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An HPLC-ESMS Study on the Solid-Phase Assembly of *C*-Terminal Proline Peptides[1]

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Abstract: DKP formation is a serious side reaction during the solid-phase synthesis of peptide acids containing either Pro or Gly at the *C*-terminus. This side reaction not only leads to a lower overall yield, but also to the presence in the reaction crude of several deletion peptides lacking the first amino acids. For the preparation of protected peptides using the Fmoc/*t*Bu strategy, the use of a ClTrt-Cl-resin with a limited incorporation of the *C*-terminal amino acid is the method of choice. The use of resins with higher loading levels leads to more impure peptide crudes. The use of HPLC-ESMS is a useful method for analysing complex samples, such as those formed when *C*-terminal Pro peptides are prepared by non-optimized solid-phase strategies. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chlorotrityl chloride resin; convergent solid-phase peptide synthesis; diketopiperazine; side reactions

INTRODUCTION

Peptide synthesis can be performed either by repetitive N^{α}-amino group deprotection and amino acid-coupling steps in a *linear approach*, or by a *convergent approach*, where protected peptide segments are used instead of single protected amino acids. Both modes can be carried out in *solution* or on *solid support* [1]. One of the most serious side reactions in any strategy is the formation of diketopiperazines (2,5-piperazinediones), which are the smallest cyclic peptides [2–10]. DKPs are formed by intramolecular aminolysis at the dipeptide level. DKP formation has been established to be either acid- [3,6] or base-catalysed [2,4–8], and is strongly dependent on the nature and the sequence of the amino acids. Thus, the reaction is favoured by the presence, in either the first or second position, of amino acids (Gly, Pro, or *N*-alkyl) that can easily adopt a *cis*-conformation in the resulting amide bond. Another favourable combination is to have one L- and one D-amino acid in the dipeptide, due to the minimal steric interference between the two side-chains [1,3]. Furthermore, rates of DKP formation differ considerably depending on the *C*-carboxyl ester protecting groups, or the structures of the peptide-resin linkages in the solid-phase approach.

The formation of DKP is most commonly seen in convergent solid-phase synthesis, since the first

Abbreviations: AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; ClTrt-Cl, chlorotrityl chloride; DHPP, 4-(1′,1′-dimethyl-1′-hydroxypropyl)phenoxyacetyl; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DKP, diketopiperazine; DMAP, *N*,*N*-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; ESMS, electrospray mass spectrometry; Et_3SiH, triethylsilane; Fmoc, 9-fluorenylmethoxycarbonyl; HAL, hypersensitive acide-labile, 5-(4-hydroxymethyl-3,5-dimethoxyphenoxy)-valeric acid; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid; HOAc, acetic acid; HPLC, high performance liquid chromatography; IRAA, internal reference amino acid; MBHA, *p*-methylbenzhydrylamine; MeOH, methanol; MS, mass spectrometry; NMM, *N*-methylmorpholine; PAM, 4-hydroxymethylphenylacetic acid; PyAOP, 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)-phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; SASRIN, super acid sensitive resin, 2-methoxy-4-benzyloxybenzyl alcohol resin; SPS, solid-phase synthesis; TFA, trifluoroacetic acid; *t*Bu, *tert*-butyl; TIC, total ion chromatogram; Trt, triphenylmethyl (trityl); amino acid symbols denote L-configuration unless indicated otherwise.

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[1] Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol Chem. 247*, 977–983 (1972).

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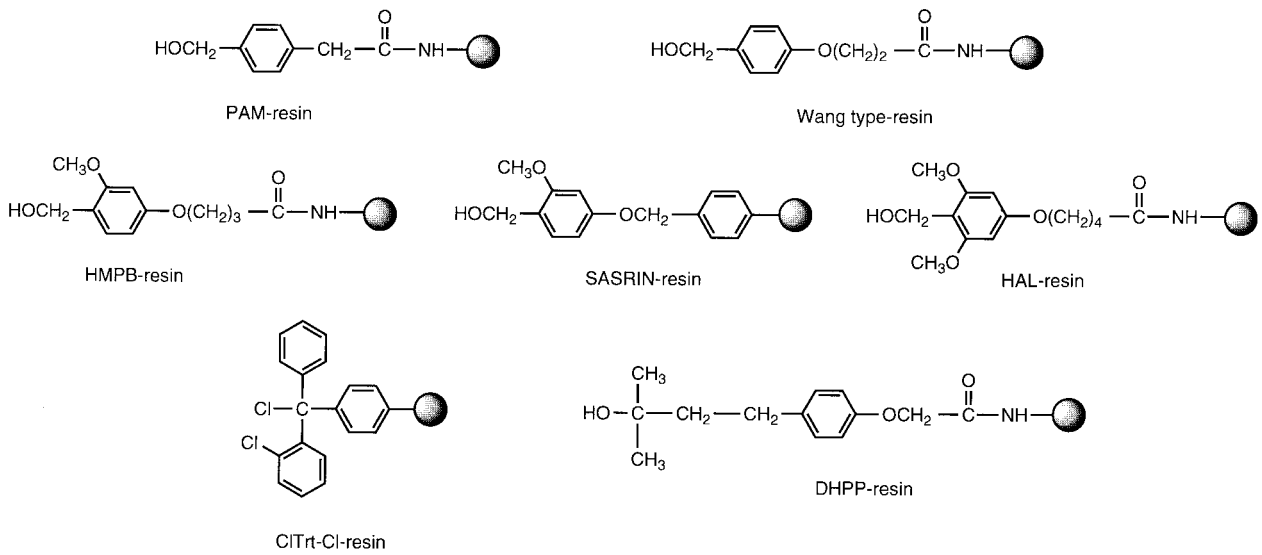

