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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS. IV. SYNTHESIS OF THE 35-43 AND 32-34 PROTECTED SEGMENTS OF THE TOXIN II OF ANDROCTONUS AUSTRALIS HECTOR.

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The protected peptide segments corresponding to the sequences 35-43 and 32-34 of toxin II of the scorpion Androctonus australis Hector have been synthesized on a p-alkoxybenzyl ester resin using the base-labile Fmoc α-amino protection and HF-labile side chain protecting groups. Crude peptides obtained after trifluoroacetic acid cleavage have been purified by solvent extraction, dimethylacetamide-water precipitation and semi-preparative reversed phase HPLC. Both synthetic and purification protocols have been optimized for good yields and high purity. Fast atom bombardment mass spectrometry has proved to be a very useful technique to characterize protected peptide segments.

The preceeding paper of this series (1) has described the synthesis of the 44-52 protected segment of the toxin II of the north african scorpion Androctonus australis Hector, which had been synthesized by stepwise elongation with TFA--labile temporary α-amino protection, HF-labile side chain protecting groups and a photolabile anchor of the peptide to the resin.

We had also explored an alternative way for the synthesis of protected peptide segments (2) with HF-labile side chain protection that combines the use of base-labile Fmoc α-amino protection (3) and a TFA-labile p-alkoxybenzyl ester peptide-resin linkage (4).

This work deals with the synthesis of the 35-43 and 32-34 protected segments of the scorpion toxin:

Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH

Fmoc-Glu(Bzl)-Ser(Bzl)-Gly-OH

The amino acids, protected in their α-amino function with the 9-fluorenylmethoxycarbonyl (Fmoc) group, have been assembled on a 4-hydroxymethylphenoxymethyl resin. Except for cysteine, which has been protected with the acetamidomethyl (Acm) group, HF-labile groups have been used to protect the side chains of trifunctional amino acids: cyclohexyl (cHex) for tyrosine (1) and benzyl (Bzl) for serine and glutamic acid. The crude protected peptides have been obtained after acidolytic cleavage of the peptide-resin bond with trifluoroacetic acid (TFA), and purification conditions have been optimized to yield highly pure peptides according to the basic requirement for a successful convergent peptide synthesis.

RESULTS AND DISCUSSION.

Synthesis of Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH.

4-Hydroxymethylphenoxymethylpolystyrene was synthesized by reaction of chloromethylpolystyrene with sodium methoxide and 4-hydroxybenzyl alcohol as

described by Wang (4). The polymer obtained was submitted to different controls prior to its use: i) gel-phase ^{13}C-NMR (5), ii) quantitation of residual chlorine content after pyridine displacement and chloride titration by the modified Volhard procedure (6), and iii) coupling of an Fmoc-amino acid to determine both incorporation and TFA cleavage yields.

The p-alkoxybenzyl ester resin was esterified with 2.5 equivalents of the symmetrical anhydride of Fmoc-glycine in the presence of 0.25 equivalents of 4-dimethylaminopyridine (DMAP) for one hr at room temperature (63% yield). The amount of diglycine formed during this process was quantified after TFA treatment of an aliquot of Fmoc-Gly-resin, Fmoc cleavage of the filtrate with piperidine and ion-exchange chromatography in an amino acid analyzer; 0.7% diglycine was found. Remaining hydroxyl groups were benzoylated.

For the stepwise elongation of the peptide chain α-amino functions were deprotected by treatment with 50% piperidine in dichloromethane (DCM) during 30 min, and the N-(9-fluorenylmethyl)piperidine (Fmp) formed was quantified at each step. Protected amino acids were coupled via their preformed symmetrical anhydrides (1.5 fold excess) except in the case of glutamine which was incorporated by a dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt)-mediated coupling (7). Completeness of couplings was monitored by the ninhydrin (8) and/or fluorescamine (9) tests, and the chloranyl test (10) was used to control the coupling of serine onto proline in the nonapeptide. In order to prevent diketopiperazine formation, the Fmoc-dipeptide-resin was deprotected by a short treatment (5 min) with 50% piperidine in dimethylformamide (DMF) (11), and the third amino acid was incorporated with a greater excess of symmetrical anhydride (2.5 fold excess). After incorporation of Fmoc-proline, an acetylation with acetic anhydride and pyridine was performed to block possible hydroxyl groups on the resin after diketopiperazine formation. If any, less than 4% diketopiperazine was formed.

