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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS. V. SYNTHESIS OF THE 1-4, 32-34, AND 53-59 PROTECTED SEGMENTS OF THE TOXIN II OF ANDROCTONUS AUSTRALIS HECTOR.

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Abstract - Two protected peptide segments corresponding to the sequence 32-34 and 53-59 of toxin II of the north African scorpion Androctonus australis Hector have been synthesized on a photolabile Nbb-resin using the TFA-labile Boc α-amino protection and HF-labile side chain protecting groups. A third protected peptide corresponding to segment 1-4 has been synthesized on the same resin but with a t-butyl group for β-protection of aspartic acid and a Z group on the lysine side chain. For this last segment a combination of Boc and Fmoc groups for α-amino protection has been used successfully on the Nbb-resin. After photolysis the crude peptides have been treated by solvent extraction and semi-preparative HPLC to yield highly purified segments. These syntheses show the flexibility of the convergent solid phase approach and how segments with different and independent protecting groups can be assembled by solid phase peptide procedure.

Current works of authors' laboratories deal with the synthesis of toxin II of the north African scorpion Androctonus australis Hector, a 64-peptide cross-linked by four disulfide bridges[1], by a "convergent solid phase approach"[2,3]. The preceding papers of this series[4,5] have described the syntheses of the two protected segments corresponding to the sequence 44-52 and 35-43 of this toxin. The side chain protecting groups are of the HF labile type. Syntheses have been carried out using two alternative strategies: i) TFA-labile temporary α-amino protection and a photolabile anchor of the peptide to the resin; and ii) base-labile α-amino protection and a TFA-labile peptide-resin linkage.

We wish to report in this paper the synthesis of the 1-4 (I), 32-34 (II) and 53-59 (III) protected segments of the scorpion toxin.

(I) Boc-Val-Lys(Z)-Asp(t-Bu)-Gly-OH

(II) Boc-Glu(Bzl)-Ser(Bzl)-Gly-OH

(III) Boc-Asp(Bzl)-His-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly-OH

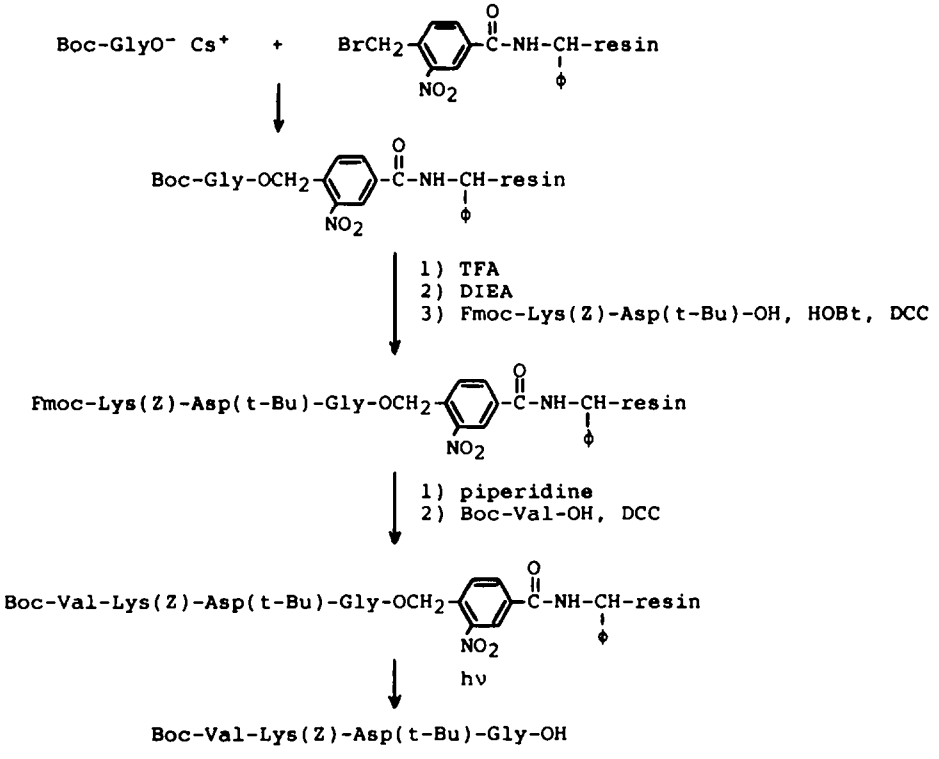
Peptides II and III have been synthesized following approach i, on a bromomethyl-Nbb-resin[6] which provides a photolabile anchoring of the peptide. This resin allows the use of TFA-labile Boc α-amino protection and HF-labile side chain protecting groups: benzyl for glutamic and aspartic acids, serine, and threonine; tosyl for arginine; and benzyloxycarbonyl for lysine. Imidazole of histidine was protected with tosyl, which could be removed by means of HOBt before purification.

