

Racemic and Quasi-Racemic X-ray Structures of Cyclic Disulfide-Rich Peptide Drug Scaffolds**

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Abstract: Cyclic disulfide-rich peptides have exceptional stability and are promising frameworks for drug design. We were interested in obtaining X-ray structures of these peptides to assist in drug design applications, but disulfide-rich peptides can be notoriously difficult to crystallize. To overcome this limitation, we chemically synthesized the L- and D-forms of three prototypic cyclic disulfide-rich peptides: SFTI-1 (14-mer with one disulfide bond), cVc1.1 (22-mer with two disulfide bonds), and kB1 (29-mer with three disulfide bonds) for racemic crystallization studies. Facile crystal formation occurred from a racemic mixture of each peptide, giving structures solved at resolutions from 1.25 Å to 1.9 Å. Additionally, we obtained the quasi-racemic structures of two mutants of kB1, [G6A]kB1, and [V25A]kB1, which were solved at a resolution of 1.25 Å and 2.3 Å, respectively. The racemic crystallography approach appears to have broad utility in the structural biology of cyclic peptides.

Highly constrained peptides show exciting potential in drug discovery applications.^[1] Of particular interest are highly constrained peptides that incorporate disulfide bonds, and are additionally stabilized by a macrocyclic backbone (Table 1). The combination of two types of topological constraints (backbone cyclization and internal cross-linking) underpins the remarkable stability of these peptides and hence their potential as drug design scaffolds.^[2] We and others have used these scaffolds for stabilizing bioactive peptide epitopes through the emerging design paradigm of molecular grafting, as highlighted in recent reviews,^[2–4] with applications including inflammatory pain,^[5] multiple sclerosis,^[6] and cancer.^[7]

Here we focus on three scaffolds. One of the smallest naturally occurring cyclic disulfide-rich peptides is sunflower trypsin inhibitor-1 (SFTI-1), a peptide consisting of 14 amino acids and containing a single disulfide bond (Cys^I-Cys^{II}), and

the most potent known peptide inhibitor of trypsin.^[8] We have also explored cyclic disulfide-rich peptides that are chemically re-engineered from natural acyclic peptides.^[2] Cyclic Vc1.1 (cVc1.1) is a 22-residue peptide containing two disulfide bonds (Cys^I-Cys^{III} and Cys^{II}-Cys^{IV}) and is re-engineered from

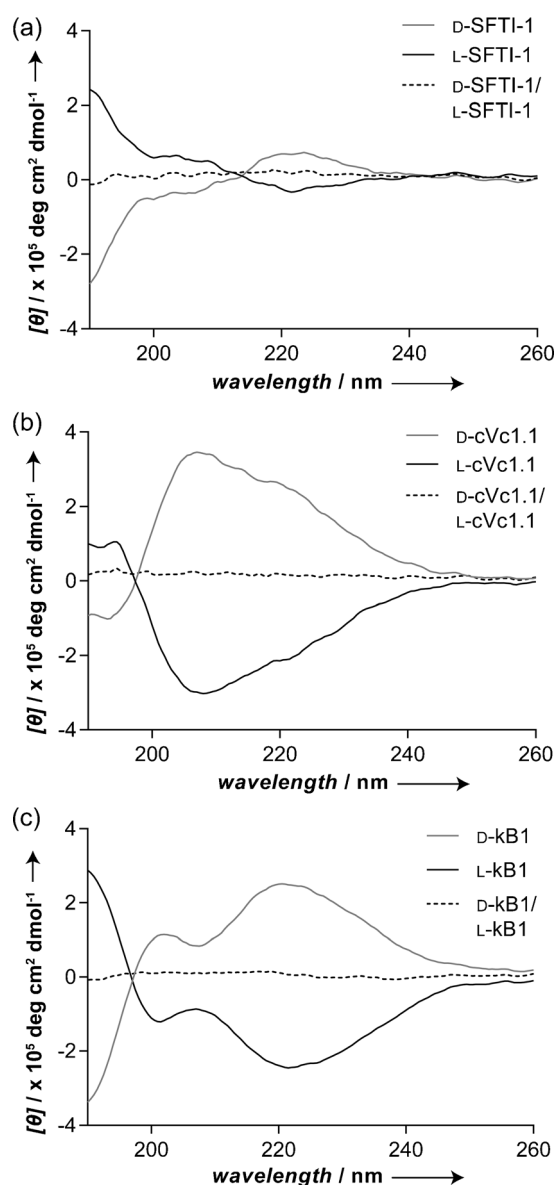


Figure 1. Circular dichroism (CD) spectra of L- and D-enantiomers of SFTI-1, cVc1.1, and kB1. CD spectra of the two a) SFTI-1 enantiomers, b) cVc1.1 enantiomers, and c) kB1 enantiomers in water. CD spectra for the two enantiomers of each peptide mixed together in equal ratios are also shown.

