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Solid-phase Synthesis of ω-Agatoxin IVA, a P-type Calcium Channel Blocker

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Abstract: ω-Agatoxin IVA, isolated from the venom of funnel web spider *Agelenopsis aperta*, blocks potently and selectively P-type calcium channels. This toxin, composed of 48 amino acids and containing 8 cysteine residues, was synthesized by the solid-phase procedure. The Cys residues were protected by acetamidomethyl (Acm) groups which were removed by mercuric acetate. During treatment with mercuric acetate, a by-product was detected, involving modification of tryptophan residues by the Acm groups. This side reaction can be completely prevented by addition of an excess of tryptophan in the reaction medium during Acm deprotection.

The resulting peptide was submitted to an oxidative refolding, in different conditions, in order to determine the most favourable protocol. After formation of the four disulphide bonds, the toxin was purified by successive preparative HPLC, on two different supports, and fully characterized by analytical HPLC, capillary electrophoresis, amino acid analysis, mass spectrometry and Edman degradation. It was found to block the P-type calcium channel with a similar biological potency as described for the natural product.

Keywords: solid-phase synthesis; calcium channel blocker; spider toxins; side reaction; peptide folding

INTRODUCTION

The venom of funnel web spider *Agelenopsis aperta* contains three classes of toxins. The α-agatoxins, a family of low molecular weight acylpolyamines, block the glutamate-sensitive receptor channel complex in insect muscle, inducing a reversible paralysis. The μ and ω-agatoxins are polypeptides which affect presynaptic voltage-sensitive ion channels [1,2]. The μ-agatoxins cause irreversible paralysis in lepidopteran insects and flies, and like scorpion toxins, induce repetitive activity in motor neurons and increase the transmitter release from presynaptic stores at neuromuscular junctions. The ω-agatoxins produce durable suppression of neurotransmitter release from presynaptic stores, they act antagonistically with the voltage-sensitive calcium channel.

It has recently been shown that ω-agatoxin IA (ω-Aga IVA), isolated from the venom of the funnel spider, *A. aperta*, blocks a sensitive P-type voltage-dependent calcium channel [3]. The primary structure analysis showed that ω-Aga IVA contains 48 amino acids including eight cysteine residues: H-Lys-Lys-Lys-Cys-Ile-Ala-Lys-Asp-Tyr-Gly-Arg-Cys-Lys-Trp-Gly-Gly-Thr-Pro-Cys-Cys-Arg-Gly-Arg-Gly-Cys-Ile-Cys-Ser-Ile-Met-Gly-Thr-Asn-Cys-Glu-Cys-Lys-Pro-Arg-Leu-Ile-Met-Glu-Gly-Leu-Gly-Leu-Ala-OH. Recently, the synthesis of this toxin was described by Nishio *et al.* [4,5] using a solution procedure. A solid-phase procedure was described in a preliminary report by Ahlijanian *et al.* [6] but with a very low yield (0.5% based on the peptide–SH).

In this work we report the detailed solid-phase synthesis of ω-Aga IVA using a Boc/benzyl strategy, and the optimization of the folding and reoxidation steps. We also report a side reaction that occurs between tryptophan residues and Acm groups during the mercuric acetate deprotection step, together with a procedure to avoid it.

Abbreviations: Acm, acetamidomethyl; DIEA, diisopropylethylamine; DCM, dichloromethane; GSH, reduced glutathione; GSSG, oxidized glutathione; NMP, *N*-methylpyrrolidone; PDMS, plasma desorption mass spectrometry.

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MATERIALS AND METHODS

Materials

Boc-protected amino acids were purchased from Propeptide (Vert-le-Petit, France), Boc-Ala-phenylacetamidomethyl–resin (Boc-Ala-PAM) was from Applied Biosystems Inc. (Foster City, CA), *N*-methylpyrrolidone (NMP), *p*-cresol, oxidized and reduced glutathione were from Aldrich (Steinheim, Germany), *p*-thiocresol and hydroxybenzotriazole (HOBt) were from Janssen Chimica (Geel, Belgium) and HF was from Union Carbide (Oevel, Belgium). All chemical solvents were from Janssen Chimica and Aldrich. The peptide chains were assembled on an ABI 431A peptide synthesizer, Applied Biosystems Inc. (Foster City, CA). The preparative HPLC was performed on a Shimadzu Liquid Chromatography Model LC-6A and analytical HPLC analysis was performed on a system Gilson Model 302. Capillary electrophoresis was performed on an Applied Biosystems Model 270 A, Inc. (Foster City, CA) under the following conditions: field, 25 kV; current, 28 μA; capillary length, 50 cm; capillary diameter, 50 μm; fused silica. The separation was performed using a buffer solution of 20 mM sodium citrate, pH 2.5 and a temperature of 30°C. The detection was performed at 200 nm.