The synthesis of peptide I shows a clear example of how the convergent solid phase strategy can be fine-tuned to circumvent several synthetic problems. Yang and Merrifield[7] have reported that in peptides containing the sequence -Asp(Bzl)--Gly- up to 99% of peptide material can be transformed into aminosuccinyl derivative after the HF treatment. The same authors proposed the use of a temporary protecting group for the β-carboxyl group of aspartic acid which could be selectively removed after the assembling of peptidic chain before HF treatment. The most commonly used temporary protection of carboxylic acids is as t-butyl esters[8]. However, due to its lability towards trifluoroacetic acid, this protection was not directly compatible with the synthetic strategy i. We show in this paper how the general approach could be adapted in order to get the specific set of protecting groups which best suits each particular peptide segment. It is illustrated by the synthesis of peptide I, bearing a t-butyl protection at the β-carboxyl group of aspartic acid and a Z group on lysine side chain, on a Nbb-resin, via the combined use of acid-labile Boc groups and base-labile Fmoc groups for the temporary protection of the α-amino groups of the growing peptide chain.

RESULTS AND DISCUSSION

Synthesis and purification of Boc-Val-Lys(Z)-Asp(t-Bu)-Gly-OH (I)

The tetrapeptide was prepared according to the following synthetic scheme:



The Boc-Gly-oxymethyl-Nbb-resin was obtained by the cesium salt procedure[9]. The reaction was carried out in DMA at 25°C and anchoring yield was 74%.

Since t-butyl protection of the β-carboxyl of aspartic acid is incompatible with Boc α-amino protection, the Fmoc group was instead chosen for this peptide synthesis. As we have recently reported[10], piperidine, the reagent of choice to deprotect Fmoc groups, is an excellent catalyst for the undesired reaction of diketopiperazine formation. This secondary reaction, already a source of problems when Fmoc-aminoacids are used in conjunction with a p-alkoxybenzyl ester resin, is specially serious when one intends to perform peptide synthesis with Fmoc amino acids on a Nbb-resin, which is particularly prone to diketopiperazine formation[11]. Preliminary experiments showed that for the sequence Fmoc-Asp(t-Bu)-Gly-, even using mild Fmoc deprotection conditions (20% piperidine-DMF, 1x8 +1x2 min), 38% of peptide chains were lost as determined by hydrolysis and amino acid analysis of the filtrates. Incorporation of the second and third amino acids as a dipeptide was the safest way to circumvent diketopiperazine formation[12]. Fmoc-Lys(Z)-Asp(t-Bu)-OH was synthesized in solution with excellent yield from Fmoc-Lys(Z)-OSu and Asp(t-Bu)-OH. The coupling of the dipeptide on the glycyl-OCH_2-Nbb-resin was carried out in DMA with DCC and HOBt. 1.88 fold excess was used and after 2 h at 0°C and 16 h at 25°C, the ninhydrin[13] test was negative.

Compatibility of Fmoc-amino acids with Nbb resin still faces another problem arising from the lability of the o-nitrobenzyl ester peptide-resin bond to nucleophiles like piperidine[14]. In the synthesis of peptide I before incorporation of Boc-Val-OH, the Fmoc group was removed by treatment with 20% piperidine-DMF (1x8 min + 1x2 min). These conditions deprotect the α-amino with a 5% loss of peptide-chain as determined by hydrolysis and amino acid analysis of the filtrates. Further experiments have shown that use of 50% piperidine-CH_2Cl_2 (2x1 min) insures a complete α-amino deprotection and minimizes the side reaction (1% of loss determined as above).

After photolysis (yield 72%) and in spite of the high homogeneity of this peptide by HPLC (figure 1A), a purification step was carried out by a semi-preparative HPLC on an Ultrasphere column run in a mixture of DMF/CH_3CN/H_2O/propionic acid (25/39/36/0.1). Pure peptide (figure 1B) was obtained with 41% recovery and had the correct amino acid composition and 200-MHz ^{1}H-NMR spectra (figure 5A).

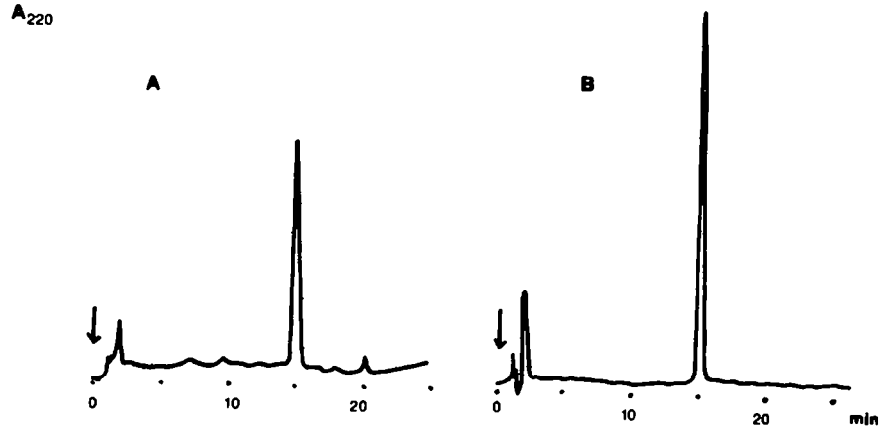


Figure 1. Analytical HPLC profiles on reversed phase C_{18}. Elution was with a linear gradient from 15% to 45% B in A during 7 min (see Experimental Part); flow rate 1.5 mL/min. A) crude peptide I; B) purified peptide I.

