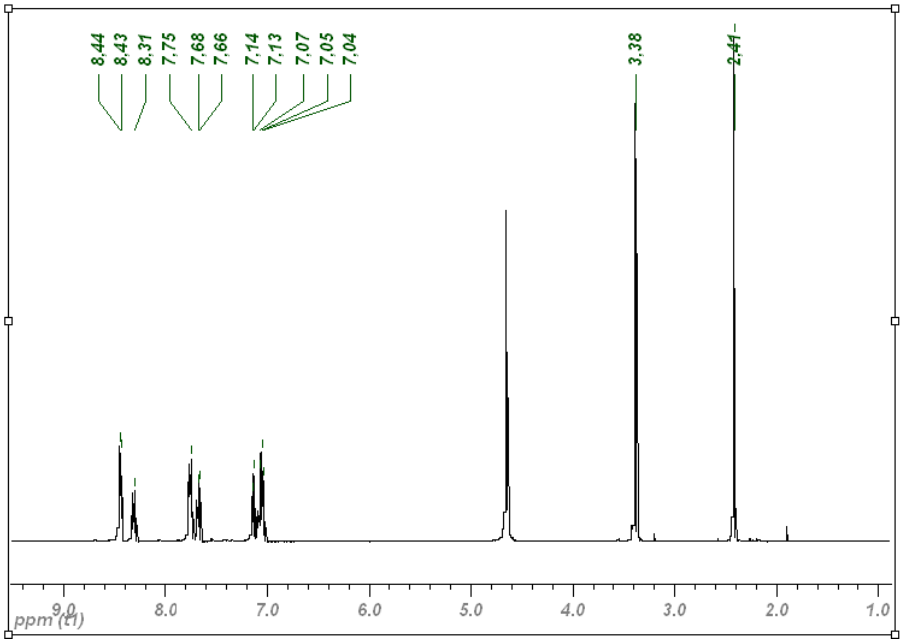

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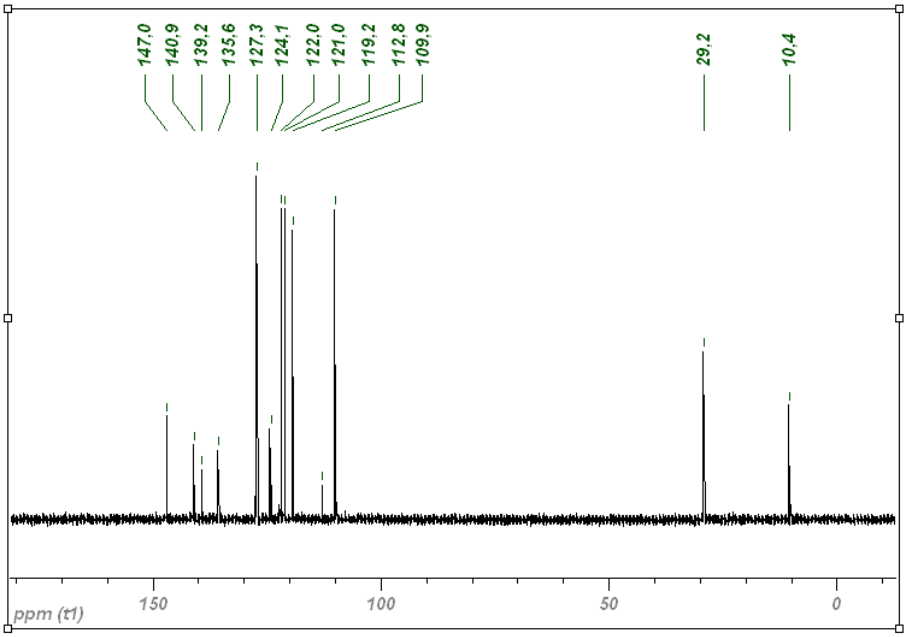

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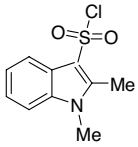