The molecular mass was determined using a Bio-Ion 20 time of flight Plasma Desorption Mass Spectrometer (PDMS) (Uppsala, Sweden) in positive mode using ^{252}Cf as a source of fission fragments or using an API (Perkin-Elmer Sciex) of a simple-quadrupole ion electrospray mass spectrometer, equipped with an ion-spray (nebulizer-assisted electrospray) source (Sciex, Toronto, Canada).

Amino acid analysis was performed using a Beckman 6300 amino acid analyser (Beckman instruments, Fullerton, CA), after hydrolysis with 6N HCl containing 0.25% phenol at 110°C for 24 h. Edman degradation was performed using an Applied Biosystems Inc. 470 A gas-phase sequencer (Foster City, DA) and the 03RPTH program. Phenylthiohydantoin derivatives of amino acid were identified with an Applied Biosystems 120 A on-line phenylthiohydantoin amino acid analyser.

Methods

Solid-phase Peptide Synthesis. Solid-phase peptide synthesis was carried out on an automated synthesizer ABI 431A (Applied Biosystems Inc.) using a standard Boc/Benzyl strategy starting with 0.5 mmol of Boc-Ala-PAM–resin (0.75 mmol/g). The standard cycle was as follows: deprotection with 25% TFA in DCM for 3 min and 50% TFA in DCM for 16 min, neutralization with 5% DIEA in DCM for 4 min. Each amino acid (4 equiv.) was coupled twice by dicyclohexylcarbodiimide/hydroxybenzotriazole (4 equiv.) for 2 × 60 min and followed by a capping step of the unreacted amino groups, with 10% acetic anhydride and 5% DIEA in NMP for 9 min. Side-chain protecting groups were as follows: Arg(Tos), Asp(O-chex), Cys(Acm), Glu(Ochex), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Met(O), Trp(Formyl) and Tyr(Br-Z). Following assembly of the 48 residues, 4.6 g of protected peptide resin were obtained (expected: 4.67 g).

The formyl group on Trp 14 was removed from the peptide, prior to the cleavage from its support by treatment with 10% piperidine in NMP for 2 × 2 h. The peptidyl resin was then treated with 50% TFA in DCM for 20 min to remove the amino-terminal Boc, and the peptide was cleaved from the resin and simultaneously deprotected according to a low and high HF procedure [7]: the resin (1 g) was treated with anhydrous HF (2.5 ml) in the presence of *p*-cresol (0.75 g), *p*-thiocresol (0.25 g) and dimethylsulphide (6.5 ml) at 0°C. After 3 h, hydrogen fluoride and dimethylsulphide were removed by vacuum evaporation and the residual scavengers and by-products were extracted with diethyl ether. The reaction vessel was then recharged with *p*-cresol (0.75 g), *p*-thiocresol (0.25 g) and 10 ml of anhydrous HF, and the mixture was allowed to react at 0°C for 1.5 h. Hydrogen fluoride was removed by evaporation and the residue was triturated with diethyl ether. The residue was filtered off, washed with diethyl ether and extracted with 200 ml of 5% aqueous acetic acid and lyophilized. The crude product was analysed by reversed-phase HPLC on a Vydac C18 column (4.6 × 250 mm, 5 μm, 100 Å) using a 60 min linear gradient from 0 to 100% Buffer B (Buffer A, 0.05% TFA in H_2O, and Buffer B 0.05% TFA, 60% CH_3CN in H_2O) at a flow rate of 0.7 ml/min and detection was performed at 215 nm.

The S-Acm protected peptide was purified by preparative reversed-phase HPLC on a Vydac C18 column (20 × 200 mm, 7 μm, 300 Å) using the buffers described above: from 0 to 40% Buffer B in 15 min, 40 to 60% Buffer B in 40 min. The flow rate was 3 ml/min and detection was performed at 280 nm.

Disulphide Bond Formation and Purification.

(1) Deprotection of S-Acm peptide with mercuric acetate: to 200 mg of the peptide dissolved in 5%

aqueous acetic acid (40 ml), mercuric acetate (1.1 eq./Acm) was added under N_2, and the solution was stirred at room temperature for 4 h [8]. The solution was diluted with water (60 ml) and the free mercuric ions were precipitated by bubbling hydrogen sulphide through the solution for 10 min. The pH was adjusted to 8 with diluted ammonium hydroxide and the solution was filtered to give a fully deprotected peptide.

