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SOLID PHASE SYNTHESIS AND HPLC PURIFICATION OF THE PROTECTED 1-12 SEQUENCE OF APAMIN FOR RAPID SYNTHESIS OF APAMIN ANALOGUES DIFFERING IN THE C-TERMINAL REGION.

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ABSTRACT

Apamin is a bee neurotoxin, active in the central nervous system. It is an 18-peptide whose amino acid residues 13 and 14 play an essential role for binding to its receptor and for displaying toxicity. In order to accelerate the preparation of apamin analogues differing in the C-terminal region, a new strategy was set up involving solid phase synthesis of the 1-12 segment, which, after purification, can be solid phase coupled with different 13-18 sequences. The formula of the 12-membered protected peptide is: Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-Glu(Bzl)-Thr(Bzl)-Ala-Leu-Cys(Acm)-Ala-OH. It has been assembled on the photosensitive resin, α-(4-bromomethyl-3-nitro-benzamido)benzylcopoly(styrene-1%divinylbenzene) by conventional solid phase technique with Boc-amino acids. Boc-Leu was coupled by the method of Suzuki. Photolysis of batches of 1 gram of peptide-resin in trifluoroethanol/methylene chloride (20:80) yielded 89% of cleavage. The procedure for segment purification : organic extractions (ether, chloroform and precipitation from DMF with water), gel filtration on Sephadex LH-60, and finally semi-preparative HPLC on C_{18} in DMF/H_2O (82:18) gave excellent results and an overall purification yield of 55%.

After characterization, the purified 1-12 segment was coupled with three analogous 13-18 apamin sequences assembled on benzhydrylamine resins with yields of 77, 94, and 96%.

After HF cleavage, deprotection and oxidation of the cysteines, the three peptides, apamin, p-aminophenylalanine-13-apamin, and p-aminophenylalanine-14-apamin were purified on carboxymethylcellulose CM-52 and C_{18} HPLC. The purified peptides (yield 14-17%), after chemical characterization, were tested for toxic activity on mice and binding on synaptic membranes. The two analogues were about 100 times less toxic to mice than apamin and about 1000 times less potent in the binding assay.

Apamin is an 18-peptide (Fig. 1)[1,2] isolated from bee venom, whose toxic action takes place in the central nervous system after crossing the blood brain

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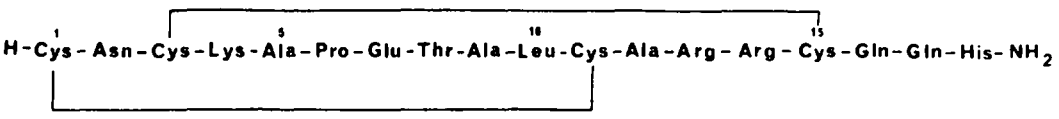

Figure 1 : Covalent structure of apamin[1,2].

barrier[3,4,5]. Recent observations suggested that apamin may be a marker of a calcium dependent potassium channel[6,7]. Structure-activity relationship studies have started either by chemical modification of the native molecule[8,9,10] or by synthesis of analogues[11,12,13]. The crucial sequence for activity appeared to be located at the two arginines in positions 13 and 14 and synthesis of new analogues became desirable for better definition of the interaction between the arginines and the receptor. It was found[11] that it was necessary to have two contiguous positive charges at positions 13 and 14 and prefentially one guanidinium group. Substitution of one of the two arginines by a lysine residue did not alter the biological activity too much (loss of 20 to 35 %), but it was also found that the distance of the positive charge to the peptide backbone was crucial for activity as shown by the noticeable drop of activity due to substitution of arginine by ornithine or by homoarginine. We chose to synthesize two new analogues of apamin, p-aminophenylalanine-13- and p-aminophenylalanine-14-apamin. Both of them have one arginine residue in the region and a second residue that places a primary amine at same distance of the peptide backbone as lysine. Besides the structure-activity relationship study, another advantage of these analogues, as far as they are active, may be the possibility to modify them into their p-azidophenylalanine derivatives which, if they still retain activity, would be photoactivable markers of apamin receptor.

In the past, synthesis of apamin was achieved either by standard stepwise synthesis[14,15,16,17] or by fragment condensation in solution[18,19]. Either way of synthesis for obtaining analogues is a demanding task. Our group has been lately concerned with convergent solid phase synthesis of peptides[20,21] which involves solid phase synthesis of segments and assembling on a resin. The main advantage over the classical fragment condensation method is the overcoming of the solubility problems often encountered after several segment couplings. The experience with solid phase synthesis of segments is still very limited especially for their purification. The work described in the present paper concerns solid phase synthesis with Boc-amino acids on a photosensitive resin of a twelve membered segment with its intensive purification and characterization. We also report solid phase couplings of this segment on three different 13-18 apamin sequences to achieve synthesis of native apamin and of the two analogues, p-amino phenylalanine-13 and-14 apamin.

RESULTS AND DISCUSSION

Assembling of the protected 1-12 sequence of apamin by standard solid phase procedure[22,23] on the photosensitive NBB-resin (Fig. 2) proceeded smoothly (details in Experimental Part). With this resin care had to be taken for coupling of the third amino acid, Boc-Leucine, to avoid formation of diketopiperazine[24].

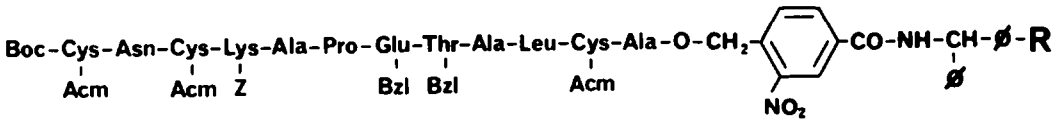

Figure 2 : Covalent structure of the 1-12 apamin segment on the NBB-resin.