Figure 1 Chemical structures of the most common handles used for the preparation of peptide acids.

criterion to be considered when a synthesis is planned is to have residues of Gly or Pro, which are the most prone to give DKPs as discussed above, at the *C*-terminus of the protected peptides in order to minimize the risk of epimerization during the coupling reaction [1,11]. The main consequence of the formation of DKPs was thought to be a decrease in the overall yield of the final peptide, since the hydroxyl sites formed on the polymer were considered to be poor nucleophiles, and therefore not predisposed to suffer acylation by the following protected amino acids, which would have started new peptide chains. Here, we demonstrate that the formation of DKPs not only lead to a lower overall yield, but also to the presence in the reaction crude of several peptides lacking the first amino acid residues. Thus, when peptides containing either Gly or Pro at the *C*-terminus are to be synthesized, methods which minimize or avoid this reaction completely should be employed. Furthermore, the optimal loading of the starting resin for the solid-phase synthesis is discussed.

Traditionally, the most common peptide-resin anchorage used for solid-phase synthesis of peptide *C*-terminal acids is the substituted benzyl ester [1,12]. The presence of certain substituents in the aromatic ring can modulate cleavage of the peptide from the resin. Thus, an electron-withdrawing carboxamidomethyl group in the *para* position makes the benzyl ester stable to TFA (PAM-resin) [13], and therefore compatible with the Boc/Bzl strategy where the final unprotected peptide is released with HF. For the Fmoc/*t*Bu approach, the presence of an electron-donating alkoxy group in the *para* position makes the benzyl ester labile to TFA (Wang type resins) [14,15]. If additional methoxy groups are situated in *ortho* positions, the corresponding benzyl esters are labile to very dilute solutions of TFA, allowing the preparation of Fmoc/*t*Bu protected peptides. Examples of these kinds of resins are HMPB- [16] and SASRIN-resins [17], which incorporate one methoxy group, and the HAL-resin [18] with two methoxy groups. Protected peptides from the two first resins can be cleaved with 1% TFA in CH_2Cl_2, while even more diluted solutions (0.2–0.5% TFA in CH_2Cl_2) release protected peptides from HAL (Figure 1).

We have recently published a method to minimize the DKP formation when alkoxy benzyl based resins are used [19]. Experimentally, this was accomplished by: (i) incorporation of the penultimate residue as its N^{α}-trityl (Trt) derivative; (ii) detritylation with dilute TFA; and (iii) incorporation of the third residue as its N^{α}-Fmoc derivative under *in situ* neutralization/coupling conditions mediated by PyAOP/DIEA in DMF. While this method is completely safe when used with Wang type resins, 5% loss of dipeptide has been detected when HMPB was used, due to premature acidolytic cleavage during the removal of trityl group, even when the mildest conditions (0.2% TFA in CH_2Cl_2–H_2O (99:1)) were employed.

Alternatively, the use of the hindered *t*-alcohol resin, DHPP, avoids DKP formation during the

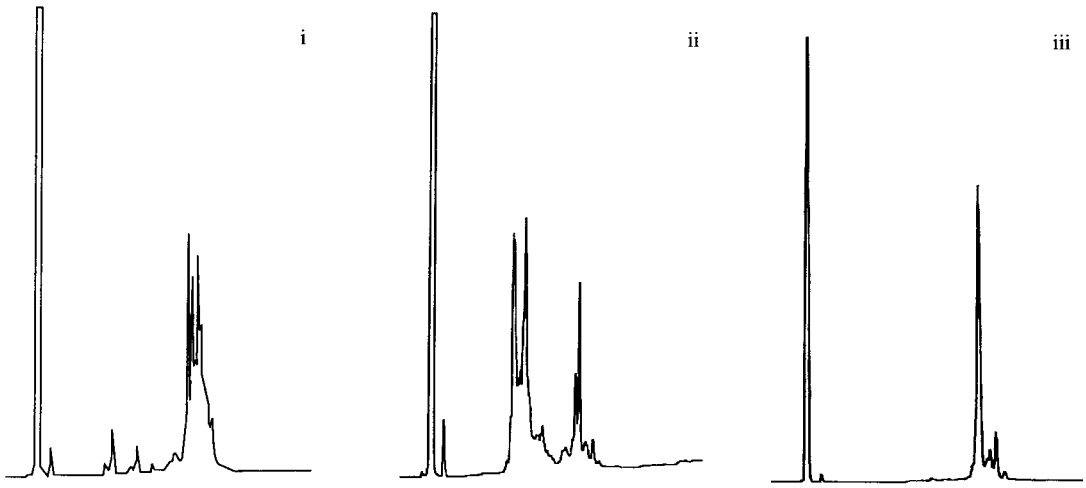

Figure 2 HPLC of crude peptides directly after lyophylization. (i) HMPB-resin synthesis; (ii) ClTrt-Cl-resin (high loading) synthesis; (iii) ClTrt-Cl-resin (low loading) synthesis.

preparation of unprotected peptides [20,21]. Furthermore, the hindered 2-chlorotrityl chloride resin (ClTrt-Cl-resin) [22,23], is also convenient for avoiding this side reaction [24,25].

