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Synthesis and Biological Characterization of a Series of Analogues of ω-Conotoxin GVIA

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Abstract: The 27-residue polypeptide ω-conotoxin GVIA (ω-CgTx), from the venom of the cone shell *Conus geographus*, blocks N-type neuronal calcium channels. It contains three disulphide bridges. We report here the synthesis and biological characterization of a series of analogues in which one disulphide has been replaced by substitution of appropriate Cys residues with Ser, viz. [Ser^{1,16}]-ω-CgTx, [Ser^{8,19}]-ω-CgTx, [Ser^{15,26}]-ω-CgTx, [Ser^{16}]-ω-CgTx$_{8-27}$ and [Ser^{15}]-ω-CgTx$_{1-19}$. All syntheses were conducted manually using either Boc or Fmoc methodology. Deprotected peptides were oxidized to their bridged forms using either aerial oxidation or aqueous dimethyl sulphoxide. Peptides were purified using RP-HPLC, and their purity and identity were checked by RP-HPLC, capillary electrophoresis and mass spectrometry. Inhibition of neuronal N-type calcium channels was assessed as the inhibition of the twitch responses of rat vas deferens stimulated with single electrical pulses at 20 second intervals. None of these analogues was biologically active, suggesting that the disulphides play an important role in maintaining biological activity.

Keywords: Conotoxin; analogues; disulphide deletions; calcium channel

INTRODUCTION

ω-Conotoxin GVIA (ω-CgTx), a peptide component of the fish hunting cone shell *Conus geographus* has been isolated and biochemically characterized [1]. The total synthesis has been reported using both solid-phase and solution-phase methods [2, 3]. The native peptide is a 27 residue structure with six cysteine residues which form three internal disulphide bridges between Cys[1] and Cys[16], Cys[8] and Cys[19] and between Cys[15] and Cys[26]. This peptide also contains three hydroxyproline residues at positions 4, 10 and 21 and is C-terminally amidated. The primary sequence is as follows:

H-Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys-Tyr-CONH2

The solution structure of chemically synthesized ω-conotoxin GVIA has been determined recently by 2D ^{1}H-NMR spectroscopy [4–6]. The secondary structure adopts a triple stranded, antiparallel β-sheet with several reverse turns.

The literature on the ω-conotoxins indicates that among the several native sequences, the conserved features are the positioning of the cysteine residues and therefore the disulphide bridging, the position of the Glycine[5] and the presence of the carboxamide at the C-terminus [7]. A preliminary report of alanine replacement studies has suggested that the disulphide bridges between Cys[8]-Cys[19] and Cys[15]-Cys[26] are critical for biological activity while the bridge between Cys[1]-Cys[16] is less crucial [8], but a full report of this work has not yet been published. In

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addition, bioassay of these analogues was conducted using whole animal lethality assays, which may not necessarily represent activity at the specific target of ω-CgTx, the N-type calcium channel [9, 10].

In order to investigate the role of the disulphide bridges in maintaining the tertiary structure, and thereby biological activity, of ω-CgTx, three analogues [$Ser^{1,16}$]-ω-CgTx, [$Ser^{8,19}$]-ω-CgTx and [$Ser^{15,26}$]-ω-CgTx were synthesized in which one of the disulphide bridges was removed and the cysteine residues of the deleted bridges replaced by the non-bridging but isoelectronic serine. In addition, deletion of the disulphide bridge in the first and third of these analogues leaves a free 'tail', which is no longer constrained. In order to test the effect on potential biological activity of removing these 'tails' a further two analogues, [Ser^{16}]-ω-$CgTx_{8-27}$ and [Ser^{15}]-ω-$CgTx_{1-19}$, where also synthesized and tested. All of these analogues were tested for activity in blocking the N-type calcium channel using a specific *in vitro* bioassay. We report here the synthesis and testing of these analogues.

MATERIALS AND METHODS

General

N-α-protected amino acids were obtained from Auspep Pty. Ltd, Bachem or Millipore and were of the L-configuration except for Gly. N^{α}-9-Fluorenyl-methoxycarbonyl (Fmoc) amino acids included: Cys(trityl), Arg(2, 2, 5, 7, 8-pentamethylchroman - 6 - sulphonyl), Ser(*t*-butyl), Lys(*t*-butyloxycarbonyl), Tyr (*t*-butyl), Thr(*t*-butyl), Hyp(*t*-butyl) and Asn(trityl). N^{α}-*t*-Butyloxycarbonyl (Boc) amino acids included: Arg(Tosyl), Cys(paramethoxybenzyl), Lys(2-chlorobenzyl), Ser(*O*-benzyl), Tyr(2-bromobenzyl), Thr(*O*-benzyl). Boc-Asparagine and Boc-hydroxyproline were incorporated into the peptides with unprotected sidechains. All reagents and solvents (Fluka, Auspep, BDH, Ajax Chemicals) for peptide synthesis were reagent grade or better and used without further purification. The *p*-methylbenzhydrylamine resin was prepared in house by the method of Rivier *et al* [11, 12]. The polyethylene glycol–polystyrene (PAL-PEG-PS) resin was purchased from Millipore.

