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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS—III

SYNTHESIS OF THE 44–52 PROTECTED SEGMENT OF THE TOXIN II OF *ANDROCTONUS AUSTRALIS HECTOR*

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Abstract—The synthesis of the fully protected peptide

Boc–Asn–Ala–Cys(Acm)–Tyr(cHex)–Cys(Acm)–Tyr(cHex)–Lys(Z)–Leu–Pro–OH

which corresponds to the 44–52 sequence of the *Androctonus australis Hector* toxin II is described. The synthesis has been carried out on a bromomethyl–Nbb–resin which provides a photolabile anchorage of the peptide. The experimental conditions of the photolysis reaction are very critical in order to obtain satisfactory cleavage yields. The influence of the solvent in this reaction is discussed taking into account the swelling properties of different solvent mixtures and the formation of coloured side-reaction products. Finally, dimethylformamide/water precipitation followed by reverse phase HPLC is shown to be a very convenient approach to the purification of fully protected peptides.

The toxic components of scorpion venoms are small basic proteins of about 7000–8000 daltons[1] which slow down the sodium channel inactivation process on excitable membranes of nervous cells.[2] One of the toxins that has been characterized through chemical, immunological and biological studies is the toxin II isolated from venom of the north African scorpion *Androctonus australis Hector* (AaH–toxin II). It is a 64 residue long polypeptide cross-linked by four disulfide bridges,[3,4] well recognized by a rather specific and sensitive radioimmunoassay[5] and whose binding on sodium channels is easily measurable by a radioreceptor assay.[6] Due to its structural complexity this molecule appears to be an appropriate target compound to test convergent solid phase synthesis.[7,8]

We wish to report in this paper the solid phase synthesis of the fully protected nonapeptide

Boc–Asn–Ala–Cys(Acm)–Tyr(cHex)

–Cys(Acm)–Tyr(cHex)–Lys(Z)–Leu–Pro–OH

which corresponds to the 44–52 sequence of AaH–toxin II. The solid support is an Nbb–resin[8] which, on a polystyrene core, provides a photolabile anchorage of the peptide. N^{α}–Boc temporary protection is used for the amino acids. The side chains are protected by HF labile groups except for cysteine. Acetamido (Acm) protection of the thiol group has been chosen due to the good results obtained when this protector was applied in the apamine synthesis.[9] The use of the benzyloxycarbonyl (Z) group to protect the α-amino side chain is justified in spite of its incomplete stability toward trifluoroacetic acid due to the limited number of these treatments needed. The entire *Androctonus australis Hector* toxin II molecule contains seven tyrosine residues therefore attention must be paid to the correct choice of protecting group. The problem of protection of tyrosine is not completely solved. Variable amounts of C-alkyl rearranged products have been reported after the final HF treatment. Nevertheless use of *o*-cyclohexyl has been reported to minimize the above rearrangement reaction.[10,11]

Low yields have been reported in some instances for the photolytical cleavage of peptides from *o*-nitrobenzyl polymeric supports.[12,13] Therefore, we have tried to establish in a systematic way the relationship between yields and experimental conditions in this photochemical reaction.

The convergent synthesis approach would not make sense without the possibility of obtaining highly pure protected peptide segments. Attempts have been made to isolate in the best conditions the protected nonapeptide from some by-products which arose from two main side reactions that occurred at specific steps during the building up of the segment.

RESULTS AND DISCUSSION

Synthesis of Boc – Asn – Ala – Cys(Acm) – Tyr(cHex) – Cys(Acm) – Tyr(cHex) – Lys(Z) – Leu – Pro – oxymethyl –Nbb–resin

The title nonapeptide was assembled on a bromomethyl–Nbb–resin[8] that offers a photolabile anchorage compatible with the use of HF labile side chain protection and TFA labile α-amino temporary protection. The initial Boc–Pro–oxymethyl–Nbb–resin was obtained by the cesium salt procedure.[14] The

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Table 1. Influence of the solvent in the photocleavage yield

Solvent	*Yield(%)*[a]
dichloromethane	21
N,N-dimethylformamide (DMF)	28
2,2,2-trifluoroethanol (TFE)	26
methanol	8
20% TFE/dichloromethane	45
20% methanol/dichloromethane	35
20% TFE/DMF	11
20% methanol/DMF	12

[a]Calculated by hydrolysis and amino acid analysis after 9.5 hr of simultaneous irradiation on screw cap tubes. Better yields were obtained when samples of peptide-resin were irradiated alone in a three-necked reaction vessel (see experimental part).

