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Assignment of the three disulfide bonds in ShK toxin: A potent potassium channel inhibitor from the sea anemone *Stichodactyla helianthus*

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SUMMARY

ShK toxin, a 35-residue peptide isolated from the Caribbean sea anemone *Stichodactyla helianthus*, is a potent inhibitor of the Kv1.3 potassium channel in lymphocytes. The natural toxin contains three disulfide bonds. The disulfide pairings of the synthetic ShK toxin were elucidated as a prerequisite for studies on its structure–function relationships. The toxin was fragmented at pH 6.5 using either thermolysin or a mixture of trypsin and chymotrypsin followed by thermolysin. The fragments were isolated by RP-HPLC and were identified by sequence analysis and MALDI-TOF mass spectrometry. The three disulfides were unambiguously identified in either proteolytic digest: Cys^{3} to Cys^{35}, Cys^{12} to Cys^{28} and Cys^{17} to Cys^{32}. The Cys^{3}-Cys^{35} disulfide, linking the amino- and carboxyl-termini, defines the characteristic cyclic structure of the molecule. A similar disulfide pairing motif is found in the snake venom-derived potassium channel blocker dendrotoxin and the mammalian antibiotic peptide defensins.

INTRODUCTION

ShK toxin is a 35-amino acid residue peptide isolated from the sea anemone *Stichodactyla helianthus* [1]. This toxin is a potent inhibitor of brain potassium channels [1]. Recently, we have succeeded in the chemical synthesis of ShK toxin and in further characterization of its biological properties [2]. In our synthesis report, we determined that ShK toxin blocks the Kv1.3 potassium channel of lymphocytes. In addition, we found that ShK has a potency of more than 20 times that of charybdotoxin, a similar sized toxin isolated from venom of the scorpion *Lieurus quinquestriatus hebraeus*, which has been studied more thoroughly than any other potassium channel toxin [3]. The small size of ShK toxin makes it an ideal molecule for studying structure–function

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R-S-C^3-I-D-T-I-P-K-S-R-C^{12}-T-A-F-Q-C^{17}-K-H-S-M-K-Y-R-L-S-F-C^{28}-R-K-T-C^{32}-G-T-C^{35}

Scheme 1. Amino acid sequence of ShK toxin. The positions of the cysteine residues are indicated.

relationships. Thus, it is desirable to determine its disulfide bonding pattern in order to gain insight into the structure of this molecule.

The toxin contains six cysteine residues, located at positions 3, 12, 17, 28, 32 and 35, which form three intramolecular disulfide bonds [1] (for complete sequence see Scheme 1). In this report, we have determined the disulfide bonding pattern of ShK toxin using a procedure which utilized Edman degradation and MALDI-TOF mass spectrometry on the RP-HPLC purified, proteolytically derived peptide fragments.

MATERIALS AND METHODS

Synthetic ShK toxin was prepared as described by Pennington et al. [2]. Sequencing-grade and HPLC-grade solvents and reagents were obtained from Applied Biosystems (Foster City, CA), *Achromobacter* lysyl endoproteinase from Wako Bioproducts (Richmond, VA) and thermolysin TLCK-α-chymotrypsin and TPCK-trypsin from Boehringer-Mannheim (Indianapolis, IN). All other reagents were of the highest grade commercially available.

Proteolytic cleavages

(i) ShK toxin (60 μg) was dissolved in 0.1 M Tris-HCl, pH 8.5, containing 2 M urea (60 μl) and was digested with lysyl endoproteinase (E : S = 1 : 50 w/w, 30 °C). Aliquots (1.5 μl) of the reaction mixture were withdrawn at time intervals from 20 min to 29 h, acidified in 0.1% aqueous TFA (50 μl) and the ShK fragments were purified by RP-HPLC and characterized as described below.

(ii) ShK toxin (15 μg) was dissolved in 0.05 M HEPES, pH 6.5, containing 10 mM $CaCl_2$ (30 μl) and was digested with thermolysin (E : S = 1 : 20, w/w, 25 °C, 3.5 h), or with a mixture of trypsin and chymotrypsin (E : S = 1 : 1 : 50, w/w, 30 °C, 6 h). The digestion was terminated by acidification with 10% aqueous TFA (3 μl), and the solution was centrifuged (13 000 g, 5 min). The supernatant was directly fractionated by RP-HPLC. Selected HPLC-purified tryptic–chymotryptic peptides of ShK toxin were reconstituted in 0.05 M HEPES, pH 6.5, and 10 mM $CaCl_2$ and were subdigested with thermolysin (E : S = 1 : 150, 25 °C, 2 h) before fractionation by RP-HPLC.