Synthesis and purification of Boc-Glu(Bzl)-Ser(Bzl)-Gly-OH (II)

The anchoring of Boc-Gly-OH onto the resin was carried out by the same procedure as described above with similar yield. In order to avoid the formation of diketopiperazines, Boc-Glu(Bzl)-OH was incorporated using the method described by Suzuki et al.[12] No decrease in substitution degree was found by picric acid titration[15].

The protected peptide was obtained by photolysis (59% yield) and showed a homogeneity of 95% by HPLC. After a treatment with hexane to remove non peptidic material, pure peptide (figure 2) was obtained in 73% recovery by semi-preparative reverse phase HPLC and characterized by amino acid analysis, 200-MHz ^{1}H-NMR (figure 5B) and TLC in two different systems.

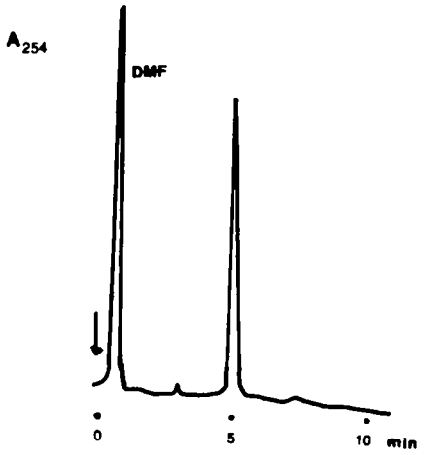


Figure 2. Analytical HPLC on C_{18} of purified peptide II. Linear gradient from 50% to 65% B in A during 5 min (see Experimental Part); flow rate 1.5 mL/min.

Synthesis and purification of Boc-Asp(Bzl)-His-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly--OH (III)

The initial Boc-Gly-oxymethyl-Nbb-resin was obtained by the cesium salt procedure. In this case, the reaction was carried out at 50°C and the yield was 75%. Boc-Thr(Bzl)-OH was incorporated by the Suzuki method and diketopiperazine formation was not detectable by a change of the substitution level of the tripeptide on the resin as assessed by the picric acid titration method. The remaining amino acids were assembled by standard solid phase procedures[16,17] (details in Experimental Part), and completeness of coupling was assessed by the ninhydrin test. An acetylation step was necessary after attachment of Boc-His(Tos)-OH.

Photocleavage is a critical step in this approach. Yields seem to depend rather on the nature of the C-terminal amino acid. Segments with C-terminal Ala (12 amino acids)[18], Pro (6 a.a.)[3] or Val (5 a.a.)[6] gave 89, 65, and 24% photocleavage yields respectively. On the other hand, with the same C-terminal amino acid the yield can depend on the length of the peptide (another peptide with C-terminal Pro (9 a.a.)[4] gave a 45% yield), so the larger the peptide the more crucial it is to optimize the cleavage conditions. Sonication[18] and the right choice of solvent

help in improving the yields. Although preliminary experiments[3,4,18] suggested that 20% TFE-CH_2Cl_2 is the most suitable solvent, other solvents have also been explored. So, with this peptide, DMF gave a yield of 18%, 20% TFE-DMF a yield of 42%, 20% MeOH-DMF a yield of 51% and finally 20% TFE-CH_2Cl_2 a yield of 57%. The overall cleavage yield for 5.0 g of peptide-oxymethyl-Nbb-resin in 20% TFE-CH_2Cl_2 treated in seven fractions was 53% giving 0.48 mmol of crude protected peptide.

Before purification, the tosyl protecting group of histidine had to be removed, because tosyl histidine-containing peptides can be unstable to further steps of purification[19,20]. This deprotection was performed with HOBt (10 fold excess)[21].

Protected peptides obtained by solid phase procedures need a thorough purification and characterization before further use. For such peptides, a three step purification: solvent extraction, gel filtration, and semi-preparative HPLC has given excellent results[4,5,18], each step removing a different type of impurities. In the first step, treatment with ethyl ether extracted non peptidic material. The solid residue was then dissolved in DMF, precipitated by addition of H_2O and the peptide recovered by centrifugation (83% yield). The second step of purification was a Sephadex LH-20 column run in $CHCl_3$-MeOH (8:2) (figure 3). The amino acid analysis showed that the first peak corresponded to title peptide (70% yield) and that the second one contained Boc-Asp(Bzl)-OH which remained non-covalently bonded to the resin after the last coupling. U.V. spectra indicated that the third peak corresponded to HOBt derivatives.

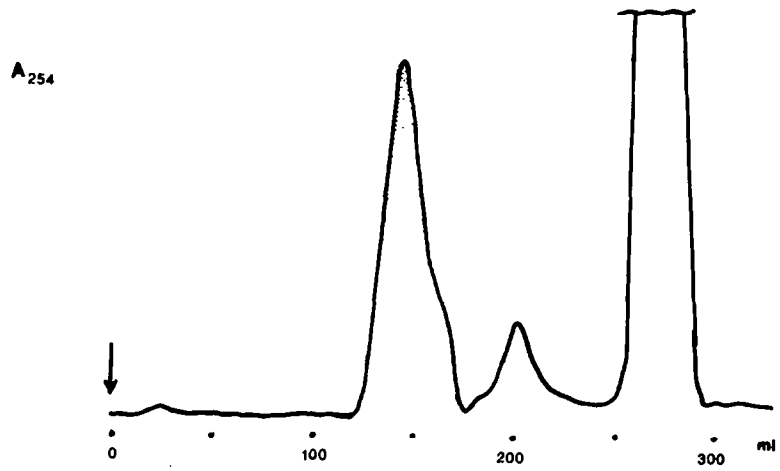


Figure 3. Chromatography on a Sephadex LH-20 column (1x150 cm) in $CHCl_3$-MeOH (8:2) of the peptide III obtained after solvent extractions; flow rate 22 mL/h.