The method of Suzuki[25] was very satisfactory as no detectable loss of functionalization was found by the quantitative ninhydrin test[26] and the ratio Leu/Ala given by amino acid analysis of a portion of peptide-resin was very close to 1. Along the assembling all amino acid derivatives could be incorporated to completeness. A few needed a third coupling reaction because the ninhydrin test[27] was slightly positive. The only acetylation that was performed was not for incomplete coupling but rather for security to block eventual secondary amines after coupling of Ala onto Pro. The constant level of substitution along the synthesis indicated that there was no blocking of a detectable amount of chains. The amino acid analysis at different steps of assembling were as expected and the final analysis of the completed sequence on the resin showed only a variation of about 5 % in the ratios of the stable residues (Table I). We used the quantitative ninhydrin test and found that although precision was not as high as one would have liked, due to variations in the absorption coefficient with the different amino acid residues and also due to a slightly high blank, it was however very useful to follow the course of the synthesis either for calculating the number of growing chains or for estimating the amount of non-coupling in case of incompleteness. Rapidity and easiness were appreciated and also the low amount of resin needed (1 to 4 mg).

	Thr*	Glu	Ala	1/2 Cys	Leu	Asp	Pro	Lys
Boc-(1-6) apamin-OCH_2 NBB-resin**	0.72	1.03	1.99	0.59	0.99			
Boc-(1-12) apamin-OCH_2 NBB-resin**	1.11	0.98	3.07	0.83	1.03	0.96	1.02	0.96
Boc-(1-12) apamin-OH***	1.18	1.03	3.00	1.44	0.99	1.00	1.01	0.97

*) The Thr value is uncorrected and includes a degradation product of Cys (Acm).
**) Peptide-resins were hydrolyzed with 12 N HCl-AcOH 1/1 for 44 hrs at 110 °C.
***) The segment was hydrolyzed with 6 N HCl for 20 hrs at 110 °C.

TABLE 1. Amino acid compositions of peptide resin combination at different steps of the assembling of the 1-12 apamin segment, and of the final purified segment.

Photolysis of this 1-12 apamin segment on the NBB-resin gave excellent yields varying from 82 to 99 % with batches of near one gram of peptide-resin. Sonication and the right choice of solvent helped in improving the yields. For this polystyrenic resin a mixture of methylene chloride and trifluoroethanol usually gave the best results[21]. DMF has been tried with no much success and gave an increased level of impurities. Photocleavage yield seems to depend rather on the nature of the first C- terminal amino acid than on the length of the peptide. This 12-membered segment gave good yields with an alanine in the COOH position, other shorter segments (6 a.a., 5 a.a. and 7 a.a.) terminated by Pro[21], Val[28] or Gly (unpublished results) gave lower yields (65, 24 and 57 % respectively). The changes in colour of the resin which became slowly reddish and opaque seem to follow the progress of the cleavage reaction. Kinetics of the photolysis are shown on Fig. 3. The overall cleavage yield for 2.38g of peptide-resin treated in three fractions was of 89 % giving 396 μmoles of crude segment as a yellowish oil.

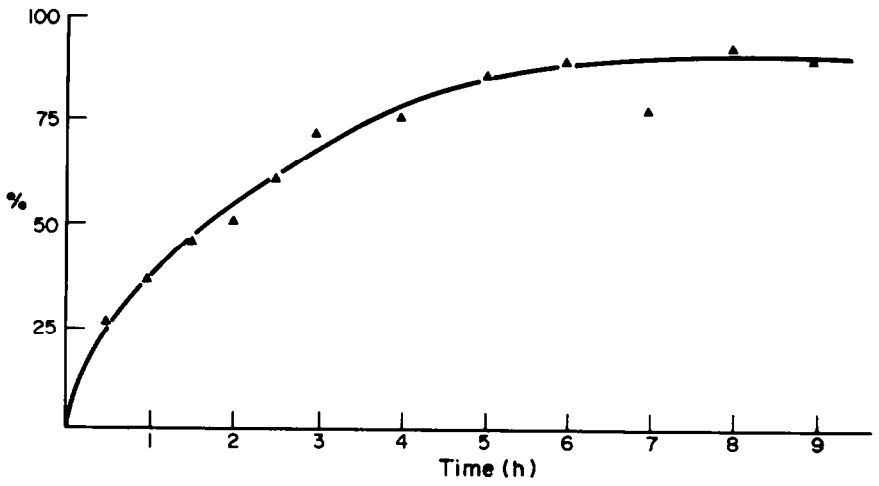

Figure 3 : Kinetics of the photocleavage reaction expressed in percent of protected Boc (1-12) apamin-OH cleaved from 870 mg of peptide resin (see Experimental Part) as a function of time. Quantity of segment liberated was determined by amino acid analysis of an aliquot part.

Purification of protected peptides has always been a subject of research (see [29,30] for example) but the methodology is still not so well established as for free peptides. Nevertheless segments obtained by solid phase procedure need a critical purification and characterization before further use. We found that a three step purification, solvent extractions, gel filtration and reverse phase HPLC gave good results, each step removing specifically some impurities. The first step was treatment by organic solvents, ether and chloroform, which extracted

mainly non peptidic material. The chloroform extract, however, contained some peptides as it showed, after concentration and elution on a Sephadex LH 60 column in DMF, a major peak with the correct amino acid composition and a few shoulders with compositions lacking some amino acids.It seemed that chloroform did extract preferentially some of the shorter peptides. The residue was then solubilized in a minimum of DMF and precipitated with water (overall yield of the extractions over crude peptide : 89%). The second step of purification was a Sephadex LH 60 column run in DMF (Fig. 4). The peptide eluted in the third peak (yield 81 %). The two first peaks did not contain any peptidic material as shown by amino acid analysis. The third purification step was semi-preparative HPLC on a C_{18} column run in DMF/water[31]. The elution pattern is shown on Fig. 5. The main peak was collected and evaporated to dryness (yield 77 %). Characterization of the purified protec ted 1-12 sequence of apamin is described in the Experimental Part. The changes in the physical aspect of the segment seemed to follow the progress of the purification : it was first a yellowish oil, then an almost white film, and finally a clear white crackled substance with a neat melting point. Each purification step gave high yields (77 to 89%) although no attempt was made to optimize recovery of the segment. Our first aim was not so much the quantity than the quality. The overall yield of purified segment (218 µmol) over crude was 55 %. Homogeneity of the segments is, of course, an essential feature when considering their assembling into large peptides. A major improvement has been the use of semi-preparative HPLC.