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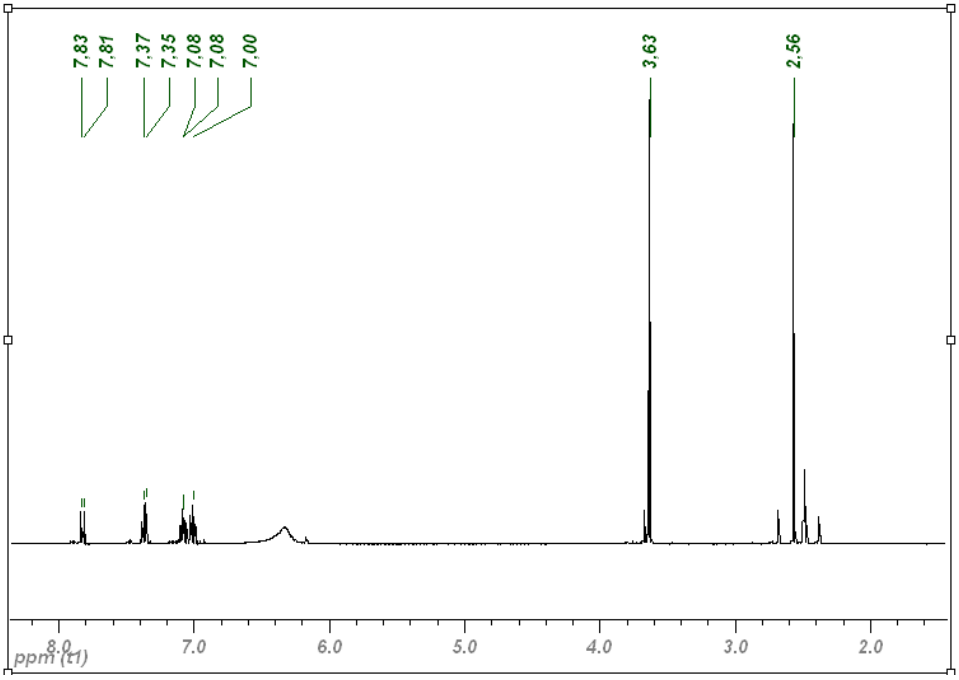

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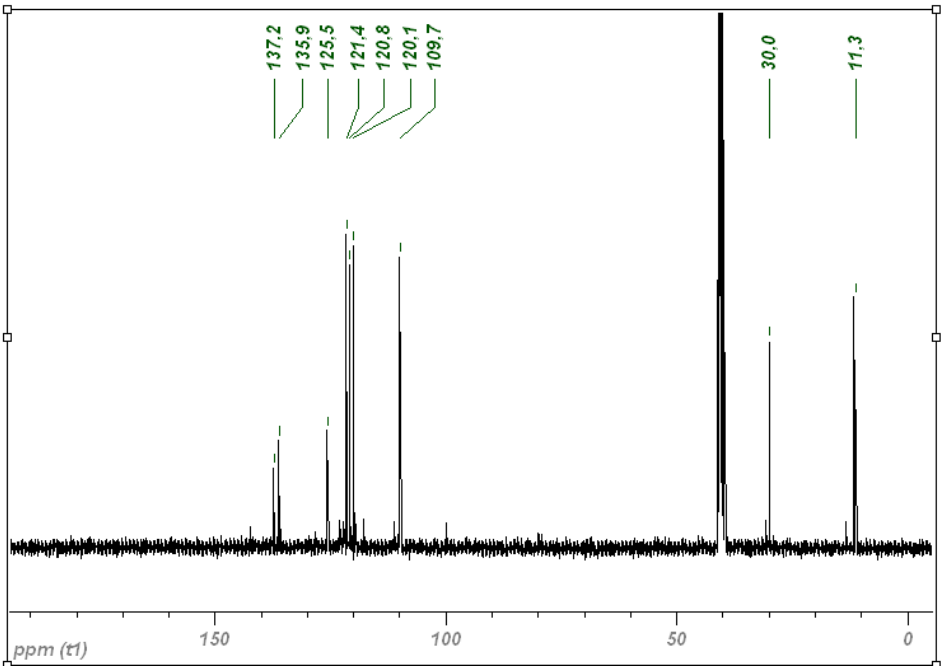