RESULTS AND DISCUSSION

With this information in mind, the synthesis of Fmoc-Lys(Boc)-Val-Leu-Thr(*t*Bu)-Lys(Boc)-Cys(Trt)-Gln(Trt)-Glu(*t*Bu)-Glu(*t*Bu)-Val-Ser(*t*Bu)-His(Trt)-Ile-Pro-OH, corresponding to a fragment of a domain of the p41 form of Ii protein, was attempted using a commercial ClTrt-Cl-resin. Several syntheses were carried out with different purity of final products in each case (results not shown). Thus, a comparative study was carried out where parallel synthesis of the peptide were performed in a multiple peptide synthesizer on an HMPB-resin, and with two ClTrt-Cl-resins of different loadings. At the end of the synthesis, in order to investigate the synthetic process, aliquots of the protected resins were treated with neat TFA in the presence of scavengers to afford the partially protected peptide (Fmoc-Lys-Val-Leu-Thr-Lys-Cys-Gln-Glu-Glu-Val-Ser-His-Ile-Pro-OH). The purity of the three batches of peptide was assessed first by HPLC (Figure 2) and amino acid analysis (AAA). Although these methods were conclusive in showing that the best quality synthesis was that carried out with the ClTrt-Cl-resin having the lowest loading, the complexity of the first two synthesis made it impossible to assess product purity until HPLC-ESMS was used.

HMPB-resin was prepared from the commercially available bifunctional spacer HMPB and a MBHA-resin (0.45 mmol/g), using an Ala residue as the internal reference amino acid (IRAA). Fmoc-Pro-OH was incorporated by the DIPCDI–DMAP coupling method to give Fmoc-Pro-O-HMPB-resin with a loading of 0.35 mmol/g, which indicates a quantitative yield. The HPLC of the crude product was rather difficult to interpret, even to the extent of doubting the presence of the title peptide (Figure 2(i)). Furthermore, the gain of weight was lower than theoretical, thereby indicating that either a termination side reaction or a premature release of peptide from the resin had taken place.

The first synthesis on ClTrt-Cl-resin (nominal loading of 1.35 mmol/g) was initiated by the incorporation of 4 equivalents of Fmoc-Pro-OH in the presence of DIEA to give Fmoc-Pro-ClTrt-resin with a loading of 0.83 mmol/g, which indicates a 86% yield with respect to the nominal loading of the resin. HPLC of the crude product does not show any major product, with other peaks corresponding to much less hydrophobic peptides, which may indicate the absence of the Fmoc group (Figure 2(ii)).

The second synthesis on ClTrt-Cl-resin was started with limited incorporation of Fmoc-Pro-OH (0.5 equivalents) in the presence of DIEA. The initial Fmoc-Pro-O-ClTrt-resin presented a loading of 0.49 mmol/g, which indicates the substitution of 50% of theoretically available leaving Cl atoms (with respect to Fmoc-Pro-OH, this value represents 80% incorporation). Unreacted chlorine sites were capped with MeOH-DIEA. In this case, the HPLC

(Figure 2(iii)) clearly shows a major peak with small, more hydrophobic impurities.

HPLC-ESMS analysis of the crude product obtained with the HMPB-resin showed that the desired peptide Fmoc-(1–14) was clearly a minor product, contaminated with other small peptides corresponding to sequences (Figure 3: (a) Fmoc-(1–12); (b) Fmoc-(1–11), Fmoc-(1–9), Fmoc-(1–8), and Fmoc-(1–7); (c) Fmoc-(1–10)]. These results indicate that during the deprotection of the second residue (Ile) formation of the DKP (*cyclo*(Ile-Pro)) had occurred. Furthermore, the presence of this peptide ladder series demonstrated clearly that the hydroxyl sites formed on the polymer were acylated by the following protected amino acids, thus starting new peptide chains. These results were corroborated by AAA, which showed a lower value for amino acids corresponding to the *C*-terminal part of the sequence, Pro (0.33 (1)) and Ile (0.25 (1)), with respect to those of the *N*-terminal part, Lys (1.96 (2)), Leu (1.00 (1)).

The analysis by HPLC-ESMS of the crude product obtained with the ClTrt-Cl-resin (high loading) shows additional peaks, corresponding to sequences H-(8–14) (Figure 4(a)) and H-(2–14) (Figure 4(b)) together with other unidentified products. This finding shows clearly that the use of this high load resin leads to the formation of termination peptides, where the Fmoc group has been removed from the peptide-resin, but without further incorporation of subsequent amino acids. Again, these results agreed with those obtained by AAA. Thus, the value for amino acids corresponding to the *C*-terminal part of the sequence, Pro (1.14 (1)), Ile (0.89 (1)), and His (0.97 (1)), were higher than those corresponding to the *N*-terminal part, Lys (0.89 (2)) and Leu (0.48 (1)).