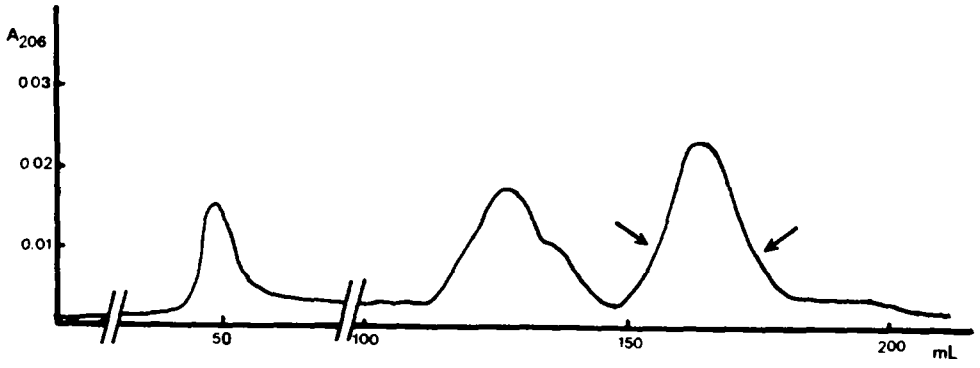

Figure 4 : Chromatography on a Sephadex LH-60 column (1 x 150 cm) in DMF of 115 µ moles of the protected Boc (1-12) apamin-OH obtained after organic extractions (see text). The flow rate was 13 ml/hr and the volume of samples collected 1 ml.

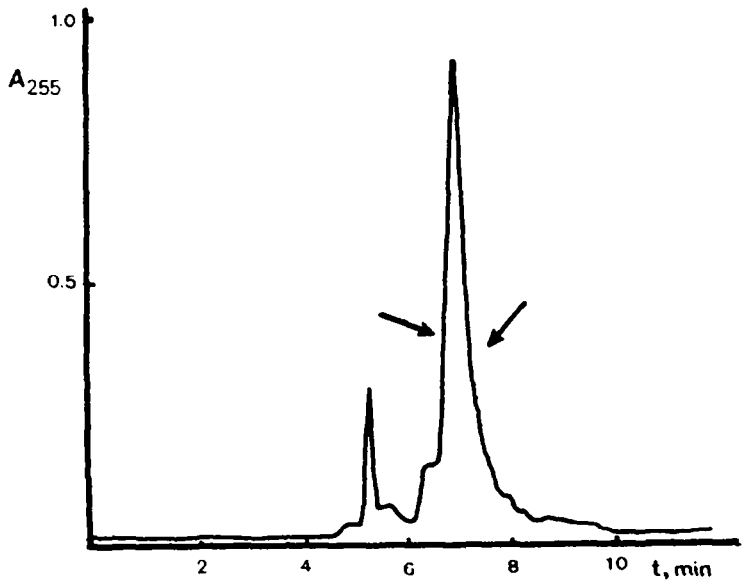

Figure 5 : Semi-preparative HPLC of the fraction of protected Boc (1-12) apamin-OH (5 μmol) obtained after Sephadex LH-60 chromatography (Fig. 4) on an Ultrasphere ODS Altex column (1 x 25 cm) run in DMF/H_2O (82:18) . The flow rate was 1.5 ml/min and the pressure 4000 p.s.i.

On three samples of a benzhydrylamine resin prepared in our laboratory (see Experimental Part), the sequences 13-18 apamin, p-amino-Phe[13] 13-18 apamin and p-amino-Phe[14] 13-18 apamin were built up. On each of the three samples a one step coupling of the 1-12 apamin segment in DMF with DCC and HOBt gave high yields (77, 94 and 96 % respectively). Incorporation was checked by amino acid analysis (Table 2) and quantitative ninhydrin test. The two excellent yields obtained with the analogues were probably due to higher concentration of the coupling mixture. The coupling for native apamin was done with a two fold excess (81 μ moles of segment for 41 μmoles of peptide-resin) in 7 ml DMF (yield 77 %). The couplings for the analogues were done with a 1.3 excess (67 μ moles of segment for 51 μmoles of peptide-resin) in 1.5 ml DMF (yields 94 and 96 %). It is encouraging to note for further studies on the convergent solid phase synthesis of large peptides[20,21] that it is possible to synthesize rapidly a segment of 12 residues long by solid phase procedure, to purify it extensively, and to couple it onto a peptide-resin with such high yields.

The hydrogen fluoride reaction proceeded with higher yields (77, 84 and 90 %) than usually for apamin (in the range of 50-60 %). This might be due to the lower amounts of peptide-resin treated each time (0.2 g instead of 2-3 g). The high values are in better accordance with those obtained by Matsueda and Stewart[32]

	Asp	Thr	Glu	Pro	Ala	1/2 Cys	Leu	His	Lys	Arg	p-NH_2-Phe
Boc-apamin-NH-CH-R* (Ø)	1.19	1.27	3.65	1.02	3.05	1.48	0.96	1.10	0.99	2.59	
apamin**	1.01	1.03	3.07	1.01	2.95	3.30	0.94	0.96	0.99	2.07	
Boc-(p-NH_2Phe)^{14}-apamin-NH-CH-R* (Ø)	1.11	1.30	3.27	1.01	3.06	1.06	0.95	0.66	0.98	1.06	1.00
(p-NH_2Phe)^{14}-apamin**	1.01	1.02	2.94	1.02	2.95	3.25	1.04	0.96	1.03	1.03	1.02
Boc-(p-NH_2Phe)^{13}-apamin-NH-CH-R* (Ø)	1.04	1.45	3.23	1.00	3.06	0.87	0.95	0.71	1.04	1.05	0.98
(p-NH_2Phe)^{13}-apamin**	1.00	0.97	2.97	1.00	2.99	3.38	1.02	0.98	1.03	1.02	1.03

*) Peptide-resins were hydrolyzed with 12 N HCl-AcOH 1/1 for 44 hrs at 110 °C.
**) Peptides were hydrolyzed with 6 N HCl for 20 hrs at 110 °C.

TABLE 2. Amino acid compositions of the three peptide-resin combinations after coupling of the 1-12 apamin segment on the different 13-18 apamin sequences. The values of Asp and Thr are high due to a by-product resulting of Cys(Acm) hydrolysis. Amino acid composition of the purified peptides apamin and the two analogues (p-NH_2-Phe)^{13} apamin and (p-NH_2-Phe)^{14} apamin.

who found that histidine is one of the best leaving amino acids on benzhydrylamine resin.