remaining amino acids were coupled following the protocol described in the Experimental. In order to prevent the formation of the cyclic dipeptide and hexapeptide, Boc–Lys(Z)–OH and Boc–Cys(Acm)–OH were incorporated using the method described by Suzuki *et al.*[15] The attachment of Boc–Asn–OH was carried out by previously forming the active ester with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide.[16] The extention of the coupling reactions was systematically monitored by the ninhydrin test[17] except for the attachment of Boc–Leu–OH to H–Pro–OCH_2–Nbb–resin that was controlled by the chloranil test.[18] When, after repeating the coupling reaction, the ninhydrin test was still slightly positive the free amino groups were acetylated. A third coupling reaction was needed for the incorporation of Boc–Asn–OH. The amine content of the polymer was also checked at several stages of the synthesis by picric acid titration.[19] After the incorporation of the last amino acid the peptide–resin gave the following amino acid analysis after acid hydrolysis: $Pro_{1.11}$, $Leu_{1.00}$, $Lys_{0.71}$, $Tyr_{0.44}$, $Cys_{0.74}$, $Ala_{0.78}$, $Asp_{0.76}$. The low values of Cys and Tyr were imputed to incomplete conversion to cystine and to alkylation, respectively, during the acid hydrolysis. These side reactions appear to be particularly important when Cys(Acm) and Tyr(cHex) are both present in the peptide to be hydrolyzed. Better values for tyrosine could be obtained when hydrolysis was performed in the presence of phenol. This procedure has been applied to the purified nonasegment (Experimental and Table 3).

Photolysis of Boc–peptide–oxymethyl–Nbb–resin

o-Nitrobenzyl derivatives have been successfully utilized as photosensitive protecting groups on peptide,[20] glycoside[21] and nucleotide[22] syntheses. The use of *o*-nitrobenzyl esters attached to a resin for the synthesis of protected peptides was introduced by Rich and Gurwara.[23] The results obtained by these authors have motivated the study of new resins which contain the *o*-nitrobenzyl group.[8,13,24–27] The photolyses of these resins were carried out in all cases by irradiation with light of wavelength longer than 320–350 nm in an inert atmosphere (N_2, Ar). There is a wide variation in the reported yields (90,[26] 50–60,[24,25] 40,[13] 24%[12]) which has been attributed to the nature of the peptide as well as to some experimental conditions such as solvent/resin ratio, or the nature of the solvent.

We have utilized the Boc–nonapeptide–OCH_2–Nbb–resin as a model to study the influence of experimental conditions on the yields of photolysis using monochromatic light of 350 nm. The solvent used to suspend the resin during the photolysis plays an important role. Preliminary experiments suggested that 20% 2,2,2-trifluoroethanol (TFE)/CH_2Cl_2 was the most suitable but we have also explored the use of CH_2Cl_2, N,N-dimethylformamide (DMF), methanol (MeOH), TFE and binary mixtures of these solvents. The photolysis yields (Table 1) seem to be related to the swelling of the peptide–resin (8% in MeOH and 28% in DMF) and the best results were obtained with 20% TFE/CH_2Cl_2. In order to check the influence of the swelling of the resin we have measured the diameter of the peptide–resin and polystyrene beads in different solvents by direct microscopic examination. As is shown in Table 2 the swelling of polystyrene beads decreased progressively when MeOH and TFE were added to CH_2Cl_2 or DMF whereas the peptide–resin beads presented a maximum of their swelling in the region of 10–20% MeOH or TFE. These maxima are due to the contribution of the peptide part to the swelling of the beads. This effect is very important in the case of 20% TFE/CH_2Cl_2 and it could be attributed to the formation of hydrogen bonds between trifluoroethanol and amide groups. The different swelling behaviour of peptide–resin beads in front of polystyrene can be qualitatively appreciated in Fig. 1.

While the photolysis of 115 mg of Boc–nonapeptide–OCH_2–Nbb–resin in 20% TFE/CH_2Cl_2 gave a yield of 76%, an eight-fold increase in the quantity of peptide–resin resulted on a reduction of the photolysis yield to one half of the previous value. The colour of the resin that initially is yellowish becomes gradually red upon irradiation and the intensity of the colour seems to follow the course of the photolysis reaction. This effect can be attributed to the formation of azo compounds,[20] secondary photoproducts of the resulting *o*-nitrosobenzaldehyde (Fig. 2). The absence of colour in the resin when DMF was used as solvent, can be explained by the presence of dimethylamine produced by photochemical decomposition of DMF. This amine could act as a scavenger of *o*-nitrosobenzaldehyde but could also induce undesired side reactions. The azo

Table 2. Average diameter of poly(styrene-*co*-1%-divinylbenzene) and peptide–OCH_2–Nbb–resin beads after swelling in several solvents. Values are expressed in micrometers ± standard deviation (more than 50 measurements were done for each solvent). The ratio between the diameter of the swollen and dry beads is given in parentheses