The analysis by HPLC-ESMS of the crude product obtained with the ClTrt-Cl-resin (low loading) shows a major peak corresponding to the target peptide with an excellent purity (Figure 5(a) $>98\%$ of sequence elongation; small peaks correspond to the *tert*-butylated correct sequence, formed during the cleavage reaction (b)). In this case, the AAA showed a correct relation among the different amino acids.

These results agree with commonly held views in the SPPS field. Thus, it is not recommended to work with loadings higher than 1 mmol/g. Furthermore, Merrifield and coworkers [26] have recommended that during chain assembly the weight of the protected peptide should not be superior to that of the starting polymer. The rationale for these two recommendations is the following: (i) To try a minimization of the interactions between the growing peptide chains. This type of non-covalent interaction might decrease the swelling of the resin and therefore the accessibility of the reactive *N*-terminal amino groups. (ii) To avoid changes in the physical properties of the polymer due to the differences in polarity between the growing peptide chain and the starting polymeric matrix. This change in properties can, in some cases, be deleterious to the quality of the peptide assembly.

CONCLUSIONS

In summary, DKP formation is a side reaction that should be avoided totally because, in addition to causing lower overall yields, the hydroxyl sites formed may suffer acylation by the following protected amino acids and start new peptide chains. Thus, not only the $n-2$ peptide is obtained, but also the $n-3$, $n-4$, $n-5\ldots$ peptides, are present in the crude mixture. This observation may also have consequences when the incorporation of protected amino acids into hydroxymethyl resins is not quantitative. Alternatively, either the use of halogenomethyl resins, where the first amino acid is incorporated via a nucleophilic substitution, or the use of preformed handles isolated, purified, and characterized in a step preceding the incorporation into the polymer, are advisable [1,12,15]. Furthermore, the loading of the starting polymer has importance with regard to the quality of the final product. Thus, the ClTrt-Cl-resin with a limited incorporation of the first amino acid is suggested to be the method of choice for the preparation of protected Fmoc/*t*Bu peptides.

The present study is also a good example of the advantages that the combination of liquid chromatography and mass spectrometry offers. This technique takes advantage of both liquid chromatography as a separation method and mass spectrometry as an identification method and allows the analysis of complex samples which are difficult, and in some cases impossible, to analyse by traditional techniques.

MATERIALS AND METHODS

Materials, solvents, instrumentation, and general methods were essentially as described in previous publications [10,18,19]. Organic and peptide synthesis transformations and washes were performed at 25°C unless indicated otherwise.