RP-HPLC

The peptides were fractionated using a microbore RP-HPLC system consisting of Applied Biosystems 140A pumps and a 1000S diode-array detector (2.3 μl flow cell, 0.0025 inch i.d. tubing). Fractionation of the thermolytic and tryptic–chymotryptic peptides was performed on a Zorbax-SB C_{18} column (1 × 150 mm, d_p ~ 5 μm, Microtech Scientific, Saratoga, CA) equilibrated in 0.1% aqueous TFA, and eluted at a flow rate of 80 μl/min using a linear gradient of acetonitrile/water/TFA (80 : 20 : 0.1). The column effluent was monitored at 215 nm. The thermolytic peptides, generated by subdigestion of the tryptic–chymotryptic peptides, were reconstituted in 0.1% heptafluorobutyric acid (HFBA) and further purified on the same column, equilibrated in 0.1% aqueous HFBA, using a linear gradient of acetonitrile/water/HFBA (80 : 20 : 0.1). The column eluent was manually collected and stored at −20 °C until analysis.

Sequence analysis

Automated Edman degradation of the peptides was performed on Applied Biosystems pulsed-liquid 477A/120A, gas phase 470A/120A, and 491A/140S Procise sequencing systems, as described previously [4]. Reagent 3 (TFA) and reagent 4 (25% aqueous TFA) contained 0.002% dithiothreitol. HPLC separation of the PTH amino acids was performed on-line. The solvent system for PTH separation [4] was modified by replacing sodium acetate, pH ~3.95 in solvent A (3.5%, v/v, aqueous tetrahydrofuran) with the Premix buffer