The analytical chromatographic systems used were either a Hewlett Packard 1050 liquid chromatograph equipped with a multi-wavelength diode array-based detector (detection was a 220 nm), or a Waters Associates liquid chromatograph consisting of two M-45 pumps, an automated gradient controller and a variable wavelength detector (detection was at 214 nm). Preparative chromatography was conducted on either the Hewlett Packard 1050 LC or a Waters 600E Prep LC equipped with a Waters 486 Tunable Absorbance Detector (detection was at 230 nm). Analytical chromatography was conducted on the following columns. Alltech Econosil C_{18} column (250 × 4.6 mm, 10 μm particle size); Alltech Hypersil C_{18} column (250 × 2.1 mm, 5 μm particle size); Vydac C_{18} column (250 × 4.6 mm, 5 μm particle size); Waters C_{18} Delta Pak (100 × 8 mm radial compression cartridge, 15 μm particle size). Preparative RP-HPLC was conducted on either an Alltech Econosil ODS column (250 × 22.5 mm, 10 μm particle size) or a Waters C_{18} Delta Pak radial compression cartridge (100 × 40 mm, 15 μm particle size). Mobile phase systems consisted of either 0.1% trifluoroacetic acid (TFA) in H_2O/0.1% TFA in acetonitrile (MeCN), 0.1% heptafluorobutyric acid (HFBA) in H_2O/0.1% HFBA in MeCN or 0.1% triethylammonium phosphate at pH 2.25 (TEAP 2.25)/40% TEAP in MeCN. Unless otherwise stated, it can be assumed that the preparative and analytical RP-HPLC were performed using gradient elution with the 0.1% TFA-based buffers. Hereafter, these mobile phase systems will be abbreviated to 0.1% TFA/MeCN, 0.1% HFBA/MeCN and TEAP 2.25/MeCN. Matrix-assisted laser desorption-mass spectrometry (MALD-MS) was performed using a Finnigan Lasermat. The absorbing matrix used was α-cyano-4-hydroxy-cinnamic acid. Capillary zone electrophoresis (CZE) was carried out on an Applied Biosystems Model 270A instrument, using the following conditions: applied potential 30 kV; sodium citrate buffer (20 mM, pH 2.5); capillary length 72 cm; capillary diameter 50 μm; temperature 30 °C.

Biological Evaluation

Male Sprague Dawley rats were killed by carbon dioxide anaesthesia followed by decapitation, and the vasa deferentia were removed and mounted in 5 ml organ baths filled with Krebs' solution (composition in mM: Na^+ 144, K^+ 5.9, Mg^{++} 1.2, Ca^{++} 2.5, HPO_4^- 1.2, Cl^- 129, SO_4^- 1.2, HCO_3^- 25, glucose 11, EDTA 0.026) bubbled with 5% CO_2 in oxygen at 37 °C. The top of each tissue was attached to an isometric force transducer (Grass FT03), and the bottom attached to a movable support and straddled with platinum stimulating electrodes. The vasa were stretched to produce a passive force of about 10 mN and stimulated with single electrical field pulses (100 V, 0.2 ms duration) every 20 s. The resulting twitch responses

were mediated by sympathetic nerves, being sensitive to inhibition by guanethidine or tetrodotoxin, and were recorded on a chart recorder. Cumulative concentration–response curves for ω-CgTX were constructed by addition of the peptide to the solution bathing the vas deferens in 10-fold concentration increments from 0.1 nM, with the time between concentration increments of 20 min. The peptide analogues of ω-CgTX were tested in the same way, and after the highest concentration (1 μm) an ω-CgTX concentration–response curve was constructed in the presence of the analogue. A single protocol was followed on each vas deferens such that each tissue received only a single ω-CgTX concentration–response curve.