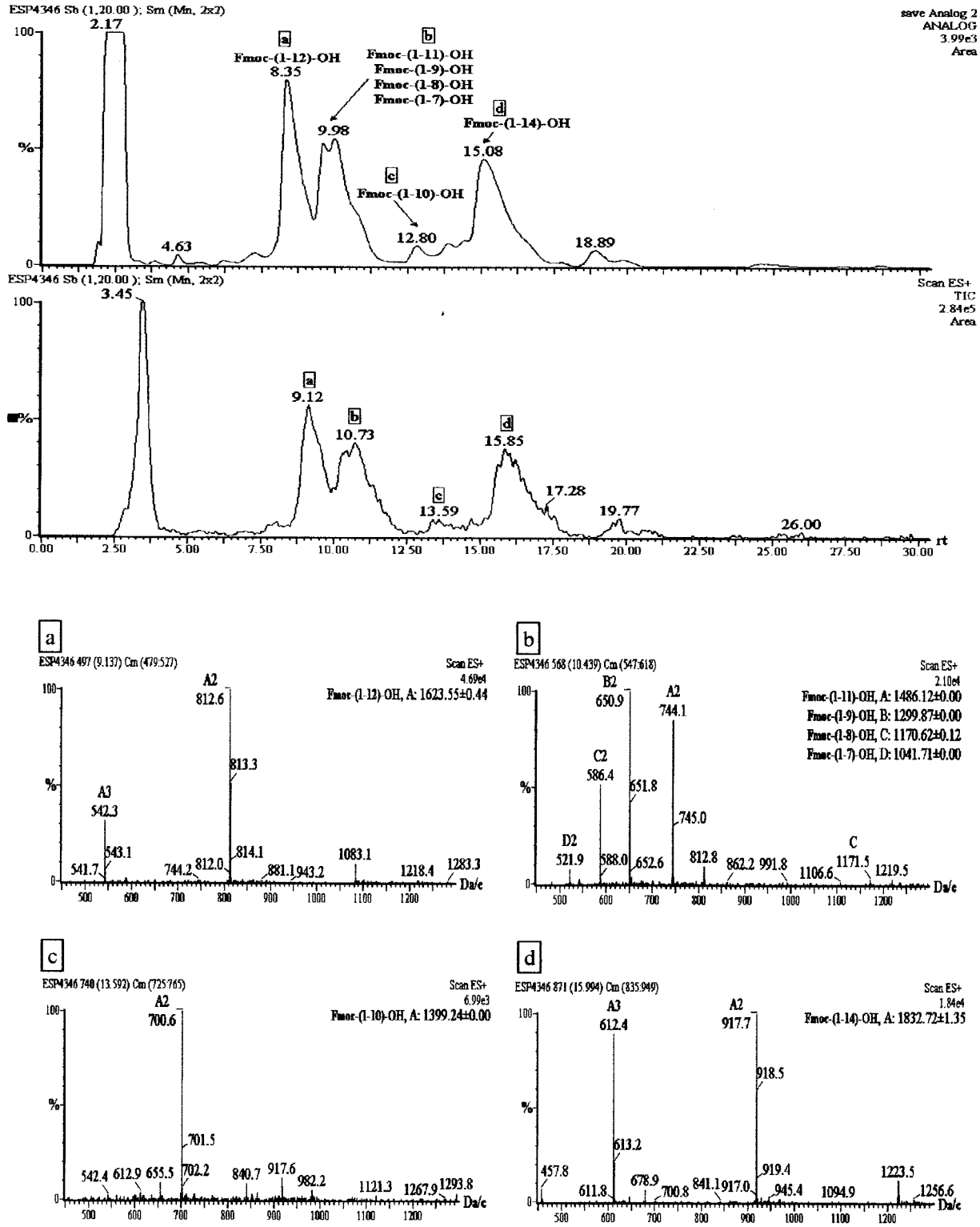

Figure 3 HPLC-ESMS analysis of the crude product corresponding to the HMPB-resin synthesis. Figure shows the chromatograms with UV (top) and TIC (bottom) detection through the recording time and ESMS spectra (a, b, c, d) of the significant peaks in the chromatograms. (a) Peptide corresponding to sequence (1–12); (b) mixture of peptides corresponding to sequences (1–11), (1–9), (1–8), (1–7); (c) peptide corresponding to sequence (1–10); (d) desired peptide (1–14). Linear gradient from 32 to 37% of B.