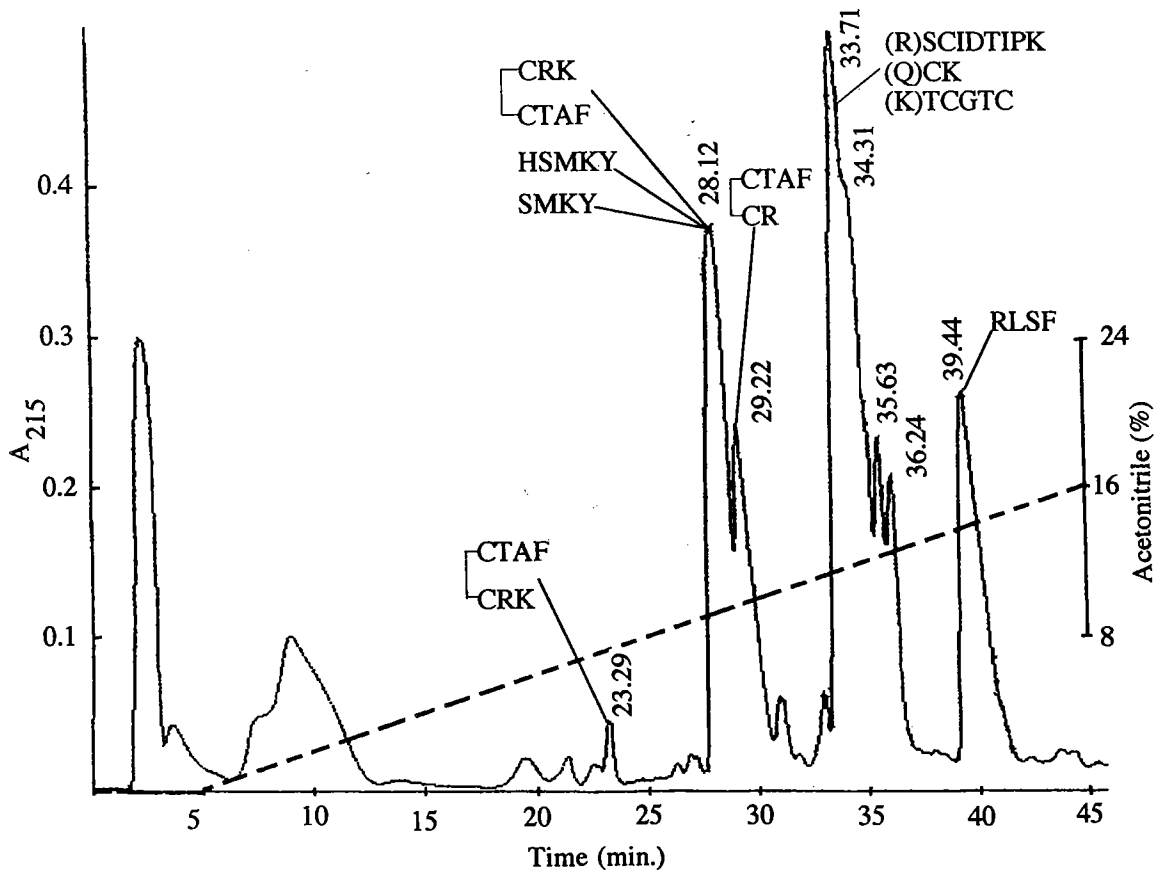

Fig. 1. RP-HPLC profile of chymotryptic–tryptic digest of ShK toxin at pH 6.5. Residues in parentheses represent N-terminally truncated species also present.

(13.5 ml/l). On all three sequencer systems, di-PTH-Cys was recovered and identified as the peak coeluting with PTH-Tyr [5,6]. PTH-Tyr coelution with diPTH-Cys was not problematic, since none of the disulfide-linked peptides contained tyrosine. In addition to diPTH-Cys, PTH-Ser' (PTH-dehydroalanine adduct with DTT) and PTH-Ser (formed by rehydration of PTH-dehydroalanine) were also present as side products in the cycles containing cystine (see Ref. 4 for details).

MALDI-TOF mass spectrometry

The peptides were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI) using a Kratos KOMPACT MALDI III mass spectrometer (Manchester). Each fraction (0.3 μl) was spotted on a target site of a 20-sample slide, followed by addition of 0.3 μl matrix (saturated α-cyano-4-hydroxycinnamic acid; Aldrich, Milwaukee, WI) dissolved in 1 : 1 ethanol/water. The sample matrix was allowed to dry at room temperature for 5 min. Each sample was desorbed with 50 laser shots, each giving a spectrum. The shots were averaged to give the final spectrum. The instrument was calibrated using external standard peptides.

FAB mass spectrometry analysis

FAB-MS analysis of synthetic ShK toxin was performed by M-Scan (West Chester, PA) on a ZAB 2-SE high-field mass spectrometer. The

sample was dissolved in 5% AcOH and a matrix of *m*-nitrobenzyl alcohol was used. A cesium ion gun was used to generate ions for the spectra, which were recorded using a PDP 11-250J data system. Mass calibration was performed using CsI.

RESULTS

Due to the limited amount of natural material available, the disulfide bonds of the synthetic ShK toxin were determined. The chemical identity and biological activity of the synthetic toxin were found to be identical to those of the natural material [2].

FAB mass spectral analysis of synthetic ShK toxin determined an average mass of 4055 ± 1. The calculated mass for ShK toxin is 4061, a difference of six mass units indicating that all six cysteine residues formed disulfide bonds. The absence of free sulfhydryls was confirmed using Ellman's reagent [7]. We determined that less than 0.1% free sulfhydryls are titratable in ShK toxin dissolved in 0.1 M sodium phosphate, pH 7.6, or in the same buffer containing 4 M urea.

The six cysteine residues in the ShK toxin amino acid sequence can form three intramolecular disulfide bridges in 15 different ways. Therefore, the ShK polypeptide was cleaved into fragments using different proteases and the disulfide-linked peptides were purified and identified by sequencing and mass spectrometry.

In the initial experiments, ShK was cleaved at the carboxyl side of its four lysine residues using lysyl endoproteinase. HPLC analysis of the time course of the cleavage revealed rapid formation ($\tau < 20$ min) of a single predominant species (data not shown). Sequence analysis of this material identified the presence of four ShK fragments, [1–9], [10–18], [23–30] and [31–35], in approximately equimolar initial sequencing yields; mass analysis identified a species of mass 3624.9. We concluded that this is the disulfide-linked cleaved ShK that is missing the HSMK tetrapeptide [19–22] (expected mass = 3625.26). On prolonged incubation ($\tau = 2$ h to 24 h, pH 8.5, 25 °C), this pep-