The quantitation of Fmp formed at each step of the synthesis did not show any important loss of peptide chains along the synthesis apart from the 4% loss detected at the level of the third amino acid. The overall yield of the synthesis was 90%, which implies an average coupling yield of 98.8%.

Cleavage of the peptide-resin bond was carried out with 55% TFA in DCM in the presence of 1% anisole (20 fold excess with respect to reactive sites) as scavenger to capture benzyl cations formed on the resin during the acidolysis and 2% β-mercaptoethanol to avoid tryptophan oxidation. The crude peptide was obtained after filtration and washing with DCM. In the event that the peptide were not soluble in DCM, in a first assay the resin was also washed with methanol and this filtrate combined with the other filtrates and washings before TFA was completely eliminated. Under these conditions, the target nonapeptide was found to be accompanied by a certain amount of its methyl ester. Washing the resin with DCM only allowed to obtain a purer peptide.

Purification of Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH.

The purification of this peptide was planned as a two step process, with a first rough separation and a second step to accurately eliminate impurities.

For the first part of the purification we compared the yields, analytical HPLC profiles and amino acid analyses of the products obtained after either Sephadex LH-60 (10% DMA in methanol), Sephadex LH-20 (same solvent system) or solvent extractions followed by dimethylacetamide/water precipitation. Although the three methods gave similar results we chose the third one because it yielded the peptide much more easily, in somewhat purer form and better yield. Thus, the crude peptide was first treated with anhydrous hexane and then with anhydrous

ether. The hexane extract contained the majority of anisole and β-mercaptoethanol that still remained after the TPA treatment, and both organic solutions contained only small amounts of peptide material whose amino acid composition did not correspond to any defined deletion or truncated sequence (see experimental part). The solid residue was dissolved in dimethylacetamide (DMA) and the peptide was was precipitated by addition of water (78% yield).

The second step of the purification was a semi-preparative reversed phase chromatography on an Ultrasphere C_{18} column (Beckman, 25x1 cm, 5 μm), eluting with a mixture of acetonitrile, water, DMF (to allow a higher loading of product, not soluble in CH_3CN/H_2O) and 0.5% propionic acid. We carried out a first chromatography of this peptide at an analytical scale to find isocratic elution conditions, and then the purification was attempted on the Ultrasphere column eluting with DMF/CH_3CN/H_2O/propionic acid 30/52.5/17.5/0.5. The limited loading capacity of this chromatographic system (injection of more than 4-5 mg gave a completely distorted elution profile) forced us to consider another elution system with more DMF (DMF/CH_3CN/H_2O/propionic acid 65/18/17/0.1). Though the addition of more DMF increased the solubility of the peptide in the solvent system, it provoked a change in the chromatographic performance so that the product obtained was less pure and with no significant increase in the loading capacity. So the initial chromatographic conditions (DMF/CH_3CN/H_2O/propionic acid 30/52.5/17.5/0.5, 4 mL/min) were chosen (see figure 1), leading to satisfactory yields (50%). The peptide was totally stable to the 30% DMF chromatographic conditions and to the subsequent process of solvent removal.

