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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS. II. SYNTHESIS OF THE 1-6 APAMIN PROTECTED SEGMENT ON A NBB-RESIN. SYNTHESIS OF APAMIN

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ABSTRACT - The article deals with the use of the NBB-resin for synthesis of protected segments followed by solid phase segment condensation. Solid phase synthesis on a NBB-resin of the segment 1-6 of apamin yielded either the (1-6) apamin-OH segment after photolysis or (1-6) apamin-$NH-NH_2$ after hydrazinolysis. The two protected segments were purified on Sephadex LH-20 followed by Bio-Beads S-X1 chromatography and respectively coupled onto a resin on which the 7-18 sequence of apamin was assembled stepwise with the standard solid phase procedure. On a portion of the resin, stepwise synthesis was continued to complete apamin. After HF treatment, deprotection of the cysteines, formation of the disulfide bonds and purification, biologically active apamin was obtained in the three cases.

As discussed in the introduction of the preceeding paper (1), several methods have been recently described for the solid phase synthesis of protected segments. Among them, the use of a photolabile bridge between the protected peptide and the resin have been more or less successfully explored by Rich and Gurwara (2), Wang (3), Tam *et al.* (4) and Tjoeng *et al.* (5). Our group has recently described synthesis of a new polymeric support, α-(4-bromomethyl-3-nitrobenzamido)benzylcopoly(styrene-1%-divinylbenzene) or bromomethyl-NBB-resin (6) which allows assembling of peptidic chains while using the most current protection scheme in solid phase peptide synthesis, *i.e.* Boc α-amino protection and HF-labile side chain protection but provides a peptide resin linkage photocleavable under mild conditions. In order to test the feasibility of use of this NBB-resin for synthesis of protected segments, the 1-6 sequence of the bee venom toxin apamin was chosen as model for subsequent solid phase fragment condensation to yield entire apamin, whose covalent structure (7) is as follows :

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1                                          10
H-Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-
  /        \                    18
Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH2
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RESULTS AND DISCUSSION

Synthesis of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH and of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-$NHNH_2$

Synthesis of Boc-Prolyl-OCH_2-NBB-resin

The polymeric support, α-(4-bromomethyl-3-nitrobenzamido) benzylcopoly(styrene-1%-divinylbenzene) ($BrCH_2$—NBB-resin) was synthesized by reaction of the anhydride of 4-bromomethyl-3-nitrobenzoic acid with α-amino benzylcopoly(styrene-1%-divinylbenzene) named also benzhydrylamine resin (Fig. 1). In our previous synthesis of $BrCH_2$-NBB-resin (6a) a direct dicyclohexylcarbodiimide (DCC) mediated coupling of

4-bromomethyl-3-nitrobenzoic acid onto α-a-minobenzyl-resin was performed but careful analysis of the resulting polymer showed presence on the resin of basic sites titrable by picric acid (8). Most probably, these unexpected basic groups arose from the unwanted N-alkylation of the resin. The use of the preformed symetric anhydride enhances the rate of the acylation reaction enough to avoid this secondary reaction.

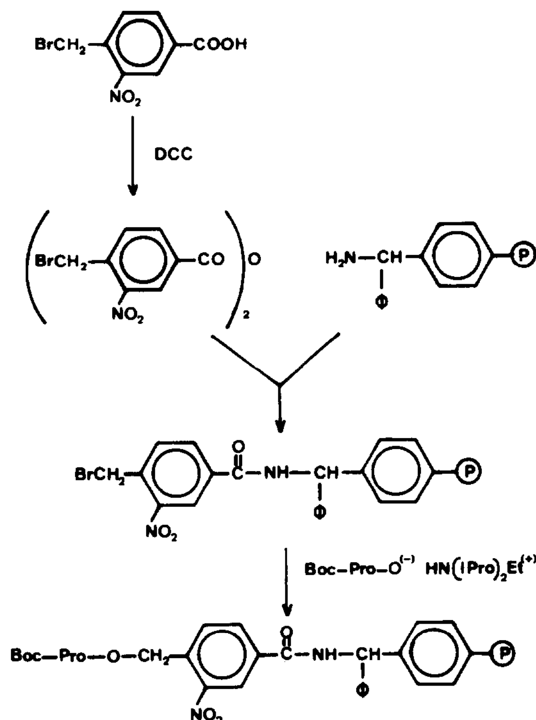

Fig.1. Scheme of the synthesis of Boc- Prolyl-OCH_2-NBB-resin

The C-terminal amino acid N^{α}-t-butoxycarbonylproline (Boc-Pro-OH) was incorporated to the resin by reaction of its diisopropylethylammonium salt in ethyl acetate for 48 hrs. at reflux. The substitution level determined by picric acid titration after deprotection with trifluoroacetic acid was 0.78 mmol/g of Boc-Pro-OCH_2 -NBB-resin. The picric acid titration of the undeprotected Boc-Pro-OCH_2 —NBB-resin afforded an amine content value virtually equal to zero, so excluding the presence of N- alkylated sites on the resin. Similar results were obtained when Boc-Pro-OH was incorporated to the $BrCH_2$ -NBB-resin by reaction of its caesium salt (9) in DMF at 45°C for 18 hrs.