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Table 1: Sequences and activity of cyclic disulfide-rich peptides studied in this work.

Peptide (SS bonds)	Sequence ^[a]	Peptide Class	Activity/Applications
SFTI-1 (1 SS)	<i>cyclo</i> (GR ^I C ^I TKSIPPIC ^{II} FPD) loop 1 loop 2	BBI-like trypsin inhibitor (<i>H. annuus</i>)	trypsin inhibition, ^[8] drug design scaffold
cVc1.1 (2 SS)	<i>cyclo</i> (GC ^I C ^{II} SDPRC ^{III} NYDHPEIC ^{IV} GGAAGG) loop 1 loop 2 loop 3	Conotoxin (<i>synthetic</i>)	oral activity against neuropathic pain, ^[9] drug design scaffold
kB1 (3 SS)	<i>cyclo</i> (GLPV ^I C ^{II} GETC ^{III} IVGGTC ^{IV} NTPGC ^V IVTC ^{VI} SWPVC ^{VI} TRN) loop 1 loop 2 loop 3 4 loop 5 loop 6	Cyclotide native (<i>O. affinis</i>)	pesticidal, ^[11] anti-HIV, ^[13] other activities, ^[12,14–15] drug design scaffold
[G6A]kB1 (3 SS)	<i>cyclo</i> (GLPV ^I C ^{II} AETC ^{III} IVGGTC ^{IV} NTPGC ^V IVTC ^{VI} SWPVC ^{VI} TRN) loop 1 loop 2 loop 3 4 loop 5 loop 6	Cyclotide (<i>synthetic</i>)	drug design scaffold
[V25A]kB1 (3 SS)	<i>cyclo</i> (GLPV ^I C ^{II} GETC ^{III} IVGGTC ^{IV} NTPGC ^V IVTC ^{VI} SWPAC ^{VI} TRN) loop 1 loop 2 loop 3 4 loop 5 loop 6	Cyclotide (<i>synthetic</i>)	drug design scaffold

[a] Cysteines are labeled with Roman numerals. The residues that have been mutated in [G6A]kB1 and [V25A]kB1 are shaded.

