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Optimizing the connectivity in disulfide-rich peptides: α-conotoxin SII as a case study

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Abstract

We describe a strategy for the efficient, unambiguous assignment of disulfide connectivities in α-conotoxin SII, of which ~30% of its mass is cysteine, as an example of a generalizable technique for investigation of cysteine-rich peptides. α-Conotoxin SII was shown to possess 3–8, 2–18, and 4–14 disulfide bond connectivity. Sequential disulfide bond connectivity analysis was performed by partial reduction with Tris(2-carboxyethyl)phosphine and real-time mass monitoring by direct-infusion electrospray mass spectrometry (ESMS). This method achieved high yields of the differentially reduced disulfide bonded intermediates and economic use of reduced peptide. Intermediates were alkylated with either *N*-phenylmaleimide or 4-vinylpyridine. The resulting alkyl products were assigned by ESMS and their alkyl positions sequentially identified via conventional Edman degradation. The methodology described allows a more efficient, rapid, and reliable assignment of disulfide bond connectivity in synthetic and native cysteine-rich peptides.

Keywords: Disulfide connectivity; Mass spectrometry; MS/MS; Conotoxin; *N*-Phenylmaleimide

The analysis and sequential assignment of disulfide bonds in peptides and proteins is beset with problems and technical difficulties. Yet the importance of establishing specific connectivity between cysteine moieties is vital to understanding the structure and function of peptides and proteins [1]. This is particularly important for biologically active peptides derived from venomous animals and toxin-producing bacteria, where peptide toxins can possess multiple disulfide bonds.

Disulfide bonding within pharmacologically related peptide toxin families plays a fundamental role in defining their three-dimensional structure, as seen with a frequent adherence to highly conserved cysteine "frameworks" or "spacing patterns." This in turn often provides a common structural scaffold or platform for toxin diversification. Such a constrained structural framework is instrumental in determining the highly selective nature of the toxin's biological activity [2].

One such family of bioactive peptides are the conotoxins (or conopeptides) derived from marine snails of the genus *Conus*. Conopeptides represent a vast pharmacopoeia of small (10–40 amino acid residues) peptides, typically containing one to three disulfide bonds. Conopeptides have defined tertiary structures formed and stabilized by these disulfide bonds [3]. In particular, the smaller α-conotoxins (12–19 amino acids; see Table 1), which target isoforms of the nicotinic acetylcholine

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Table 1
Comparative amino acid sequence and disulfide framework spacing of the α-, αA-, and λ-conotoxin families

	Sequence	Disulfide framework spacing	Original toxin reference	Method for S–S connectivity assignment	Reference for S–S connectivity assignment
α-Conotoxins					
α-GI	ECCNPACGRHYSC*	3:5	[29]	NMR	[30]
				X-ray crystallography	[31]
α-GIA	ECCNPACGRHYSCGK*	3:5	[29]	ND	—
α-GIB	ECCNPACGRHYSCKG*	3:5	[32]	ND	—
α-GII	ECCHPACGKHFSC*	3:5	[29]	ND	—
α-MI	GRCCHPACGKNYSC*	3:5	[33]	Retro-selective synthesis	[34]
α-MIA	GRCCHPACAKHFNC*	3:5	[32]	ND	—
α-MIB	NGRCCHPACARKYNC	3:5	[32]	ND	—
α-SI	ICCNPACGPKYSC*	3:5	[35]	NMR	[36]
α-SIA	YCCHPACGKNFDC*	3:5	[37]	ND	—
α-SII	GCCCNPACGPNYGCGTSCS	3:5:3	[38]	This work—partial reduction	This work
α-CnIA	GRCCHPACGKYYSC*	3:5	[39]	NMR/retro-selective synthesis	[39]
α-CnIB	CCHPACGKYYSC*	3:5	[39]	NMR/retro-selective synthesis	[39]
α-MII	GRCCSNPVCHLEHSNLC*	4:7	[40]	Retro-selective synthesis	[40]
				NMR	[41]
				NMR	[42]
α-GIC	GCCSHPACAGNNQHIC*	4:7	[32]	Retro-selective synthesis	[32]
α-GID	IRDγCCSNPACRVNNOHVC	4:7	[43]	NMR	[43]
α-EI	RDOCCYHPTCNMSNPQIC*	4:7	[44]	Retro-selective synthesis	[44]
				NMR	[45]
α-EpI	GCCSDPRCNMNNPDYC*	4:7	[16]	Partial reduction	[16]
	α-Conotoxin [tyr^{15}]-Epi	4:7	[46]	NMR	[46]
α-PnIA	CCSLPRCAANNPDYC*	4:7	[47]	ND	—
	α-Conotoxin [tyr^{14}]-PnIA	4:7	[48]	X-ray crystallography	[49]
α-PnIB	GCCSLPRCALSNPDYC*	4:7	[47]	Retro-selective synthesis	[47]
	α-Conotoxin [tyr^{14}]-PnIB	4:7	[48]	X-ray crystallography	[50]
α-AuIA	GCCSYPRCFATNSDYC*	4:7	[51]	ND	—
α-AuIC	GCCSYPRCFATNSGYC*	4:7	[51]	ND	—
α-AuIB	GCCSYPRCFATNPDC*	4:6	[51]	Retro-selective synthesis	[51]
				NMR	[52]
α-ImI	GCCSDPRCAWRC*	4:3	[53]	Retro-selective synthesis	[53]
				NMR	[54]
				NMR	[55]
				NMR	[56]
α-ImII	ACCSDRRCRWRC*	4:3	[57]	Retro-selective synthesis	[57]
αA-OIVA	CCGVONAACHOCVCKNTC*	6:2:1:3	[5]	Partial reduction	[5]
λ-Conotoxins					
λ-MrVIA	VCCGYKLCHOC	4:2	[4]	NMR/retro-selective synthesis	[4]
λ-MrVIB	NGVCCGYKLCHOC	4:2	[4]	NMR/retro-selective synthesis	[4]
λ-MrX	GICCGVSFCYOC	4:2	[4]	NMR/retro-selective synthesis	[4]

ND, not determined; *, C-terminal amidation; γ, γ-carboxylglutamic acid; O, 4-*trans*-hydroxyproline; Y, sulfated tyrosine.

receptor (nAChR),[2] are perhaps the most highly constrained biologically active peptides known. The cysteine content of these α-conotoxins may account for 20–30% of the total peptide sequence.

Reference to the α-conotoxin family (see Table 1) reveals that they adhere to the generic amino acid sequence $(X)_{n1}C^{i}C^{ii}(X)_{n2}C^{iii}(X)_{n3}C^{iv}(X)_{n4}$, where $n1 =$ 1–4 amino acids, $n2 = 3$ or 4 amino acids, $n3 = 3$–7 amino acids, and $n4 = 0$–4 amino acids, with the cysteinyl linkages being hypothesized (or verified) as C^{i}–C^{iii} and C^{ii}–C^{iv}, in the above generic sequence.