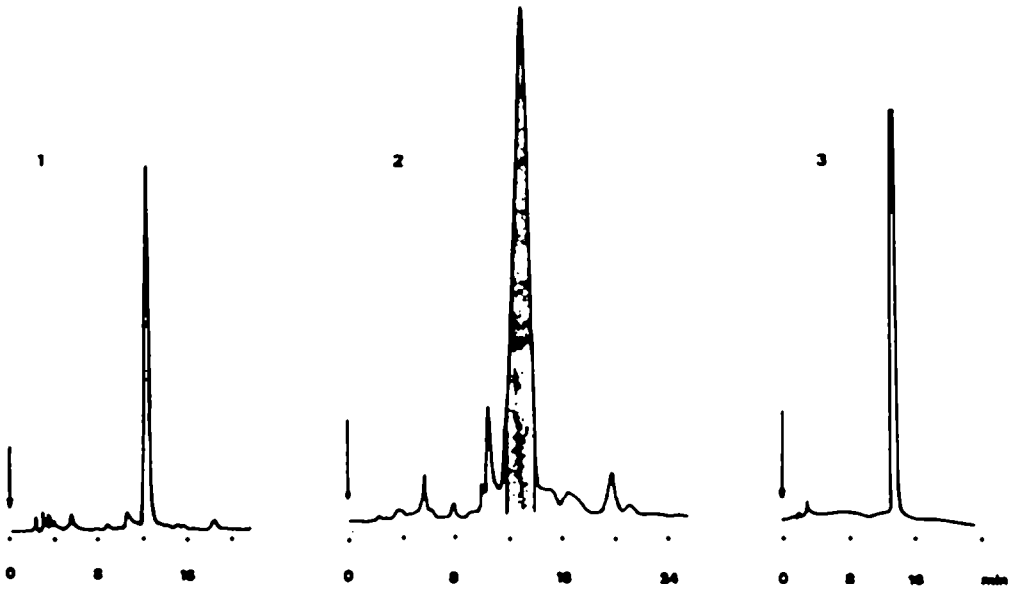
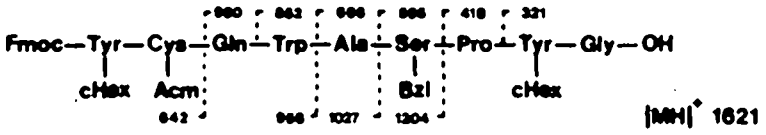


Figure 1. HPLC profiles on reversed phase C_{18} (Ultrasphere, 25x1 cm, 5 μm) DMF/CH_3CN/H_2O/propionic acid 30/52.5/17.5/0.5, 4 mL/min. 1) Analytical HPLC of crude peptide; 2) semi-preparative chromatography; 3)analytical HPLC of purified nonapeptide.

The peptide obtained after semi-preparative HPLC was homogeneous by analytical HPLC (figure 1) and TLC, and gave the following amino acid analysis: Ser 0.92, Glu 0.99, Pro 0.97, Gly 1.01, Ala 1.03, Cys 0.58, Tyr 1.63. It was also characterized by ^{1}H-NMR (200 MHz) and positive fast atom bombardment mass

spectrometry. With this technique, the following fragmentation pattern according to Morris et al. (12) was obtained:



Synthesis and purification of Pmoc-Glu(Bzl)-Ser(Bzl)-Gly-OH.

The synthesis of this tripeptide was carried out by the same procedure described for the synthesis of the nonapeptide. There were only small differences in the synthesis of the p-alkoxybenzyl ester resin, which was obtained by the method described by Lu et al. (13), and in the anchoring of Pmoc-glycine to the resin. This esterification was performed with 2.5 equivalents of both the symmetrical anhydride of Pmoc-glycine and DMAP for 30 min at room temperature, and although the amount of diglycine detected was only 0.5%, the coupling yield (25%) was much lower than in the case of the nonapeptide. 0.76 and 0.75 mmol Pmp were quantitated after the deprotections of glycine and serine respectively, and the piperidine treatment of an aliquot of peptide-resin at the end of the synthesis and Pmp quantification did not show any loss of peptide chains due to diketopiperazine formation. Anisole was the only scavenger added in the TFA treament to cleave the peptide-resin bond.

The purification of this peptide was made in the same way as for the nonapeptide: the crude peptide was first treated with hexane and a mixture of hexane/ether 1/4, and after solubilization in DMA it was precipitated by addition of water. It was finally purified by reversed phase HPLC on the same Ultrasphere column, eluting with DMF/CH_3CN/H_2O/propionic acid 24.5/45.5/30/0.1 at 3.5 mL/min (see figure 2). The overall purification yield was 75%.