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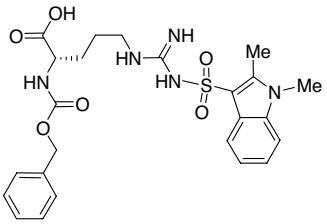

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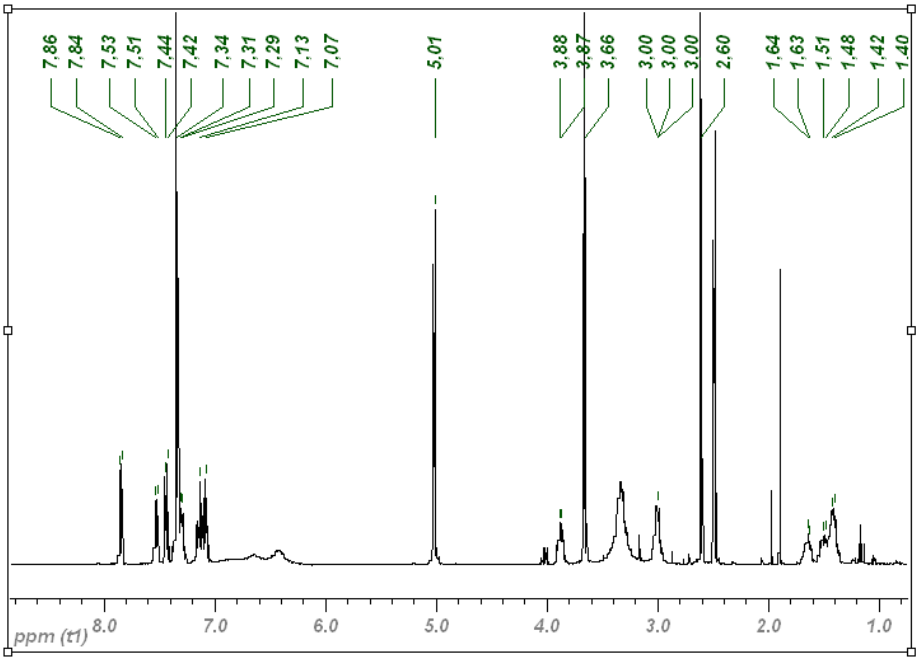

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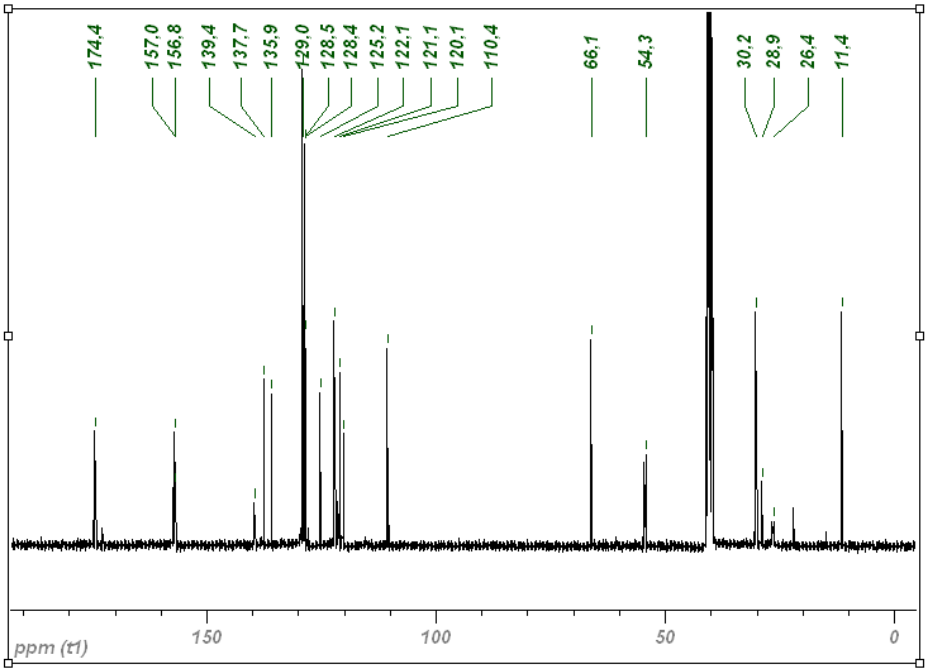

