S-Phenylacetamidomethyl (Phacm): an orthogonal cysteine protecting group for Boc and Fmoc solid-phase peptide synthesis strategies †

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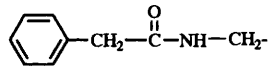
The reliability of the *S*-phenylacetamidomethyl-L-cysteine Cys(Phacm), a versatile derivative compatible with both Boc and Fmoc protection schemes, has been examined for solid-phase peptide synthesis. The Phacm group has the same properties as acetamidomethyl (Acm) and in addition is cleaved by the action of the enzyme penicillin amidohydrolase. Thus, Phacm is orthogonal with the common cysteine-protecting groups, such as 4-methylbenzyl (*p*-MeBzl), trityl (Trt) and fluorenylmethyl (Fm).

Introduction

It is well known that the side-chain of cysteine requires protection during the coupling steps in solid-phase peptide synthesis.[1,2] The synthesis of peptides with all cysteine residues as free thiols or with one or more disulfide bridges formed in a controlled fashion is related to the availability of appropriate readily orthogonal,[3] removable protecting groups for the thiol function. In addition to the common protecting groups that can be removed by chemical methods,[2] it would be important to have a protecting group that can be removed orthogonally by an independent and different approach, such as an enzymatic one.

During the last few decades, enzymic methods have been used more frequently to remove blocking groups from α-amino and α-carboxyl groups rather than side-chain functionalities in peptide synthesis.[4] Enzymes often operate at neutral, weakly acidic or basic pH-values and in many cases combine a high selectivity for the reactions and structures that are catalysed and recognized with broad substrate specificity. Therefore, the application of these biocatalysts to effect the removal of suitable protecting groups promises to be a viable alternative to classical chemical methods. Hermann and Hoffmann[5] have found that sulfur-protecting groups of the acylamidomethyl type could be removed in a two-step reaction by first eliminating the acyl component and then by generating the SH group by spontaneous hydrolysis of the intermediate aminomethylsulfanyl compound. One of the most common cysteine-protecting groups in solid-phase peptide synthesis of this type is acetamidomethyl (Acm),[6] which has the great advantage of being stable to all the standard reagents in the synthesis of either free peptides or protected segments in a convergent strategy.[7] Initial attempts to remove the acetyl group in Cys(Acm) and subsequently to liberate the β-mercapto group of cysteine involved the use of an ω-aminoacylase from chicken kidney. Unfortunately, the enzymic cleavage was not effective on N-protected derivatives of Cys(Acm) and this approach could not be used for deprotection of the Acm group in peptide synthesis.[4a] Hermann and Greiner[8] obtained more promising results when a phenylacetamidomethyl group (Phacm) was used as a cysteine-protecting group and deprotected by means of the penicillin G acylase from *E. coli* (EC 3.5.1.11) with a P_1 specificity for the phenylacetyl residue. After penicillin acylase-catalysed hydrolysis of the amide incorporated in the acylated thioaminal, the labile *S*-aminomethyl compound was formed and immediately liberated the desired thiol. The selective enzymic deprotection of the *S*-phenylacetamidomethyl group is orthogonal to several protecting groups used for the thiol side-chain of cysteine in peptide synthesis. The use of Cys(Phacm) for the solution synthesis of several peptides has recently been reported.[9] Therefore, we were encouraged to explore the use of Phacm as a cysteine-protecting group in solid-phase peptide synthesis.



Phacm

Results and discussion

Preparation and properties of Cys(Phacm) derivatives

The Phacm group was introduced onto cysteine by the same one-pot protocol as used for the Acm group[6a,10] (Scheme 1). First, *N*-(hydroxymethyl)phenylacetamide **1** was synthesized from formaldehyde and phenylacetamide, which was prepared by acid hydrolysis of benzyl cyanide.[11] Then, L-cysteine was treated with *N*-(hydroxymethyl)phenylacetamide **1** in the presence of trifluoromethanesulfonic acid (TFMSA), and subsequent treatment with di-*tert*-butyl dicarbonate[12] or Fmoc-succinimide[13] to give the appropriate fully protected derivatives **2**, **3** in an overall yield of 56 and 67%, respectively.§

In order to test the compatibility of this protecting group with Boc/Bzl and Fmoc/Bu^t¶ solid-phase peptide strategies, the stability of Cys(Phacm) was examined under several representative conditions employed in these methodologies, Cys(Phacm) was completely stable to DIEA–CH_2Cl_2 (1:19), TFA–CH_2Cl_2 (4:6), piperidine–DMF (2:8), 0.1 mol dm^{-3} TBAF in DMF, and DBU–DMF (1:19)¶ for 24 h at 25 °C, conditions commonly used for the solid-phase peptide elongation when using Boc and Fmoc strategies.[1d] In addition, Cys(Phacm) was also stable to HF–anisole or *p*-cresol (9:1) for 1 h at 0 °C, and

† Taken in part from the PhD thesis of M. R., University of Barcelona, March 1994.

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§ In our hands, N-protected Phacm derivatives of cysteine were found to be more readily available in crystalline form than were compounds with Acm as their protecting group.

¶ Boc = *tert*-butoxycarbonyl, Fmoc = fluoren-9-ylmethoxycarbonyl, DIEA = diisopropylethylamine, TFA = trifluoroacetic acid, DMF = dimethylformamide, TBAF = tetrabutylammonium tetrafluoroboranuide, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TEAOAc = tetraethylammonium acetate, PEG–PS = polyethylene glycol–polystyrene, PAL = 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy] valeric acid, DIPCDI = diisopropylcarbodiimide and MBHA = methylbenzhydrylamine; Dnpe = dinitrophenylethyl.