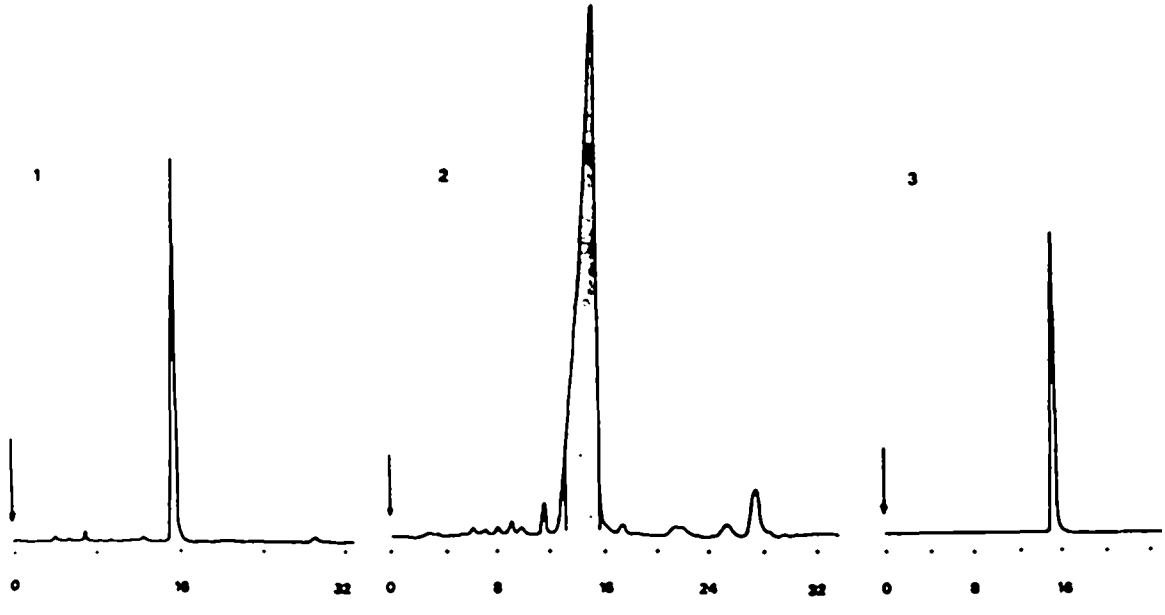
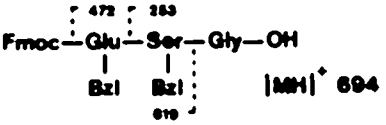


Figure 2. 1) Analytical HPLC of crude peptide; 2) semi-preparative HPLC; 3) analytical HPLC of purified tripeptide. Ultrasphere C_{18} (25x1 cm, 5 µm), DMF/CH_3CN/H_2O/propionic acid 24.5/45.5/30/0.1, 3.5 mL/min.

The purified peptide (Ser 0.88, Glu 1.01, Gly 1.11) was homogeneous by HPLC (figure 2) and TLC, and its ^{1}H-NMR spectrum (200 MHz) and positive fast atom bombardment mass spectrum were recorded. The fragmentation pattern obtained in the latter case was as follows:



Discussion.

The comparison between the results presented in this paper and those previously reported for the synthesis of two protected segments of the decapeptide luliberin (2) makes evident that there has been an improvement in our synthetic methodology as well as in our purification procedures of protected peptides.

In the first place, we consider very important to control the quality of any batch of p-alkoxybenzyl ester resin prior to a synthesis by all the three methods described above, though gel-phase ^{13}C-NMR spectra are only valuable when compared between each other. Concerning the method to prepare 4-hydroxymethylphenoxymethylpolystyrene, the two procedures described in the literature (4, 13) give, in our hands, resins of quite similar quality. We have found that the the resins obtained following the protocol of Lu et al. have lower percentages of hydroxymethylpolystyrene (2-5% instead of 9%), but the reaction of 4-hydroxybenzyl alcohol with chloromethyl groups is not always quantitative under these conditions.