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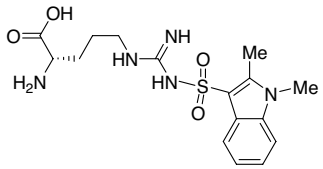

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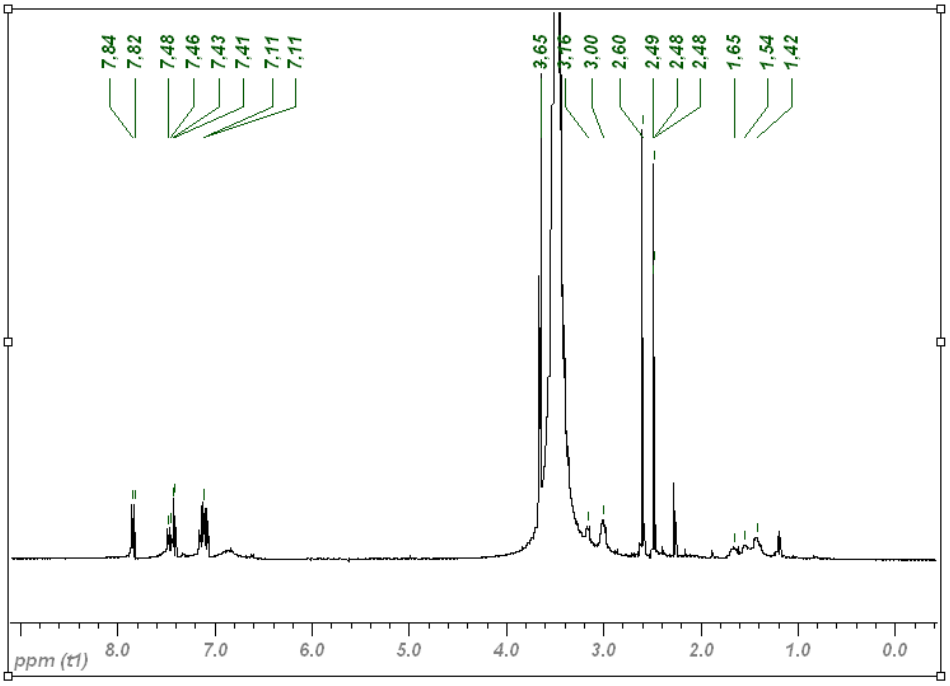

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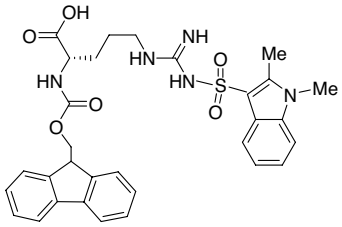