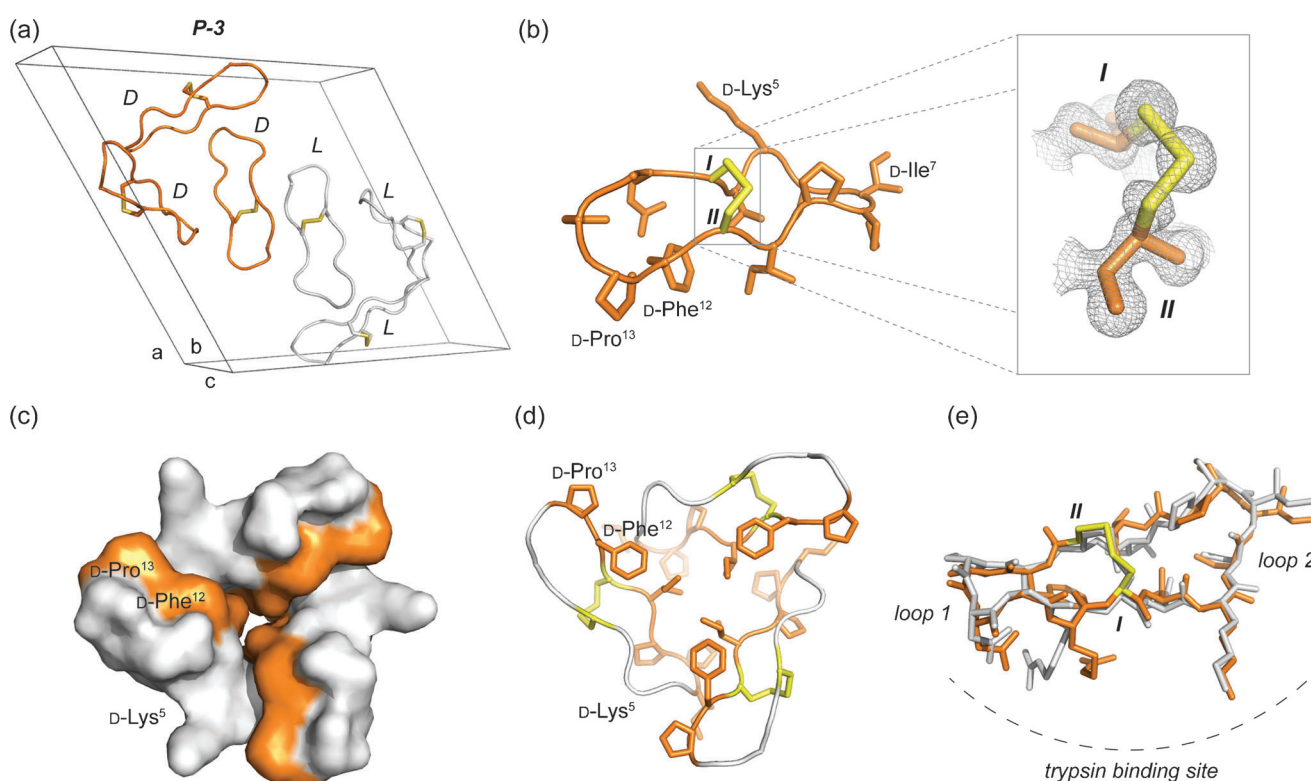


Figure 2. Racemic crystallography of SFTI-1. a) Unit cell of the true racemate in space group $P\bar{3}$ with ribbon representations of the cyclic peptide molecules. The D-enantiomers (in orange) and the L-enantiomers (in grey) are labeled. The disulfide bonds are shown in yellow as stick representations. b) Ribbon representation of the D-SFTI-1 (orange) with the disulfide bond shown (yellow). Inset: $2F_o - F_c$ electron density map of the Cys^I-Cys^{II} disulfide bond shown contoured at a σ level of 1.0. c) Surface representation of the trimer of D-SFTI-1 with the hydrophobic residues colored in orange. d) Ribbon representation of the trimer of D-SFTI-1 with the side chains of hydrophobic residues shown in orange. e) Superposition of L-SFTI-1 determined here (in orange) with the trypsin-bound structure (in grey; PDB ID: 1SFI).

a cone snail venom peptide.^[9] Orally delivered, it shows activity in a rat model of neuropathic pain and is more than two orders of magnitude more potent than gabapentin, the current leading oral therapy for neuropathic pain.^[9] Kalata B1 (kB1) is the prototypical and one of the most well-studied cyclotides, a family of naturally occurring cyclic disulfide-rich peptides that are characterized by a knotted disulfide bond

arrangement (Cys^I-Cys^{IV}, Cys^{II}-Cys^V, and Cys^{III}-Cys^{VI})^[10] and a wide variety of biological activities,^[11–16] with many potential agrochemical and pharmaceutical applications.

In view of the growing interest in cyclic disulfide-rich peptides, we sought to obtain high-resolution structural information for the three prototypical scaffolds, SFTI-1, cVc1.1, and kB1. However, crystallization of small peptides

is notoriously challenging. A robust method that can be used to facilitate the elucidation of high-quality atomic resolution structures would therefore be a valuable tool. Herein, we explored the emerging approach of racemic crystallography, which has been used for overcoming the crystallization “bottleneck” for a range of proteins,^[17–22] but has not yet been applied to cyclic disulfide-rich peptides. Using this approach, we obtained high-resolution structures of a range of cyclic disulfide-rich peptides with a varying number of disulfide bonds and amino acids.

We took advantage of modern approaches for chemical synthesis, cyclization, and oxidative folding of cyclic disulfide-rich peptides, which have generally made the production of large amounts of pure L- and D-peptides affordable.^[23] Synthesis of SFTI-1, cVc1.1, and kB1, as well as their mirror-image forms was carried out using reported procedures with slight modifications (for details, see the Supporting Information). The mirror-image symmetry of the D-peptide forms with their respective L-peptide forms was confirmed in circular dichroism spectra, which showed equal but opposite optical rotation of each enantiomeric peptide pair. When each pair of isomers was mixed in equal proportions, the circular dichroism signal of one of the isomers was cancelled by the other (Figure 1).