The amount of dipeptide formed during attachment of the C-terminal amino acid to the resin has almost been suppressed (0.5-0.7%) in comparison with our previous results (9%). Shortening the reaction time from one hour to 30 minutes has lowered considerably the coupling yield (from 63% for the nonapeptide to 25% for the tripeptide) in spite of the greater amount of DMAP present in the reaction medium. In other words, our best results have been obtained in the esterification with 10% DMAP with respect to the anhydride and one hour reaction time.

The synthetic protocol for the assembly of the other amino acids has been essentially the same as in our synthesis of the luliberin protected segments (2): Fmoc-amino acids have been coupled as symmetrical anhydrides, and at each deprotection step the amount of Fmp formed has been quantitated, as it is a useful way to monitor the progress of the synthesis. The protocol for the incorporation of the third amino acid has been modified (rapid deprotection and coupling with a greater excess of symmetrical anhydride) to minimize diketopiperazine formation (11). The yields of the two syntheses have been very good, as well as the quality of the peptides obtained (see the analytical HPLC profiles of the crude peptides in figures 1 and 2). These results are also related to the quality of the Fmoc-amino acids employed, which had been carefully controlled before their use and purified (washed or recrystallized) when necessary. In any case, the use of highly pure Fmoc-amino acids, free of dipeptide contamination (14, 15) should be strongly recommended.

Acidolysis of the peptide-resin bond in the presence of anisole as scavenger has taken place with good yields (80-85%), much better than in our previous syntheses. This improvement can be related to the use of resins of better quality.

Many problems had been found in the purification of protected peptides with preparative chromatography on silica gel (2). The results we report in this paper

clearly show that the use of reversed phase HPLC (C_{18}) allows to overcome those problems: once the elution system composition has been carefully adjusted, it is possible to separate the target peptide in high purity, as can be seen in the analytical HPLC profiles in figures 1 and 2. Our confidence in this chromatographic system is further supported by the fact that, under very close conditions, we were able to separate the nonapeptide (and the tripeptide) from all their synthetic precursors (16). Since DMF is necessary to solubilize the peptide in the solvent system, the presence of an Fmoc-group at the N-terminus allows to monitor HPLC purification at 301 nm and to use DMF gradient systems without interferences nor shift in the base line.

Finally, fast atom bombardment mass spectrometry has proved to be an excellent technique to characterize protected peptide segments, since in both cases the molecular ion peak has been detected and almost all peptide bonds have been cleaved to give either an acylium or an ammonium ion, or both.

Our conclusion is that the improved methodology here described makes possible to synthesize and purify protected peptide segments in good yields and high purity, which are necessary requirements for a successful convergent peptide synthesis.

EXPERIMENTAL PART

Dichloromethane was dried over anhydrous K_2CO_3 and distilled over it immediately before use. DMF was vacuum distilled over P_2O_5 and ninhydrin under N_2, stored over 4Å molecular sieves and freed of amines by N_2 bubbling until negative 1-fluoro-2,4-dinitrobenzene test (6). DMA was vacuum distilled over CaH_2 and stored over 4Å molecular sieves. The amine content was also checked by the 1-fluoro-2,4-dinitrobenzene test. Piperidine was distilled under N_2 from NaOH pellets and redistilled under N_2. 2-propanol was dried over $CaCl_2$ and BaOH prior to distillation. TFA was distilled before use. Peroxide-free dioxane was used. Sephadex LH-60 and LH-20 were purchased from Pharmacia Fine Chemicals. All other solvents and chemicals were reagent grade.