(2) Oxidative folding reaction. The preceding solution of fully deprotected peptide was divided into 12 equal small samples (5 ml) which were submitted to an oxidative folding reaction. Several attempts were made in order to determine the most suitable oxidation conditions. In each case, mentioned below, the oxidative folding of the deprotected peptide was monitored by reversed-phase HPLC.

(a) Air oxidation. The octa-SH peptide was oxidized in NH_4OAc Buffer at different concentrations ranging from 0.1 to 2 M, at peptide concentrations of 0.1 mg/10 ml and 1 mg/10 ml, at pH 6 and at pH 8, in the presence or absence of reduced and oxidized glutathione (GSH and GSSG) with the following ratio: 60/6. All oxidations were performed both at 4°C and at room temperature.

(b) Dimethylsulphoxide oxidation. The octa-SH peptide was diluted in 5% aqueous acetic acid at a final peptide concentration of 0.5 mg/ml (9.6×10^{-4} M), the pH was adjusted to 6.5 with ammonium bicarbonate and dimethylsulphoxide (20% of peptide solution volume) was added. The resulting mixture was stirred for 10 h at room temperature [9].

(c) Potassium ferricyanide oxidation. The octa-SH peptide was diluted in water at a peptide concentration of 1 mg/10 ml (2×10^{-5} M) and the pH was adjusted to 8.5 with diluted ammonium hydroxide. The thiol groups were oxidized to disulphides by dropwise addition of a potassium ferricyanide 1 mM solution until a yellow colour persisted (32 equiv./peptide) [10]. The mixture was stirred for 5 h at room temperature. Then the pH was adjusted to 5 with acetic acid. An anion exchange ion resin (70 equiv.) (Bio-Rad, AG 2-X10, chloride form) was added and the mixture was stirred for 1 h. The resin was removed by filtration.

(3) Simultaneous cleavage of Acm groups and disulphide bond formation with thallium(III) trifluoroacetate [11]. The S-Acm peptide (20 mg, 5.5 μM) was dissolved in trifluoroacetic acid (3 ml) and treated with $Tl(CF_3CO_2)_3$ (1.2 equiv.) in the presence of anisole alone (50 μl) or anisole (25 μl) and glycerol (25 μl) in an ice bath for 1 h [11]. Trifluoroacetic acid was removed by vacuum evaporation. The oxidized peptide was precipitated in dry ether and the resulting precipitate was collected by centrifugation, dissolved in 5% aqueous acetic acid and lyophilized.

Synthesis and Characterization of ω-Aga IVA Under Optimal Conditions. According to the previous experiments, the following optimal conditions were used: the S-Acm peptide (200 mg) was deprotected with mercuric acetate as described above in the presence of tryptophan (10 molar excess over the peptide). The octa-SH peptide was dissolved in 0.2 M NH_4OAc pH 8 at a peptide concentration of 2×10^{-5} M, in the presence of reduced and oxidized glutathione; the ratio of peptide: GSH:GSSG was 1:60:6. The solution was stirred at 4°C for 48 h and the reaction was stopped by acidification to pH 2.5 with TFA. The solution was filtered on a Millipore System 0.22 μm and pumped directly at 4°C onto a preparative reversed-phase HPLC Vydac 218TP C18 column (10 × 500 mm, 5 μm, 100 Å). The column was then eluted using buffers described above and gradient of 0–40% Buffer B in 15 min, 40–40% Buffer B in 10 min and 40–60% Buffer B in 80 min, at a flow rate of 2 ml/min and the detection was performed at 280 nm. This was followed by purification on HPLC Machery Nagel Nucleosil 5 CN (10 × 500 mm, 5 μm, 100 Å) using the same eluants and a gradient of 0–50% Buffer B in 20 min, 50–50% Buffer B in 10 min and 50–80% Buffer B in 100 min, at flow rate of 2 ml/min and the detection was performed at 225 nm.

The amino acid composition of the purified peptide was determined as described above. The molecular mass of the synthetic peptide was determined by a Bio-Ion 20 time of flight plasma desorption mass spectrometer (PDMS). The homogeneity of the purified product was tested by two different systems. It was analysed by reversed-phase HPLC on a Vydac C18 column (4.6 × 250 mm, 5 μm, 100 Å) using a 60 min linear gradient from 0–100% Buffer B (Buffer A: 0.05% TFA in H_2O and Buffer B: 0.05% TFA, 60% CH_3CN in H_2O) at a flow rate of 0.7 ml/min and detection was performed at 215 nm. It was also analysed on an Applied Biosystems Model 270 A capillary electrophoresis in the conditions previously mentioned.