The higher probability of obtaining crystals from racemic mixtures compared to the L-form alone has a sound theoretical basis that is supported by several recent experimental

observations.^[24] We were able to obtain diffraction-quality crystals and the crystal structures were determined using the molecular replacement method (see the Supporting Information, data collection and refinement statistics are given in Table S1). The final refined models have been deposited in the Protein Data Bank with the codes 4TTK (SFTI-1 racemate), 4TTL (cVc1.1 racemate), 4TTM (kB1 racemate), 4TTN ([G6A]kB1 quasi-racemate), and 4TTO ([V25A]kB1 quasi-racemate).

The X-ray structure of the SFTI-1 true racemate was solved in the centrosymmetric space group $P\bar{3}$ (Figure 2a). This result was initially surprising, because theoretical calculations predict that $P\bar{1}$ is the most likely space group for obtaining racemate structures^[24,25] and, also, most current examples of protein/peptide racemate structures have been solved in space group $P\bar{1}$,^[20,21,26–31] with $P1$,^[17,32] $P2_1/c$,^[33] and $I41/a$ ^[34] being the other reported examples. The asymmetric unit cell contains a single molecule of the D-form of SFTI-1 (Figure 2b), which adopts a compact tertiary fold that is identical to its inverted L-form. The structures of both D- and L-forms of SFTI-1 are stabilized by a series of hydrogen bonds (e.g., D-Phe¹² HN to D-Arg² CO, D-Thr⁴ HN to D-Ile¹⁰ CO) and a disulfide bond (Cys^I-Cys^{II}, Figure 2b inset) that compresses the overall topology of the cyclic peptide. It has been shown that both the disulfide bond and the cyclic backbone are required to maintain the potent trypsin-inhibitory activity of SFTI-1 and its resistance to proteoly-