Finally, the last purification step was a semi-preparative HPLC on a silica gel column in $CHCl_3$-MeOH-H_2O (72:28:1) (figure 4A). The purified peptide (figure 4B) obtained in 57% yield had a correct amino acid analysis (see Experimental Part), 200-MHz ^1H-NMR spectra (figure 5C), and showed a single spot in two different TLC systems.

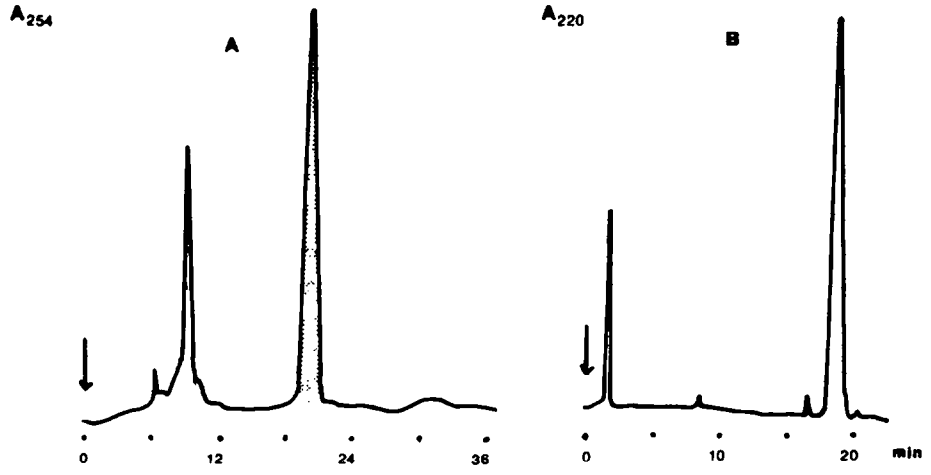


Figure 4. A) Semipreparative HPLC on a Silica Gel column (see Experimental Part) of the fraction of peptide III obtained after Sephadex LH-20 chromatography; B) Analytical HPLC on C_{18} of purified peptide III. Linear gradient from 60% to 75% B in A (see Experimental Part) during 20 min; flow rate 1.5 mL/min.

CONCLUSIONS

The combination of Boc α-protection, HF-labile side chain protection, and bromomethyl-Nbb-resin provides a useful strategy to prepare highly pure protected peptide segments.

The C-terminal Boc-amino acid can be incorporated rather smoothly onto the resin by the cesium salt procedure. No high temperatures are necessary (same yields at 50°C than at 25°C). With Nbb-resin care has to be taken during coupling of the third amino acid to avoid diketopiperazine formation, but the method of Suzuki provides very satisfactory results. The remaining amino acids can be incorporated following standard solid phase procedures[16,17], taking advantage of improvements achieved during the last years in this field[22].

A necessary requirement for a successful convergent peptide synthesis is high purity of the different protected peptides. So, a careful attention has to be paid to the purification step. The conjugation of traditional methods (extraction with organic solvents, precipitation, gel filtration chromatography) together with semi-preparative high performance liquid chromatography on either C_{18} or silica gel column has shown to give very good results. The use of C_{18} packings has some advantages over the silica gel (high reproducibility, easier handling) but in some cases, depending on the solubility of the peptide, silica gel columns can be useful.

The protected peptides have been characterized and proved to be homogeneous by amino acid analysis, 200-MHz ^{1}H-NMR, analytical HPLC and TLC. ^{1}H-NMR is a specially useful technique to characterize protected peptides, because protecting groups are removed during the acid hydrolysis and can not be visualyzed by amino acid analysis. The NMR spectra of these peptides (figure 5) show, for instance, how it is possible to distinguish between $-CH_2-$ signals corresponding to benzyl