Biological Activity. The measurement of voltage-clamped calcium currents in slices from young rat Purkinje cells was obtained from a cerebellar slice of a 9-day-old rat. This slice was superfused at room temperature with a bicarbonate buffered saline (BBS) containing: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$,

1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 25 mM glucose. This solution was pre-equilibrated with oxygen and bicarbonate, bringing its pH to 7.4, and was supplemented with 4 mM tetraethylammonium-chloride and 0.5 μM tetrodotoxin to block voltage-dependent K^+ and Na^+ currents respectively. A tight-seal whole-cell recording was obtained with a patch-clamp pipette containing: 155 mM CsCl, 10 mM Cs-HEPES, 0.4 mM Na-GTP, 4 mM Na-ATP, 2 mM $MgCl_2$ and 0.1 mM fura-2 (pH 7.3).

The control current records were obtained by stepping the membrane potential from −70 mV to various test potentials ranging from −50 to +50 mV, in 20 mV increments. The inhibition by ω-Aga IVA of current was recorded in the same conditions after superfusion of the slice with 160 nm of ω-Aga IVA. (Reproduced with permission from Llano *et al.* and *Neuron* journal [12].)

RESULTS AND DISCUSSION

The synthesis of ω-Aga IVA was performed in the solid phase using Boc/benzyl chemistry. After deprotection (on the resin) of the formyl group from the tryptophan side chain with piperidine, all protecting groups, except the Acm groups on cysteine residues, were removed by hydrogen fluoride treatment (Figure 1). Acm protection of cysteine was used in order to allow easy purification of the crude S-protected

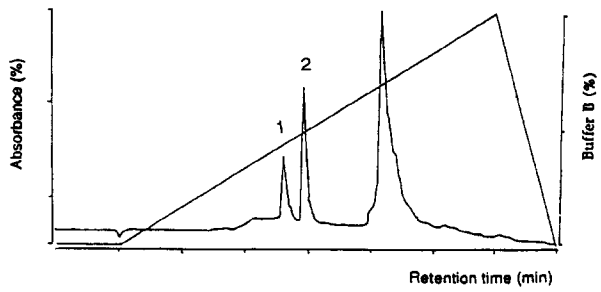

Figure 1 Reversed-phase HPLC of crude product from HF cleavage, performed on a nucleosil C18 column (4.6 × 250 mm) using as Buffer B: 0.05% TFA in CH3CN–water. Peaks 1 and 2 are not peptidic materials. The flow rate was 0.7 ml/min and detection was at 215 nm.

peptide prior to the oxidation step. The determination of the molecular weight of the crude S-Acm products, obtained after HF treatment, by ion electrospray mass spectrometry showed several peaks (Figure 2). One corresponds to the expected M + 8Acm while other peaks correspond to M+7Acm, M+6Acm and M+5Acm, providing evidence for partial loss of Acm during HF cleavage of the peptidyl–resin. Although the mass distribution does not exactly fit a binomial expression (indicating that the successive deprotections cannot be considered as completely independent events), it corresponds roughly to 10% individual deprotection of the Acm groups. Instability of Acm groups has been previously reported by Nutt *et al.* [13], Brady *et al.* [14] and Lyle *et al.* [15], and