Stepwise synthesis of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OCH_2-NBB-resin

All the N^{α}-amino groups were protected in form of Boc-derivatives. Acetamido (Acm) and benzyloxycarbonyl (Z) groups were used to protect the thiol and ε -amino functions of cysteine and lysine respectively. Asparagine was incorporated through its *p*-nitrophenyl ester (ONp).

The synthetic program followed for the attachment of each amino acid and other details of the synthesis are described in the experimental part. The synthesis was controled by the ninhydrin test (10). In order to prevent the formation of diketopiperazine, the third amino acid, Boc-Lys(Z)-OH, was incorporated by the Suzuki method (11). In these conditions the formation of diketopiperazine was virtually zero as shown by the level of the substitution of the tripeptide on the resin as assessed by the method of Gisin (8). After incorporation of the last cysteine, picric acid titration showed on a portion of the resin treated by TFA, an amine content of 0.73 mmole/g Boc-Pro-OCH_2-NBB-resin which compares well with the original substitution.

Preparation of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH

In our previous work (6a) the photolysis of the peptide-NBB-resin bond was performed by irradiation of the resin suspension with a 125 W HPK Philips high pressure lamp. In order to improve the selectivity of the photochemical treatment, in the present work, the 125 W HPK lamp was substituted by a H 125 BL Eye blacklight mercury lamp whose emission is practically nil below 340 nm. The choice of the solvent to be used during the irradiation has revealed to be very critical. For the 1-6 apamin protected fragment, the best results were achieved using trifluoroethanol-dichloromethane 80:20 with a photolytical yield of 65-70 %.

The crude product after photolysis gave the following amino acid analysis : Asp 0.93, Pro 1.06, Ala 0.97, Lys 1.07. The

protected peptide was purified by chromatography on a Sephadex LH-20 column in methanol followed by a Bio-Beads S-X1 chromatography in dichloromethane/methanol 95:5 to give with a 56 % purification yield a product homogeneous by TLC in two different solvent systems and the following amino acid composition : Asp 0.99, Pro 0.99, Ala 0.99, Lys 1.03.

Preparation of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-$NHNH_2$

The same resin-peptide combination was treated with hydrazine in DMF to yield 88 % of the protected 1-6 apamin hydrazine. Purification on LH-20 in methanol followed by a S-X1 column in methylene chloride and methanol (90:10) permitted recovery of 69 % of homogeneous 1-6 apamin hydrazine as checked by TLC in two systems (yield of purification 69 %). The amino acid analysis gave the following ratios : Asp 0.98, Pro 0.97, Ala 0.98, Lys 1.07.

Synthesis of apamin (Fig. 2).

Stepwise synthesis of (7-18) apamin-aminobenzyl-resin

The C-terminal part of apamin was assembled on a α-aminobenzyl-resin following a synthetic program similar to that used for the synthesis of the N-terminal fragment. All the α-amino groups were protected in form of Boc derivatives, the guanidino and imidazole functions of arginine and histidine as tosyl (Tos) derivatives and the hydroxy and carboxy groups of threonine and glutamic acid as benzyl ether (Bzl) and benzyl ester (Bzl) respectively. The synthesis was controlled by the fluorescamine test. At the end of the synthesis one half of the (7-18) apamin-aminobenzylresin was reserved for the couplings of the protected (1-6) apamin fragments and on the other half the synthesis of apamin was continued in a stepwise manner for comparative purposes.