Ac-Cys-Pro-D-Val-Cys-NH_2 H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-NH_2

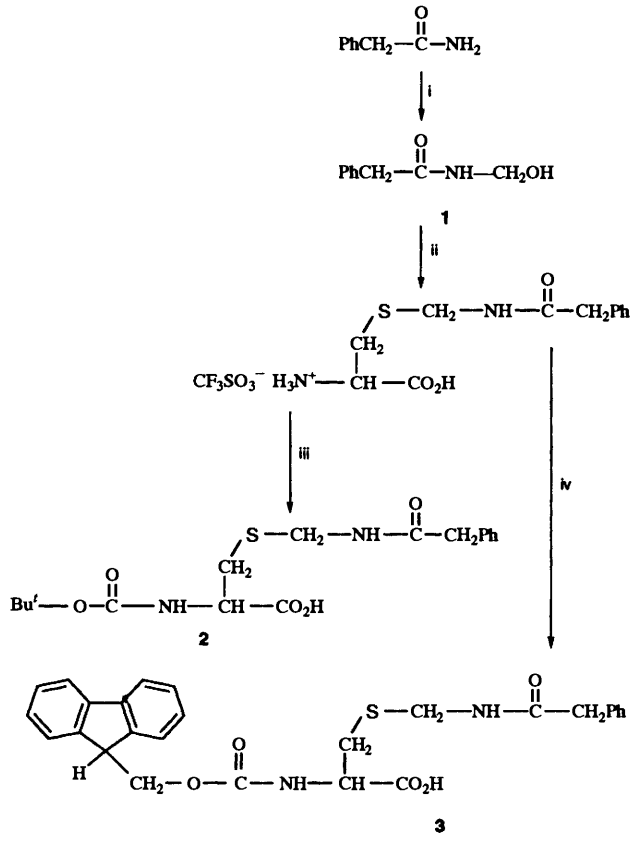
β-turn model peptide, **4** [Lys^8]vasopressin, **5**

H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-NH_2 Ac-Cys-Pro-D-Val-Cys-NH_2

somatostatinamide, **6** Ac-Cys-Pro-D-Val-Cys-NH_2

dimer β-turn model peptide, **7**

Scheme 2 Synthesis of cystine-containing peptides



Scheme 1 *Reagents and conditions:* (i) HCHO, KOH, 70 °C to 25 °C, overnight; (ii) L-cysteine·HCl, TFMSA–TFA–water (2:38:60), 0 °C, 6 h; (iii) Boc_2O–Na_2CO_3, pH 9–9.5, Bu^{t}OH–water (2:1), 25 °C, 24 h; (iv) FmocOSu–Na_2CO_3, pH 9–9.5, 1,4-dioxane, 16 h

TFA–scavengers (phenol, ethane-1,2-dithiol, *p*-cresol, anisole) (9:1) for 2 h at 25 °C, which are reagents used for the final cleavage of the peptide from the resin. On the other hand, it was only partially stable (> 80%) in the presence of TFMSA–TFA-*p*-cresol (1:10:0.3) for 2 h at 25 °C, a cocktail used in the Boc strategy to cleave the peptide from the resin.[14] Finally, Cys(Phacm) was also stable to piperidine–DMF–β-mercaptoethanol (βME) (10:10:0.7), a reagent used for the removal of the Fm group from the thiol of Cys.[15] These results indicated that the Phacm protecting group was stable to all the standard reagents used in the synthesis, and therefore the Cys can be deprotected independently at the end of the synthesis, once all other protecting groups have been removed.

The Cys(Phacm) derivative was quantitatively converted into cystine by either the penicillin G acylase (PAH) in solution, immobilized on dry fibre or on acrylic beads (Eupergit C),‖ in Na_2HPO_4–NaH_2PO_4 (0.02 mol dm^{-3}) buffer at pH 7.8, at 35 °C over a period of 30 min–2 h. Reactions carried out in triethylammonium acetate (TEAOAc) (0.02 mol dm^{-3}) or NH_4OAc (0.05 mol dm^{-3}) buffers at pH 7.8, at 35 °C under identical pH and temperature conditions, also gave complete deprotection. If the enzymic hydrolysis was conducted in the presence of βME (2%), the free cysteine derivative was isolated; in the absence of external thiols, direct oxidation to the cystine derivative occurs. Although the enzymic deprotection was more effective in aqueous media, it could be carried out in the presence of organic cosolvents such as DMF and *N*-methylpyrrolidine (NMP) (up to 25%), or MeOH (up to 30%). In these cases, a reaction time of 48 h was required for complete deprotection and oxidation. Since Phacm is an acylamidomethyl-type sulfur-protecting group, it is labile to oxidative conditions such as I_2 in aq. HOAc (80%) similar to those used for the deprotection of Cys(Acm).[6b,16] Under these conditions, the formation of disulfide bridges is accomplished.

In summary, the Phacm group has the same stability/lability properties as Acm, but Phacm is additionally cleaved by the action of penicillin amidohydrolase. The Phacm group is orthogonal with the base-labile protecting groups Fm[15,17] and Dnpe,[18] the acid-labile *p*-MeBzl,[19] Trt,[20] and Tmob[21] groups, and is compatible with Acm under proper conditions (initial enzymic deprotection of Phacm followed by deprotection of Acm with iodine or thallium salts[22]).

Use of Cys(Phacm) in solid-phase peptide synthesis

To demonstrate the usefulness of this protecting group in solid-phase synthesis, several cystine-containing peptides were synthesized (Scheme 2).

Starting with an MBHA-resin, assembly of the bis(Phacm) sequence of **4**[23] and [Lys^8] vasopressin **5** by Boc chemistry was performed manually and on an ABI batch synthesizer, respectively. Side-chains of Lys and Tyr were protected with ClZ and BrZ, respectively (Z = benzyloxycarbonyl). Treatment of the peptide–resins with anhydrous HF–anisole (9:1) for 1 h at 0 °C provided the crude bis(Phacm)-protected peptides (82 and 84% yield, respectively). The partially protected peptides were incubated with immobilized PAH at 35 °C for 24 h under the conditions outlined in Tables 1 and 2. For both peptides, deprotections carried out in NH_4OAc buffer (Figs 1 and 2) presented slightly better results in both overall yield and purity of the final product.

The synthesis of somatostatinamide **6** was accomplished automatically on a Millipore continuous-flow synthesizer according to a standard Fmoc/Bu^t strategy by using PAL-handle[25] on a PEG-PS-resin.[26] Threonine and serine were protected as their Bu^t ethers, asparagine with a Trt group, lysine with a Boc group, whereas tryptophan was unprotected. Bis(Phacm)-protected peptide was cleaved from the resin in 41% yield with TFA–water (9:1)** and subsequently was incubated with immobilized PAH by using conditions described in Table 3.†† The final product was obtained in both cases with good yield and purity (Fig. 3).

‖ Immobilized PAH is currently used for the industrial production of penicillin, so this approach can be used for large-scale of synthesis cystine-containing peptides.

** Similar yields were obtained when the same peptide was synthesized with Trt protection for Cys, showing that this low yield is sequence dependent rather due to the protecting group of Cys.