In recent years the hazards of automatic assumption of a universal disulfide framework for this group of peptides has been brought into question with the isolation of the lambda (λ) class of conotoxins from the molluscivorous snail *Conus marmoreus* [4] and furthered by a recent example of αA-conotoxin OIVA isolated from *C. obscurucus* [5] (see Table 1). From a sequence

[2] *Abbreviations used:* nAChR, nicotinic acetylcholine receptor; TCEP, Tris(2-carboxyethyl) phosphine; ESMS, electrospray MS; TFA, trifluoroacetic acid; CID, collision-induced dissociation; *t*-Boc, *t*-butoxycarbonyl; PTH, phenylthiohydantoin.

perspective, the *C. marmoreus* peptides (λ-conotoxins MrVIA, MrVIB, and MrX) are typical of the "α-conotoxin-like" family, possessing the same characteristic disulfide framework spacing; however, these conotoxins are not pharmacologically related to them. The disulfide bond connectivity of these peptides was demonstrated to be different from that of the "classical" α-conotoxins by use of retro-selective synthesis (selective sequential disulfide formation) and NMR spectroscopy [4]. The connectivity of the λ-conotoxins was established as being C^{i}–C^{iv} and C^{ii}–C^{iii}, while maintaining the generic sequence (3)CC(4)C(2)C(0)—the latter framework being within the previously described criteria of the α-conotoxin family [6].

In contrast, the disulfide framework of αA-conotoxin OIVA was shown to be an extension of the classical α-conotoxin-like cysteine framework, with an additional C-terminal loop as verified by partial reduction disulfide analysis [5]. The connectivity of the αA-conotoxin OIVA was established as C^{i}–C^{iii} and C^{ii}–C^{v}, with the encompassing loop being C^{iv}–C^{vi}, in an amino acid sequence of (0)CC(6)C(2)C(1)C(3)C. Furthermore, this peptide was shown to target vertebrate neuromuscular nAChRs.

These findings sound caution relative to the assumed disulfide connectivity of newly discovered α-conotoxin-like sequences, whether by classically or genomically derived routes. There remains a specific need for methods to establish unambiguous assignment of disulfide bond connectivity.

Various methodologies have been used to assign disulfide bond connectivity for conotoxins (see Table 1). From retro-selective synthesis and native comparison [7] to analysis via enzymatic digestion and sequential Edman degradation [8], none is more powerful than the use of Tris(2-carboxyethyl)phosphine (TCEP) to generate partially reduced peptide intermediates that enable sequential opening of the disulfide bonds [9]. Yet this approach is rarely used, due to the quantity of material and the intensive kinetic analysis (i.e., time scale of partial reduction) required to generate the partially reduced products.

In this study we have made use of TCEP to determine the disulfide connectivity of a 19-amino-acid, three-disulfide-bonded conotoxin, α-conotoxin SII. In the present application real-time analysis, by electrospray mass spectrometry (ESMS), has been used to monitor the formation and hence optimize the proportion of the required selectively reduced intermediates. This has simplified the most difficult step in the process, namely, the identification of the sites of paired reduction/alkylation, allowing the assignment of disulfide bond connectivity. It has also greatly reduced the amount of starting material required. We have also introduced the use of the differential alkylation agent *N*-phenylmaleimide to provide a rapid and selective means of thiol alkylation. This particular reagent has greatly assisted in the assignment of disulfide bonds. The work here illustrates a multifaceted approach that is both rapid and reproducible for the analysis of complex, multiply disulfide-bonded peptides and the assignment of disulfide connectivity.

Materials and methods

Milking of Conus striatus

The milking apparatus consisted of a 1.8-mL Eppendorf tube, with the cap removed and replaced with a latex membrane. The modified Eppendorf tube was held in front of the gastropod, *C. striatus*, which was enticed with a live goldfish (*Carassius auratus*) to sting through both the thin tail of the fish and the latex membrane into the Eppendorf tube. The tube was sealed, centrifuged at 10,000*g*, and stored at −20 °C until required.

Analytical RP-HPLC/UV

Milked venom components and synthetic peptides were separated either by C_{18} or C_4 analytical reverse-phase high-performance liquid chromatography/ultraviolet detection (Vydac; 5 μm, 300 A, 4.6 × 250 mm). Eluants were monitored at 214 or 230 nm. Samples were eluted using a standardized linear 1%/min gradient of organic (90/10 CH_3CN/0.08% aq. TFA, Solvent B) against 0.1% aq. TFA (Solvent A) at a flow rate of 1 mL/min.

Direct ESMS infusion

A PE-Sciex API III triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) was used. Samples were directly delivered via a 30-cm, 50-μm-i.d. fused silica capillary (Polymicro Technology, Phoenix, AZ, USA) into the atmospheric pressure ionization source of the mass spectrometer interfaced with a Rheodyne 8125 injector (5 μL external loop; Rheodyne, Cotati, CA, USA). Carrier solvent (0.09% aq. TFA) was provided by either an ABI 140B dual-syringe pump or by a microsyringe infusion pump (Harvard Apparatus, South Natick, MA, USA). Both systems permitted varying flow rates (5–200 μL/min).

With the ionspray interface sprayer component operating at a positive potential of 5.6 kV for the detection of protonated molecular species, ions generated by the ion evaporation process entered the analyzer of the mass spectrometer through the interface plate and subsequently via a 100-μm hole in the sampling orifice (with 60–70 V applied). These two components of the

ionspray apparatus were at progressively lower potentials with respect to the interface sprayer to attract the highly charged ions. To aid desolvation of the charged droplets and to prevent particulate matter entering the analyzer region, a curtain of nitrogen gas (1–2 L/min; Ultra Pure; BOC Gases, Australia) was applied between the interface plate and the sampling orifice. The analyzer region of the mass spectrometer was maintained at $\sim 1 \times 10^{-5}$ Torr by the use of a helium cryopump system (closed-cycle helium refrigerator). Full-scan single MS experiments were typically obtained by scanning quadrupole-3 (Q-3) from m/z 400–2200 in 3–5 s with a scan step size of 0.2–0.5 Da. Data were acquired using Mac Tune v 2.4 (PE Sciex, Thornhill, Ontario, Canada).

MS/MS

For tandem mass spectrometry (referred to as MS/MS) experiments, collision-induced dissociation (CID) of the parent precursor ions was effected by bombardment with argon (Ultra High Pure; BOC Gases). Bombardment was confined to quadrupole-2 (Q-2) with a collision cell gas thickness of 3×10^{14} atoms/cm^2 and a collision energy (Q-0 to Q-2 rod offset voltage) typically set at ~20–40 eV. The resulting CID (daughter ion) spectra were obtained by scanning quadrupole-3 (Q-3) from m/z 50–2400 in 5 s with a step size of 0.2 Da [10]. MS/MS data analysis was assisted with the use of Mac BioSpec v1.01 (PE Sciex).

Conotoxin synthesis

Synthetic α-conotoxin SII was manually assembled on 0.5 mmol scale, using the standard *t*-butoxycarbonyl (*t*-Boc) solid-phase peptide chemistry procedures essentially as described by Steward and Young [10] incorporating the modifications of Alewood et al. [11] and Schnölzer et al. [12,13], using a Boc-Ser-Pam Resin (0.84 eqm/g) to achieve the required C-terminal free acid. Cysteine protection was achieved by the incorporation of 4-methylbenzyl groups (cysteine-4-MeBlz; Peptide Institute, Japan). The rationale for the choice of other side chain protection groups is as discussed by Schnölzer et al. [13]. Average coupling yields were maintained above 99.5% as indicated by ninhydrin analysis. Cleavage of the peptide from the support resin, typically 200–300 mg scale, was effected by HF (20 mL, −5 °C for 1.5 h) with either *p*-thiocresol and/or *p*-cresol (Aldrich, Milwaukee, WI, USA) added as protecting group scavengers (400 μL). The cleaved peptide was precipitated with ether and recovered by acetic acid extraction (20–50% v/v, 25–100 mL) and freeze dried. Cleavage yield was then calculated, followed by C_{18} analytical RP-HPLC/UV analysis of 50 μg of crude material.