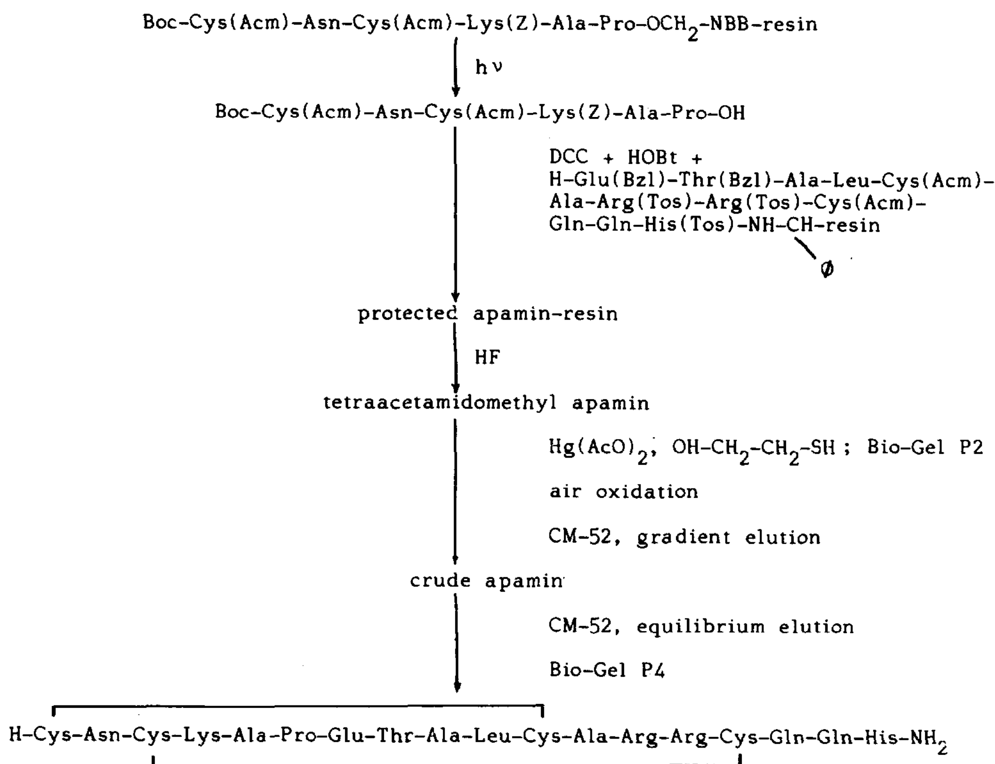

Fig. 2. Scheme of the synthesis of apamin

Coupling of the (1-6) apamin-OH segment onto (7-18) apaminyl-aminobenzyl-resin

The (1-6) apamin-OH segment (1.25 excess) was coupled to the (7-18) apamin-resin by reaction with DCC and hydroxybenzotriazole (HOBt). After the single coupling for 48 hrs, the ninhydrin test was negative and the apamin-aminobenzyl-resin gave an amino acid analysis showing that the coupling reaction has proceeded with a very good yield.

Coupling of the (1-6) apamin-NHNH$_2$ segment

Coupling of the (1-6) apamin-NHNH$_2$ segment onto the (7-18) apamin-resin proceeded through the azide after reaction of the peptide hydrazide with HCl and tBuONO at -20°C in DMF followed by neutralization with diisopropylethylamine (13). After three days the resin was washed with DMF, CH_2Cl_2 and MeOH and amino acid analysis showed a coupling ratio of about 65 %. A second coupling of the peptide did not improve the yield (67 % by amino acid analysis).

Cleavage from the resin, deprotection and oxidation

The protected apamin-aminobenzyl resins were treated the same way. For apamin-aminobenzyl-resin obtained by coupling of the (1-6) apamin-OH segment, results show that treatment with HF/10% anisole for 1 hr at 0°C gave crude tetraacetamidomethyl apamin in 58 % yield. This material was treated with mercury acetate at pH 4 for 70 min and afterwards with mercaptoethanol. The reduced apamin was then desalted by chromatography on a Bio-Gel P2 column and air oxidized in 2 l of a 0.1 M Tris-HCl pH 8.0 buffer solution (peptide concentration = 10^{-5} M) during 48 hrs. It has been shown by amino acid analysis after enzymatic hydrolysis of the reduced apamin that deprotection of the cysteines was complete. After an ionic strength gradient chromatography on a carboxymethylcellulose CM-52 column oxidized apamin was obtained. This product was further purified by chromatography on carboxymethylcellulose at equilibrium followed by a gel filtration chromatography on Bio-Gel P4. Pure apamin was thus obtained with a global deprotection, oxidation and purification yield of 16 %. The product was homogeneous by high voltage paper electrophoresis and gave the following amino acid analysis : Asp 0.96, Thr 0.99, Glu 2.92, Ala 3.01, Leu 1.03, His 0.98, Lys 1.03, Arg 2.04, Pro 1.06, Cys 3.66. Subcutaneous LD_{50} in mouse (50 µg per 20 g of mouse) and intracerebroventricular LD_{50} (9.5 ng per 20 g of mouse) were the same as those measured for natural apamin and for previously synthesized apamin (14). Crude tetraacetamidomethyl apamin coming from the HF treatment of the protected apamin-aminobenzyl-resin prepared by the stepwise synthesis were deprotected, oxidized and purified following exactly the same procedure described above. Pure apamin was also obtained in this case with a global yield slightly inferior (11 %). Amino acid analysis was as follows : Asp 0.98, Thr 1.01, Glu 2.96, Ala 3.08, Leu 1.01, His 0.98, Lys 1.02, Arg 1.95, Pro 1.06, Cys 3.74 and the subcutaneous LD_{50} in mice was 45 µg per 20 g of mouse and the intraventricular LD_{50} 9.3 ng per 20 g of mouse. Pure apamin was also obtained from the protected apamin-aminobenzyl-resin prepared by coupling the (1-6) apamin-NHNH$_2$ segment on the (7-18) apamin-aminobenzyl-resin. Yield of apamin was of 9.8 % and amino acid analysis was as follows : Asp 0.96, Thr 0.96, Glu 3.09, Ala 3.10, Leu 0.98, His 0.93, Lys 0.96, Arg 2.03, Pro 0.97, Cys 2.89. Too little material was obtained to accurately determine the LD_{50} in mice but activity screening showed correct symptomatology of envenomation for the different doses.