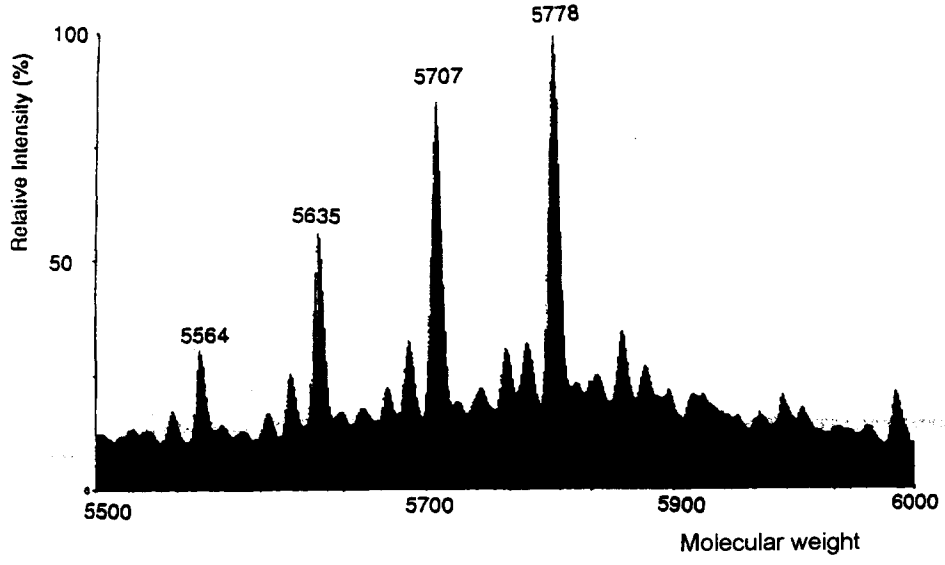

Figure 2 Ion electrospray mass spectra of S-Acm crude product after HF cleavage. The peak at MW = 5778 corresponds to the expected M+8Acm, the other peaks correspond to M+7Acm, M+6Acm and M+5Acm.

was expected, owing to the use of a thiol scavenger (*p*-thiocresol in our case) during HF cleavage. However, it proved to be more extensive in this case than in other similar syntheses performed in our laboratory [16,17]. The presence of these peaks is not due to the cleavage of the S-Acm groups during the process of mass measurement as this was not observed when pure fully S-Acm protected peptides were examined following the same procedure. The crude S-Acm peptide was submitted to preparative reversed-phase HPLC, yielding three main fractions which were submitted to amino acid analysis. The three fractions demonstrated the expected amino acid composition. Owing to the fact that mass spectrometry and amino acid analysis seemed to indicate that the loss of the Acm group(s) had not been accompanied by any other modification, the three Acm-peptide fractions were pooled and treated with mercuric acetate to remove the Acm groups completely.

In order to optimize the oxidation step, a large number of different conditions were systematically tested by comparison of the HPLC profile of the reaction medium. Disulphide bond formations from the Cys(Acm) peptide were carried out according to two different strategies. In the first case, a two-step protocol was used in which deprotection of Cys(Acm) with mercuric acetate was followed by disulphide bond formations. Different oxidation conditions were tested. Thiols groups have been oxidized using the procedure described by Tam *et al.* [9] using 20% DMSO, but this method led to an equilibrium between several configurations and did not allow the right folding to be obtained, even after several days. Oxidation in the presence of potassium ferricyanide gave us mainly aggregates and did not seem to be useful in this case. We mainly used air oxygen, checking the importance of several experimental conditions, such as the presence or absence of the redox system, different concentrations of NH_4OAc, different pHs at room temperature and 4°C (Figure 3Aa and 3Ab).

The second strategy involved concomitant Acm cleavage and disulphide bond formations with thallium(III) trifluoroacetate. In each case, the oxidation medium was checked at increasing times, from one hour to one week and HPLC demonstrated the formation of aggregates when the conditions were not favourable to the right folding of the toxin. Finally, the results have shown that the oxidation conditions which give the best results, as judged by the HPLC profile of the oxidative reaction medium, are: deprotection with mercuric acetate followed by an oxidative folding reaction of the octa-SH peptide in