Amino acid analyses were run either on Beckman 120C or Biotronik model 6000 autoanalyzers. Hydrolysates from peptide samples were prepared by treatment with 6N HCl or 12N HCl/phenol/acetic acid (2/1/1) for 20 hr at 110°C in vacuum degassed sealed tubes. Thin layer chromatography was carried out on precoated silica gel 60 (F-254) plates (Merck) with the solvent system indicated at every case. HPLC was performed in a Waters Associates apparatus with two solvent delivery systems (models 6000 A and M-45), a U6K injector, a model 660 solvent programmer and a model 450 variable wavelength UV detector. All chromatographic profiles were recording absorbance at 301 nm.

Protected amino acids were purchased either from Bachem or UCB, except Fmoc-Cys(Acm)-OH and Fmoc-Tyr(cHex)-OH which were synthesized in our laboratory. H-Tyr(cHex)-OH was obtained according to the procedure of Engelhard and Merrifield (17) with minor modifications, and the Fmoc group was introduced as previously described (18). Fmoc-Cys(Acm)-OH was obtained by reaction of commercial H-Cys(Acm)-OH and Fmoc-N_3 (19).

Chloromethylpolystyrene.

6.0 g of polystyrene were suspended in 70 mL of anhydrous $CHCl_3$ and stirred for 1 hr at 25°C, then cooled in an ice bath. A mixture of $SnCl_4$ (0.5 mmol/g resin) and $ClCH_2OCH_3$ (25 mmol/g resin) was added dropwise, the suspension was stirred for 30 min at 0°C and immediately filtered and washed (6). The polymer obtained by this procedure had a chlorine content of about 0.5-0.6 mmol/g (results from different assays were not always very reproducible) as determined by the modified Volhard procedure, and no hydroxymethyl groups were detected by gel-phase ^{13}C-NMR.

4-hydroxymethylphenoxymethylpolystyrene.

4-hydroxymethylphenoxymethylpolystyrene was obtained from chloromethylpolystyrene by reaction with 4-hydroxybenzyl alcohol and sodium methoxide following the two procedures described in the literature (4, 13).

According to the first procedure (4), 5.1 g of chloromethylpolystyrene (3.1 mmol) were suspended in 150 mL of DMA and 0.74 g of 4-hydroxybenzyl alcohol (5.9 mmol) and a methanolic solution of $NaOCH_3$ (6.1 mmol) were added. The suspension was stirred for 24 hr at 80°C and then filtered and washed (3xDMF, 3xdioxane, 3xDCM and 5xmethanol). The resin obtained had a substitution degree of 0.61 mmol/g.

According to the second procedure (13), 9.1 g of chloromethylpolystyrene

(4.2 mmol) and 1.56 g of 4-hydroxybenzyl alcohol (12.6 mmol) were suspended in DMA and a methanolic solution of $NaOCH_3$ (12.6 mmol) was added. The suspension was stirred for 8 hr at 50°C and then filtered and washed (4xdioxane, 6xdioxane/H_2O 1/1, 4xdioxane and 4xmethanol). A substitution degree of 0.40 mmol/g was obtained.

Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH.

Synthesis.

The symmetrical anhydride of Fmoc-glycine was prepared from 5.2 g of Fmoc-Gly-OH (17.5 mmol) and 1.99 g of DCC (9.6 mmol) in DCM/DMF 4/1. After 30 min stirring at 0°C, the mixture was filtered and the filtrate was added to 5.74 g of $HO-CH_2-C_6H_4-O-CH_2$-polystyrene (3.5 mmol, synthesized by the method of Wang (4)) together with 107 mg of DMAP (0.88 mmol) dissolved in the same solvent (the total volume of solvent added was 100 mL). The suspension was stirred for 1 hr at room temperature and then filtered and washed 3 times with each DCM, DMF, 2-propanol, DCM, DMF, 2-propanol and methanol.

An aliquot of Fmoc-Gly-resin was treated with piperidine, and the quantitation of Fmp formed corresponded to a substitution degree of 0.33 mmol/g Fmoc-Gly--resin.

Unreacted hydroxymethyl sites were blocked by two successive reactions with benzoyl chloride and pyridine (30 and 33 equivalents excess with respect to free hydroxymethyl groups) 30 min at 0°C and 30 min at room temperature.