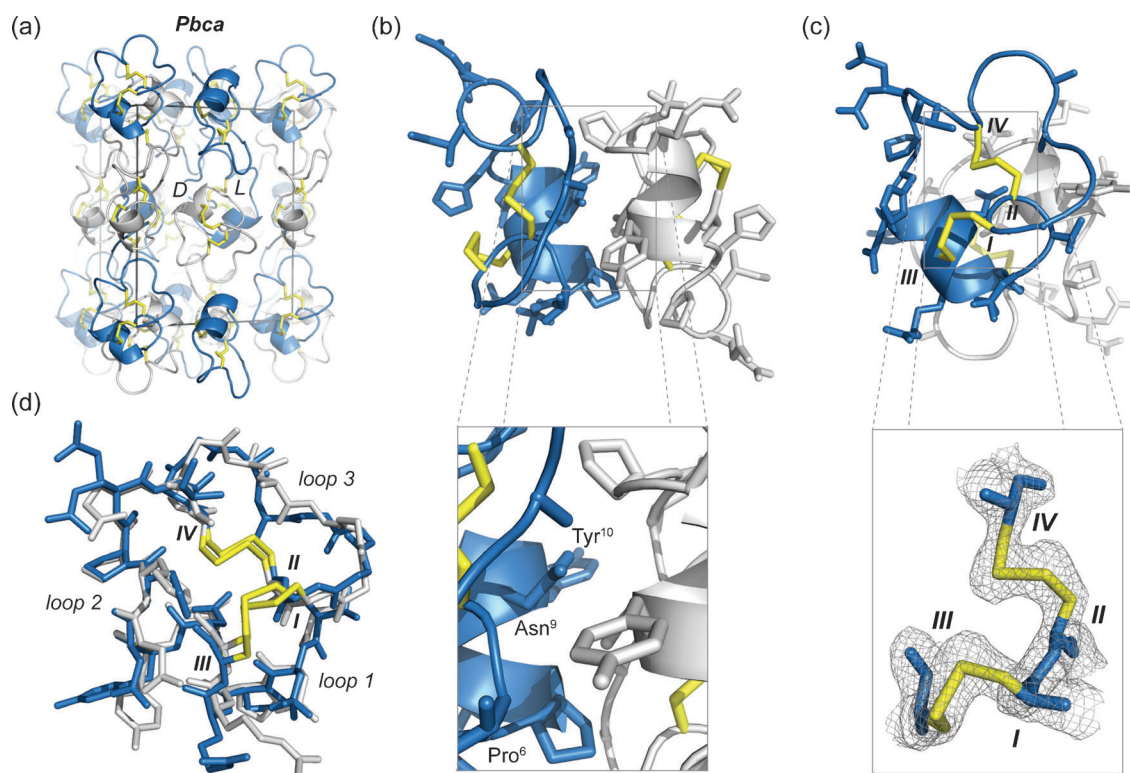


Figure 3. Racemic crystallography of cVc1.1. a) Unit cell of the true racemate in space group $P6_3$ with ribbon representations of the cyclic peptide molecules. A pair of the L-enantiomer (in blue) and D-enantiomer (in grey) is labeled. The disulfide bonds are also shown as stick representations in yellow. b and c) Ribbon representations of L-cVc1.1 (blue) and D-cVc1.1 (grey). Inset of b: the packing interface of the D- and L-enantiomers; inset of c: $2F_o - F_c$ electron density map of the Cys^I-Cys^{III} and Cys^{II}-Cys^{IV} disulfide bonds shown contoured at a σ level of 1.0. d) Superposition of L-cVc1.1 (blue) with the solution NMR structure of L-cVc1.1 (grey). The cysteines are labeled with Roman numerals and the loops are also labeled.

sis.^[35] Interestingly, the unit cell contains three molecules of each enantiomer as shown in Figure 2a; each triplet of enantiomers forms a supramolecular complex that has three-fold symmetry and is stabilized by hydrophobic interactions, involving residues D-Ile¹⁰ and D-Phe¹² for the trimer of D-forms, for example, as shown in Figure 2c and d. To our knowledge, this is the first description of a possible supramolecular complex of SFTI-1, and we postulate that this quaternary structure might have implications for its storage state, and possibly its long-term stability, in sunflower seeds. Figure 2e shows a superposition of our structure of L-SFTI-1 with the structure of the trypsin-bound L-SFTI-1 (the only other reported X-ray structure of SFTI-1), showing that the overall fold is maintained, though deviations in the precise positions of several atoms are observed. Generally, this result highlights the rigidity of the SFTI-1 fold and also provides validation of the racemic crystallography approach for obtaining high-resolution structures of cyclic disulfide-rich peptides.

To examine whether racemic crystallography can be applied to other cyclic disulfide-rich peptides, we elucidated the X-ray structure of the cVc1.1 true racemate, which was solved in space group *Pbca*. As shown in Figure 3a, the L- and D-enantiomers are tightly packed in an ordered array. Figure 3b shows a pair of the L- and D-enantiomers and highlights some of the residues involved in the packing interface (e.g. Tyr¹⁰, Asn⁹, and Pro⁶; inset). Within the asymmetric unit cell is one L-form of cVc1.1, which is identical in structure to the inverted D-form. The structure of L-cVc1.1 displays an α -helix spanning residues Arg⁷ to Asp¹¹; conservation of this structural motif is considered to be important for retaining biological activity.^[36] Furthermore, the structure is stabilized by two disulfide bonds (Cys^I-Cys^{III} and Cys^{II}-Cys^{IV}), which are clearly resolved by the electron density (Figure 3c). Residues Gly¹⁷ to Gly²² of cVc1.1 form a “linker” sequence that was introduced to native Vc1.1 to connect its free N- and C-termini to enhance its stability and biopharmaceutical properties. Comparison of our structure of the L-enantiomer of cVc1.1 with the solution NMR structure of the same peptide identifies some differences in the arrangement of the Cys^I-Cys^{III} disulfide bond as well as the conformation of the linker sequence. As the first X-ray structure of cVc1.1 (both L- and D-forms), it could be used as a starting point for comprehensive molecular dynamics simulations to investigate binding mechanisms to cell surface receptors or as a template for in silico re-engineering.