Air oxidation

To facilitate the formation of intra-disulfide bonds, synthetic peptides (0.02–0.1 mM) were oxidized in 0.1 M NH_4HCO_3, pH 8.0, stirred continuously, and maintained at room temperature or 4 °C for a period of 5 days. On completion, a small aliquot of oxidized material was analyzed using Ellman's reagent and/or acidified and then freeze-dried followed by direct ESMS infusion. When oxidation was complete, the bulk of the oxidized material was acidified drop-wise with acetic acid (to pH ~4) and freeze-dried prior to C_{18} RP-HPLC/UV purification. In a number of cases 0.33 M NH_4OAc, 0.5 M guanidine–HCl (GnHCl), pH 7.5, was substituted for NH_4HCO_3.

Glutathione redox system

Peptide concentrations of 0.02–0.1 mM in 0.33 M NH_4OAc, 0.5 M GnHCl, pH 7.5, were used. Glutathione-oxidized (GSSG; Sigma, St. Louis, MO, USA) and glutathione-reduced (GSH; BDH Chemicals, Poole, UK) were added 1:10 with respect to peptide concentration, essentially as described by Jaenicke and Rudolph [14]. Oxidizing solutions were stirred continuously at 4 °C for a period of 5 days and analyzed as above.

Disulfide connectivity analysis

To enable the controlled reduction of individual disulfide bonds, 30 mM TCEP (Strem Chemicals, USA) in 25 mM NH_4OAc, pH 4.5, was used. Observation of the extent of reduction was made using real-time analysis via direct ESMS infusion. Typically 1–10 nmol of fully oxidized peptide (in 20–40 μL of 100 mM NH_4OAc, pH 4.5) was partially reduced by the addition of small aliquots (1–3 μL) of the TCEP solution above. The reduction process was assisted by exposure to 60 °C (1–2 min). The reduction of a single disulfide bond was monitored by direct ESMS infusion of ~1 μL of sample and scanning within a narrow mass range (±10 Da) of the mono-isotopic parent molecule, MH^+.

On observing the mass shift in the isotopic distribution profile (+2 Da for the MH^+ for each disulfide reduced) to the stage that represents 50–70% of the sample, the reduction was stopped by acidification (TFA neat, 1:2 v/v). pH was adjusted to 4.5 with 100 mM NH_4OAc (usually 2:1 v/v) and directly alkylated with *N*-phenylmaleimide (typically 50- to 100-fold excess w/w 100 mM in isopropanol; Fluka, Switzerland). The resulting partially alkylated product was rechecked by ESMS and purified by C_4 RP-HPLC/UV. Formula masses of peptides in these fractions were assigned by ESMS. The peptide corresponding to the addition of two alkyl groups to the parent molecule was subjected to Edman degradation.

Further TCEP reduction of the reconstituted, C_4 RP-HPLC/UV purified, partially alkylated product (typically in 20–40 μL of 100 mM NH_4OAc, pH 4.5) was undertaken. On observing a further shift in the molecular mass, extending to 50–70% of the original alkylated sample, the reaction was acid quenched and the pH readjusted to pH 4.5. Alkylation at this stage was via freshly distilled 4-vinylpyridine (stored under argon, neat; Aldrich). The products of this reaction were purified by C_4 RP-HPLC/UV. The product with molecular mass corresponding to the addition of a further two alkylation groups was subjected to Edman degradation. Identification of the variously alkylated Cys-PTH derivates in the sequential chemical degradation of the peptide allowed disulfide assignment.

Muscle-type nicotinic receptor assay

Electrically stimulated muscle twitch was analyzed at the neuromuscular junction in a rat phrenic nerve/hemidiaphragm preparation as previously described [15–17]. Incubations of the muscle tissue with α-conotoxin SII (1–10 μM) in buffer (Krebs Henseleit, pH 7.4) were for 60 min, after which the toxin was removed by washing several times with buffer alone and the preparation was allowed 30 min to recover prior to treatment with the next higher toxin concentration. Data analyses were performed as previously described [16].

Neuronal-type nicotinic receptor assay

The action of α-conotoxin SII on neuronal-type nicotinic receptors was assayed by measuring the nicotine-evoked release of catecholamines from bovine adrenal chromaffin cells as previously described [18]. Catecholamines were separated by HPLC and measured by electrochemical detection [19] after stimulation by nicotine (4 μM) in the presence or absence of α-conotoxin SII.

Results

Characterization of α-conotoxin SII within the milked venom of C. striatus

A fraction (peak 3, R_t 36.1 min; Fig. 1 and Table 2), corresponding to the mono-isotopic molecular mass (MH^+) of α-conotoxin SII, 1790.3 Da (calc. MH^+

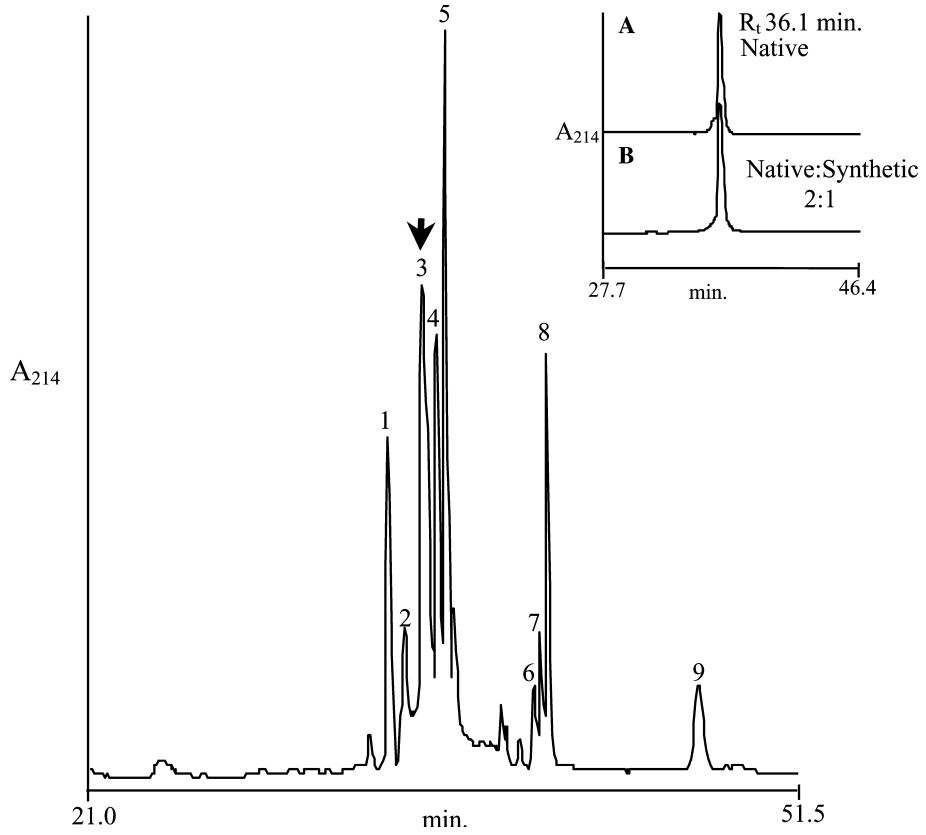


Fig. 1. C_{18} RP-HPLC/UV of pooled milked venom derived from *C. striatus*. The position of eluted α-conotoxin SII is indicated by the arrow at peak 3. Peak numbers refer to Table 2. (Inset) C_{18} RP-HPLC comparison of (A) the native α-conotoxin SII and (B) a 2:1 mixture of native and synthetic α-conotoxin SII. Crude venom was eluted with a standardized RP-HPLC linear gradient 1%/min of 90/10 CH_3CN/0.08% aq. TFA against 0.1% aq. TFA at a flow rate of 1 mL/min. Fractions were monitored at 214 nm and collected by hand for further analysis.