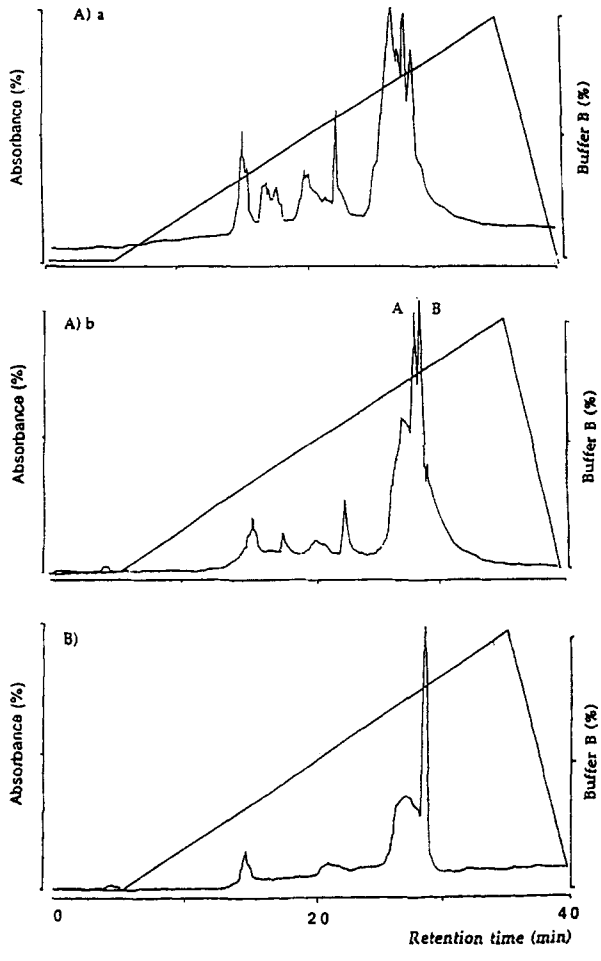

Figure 3 Reversed-phase HPLC after 48 h of oxidative refolding using a nucleosil C18 column (4.6 × 250 mm). (A) Deprotection of Cys-Acm was performed with mercuric acetate without the addition of tryptophan and the oxidation was performed in 0.1 M ammonium acetate at pH 8 in the presence of reduced and oxidized glutathione: (a) at room temperature; (b) at 4°C; peak A corresponds to the side product (with modified Trp) and peak B corresponds to the ω-Aga IVA. (B) Deprotection of Cys-Acm was performed with mercuric acetate in the presence of tryptophan (10 molar excess over the peptide) and the oxidation was performed at 4°C in the presence of reduced and oxidized glutathione in 0.2 M ammonium acetate at pH 8.

0.1 M NH_4OAc buffer (pH 8), at a peptide concentration 2×10^{-5} M, in the presence of reduced and oxidized glutathione, at 4°C for 48 h. The HPLC analysis of the reaction mixture showed two major products, A and B (Figure 3Ab). The amino acid compositions of products A and B were identical (Trp was not determined). However, molecular weight determination by ion electrospray showed that

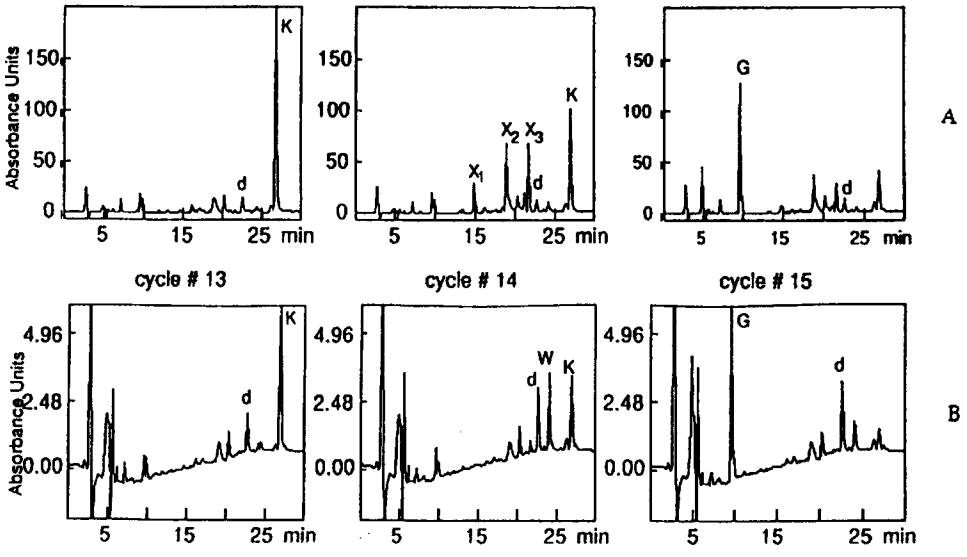

Figure 4 Edman degradation profiles, d = diphenylthiourea. (A) Edman degradation of product B: cycle 13 corresponds to PTH-Lys, cycle 14 corresponds to PTH-Trp (and the overlap from PTH-Lys) and cycle 15 corresponds to PTH-Gly. (B) Edman degradation of product A: cycle 13 corresponds to PTH-Lys, cycle 14 corresponds to the presence of non-identified peaks (and the overlap from PTH-Lys), cycle 15 corresponds to PTH-Gly.

product B had the expected mass, while product A showed an excess of 71 mass units, which corresponds to the mass of an Acm group. Thiol determination according to the Ellman test [18], performed on product A, yielded a negative result. This was not consistent with our initial hypothesis that one of the S-Acm groups had remained undeprotected as, in this case, only three disulphides could have been formed, leaving one cysteine with a free sulphydryl group (the molecular weight determination excluded the formation of a disulphide-linked dimer). This led us to suspect that the Acm group could have migrated on another side chain, allowing the formation of the four disulphides. In order to check this hypothesis, both peptides were submitted to Edman degradation. This method allowed us to confirm that product B had the expected sequence for ω-Aga IV A. In contrast, during the sequencing of product A, no PTH-Trp could be detected at cycle 14, while three unidentified peaks (X1, X2, X3) were observed (Figure 4).