	A = CH_2Cl_2 B = TFE		A = CH_2Cl_2 B = MeOH		A = DMF B = TFE		A = DMF B = MeOH	
Protected nonapeptide–OCH_2–resin:								
100 % A	127 ± 24	(1.8)	127 ± 24	(1.8)	116 ± 19	(1.6)	116 ± 19	(1.6)
95 % A + 5 % B	133 ± 36	(1.9)	117 ± 32	(1.7)	109 ± 25	(1.5)	103 ± 16	(1.5)
90 % A + 10 % B	161 ± 20	(2.3)	121 ± 36	(1.7)	113 ± 26	(1.6)	114 ± 23	(1.6)
80 % A + 20 % B	165 ± 26	(2.3)	111 ± 16	(1.6)	109 ± 20	(1.5)	113 ± 28	(1.6)
50 % A + 50 % B	151 ± 46	(2.1)	91 ± 18	(1.3)	61 ± 13	(0.9)	84 ± 17	(1.2)
100 % B	71 ± 15	(1.0)	74 ± 16	(1.0)	71 ± 15	(1.0)	74 ± 16	(1.0)
Poly(styrene-co-1%-divinylbenzene):								
100 % A	106 ± 18	(1.8)	106 ± 18	(1.8)	91 ± 20	(1.6)	91 ± 20	(1.6)
80 % A + 20 % B	78 ± 17	(1.3)	81 ± 18	(1.4)	65 ± 14	(1.1)	69 ± 16	(1.2)
100 % B	49 ± 9	(0.8)	50 ± 9	(0.8)	49 ± 9	(0.8)	50 ± 9	(0.8)

compounds formed during the photolysis can filter UV light, decreasing the reaction rate especially for the photolysis of large amounts of resin. We have determined by actinometry with uranyl oxalate that 300 mg of previously irradiated resin reduced the available amount of light to one third. Grinding the resin in a ball-mill before the photolysis reaction, produces a partial solubilization of the peptide–resin and could provide a solution to this problem. Photolysis of the ground resin suffers from the problem that an additional purification step with Sephadex LH-20 is needed to separate the protected peptide from the unreacted "solubilized" resin. This fact combined with the small increment observed in the photolysis yield

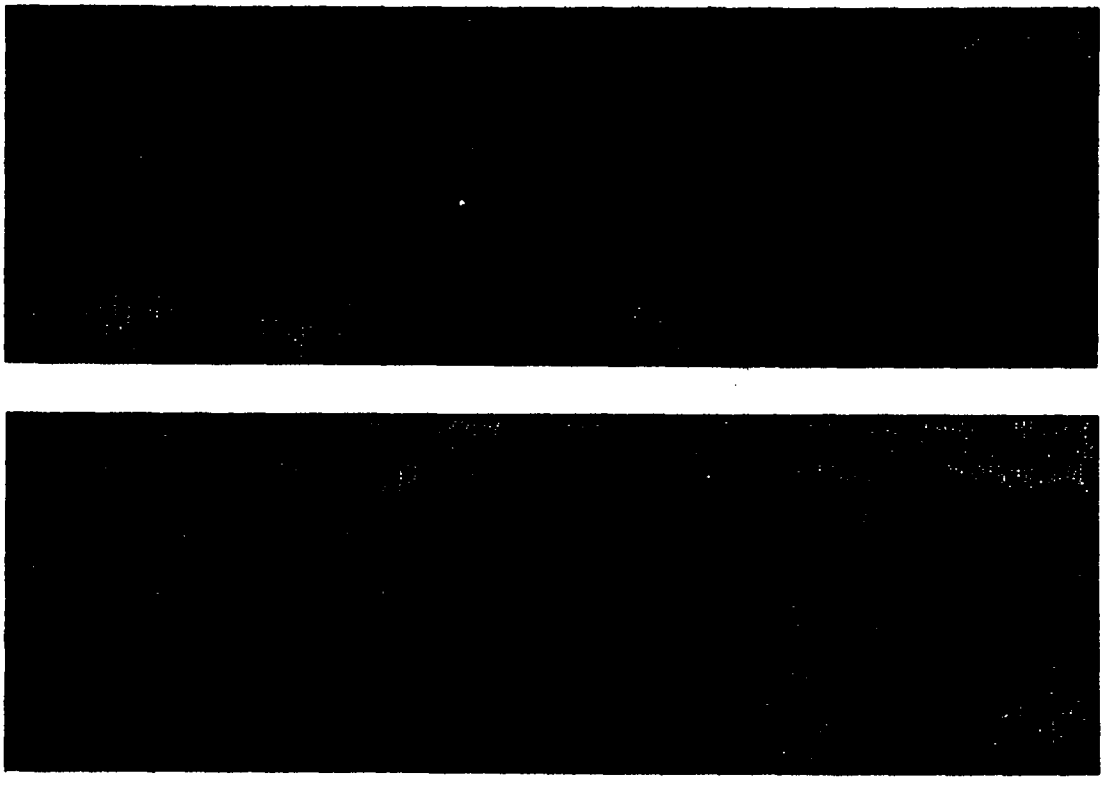

Fig. 1. Micrographs of poly(styrene-*co*-1%-divinylbenzene) and peptide–OCH_2–Nbb–resin beads after swelling in different solvents.