Table 2
RP-HPLC peaks and relative abundance of known conotoxins within the milked venom of *Conus striatus*

Peak	R_t (min)	% Area	Conotoxin[a]
1	32.7	5.6	
2	34.4	2.2	ω-SVIA
3	36.1	9.6	α-SII
4	37.4	13.4	
5	38.2	12.0	κ-SIVA
6	46.8	1.1	
7	47.3	2.6	
8	47.9	7.7	
9	62.6	3.5	

[a] As assigned by ESMS and Edman sequencing (data not shown).

1790.6 Da), was purified (C_{18} RP-HPLC/UV) from the milked venom derived from captive specimens of *C. striatus* and represented 9.6% of the whole milked venom profile by UV. To confirm the assignment of this molecular mass, ESMS analysis was undertaken using native and fully TCEP-reduced C_{18} RP-HPLC/UV-purified materials. This component demonstrated the presence of three disulfide bonds (observed native MH^+ 1790.3 Da; Observed TCEP reduced form MH^+ 1796.3 Da, reduced calc. MH^+ 1796.6 Da). Finally MS/MS analysis was carried out on the fully TCEP-reduced, purified native material to verify this assignment. Fig. 2 illustrates the generated fragment spectrum observed with CID. The observed ions directly correlated to the predicted fragments of the published amino acid sequence of α-conotoxin SII. The sequence data were obtained simultaneously from both N and C termini.

As indicated in Fig. 2, the sequential order of a number of amino acid regions within the native sequence could not be established; however, their combined mass correlated specifically to designated peptide fragments. For example, the observed difference with the ions of *m/z* 777.4 and 988.4 as analyzed with the N-terminal fragmentation assignment is 211 Da, which correlates with the dipeptidyl Pro [MH^+ 97 Da] and Asn [MH^+ 114 Da] in unspecified order (i.e., either -Pro-Asn- or -Asn-Pro-). Purified synthetic TCEP-reduced material was analyzed in the same manner by MS/MS and generated the identical CID fragmentation pattern and assignment observed with the native material (data not shown).

Synthesis of α-conotoxin SII

Independent confirmation for the amino acid sequence of α-conotoxin SII was obtained by our *t*-Boc solid-phase peptide synthesis of the published sequence [32]. Synthetic material was manually assembled (0.5 mmol scale) and aliquots of HF-cleaved C_{18} RP-HPLC/UV-purified material (2 mg) were subjected to a number of test oxidative environments to maximize yield. Typically, as shown in Fig. 3, a number of isomers were produced, these being independent of the four oxidative methods employed. Molecular mass analysis of these individual peaks demonstrated identical molecular composition. Oxidation in 0.1 M NH_4HCO_3 at 4 °C was chosen for a preparative-scale oxidation of cleaved material (30 mg), this being the simplest approach. Analytical C_{18} RP-HPLC/UV of the preparative-scale oxidation demonstrated an abundance of unwanted isomeric material (Fig. 3, Table 3). The major peak, peak (iv) (37% of the total yield by weight; Fig. 3), was identified as the correctly folded material by C_{18} RP-HPLC/UV co-injection of the native (A) and synthetic (B) materials in a 2:1 ratio (Fig. 1, inset)

Effect of α-conotoxin SII on muscle-type nicotinic response

α-Conotoxin SII was active at the neuromuscular junction, inhibiting the electrically stimulated response of the rat phrenic nerve hemidiaphragm preparation. Both pre- and postjunctional parameters were inhibited. A concentration of 5 μM α-conotoxin SII produced complete tetanic fade and 2 μM peptide produced approx. 50% fade. The train of four responses was inhibited to 80% of control level with 5 μM α-conotoxin SII. Tetanic amplitude was reduced to 45% of control levels and single twitches were inhibited to 85% (Fig. 4). Tetanic and train of four fades are measures of presynaptic nicotinic receptors whereas tetanic and twitch amplitude fade are postsynaptic events. These effects were dose dependent and reversible upon washing the muscle preparation with buffer (Fig. 4). The biological activity of the synthetic α-conotoxin SII demonstrated here is consistent with that of the native toxin.

Effect of α-conotoxin SII on the neuronal-type nicotinic response

α-Conotoxin SII had no effect on the release of catecholamines evoked by nicotine (4 μM) from monolayer cultures of bovine chromaffin cells, a neuronal nicotinic receptor preparation, indicating that α-conotoxin SII did not affect the neuronal nicotinic receptor response (data not shown).

Disulfide connectivity analysis of α-conotoxin SII aided by MS analysis

Exposing the α-conotoxin SII (a 2:1 mixture of native and synthetic material) to small aliquots of TCEP followed by direct ESMS infusion MS revealed the parent peptide in various stages of reduction. The proportional distribution of these forms could be varied with increased exposure to TCEP or by the application of heat (see Materials and methods). The progressive reduction