The synthesis was continued with 5.8 g of Fmoc-Gly-resin. The general protocol for each synthetic cycle was as previously described (2), with the exception of the second synthetic cycle and the coupling of glutamine. Fmoc-Tyr(cHex)-Gly-resin was deprotected as follows: 1) 3xDCM, 2) 1xDMF, 3)1x1 min 50% piperidine/DMF, 4) 1x4 min 50% piperidine/DMF, 5) 2xDMF, 6) 4xDCM, to prevent diketopiperazine formation. The rest of the washings were as usual. Coupling of Fmoc-Pro-OH was carried out with 2.5 fold excess of anhydride in the presence of 1 eq of ethyldiisopropylamine. Possible free hydroxyl groups were blocked by reaction with acetic anhydride and pyridine. The active HOBt ester of Fmoc-glutamine was prepared as follows: protected amino acid (3 eq) and an equimolar amount of HOBt dissolved in DMF were stirred for 10 min at 0°C. After addition of a solution of DCC (3 eq) in DMF, the mixture was stirred for 10 min at 0°C and then poured onto the resin. Coupling was performed for 2 hr.

In spite of a negative ninhydrin or chloranyl test, an acetylation (acetic anhydride/ethyldiisopropylamine) was performed after the first coupling of tyrosine and serine. For the other residues, a negative test was obtained after a second coupling with 1 eq of acylating reagent.

Deprotection of an aliquot of Fmoc-nonapeptide-resin gave a substitution degree of 0.24 mmol/g Fmoc-nonapeptide-resin.

The acidolysis of the peptide-resin bond was carried out with small batches (500 mg) of Fmoc-peptide-resin, by treatment with 25 mL of 55% TFA/DCM, 0.25 mL of anisole and 0.5 mL of β-mercaptoethanol. 543 mg of crude peptide (72.2 μmol of peptide material) were obtained by evaporation of filtrates and washings with DCM. The cleavage yield was 80%, as determined by comparison between the mmol of Fmp quantitated in the last deprotection step of the synthesis and the amount of Fmp found in the deprotection of an aliquot of resin after the TFA treatment.

Purification.

543 mg of crude peptide were treated with 20 mL of anhydrous hexane and sonicated for 1 min. After sedimentation of the insoluble product the solvent was removed by suction, filtered through a Millipore PH filter (0.5 μm), evaporated and analyzed. The peptide solid residue was further sonicated with 20 mL of anhydrous ether for 90 s, and the organic solution was treated as before. The ether-insoluble solid residue was dissolved in 4 mL of DMA and the peptide was precipitated by the addition of 12 mL of water. After 3-4 hr at 4°C the precipitated peptide (78% yield) was filtered (Millipore GSWP, 0.22 μm) and washed with water. The DMA/water solution was lyophilysed and analyzed. Amino acid analyses of the different fractions as well as their weight and peptide content are shown in table 1.

Table 1. Amino acid analyses at different stages of the purification of nonapeptide.

	Crude	Hexane solution	Ether solution	DMA/H_2O solution	Precipitate
Ser	0.90	1.10	0.91	0.89	0.97
Glu	0.97	0.83	0.24	1.02	1.01
Pro	1.14	-	1.09	-	0.88
Gly	1.05	3.10	1.37	1.63	1.08
Ala	0.98	1.08	-	0.94	1.07
Cys	0.13	3.57	0.40	0.82	0.47
Tyr	1.06	-	0.05	1.70	1.36
Weight (mg)	543	259	50	12	118
Peptide content (μmol)	72.2	0.04	0.15	3.4	56.3

The white solid residue obtained after precipitation was dissolved in 2.9 mL of DMA and purified on an Ultrasphere C_{18} column (Beckman, 25x1 cm, 5 μm) eluting

with the mixture DMP/CH_3CN/H_2O/propionic acid 30/52.5/17.5/0.5 at 4 mL/min. 90 µL fractions (3.5 mg, 1.67 µmol) were injected at each run. 36 µmol of pure peptide were obtained (50% overall purification yield), $[\alpha]_D$=-38.6±2.4 (c 0.58, DMA) and Rf=0.39 ($CHCl_3$/CH_3OH/acetic acid 73/25/2). The most significant signals in the ^{1}H-NMR spectrum of the peptide (DMSO-d_6, 200 MHz) were: δ8.6, t, NH Acm; 8.1, t, NH Gly; 7.3, s, Ar Bzl; 4.5, s, CH_2 Bzl; 1.8, s, CH_3 Acm; 1.2, d, CH_3 Ala.