†† Deprotection of [Lys^8]vasopressin and Ac-Cys(Phacm)-Pro-D-Val-Cys(Acm)-NH_2 was attempted in the presence of organic co-solvents (25% of MeOH, DMF, and NMP) with negative results in all cases. These results corroborate those found by Hermann in the synthesis of oxytocin.[4a]

Table 1 Enzymic deprotection Ac-Cys(Phacm)-Pro-D-Val-Cys(Phacm)-NH_2 using different buffers

	NaH_2PO_4–Na_2HPO_4 (0.02 mol dm^{-3})	NH_4OAc (0.05 mol dm^{-3})	TEAOAc (0.02 mol dm^{-3})
Peptide (μmol)	6.23	6.25	7.1
Peptide concentration (mol dm^{-3})	1.25×10^{-3}	1.25×10^{-3}	1.4×10^{-3}
EU/μmol of Phacm	0.59	0.72	0.34
Recovery yield (%)	87	84	65
Overall yield (%)[a]	69	92	88

[a] The absolute yield of the peptide was determined by comparing the HPLC peak area of the crude peptide with that of a standard of pure peptide of known concentration, as described previously.[24]

Table 2 Enzymic deprotection bis(Phacm)-[Lys^8]vasopressin using different buffers

	NaH_2PO_4–Na_2HPO_4 (0.02 mol dm^{-3})	NH_4OAc (0.05 mol dm^{-3})	TEAOAc (0.02 mol dm^{-3})
Peptide (μmol)	1.3	1.2	1.4
Peptide concentration (mol dm^{-3})	8.7×10^{-5}	8×10^{-5}	9.3×10^{-5}
EU/μmol of Phacm	1.49	1.65	1.12
Recovery yield (%)	61	93	59
Overall yield (%)[a]	90	93	90

[a] The absolute yield of the peptide was determined by comparing the HPLC peak area of the crude peptide with that of a standard of pure peptide of known concentration.

Table 3 Enzymic deprotection bis(Phacm)-somatostatinamide using different buffers

	NH_4OAc (0.05 mol dm^{-3})	TEAOAc (0.02 mol dm^{-3})
Peptide (μmol)	0.6	0.6
Peptide concentration (mol dm^{-3})	2×10^{-4}	2×10^{-4}
EU/μmol of Phacm	1	1
Recovery yield (%)	84	89
Overall yield (%)[a]	80	85

[a] The absolute yield of the peptide was determined by comparing the HPLC peak area of the crude peptide with that of a standard of pure peptide of known concentration.

In order to demonstrate the compatibility of Phacm with Acm, the cyclic dimer peptide **7** [27] was prepared (Scheme 3). Ac-Cys(Phacm)-Pro-D-Cys(Acm)-NH_2, which was synthesized manually *via* a Boc strategy, was incubated with immobilized PAH under conditions shown in Table 4, to give the open dimer (**8**, Fig. 4). The second disulfide bond was formed upon treatment of the bis-*S*-Acm peptide with I_2 (10 mol equiv.) in HOAc–water (4:1) for 2 h at 25 °C to render the target peptide in excellent yield.

Conclusions

The use of phenylacetamidomethyl (Phacm) group as a protecting group for the thiol function of cysteine has been demonstrated to be compatible with both Boc/Bzl and Fmoc/Bu'-based solid-phase peptide-synthesis strategies. The Phacm group has the same stability/lability properties than Acm, but Phacm is also cleaved by the action of penicillin amidohydrolase at neutral pH. Owing to these mild properties of the Phacm group, the peptide-synthesis strategies involving Phacm are more flexible than those using only chemically removable protecting groups. The enzymically removable Phacm can be used in combination with protecting groups such as Fm–Dnpe and *p*-MeBzl–Trt–Tmob cleaved by chemical methods. In addition, the Phacm group can also be compatibly applied with Acm (initial enzymic deprotection of Phacm followed by chemical deprotection of Acm). Furthermore, a proper combination of Phacm protecting group with chemically removed cysteine-protecting groups may facilitate regiospecific synthesis of target peptides containing two or more disulfide bridges.

Experimental

General procedures

Materials, solvents, instrumentation and general methods were described in previous publications of these laboratories.[7,10,15,16,18,25,27] PAH immobilized on acrylic beads (Eupergit-PcA) was supplied by Röhm Pharma (Weiterstad, Germany) and showed an enzymic activity of 108 u/g wet weight. *N*-Phenylacetamide was prepared in 72% yield according to a previous, well established method.[11] ^1H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Varian XL-200 spectrometer. *J*-Values are given in Hz. TLC was performed on Merck silica gel plates (200 μm, 10 × 20 cm), developed with the BAW system: butan-1-ol–acetic acid–water (10:2:3). Compounds were visualized by (1) fluorescence quenching; (2) spray with 0.03% (w/v) ninhydrin in acetone; (3) spray with 5,5'-dithiobis(2-nitrobenzoic acid) (0.01 mol dm^{-3}) in pH 7.0 buffer followed by spray with 0.1 mol dm^{-3} [tris(hydroxymethyl)methyl]ammonium chloride (Tris·HCl) buffer at pH 8 (free thiol indicated by immediate formation of yellow spot, Ellman reagent). A pH-meter was used to determine pH values.

N-(Hydroxymethyl)phenylacetamide 1

Phenylacetamide (10 g, 74 mmol) and potassium hydroxide (0.5 g, 8.7 mmol) were dissolved in aq. formaldehyde (35%) (5.9 cm^3, 75 mmol). The mixture was stirred for 5 min at 70 °C and then overnight at 25 °C, and then was acidified to pH 7 with aq. 1 mol dm^{-3} HCl. The solution was concentrated by rotary evaporation, and the residue was dissolved in acetone (50 cm^3), dried over $MgSO_4$, and concentrated under reduced pressure to give an NMR-pure solid, which was used without further purification (12.2 g, 99%); mp 75–77 °C; $\delta_H[(CD_3)_2SO]$ 8.64 (1 H, t, *J* 5.9), 7.26 (5 H, m), 5.56 (1 H, *J* 6.8), 4.47 (2 H, t, *J* 6.6 and 3.39 (2 H, s); $\delta_C[(CD_3)_2]$ 170.2 (C=O), 136.1 (aryl C-1), 129.1, 128.2