One hypothesis to account for this side reaction, consistent with the loss of Acm groups observed on the crude peptide obtained after hydrogen fluoride treatment, was that the indole ring had been modified during the synthesis or HF cleavage by electrophilic species (such as $^{+}CH_2-NH-CO-CH_3 \longleftrightarrow CH_2=NH^{+}-CO-CH_3$). Another possibility was that this modification had occurred during the cleavage of the Acm by mercuric acetate. In order to choose between these two possibilities, an excess of tryptophan (10 molar excess over the peptide) was added during the treatment with mercuric acetate, to trap alkylating species. Reversed-phase HPLC of the oxidation reaction medium showed the formation of a major peak (Figure 3B), corresponding to product B, and the complete disappearance of peak A, indicating that the migration of the Acm group on tryptophan occurs during the mercuric acetate deprotection step. Recently, another modification involving the side chain of tryptophan during Acm deprotection by mercuric acetate has been reported by Nishio *et al.* which consists of the incorporation of a mercaptoethanol moiety in various positions of the indole ring [19]. These observations indicate that great care must be exercised during Acm deprotection of tryptophan-containing peptides and that addition of tryptophan as a scavenger can greatly improve this step. The small quantities of by-product isolated did not allow NMR studies; moreover, it is highly probable that modification of tryptophan occurs at several positions of the indole ring, leading to a heterogeneous product which, because of the size of the molecule, cannot be resolved.

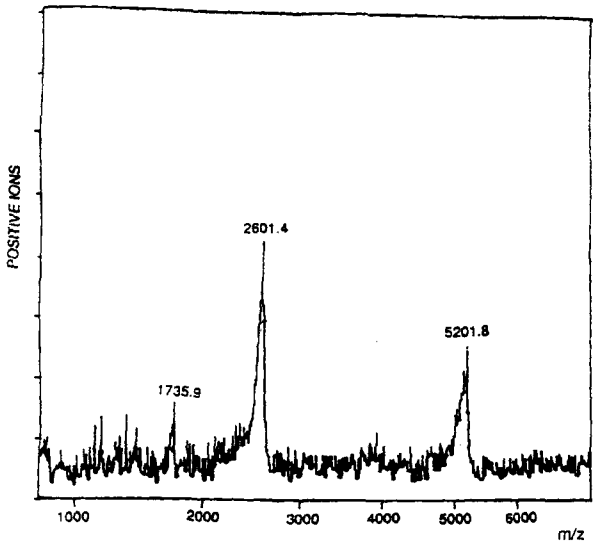

Figure 5 Plasma desorption mass spectra in positive mode of synthetic ω-Aga IVA. The peak at MW = 5201.8 corresponds to the expected $(M+H)^+$ ion. The additional peaks at 2601.4 and 1735.9 correspond respectively to $(M+2H)^{2+}$ and $(M+3H)^{3+}$ ions.

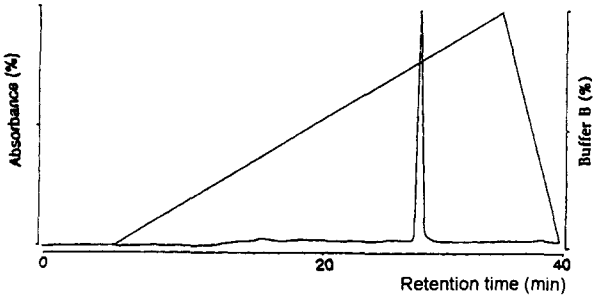

Figure 6 Reversed-phase HPLC of synthetic and purified ω-Aga IVA on a nucleosil C18 column (4.6 × 250 mm) using as Buffer B: 0.05% TFA in CH_3CN–water. The flow rate was 0.7 ml/min and detection was performed at 215 nm.

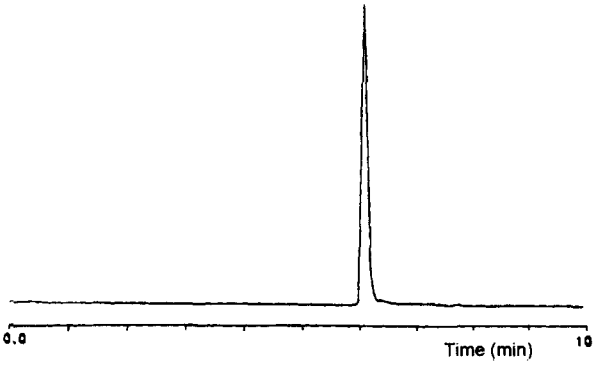

Figure 7 Capillary electrophoresis profile of purified synthetic ω-Aga IVA. Analysis was performed on a 50 cm coated capillary, in 20 mM sodium citrate. pH 2.5. Injection: 1s. Temperature: 30°C. Detection: 200 nm.