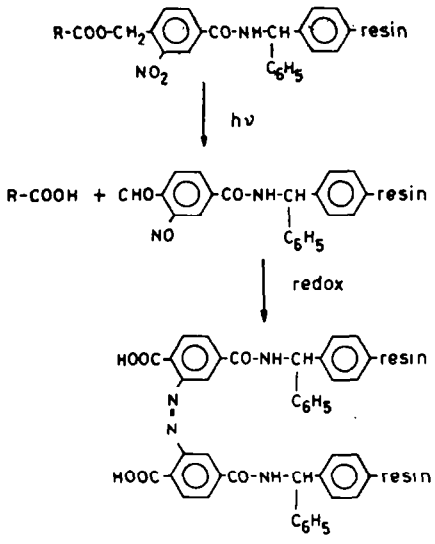

Fig. 2. Formation of resin-bound azo compounds during the photolysis of the peptide–resin bond.

makes this procedure inadvisable. Photolysis of a solid solution of the Boc–nonapeptide–resin in KBr gave the peptide in poor yield (23%).

Photolysis of large quantities of peptide–resin was carried out in 20% TFE/CH_2Cl_2 in *ca* 500 mg batches. Sonication of the suspended resin before photolysis increased the yield slightly. Following the general procedure described in the Experimental we obtained an average yield of 45%.

Purification of Boc – Asn – Ala – Cys(Acm) – Tyr(cHex) – Cys(Acm) – Tyr(cHex) – Lys(Z) – Leu – Pro – OH

We first planned a purification scheme based on molecular filtration followed by preparative HPLC on silica columns. Chromatography of the photolysis crudes on both Sephadex LH-20 and Sephadex LH-60 columns eluted with CH_2Cl_2/MeOH gave three overlapping broad peaks. The analysis of the different fractions by analytical HPLC showed that the three peaks contained several components. Using a Sephadex LH-20 column, the desired peptide was eluted in the first fractions and a small sample containing rather pure peptide could be obtained. The remaining fractions containing the desired peptide were rechromatographed on a HIBAR silica gel semipreparative column. A typical chromatogram is shown in Fig. 3. The product in fractions II_D corresponded to the protected nonapeptide and presented a major peak on analytical HPLC with two minor impurities on both peak flanks. The main disadvantage of this purification is the low recovery of chromatographed product probably due to the poor solubility of the protected nonapeptide in the organic solvents used.

In the light of these results we have considered a new purification scheme based on solvent extractions, DMF/water precipitation and reverse phase HPLC eluted with DMF, a solvent in which the peptide is very soluble. The solubility characteristics of the protected peptide have been utilized to remove selectively non-peptidic impurities and some shorter peptides. The photolysis crude was treated with diethyl ether and acetone. The ethereal extract (8.8% of the initial weight) was not peptidic. The acetone extract (10% in weight) contained mainly truncated sequences. The residue that was not soluble in any of the previous solvents was dissolved in DMF and was precipitated with a four-fold excess of water (69%). An aliquot of the DMF/H_2O precipitate was chromatographed on a Sephadex LH-20 column eluted with DMF giving a single peak that showed the same analytical chromatogram as the DMF/H_2O precipitate. This material along with the rest of the precipitate was further chromatographed on an Ultrasphere (C_{18}) semipreparative column eluted with DMF/H_2O/AcOH (87:13:1).[28] In such optimized conditions, the product of the major peak was isolated and fully characterized as the desired sequence (see Experimental). The overall purification yield was 28%. The good chromatographic separation obtained allowed the isolation of the major components of the photolysis crude. The characterization of these products was achieved by amino acid analysis ^{1}H- and ^{19}F-NMR. The structures are presented in Fig. 4. The presence of these compounds can be explained as a result of two side reactions produced during the

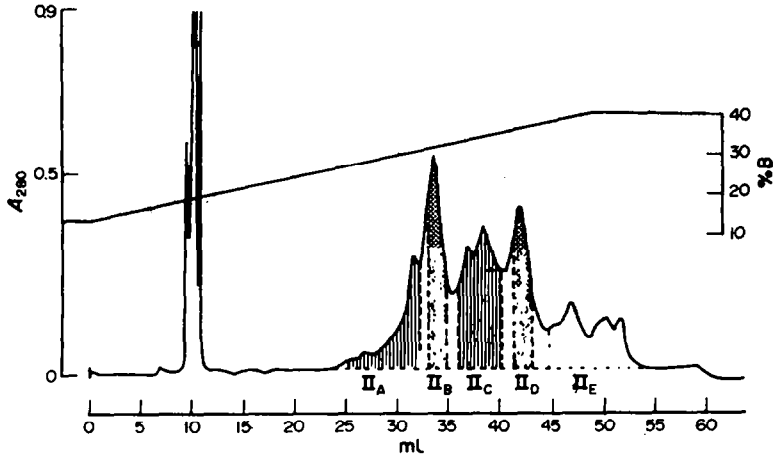

Fig. 3. Adsorption chromatography of the protected nonapeptide on a Hibar (Merck) (1 × 25 cm) column filled with LiChrosorb Si60 silica gel (7 μm) and eluted with a chloroform (A) 3% water/MeOH (B) gradient.