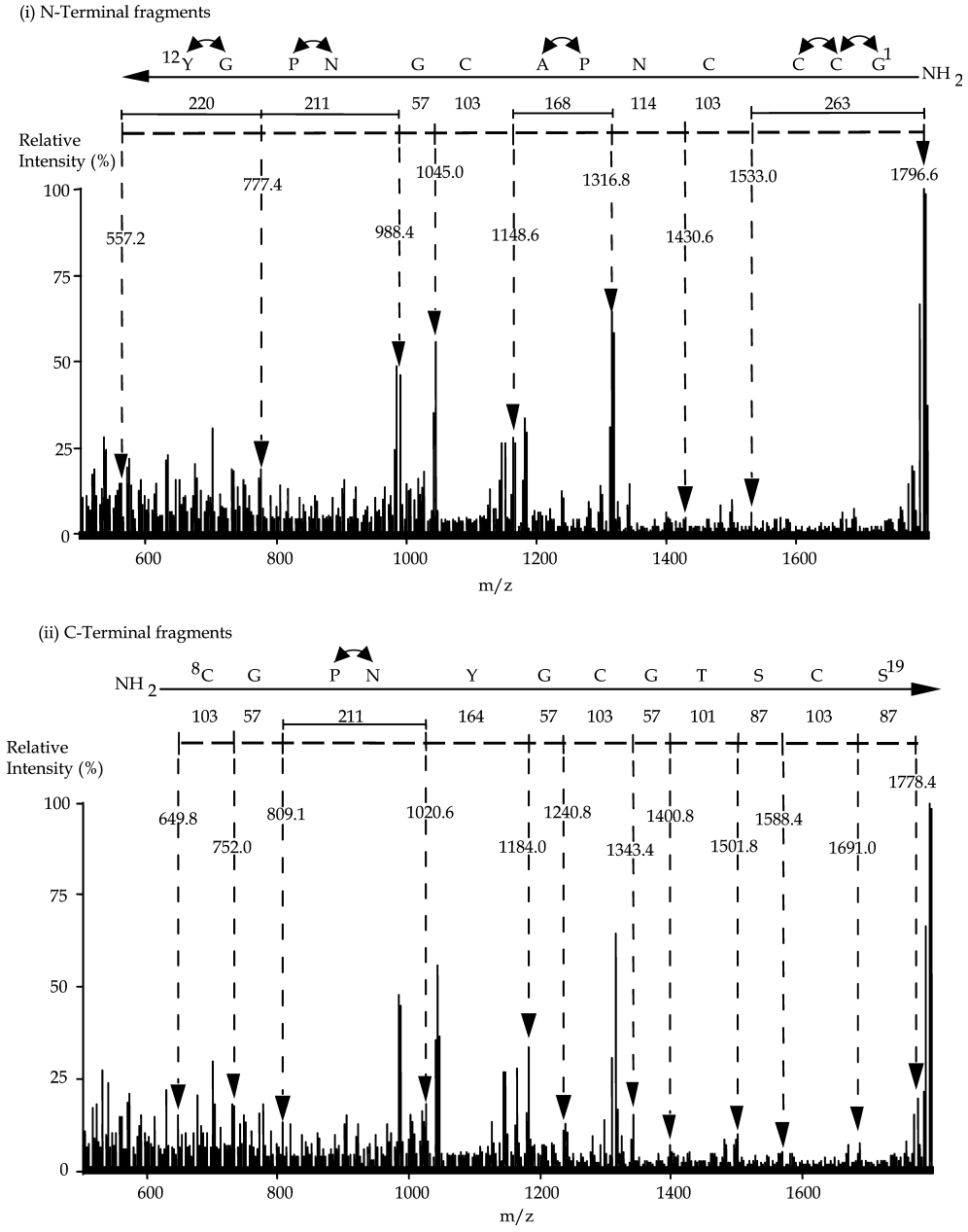


Fig. 2. CID fragmentation spectra of the TCEP-reduced milked-venom-derived peptide that corresponds to the mass of fully reduced α-conotoxin SII (calc. MH^+ 1796.3 Da). Sequence fragmentation ions are generated from both C and N termini simultaneously. Assignment of mono-isotopic peaks is by examination of mass differences between neighboring peaks and correlation with known masses of peptidyl fragments. Ions of interest are labeled, as are the corresponding differences between them. The mono-isotopic mass differences between consecutive ions are assigned as amino acid residue(s). The resulting sequential order of fragments and individual amino acids directly correlates with the published sequence of α-conotoxin SII [38].

of α-conotoxin SII was monitored in real time and was represented by the broadening of the mono-isotopic distribution for the MH^+ ion. The observed parent ion masses for reduced products were MH^+ 1792.2 Da (one disulfide reduced, calc. MH^+ 1792.6 Da), MH^+ 1794.6 Da (two disulfides reduced, calc. MH^+ 1794.6 Da), and MH^+ 1796.3 Da (fully reduced, calc. MH^+ 1796.6 Da). The reaction was stopped at the first appearance of the fully reduced material (MH^+ 1796.3 Da); then reduced materials were alkylated with *N*-phenylmaleimide. C_4 RP-HPLC/UV isolation of the resulting alkylated forms followed by mass analysis and deconvolution demonstrated the presence of three alkylated derivatives, observed average molecular mass (M_r) 2139.9 Da (Compound A), 2486.6 Da (Compound B), and 2835.6 Da (Compound C), as illustrated in Fig. 5, representing two different partially reduced Compounds A and B (one- and two-disulfide bridges in

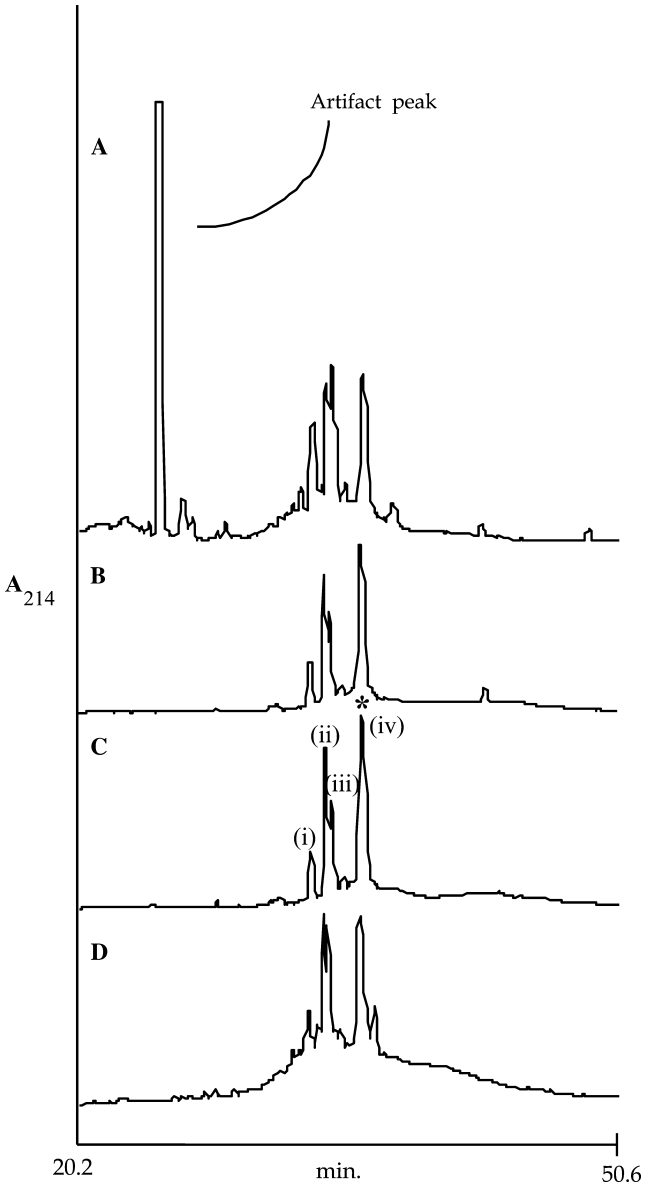


Fig. 3. Oxidation conditions for the preparation of α-conotoxin SII from the fully reduced cleavage product. Test oxidations and C_{18} RP-HPLC profiling of 0.05 mM α-conotoxin SII at (A) 0.33 M NH_4OAc, 0. 5 M GnHCl, GSH:GSSG (10:1), pH 7.5, 4 °C; (B) 0.33 M NH_4OAc, 0.5 M GnHCl, pH 7.5, 4 °C; (C) 0.1 M NH_4HCO_3, pH 8.0, 4 °C; and (D) 0.1 M NH_4HCO_3, pH 8.0, 24 °C. All samples were continuously stirred for 5 days. Asterisk indicates the native-like peptide. Sequential yields of α-conotoxin SII did not significantly differ with changing oxidative environments. Relative abundances of the native form and nonnative isomers are shown in Table 3 from the oxidation using conditions (C). Each profile represents the same quantity of crude oxidized peptide loaded onto RP-HPLC.