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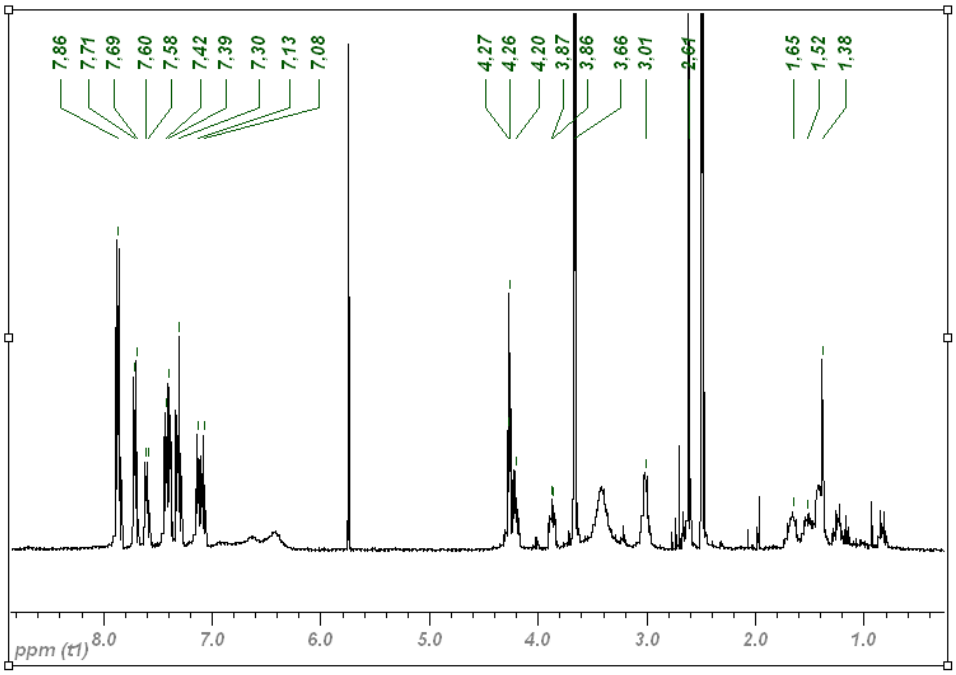

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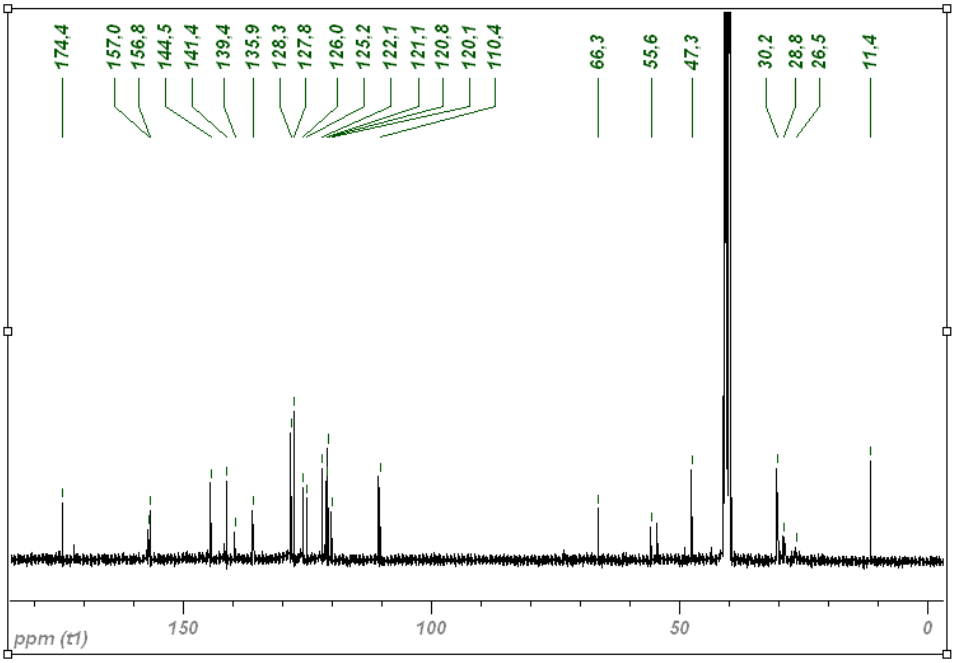
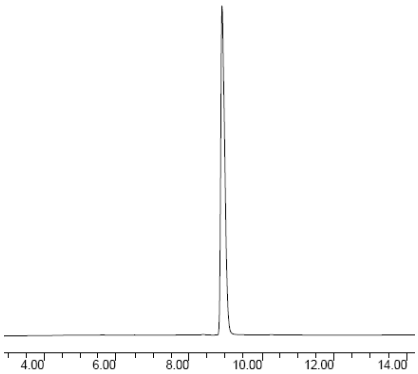