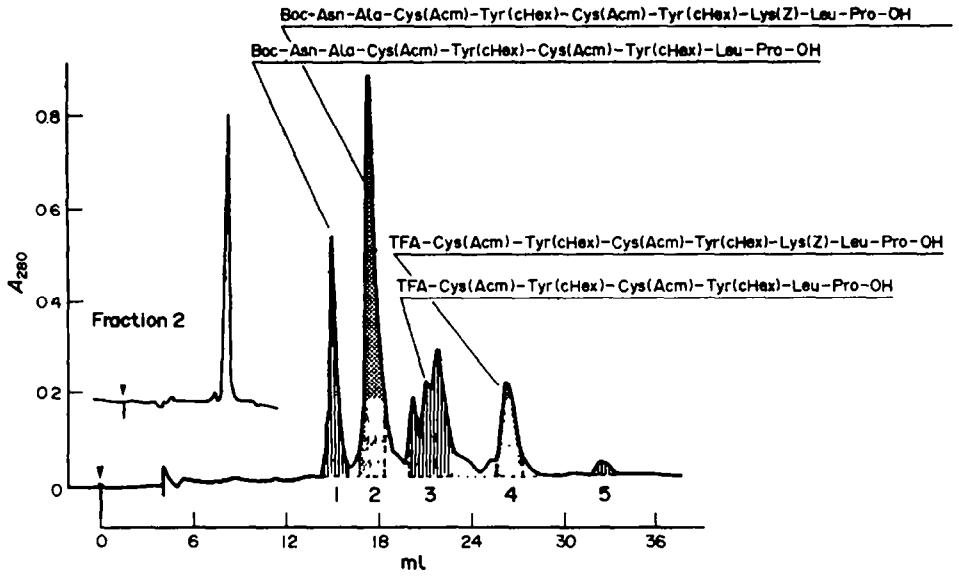

Fig. 4. Reverse phase chromatography of the protected nonapeptide on an Ultrasphere (C_{18}) semipreparative column (elution conditions in the Experimental) and analytical HPLC of fraction 2 (insert) which corresponds to the target peptide.

synthetic process: the incomplete deprotection of the Boc–amino protecting group of the second amino acid by 4 N HCl/dioxane, used in the coupling method of Suzuki *et al.*, and the trifluoroacetylation of the Cys(Acm) at position 7. The blockage of the growing peptide chains by trifluoroacetylation during the solid phase synthesis of peptides has been described[29] but the high level of trifluoroacetylation in practically only one synthetic step found in our synthesis is hard to explain by any of the reported mechanisms. ^{19}F-NMR[30,31] is from our point of view the analytical technique of choice to study this secondary reaction.

EXPERIMENTAL

Abbreviations: Acm, acetamidomethyl; AcOH, acetic acid; Ac_2O, acetic anhydride; Boc–, t-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DIEA, ethyldiisopropylamine; DMF, N,N-dimethylformamide; EtOH, ethanol; cHex, cyclohexyl; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; Nbb–, nitrobenzamidobenzyl; NMF, N-methylmorpholine; –resin, poly(styrene-*co*-1%-divinylbenzene); TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Z, benzyloxycarbonyl.

CH_2Cl_2 was dried over $CaCl_2$ and distilled over K_2CO_3 immediately before use. DMF was stored over 4 Å molecular sieve and freed by amines by N_2 bubbling until negative 1-fluoro-2,4-dinitrobenzene test.[32] The protected amino acids were from Protein Research Foundation (Osaka, Japan) and Beckman (Palo Alto, U.S.A.) except Boc–Tyr(cHex)–OH · DCHA that was synthesized in our laboratory following the procedure described by Engelhard and Merrifield[10] with minor modifications.[11] Bromomethyl–Nbb–resin was prepared starting from poly(styrene-*co*-1%-divinylbenzene) 200–400 mesh from Bio-Rad Laboratories (Richmond, California), as previously described.[8,28] Trifluoroacetic acid and N-methylmorpholine were distilled before use. Peroxide-free dioxane was used. Sephadex LH-20 and LH-60 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other solvents and chemicals were of reagent grade.

The peptide synthesis was performed with a Beckman model 990 peptide synthesizer. The photochemical cleavage was carried out with two lamps (Eye H 125 BL or Philips HPW 125W) on an apparatus previously described.[8] The light intensity at 350 nm was determined by actinometry with uranyl oxalate and was of 6.8×10^{-5} Einsteins min^{-1}. It should be stressed that this value is highly dependent on the correct choice of the lampshade. Amino acid analyses were done on a Biotronik model LC 7000 or model 6000. Peptides were hydrolyzed with 6 N HCl or with 12 N HCl/phenol/AcOH (2:1:1)[33] in sealed evacuated tubes for 24 hr at 110°. Peptide–resins were hydrolyzed in 12 N HCl/AcOH (1:1) at 110° for 48 hr. ^{1}H- and ^{19}F-NMR spectra were recorded on a Varian XL-200 spectrometer using TFA as an external reference. High-performance liquid chromatography was carried out in a Waters Associates apparatus with two solvent delivery systems (models 6000A and M-45), a model U6K injector, a model 660 solvent programmer and a model 450 variable-wavelength UV detector. TLC was carried out on precoated silica gel 60 (F-254) plates 0.2 mm (Merck) in the following solvent systems: (A) $CHCl_3$/EtOH (9:1); (B) $CHCl_3$/MeOH/AcOH (90:5:5); (C) Me_2CO/AcOH (98:2). Spots were visualized with the hipochlorite/*o*-tolidine reagent. Boc–nonapeptide–resin beads were ground on an agate ball-mill model (Riik, RM-100).