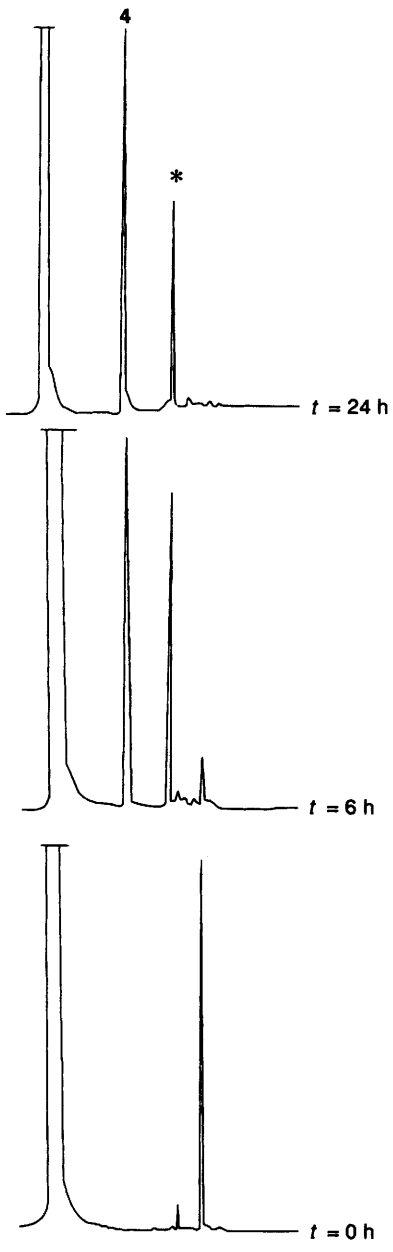


Fig. 1 Analytical HPLC of samples from enzymical deprotection and posterior oxidation of Ac-Cys(Phacm)-Pro-D-Val-Cys(Phacm)-NH_2 at different times. The reaction was carried out in NH_4OAc buffer (0.05 mol dm^{-3}). HPLC was performed on a Vydac C-18 reversed-phase column (5 μm, 4.6 × 250 mm); linear gradient over a period of 20 min using MeCN and water containing, respectively, 0.036% and 0.045% of TFA, from 1:19 to 13:7, flow rate 1.0 cm^3 $min.^{-1}$ The peak (*) corresponds to phenylacetic acid.

Fig. 2 Analytical HPLC of samples from enzymatical deprotection and posterior oxidation of bis(Phacm)-[Lys^8] vasopressin at different times. The reaction was carried out in NH_4OAc buffer (0.05 mol dm^{-3}). HPLC conditions were nominally the same as for Fig. 1. The peak (*) corresponds to phenylacetic acid.

and 126.3 (aryl C-2, -3 and -4), 62.4 (CH_2OH) and 42.5 (CH_2-aryl); ν_{max}(KBr)/cm^{-1} 3300, 3060, 3030, 2950, 1660, 1545, 1065, 1020, 890, 765, 750 and 700; *m/z* (EI) 165 (M^+, 3%), 148 (M^+ − 17, 1, $C_9H_{10}NO^+$), 135 (M^+ − 30, 5, $C_8H_9NO^+$), 92 (M^+ − 73, 100, $C_7H_8^+$), 91 (M^+ − 74, 100, $C_7H_7^+$) and 65 (M^+ − 100, 30, $C_5H_5^+$) (Found: C, 65.5; H, 6.65; N, 8.1. Calc. for $C_9H_{11}NO_2$: M, 165.2; C, 65.44; H, 6.71; N, 8.48%).

N^{α}-(*tert*-Butoxycarbonyl)-*S*-(phenylacetamidomethyl)-L-cysteine [Boc-Cys(Phacm)-OH] 2

N-(Hydroxymethyl)phenylacetamide **1** (5 g, 30.3 mmol) and L-cysteine hydrochloride (3.9 g, 25.0 mmol) were dissolved in distilled water (30 cm^3). The mixture was cooled in an ice-bath and TFMSA–TFA (1:19) (39 cm^3) was added. After being stirred under Ar for 6 h, TLC (BAH) indicated that all cysteine had reacted. The solvent was removed by rotary evaporation, including chasing with Et_2O (8 × 50 cm^3), to give an oil. The oil was suspended in Bu^{t}OH–water (2:1) (150 cm^3), cooled in an ice-bath, and a solution of Boc_2O (6 g, 27.5 mmol) in Bu^{t}OH (49 cm^3) was added. The pH was brought to 9–9.5 with aq. Na_2CO_3 (10%). After being stirred on an ice bath for 2 h, the reaction mixture was maintained at 25 °C for 24 h, with the pH being maintained at 9.0–9.5 by addition of aq. Na_2CO_3. The mixture was washed with hexane (2 × 50 cm^3) and the aqueous phase was acidified with aq. HCl (1 mol dm^{-3}) to pH 2 and extracted with EtOAc (3 × 50 cm^3). The organic layers were combined, washed and saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to provide a pale yellow oil, which was crystallized from EtOAc–hexane to give a solid (5.2 g, 56% calculated from the L-cysteine hydrochloride); mp 100–101 °C; $\delta_H(CD_3OD)$ 7.40 (5 H, m), 4.43 (3H, br s), 3.62 (2 H, s),

Table 4 Enzymic deprotection Ac-Cys(Phacm)-Pro-D-Val-Cys(Acm)-NH_2 using different buffers

	NaH_2PO_4–Na_2HPO_4 (0.02 mol dm^{-3})	NH_4OAc (0.05 mol dm^{-3})	TEAOAc (0.02 mol dm^{-3})
Peptide (μmol)	5.6	5.7	5.45
Peptide concentration (mol dm^{-3})	8.3×10^{-4}	9.5×10^{-4}	9.1×10^{-4}
EU/μmol of Phacm	1.56	2.12	1.88
Recovery yield (%)	77	79	77
Overall yield (%)[a]	84	95	94

[a] The absolute yield of the peptide was determined by comparing the HPLC peak area of the crude peptide with that of a standard of pure peptide of known concentration.