2. HPLC chromatograms (λ= 220 nm)

2.1 Arginine derivatives:

Z-L-Arg(MIS)-OH (3)



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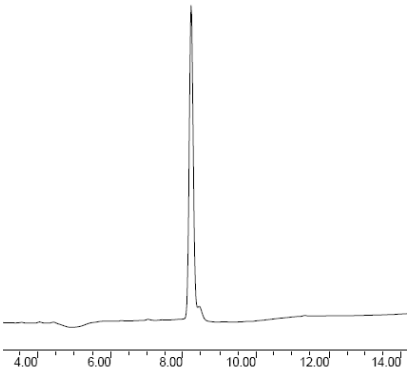
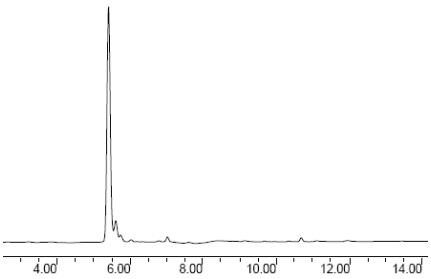
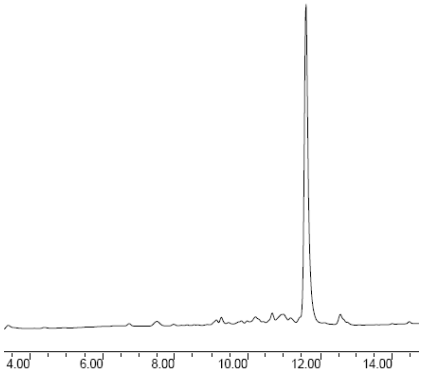


H-L-Arg(MIS) (4)

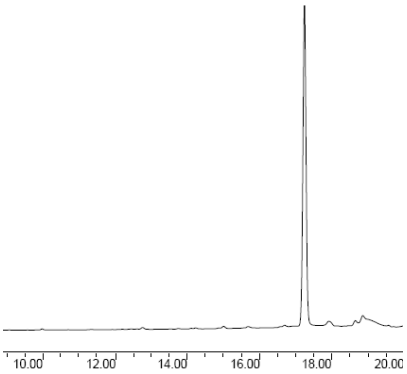


2.2 Multiarginine containing peptides:

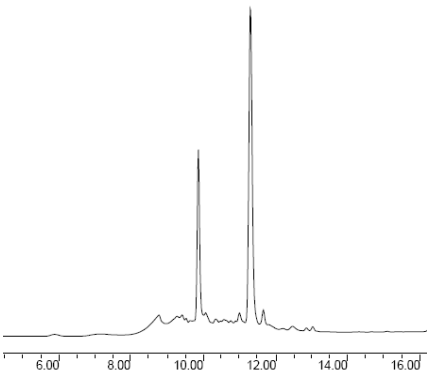
Ac-Phe-Arg(MIS)-Arg(MIS)-Arg(MIS)-Arg(MIS)-Val-NH_2 (peptide 1)



Ac-Phe-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Val-NH_2 (peptide 2)

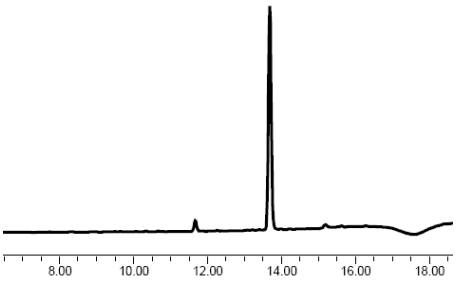


Ac-Phe-Arg-Arg-Arg-Arg-Val-NH_2 from Ac-Phe-Arg(MIS)-Arg(MIS)-Arg(MIS)-Arg(MIS)-Val-NH_2 using 50% of TFA and 10% of 3,4-dimethoxyphenol in DCM as cleavage cocktail, 1h (*R* t= 10.2 min: target peptide, *R* t= 11.7 min MIS-OH).