Boc – Asn – Ala – Cys(Acm) – Tyr(cHex) – Cys(Acm) – Tyr(cHex) – Lys(Z) – Leu – Pro – OCH_2 – Nbb – resin

3.63 g of $BrCH_2$–Nbb–resin (0.7 mmol Br/g resin; 2.54 mmol Br) were suspended on 35 ml of DMF together with the equivalent quantity of cesium Boc–prolinate (prepared with 546 mg of Boc–Pro–OH and Cs_2CO_3).[14] After 15 hr of magnetic stirring at 50°, the suspension was filtered and the resin was washed several times with DMF, DMF/H_2O (9:1), DMF, CH_2Cl_2, EtOH, CH_2Cl_2, EtOH, and MeOH. The loading was 0.61 mmol NH_2/g of Boc–Pro–OCH_2–Nbb–resin by picric acid titration,[19] that represents an incorporation yield of 94%.

The remaining amino acids (except the third and the seventh) were assembled using the following procedure: (1) CH_2Cl_2, 4 × 1.5 min; (2) 30% TFA/CH_2Cl_2, 2 × 1.5 min; (3) 30% TFA/CH_2Cl_2, 1 × 30 min; (4) CH_2Cl_2, 9 × 1.5 min; (5) 5% DIEA/CH_2Cl_2, 2 × 1.5 min; (6) 5% DIEA/CH_2Cl_2, 1 × 2 min; (7) CH_2Cl_2, 5 × 1.5 min; (8) Boc–amino acid in CH_2Cl_2, after 2 min add the equivalent amount of DCC in CH_2Cl_2, shake 180 min; (9) CH_2Cl_2, 4 × 1.5 min; (10) 5% DIEA/CH_2Cl_2, 2 × 1.5 min; (11) DMF, 4 × 1.5 min; (12) Boc–amino acid in CH_2Cl_2,

after 2 min add the equivalent amount of DCC in CH_2Cl_2, shake 480 min; (13) CH_2Cl_2, 4 × 1.5 min; (14) DMF, 4 × 1.5 min; (15) CH_2Cl_2, 4 × 1.5 min; (16) EtOH, 4 × 1.5 min. For the incorporation of Boc–Leu–OH, Boc–Ala–OH and Boc–Asn 2.5 equiv of Boc–amino acid were utilized in each coupling reaction, for Boc–Lys(Z)–OH, Boc–Cys(Acm)–OH and Boc–Tyr(cHex)–OH the first coupling reaction was carried out with 2.5 equiv and the second with 1.25 equiv. Boc–Lys(Z)–OH and Boc–Tyr(cHex)–OH were liberated from their salts with H_2SO_4 2 N before use. The coupling solution of Boc–Asn was prepared as follows:[16] 1.36 of Boc–Asn–OH (2.5 equiv) and 0.79 g of HOBt (2.5 equiv) were dissolved in DMF and kept in ice at 0° for 10 min DCC (2.5 equiv) in DMF was added and the mixture was maintained at 0° for 10 min and poured into the reaction vessel. In this case, a third coupling reaction was necessary which was carried out in the same conditions.

The third and the seventh protected amino acids (Boc–Lys(Z)–OH and Boc–Cys(Acm)–OH, respectively) were incorporated following Suzuki *et al.*'s method:[15] (1) CH_2Cl_2, 3 × 1.5 min; (2) dioxane, 3 × 1.5 min; (3) dioxane/HCl 4 N, 2 × 1.5 min; (4) dioxane/HCl 4 N, 1 × 30 min; (5) DMF, 3 × 1.5 min; (6) CH_2Cl_2, 3 × 1.5 min; (7) DCC in CH_2Cl_2, after 2 min add Boc–amino acid N-methylmorpholinium salt, 1 × 150 min; (8) CH_2Cl_2, 8 × 1.5 min; (9) DMF, 4 × 1.5 min; (10) DCC in CH_2Cl_2, after 2 min add Boc–amino acid N-methylmorpholinium salt, 1 × 300 min; (11) CH_2Cl_2, 4 × 1.5 min.