Synthesis of Full Length Analogues

Peptide Synthesis. [$Ser^{1,16}$]-ω-Conotoxin GVIA, [$Ser^{8,19}$]-ω-conotoxin GVIA and [$Ser^{15,26}$]-ω-conotoxin GVIA were synthesized manually by continuous flow Fmoc solid-phase methodology, using a Cambridge Research Biochemicals peptide synthesizer, at 0.2 mmol scale on PAL-PEG-PS resin (0.16 mmol/g). Fmoc amino acids (3 eq.) were activated using 2-(1H-benzotriazol-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3 eq.) with diisopropylethylamine (DIPEA) (6 eq.) used to initiate the *in situ* reaction. After completion of the synthesis, peptide resins were washed with dichloromethane, methanol and diethylether and were then dried. Peptides were cleaved from the resin, and all protecting groups removed by treatment with a solution containing TFA (10 ml), ethane-1,2-dithiol (EDT) (0.25 ml), thioanisole (0.5 ml), H_2O (0.5 ml) and phenol (0.8 g) [13]. After 4–15 h resins were removed by filtration and the peptide solutions were added to distilled H_2O (50 ml) and extracted several times with diethyl ether (50 ml). The aqueous extracts were lyophilized to give the crude peptides. The crude, linear (reduced) peptides were typically purified by preparative RP-HPLC prior to oxidation. Fractions containing the purified material were pooled and lyophilized to give pure tetrasulphydryl peptides.

Oxidation Using Dimethyl Sulphoxide. Each of the purified tetrasulphydryl peptides was dissolved in 5–20% dimethyl sulphoxide (Me_2SO) in aqueous ammonium acetate (NH_4OAc) buffer (0.1 M, pH 7) at a concentration of 0.5 mg/ml [14]. The oxidation was monitored by analytical RP-HPLC and the Ellman test with 5,5′-dithiobis-(2-nitrobenzoic acid) [15]. The oxidized peptides were purified by preparative RP-HPLC using either gradient or isocratic elution. Fractions containing the purified peptide were pooled and lyophilized (see Table I for yields and mass spectral data).

Peptide Characterization. Purified peptides were subjected to RP-HPLC analysis in two mobile phase systems: 0.1%-TFA/MeCN and 0.1%- HFBA/MeCN. Further evidence for homogeneity was provided by CZE and the identity of the purified products were confirmed by MALD-MS.

Synthesis of Truncated Analogues

Peptide Synthesis. [Ser^{16}]-ω-Conotoxin $GVIA_{8-27}$ and [Ser^{15}]-ω-conotoxin $GVIA_{1-19}$ were synthesized manually using boc-benzyl protecting group strategy with stepwise assembly of the peptide on 2.0 g of *p*-methylbenzhydrylamine resin (0.52 mmol/g) and 2 eq. of protected amino acid. Trifluoroacetic acid (70% in dichloromethane (DCM) and EDT (2%) were utilized for Boc group removal, and triethylamine (10% in DCM) for neutralization. Resin washing was performed with methanol and dichloromethane. Coupling was mediated by dicyclohexylcarbodiimide (DCC) in either dichloromethane or dimethylformamide depending on the solubility of the BOC amino acid. *t*-Butyloxycarbonyl-asparagine was coupled in dimethylformamide in the presence of 1-hydroxybenzotriazole (4 eq.) plus DCC (1 eq.). After comple-

Table I. Mass Spectral Data and Synthesis Yields for Ser Substituted Analogues

ω-CgTx analogue	Calculated MH[a]	Observed MH[a]	Crude yield[b] (%)	Final yield[b] (%)
[$Ser^{1,16}$]-ω-CgTx	3008.3	3008.2	45	5.2
[$Ser^{8,19}$]-ω-CgTx	3008.3	3008.7	63	1.9
[$Ser^{15,26}$]-ω-CgTx	3008.3	3008.0	55	2.0
[Ser^{16}]-ω-$CgTx_{8-27}$	2361.6	2361.7	75	1.5
[Ser^{15}]-ω-$CgTx_{1-19}$	1983.2	1983.4	65	1.8

[a]Yield of linear peptide expressed as % of theoretical yield from resin substitution.
[b]Yield of oxidized final product expressed as % of crude linear peptide.

tion of chain assembly, the protected peptide–resins were cleaved in anhydrous hydrogen fluoride (HF) in presence of anisole at 0 °C for 60 min. Upon removal of the HF under high vacuum, the resins were washed with diethyl ether and the peptide was extracted from the resin with distilled H_2O and lyophilized. Reduced peptides were not purified before oxidation.