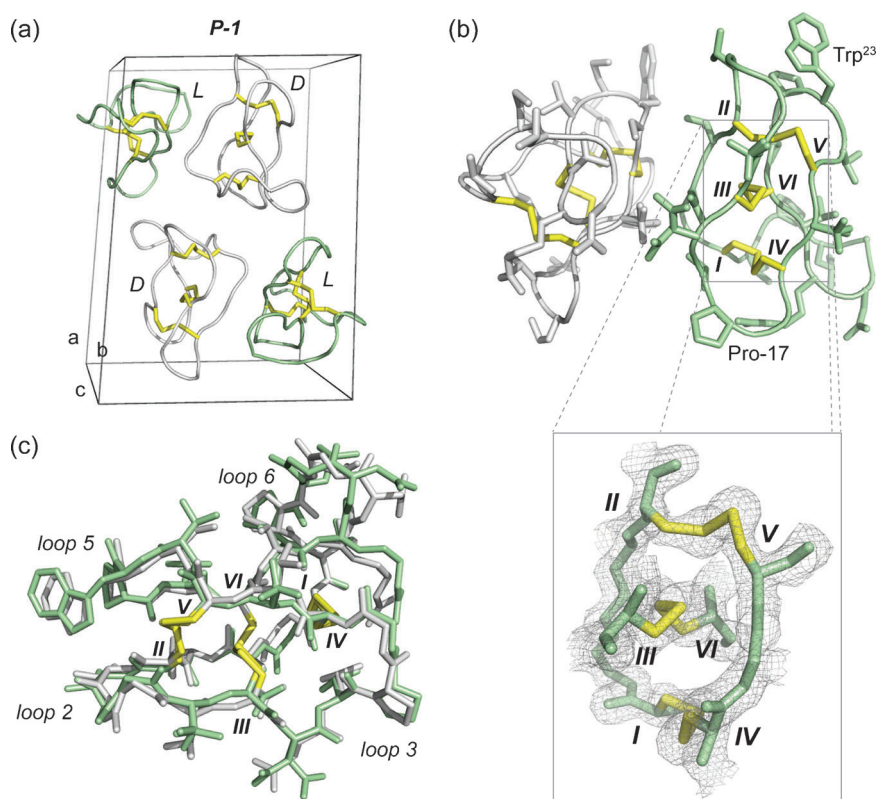


Figure 4. Racemic crystallography of kB1. a) Unit cell of the true racemate in space group $P\bar{1}$ with ribbon representations of the cyclic peptide molecules. The L-enantiomer (in green) and D-enantiomers (in grey) are labeled. The disulfide bonds are also shown as stick representations in yellow. b) Ribbon representations of L-kB1 (green) and D-kB1 (grey). Inset: $2F_o - F_c$ electron density map of the cystine knot shown contoured at a σ level of 1.0. c) Superposition of L-kB1 (green) with the solution NMR structure of L-kB1 (grey; PDB ID: 1NB1). The cysteines are labeled with Roman numerals and the loops are also labeled.

The X-ray structure of the true racemate of cyclotide kB1 was solved in space group $P\bar{1}$ and is shown in Figure 4a. The unit cell contains two molecules of each enantiomer; a pair of L- and D-enantiomers of kB1, which occupy the asymmetric unit cell, is shown in Figure 4b. A comparison of the structures of the L- and (inverted) D-forms of kB1 shows that there are slight differences in the side-chain orientations of some residues (e.g. orientation of Arg²⁸ compared to that of D-Arg²⁸), though the backbone conformation is largely invariant between the two enantiomers. Aside from a network of hydrogen bonds, the structure of kB1 is stabilized by the cystine knot motif, which comprises an embedded ring formed by two disulfide bonds (Cys^I-Cys^{IV} and Cys^{II}-Cys^V) and their connecting backbone segments as well as a third disulfide bond (Cys^{III}-Cys^{VI}) that threads through the embedded ring (Figure 4b inset). Figure 4c shows a comparison of our structure of L-kB1 with the most recent solution NMR structure of kB1 (PDB ID: 1NB1), showing that the overall structures are similar, with minor differences in the precise position of loop 3 (residues Asn¹⁵ to Gly¹⁸) and loop 6 (Thr²⁷ to Val⁴). Until now, structural studies on kB1 have been performed using NMR spectroscopy; initially, some structural features of kB1, such as the cystine knot topology,^[37,38] were difficult to unambiguously define. Although the connectivity

has been resolved on the basis of an analysis of high-field NMR data relating to the side-chain angles of the Cys residues,^[39] and by a chemical analysis of partially reduced species,^[40] the crystal structure of kB1 presented here provides conclusive evidence of the disulfide bond topology. It is worth noting that the knotted disulfide bond arrangement in combination with the cyclic backbone has been shown through mutational studies to underpin the exceptional stability of kB1, which is resistant to chaotropic agents, high temperature, acids, and a range of proteases, including conditions under which most proteins denature or degrade.^[41] Overall, the remarkable stability of kB1 together with the possibility to introduce combinatorial variation within these sequences, make kB1 and other cyclotides promising scaffolds for design and development of peptide-based therapeutics.