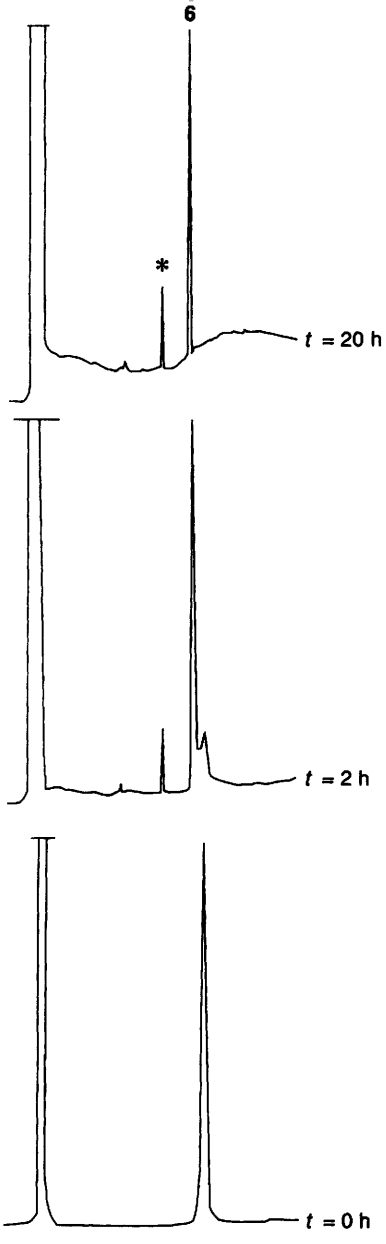
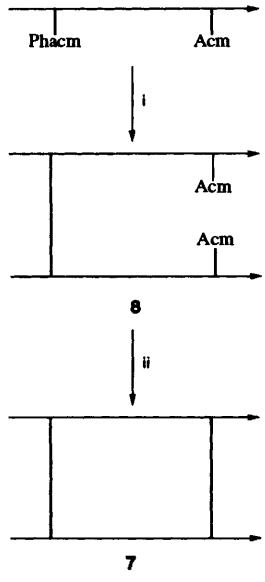


Fig. 3 Analytical HPLC of samples from enzymatical deprotection and posterior oxidation of bis(Phacm)-somatostatinamide at different times. The reaction was carried out in TEAOAc buffer (0.02 mol dm^{-3}). HPLC conditions were nominally the same as for Fig. 1. The peak (*) corresponds to phenylacetic acid.



Scheme 3 *Reagents and conditions:* i, Penicillin amidohydrolase, pH 7.8, 38 °C, 30 h; (ii) I_2, AcOH–water (4:1), 2 h

3.15 (1 H, dd, *J* 4.6 and 14.2), 2.93 (1 H, dd, *J* 8.7 and 14.2 and 1.54 (9 H, s); δ_C[$(CD_3)_2SO$] 172.9 (CO_2H), 170.6 (phenylacetamidomethyl carbonyl), 155.7 (Boc carbonyl), 136.3 (aryl C-1), 129.3, 128.5 and 126.7 (aryl C-2, -3 and -4), 78.5 (Boc C), 53.9 (C-α), 42.5 (CH_2Ph), 41.0 (CH_2-phenylacetamido), 32.0 (CH_2-β) and 28.4 (Boc Me); *m/z* (EI) 368 (M^+, 5%), 312 ($M^+ - 56, 1, M^+ - C_4H_8$), 148 ($M^+ - 101 - 119, 24, C_4H_8N_2O_2S^+$), 91 ($M^+ - 277$, 100, $C_7H_7^+$) and 57 ($M^+ - 311$, 59, $C_4H_9^+$) (Found: C, 55.3; H, 6.5; N, 7.6; S, 8.5. Calc. for $C_{17}H_{24}N_2O_5S$: M, 368.5; C, 55.42; H, 6.56; N, 7.60; S, 8.70%).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*S*-(phenylacetamidomethyl)-L-cysteine [Fmoc-Cys(Phacm)-OH] 3

S-(Phenylacetamidomethyl)-L-cysteine trifluoromethanesulfonate salt, prepared as described above from *N*-(hydroxymethyl)phenylacetamide **1** (6.3 g, 38.3 mmol) and L-cysteine hydrochloride (5.0 g, 31.7 mmol), was dissolved in aq. Na_2CO_3 (10%); (65 cm^3) and the pH was brought to 9.5 with the same solution. The solution was cooled in an ice-bath and a solution of fluoren-9-ylmethyl succinimido carbonate (Fmoc-OSu) (9.4 g, 27.7 mmol) in 1,4-dioxane (82 cm^3) was added. After being stirred for 2 h in the ice-bath, the mixture was kept at 25 °C for 16 h, with the pH being maintained at 9.0–9.5 by addition of aq. Na_2CO_3. The mixture was then diluted with distilled water (350 cm^3) and extracted with Et_2O (2 × 50 cm^3). The aqueous layer was acidified with aq. 6 mol dm^{-3} HCl to pH 2, and then extracted with EtOAc (3 × 100 cm^3). The combined organic layers were washed with saturated aq. NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to provide an oil, which was crystallized from aq. MeOH to give the title compound as a solid (10.4 g, 67% calculated from the L-cysteine hydrochloride), mp 108–110 °C; δ_H[$(CD_3)_2CO$] 7.80 (2 H, d, *J* 7.0), 7.68 (2 H, d, *J* 7.2), 7.4–7.1 (9 H, m), 4.5–4.1, (6 H, m), 3.52 (2 H, s), 3.09 (1 H, dd, *J* 4.5 and 14.1) and 2.88 (1 H, dd, *J* 8.8

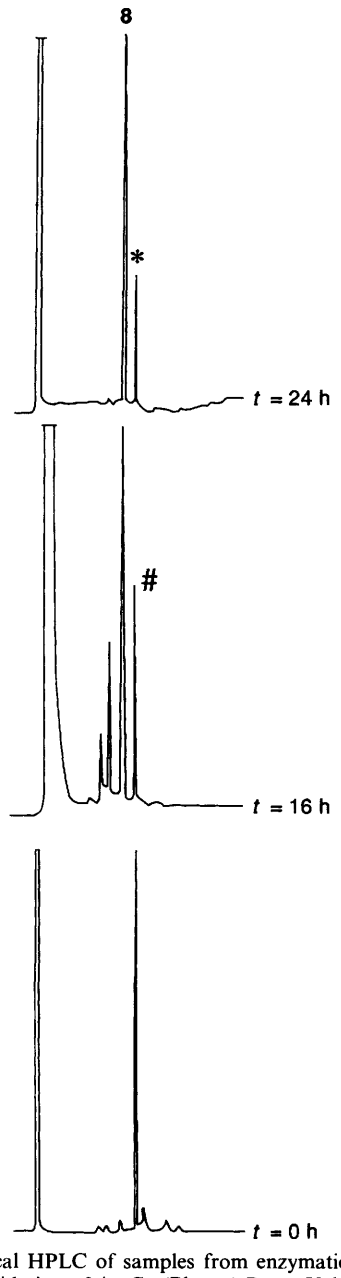


Fig. 4 Analytical HPLC of samples from enzymatical deprotection and posterior oxidation of Ac-Cys(Phacm)-Pro-D-Val-Cys(Acm)-NH_2 at different times. The reaction was carried out in NH_4OAc buffer (0.05 mol dm^{-3}). HPLC conditions were nominally the same as for Fig. 1. Peak (#) corresponds to a mixture of starting protected peptide and phenylacetic acid; (*) corresponds to phenylacetic acid.