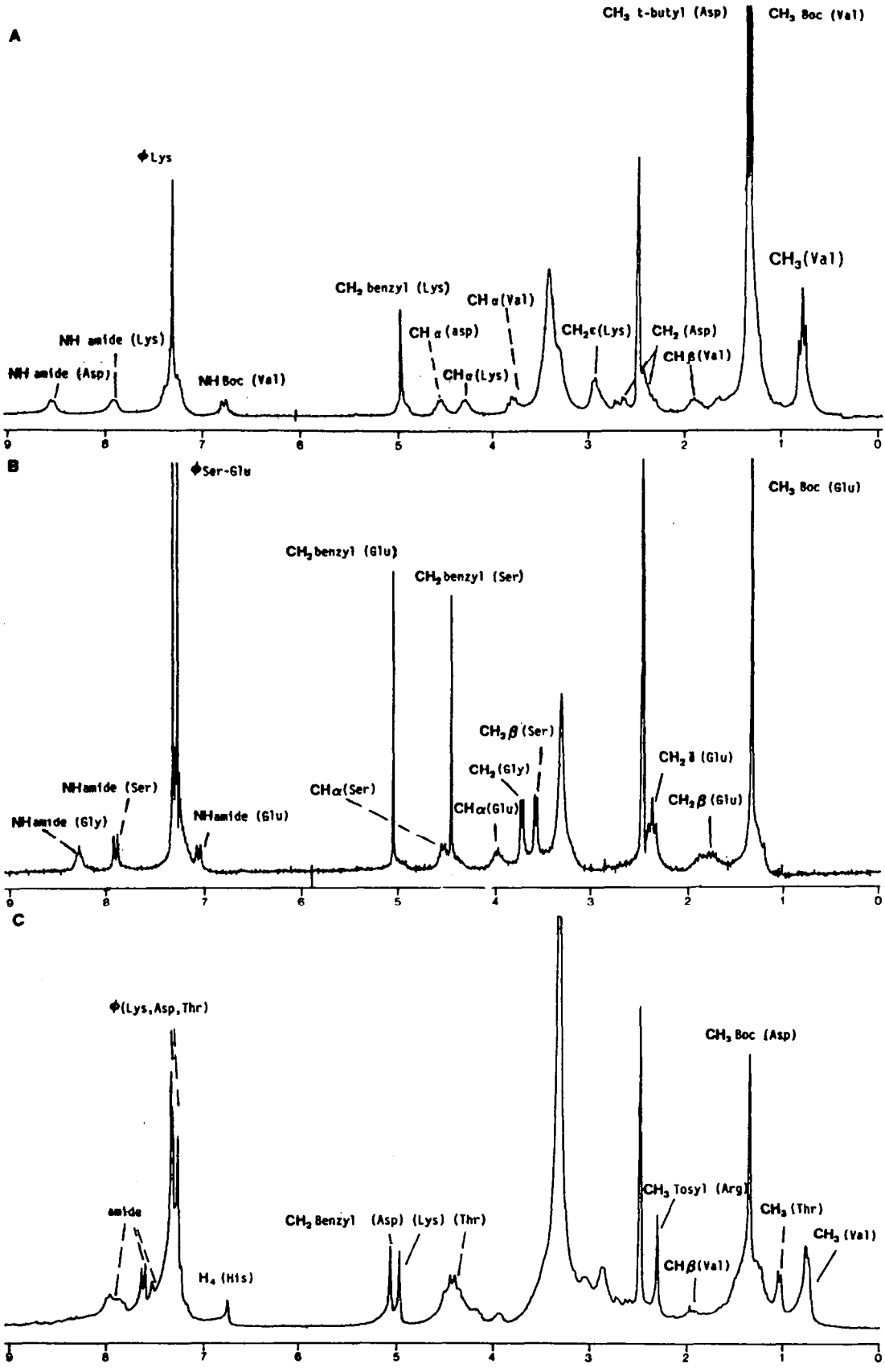


Figure 5. ^{1}H-NMR spectra observed at 200 MHz of purified peptides I (A), II (B) and III (C).

groups of protected Asp, Glu, Ser, Thr, and Lys.

Finally, these syntheses clearly show the flexibility of the convergent solid phase peptide methodology. Each segment can be adapted to different and independent protecting groups in order to avoid any expected side reactions and thus perform the synthesis in the most optimal conditions. More than one protecting group can be associated to the same function depending on the sequence of the segment and the methodology used for assembling the peptide.

EXPERIMENTAL PART

Abbreviations: AcOH, acetic acid; Boc-, t-butoxycarbonyl; BuOH, n-butanol; t-Bu, t-butyl; Bzl, benzyl; DCC, N,N'-dicyclohexylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMA, N,N'-dimethylacetamide; DMF, N,N'-dimethylformamide; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; iPrOH, isopropanol; MeOH, methanol; Nbb-, nitrobenzamidobenzyl; NMM, N-methylmorpholine; Su, succinimidyl; -resin, poly(styrene-co-1%-divinylbenzene); TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tos, tosyl; Z, benzyloxycarbonyl. Amino acid symbols denote the L configuration where applicable.

The protected amino acids were from Protein Research Foundation (Osaka, Japan) and Nova Biochem AG (Läufelfingen, Switzerland). Their purity was checked by TLC. Bromomethyl-Nbb-resins were prepared starting from poly(styrene-co-1%-divinylbenzene) 200-400 mesh from Bio-Rad Laboratories (Richmond, California), as previously described[3]. Amino acid analyses were done either on a Biotronik model LC 7000 or model 6000. Peptides and Peptide-resins I and II were hydrolyzed at 110°C with 6N HCl and 12N HCl/AcOH for 24 h and 48 h respectively in vacuum degassed sealed tubes. Peptide and Peptide-resin III needed 60 h at 110°C in order to achieve complete hydrolysis of Arg-Val-His bonds. ^{1}H-NMR spectra were recorded on a Varian XL-200 spectrometer. High-performance liquid chromatography was carried out in a Water Associates apparatus with two solvent delivery systems and a variable wavelength UV monitor. The reverse phase C_{18} column used for purification of protected segments I and II was a semi-preparative Ultrasphere Altex column (1x25 cm, 5 µm). The silica gel column used for purification of the protected segment III was a semi-preparative LiChrosorb Merck Silica Gel 60 column (1x25 cm, 7 µm). Analytical C_{18} HPLC column was a Spherisorb ODS-2 (0.39x30 cm, 10 µm). Solution A was 0.045% TFA in H_2O and B, 0.036% TFA in CH_3CN. Solvent systems for TLC on silica gel 60(F-254) plates 0.2 mm (Merck) were CMA-I, $CHCl_3$-MeOH-AcOH (73:25:2); CMA-II, $CHCl_3$-MeOH-AcOH(85:14:1); CMA-III, $CHCl_3$-MeOH-AcOH (70-20-5); BAH, BuOH-AcOH-H_2O (4:1:1), and CMN, $CHCl_3$-MeOH-NH_3 (20-10-1).