Aside from being of interest because of its stability, kB1 has various bioactivities that are of interest from an agrochemical and/or pharmaceutical perspective. These include insecticidal,^[11] uteronic,^[12] HIV inhibitory,^[13] antimicrobial,^[14] cancer-cell toxicity,^[15] and immunosuppressant activities.^[16] We have found that the activities of kB1 can be fine-tuned by single-point mutations.^[42] For example, the analogue [G6A]kB1 is inactive in insecticidal assays, whereas [V25A]kB1 maintains activity comparable to the native peptide.^[42] We were interested in determining the structures of kB1 analogues that have different activity profiles, and therefore investigated the use of quasi-racemic crystallography. The X-ray structures of the [G6A]kB1 and [V25A]kB1 quasi-racemates were both solved in space group *P* $\bar{1}$ and are shown in Figure 5 a and b, respectively. Interestingly, their unit cells are almost identical to that of the true racemate.

To summarize, we have determined the X-ray crystal structures of three prototypical cyclic disulfide-rich peptides as well as two quasi-racemic structures. X-ray diffraction data were acquired to 1.25 Å resolution for the racemic mixture of SFTI-1, 1.70 Å resolution for the racemic mixture of cVc1.1, and 1.90 Å resolution for the racemic mixture of kB1, 1.25 Å resolution for the quasi-racemic mixture of [G6A]kB1, and 2.30 Å resolution for the quasi-racemic mixture of [V25A]kB1. Inspection of the structures revealed that the peptides form compact tertiary folds that are stabilized by disulfide bonds, sometimes forming an intricate topology.

In addition to providing high-resolution structural information, the racemic crystallography approach made it possible to crystallize a range of cyclic disulfide-rich peptides and to elucidate the structures of both enantiomeric forms. SFTI-1, cVc1.1, and kB1 represent a series of cyclic disulfide-rich peptides with increasing size and complexity, that is, from a 14-mer peptide with one disulfide bond to a 29-mer peptide with three knotted disulfide bonds. Therefore, the racemic crystallization strategy is likely to be useful for cyclic disulfide-rich peptide scaffolds between and beyond the examples used here. Additionally, the quasi-racemic crystallography approach was also successful. We postulate that the quasi-racemic crystallization strategy is likely to be useful for other chemically engineered peptides. For example, it might be possible to use quasi-racemic crystallization to determine the X-ray structure of a grafted cyclic disulfide-rich peptide by partnering the L-grafted peptide with the D-peptide

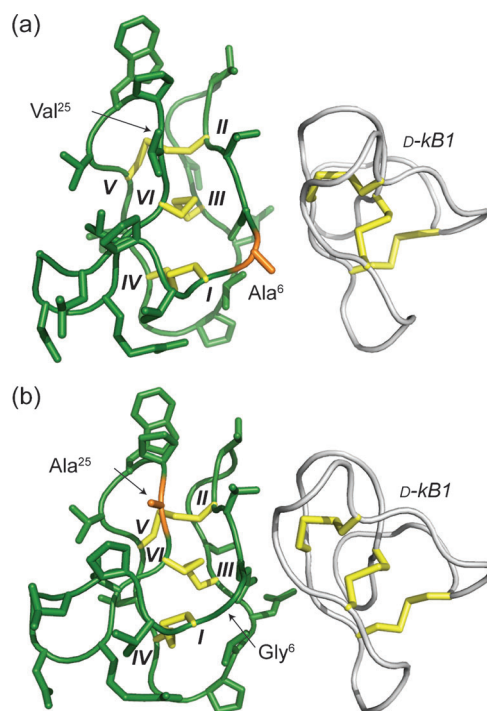


Figure 5. Quasi-racemic crystallography of [G6A]kB1 and [V25A]kB1. a) Ribbon representations of L-[G6A]kB1 (dark green) and D-kB1 (grey). b) Ribbon representations of L-[V25A]kB1 (dark green) and D-kB1 (grey). The disulfide bonds are shown in yellow and the cysteines are labeled with Roman numerals. The residues that were mutated are indicated.

scaffold, or a cyclic disulfide-rich peptide with a chemical conjugate (e.g. to be used as a diagnostic contrasting agent) as has been attempted recently for a glycosylated protein.^[32] We believe that racemic and quasi-racemic crystallography of cyclic disulfide-rich peptides can be widely applied in the field of cyclic peptide structural biology.