TABLE I
IDENTIFICATION OF PEAKS GENERATED BY CHYMOTRYPTIC–TRYPTIC AND THERMOLYTIC DIGESTION OF SYNTHETIC ShK TOXIN

Disulfide bridge	ShK position	Sequence found by Edman degradation	MH^+ observed[a]	MH^+ MW
C^1-C^6	(C^3-C^{35})	RSC TC	585.2	584.69
C^2-C^4	(C^{12}-C^{28})	IDTIPKSRC FCRK	1585	1582.93[b]
		CTAF CR	715.9	715.87
C^3-C^5	(C^{17}-C^{32})	KTC QCK	727.0	725.90
		FQCKH TCG	944.0	939.11[b]
		KTCG QCK	783.7	782.95
		TCG QCK	652.2	654.80
C^3 C^1 C^5 C^6	{C^{17} C^3} {C^{32} C^{35}}	QCKH RSC TCGTC	1360.9	1358.52[b]

[a] MH^+ = protonated molecular ion = (mass/charge) + H^+. In all sequences the charge was one.
[b] Peptide derived from thermolysin digest of ShK toxin.

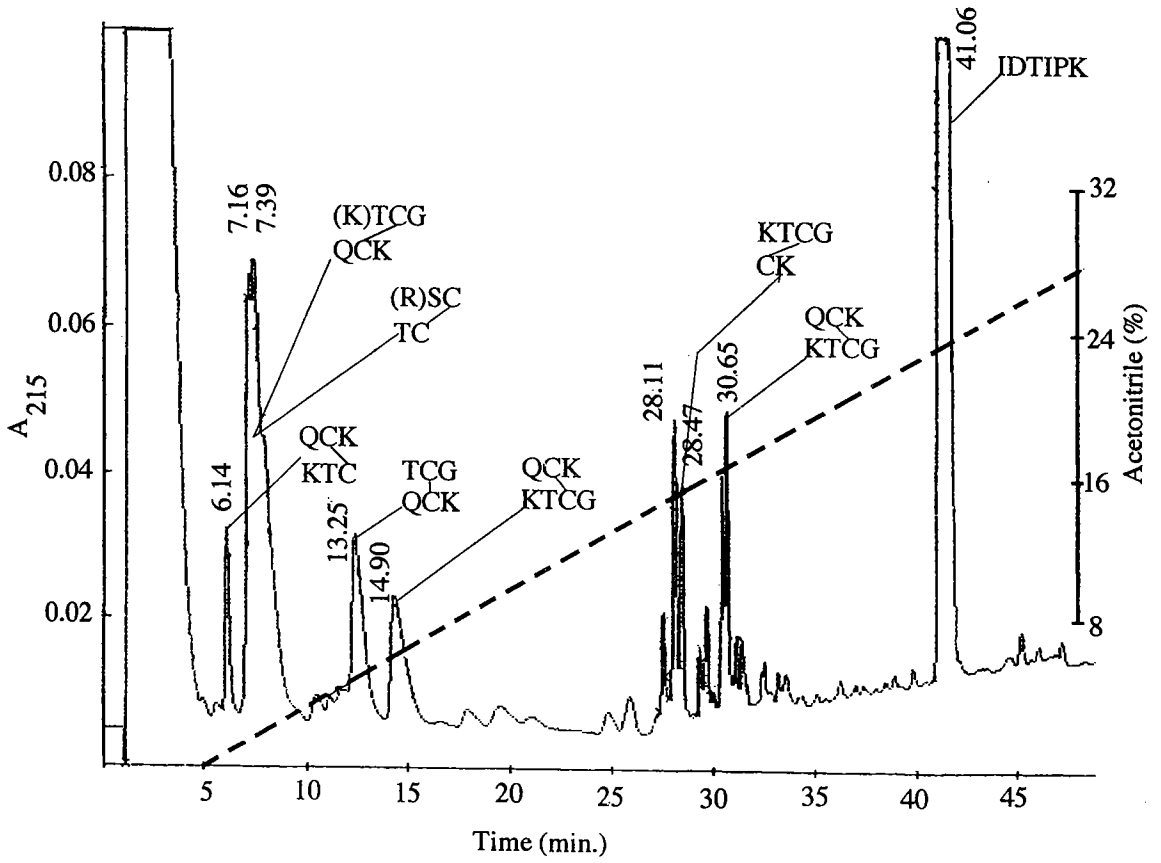

Fig. 2. RP-HPLC profile of thermolysin digest (pH 6.5) of the tryptic–chymotryptic disulfide cluster (33.71 min peak in Fig. 1). Residues in parentheses represent N-terminally truncated species also present.

tide cluster gradually disappeared from the chromatograms and was replaced by multiple peptides which were mostly less retained on the reversed-phase column (not shown). These fragments were later identified as being either pure [1–9], [10–18], [23–30] and [31–35], or their disulfide-linked combinations. Therefore it could be concluded that, at an alkaline pH of 8.5, extensive disulfide interchange occurred (see, e.g., Ref. 8); lysyl endoproteinase was not used in subsequent experiments.