and 14.1); δ_C[$(CD_3)_2SO$] 172.6 (CO_2H), 170.6 (phenylacetamidomethyl carbonyl), 156.3 (Fmoc carbonyl), 144.1 and 141.0 (Fmoc-aryl C), 136.3 (aryl C-1), 129.3, 128.5 and 126.7 (aryl C-2, -3 and -4), 127.9, 127.4, 125.6 and 120.4 (Fmoc-aryl CH), 66.0 (Fmoc-CH_2), 54.3 (C-α), 46.9 (fluorenyl C-9), 42.5 (CH_2-aryl), 41.0 (CH_2-phenylacetamido) and 32.0 (CH_2-β); *m/z* (EI) 178 (M^+ − 312, 100%, $C_{14}H_{10}^+$), 165 (M^+ − 325, 41, $C_9H_{11}NO_2^+$), 92 (M^+ − 398, 16, $C_7H_8^+$) and 91 (M^+ − 399, 93, $C_7H_7^+$) (Found: C, 65.8; H, 5.3; N, 5.7; S, 6.5. Calc. for $C_{27}H_{26}N_2O_5S$: M, 490.6; C, 66.11; H, 5.34; N, 5.71; S, 6.54%).

Studies on stability and removal of Phacm

Solutions (1 mmol dm^{-3}) of protected amino acids **2**, **3** in different reagents/solvent mixtures were prepared. Experiments with basic reagents were carried out with the Boc derivative **2** and the rest with the Fmoc compound **3**. Aliquots of the solution (20 mm^3) were removed at different times and checked by TLC (BAW) with fluorescence quenching, ninhydrin and Ellman detection and/or HPLC (linear gradient over 20 min of MeCN–water containing 0.035 and 0.045% TFA, respectively, from 1:9 to 1:0, 1 cm^3 min^{-1}). Aliquots removed from TFA and TFMSA cocktails were neutralized with aq. Na_2CO_3 (10%). Reaction with HF was carried out in the presence of anisole or *p*-cresol (9:1) at 0 °C for 1 h, and after evaporation the residue was checked. After reaction with I_2, the mixture was quenched with ascorbic acid.

Enzymic removal of Phacm

Solutions (1 mmol dm^{-3}) of Boc-Cys(Phacm)-OH **2** were incubated with different quantities (0.13, 1,4, and 5 ue/μmol Phacm) of PAH in solution or immobilized on dry fibre or on acrylic beads (Eupergit C), in Na_2HPO_4–NaH_2PO_4 (0.02 mol dm^{-3}) buffer at pH 7.8, at 35 °C for 24 h. Other aq. buffers such as TEAOAc (0.02 mol dm^{-3}) and NH_4OAc (0.05 mol dm^{-3}) were also studied, as well as addition of βME (2%), NMP (25%), MeOH (30%), and DMF (25%) to Na_2HPO_4–NaH_2PO_4 buffer. Aliquots of the solution (20 mm^3) were removed at different times and checked by HPLC as described above.

General procedure for the solid-phase synthesis of peptides

Boc strategy. Peptide syntheses performed manually were carried out with MBHA-resin (0.15 g, 0.12 mmol) in a 50 cm^3 polypropylene syringe fitted with a polyethylene disc. Boc-amino acids were assembled using the following protocol: (1) CH_2Cl_2 (4 × 0.5 min); (2) TFA–CH_2Cl_2 (4:6) (1 × 1 min, 1 × 30 min); (3) CH_2Cl_2 (3 × 0.5 min); (4) DIEA–CH_2Cl_2 (1:19) (3 × 0.5 min); (5) CH_2Cl_2 (4 × 0.5 min); (6) Boc-amino acid (3 mol equiv.) in CH_2Cl_2; after 2 min, add the equivalent amount of DCC, store 90 min at 25 °C with occasional agitation; (7) CH_2Cl_2 (4 × 0.5 min). The final acetylation when required was carried out with HOAc and dicyclohexylcarbodiimide (DCC) (2.5 mol equiv. of each) in CH_2Cl_2 for 30 min. Qualitative ninhydrin [28] and chloranil [29] tests were used to monitor the synthesis; if the test was positive the protocol was repeated from step (4).

Peptide synthesis performed on a batch ABI 430A synthesizer was carried out with MBHA-resin (0.12 g, 0.1 mmol), using the following protocol: Boc deprotection with neat TFA (5 min), DMF washings (40 s), neutralization with DIEA–CH_2Cl_2 (1:4) (1 min), DMF washings (40 s), and couplings with Boc-amino acids (10 mol equiv.) and DCC (5 mol equiv.) in DMF–CH_2Cl_2 for 10 min. For Asn and Gln, *N*-hydroxybenzotriazole (HOBt) (5 mol equiv.) was added and the coupling was extended to 20 min.

Peptide–MBHA-resins (0.5 g) were treated with HF–*p*-cresol (9:1; 5 cm^3) at 0 °C for 1 h. After removal of HF by evaporation, the residue was washed with Et_2O (4 × 30 cm^3) and the precipitated peptides were solubilized with aq. HOAc (10%) and lyophilized.

Fmoc strategy. Chain assembly was carried out on a continuous-flow Millipore 9050Plus automated synthesizer, starting with Fmoc-PAL-PEG-PS-resin (0.4 g; 0.24 mmol g^{-1}). The flow rate of the unit pump was set at 5.0 cm^3 min^{-1} and the following reaction schedule was used: Fmoc-group deprotection with piperidine–DMF (2:8) (7 min), DMF washing (12 min), amino acid coupling (30 min), and DMF washing (12 min). Fmoc-amino acids (4 mol equiv.) and HOBt (4 mol equiv.) were dissolved to a final concentration of 0.3 mol dm^{-3} with a solution of DIPCDI (0.3 mol dm^{-3}) in DMF.