After deprotection and oxidation of the cysteines, purification of apamin and of the two analogues proceeded as described before[33,21] by two chromatographies on carboxymethylcellulose CM-52, first with a gradient elution, and then in equilibrium conditions. The last step was not a filtration on Bio-Gel P_4 as usually but a HPLC purification on a reverse phase C_{18} column run in ammonium acetate and ethanol. It helped in removing small amounts of by-products (Fig. 6). It was demonstrated earlier that oxidation of reduced apamin and purification led to isolation of the peptide with the correct disulfide pairing[14]. Amino acid ratios of the purified peptides were in excellent agreement with the expected values (Table 2). Total purification yields were in the same range as usually (14-17 % from crude to purified), although the amounts of peptides treated were small (19 to 42 µmol of crude peptide) and no correction was made for all the samples taken for analysis and trials on HPLC. By using the fragment condensation method with a purified 1-12 segment, we were not expecting so much to increase the yields of purification as compared to those obtained by the standard solid phase procedure[33,11], because this method was already found to be very effective in assembling the peptide on the resin with an average incorporation yield of the amino acid derivatives of about 99.3 %[14]. The aim was rather to reduce the number of steps necessary for synthesis of analogues of apamin modified in their C-terminal region and, in the same time, to gain more experience in handling solid phase synthesis of segments.

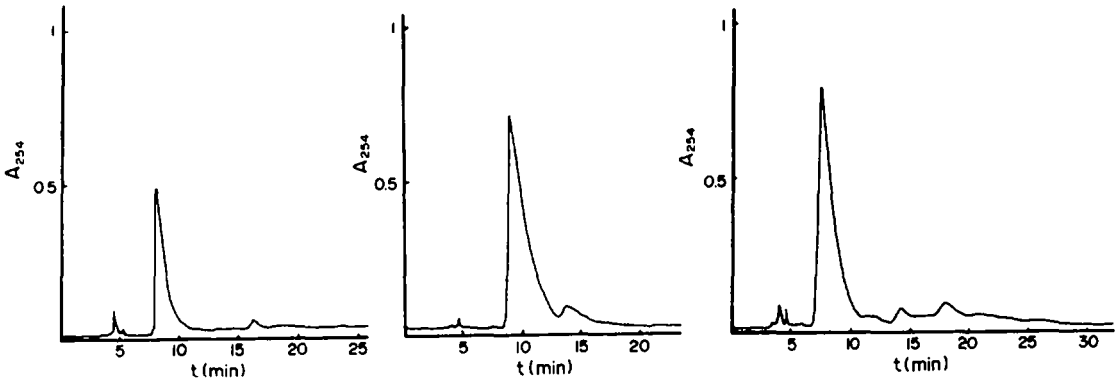

Figure 6 : Semi-preparative HPLC on an Ultrasphere ODS Altex column (1 x 25 cm) of apamin (Fig. 6a) and of the two analogues (p-NH_2-Phe^{14})-apamin (Fig. 6b) and (p-NH_2-Phe^{13})-apamin (Fig. 6c). Elution was with 10 mM ammonium acetate pH 4.5 and 15 % ethanol (Fig. 6a,b) or 23 % ethanol (Fig. 6c). Flow rate was 3 ml/min and pressure 2600 p.s.i. (Fig. 6a,b) and 3000 p.s.i. (Fig. 6c). Sample injections were approximately of 300 nmol.

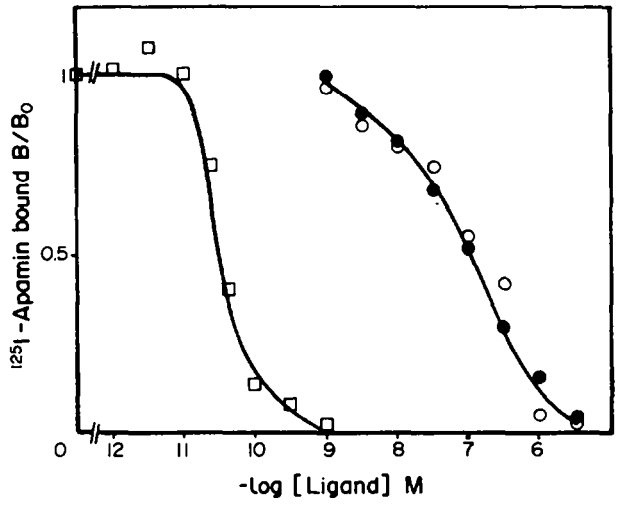

Figure 7 : Displacement curves of labelled apamin by synthetic apamin and the two analogues (p-NH_2-Phe^{13})-apamin and (p-NH_2-Phe^{14})-apamin. Crude synaptic membranes were incubated with 6 pM ^{125}-apamin in the presence or absence of apamin (□), (p-NH_2-Phe^{13})-apamin (●) or (p-NH_2-Phe^{14})-apamin (○). See methods.
B = bound, Bo = bound in the absence of apamin and analogues.

The two analogues of apamin in which one arginine was replaced by a p-aminophenylalanine residue showed low toxicity to mice and also a reduced affinity for apamin receptors in binding experiments (0.1 to 1 % compared to apamin). The pK of the side chain amino group of p-aminophenylalanine (less than 6) is lower than that of lysine, and may explain the drop of activity of the analogues compared to that of Lys^{13}-apamin and Lys^{14}-apamin which possessed still 65 to 80 % toxicity and binding potency[11,12,13]. Still, the toxic symptomatology remained the same and the displacement of radiolabelled apamin was complete at high doses (Fig. 7), showing that these analogues are weak agonists of apamin.

EXPERIMENTAL PART

Amino acid symbols follow mostly IUPAC-IUB recommendations (Eur. J. Biochem. 27, 201, 1972). Abbreviations not previously defined are as follows : DMF, Dimethylformamide ; DCM, Dichloromethane ; DIEA, Diisopropylethylamine ; TFE, Trifluoroethanol; HOBt, 1-Hydroxybenzotriazole ; Acm, Acetamidomethyl ; p.NH_2-Phe, para-aminophenylalanine ; NBB-resin, α-(4-bromomethyl-3-nitrobenzamido) benzyl-copoly(styrene- 1%- divinylbenzene) ; LD_{50}, lethal dose 50 % ; mS, milliSiemens (1 mS = $10^{-3}\ \Omega^{-1}$).