Acetylations were performed after incorporation of Lys(Z) at position 3, Tyr(cHex) at position 4 and Cys(Acm) at position 5 with the following program: (1) CH_2Cl_2, 4 × 1.5 min; (2) 5% DIEA/CH_2Cl_2, 2 × 1.5 min; (3) CH_2Cl_2, 3 × 1.5 min; (4) Ac_2O/DIEA/DMF (1 ml/1.7 ml/15.3 ml), 1 × 60 min; (5) CH_2Cl_2, 4 × 1.5 min.

At the end of the synthesis the weight of dry peptide–resin was 5.96 g. Picric acid titration after deprotection gave 0.32 mmol/g resin. Amino acid analysis gave $Pro_{1.11}$, $Leu_{1.00}$, $Lys_{0.71}$, $Tyr_{0.44}$, $Cys_{0.74}$, $Ala_{0.78}$, $Asp_{0.76}$.

Photolysis of Boc – Asn – Ala – Cys(Acm) – Tyr(cHex) – Cys(Acm) – Tyr(cHex) – Lys(Z) – Leu – Pro – oxymethyl – Nbb – resin

The photolysis was carried out in *ca* 500 mg batches. The Boc–nonapeptide–OCH_2–Nbb–resin was suspended in a three-necked reaction vessel with 100 ml of a soln of 20% TFE/CH_2Cl_2 and the suspension was sonicated for 15 min, at 0°. The reaction vessel was submerged in MeOH refrigerated by a cryostat.[8] The suspension was cooled, purged with Ar and irradiated for 10 hr, keeping the temp inside the vessel below 20°. The resin was then filtered, washed with CH_2Cl_2, CH_2Cl_2/MeOH, and MeOH and the filtrates evaporated to dryness. The hydrolysis and amino acid analysis of an aliquot of the filtrates gave an average cleavage yield of 45% ($Pro_{1.31}$, $Leu_{1.18}$, $Lys_{0.79}$, $Tyr_{0.55}$, $Cys_{0.85}$, $Ala_{0.86}$, $Asp_{0.90}$).

Photolytical assays on peptide–resin (approx 20 mg with 6 ml of solvent) were performed in 10 × 1.2 cm screw cap tubes mounted on a rotatory device which allowed a homogeneous irradiation of all the samples. Before photolysis the suspensions were deaerated by N_2 bubbling and sonicated for 20 min.

The solid-phase photolysis was done with pellets obtained from the compression of 25 mg of peptide–resin plus 50 mg of KBr anhyd. These pellets were placed on a small UV-transparent plastic box at about 3 cm from the light source. After photolysis the resin was separated from the KBr by flotation on CH_2Cl_2.

The photolysis of the agate ball-mill ground peptide–resin was carried out on a screw cap test tube. Ground resin (15 mg) was suspended in 6 ml of 20% TFE/CH_2Cl_2. After 10 hr irradiation, the coloured soln was filtered through Millipore and evaporated. The resulting solid was chromatographed on an LH-60 column (130 × 0.55 cm) with 1:3 MeOH/CH_2Cl_2 (v/v) yielding two different peaks. The fraction that was eluted first contained resin, as seen by ^{1}H-NMR. The second fraction contained the liberated peptide.

Purification of Boc – Asn – Ala – Cys(Acm) – Tyr(cHex) – Cys(Acm) – Tyr(cHex) – Lys(Z) – Leu – Pro – OH

430 mg of photolysis crude (143 μmol of peptidic material) were sonicated with 15 ml of diethyl ether for 5 min. After 30 min at room temp, the solvent was removed and filtered through sintered glass funnel (grade 3). The weight of the residue after evaporation of the ethereal soln was 38 mg (9% of the initial weight). The product insoluble in ether was treated in the same way with 5 ml of acetone. Evaporation of the acetone soln gave a solid weighing 43 mg (10%). The residue that was not soluble