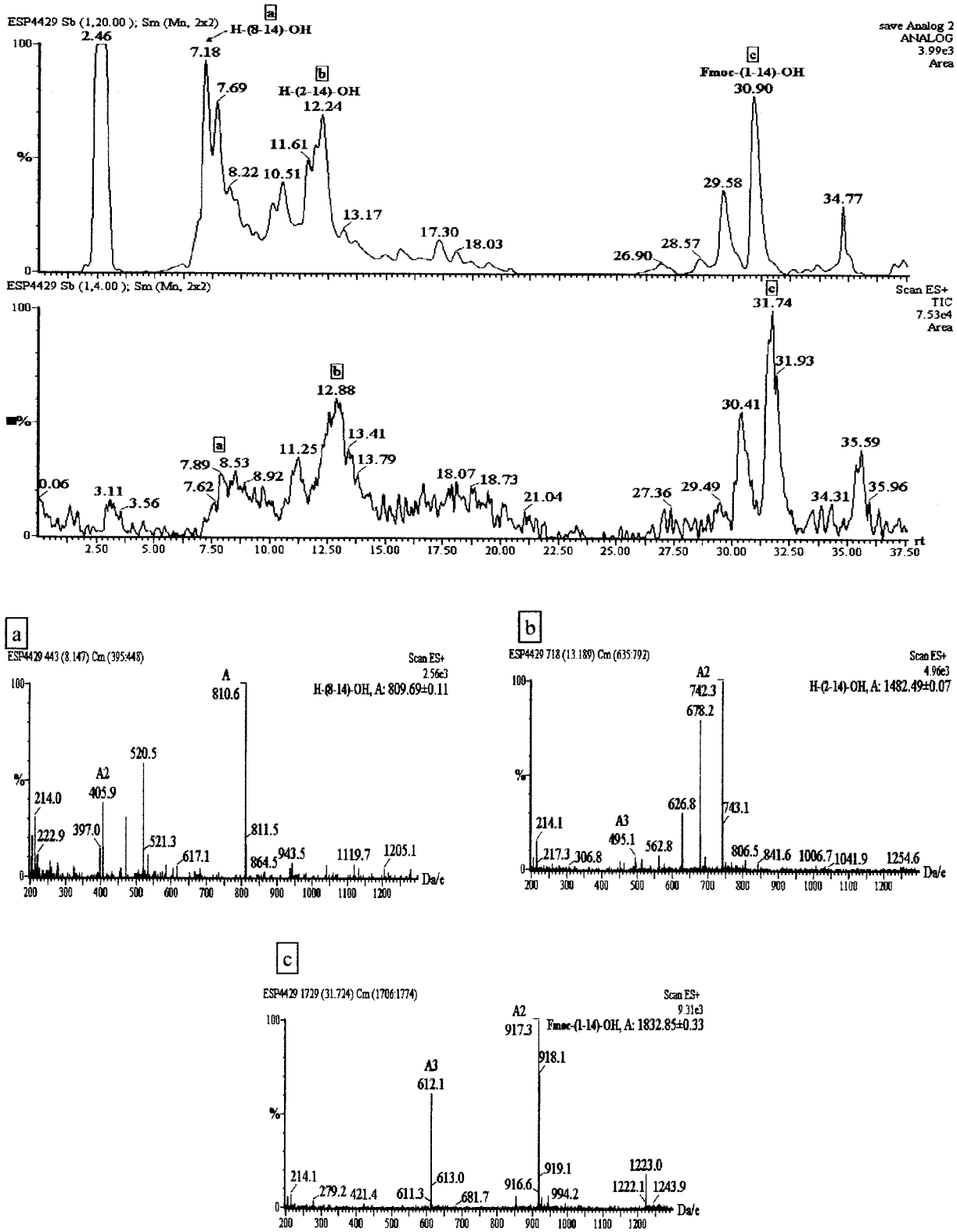

Figure 4 HPLC-ESMS analysis of the crude product corresponding to the ClTrt-Cl-resin (high loading) synthesis. Figure shows the chromatograms with UV (top) and TIC (bottom) detection through the recording time and ESMS spectra (a, b, c) of the significant peaks in the chromatograms. (a) Peptide corresponding to sequence (8–14); (b) peptide corresponding to sequence (2–14); (c) desired peptide (1–14). Linear gradient from 15 to 40% of B.

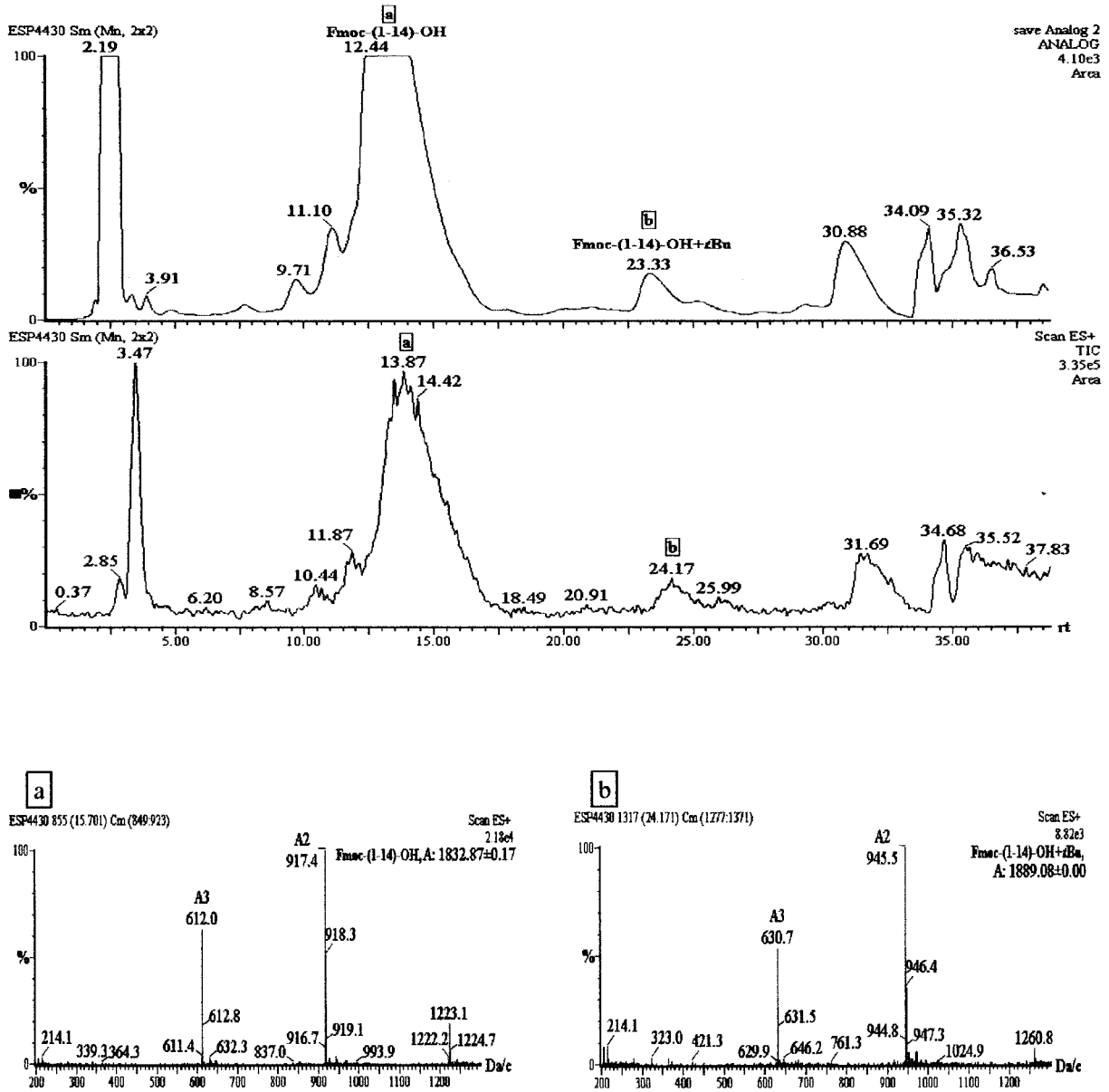

Figure 5 HPLC-ESMS analysis of crude product corresponding to the ClTrt-Cl-resin (low loading) synthesis. Figure shows the chromatograms with UV (top) and TIC (bottom) detection through the recording time and ESMS spectra (a, b) of the significant peaks in the chromatograms. (a) Desired peptide (1–14); (b) *tert*-butylated peptides corresponding to the correct sequence (1–14). Linear gradient from 32 to 37% of B.