Aerial Oxidation. The analogues were oxidized by dissolving the crude peptides in ammonium bicarbonate (NH_4HCO_3) buffer (25–50 mM, pH 8) at a concentration of 1 mg/ml and allowed to stir. At the completion of cyclization (as determined by the Ellman test) the analogues were shell frozen and lyophilized.

Peptide Purification. The crude (oxidized) lyophilized peptides were dissolved in TEAP 2.25, loaded batchwise on to a C_{18} 100 × 40 mm preparative cartridge, and eluted with a linear gradient of TEAP 2.25/MeCN [16,17]. Appropriate fractions (>95% pure) were pooled, diluted and reloaded on to the preparative cartridge. The peptide was eluted with a linear gradient of 0.1% TFA/MeCN. Fractions containing the purified peptide were pooled and lyophilized (see Table I for yields and mass spectral data).

Peptide Characterization. Purified peptides were subjected to RP-HPLC analysis in two mobile phase systems: 0.1% TFA/MeCN and TEAP 2.25/MeCN. Further evidence for homogeneity was provided by CZE and the identity of the products was confirmed by MALD-MS.

RESULTS AND DISCUSSION

The amino acid sequences of [$Ser^{1,16}$]ω-CgTx, [$Ser^{8,19}$]ω-CgTx, [$Ser^{15,26}$]ω-CgTx [Ser^{16}]ω-$CgTx_{8-27}$ and [Ser^{15}]ω-$CgTx_{1-19}$ were successfully assembled as described in detail above. The tetrasulphydryl peptides were obtained after cleavage and deprotection. The three full-length peptides were synthesized by Fmoc methodology, and the two truncated peptides by Boc methodology. In general the linear crude products obtained via each methodology, after deprotection and cleavage from the resin, were comparable in purity and yields (see Table I). Hydroxyproline was fully protected as the *t*-butyl ether during Fmoc syntheses, but was incorporated without sidechain protection in Boc syntheses. The lack of sidechain protection in the Boc syntheses seemed to have no deleterious effects, with no evidence being found for dehydration products or other side reactions in the final peptides.

These peptides were oxidized using either aqueous dimethylsulphoxide (for the full-length analogues) or aerial oxidation (for the truncated analogues). Both oxidation procedures gave similar HPLC profiles of the crude products in the reaction mixtures (data not shown). Generally, there was one major product, with multiple poorly resolved minor products, usually eluting after the main peak. Although the identity of these minor products was not established, they may represent either alternatively bridged monomeric species or intermolecular disulphide bridging to give oligomeric species. These minor products were more abundant in DMSO-mediated oxidations, but aerial oxidations gave much greater amounts of precipitated material in the oxidation mixture. Thus DMSO may have served to keep these oligomers in solution. In both techniques the kinetics of oxidation appeared to be similar, with oxidation being complete over a similar time course. DMSO-mediated oxidation did give slightly higher yields of the major product. The major component from the oxidation mixtures was purified using preparative RP-HPLC. These peptides were shown to be >98% pure by analytical RP-HPLC (see Figure 1). Homogeneity was confirmed by RP-HPLC in a second buffer system and by CZE (data not shown). Yields and mass spectral data are recorded in Table I.

With regard to disulphide bond connectivities within these peptides our preliminary data with NMR indicate that the bridging patterns of the remaining disulphides are the same as those in the parent molecule. The rather extensive experimental evidence will be reported separately.

The previous study in which disulphide bridges were deleted purported to show some residual biological activity when the $Cys^{1,16}$ disulphide bridge was deleted [8]. However, these results were obtained from an *in vivo* lethality assay, and may not necessarily represent action of the analogue at the specific target of ω-conotoxin GVIA. Voltage-operated calcium (Ca^{2+}) channels are an essential component of all excitable cell membranes and may be subdivided into T-, L-, N- and P-type Ca^{2+} channels [18], based on differences in gating properties, ionic conductance, pharmacology and tissue distribution [19, 20]. N-type Ca^{2+} channels are found only on neuronal tissue, both in the brain and in the periphery of vertebrates [10, 21]. The Ca^{2+} entry required for neurotransmitter release has been demonstrated to be principally, if not exclusively,