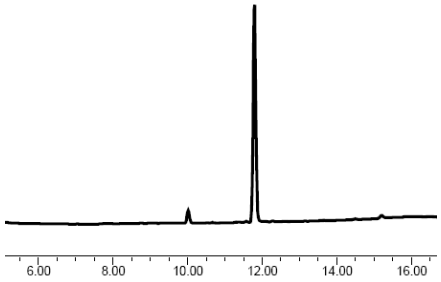


2.3 Tryptophan containing peptides:

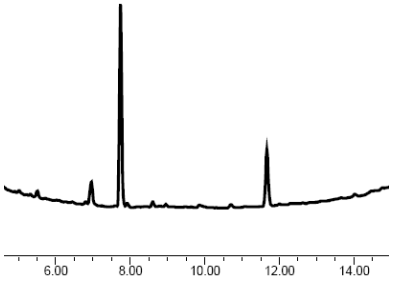
Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2



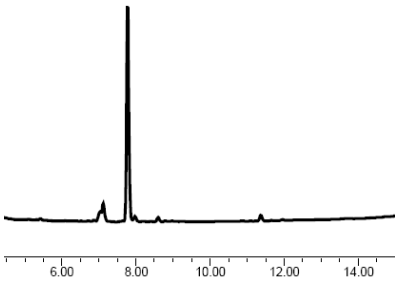
Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2



Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 (*R* t= 7.7 min: target peptide, *R* t= 11.7 min Pbf protected peptide).



Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2



CONCLUSIONS

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El treball realitzat durant aquesta Tesi Doctoral ha permès desenvolupar una sèrie de grups protectors que resolen alguns dels problemes que presenta la síntesi de pèptids en l'actualitat o si més no donen opcions diferents a les ja existents augmentant la versatilitat de la química de pèptids. Concretament:

- El nou agent protector desenvolupat, l'Fmoc-2-mercaptobenzotiazole (Fmoc-MBT), permet la síntesi d'Fmoc aminoàcids evitant les reaccions secundàries associades als reactius actuals.
- Les condicions d'eliminació del grup *p*NZ com a protector tan de la funció α-amino, com de les cadenes laterals d'Orn i Lys, millorades en aquesta tesi, el fan ortogonal als grups protectors més usats en síntesi de pèptids (Boc, Fmoc i Alloc) i minimitzen reaccions secundàries associades a la síntesi de pèptids en fase sòlida mitjançant l'estratègia Fmoc/^{t}Bu, com la formació de dicetopiperazines, aspartimides i l'eliminació no desitjada del grup α-Fmoc. L'ús del grup *p*NZ permet l'accés a estructures peptídiques inaccessibles d'una altra manera i el desenvolupament de noves estratègies de síntesi de pèptids d'elevat interès biològic com el pèptid antitumoral Kahalalide F.
- El nou espaïador bifuncional desenvolupat, l'àcid *p*-nitromandèlic (*p*NMA) és útil per a la síntesi de pèptids i depsipèptids tan lliures com amb protectors de tipus benzílic mitjançant química Boc/Bn.
- Els dos nous protectors d'amides de l'esquelet peptídic (3,4-etilendioxi-2-tenil (EDOTn) i l'1-metil-3-indolilmetil (MIM)) milloren algunes de les propietats dels protectors actuals, com la seva difícil eliminació i el seu impediment estèric.
- La sèrie de grups protectors d'àcids carboxílics (tan estàndar com de tipus espaïador bifuncional) derivats del 3,4-etilendioxitiofè, són làbils a concentracions molt baixes de TFA i per tant potencialment útils per a la síntesi de pèptids sensibles a medi àcid.
- El nou protector per a la cadena lateral de l'Arg, l'1,2-dimetilindole-3-sulfonil, és molt més làbil a medi àcid que els protectors usats en l'actualitat, així com compatible amb pèptids que continguin Trp.