Materials

Copoly-(styrene-1 %-divinylbenzene) (200-400 mesh) was purchased from Bio Rad Laboratories (Richmond, California) and was extensively washed as described previously[23]. The protected amino acids were from Protein Research Foundation (Osaka, Japan) and Bachem (Bubendorf, Schweiz). Their purity was checked by thin-layer chromatography. DCM was dried over anhydrous K_2CO_3 and distilled over it immediately before use[34]. DMF was stored over 4 Å molecular sieves and freed of amines by nitrogen bubbling until negative 1-fluoro-2,4-dinitrobenzene test (less than 0.15 absorbance unit)[35]. Diisopropylethylamine and pyridine were distilled over ninhydrin under vacuum. Nitrobenzene was distilled under vacuum before use. Trifluoroacetic acid was refluxed over Cr_2O_3 overnight and distilled. Peroxide-free dioxane was used. Sephadex LH-60 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Bio-Gel P_2 from Bio Rad Laboratories and CM-52 from Whatman (Springfield Mill., England). HF was from Air Liquide (Paris, France). Water was ion free and distilled over quartz. For use in HPLC chromatography water was passed through a column of C_{18} beads. All other solvents and chemicals were of the best commercial grade.

Methods

The peptide syntheses were performed manually with mechanic stirring in a teflon reaction vessel fitted with a polyethylene fritted disc. The photochemical cleavage was carried out with two H 125 BL Eye lamps on a similar apparatus, with minor differences, to the one described previously[21]. The resin before photolysis was sonicated (7 x 5 min) with an Ultrason-Annemase apparatus. HF reactions were performed in a Toho-Kasei Co installation (Japan).

For amino acid analyses, hydrolysates of peptides were prepared by treatment in sealed vacuum evacuated tubes with 6N HCl for 20 hrs (110 °C) and hydrolysates of peptide-resin samples by treatment with 12N HCl/AcOH (1:1) for 44 hrs (110 °C). For routine checkings the temperature of hydrolysis was raised to 160 °C and the time reduced respectively to 0.5 hr and 1 hr. Analyses were run indistinctly on a Beckman 120C or a Biotronik LC 7000 autoanalyzer.

Thin-layer chromatographies were carried out on precoated Silica Gel 60 (F-254) and HPTLC RP-18 (F-254) plates (Merck). Monitoring of liquid chromatography at 206 nm was carried out on line with a LKB apparatus and at 240 nm on a Jobin Yvon 201 spectrophotometer tube by tube. HPLC was performed on a Waters ALC/GPC 205 U System with a Schoeffel variable wavelength UV monitor. The reverse phase C_{18} column used for both purifications of the protected segment and of the final peptides was a semi-preparative Ultrasphere ODS Altex column (1 x 25 cm, 5 µm) purchased at Beckman-France.

The following measures and spectra were performed at the Departament de Quimica Organica, Facultat de Quimica, Barcelona, Spain: optical rotations on a Perkin Elmer 141, melting point on a Kofler microscope (uncorrected), infrared spectra on a Perkin Elmer 457 spectrometer, and the NMR spectrum of the purified segment on a Varian 200 MHz spectrometer.

Determination of the LD_{50} for mice by intraventricular injection was performed as previously described[3].

The binding assays were performed as follows. Crude synaptic membranes were prepared from rat brains[36]. 25 nmol of apamin were iodinated with 1 mCi $Na^{125}I$ (Amersham) in the presence of 75 nmol iodogen (Pierce), and a pure ^{125}I monoiodoapamin derivative with a specific radioactivity of 2000 Ci/mmol was separated by SP-Sephadex C_{25} chromatography as previously described[37]. The binding medium contained 25 mM Hepes, 5 mMKCl, and 0.25 % bovin serum albumin adjusted to pH 7.2 with Tris base. 6 pM ^{125}I-apamin was incubated in 500 µl buffer with 250 µg of brain membrane protein in the presence or absence of apamin or its analogues. After 60 minutes incubation at 4°C the reaction was stopped by rapid filtration on Millipore EH filters, followed by 3 washes of 1 ml binding buffer, and the filter associated radioactivity was estimated by gamma counting with 60 % efficiency. Membrane protein was assayed by a modified Lowry method[38].

Bromomethyl-NBB-resin

Synthesis of α-aminobenzylcopoly-(styrene-1 %-divinyl-benzene) resin has been described by several authors[39,40,32] and this solid support is commercially

available. Still, quality seemed to vary from one batch to another and very often the resin is yellowish if not brown. Details of the synthesis are described here which allowed us to obtain a white and efficient resin.*[1]

40 g of copoly-(styrene-1 %-divinylbenzene) were suspended in 300 ml of nitrobenzene in a five-necked round bottom flask fitted with a thermometer, a $CaCl_2$ drying tube and a dropping funnel. While stirring the suspension was cooled to 5 °C, 14 ml of benzoylchloride (120 mmoles) were carefully added in 15 min, then 32 g of $AlCl_3$ (240 mmoles) in 30 min and the mixture was stirred for another 2 hrs at 5 °C. The resin was filtered and extensively washed with nitrobenzene, EtOH, EtOH/H_2O (1:1), and DCM. It was dried overnight under vacuum over P_2O_5 and gave 48 g of resin which represents 1.58 meq of ketone per gram of resin. Infrared spectrum showed a strong ketone band at 1660 cm^{-1}. The 48 g of benzoyl-copoly-(styrene-1%-divinylbenzene) were suspended in 300 ml of nitrobenzene and placed in a five-necked round bottom flask fitted with a thermometer, a Dean-Stark trap and a dropping funnel. The suspension was mechanically stirred and 202 g of ammonium formate (3.04 moles), 223 ml of formamide (5.32 moles), 303 ml of 88 % formic acid (7.6 moles) and 1.64 g of magnesium chloride (7.6 mmoles) were added. The mixture was heated and kept at 175 °C for 5 hrs. After the second hour, 60 ml of 88 % formic acid were added every hour. The resin was cooled, filtered and carefully washed with nitrobenzene, EtOH, EtOH/H_2O (1:1), and DCM. Infrared spectrum showed a formyl group absorption at 1680 cm^{-1}. Deformylation was performed by treatment with 450 ml of 12 N HCl/EtOH (2:8) for 2 hrs at 120 °C. Finally the resin was filtered, washed with EtOH until the filtrate gave a negative silver chloride test and dried under vacuum over P_2O_5 to give 47.5 g of white resin. Picric acid titration[41] gave 0.86 meq NH_2/g resin.