Peptide-PAL-PEG-PS-resin (0.5 g) was treated with TFA–water (19:1; 3 cm^3) at 25 °C for 2 h in a 10 cm^3 polypropylene syringe fitted with a polyethylene disc. The solution was filtered and the resin was washed with further TFA–water (2 × 0.5 cm^3) and the peptide was precipitated from the combined

filtrates and washed by addition of 10 volumes of cold anhydrous Et_2O. The precipitates collected by centrifugation were washed with cold anhydrous Et_2O (4 × 20 cm^3), dissolved in glacial HOAc, and lyophilized.

General procedure for the enzymic deprotection

The Phacm-protected peptides were incubated with penicillin amidohydrolase immobilized on acrylic beads (Eupergit C) in different buffers, pH 7.8, at 35 °C, and the reactions were followed by HPLC. After completion of the deprotection and oxidation, HOAc–water (9 : 1) was added and the mixtures were filtered through a disposable pipette with a glass wool plug and lyophilized.

β-Turn model peptide 4. HF-induced cleavage of the peptide–resin (0.22 g, 86 μmol) rendered the protected peptide, Ac-Cys(Phacm)-Pro-D-Val-Cys(Phacm)-NH_2, in 82% yield. This peptide showed a single peak on HPLC, and a correct FAB-MS (glycerol matrix): 778 [M + Na^+], 756 [M + H^+], 714 [M + H^+ − CH_3CO] and 609 [M + H^+ − Phacm]. After enzymic deprotection of the protected peptide with immobilized PAH (Table 1), HOAc was added to pH 3–4 and the solution was filtered. The product obtained showed a single peak on HPLC, and a correct FAB–MS (glycerol matrix): 460 [M + H^+] and amino acid analysis: Pro, 0.99; Val, 1.00; Cys, N.D.‡‡

[Lys^8] Vasopressin 5. HF-induced cleavage of the peptide–resin (0.21 g, 119 μmol) gave the protected peptide in 84% yield. The peptide showed a single peak on HPLC, and a correct FAB-MS (thioglycerol matrix): 1374 [M + Na^+], 1352 [M + H^+], 1205, [M + H^+ − Phacm]. After enzymic deprotection of the protected peptide with PAH as described in Table 2, HOAc was added to pH 3–4 and the solution was filtered. The product obtained showed a single peak on HPLC, and a correct FAB-MS (glycerol matrix): 1078 [M + Na^+], 1056 [M − H^+], and amino acid analysis: Asx, 1.00; Glx, 1.04; Gly, 1.10; Phe, 0.79; Pro, 1.19 Lys, 1.06; Tyr, 0.71; Cys, N.D.‡‡

Somatostatinamide 6. After treatment of the peptide–resin (0.64 g, 51 μmol) with the TFA cocktail, the protected peptide was obtained in 41% yield. The peptide showed a single major peak on HPLC (>90% purity) and a correct FAB–MS (thioglycerol–water matrix): 1954 [M + Na^+] and 1932 [M + H^+]. After enzymic deprotection of the protected peptide with PAH immobilized as described in Table 3, HOAc was added to pH 3–4 and the solution was filtered. The product obtained showed a single peak on HPLC, and a correct FAB–MS (thioglycerol matrix): 1637 [M + H^+], and amino acid analysis: Ala, 1.04; Asx, 0.96; Cys, 1.22; Gly, 0.98; Lys, 2.07; Phe, 2.99; Ser, 0.21; Thr, 1.35; Trp, N.D.‡‡

Dimer β-turn model peptide 7. HF-induced cleavage of the peptide–resin (0.20 g, 73 μmol) gave the protected peptide in 93% yield. The peptide showed a single peak on HPLC, and a correct FAB–MS (thioglycerol matrix): 702 [M + Na^+], 680 [M + H^+], 609 [M + H^+ − Acm], 533 [M + H^+ − Phacm] and 462 [M + H^+ − (Acm + Phacm)]. Enzymic deprotection of Ac-Cys(Phacm)-Pro-D-Val-Cys(Acm)-NH_2 with immobilized PAH (Table 4) for 30 h and work-up rendered (^{1}Cys-^{1}Cys)-dithiobis[Ac-^{1}Cys-Pro-D-Val-^{4}Cys(Acm)-NH_2] **8**, which showed a single peak on HPLC, and correct FAB-MS (thioglycerol matrix): 1063 [M + H^+], and amino acid analysis: Pro, 1.02; Val, 0.98, Cys, N.D.‡‡ The open dimer was dissolved in 12 mL of HOAc–water (4 : 1; 12 cm^3) and then I_2 (40 μmol, 10 mol equiv.) was added in one portion. Aliquots of the solution (20 mm^3) were removed at different times, quenched with saturated aq. ascorbic acid (20 mm^3) and checked by HPLC (see conditions in Fig. 4). After 2 h, all starting material had been deprotected and oxidized, and the solution was diluted with water (20 cm^3), extracted with CCl_4 (5 × 10 cm^3), concentrated to remove the remaining CCl_4, and lyophilized. The product obtained co-eluted with another sample obtained by an alternative method [27] and showed a correct FAB-MS: 919 [M + H^+] and 941 [M + Na^+], and amino acid analysis: Pro: 1.02, Val: 0.98, Cys: N.D.‡‡

‡‡ Not determined.

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References

1 Review on solid-phase peptide synthesis: (*a*) G. Barany, N. Kneib-Cordonier and D. G. Mulle, *Int. J. Pept. Protein Res.*, 1987, **30**, 705; (*b*) E. Atherton and R. Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford, 1989; (*c*) G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, 1990, **35**, 161; (*d*) G. B. Fields, Z. T. Tian and G. Barany, in *Synthetic Peptides: A User's Guide*, ed. G. Grant, Freeman, New York, 1992, pp. 77–183.