Fmoc-protected amino acids, PyBOP, and MBHA resin (0.45 mmol/g) were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland), Calbiochem-Novabiochem (Läufelfingen, Switzerland) or Neosystem (Strasbourg, France). Calbiochem-Novabiochem also supplied ClTrt-Cl-resin (1.35 mmol/g). After incorporation of Fmoc-Pro-OH, the Fmoc group was removed and resins were stored with the free amino group. The corresponding Fmoc-derivative has been shown to be less stable [27]. In the synthesis performed on the HMPB (Calbiochem-Novabiochem) handle, to accurately determine anchoring, coupling, and cleavage yields, resins were extended further with an IRAA [18,28], introduced as its Boc derivative by standard coupling methods, at a point before the introduction of the handle. Solvents for peptide synthesis (DMF and CH_2Cl_2) and HPLC supplies (CH_3CN and Nu-

cleosil C_{18} reversed-phase columns, 4×250 mm, 10 μm) were obtained from Scharlau (Barcelona, Spain). DMF was bubbled with nitrogen to remove volatile contaminants and stored over activated 4 Å molecular sieves.

Parallel peptide synthesis were carried out using a simultaneous multiple peptide synthesizer (AMS 422, Abimed Analysentechnik, Langenfeld, Germany). Several batches of the peptide were carried out on the same resin to overcome the position factor in the synthesizer. Peptide-resin samples were hydrolysed in 12 N aqueous HCl–propionic acid (1:1) at 155°C for 1–3 h. Subsequent amino acid analyses were performed on a Beckman System 6300 autoanalyser (Palo Alto, CA).

Analytical HPLC was carried out on a Shimadzu instrument (Kyoto, Japan) comprising two solvent delivery pumps (model LC-6A), automatic injector (model SIL-6B), variable wavelength detector (model SPD-6A), system controller (model SCL-6B) and plotter (model C-R6A). Peptides were eluted by a linear gradient over 30 min of 0.035% TFA in CH_3CN and 0.045% aqueous TFA from 1:19 to 19:1, flow rate 1.0 mL/min, with UV detection at 220 nm.

HPLC-ESMS analysis of peptide samples were performed using the previously described Nucleosil C_{18} reversed-phase column operating with a LC-9A Shimadzu HPLC in a conventional manner. Linear gradients in 30 min from mixtures of A (0.045% aqueous TFA) and B (0.035% TFA in CH_3CN), flow rate of 1 mL/min, with UV detection at 220 nm (see legends for Figures 3–5). The column effluent was connected to a variable wavelength detector (micro UVIS 20, Carlo Erba Instrument, Milan, Italy) and the solvent diverted with a VALCO split tee (1:50) in order to use a 20 μL/min flow rate into the electrospray system. Electrospray mass spectra of the eluting products were obtained using a VG-Quattro double quadrupole mass spectrometer (Fisons Instrument, VG Biotech, UK) working in the positive mode, using nitrogen as the nebulizing and drying gas (10 and 450 L/h, respectively) with a source temperature of 80°C, a capillary voltage of 3.5 kV and a focusing voltage of 57 V. Spectra were scanned at a speed of 1 s over the mass range m/z 200–1300 and 450–1300 and were recorded and processed using the MassLynx software, version 1.03. Mass calibration was performed by multiple-ion monitoring of horse-heart myoglobin signals.

Fmoc-Pro-O-HMPB-Ala-resin

MBHA resin (0.33 g, 0.57 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), treated with TFA–CH_2Cl_2 (2:3, v/v, 1×1 min, 1×20 min), and washed with CH_2Cl_2 (5×0.5 min). The trifluoroacetate salt was neutralized with DIEA–CH_2Cl_2 (1:19, v/v, 3×1 min), and the resin washed again with CH_2Cl_2 (5×0.5 min) and DMF (5×0.5 min). Boc-Ala-OH (113 mg, 0.6 mmol, 4 equivalents) was coupled in the presence of equimolar amounts of DIPCDI in DMF at 25°C for 1 h (the resin was negative to the Kaiser ninhydrin test) [29]. The resin was washed with DMF (5×0.5 min) and CH_2Cl_2 (3×0.5 min). The resultant Boc-Ala-MBHA-resin (Ala serving as IRAA) was deprotected with TFA–CH_2Cl_2 (2:3, v/v, 1×1 min, 1×20 min), washed with CH_2Cl_2 (5×0.5 min), neutralized with DIEA–CH_2Cl_2 (1:19, v/v, 3×1 min), and washed with CH_2Cl_2 (5×0.5 min) and DMF (5×0.5 min). The bifunctional spacer HMPB (144 mg, 0.6 mmol, 4 equivalents) was then coupled in the presence of equimolar amounts of DIPCDI (93 μL, 0.6 mmol) in DMF at 25°C for 16 h (negative to the Kaiser ninhydrin test). The HMPB-resin was washed with DMF (5×0.5 min) and Fmoc-Pro-OH (506 mg, 1.5 mmol) in DMF (1 mL), DIPCDI (232 μL, 1.5 mmol), and DMAP (73 mg, 0.15 mmol) were added, and the mixture was stirred for 1 h. Finally, the resin was washed with DMF (5×0.5 min), CH_2Cl_2 (3×0.5 min), and dried over vacuum.