Coupling of 4-bromomethyl-3-nitrobenzoic acid (1.74 g, 6.73 mmoles) on 3.13 g of this α-amino benzylcopoly-(styrene-1 %-divinylbenzene) (2.69 meq NH_2) was performed as described before[21] by preformation of the anhydride in DCM with 1.38 g of DCC (6.73 mmoles). After 2 hrs of coupling the ninhydrin test was negative and picric acid titration gave a value of 0.07 meq NH_2/g resin. After acetylation by AcOH (38 µl) and DCC (0.138 g) in DCM, the resin was washed with DCM, DMF, DCM, 30 % TFA/DCM, DCM and MeOH. After drying under vacuum over P_2O_5 there was 3.68 g of bromomethyl-NBB-resin which is in good agreement with the expected weight.

Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-Glu(Bzl)-Thr(Bzl)-Ala-Leu-Cys(Acm)-Ala-OCH_2-NBB-resin

3.39 g of NBB-resin (2.47 meq CH_2Br) were reacted with mechanic stirring during 18 hrs at 50 °C in DMF with 1.53 g of cesium Boc-alaninate (4.96 mmol) previously prepared according to the procedure described by Gisin[42]. After several washings with DMF, DMF/H_2O (7:3), DMF, MeOH, DCM and MeOH the picric acid titration[41] gave 0.005 meq/g of basic sites before Boc-deprotection and 0.60 meq/g Boc-Ala-OCH_2-NBB-resin after 30 min deprotection with 30 % TFA/CH_2Cl_2. This value, corrected for the weight increase of the resin, represents an incorporation yield of 89 % or a total of 2.20 meq of Ala.

Boc-Cys(Acm)-OH, Boc-Ala-OH, Boc-Thr(Bzl)-OH, Boc-Glu(Bzl)-OH, Boc-Pro-OH and Boc-Lys(Z)-OH were incorporated following the standard solid phase program described in Table 3. For the incorporation of Boc-Leu-OH, the Boc-

Step	Reagent	Times	Min
1	DCM	4	1.5
2	30 % TFA/DCM	2	1.5
3	30 % TFA/DCM	1	30
4	DCM	8	1.5
5	5 % DIEA/DCM	2	1.5
6	5 % DIEA/DCM	1	10
7	DCM	5	1.5
8	protected amino acid (5.3 mmol)/DCM	-	10
9	DCC (5.3 mmol)/DCM	1	90
10	DCM	4	1.5
11	5 % DIEA/DCM	2	1.5
12	5 % DIEA/DCM	1	10
13	DCM	4	1.5
14	DMF	4	1.5
15	protected amino acid (5.3 mmol)/DCM	-	10
16	DCC (5.3 mmol)/DCM	1	90
17	DCM	4	1.5

TABLE 3: protocol for solid phase assembling of the segment.

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[1]Synthesis was performed by one us (F. Albericio) at the Department de Quimica Organica, Facultat de Quimica, Universitat de Barcelona, Spain.

Cys(Acm)-Ala-OCH$_2$-NBB-resin was treated with : 1) 4 x CH_2Cl_2 1.5 min ; 2) 4 x dioxane 1.5 min ; 3) 2x 4N HCl/dioxane 1.5 min ; 4) 2 x 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 (2.5 excess)/DCM 10 min ; 9) N-methylmorpholine Boc-leucinate (2.5 excess)/CH_2Cl_2 300 min ; 10) 4 x DCM 1.5 min and repetition of steps 6 to 10. Boc-asparagine was coupled through its hydroxybenzotriazole ester to avoid formation of cyanoalanine. The ester was preformed[43] with Boc-Asn-OH (2.5 excess) and HOBt (2.5 excess) in presence of DCC (2.5 excess) in DMF during 60 min at 0 °C and the N,N'-dicyclohexylurea was filtered before addition to the peptidyl resin. After the incorporation of Cys(Acm)-11, Ala-9, Thr(Bzl), Pro, and Cys(Acm)-1, the ninhydrin test was slightly positive and a third coupling was carried out with Boc-amino acid hydroxybenzotriazole esters in DMF. Acetylation with AcOH (2.5 excess) and DCC (2.5 excess) was performed after incorporation of Ala-5 in order to block eventual secondary amines undetectable by the ninhydrin test. The synthesis was also monitored by quantitative ninhydrin test before the incorporation of Ala-9, Glu(Bzl) and Lys(Z) and there was no detectable decrease of the level of substitution. At the end of the synthesis the resin was washed carefully with DMF, EtOH, and DCM, dried in vacuum over P_2O_5 to give 6.59 g of peptide resin (expected for 2.20 meq of peptide : 6.72 g). Amino acid analysis is shown in Table 1.

Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-Glu(Bzl)-Thr(Bzl)-Ala-Leu-Cys(Acm)-Ala-OH

The peptide resin was sonicated (7 x 5 min) in 100 ml of solvent in a three-necked cylindrical reaction vessel externally cooled with ice. Oxygen was removed at 0°C by alternated connection to vacuum and nitrogen lines for about 1 hr. The photochemical reaction was carried out in the same vessel which was externally refrigerated by cold ethanol flowing through a solution of dry ice in aceto ne[20]. During the irradiation the temperature inside the vessel was kept below 20°C. In order to check the course of the radiation, samples were withdrawn through a neck fitted with a rubber septum.

To find out the best conditions of cleavage two solvents were tested first on 600 mg of peptidyl-resin for 4 hrs : MeOH/DMF (1:5) gave a yield of 44 % and TFE/DCM (1:5) a yield of 77 %. The rest of peptide resin was photocleaved in aliquots of 700-900 mg in this second solvent for 9 hrs with yields varying between 82 % and 99 %. The best yields were obtained with new lamps. After irradiation, the resin was filtered and carefully washed with DCM, DMF, MeOH, DMF and MeOH. The combined filtrates and washings (overall cleavage yield: 89%) were evaporated under reduced pressure and gave an oil with the following amino acid analysis : Asp 0.94, Thr 1.16, Glu 1.00, Ala 3.00, Pro 1.03, Cys 1.29, Leu 1.06, Lys 0.94.