General Procedure for Solid-Phase Assembling of Peptides

Peptide syntheses were performed manually either in a teflon reaction vessel or in a 50 mL polypropylene syringe both fitted with a polyethylene disc.

Boc-aminoacids (except the first and the third) were assembled using the following protocol: 1) CH_2Cl_2, 4x1.5 min; 2) 30% TFA/CH_2Cl_2, 2x1.5 min + 1x30 min; 3) CH_2Cl_2, 4x1.5 min; 4) 5% DIEA/CH_2Cl_2, 2x1.5 min + 1x20 min; 5) CH_2Cl_2, 5x1.5 min; 6) Boc-aminoacid (2.5 equiv.) in CH_2Cl_2, after 2 min add the equivalent amount of DCC in CH_2Cl_2, stir 90 min; 7) CH_2Cl_2, 4x1.5 min; 8) steps 4-7; 9) DMF, 4x1.5 min; 10) CH_2Cl_2, 4x1.5 min.

The third protected amino acids were incorporated following Suzuki et al.'s method: 1) CH_2Cl_2, 3x1.5 min; 2) dioxane, 3x1.5 min; 3) dioxane/HCl 4N, 2x1.5 min + 1x30 min; 4) dioxane, 2x1.5 min; 5) CH_2Cl_2, 3x1.5 min; 6) DCC (2.5 equiv.) in CH_2Cl_2, after 2 min add the equivalent amount of Boc-amino acid N-methylmorpholinium salt, stir 180 min; 7) CH_2Cl_2, 4x1.5 min; 8) DMF, 4x1.5 min; 9) steps 6-7.

Acetylations where necessary were done using the following procedure: 1) CH_2Cl_2, 4x1.5 min; 2) 5% DIEA/CH_2Cl_2, 2x1.5 min; 3) CH_2Cl_2, 3x1.5 min; 4) HOAc (5 mmol) in CH_2Cl_2, after 2 min add the equivalent amount of DCC in CH_2Cl_2, stir 15 min; 5) CH_2Cl_2, 4x1.5 min.

General Procedure for Photolytic Cleavage of o-Nitrobenzyl Ester Anchoring linkages of Peptide-Nbb-resins

Peptide-Nbb-resins were placed in a three-necked cylindrical reaction vessel and suspended in TFE-CH_2Cl_2 (2:8) (100 mL). The microtip of an Ultrason-Annemase

ultrasonicator was then inmersed into this suspension, which was externally chilled with an ice bath, and sonication was applied (7x5 min). Oxygen was removed at 0°C by alternated connection to vacuum and nitrogen lines for about 30 min. The photochemical reaction was carried out for 9-14 h with two H 125 BL Eye lamp on a similar apparatus, with minor differences, to the one described previously[2]. The cleaved peptide-resin was filtered and washed with TFE-CH_2Cl_2 (2:8) (2x10 mL), CH_2Cl_2 (2x10 mL), and MeOH (2x10 mL), and the combined filtrates and washings were evaporated to dryness at 25°C.

Fmoc-Lys(Z)-OSu

A mixture of Fmoc-Lys(Z)-OH (5.0 g, 10.2 mmol), N-hydroxysuccinimide (1.2 g, 10.2 mmol) and DCC (2.1 g, 10.2 mmol) in dioxane (200 mL) was allowed to stir overnight at 25°C. After this time, N,N'-dicyclohexylurea was filtered off and the solution was evaporated to dryness. The residue was recrystallized from ethyl acetate-hexane. Yield 4.1 g (70%). mp 115°C; $[\alpha]_D$ =-21.9 (c= 1, MeOH); R_f (CMA-I), 0.80; NMR ($CD_3S(O)CD_3$) 8.08 (d, NH), 7.1-7.9 (m, Fmoc), 7.1-7.5 (m, benzyl aromatic), 5.00 (s, benzyl CH_2), 4.1-4.5 (m, Lys -CH ; Fmoc CH-CH_2), 2.9-3.1 (m, Lys ε-CH_2), 2.80 (s, Su CH_2), 1.3-1.9 (m, Lys β, γ, δ -CH_2). Anal. Calcd. for $C_{33}H_{33}N_3O_8$(Mol. wt. 599): C, 66.10; H, 5.55; N, 7.01. Found: C, 65.27; H, 5.47; N, 6.79.