Table 3
Identification of oxidized products from α-conotoxin SII

Peak	R_t (min)	% Area	
(i)	33.2	7.2	Isomer 1
(ii)	34.1	17.2	Isomer 2
(iii)	34.3	10.6	Isomer 3
(iv)	36.1	44.2	Native form

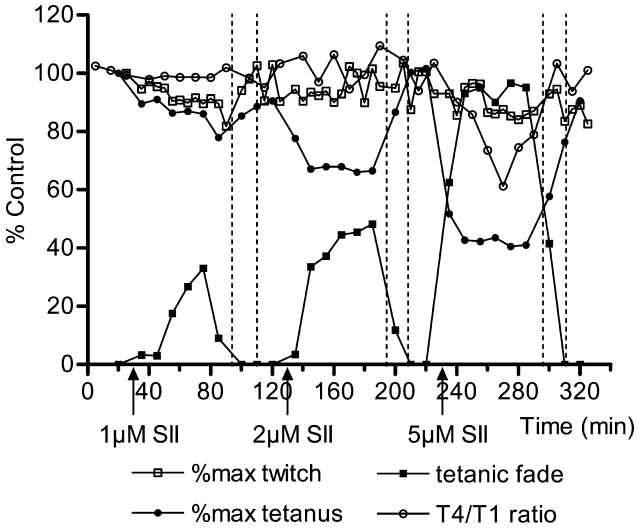
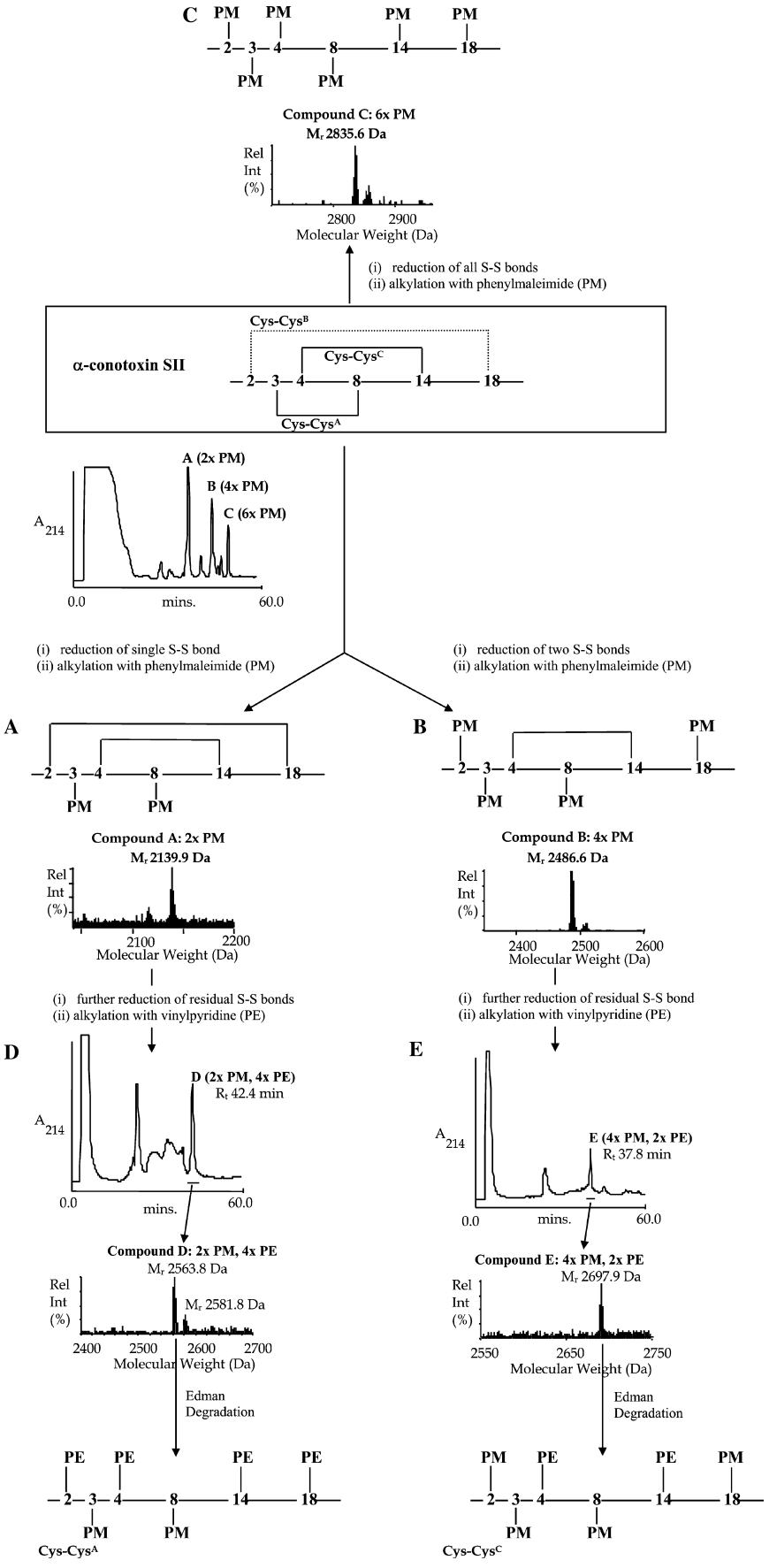
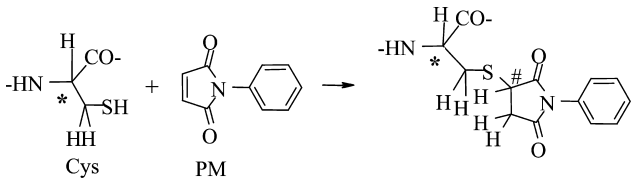


Fig. 4. Biological activity of the synthetic α-conotoxin SII showing time course of action of α-conotoxin SII (1, 2, and 5 μM) on the electrically stimulated rat phrenic nerve hemidiaphragm muscle preparation (assay of muscle-type nicotinic receptor response). The phrenic nerve was stimulated via a bipolar electrode with pulses of 0.5 ms duration and supramaximal voltage. Single twitches were produced by continuous stimulation at 0.1 Hz, interspersed at 5-min intervals by train of four twitches (2 Hz for 1.9 s) or tetanic contractions (50 Hz for 2 s), measured alternately [16]. The effects of α-conotoxin SII on presynaptic (tetanic and train of four fade) and postsynaptic (tetanic and twitch amplitude) measures are shown over the duration of the experiment [15]. The graph shows the concentration dependence of the inhibition and its reversibility following washout (indicated by dashed vertical lines).