Pmoc-Glu(Bzl)-Ser(Bzl)-Gly-OH.

Synthesis.

This tripeptide was obtained by the same synthetic protocols used for the nonapeptide. Only differences between each other are reported here.

4-hydroxymethylphenoxymethylpolystyrene (7.64 g, 3.06 mmol), synthesized as described by Lu et al. (13), was esterified with 7.64 mmol of the symmetrical anhydride of Pmoc-glycine (4.54 g of Pmoc-Gly-OH and 1.73 g of DCC) and an equimolar amount of DMAP (932 mg) for 30 min at room temperature. A substitution degree of 0.11 mmol/g Pmoc-Gly-resin was determined after deprotection of an aliquot of Pmoc-glycine-resin and spectrophotometric quantitation of Pmp.

A single coupling with 1.5 eq of the symmetrical anhydride of Pmoc-Ser-(Bzl)-OH afforded a negative ninhydrin test. After deprotection with 50% piperidine/DMP, 2.5 eq of symmetrical anhydride of Pmoc-Glu(Bzl)-OH were used for the first coupling, but a second coupling with 0.75 eq of symmetrical anhydride was necessary to obtain a negative ninhydrin test.

Deprotection of an aliquot of Pmoc-tripeptide-resin gave a substitution degree of 0.10 mmol/g Pmoc-peptide-resin.

Batches of 1.2 g of Pmoc-peptide-resin were treated with 29 mL of 55% TFA/DCM in the presence of 0.28 mL of anisole for 1 hr at room temperature. After evaporation of filtrates and washings with DCM 111 mg of crude peptide were obtained (86% yield).

Purification.

111 mg of crude peptide (85.8 µmol) were first extracted with 20 mL of anhydrous hexane for 40 s and then with 15 mL of hexane/ether 1/4 for 40 s. The solid residue was dissolved in 4 mL of DMA and 20 mL of water were added to precipitate the peptide, which was filtered and washed after one day at 4°C (80% yield). Amino acid composition of the different fractions, together with their weight and peptide content, are shown in table 2.

Table 2. Amino acid analyses at different stages of the purification of tripeptide.

	Crude	Hexane solution	Ether solution	DMA/H_2O solution	Precipitate
Ser	0.93	0.53	0.74	0.85	0.86
Glu	1.03	0.85	1.02	0.47	1.03
Gly	1.16	1.62	1.23	1.68	1.11
Weight (mg)	111	7	11	5	63
Peptide content (µmol)	85.8	0.05	2.7	3.1	69

The precipitated peptide was dissolved in 2 mL of DMA, and 100 µL fractions (3.2 mg, 3.1 µmol) were injected on an Ultrasphere column and eluted with DMP/CH_3CN/H_2O/propionic acid 24.5/45.5/30/0.1 at 3.5 mL/min. 64.1 µmol of pure peptide were obtained (75% overall purification yield), $[\alpha]_D$=2.6±0.3 (c 0.89, DMA) and Rf=0.60 ($CHCl_3$/CH_3OH/acetic acid 73/25/2). The most significant signals in the ^{1}H-NMR spectrum of the peptide (DMSO-d_6, 200 MHz) were: δ8.3, t, NH Gly; 8.1, d, NH Ser; 7.4, s, Ar Bzl; 5.1, s, CH_2 Bzl Glu; 4.5, s, CH_2 Bzl Ser; 3.6, d, $C^\beta H_2$ Ser; 3.7, d, CH_2 Gly.

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