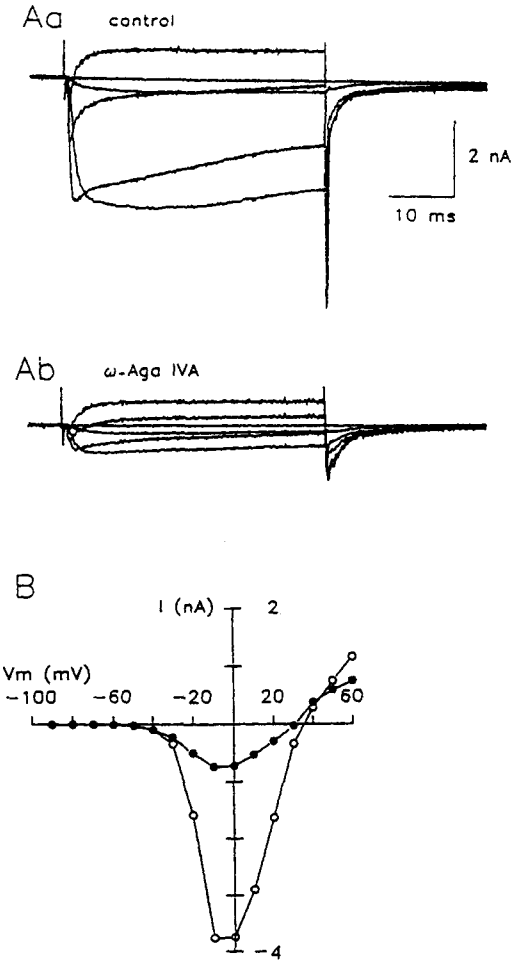

Figure 8 Effects of ω-Aga IVA on Ca2+ currents of cerebellar Purkinje cells. Recording obtained from a cerebellar slice of a 9-day-old rat. (Aa) Control current records obtained by stepping the membrane potential from −70 mV to various test potentials ranging from −50 to +50 mV, in 20 mV increments. (Ab) Responses to the same voltage steps after superfusion of the slice with 160 nM of ω-Aga IVA. (B) I–V curves in control BBS (open symbols) and in the presence of ω-Aga IVA (filled symbols). Reproduced with permission from Llano *et al.* and *Neuron* journal (12).

In order to obtain large quantities of ω-Aga IVA, the deprotection with mercuric acetate was performed on 200 mg of S-Acm protected peptide, followed by the oxidation under the optimal conditions (in 0.2 M NH_4OAc pH 8 at peptide concentration of 2×10^{-5} M, in the presence of GSH/GSSG, at 4 °C) and the main product was isolated to homogeneity. Two steps of preparative HPLC on two different supports (octadecylsilane and cyanoethyl) afforded 20 mg of pure ω-Aga IVA (determined by quantitative

amino acid analysis). This corresponds to 10% for the oxidation step, based on the S-Acm peptide and to an overall yield of 5% based on the load of the starting peptidyl–resin. The amino acid composition of the final product was determined as follows: Asp 1.9 (2), Thr 1.9 (2), Ser (0.9 (1), Glu 2.2 (2), Pro 1.9 (2), Gly 8.0 (8), Ala 2 (2), Cys 6.0 (8), Met 1.8 (2), Ile 3.8 (4), Leu 3.1 (3), Tyr 0.8 (1), Lys 5.9 (6), Arg 3.8 (4). The correct molecular weight was confirmed by mass spectrometric analysis (Figure 5) which showed 5201.8 as $(M+H)^+$, 2601.4 as $(M+2H)^{2+}$ and 1735.9 as $(M+3H)^{3+}$. The purity was controlled by reversed-phase HPLC and capillary electrophoresis (Figures 6 and 7). Synthetic ω-Aga IVA was tested for its capacity to block P-type calcium channel in Purkinje cells as shown in (Figure 8). The potency and the selectivity of the synthetic toxin were similar to those reported for the natural product. The Acm-modified product (product A, Figure 3Ab) was shown to induce a leak of Ca^{2+}.

In conclusion, we described an optimized preparation of ω-Aga IVA, using a solid-phase method which will allow the synthesis of various analogues in order to perform structure–activity studies. A major improvement during this synthesis was the addition of an excess of tryptophan during the mercuric acetate treatment which prevents a side reaction involving the side chain of tryptophan.

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