Of course, the advantadges for use of the convergent approach to solid phase peptide synthesis can be reflected only in synthesis of peptide sequences longer than that of apamin, but the aim of this work was to test the suitability of this approach. Nevertheless, even for apamin

the global purification yield for the fragment condensation synthesis was slightly higher than that of the stepwise synthesis, due mainly to uncomplete couplings of the amino acids at positions 1 to 3 in that latter synthesis. This was reflected with the amino acid analysis of the protected apamin-resin and the crude tetraacetamidomethyl-apamin (see experimental part).

Use of NBB-resin is surely not the single possibility for solid phase synthesis of protected peptides : interesting results using photolabile anchorings to soluble polymeric supports have been recently described (5) and Tam et al. have proposed an elegant approach that could in principle solve also this problem in a very versatile way (4). Still the NBB-resin fullfilled this requirement in a rather simple way while using a methodology very close to that followed in the standard solid phase synthesis of unprotected peptides.

EXPERIMENTAL PART

Abbreviations not previously defined are as follows : AcOH, acetic acid ; AcOEt, ethyl acetate ; TFE, trifluoroethanol; THF, tetrahydrofuran.

The protected amino acids were from Beckman and Bachem. 4-Bromomethyl-3-nitrobenzoic acid was prepared by bromination and nitration of *p*-toluic acid as previously described (6a). α-amino-benzyl resins from Beckman and own synthesized using the Leuckart reaction (15) were used indistinctly along this work. CH_2Cl_2 was dried over anhydrous K_2CO_3 and distilled over it immediately before used. DMF was dried over 4 Å molecular sieve and freed of amines by nitrogen bubbling until negative 1-fluoro-2,4-dinitrobenzene test (16). Peroxide-free dioxane was used. All other solvents and chemicals were reagent grade. The peptide syntheses were carried out on a Beckman 990 synthesizer. HF reactions were performed in an installation purchased from Toho Kasei Co. (Japan). The lamps used for the photolysis, H 125 BL Eye can be replaced by any other standard black light lamp. Hydrolysates for amino acid analyses from peptide or peptide-resin samples were prepared by treatment with 6 N HCl for 25 hrs or 12 N HCl/AcOH (1:1) for 48 hrs respectively in vacuum evacuated sealed tubes at 110°C. Amino

Step	Reagent	Times	Min
1	CH_2Cl_2	4	1.5
2	30 % TFA/CH_2Cl_2	2	1.5
3	30 % TFA/CH_2Cl_2	1	30
4	CH_2Cl_2	8	1.5
5	5 % DIEA/CH_2Cl_2	2	1.5
6	5 % DIEA/CH_2Cl_2	1	10
7	CH_2Cl_2	5	1.5
8	protected amino acid (4.0 mmol)/CH_2Cl_2	-	10
9	DCC (4.0 mmol)/CH_2Cl_2	1	120
10	CH_2Cl_2	4	1.5
11	5 % DIEA/CH_2Cl_2	2	1.5
12	5 % DIEA/CH_2Cl_2	1	10
13	CH_2Cl_2	4	1.5
14	DMF	4	1.5
15	protected amino acid (4.0 mmol)/CH_2Cl_2	-	10
16	DCC (4.0 mmol)/CH_2Cl_2	1	120
17	CH_2Cl_2	4	1.5

Table 1 . Repetitive procedure for incorporation of amino acid derivatives.

acid analyses were run on a Beckman 120 C autoanalyzer. Thin layer chromatography was carried out on precoated Silica Gel 60 (F-254) plates (Merck). High-voltage paper electrophoreses were performed in pyridine-AcOH-water (25:1:225) pH 6.48 buffer for 2 hrs at 4000 V, using a Savant HV-5000 A power supply.

Bromomethyl-NBB-resin

5.35 g of α-aminobenzyl-resin (1.15 mmol NH_2/g) were treated in a 60 ml polypropylene syringe fitted with a porous polyethylene disc with CH_2Cl_2 , TFA , CH_2Cl_2, DIEA and CH_2Cl_2 as indicated on table 2 for the normal synthetic program (steps 1 to 7). 4.0 g of 4-bromomethyl-3-nitrobenzoic acid were dissolved in 40 ml of CH_2Cl_2 and cooled to 4°C. 3.45 g of DCC dissolved in 5 ml of CH_2Cl_2 and cooled to 4°C were then added. After standing for 1 hr at 4°C the resulting solution was filtered and added to the α-aminobenzyl-resin. After 2 hrs with eventual stirring, the normal synthetic program (table 1) was pursued from step 10 to 13.
At this stage the ninhydrin test of an aliquote of the resin was negative and the picric acid spectrophotometric titration of another resin aliquot gave an amine content value of less than 0.01 mmol/g. Even so the resin was acetylated by treatment with 0.09 ml of AcOH in 40 ml of CH_2Cl_2 for 10 min plus 320 mg DCC in 5 ml of CH_2Cl_2 for 90 min followed by washing with : 4 x CH_2Cl_2 1.5 min ; 4 x DMF 1.5 min ; 4 x CH_2Cl_2 1.5 min ; 30 % TFA/CH_2Cl_2 15 min ; 4 x CH_2Cl_2 1.5 min and 2 x EtOH for 1.5 min.