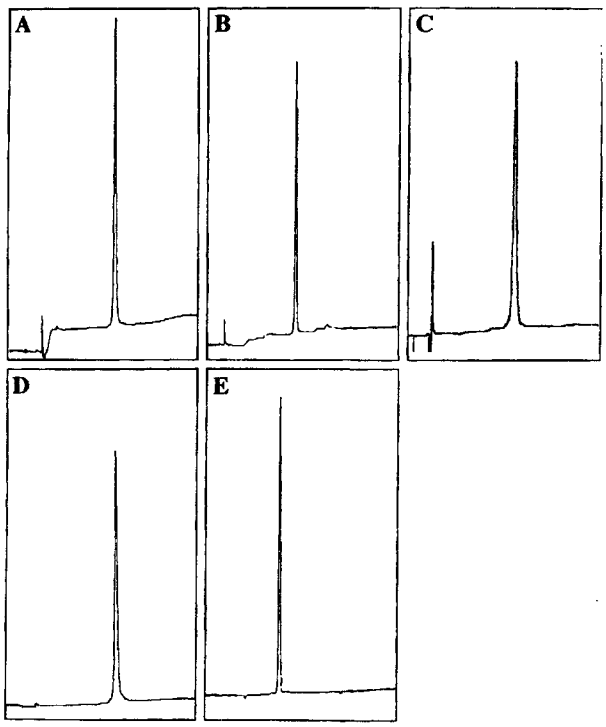

Figure 1 Analytical RP-HPLC analysis of (A) [Ser^{1,16}]ω-CgTx; (B) [Ser^{8,19}]ω-CgTx; (C) [Ser^{15,26}]ω-CgTx; (D) [Ser^{16}]ω-CgTx$_{8-27}$; and (E) [Ser^{15}]ω-CgTx$_{1-19}$. Column: 250 × 2.1 mm, Hypersil 5 μm. Buffers: A = 0.1% TFA, B = 0.1% TFA in MeCN. Flow rate: 0.3 ml/min. Gradient: 10–40% in 30 min.

through N-type Ca^{2+} channels [9, 20, 21], and ω-conotoxin GVIA is a selective inhibitor of these Ca^{2+} channels [9, 10]. Thus the responses of the preparations used to test biological activity in the current study are dependent upon the release of neurotransmitter by a Ca^{2+}-dependent mechanism mediated by N-type channels. As shown in Figure 2, ω-conotoxin GVIA completely inhibited the twitch of the vas deferens, with 50% inhibition at between 1 and 10 nM. None of the analogues tested up to a concentration of 1 μM had any noticeable effect on the twitch. Also, ω-conotoxin GVIA retained its full ability to inhibit the twitch in the presence of 1 μM of the analogues, and its potency was not reduced. Thus it appears that the analogues have little, if any, affinity for the channels that are blocked by native ω-CgTX.

CONCLUSIONS

These analogues were not biologically active, suggesting that removal of any of the disulphide bridges results in extensive disruption to the architecture of the peptide and thereby its ability to maintain its biologically active conformation. Studies are currently in progress to determine the three-dimensional structure of these analogues using 2D ^{1}H-NMR, and the results of these studies will allow us to correlate their lack of activity with the structural effects of disulphide bridge deletion.

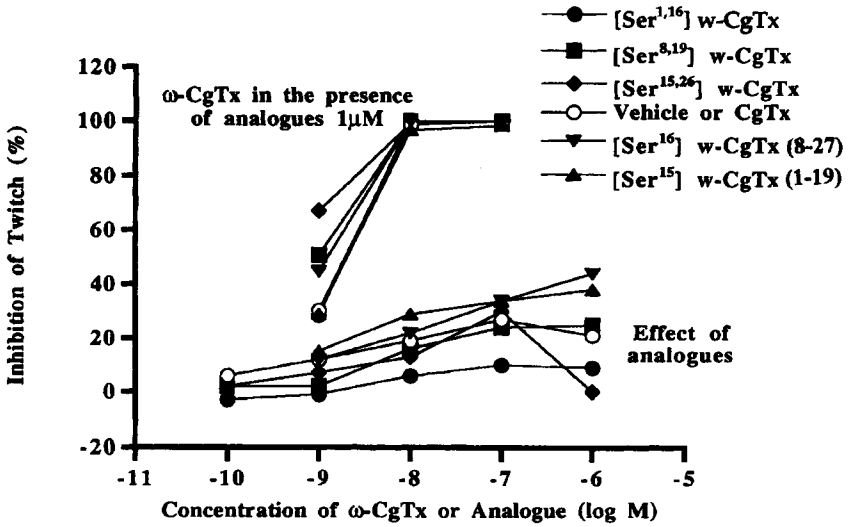

Figure 2 Effect of analogues on electrically evoked twitch responses in rat vas deferens in the presence and absence of native ω-conotoxin GVIA.

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