In order to minimize disulfide rearrangement, the following experiments were conducted at pH < 7. ShK toxin was completely resistant to proteolysis (3.5 h, 30 °C) by porcine pepsin A (E : S = 1 : 300, pH 3.0), pancreatic elastase (E : S = 1 : 20, pH 6.5) and subtilisin (E : S = 1 : 20, pH 6.5) as determined by HPLC and sequence (pepsin A digest) analysis. However, the toxin was readily cleaved at pH 6.5 with a mixture of trypsin–chymotrypsin (Fig. 1), or with thermolysin alone (not shown). The Cys^{12}-Cys^{28} disulfide was identified through isolation of CTAF-CR(K) [12–15]/[28–29(30)] from the combined tryptic–chymotryptic digest (Fig. 1 (peaks at 23.29 and 28.12 min*); Table 1). In addition, the disulfide cluster [1–9]/[16–18]/[30–35] and its truncated versions were also purified and identified by sequencing. In the

*For unknown reasons, peptide fragments with the same sequence were observed with different retention times by RP-HPLC. We have observed this phenomenon in many different protease digest experiments with other proteins (J. Pohl, unpublished observation).

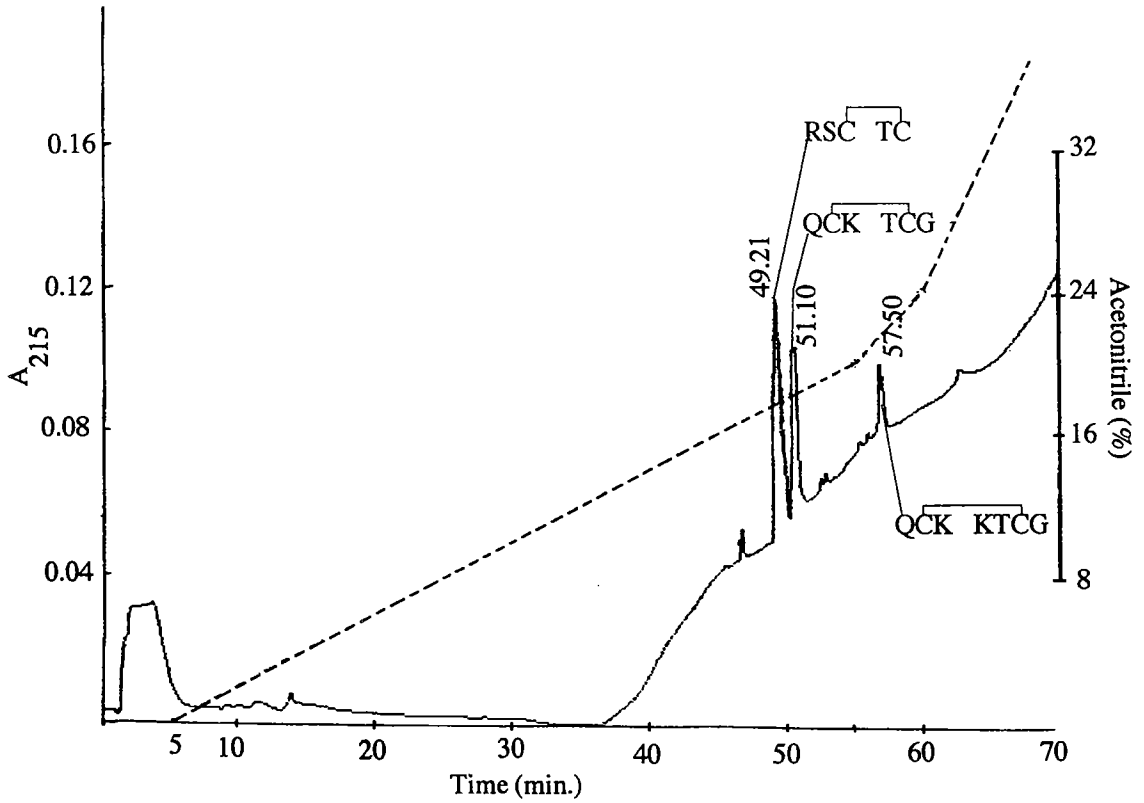

Fig. 3. RP-HPLC purification of the early eluting fragments (7–9 min peak in Fig. 2) derived from the thermolytic digest of the disulfide cluster peak at 33.71 min derived from the tryptic-chymotryptic digest. Gradient conditions are as indicated, using HFBA as the ion-pairing reagent.