Boc-Pro-OCH$_2$-NBB-resin

2 g of bromomethyl-NBB-resin were refluxed with magnetic stirring during 63 hrs with 1.7 g of Boc-Pro-OH (6.6 mmol i.e. 3.6 excess over the amine content of the original α-aminobenzyl-resin), 1.0 g of DIEA (6.6 mmol) and 25 ml of AcOEt. After thoroughly washing with AcOEt, MeOH, CH_2Cl_2 and MeOH the picric acid titration of a resin aliquot gave less than 0.01 mmol/g of basic sites before Boc-deprotection and 0.78 mmol/g Boc-Pro-OCH$_2$-NBB-resin after 30 min deprotection with 30 % TFA/CH_2Cl_2. This value, when compared with the amine content of the original aminobenzyl-resin, taking account of the increased weight of the resin, represents an incorporation yield of 98 %.

Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OCH$_2$-NBB-resin

The synthesis was carried out on 2 g of Boc-Pro-OCH$_2$-NBB-resin. Boc-Ala-OH and Boc-Cys(Acm)-OH were incorporated following the general program described in table 1. For the incorporation of Boc-Lys(Z)-OH, the Boc-Ala-Pro-OCH$_2$-NBB-resin was treated with : 1) 4 x CH_2Cl_2 1.5 min; 2) 4 x dioxane 1.5 min ; 3) 4N HCl/dioxane 1.5 min ; 4) 4N HCl/dioxane 30 min 5) 5 x dioxane 1.5 min ; 6) 4 x DMF 1.5 min ; 7) 4 x CH_2Cl_2 1.5 min ; 8) DCC (4 mmol)/CH_2Cl_2 10 min ; 9) Boc-Lys(Z)-OH N-methylmorpholine salt (4 mmol)/CH_2Cl_2 300 min ; 10) 4 x CH_2Cl_2 1.5 min ; repetition of steps 6 to 10. Boc-Asn-ONp was incorporated following the general program of table 1 but changing steps 8 and 9 by : 5 x DMF 1.5 min ; Boc-Asn-ONp (8 mmol)/DMF 240 min ; 4 x DMF 1.5 min ; and steps 15 and 16 by Boc-Asn-ONp (7.2 mmol)/DMF 600 min ; 4 x DMF 1.5 min.
The completion of the couplings was controled by the ninhydrin test. Only after the incorporation of Cys(Acm) at position 3 the test was positive and the resin was acetylated with Ac_2O (1.6 mmol) and DIEA (1.6 mmol). Acetylation was also performed after incorporation of Ala at position 5 and Lys (Z) at position 4 in order to block eventual secondary amines or hydroxyl groups undetectable by the ninhydrin test. At the end of the synthesis the resin was washed with DMF,

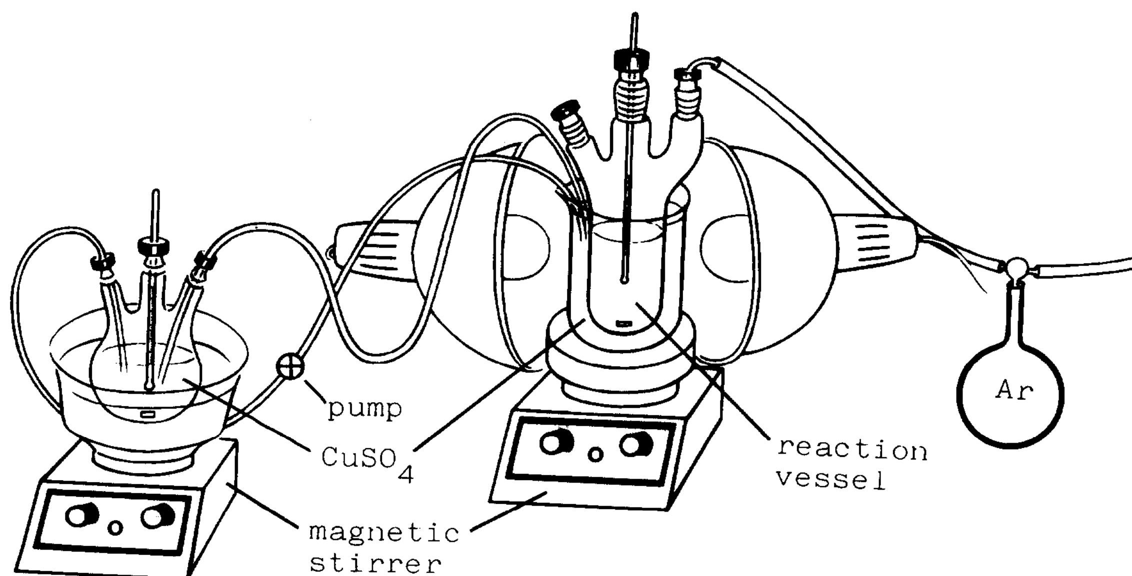

Fig.3. Assembling of apparatus for the photochemical cleavage reaction.

EtOH and CH_2Cl_2 and dried to give 3.05 g of peptide resin. Picric acid titration after deprotection gave 0.46 mmol/g, which is equivalent to 0.73 mmol/g Boc-Pro-OCH_2-NBB-resin when corrected to the weight gain of the resin. Amino acid analysis gave Asp 0.92, Pro 1.09, Ala 1.20, Lys 0.79.

Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH

The following general procedure was used for the photochemical cleavage : the peptide resin was magnetically stirred in 100 ml of solvent in a three-necked reaction vessel externally cooled with a 40 % $CuSO_4$ solution (Fig. 3). Oxygen was removed at 0°C by alternated connection to the vacuum and argon lines for about 30 min. Afterwards the two H 125 BL Eye black light mercury lamps were lighted on and attention was paid to keep the temperature inside the vessel below 20°C. In order to check the course of the reaction, samples were withdrawn through the central neck, fitted with a rubber septum.
Three solvents have been tested on small aliquots for this photochemical reaction : CH_2Cl_2 gave a yield of cleavage of 6 % ; MeOH a yield of 29 % and 20 % TFE in CH_2Cl_2 allowed a yield of near 77 %.
When 711 mg of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OCH_2-NBB-resin were irradiated for 9 hrs in 100 ml of TFE/CH_2Cl_2 1:4, 230 mg of crude protected peptide were obtained (Asp 0.93, Pro 1.06, Ala 0.94, Lys 1.07), yield 70 %. In a second experiment 744 mg of the same resin were treated the same way and 244 mg of protected peptide were recovered in solution (yield 65 %). The 230 mg of crude peptide obtained after the first photochemical cleavage were purified in the following way : 115 mg were chromatographied on a LH-20 column (1x100 cm) eluted with MeOH (5 ml/h) (Fig. 4a).
The amino acid analysis of the different fractions showed that the large peak centered at 92 ml consisted mainly of non-peptide material but that the peak centered at 50 ml gave a correct amino acid analysis. A half of this peak was further purified on a Bio-Beads S-X1 (1x100 cm) chromatographic column (Fig. 4b) eluted with CH_2Cl_2/MeOH 95:5 (22 ml/h). The main peak (eluted at 33 ml) was collected and gave the following amino acid analysis : Asp 0.99, Pro 0.99, Ala 0.99, Lys 1.03 $|\alpha|_D$ = -0.87°(c = 1.1, MeOH). TLC : Rf 0.61 ($CHCl_3$/MeOH/H_2O 27:70:5) and Rf 0.19 ($CHCl_3$/MeOH/AcOH 70:20:5). The remaining of the crude peptide was then purified by this procedure (in total two LH-20 followed by four S-X1 chromatographies) and 129 mg of pure Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH were thus obtained as determined by amino acid analysis. The overall purification yield was 56 %.

Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-$NHNH_2$

527 mg of protected (1-6) apamin NBB-resin (243 µmoles of peptide) were suspended in 5 ml of DMF and 0.5 ml of NH_2NH_2-H_2O (40 x excess) were added. After 71 hrs of magnetic stirring at 20°C the resin was washed with DMF, DMF in methanol (1:1) ; methylene chloride in methanol (1:1), and methanol. 212 µmoles of protected peptide were thus recovered as estimated by amino acid analysis (Asp 0.98, Pro 1.09, Ala 0.98, Lys 0.94) which means a yield of 88 %. Purification on Sephadex LH-20 in MeOH followed by Bio-Beads S-X1 in CH_2Cl_2/MeOH (90:10) yielded homogeneous protected (1-6) apamin-hydrazide (purification yield 69 %). The amino acid composition after acid hydrolysis is Asp 0.98, Pro 0.97, Ala 0.98, Lys 1.07.
$|\alpha|_D^{25}$ =-58.7 (c = 0.89 MeOH) TLC: Rf 0.76 (CH_2Cl_2/MeOH/H_2O 27:70:5).

(7-18) apamin-aminobenzyl-resin

The synthesis was carried out on 5 g of benzhydrylamine resin Beckman (0.25 mmol NH_2/g) following the program described in table 1 using 3.1 mmol of protected amino acids. After the second coupling of each amino acid the resin was checked by the fluorescamine test and a third coupling was carried out if the test was positive. The resin was acetylated after the coupling of Ala^{12}. HOBt (6.2 mmol) was added during the coupling of Boc-Gln-OH in DMF and the repetition of the coupling of some residues. At the end of the synthesis amino acid analysis of the peptidyl resin gave : Thr 0.81, Glu 3.66, Ala 1.92, Leu 0.83, His 0.81, Arg 1.96.