α-conotoxin SII reduced and alkylated; calc. M_r 2138.6 and 2486.6 Da, respectively) and the fully reduced and alkylated α-conotoxin SII, Compound C (calc. M_r 2834.6 Da). A mass increase of 173 Da is observed with the addition of each *N*-phenylmaleimide to a free thiol. The product Compound A represented by M_r 2139.9 Da (Fig. 5A)—formed from α-conotoxin SII by reduction of a single disulfide bond (Cys–Cys^A) and subsequent *N*-phenylmaleimide di-alkylation—underwent further TCEP reduction and was then alkylated with 4-vinylpyridine. A mass increase of 105 Da was observed with the addition of each vinylpyridine to a free thiol. This resulted in the production of Compound D with observed M_r 2563.8 Da (Fig. 5D) (calc. M_r 2562.6 Da). This mass was appropriate for the alkylated derivative 2× *N*-phenylmaleimide, 4×4-vinylpyridine-hexahydro-α-conotoxin SII. It was then repurified by C_4 RP-HPLC/UV and subjected to Edman degradation. The resulting Edman sequence on this hexa-alkylated product clearly indicated the presence of *N*-phenylmaleimide-Cys-PTH at cycles 3 and 8, as demonstrated by a unique doublet peak observed in each case within the amino acid PTH RP-HPLC/UV profile. Doublet peaks are a direct result of the addition of the substituted maleimide to the free thiols after partial reduction, producing a new


C
PM PM PM PM
2 3 4 8 14 18
PM PM
Compound C: 6x PM
Mr 2835.6 Da
Rel Int (%)
2800 2900
Molecular Weight (Da)
(i) reduction of all S-S bonds
(ii) alkylation with phenylmaleimide (PM)
α-conotoxin SII
Cys-Cys^B
Cys-Cys^C
Cys-Cys^A
2 3 4 8 14 18
A (2x PM)
B (4x PM)
C (6x PM)
A214
0.0 mins. 60.0
(i) reduction of single S-S bond
(ii) alkylation with phenylmaleimide (PM)
(i) reduction of two S-S bonds
(ii) alkylation with phenylmaleimide (PM)
A
2 3 4 8 14 18
PM PM
B
PM PM
2 3 4 8 14 18
PM PM
Compound A: 2x PM
Mr 2139.9 Da
Rel Int (%)
2100 2200
Molecular Weight (Da)
Compound B: 4x PM
Mr 2486.6 Da
Rel Int (%)
2400 2500 2600
Molecular Weight (Da)
(i) further reduction of residual S-S bonds
(ii) alkylation with vinylpyridine (PE)
(i) further reduction of residual S-S bond
(ii) alkylation with vinylpyridine (PE)
D
D (2x PM, 4x PE)
Rt 42.4 min
A214
0.0 mins. 60.0
E
E (4x PM, 2x PE)
Rt 37.8 min
A214
0.0 mins. 60.0
Compound D: 2x PM, 4x PE
Mr 2563.8 Da
Mr 2581.8 Da
Rel Int (%)
2400 2500 2600 2700
Molecular Weight (Da)
Compound E: 4x PM, 2x PE
Mr 2697.9 Da
Rel Int (%)
2550 2650 2750
Molecular Weight (Da)
Edman Degradation
Edman Degradation
PE PE PE PE
2 3 4 8 14 18
PM PM
Cys-Cys^A
PM PE PE PM
2 3 4 8 14 18
PM PM
Cys-Cys^C



* chiral centre in cysteine residue (retains its S-configuration)

new chiral centre generated in succinimide ring, can be R or S

Fig. 6. Reaction of *N*-phenylmaleimide with free cysteine. Here the production of a new chiral center within the substituted succinimide, *N*-phenylmaleimide (PM), is illustrated after its reaction with cysteine. As the S, R compound is a diastereoisomer of the S, S compound, it is likely to behave differently during RP-HPLC/UV (it is also expected to have other different physical properties, e.g., melting point). This is confirmed with a resolvable doublet peak via Cys-PM's subsequent PTH derivitization and RP-HPLC/UV analysis (not shown). The reactivity of the substituted succinimides provides rapid and sensitive means to selectively alkylate free thiols.

chiral center and therefore resulting in diastereoisomeric products, as illustrated in Fig. 6. This doublet peak feature was also observed for PTH derivatives following thiol alkylation with other substituted maleimide derivatives (e.g., *N*-methylmaleimide, *N*-ethylmaleimide, and *N*-cyclomaleimide; J.-P. Bingham, unpublished data) and provides a sensitive indicator to their presence. Here, their presence at cycles 3 and 8 indicates the presence of a disulfide bond between Cys^3–Cys^8 within the original sequence, this being the first disulfide bond reduced by TCEP, either being the most solvent accessible or the weakest within the parent structure. Edman cycles associated with Cys^2, Cys^4, Cys^{14}, and Cys^{18} demonstrated the expected pyridylethylated-Cys-PTH derivative, indicating their connectivity in an unspecified manner.

In an analogous procedure, Compound B, M_r 2486.6 Da (Fig. 5B), the product of two reduced and *N*-phenylmaleimide alkylated disulfide bonds (Cys–$Cys^{A,B}$), was fully TCEP-reduced and then further alkylated with 4-vinylpyridine. The resulting product, Compound E Fig. 5E of observed molecular mass M_r 2697.9 Da, represented the alkylated product 4× *N*-phenylmaleimide, 2×4-vinylpyridine-hexahydro-α-conotoxin SII (calc. M_r 2698.6 Da). Compound E was purified by C_4 RP-HPLC/UV and subjected to Edman degradation. The resulting sequence indicated the presence of a *N*-phenylmaleimide-Cys-PTH doublet at cycles 2, 3, 8, and 18, while at cycles 4 and 14 the standard single peak PTH-pyridylethylated cysteine derivatives were observed. This pattern of *N*-phenylmaleimide alkylation demonstrated the presence of unspecified disulfide bonds between Cys^3, Cys^8, Cys^2, and Cys^{18}, while the Cys^4–Cys^{14} pyridylethylation pattern illustrated the existence of a single disulfide bond between these residues (Cys–Cys^C), either the least solvent accessible or the most stable disulfide bond present in the original α-conotoxin SII.

These data allow the assignment of the disulfide connectivities Cys^3–Cys^8 and Cys^4–Cys^{14} (bonds Cys–$Cys^{A,C}$, respectively; Fig. 5), with Cys^2–Cys^{18} completing the connectivity assignment (Cys–Cys^B; Fig. 5), consistent with the absence of any free thiols in the original α-conotoxin SII and the measured molecular masses of both the native and the fully reduced α-conotoxin SII. This assignment (Cys–Cys^B) now verifies the connectivity of the third disulfide bond, as hypothesized by Gray [20] and Groebe et al. [21].

Discussion

The conotoxin families include some of the most highly constrained, multiply disulfide-bonded peptides known. The disulfide framework helps them to maintain their specific three-dimensional structure [3]. Of the many published α-conotoxin-like sequences possessing the CC–C–C framework, few have been directly examined with respect to their disulfide connectivity. The main approach for assignment of disulfide connectivity has been NMR spectroscopy and the use of retro-selective synthetic peptide protocols enabling controlled disulfide bond formation (see Table 1).