Fmoc-Lys-(Z)-Asp(t-Bu)-OH

Fmoc-Lys(Z)-OSu (3.6 g, 6 mmol) in dioxane (50 mL) was added to a solution of H-Asp(t-Bu)-OH (1.1 g, 6 mmol) and $NaHCO_3$ (0.5 g, 6 mmol) in H_2O (65 mL). Within 5 min a precipitate of N-hydroxysuccinimide appeared, but stirring was continued overnight. The mixture was filtered, diluted with H_2O (30 mL), adjusted to pH 3-4 with 6N HCl and extracted with ethyl acetate (3x50 mL). The combined organic phases were washed with saturated aqueous NaCl (2x50 mL), dried over $MgSO_4$, and evaporated. The resultant residue was recrystallized from MeOH-H_2O to provide a white solid (3.3 g, 82%). mp 90°C, $[\alpha]_D$ = 2.8 (c= 1, MeOH), R_f (CMA-I)= 0.60. NMR ($CD_3S(O)CD_3$) 7.1-7.8 (m, Fmoc), 7.1-7.5 (m, benzyl aromatic), 5.04 (s, benzyl CH_2), 4.0-4.5 (m, Lys, Asp -CH; Fmoc, CH-CH_2), 3.0-3.2 (m, Lys ε-CH_2), 2.5-2.8 (m, Asp CH_2), 1.38 (s, t-bu), 1.1-1.9 (m, Lys β, γ, δ -CH_2). Anal. Calcd. for $C_{37}H_{43}N_3O_9$(Mol. wt. 673): C, 65.96; H, 6.43; N, 6.24. Found: C, 65.63; H, 6.40; N, 6.25.

Boc-Val-Lys(Z)-Asp(t-Bu)-Gly-OCH_2-Nbb-resin

$BrCH_2$-Nbb-resin (3.57 g, 3.2 mmol of Br) was suspended in DMA (30 mL), cesium Boc-glycinate (prepared from 700 mg (4 mmol) of Boc-glycine and cesium carbonate) was added and the mixture was magnetically stirred for 24 h at 25°C. After this time, the resin was filtered and carefully washed with DMF (4x20 mL), CH_2Cl_2 (4x20 mL) and MeOH (4x20 mL). The loading was 0.63 mmol Gly/g of resin calculated, after removal of the Boc, by acid picric titration, that represents an incorporation yield of 74%.

Boc-Gly-OCH_2-Nbb-resin (2.73 g, 1.70 mmol of Gly) were subjected to step 1-5 of regular program of synthesis, then Fmoc-Lys-(Z)-Asp(t-Bu)-OH (2.20 g, 3.2 mmol) and HOBt (0.43 g, 3.2 mmol) in DMA (9 mL) were added. The mixture was chilled to 0°C and, after 10 min, DCC (0.66 g, 3.2 mmol) in DMA (3 mL) was added. After 2 h at 0°C and 16 h at 25°C the resin was filtered and washed (steps 9-10 of the synthetic program). The valine residue was incorporated as follows: Deprotection of Fmoc group was achieved with piperidine-DMF (2:8) (1x8 min + 1x2 min), after which the peptide-resin was washed with DMF (3x2 min), CH_2Cl_2 (4x2 min), iPrOH (2x2 min), DMF (3x2 min) and CH_2Cl_2 (3x2 min). Boc-Val-OH (1.20 g, 5.4 mmol) in CH_2Cl_2 was added to the resin and after stirring for 10 min, DCC (0.56 g, 2.7 mmol) in CH_2Cl_2 (total volume 20 mL) was added to effect the coupling (60 min). Final washes with CH_2Cl_2 (4x2 min) and MeOH (4x2 min) followed by ninhydrin test, which was negative, completed the cycle. At the end of the synthesis the weight of dry peptide-resin was 3.10 g. Picric acid titration after deprotection gave 0.48 mmol/g resin. Amino acid analysis gave $Asp_{0.99}$, $Gly_{1.36}$, $Lys_{1.00}$, $Val_{0.94}$.

Boc-Val-Lys(Z)-Asp(t-Bu)-Gly-OH

The photolysis was carried out in 300-400 mg batches up a total amount of 1.53 g (0.74 mmol); the general procedure already described provided 0.53 mmol (72%) of crude peptide, which presented the following amino acid analysis $Asp_{1.00}$, $Gly_{1.11}$, $Val_{0.98}$, $Lys_{1.00}$.

This material was dissolved in DMF (2.8 mL) and purified on an Ultrasphere C_{18} column eluting with DMF/CH_3CN/H_2O/propionic acid 25/39/36/0.1 at 3 mL/min. 100

µL fractions (26.6 µmol) were injected at each run. 0.22 mmol of pure peptide were obtained (41% yield) after evaporation, addition of H_2O and lyophilization. mp 158°C, $[\alpha]_D$= -18.0 (c= 1, DMF), R_f (CMA-I) = 0.63. Amino acid analysis: $Asp_{1.00}$, $Gly_{1.02}$, $Lys_{1.00}$, $Val_{0.96}$.

Boc-Glu(Bzl)-Ser(Bzl)-Gly-OCH_2-Nbb-resin

Br-CH_2-Nbb-resin (3.19 g, 2.55 mmol Br) was suspended in DMF (25 mL), cesium Boc-glycinate (prepared from 470 mg (2.68 mmol) of Boc-GlyOH and cesium carbonate) in DMF (5 mL) was added, and the mixture was magnetically stirred for 18 h at 25°C. After this time, the resin was filtered and washed with DMF (3x25 mL), DMF/H_2O (9:1) (4x25 mL), CH_2Cl_2 (4x30 mL), iPrOH (3x20 mL) and MeOH (1x20 mL). Picric acid titration, after deprotection of the Boc group gave a loading of 0.68 mmol NH_2/g, that represents an incorporation yield of 85%.