apamin-aminobenzyl-resin

i. by solid phase fragment condensation with protected (1-6) apamin-OH

186 mg of (7-18) apamin-resin were treated in a 10 ml polypropylene syringe fitted with a porous polyethylene disk with : 4 x CH_2Cl_2 1.5 min ; 30 % TFA/CH_2Cl_2 1.5 min ; 30 % TFA/CH_2Cl_2 30 min ; 4 x CH_2Cl_2 1.5 min ; 5 % DIEA/CH_2Cl_2 1.5 min; 5 % DIEA/CH_2Cl_2 10 min and 4 x CH_2Cl_2 2.5 min. 82 mg (81 mmol) of Boc-Cys(Acm)-Asn-Cys (Acm)-Lys(Z)-Ala-Pro-OH dissolved in 1 ml of DMF and 11 mg (81 mmol) of HOBt in 1 ml of DMF were then added to the resin at 0°C. After 10 min 17 mg (81 mmol) of DCC in 1 ml of DMF were added. After 2 hrs at 0°C and 46 hrs at room temperature the resin was washed with 4 x DMF 1.5 min ; 4 x CH_2Cl_2 1.5 min ; 4 x DMF 1.5 min ; 4 x CH_2Cl_2 1.5 min and 2 x EtOH 2.5 min. The ninhydrin test was negative and amino acid composition of the peptide-resin combination (Asp 0.89, Thr 0.50, Ser 0.33, Glu 3.12, Pro 1.02, Ala 3.15, Leu 0.97, His 0.47, Lys 0.78, Arg 1.84) showed that the ratios for the N-terminal amino acids (Asp, Pro, Lys) are very close to those of the C-terminal part of apamin, meaning a good yield of incorporation.

ii. by solid phase fragment condensation with protected (1-6) apamin-NH-NH_2

60 µmoles of Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-NH-NH_2 were dissolved in 0.5 ml of DMF at -20°C to which 0.15 ml HCl/THF (350 µmoles) were added and 0.5 ml tBuONO in DMF (87 µmoles). After 30 min stirring at -20°C the solution was

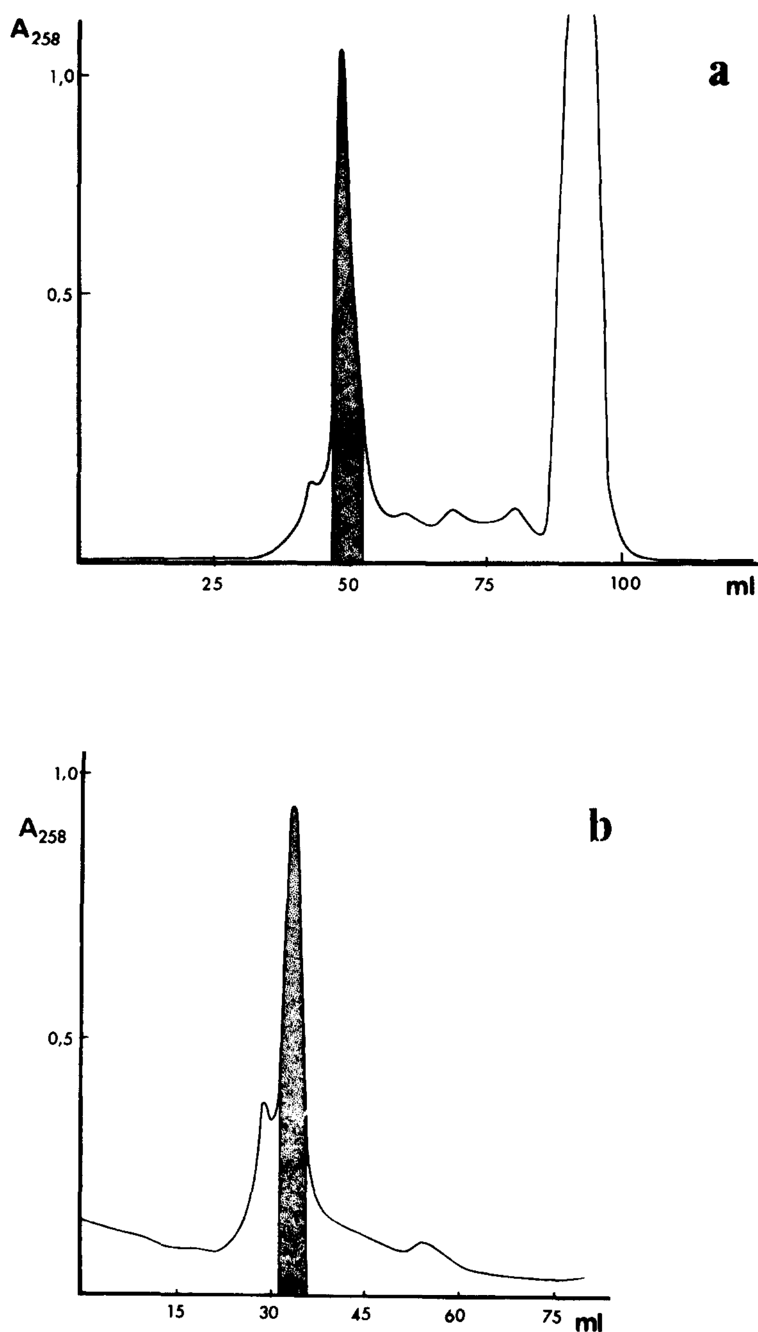

Fig.4. Purification of (1-6) apamin-OH segment. a) Sephadex LH-20 chromatography of crude segment. b) Bio-Beads S-X1 chromatography of half the petide fraction obtained on Sephadex LH-20.