Part of this residue (ca. 370 mg, 160 µmol) was then triturated with diethyl ether (3 x 15 ml) and $CHCl_3$ (3 x 15 ml) and the solvent poured off. The product was then dissolved in 1 ml DMF and precipitated by addition of water at 0°C. The 142 µmol of peptide obtained after the precipitation (yield 89 %) were chromatographied on a Sephadex LH-60 column (1 x 150 cm) eluted with DMF at a flow rate of 13 ml/h and UV monitored at 206 nm (Fig. 4). The amino acid analysis of the different fractions showed that only the third peak contained peptidic material. Fractions corresponding to this peak were pooled and evaporated to

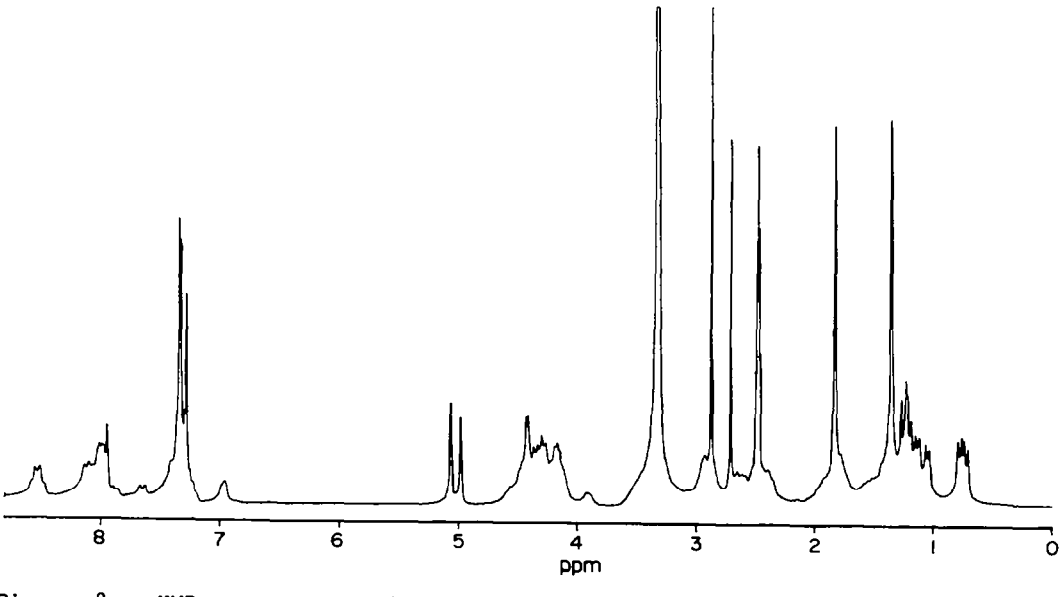

Figure 8 : NMR spectrum at 200 MHz of the protected 1 - 12 segment of apamin dissolved in D_6-DMSO.

dryness (115 µmol, yield 82 %). This product looked homogeneous in two TLC systems. Finally the product was further purified on a semi preparative reverse-phase HPLC. The peptide was eluted with DMF/H_2O (82:18) at a flow rate of 1.5 ml/min, with a pressure of 4000 psi. UV monitoring at 254 nm gave the elution pattern shown on Fig. 5. 88 µmol of the purified protected peptide were recovered (yield 76%) and characterized. Amino acid analysis is shown in Table 1. TLC (silica gel, MeOH) : single spot, Rf : 0.68. HPTLC (RP-18, MeOH/DMF 1:1): single spot, Rf : 0.60. $\alpha\ ^{25}_{D}$ =-35.7 (c, 0.6, DMF). Mp = 150-152 °C. The NMR analysis of the segment dissolved in D_6-DMSO showed a clean spectrum at 200 MHz (Fig. 8). Signals expressed by their chemical shifts in ppm, downfield from tetramethylsilane, have been assigned as follows: two doublets at 0.71 and 0.78 for δCH_3(Leu); three doublets at 1.04, 1.14, and 1.21 for $\beta\ CH_3$(Ala); a doublet at 1.26 for γCH_3(Thr); other assigned signals are singulets, at 1.36 for CH_3(Boc); at 1.86 for CH_3(Acm); at 4.97 for $\underline{CH_2}$Ø(Lys); at 5.05 for $\underline{CH_2}$Ø(Glu); at 7.28 for Ø(Thr); at 7.32 for Ø(Lys); at 7.34 for Ø(Glu).

The remaining of the crude peptide was purified by this procedure and 218 µmol were obtained. The overall yields were as follows : photocleavage 89 %, 3-step purification 55 %. No attempt was made to optimize recovery of peptides.

(13-18) apamin-aminobenzyl resin

The synthesis was carried out on 350 mg of benzhydrylamine resin (0.86 mmol NH_2/g) following the program described in Table 3 using 0.75 mmol of each protected amino acid. The incorporations of Gln-17, Gln-16 and Cys were performed via the DCC-HOBt procedure[43,44]. The synthesis was checked by the quantitative ninhydrin analysis before and after each coupling reaction. A third coupling in presence of HOBt was necessary for Gln-17, Arg-14 and Arg-13. At the end of the synthesis amino acid analysis gave : Glu 2.05, Cys 0.25, His 0.61 and Arg 1.95.

(p-NH_2-Phe^{14}) (13-18) apamin-aminobenzyl resin and (p-NH_2-Phe^{13}) (13-18) apamin-aminobenzyl resin

The synthesis was common up to Cys-15, then two portions were made. The incorporation of the successive amino acids took place on 470 mg of benzhydrylamine resin (0.86 mmol NH_2/g) following the same procedure as described for the (13-18)-apamin- amino-benzyl resin. A third coupling in presence of HOBt was necessary to obtain a negative quantitative ninhydrin test for the Gln-17 and the both Arg. Amino acid analysis gave : Glu 1.97, Cys 0.40, p-NH_2-Phe 1.01, His 0.55 and Arg 0.95 for the p-NH_2-Phe^{14} (13-18) apamin-amino-benzyl resin and Glu 1.97, Cys 0.18, p-NH_2-Phe 1.03, His 0.54 and Arg 0.97 for the p-NH_2-Phe^{13} (13-18)-apamin-amino-benzyl resin. The p-NH_2-Phe eluted at 130 min on the Beckman 120 C amino acid analyzer between Phe (124 min) and His (135 min), on the Biotronik LC 7000 amino acid analyzer it eluted together with His.