2 Reviews on protection of thiols and formation of disulfides: R. G. Hiskey, V. R. Rao and W. G. Rhodes, in *Protective Groups in Organic Chemistry*, ed. J. F. W. McOmie, Plenum, New York, 1973, pp. 235–308; I. Photaki, in *Top. Sulfur Chem.* 1976, **1**, 111; R. G. Hiskey, in *The Peptides*, ed. E. Gross and J. Meienhofer, Academic Press, New York, 1981, vol. 3, pp. 137–167; W. König and R. Geiger, in *Perspectives in Peptide Chemistry*, ed. A. Eberle, R. Geiger and T. Wieland, Karger, Basel, 1981, pp. 31–44; H. Yajima, N. Fujii, S. Funakoshi, T. Watanabe, E. Murayama and A. Otaka, *Tetrahedron* 1988, **44**, 805; F. Cavelier, J. Daunis and R. Jacquier, *Bull. Soc. Chim. Fr.*, 1989, 788; 1990, 210; D. Andreu, F. Albericio, N. A. Solé, M. C. Munson, M. Ferrer and G. Barany, in *Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols*, ed. M. W. Pennington and B. M. Dunn, Humana Press, Totowa, NJ, 1994, pp. 91–169.

3 An orthogonal system has been defined as a set of completely independent classes of protecting groups, such that each class of groups can be removed in any order and in the presence of all other classes: G. Barany and R. B. Merrifield, *J. Am. Chem. Soc.*, 1977, **99**, 7363; G. Barany and F. Albericio, *J. Am. Chem. Soc.*, 1985, **107**, 4936.

4 Reviews on enzymic deprotection: (*a*) P. Hermann, in *Wissenschaftlicher Beitrag Friedrich-Schiller-Universität, Jenaer Symposium Chemie und Biologie von Peptidwirkstoffen*, ed. B. Wilhelmi, Jena, 1986, pp. 102–112; (*b*) J. D. Glass, in *The Peptides*, ed. S. Undenfried and J. Meienhofer, Academic Press, New York, 1987, pp. 167–184; (*c*) H. Waldmann, *Kontakte* (*Darmstadt*), 1991, (2), 35–54; (*d*) P. Hermann, *Biomed. Biochim. Acta*, 1991, **50**, S19.

5 P. Hermann and G. Hoffmann, in *Peptides 1976*, ed. A. Loffet, Edition de l'Universite de Bruxelles, Bruxelles, 1976, pp. 121–122.

6 (*a*) D. F. Veber, J. D. Milkowski, S. L. Varga, R. G. Denkewalter and R. Hirschmann, *J. Am. Chem. Soc.*, 1972, **94**, 5456; (*b*) B. Kamber, A. Hartmann, K. Eiser, B. Riniker, H. Rink, P. Sieber and W. Rittel, *Helv. Chim. Acta*, 1980, **63**, 899.

7 P. Lloyd-Williams, F. Albericio and E. Giralt, *Tetrahedron*, 1993, **49**, 11065.

8 P. Hermann and G. Greiner, in *Peptides 1990, Proceedings of the Twenty-First European Peptide Symposium*, ed. E. Giralt and D. Andreu, ESCOM Science Publishers B.V., Leiden, 1991, pp. 227–278.

9 L. Vezenkov, L. Milev and A. Dimitrova, in *Peptides 1992, Proceedings of the Twenty-Second European Peptide Symposium*, ed. C. H. Schneider and A. N. Eberle, ESCOM Science Publishers B.V., Leiden, 1993, pp. 421–422.

10 F. Albericio, A. Grandas, A. Porta, E. Pedroso and E. Giralt, *Synthesis*, 1987, 271.

11 W. Wenner, *Org. Synth.*, 1963, Coll. Vol. IV, pp. 760–763.

12 L. Moroder, A. Hallet, E. Wunsch, O. Keller and G. Wersein, *Hoppe-Seyler's Z. Physiol. Chem.*, 1976, **357**, 1651.
13 P. B. W. Ten Kortenaar, B. G. Van Dijk, J. M. Peters, B. J. Raaben, P. J. H. M. Adams and G. I. Tesser, *Int. J. Pept. Protein Res.*, 1986, **27**, 398.
14 J. P. Tam and R. B. Merrifield, in *The Peptides*, ed. S. Udenfriend and J. Meienhofer, Academic Press, New York, 1987, vol. 9, pp. 185–248.
15 M. Ruiz-Gayo, F. Albericio, E. Pedroso and E. Giralt, *J. Chem. Soc., Chem. Commun.*, 1986, 1501.
16 M. Ruiz-Gayo, F. Albericio, M. Royo, C. García Echeverría, E. Pedroso, M. Pons and E. Giralt, *An. Quím. C*, 1989, **85**, 116.
17 M. Bodanszky and M. Bednarek, *Int. J. Pept. Protein Res.*, 1982, **20**, 434.
18 M. Royo, C. García-Echeverría, E. Giralt, R. Eritja and F. Albericio, *Tetrahedron Lett.*, 1992, **33**, 2391.
19 S. Akabori, S. Sakakibara, Y. Shimonishi and Y. Nobuhara, *Bull. Chem. Soc. Jpn.*, 1964, **37**, 433.
20 R. G. Hiskey and J. B. Adams, *J. Org. Chem.*, 1965, **30**, 1340.
21 M. Munson, C. García-Echeverría, F. Albericio and G. Barany, *J. Org. Chem.*, 1992, **57**, 3013.
22 N. Fujii, A. Otaka, S. Funakoshi, K. Bessho, T. Watanabe, K. Akaji and H. Yajima, *Chem. Pharm. Bull.*, 1987, **35**, 2339 and references cited therein.
23 C. García-Echeverría, F. Albericio, M. Pons, G. Barany and E. Giralt, *Tetrahedron Lett.*, 1989, **30**, 2441.
24 F. Albericio, R. P. Hammer, C. García-Echeverría, M. A. Molins, J. L. Chang, M. C. Munson, M. Pons, E. Giralt and G. Barany, *Int. J. Pept. Protein Res.*, 1991, **37**, 404.
25 F. Albericio, N. Kneib-Cordonier, S. Biancalana, L. Gera, R. I. Masada, D. Hudson and G. Barany, *J. Org. Chem.*, 1990, **55**, 3730.
26 G. Barany, F. Albericio, N. A. Solé, G. W. Griffin, S. A. Kates and D. Hudson, in *Peptides 1992, Proceedings of the Twenty-Second European Peptide Symposium*, ed. C. H. Schneider and A. N. Eberle, ESCOM Science Publishers B.V., Leiden, 1993, pp. 267–268; S. Zalipsky, J. L. Chang, F. Albericio and G. Barany, *React. Polym.*, 1994, **22**, 243.
27 C. García-Echeverría, F. Albericio, E. Giralt and M. Pons, *J. Am. Chem. Soc.*, 1993, **115**, 11663.
28 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.
29 T. Christensen, *Acta Chem. Scand. Ser. B*, 1979, **33**, 763.

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