combined tryptic–chymotryptic digest, the ShK sequence was identified with the exception of the polar tryptic dipeptide [10–11], Ser-Arg, which likely eluted early in the gradient. In order to identify the remaining two disulfides, the [1–9]/[16–18]/[30–35] cluster was subdigested with thermolysin and the peptides were purified (Fig. 2). Owing to the facile cleavage of the ~Gly^{33}-Thr^{34}~ peptide bond by thermolysin, the closely spaced Cys^{32} and Cys^{35} residues were separated, and the Cys^{17}-Cys^{32} disulfide could be identified in several pure peptides (Fig. 2, Table 1). Sequencing of the material eluting between 7 and 9 min indicated the presence of disulfide-linked peptide(s); however, we failed to obtain any mass information on this mixture. In a subsequent experiment, substantially stronger retention of these peptides was induced, and their complete resolution was in turn accomplished by chromatography of the mixture in solvents containing HFBA as the counterion

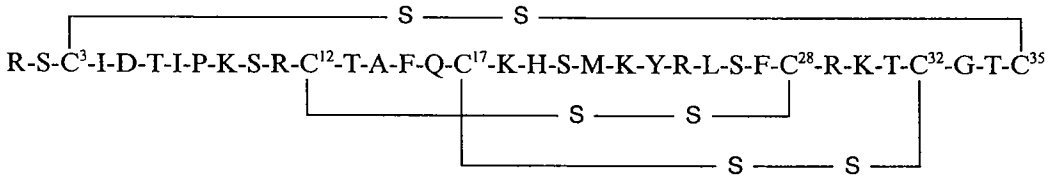

Fig. 4. Schematic representation of ShK disulfide pairings.

(Fig. 3, Table 1). The third disulfide, Cys^3-Cys^{35}, could be identified and unambiguously assigned.

In a separate experiment, ShK toxin was cleaved with thermolysin alone at pH 6.5, and the peptides were separated and identified (Table 1). The Cys^{12}-Cys^{28} and Cys^{17}-Cys^{32} disulfides were identified as pure entities. The Cys^3-Cys^{35} disulfide-linked peptide [1–3] / [34–35] was identified by sequencing. However, as it was present in the mixture with other short peptides, no attempt was made to obtain it in the pure form.

DISCUSSION AND CONCLUSIONS

Figure 4 shows the arrangement of disulfide bonds in ShK toxin. This is the first sea anemone potassium channel toxin in which the disulfide bonds have been assigned. A common structural element found in peptide toxins and protease inhibitors is a disulfide pairing arrangement which creates a knotted-type structure. In many cases, molecules with totally different biological properties have the same disulfide pairing array and tertiary structural elements [9]. In fact, some toxins, such as α-dendrotoxin, have nearly identical crystal structures as some protease inhibitors, such as bovine pancreatic trypsin inhibitor [10]. The disulfide bonds are essential in creating this type of structural identity. The knotted arrangement creates a very compact structure, orienting the disulfide bonds to the internal hydrophobic domain of the folded molecule. The disulfide pairings of ShK toxin do not fit into the cystine knot triple-strand β-sheet structural motif described by Pallaghy et al. [9]. The arrangement present in ShK still appears to create a 'knotted' type of configuration about the Cys^{12}-Cys^{28} and Cys^{17}-Cys^{32} pairings. Additionally, the other disulfide bond creates one large loop (Cys^3 to Cys^{35}), giving ShK toxin a cyclic structure as well.

Interestingly, if the cysteine residues are numbered consecutively relative to their occurrence in the peptide sequence, the same disulfide bonding pattern of C^1-C^6, C^2-C^4 and C^3-C^5 found in ShK toxin is present in the dendrotoxins, peptide potassium channel blockers derived from snake venom [11], as well as in the antibiotic peptide defensins [12]. It will be interesting to compare the solution structures of these molecules to observe whether any common structural motif arises for this pairing arrangement.

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