H-Pro-ClTrt-resin

(i) ClTrt-Cl-resin (0.11 g, 1.35 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-Pro-OH (202 mg, 0.6 mmol, 4 equivalents) and DIEA (87 μL, 0.5 mmol, 3.3 equivalents) in CH_2Cl_2 (0.5 mL) was added. The mixture was stirred for 5 min. Then, DIEA (174 μL, 1 mmol 6.6 equivalents) in CH_2Cl_2 (174 μL) was added and the mixture was stirred for 1 h. The reaction was terminated by addition of MeOH (89 μL). Then, the mixture was stirred for 10 min and filtered. The Fmoc-Pro-ClTrt-resin was subjected to the following washings/treatments: DMF (5×0.5 min), CH_2Cl_2 (3×0.5 min), DMF (3×0.5 min), piperidine–DCM–DMF (1:10:10, 1×10 min), piperidine–DMF (1:4, 1×15 min), DMF (5×0.5 min), isopropanol (2×1 min), DMF (5×0.5 min), isopropanol (2×1 min), MeOH (2×1 min), and dried in the vacuum. The loading, as

calculated by AAA, was 0.83 mmol/g. (ii) ClTrt-Cl-resin (0.55 g, 1.35 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-Pro-OH (127 mg, 0.375 mmol, 0.5 equivalents) and DIEA (218 μL, 1.25 mmol, 1.7 equivalents) in CH_2Cl_2 (1 mL) was added. The mixture was stirred for 5 min. Then, DIEA (435 μL, 2.5 mmol, 3.3 equivalents) in CH_2Cl_2 (435 μL) was added and the mixture was stirred for 1 h. The reaction was terminated by addition of MeOH (400 μL), the mixture was stirred for 10 min and filtered. The Fmoc-Pro-ClTrt-resin was subjected to the same washings/treatments indicated above. The loading, as calculated by AAA, was 0.49 mmol/g.

Fmoc-Lys(Boc)-Val-Leu-Thr(*t*Bu)-Lys(Boc)-Cys(Trt)-Gln(Trt)-Glu(*t*Bu)-Glu(*t*Bu)-Val-Ser(*t*Bu)-His(Trt)-Ile-Pro-O-resins

Chain assemblies were performed in the $C \rightarrow N$ direction in the automatic synthesizer according to the standard Fmoc/*t*Bu protocol. Fmoc-Pro-O-HMPB-Ala-resin (three batches of 50 μmol), H-Pro-ClTrt-resin (three batches of 28 μmol of 0.83 mmol/g), and H-Pro-ClTrt-resin (six batches of 50 μmol of 0.49 mmol/g) were placed in the 2 mL polypropylene syringe fitted with a polyethylene filter disk, and the following protocol was used: deprotection with piperidine–DMF (1:4, 2×10 min), washings with DMF (2 mL, 6×10 s), and coupling of the Fmoc-amino acid (200 μmol) with PyBOP (200 μmol) and NMM (400 μmol) in DMF (0.5 mL) for 45 min. After 70% of coupling time, 200 μL of DCM were added to each syringe. After each coupling, washings were carried out with DMF (2 mL, 6×1 min).

Fmoc-Lys-Val-Leu-Thr-Lys-Cys-Gln-Glu-Glu-Val-Ser-His-Ile-Pro-OH

After chain assembly, the partially protected Fmoc-peptide was cleaved from the resins by treatment with TFA–H_2O–Et_3SiH (95:2.5:2.5) (0.2 mL) for 90 min, followed by an additional treatment with 0.2 mL of fresh reagent for 30 min. The peptides were precipitated from the cleavage solution by addition of chilled methyl *tert*-butyl ether (5 mL), centrifuged, and washed four times with the same solvent, dissolved in HOAc–H_2O (1:9) and lyophilized.

Amino Acid Analysis

HMPB-resin synthesis: Pro, 0.33 (1); Ile, 0.25 (1); His, 0.62 (1); Ser, 0.47 (1); Val, 1.65 (2); Glx, 2.84 (3); Cys, 0.39 (1); Lys, 1.96 (2); Thr, 0.89 (1); Leu 1.00 (1).

ClTrt-Cl-resin (high loading) synthesis: Pro, 1.14 (1); Ile, 0.89 (1); His, 0.97 (1); Ser, 0.72 (1); Val, 1.40 (2); Glx, 2.46 (3); Cys, n.d. (1); Lys, 0.89 (2); Thr, 0.57 (1); Leu 0.48 (1).

ClTrt-Cl-resin (low loading) synthesis: Pro, 1.46 (1); Ile, 0.93 (1); His, 1.02 (1); Ser, 0.71 (1); Val, 2.01 (2); Glx, 3.33 (3); Cys, 0.22 (1); Lys, 1.83 (2); Thr, 0.90 (1); Leu 0.98 (1).

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