cooled to -25°C and 0.5 ml DIPEA in DMF (360 μ moles) were added to neutralize. The solution was then applied on 143 mg of 7-18 apamin-aminobenzyl-resin (50 μmoles) and 0.2 ml of DIEA in DMF (144 μ moles) were added. The mixture was left 60 min at -20°C, 48 hrs at +2°C and 72 hrs at room temperature. The resin was washed with DMF, CH_2Cl_2, CH_3OH . Amino acid analysis of the peptide-resin combination showed that the N-terminal amino acids were in a ratio of 65 % compared to the C-terminal amino acids. A second coupling in the same conditions did not improve the yield (67 %).

iii. by stepwise synthesis

The protected amino acids corresponding to the (1-6) apamin sequence was successively incorporated to (7-18) apamin-resin following the same procedure described for the synthesis of this peptide resin. Asparagine was incorporated as Boc-Asn-

ONp (3.1 mmol). The resin was acetylated after the coupling of Ala and Asn. At the end of the synthesis amino acid analysis gave Asp 0.66, Thr 0.61, Glu 3.10, Pro 1.14, Ala 3.08, Leu 0.94, His 0.49, Lys 0.62, Arg 1.87.

Cleavage from the resin, disulfide bridging and purification of apamin

The three peptide-resin combinations were treated the same way to yield purified apamin. Typically, taking as example the apamin obtained by fragment condensation with the (1-6) apamin-OH segment, the procedure was as follows. HF treatment in presence of 10 % anisole at 0°C for 1 hr yielded 58 % of crude peptide. To deprotect the cysteines, 68 mg of peptide (in fact 21 μ moles as determined by amino acid analysis) were treated with 204 mg of $Hg(OAc)_2$ in dilute AcOH pH 4 (1.5 ml) for 70 min. Mercaptoethanol was then added to precipitate the mercury : 4.5 ml were necessary to resolubilize the precipitate that forms early in the addition and to obtain a quite clear solution with a neat black precipitate. After overnight stirring the solution was applied on a Bio-Gel P2 column (2,5 x 150 cm) equilibrated in 0.1 M AcOH. UV detection at 230 nm showed the clear separation of the unprotected reduced apamin from the mercaptoethanol and other derivatives. The peak of apamin was poured straight away in 1.8 l of Tris-HCl buffer 0.1 M pH 8.0 with a slow stirring for 48 hrs. As a control, the reduced apamin eluting from the Bio-Gel P2 column has been hydrolyzed by enzymes and amino acid analysis showed no detectable peak at position of Cys(Acm) or Asp meaning that there was complete deprotection of the cysteines. Reoxidation of apamin could be followed by disappearance of SH groups as detected by Ellman's reagent and also by appearance of neurotoxic activity on mice after intraventricular injections of the reoxidation mixture (10 μl at first, down to 1 μl at the end). If the separation of the reduced peptide from mercaptoethanol was excellent, reoxidation occured already at 90 % after 18 hrs. On the reverse if some mercaptoethanol was mixed with the peptide, the reoxidation took much longer time as indicated by slow disappearance of the SH groups and mixed disulfide formed.
After reoxidation, the whole solution was diluted to a conductivity of 4 mS, the pH adjusted to 5.1 and then passed through a column of CM 52 (2,5 x 25 cm) equilibrated with ammonium acetate 4 mS at pH 5.1. The peptide concentrated on top of the column was then eluted by a conductivity gradient of ammonium acetate from 10 to 20 mS and pH 5.1 to 6.3. The peak containing apamin eluted at 16 mS, was lyophilized and chromatographed on a Whatman CM 52 column (1 x 100 cm) equilibrated with ammonium acetate 14 mS pH 6.0. Detection of the peptide was done at 235 nm by UV absorption. After lyophilisation the peptide was desalted on Bio-Gel P4 (2,5 x 150 cm) and characterized by amino acid analysis (Asp 0.96, Thr 0.99, Glu 2.92, Ala 3.01, Leu 1.03, His 0.98, Lys 1.03, Arg 2.04, Pro 1.06, Cys 3.66), high voltage paper electrophoresis and toxic activity in mice subcutaneously (LD_{50} = 50 μg for 20 g mouse) and intraventricularly (LD_{50} = 9.5 ng per 20 g mouse). Total purification yield 16 %.
Crude peptide obtained after HF treatment of apamin synthesized stepwise entirely showed that acetylations that occured in the N-terminal part lowered in an appreciable amount the proportion of complete apamin. This was detected by amino acid analysis of the crude peptide : Asp 0.57, Thr 0.93, Glu 3.02, Pro 0.65, Ala 2.97, Leu 0.93, His 1.01, Lys 0.85, Arg 2.08. Also yield of purification was somewhat lower : 11 %. Purified apamin showed the following amino acid composition : Asp 0.98, Thr 1.01, Glu 2.96, Ala 3.08, Leu 1.01, His 0.98, Lys 1.02, Arg 1.95, Pro 1.06, Cys 3.74 and excellent activity : subcutaneous LD_{50} in mice : 45 μ g for 20 g mouse and intraventricular LD_{50} : 9.3 ng for 20 g mouse.
Crude apamin from fragment condensation with the (1-6) apamin-$NHNH_2$ segment was also purified in a similar manner.

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