A number of new α-conotoxin-like sequences from *Conus* have been derived by gene cloning techniques

Fig. 5. Sequential disulfide connectivity assignment for α-conotoxin SII. Partial reduction of α-conotoxin SII produced three peaks: Compound (A) has an observed average molecular mass (M_r) 2139.9 Da, consistent with the reduction of a single disulfide bond and subsequent addition of two *N*-phenylmaleimide residues (PM). Compound (B) has an observed M_r 2486.6 Da, consistent with the reduction of two disulfide bonds and subsequent addition of four *N*-phenylmaleimide residues. Compound (C) has an observed M_r 2835.6 Da consistent with the reduction of all three disulfide bonds and subsequent addition of six *N*-phenylmaleimide residues. Crude partially reduced and alkylated materials were eluted with a standardized C_4 RP-HPLC linear gradient 1%/min of 90/10 CH_3CN/0.08% aq. TFA against 0.1% aq. TFA at a flow rate of 1 mL/min. Fractions were monitored at 214 nm and collected by hand for further analysis. Further reduction and differential alkylation were undertaken on Compounds (A) and (B) to produce Compounds (D) and (E), respectively. Compound (D), the product of two disulfide bond reductions of Compound (A) followed by four vinylpyridine additions, has an observed M_r 2563.8 Da as shown. Edman degradation confirmed this with the identification of *N*-phenylmaleimide-Cys-PTH at positions 3 and 8 (Cys–Cys^A). Other cysteine moieties were identified as the standard pyridethylated PTH derivative. Compound (E), the product of one disulfide bond reduction of Compound (B) followed by two vinylpyridine additions, has an observed M_r 2697.9 Da as shown. Edman degradation verified the presence of *N*-phenylmaleimide-Cys-PTH at positions 2, 3, 8, and 18 together with pyridethylated-Cys at positions 4 and 14 (Cys–Cys^C).

[2,6,23]. Establishing the disulfide connectivity in addition to the pharmacological activity has become essential in classifying these peptides as α-conotoxins. In the past, preliminary classification of newly discovered α-conotoxin-like sequences was often based solely on amino acid sequence homology with the conserved cysteine framework of proven α-conotoxins—a practice that is now unsustainable.

An interesting case in point is a comparison of the α- and λ-conotoxin families (Table 1). Although these two families have distinct pharmacology, the cysteine framework pattern of the λ-conotoxins is identical to that of the well-described and chemically characterized classical α-conotoxins (e.g., α-GI, α-GII, and α-MI). However, the λ-conotoxins are structurally different from the α-conotoxins as a consequence of their disulfide connectivity and they demonstrate little structural similarity to numerous α-conotoxins that have to date been studied by NMR [4,22].

The peptide α-conotoxin-SII is an unusual member of the α-conotoxin family, with its incorporation of an extension to the classical disulfide framework. As with other α-conotoxins it is a nicotinic acetylcholine receptor antagonist; however, in contrast to other α-conotoxins that have two disulfide bonds, α-conotoxin SII with two additional cysteine residues has three disulfide bonds. The additional disulfide link contributes to the overall folding, making α-conotoxin SII the most complex and constrained peptide of this toxin family to date. A number of additional homologues have recently been disclosed [23].

The relative abundance of α-conotoxin SII within the milked venom of *C. striatus* together with our observations of its in vitro pharmacological activity suggest its biological importance in the process of envenomation. Thus further examination and structural assignment of this molecule were important. During synthesis of α-conotoxin SII, a number of problems that are common to the random approach undertaken in the folding of multiply disulfide bonded peptides were encountered—notably the formation of isomeric and/or polymeric materials. In α-conotoxin SII this was exacerbated by the presence of the additional disulfide bridge extending the number of potential isomers to 15. However, only four products were observed, including the native peptide, indicating that only a limited number of outcomes are favored for the oxidation of the reduced conopeptide. There are two consequences of this process: (1) the yield of the desired isomer can be reduced substantially by the formation of other products and (2) unambiguous identification of the correctly folded material is made difficult without access to the native peptide or specific bioassays.

In previous studies [24] optimization of yield of the preferred material has been achieved by trial and error variation of the oxidation conditions (e.g., oxidation in the presence of various ratios of oxidized and reduced glutathione or temperature). However, in the present study these variables had little effect on the production of the preferred native isomer compared to that of other isomers.

With most α-conotoxins, the most abundant synthetic isomer resulting from random oxidation is the "native-like peptide," this being clearly distinguishable from isomeric material(s) that together account for less then 10–25% of the synthetic yield as determined by RP-HPLC/UV. Recently, a number of examples of synthetic α-conotoxin-like peptides that do not oxidize spontaneously to the native-like peptide from their cleaved fully reduced forms have been observed (J.-P. Bingham, unpublished results). This indicates that the native isomer may not always exist at the lowest energy state or may be produced under kinetic control, where folding may be template-directed by the structure of the pre-pro peptide [25]. This template-directed synthesis in nature poses a major limitation to chemical synthesis of putative native conotoxins based solely on genetically derived (or deduced) conotoxin sequences. In such cases, where there is usually no reference peptide available, there is clearly a need for a rapid, reliable, and unambiguous assay to allow disulfide connectivity to be determined. The pharmacological activity of the synthetic compound demonstrated here together with the HPLC data showing its coelution with the native toxin provide essential confirmation that the synthetic isomer used to assign connectivity was identical with α-conotoxin SII.

Although elements of our approach have been used previously in isolation, our use of ESMS to monitor the sequential reduction of disulfide bonds together with the use of two different alkylating agents has provided a powerful improvement on existing protocols. The introduction of TCEP by Burns et al. [26] enabled both partial and full reduction of disulfide bonds of conopeptides and is an excellent reagent for such reductions. This is because it allows controlled partial reduction pivotal to establishing disulfide bond connectivity. Furthermore, TCEP has good stability in aqueous solution and does not react with other common functional groups. Its chemical stability and selectivity give it a clear advantage over reducing reagents such as 2-mercaptoethanol, dithiothreitol, and dithioerythritol that require mixed disulfide participation in the reduction process.

Gray [9,27] demonstrated the application of TCEP for the assignment of disulfide connectivity in conotoxins and echistatin. He selectively reduced individual disulfide bonds capturing the reduced intermediates by alkylation with iodoacetamide and other agents. The process was monitored by RP-HPLC/UV and positional assignments followed Edman degradation (a more recent account is given in [5]). However, the process of

capturing partially reduced intermediates via RP-HPLC/UV required both extensive analysis of the kinetics and optimization of the reaction conditions for each sequential reduction, since the solvent accessibility and energy constraints within the disulfide-containing molecule are unique for each individual compound and each sequential reductive step. These processes are very time consuming and wasteful of peptide.

We perceived a need for a modified protocol to limit "thiol-scrambling" [28] and minimize the consumption of peptide, features that have limited the use of TCEP and its excellent potential in disulfide connectivity analysis. Our refinement of the partial disulfide reduction process in conjunction with ESMS and classical Edman degradation provides rapid and reproducible information not just on disulfide connectivity but also on the sequential order in which disulfide bonds are accessible to a solvent environment. The speed of direct analysis by ESMS following partial reduction, illustrated by our analysis of α-conotoxin SII, provides a direct demonstration of the utility of this approach. An additional benefit is that it can be undertaken with the limited amounts of isolated peptides available, e.g., with milked venom components (J.-P. Bingham, unpublished results). Furthermore, the risk of disulfide scrambling is minimized by the use of substituted maleimides for rapid alkylation of the semireduced products of TCEP reduction whose presence is established in real time by ESMS sampling.

The utility of our protocol extends beyond its application to conotoxins but in this field alone there are estimated to be over 50,000 peptides. This technology has proven useful in the elaboration of disulfide connectivities in a number of other conotoxins (*C. magus*, *C. textile*, *C. pennaceus*) (unpublished). There are in addition other disulfide-containing peptides where pharmacological activity and/or overall structure does not match apparently closely related members of a class. Each such peptide warrants independent investigation of disulfide connectivities. The methodology that we have described allows efficient and rapid disulfide bond assignment essential to this task.

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