The assembling of the remaining amino acids was carried out with 2.5 g (1.70 mmol) of Boc-Gly-OCH_2-resin using the programs described before. In both cases, the ninhydrin test was negative. At the end of the synthesis 3.20 g of dry peptide-resin were obtained. Picric acid titration, after deprotection of the Boc, gave 0.52 mmol NH_2/g resin. A portion was hydrolyzed and subjected to amino acid analysis: $Ser_{0.29}$, $Glu_{1.01}$, $Gly_{0.99}$.

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

The photolytic cleavage of Boc-Glu(Bzl)-Ser(Bzl)-GlyOCH_2-Nbb-resin (1.5 g, 0.75 mmol) in 200-500 mg batches provided 0.46 mmol (59%) of crude protected peptide, with the following amino acid analysis: $Ser_{0.87}$, $Glu_{1.04}$, $Gly_{0.96}$.

Part of this material (68 mg, 0.10 mmol) was treated with hexane (8 mL), sonicated for 1 min, and filtered. The solid residue was dissolved in DMF (2 mL) and purified on an Ultrasphere C_{18} column, eluting with CH_3CN/DMF/H_2O/propionic acid (44:20:36:0.5). 100 µL (5 µmol) were injected at each run. 73 µmol of pure peptide were obtained (73% overall purification yield). $[\alpha]_D$= -1.4 (c=1.7, DMF), R_f (CMA-II) 0.35, R_f (BAH) 0.75. Amino acid analysis: $Ser_{0.82}$, $Glu_{0.95}$, $Gly_{1.05}$.

Boc-Asp(Bzl)-His(Tos)-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly-OCH_2-Nbb-resin

Cesium Boc-glycinate (1.03 g, 3.48 mmol) previously prepared was added to a suspension of Nbb-resin (3.52 g, 2.56 mmol of Br) in DMF (30 mL) and was stirred for 14 h at 50°C. After this time, the resin was washed as before and the picric acid titration, after treatment with 30% TFA-CH_2Cl_2, gave 0.52 mmol NH_2/g. This value corresponds to a 76% incorporation yield.

Boc-Lys(Z)-OH, Boc-Arg(Tos)-OH, Boc-His(Tos)-OH, and Boc-Asp(Bzl)-OH were incorporated following the general solid-pase program already described. For the incorporation of Boc-Thr(Bzl)-OH, the Suzuki et al.'s procedure was used. The ninhydrin test after the coupling of Boc-His(Tos)-OH was slightly positive, and an acetylation was performed.

Once chain assembling of protected peptide-resin (6.01 g, compared to theoretical weight of 6.19 g) was finished, a portion was hydrolyzed and subjected to amino acid analysis: $Asp_{1.81}$, $Thr_{0.70}$, $Gly_{1.12}$, $Val_{0.91}$, $His_{0.93}$, $Lys_{0.95}$, $Arg_{1.00}$.

Boc-Asp(Bzl)-His-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly-OH

Boc-peptide-OCH_2-Nbb-resin (5 g, 0.92 mmol) was photolyzed in different batches (700-900 mg) to provide 0.48 mmol (53% yield) of crude protected peptide. Part of this material (0.12 mmol) was dissolved in MeOH-CH_2Cl_2 (1:9) (20 mL), HOBt (162 mg, 1.2 mmol) in MeOH (2 mL) was added, and the mixture was magnetically stirred overnight at 25°C and finally evaporated to dryness.

The residue (360 mg, 0.12 mmol of peptide) was washed with diethyl ether (3x15 mL). The solid residue was then dissolved in DMF (1 mL) and precipitated by addition of water at 0°C. The white precipitate was recovered by centrifugation, dissolved in $CHCl_3$-MeOH (8:2) (40 mL), dried over Na_2SO_4, and evaporated to dryness. The resulting solid (0.10 mmol, 83%) was chromatographed on a Sephadex LH-20 column (1x150 cm) eluted with $CHCl_3$-MeOH (8:2) at a flow rate of 22 mL/h and U.V. monitored at 206 nm. Peptide fractions were pooled and evaporated to dryness (70 µmol, 70%). Finally the product was further purified on a semipreparative Silica Gel column, with $CHCl_3$-MeOH-H_2O (72:28:1) (3 mol were injected at each run). 40 µmol (57% yield) of purified peptide were recovered and characterized. $[\alpha]_D$= -21.9 (c= 0.4, $CHCl_3$-MeOH (8:2)); mp. 175-177°C, R_f (CMN) 0.66, R_f (CMA-III) 0.51; Amino acid analysis: $Asp_{0.98}$, $Thr_{1.03}$, $Gly_{1.06}$, $Val_{1.01}$, $His_{0.94}$, $Lys_{1.02}$, $Arg_{1.00}$.

The remaining crude peptide was purified by the same procedure and 0.16 mmol

were obtained. No attempt was made to optimize recovery of peptide.

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