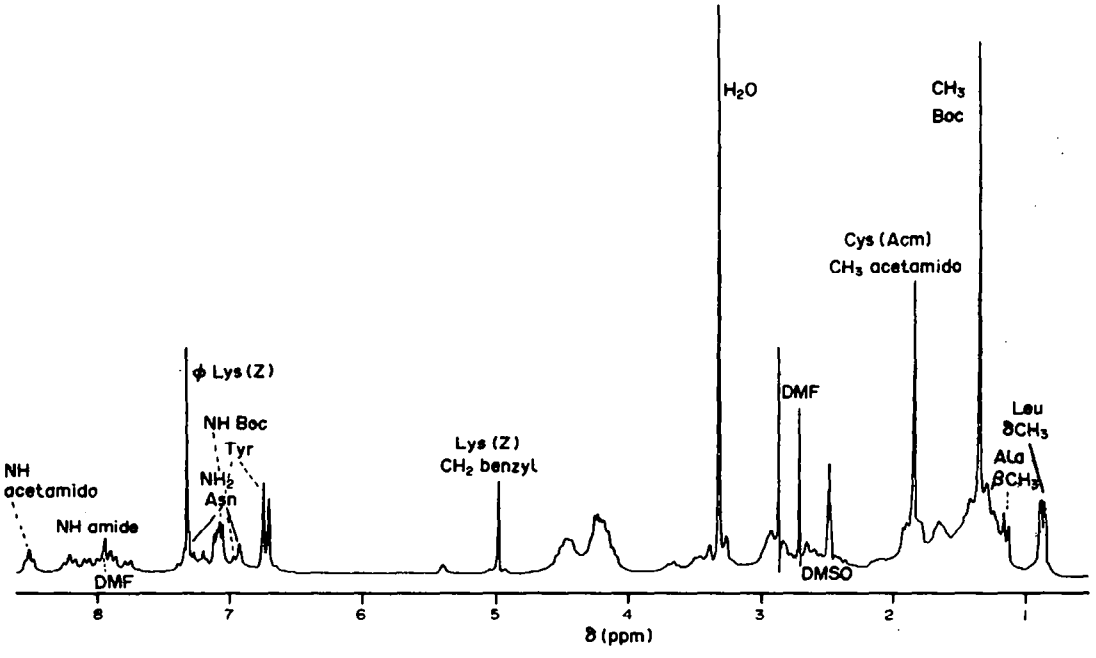

Fig. 5. ^{1}H-NMR spectrum (200 MHz) of Boc–Asn–Ala–Cys(Acm)–Tyr(cHex)–Cys(Acm)–Tyr(cHex)–Lys(Z)–Leu–Pro–OH.

Table 3. Amino acid analyses at different stages of the purification

		solvent treatments					HPLC				
	expected	phot. crude	ethereal solution	acetone solution	DMF/H_2O solution	DMF/H_2O precip.	fr.1	fr. 2	fr.3	fr.4	fr.5
Pro	1	1.00	----	1.28	1.64		1.04	1.03 1.00[&]	1.38	1.07	0.31
Leu	1	1.35	----	1.63	0.99		0.95	0.99 0.93	1.20	1.03	0.33
Lys	1	0.88	----	0.93	0.45		0.16	0.98 0.98	0.66	0.90	1.00
Tyr	2	0.49	----	0.88	0.16		0.43	0.71 1.91	0.85	0.27	0.19
Cys	2	0.79	----	----	----		0.73	1.14 1.00	0.93	0.56	0.12
Ala	1	0.89	----	0.56	0.95		1.05	0.98 1.09	0.90	0.12	0.06
Asn	1	0.88	----	0.60	0.97		0.96	1.02 1.04	0.85	0.11	0.06
μmol		143	0.5	16.6	2.0		11.9	40.1	20.8	12.9	4.1
mg		440	38	43	11	295	33	79	58	40	18

[&] AcOH/phenol/12N HCl (1:1:2)

in acetone was dissolved in 3 ml of DMF and precipitated by adding 12 ml of water yielding 295 mg (69%) of a white solid. This residue was dissolved in 3 ml of DMF and chromatographed on an Ultrasphere ODS column (1 × 25 cm) filled with 5 μm octadecylsilica eluted with DMF/H_2O/AcOH (87:13:1). Fractions containing the protected peptide were pooled and evaporated yielding 40 μmol (overall purification yield 28%). M.p. 216–218° $[\alpha]_D^{20} = -56.1$ ($c = 1$, DMF). Amino acid analysis (phenol/AcOH/12 N HCl (1:1:2)): $Pro_{1.00}$, $Leu_{0.93}$, $Lys_{0.98}$, $Tyr_{1.91}$, $Cys_{1.00}$, $Ala_{1.09}$, $Asp_{1.04}$. Mass spectroscopy (FAB^+): molecular ion $m/e = 1613$. ^{1}H-NMR (200 MHz, Cl_3CD): Fig. 5. TLC: R_f (A) 0.09; R_f (B) 0.17; R_f (C) 0.65. Analytical HPLC: Fig. 4.

The product present in fraction 1 (Fig. 4) was characterized as the protected octapeptide Boc–Asn–Ala–Cys(Acm)–Tyr(cHex)–Cys(Acm)–Tyr(cHex)–Leu–Pro–OH by amino acid analysis (Table 3) and ^{1}H-NMR (lack of benzyl protecting group signals). The product present in fraction 4 was identified as the heptapeptide N–TFA–Cys(Acm)–Tyr(cHex)–Cys(Acm)–Tyr(cHex)–Lys(Z)–Leu–Pro–OH by amino acid analysis (Table 3), ^{1}H-NMR (lack of Ala, Asn, Boc signals, appearance of an NH amide signal at 9.5–9.6 ppm) and ^{19}F-NMR (s, 4.306 ppm) typical of trifluoroacetylamides.[30,31] Fraction 3 contained three different compounds. An analytical quantity of each product was obtained by HPLC using small modifications of the above-mentioned conditions. N–TFA–Cys(Acm)–Tyr(cHex)–Cys(Acm)–Tyr(cHex)–Leu–Pro–OH was identified by ^{1}H- and ^{19}F-NMR (s, 4.34 ppm) as the second component of this fraction. One of the other components has a correct amino acid analysis. The third component lacks lysine but a complete identification of any of these products has not been possible.

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