Apamin

Steps 1-10 of the protocol described in Table 3 was applied to 207 mg of (13-18) apamin amino-benzyl resin (41 µmol by amino acid analysis) in a 50 ml polypropylene syringe fitted with a porous polyethylene disk. 81 µ mol of Boc-(1-12)-apamin dissolved in 3 ml of DMF and 11 mg of HOBt (81 µmol) dissolved in 2 ml of DMF were then added to the peptidyl resin at 0 °C. After 10 min of magnetic stirring, 17 mg of DCC (81 µmol) in 2 ml of DMF were added and the mixture was stirred for 2 hrs at 0 °C and 46 hrs at room temperature. Finally, the peptidyl-resin was filtered, washed with DMF, DCM, DMF, DCM, MeOH and dried under vacuum over P_2O_5 to give 277 mg of peptide-resin. The ratio between the value of Arg (C-terminal fragment) and that of Ala, Leu and Lys (N-terminal fragment) in the amino acid analysis (Table 2) showed a yield of coupling of 77 %, and the quantitative ninhydrine test gave an effective coupling of 75 %.

187 mg of apamin-resin (25 µmol) were treated with 10 ml of anhydrous HF in presence of 1 ml of anisole at 0 °C with strong magnetic stirring. After 1 hr the mixture was cooled at -70 °C for 30 min with dry-ice in acetone, and the HF removed under vacuum with strong stirring in open air. The residue was washed with ether (3 x 25 ml) and then treated by AcOH (2 x 25 ml) followed by H_2O (5 x 25 ml). After lyophilisation, 80 mg of crude peptide (19.2 µmol, yield 77 %) were recovered.

In order to deprotect the four cysteines, all the lyophilized powder was dissolved in 2 ml of degassed aqueous AcOH pH 4.0 and 640 mg of $Hg(OAc)_2$ (160 µmol) were added. After 70 min of reaction, mercaptoethanol was added until all of the grey precipitate that formed early in the addition solubilized (total amount : 4.5 ml). After overnight stirring the solution was run through a Bio-Gel P_2 column (1.2 x 110 cm) equilibrated in 0.1 M AcOH and monitored at 206 nm. The fraction corresponding to the unprotected and reduced apamin was poured straight into 2 l of Tris-HCl buffer 0.1 M, pH 8.0 for an air oxidation with a slow stirring maintained for 48 hrs. The solution was then diluted to a conductivity of 5 mS with water, and the pH adjusted to 5.1 with acetic acid. It was then passed through a column of CM-52 (2.5 x 25 cm) equilibrated with ammonium acetate 5 mS at pH 5.1. The peptide was eluted with a conductivity gradient of ammonium acetate (buffer 1: 10 mS, pH 5.1 and buffer 2: 25 mS, pH 6.3) with a flow rate of 40 ml/hr, monitored at 240 nm. The fraction containing apamin (6.4 µmol) eluted at 19 mS. After lyophilization it was applied to a CM-52 column (1 x 100 cm) equilibrated with ammonium acetate 18 mS, pH 6.0 at a flow rate of 20 ml/hr with a

detection at 240 nm. Finally the peptide (5.1 µmol) was further purified by reverse-phase HPLC (Fig. 6a). After lyophilisation 3.3 µmol of pure apamin were obtained (total purification yield: 17 %) and characterized by amino acid analysis (Table 2) and toxic activity in mice (LD_{50} of 11.1 ng per 20 g mouse intraventricularly).

(p-NH_2-Phe^{14})-apamin

The coupling reaction was carried out with 160 mg of (p-NH_2-Phe^{14}) (13-18)-apamin-amino-benzyl resin (51 µmol) by amino acid analysis), 67 µmol of Boc-(1-12)-apamin dissolved in 1 ml of DMF, 9.1 mg of HOBt (67 µmol) in 0.25 ml of DMF and 13.8 mg of DCC (67 µmol) in 0.25 ml of DMF also (total coupling volume : 1.5 ml). The protocol was similar to the one described for apamin in a 10 ml polypropylene syringe. After 48 hrs of coupling, the ninhydrin test was slightly positive and the amino acid analysis showed that the reaction took place with a yield of 94 %. The HF treatment of 213 mg (41 µmol) of peptide-resin (10 ml HF, 1 ml anisole, 1 hr, 0°C) led to 34 µmol of crude peptide (yield 84 %). After deprotection of sulfhydryl groups, desalting, oxidation and concentration on CM-52, the peptide was eluted with a molarity gradient of ammonium acetate (same details as those described for apamin). The peptide eluted at a conductivity of 14 mS (13.1 µmol) and was applied on a CM-52 column (1 x 100 cm) equilibrated with ammonium acetate 8.5 mS pH 6.0 before semi-preparative reverse-phase HPLC (Fig. 6b). After lyophilisation 5.1 µmol of pure p-NH_2-Phe^{14} apamin were obtained (total purification yield: 15%). Amino acid analysis is shown in Table 2. This analogue presented an LD_{50} of 612 ng per 20 g mouse intraventricularly (1.6 % of relative activity compared to that of apamin), and a much lower binding affinity (1.3×10^{-7} M) than apamin (4×10^{-11} M) as shown on Figure 7.

(p-NH_2-Phe^{13})-apamin

The coupling reaction was performed exactly in the same conditions as described for the other analogue with 152 mg of (p-NH_2-Phe^{13}) (13-18)-apamin-amino-benzyl resin (49 µmol by amino acid analysis), 67 µmol of Boc-(1-12) apamin, HOBt and DCC dissolved in DMF (total volume 1.5 ml). The ninhydrin test was negative and the yield calculated by amino acid analysis was of 96 %. The HF reaction on 210 mg (46 µmol) of peptide resin gave 42 µmol of crude peptide (yield 90 %). The peptide was treated as described for apamin. It eluted of the CM-52 column at a conductivity of 15.4 mS and, after lyophilisation, was applied to the equilibrium CM-52, but at 10 mS. Finally, the peptide was further purified by reverse-phase HPLC (Fig. 6c). After lyophilisation, 5.8 µmol of pure (p-NH_2-Phe^{13})-apamin were obtained (total purification yield : 14 %) and characterized by amino acid analysis (Table 2). This second analogue presented a toxic activity in mice intraventricularly of 732 ng per 20 g mouse (1.4 % of activity relative to apamin) and the same binding capacity than (p-NH_2-Phe^{14})-apamin (Fig. 7).

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