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- [1] R. Dharanipragada, *Future Med. Chem.* **2013**, *5*, 831–849.
- [2] S. E. Northfield, C. K. Wang, C. I. Schroeder, T. Durek, M. W. Kan, J. E. Swedberg, D. J. Craik, *Eur. J. Med. Chem.* **2014**, *77*, 248–257.
- [3] A. G. Poth, L. Y. Chan, D. J. Craik, *Biopolymers* **2013**, *100*, 480–491.
- [4] T. Rivera, L. Sanz, G. Camarero, I. Varela-Nieto, *Curr. Drug Delivery* **2012**, *9*, 231–242.
- [5] C. T. Wong, D. K. Rowlands, C. H. Wong, T. W. Lo, G. K. Nguyen, H. Y. Li, J. P. Tam, *Angew. Chem. Int. Ed.* **2012**, *51*, 5620–5624; *Angew. Chem.* **2012**, *124*, 5718–5722.
- [6] C. K. Wang, C. W. Gruber, M. Cemazar, C. Siatskas, P. Tagore, N. Payne, G. Sun, S. Wang, C. C. Bernard, D. J. Craik, *ACS Chem. Biol.* **2014**, *9*, 156–163.

- [7] Y. Ji, S. Majumder, M. Millard, R. Borra, T. Bi, A. Y. Elnagar, N. Neamati, A. Shekhtman, J. A. Camarero, *J. Am. Chem. Soc.* **2013**, *135*, 11623–11633.
- [8] M. L. Korsinczy, H. J. Schirra, D. J. Craik, *Curr. Protein Pept. Sci.* **2004**, *5*, 351–364.
- [9] R. J. Clark, J. Jensen, S. T. Nevin, B. P. Callaghan, D. J. Adams, D. J. Craik, *Angew. Chem. Int. Ed.* **2010**, *49*, 6545–6548; *Angew. Chem.* **2010**, *122*, 6695–6698.
- [10] D. J. Craik, N. L. Daly, T. Bond, C. Waine, *J. Mol. Biol.* **1999**, *294*, 1327–1336.
- [11] C. Jennings, J. West, C. Waine, D. Craik, M. Anderson, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10614–10619.
- [12] L. Gran, F. Sandberg, K. Sletten, *J. Ethnopharmacol.* **2000**, *70*, 197–203.
- [13] K. R. Gustafson, T. C. McKee, H. R. Bokesch, *Curr. Protein Pept. Sci.* **2004**, *5*, 331–340.
- [14] J. P. Tam, Y. A. Lu, J. L. Yang, K. W. Chiu, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8913–8918.
- [15] E. Svängård, U. Goransson, Z. Hocaoglu, J. Gullbo, R. Larsson, P. Claeson, L. Bohlin, *J. Nat. Prod.* **2004**, *67*, 144–147.
- [16] C. Gründemann, K. Thell, K. Lengen, M. Garcia-Kaufer, Y. H. Huang, R. Huber, D. J. Craik, G. Schabbauer, C. W. Gruber, *PLoS One* **2013**, *8*, e68016.
- [17] B. L. Pentelute, Z. P. Gates, V. Tereshko, J. L. Dashnau, J. M. Vanderkooi, A. A. Kossiakoff, S. B. Kent, *J. Am. Chem. Soc.* **2008**, *130*, 9695–9701.
- [18] K. Mandal, M. Uppalapati, D. Ault-Riche, J. Kenney, J. Lowitz, S. S. Sidhu, S. B. Kent, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 14779–14784.
- [19] K. Mandal, B. L. Pentelute, D. Bang, Z. P. Gates, V. Y. Torbeev, S. B. Kent, *Angew. Chem. Int. Ed.* **2012**, *51*, 1481–1486; *Angew. Chem.* **2012**, *124*, 1510–1515.
- [20] M. Avital-Shmilovici, K. Mandal, Z. P. Gates, N. B. Phillips, M. A. Weiss, S. B. Kent, *J. Am. Chem. Soc.* **2013**, *135*, 3173–3185.
- [21] K. Mandal, B. L. Pentelute, V. Tereshko, V. Thammavongsa, O. Schneewind, A. A. Kossiakoff, S. B. Kent, *Protein Sci.* **2009**, *18*, 1146–1154.
- [22] J. R. Banigan, K. Mandal, M. R. Sawaya, V. Thammavongsa, A. P. Hendrickx, O. Schneewind, T. O. Yeates, S. B. Kent, *Protein Sci.* **2010**, *19*, 1840–1849.
- [23] R. J. Clark, D. J. Craik, *Biopolymers* **2010**, *94*, 414–422.
- [24] T. O. Yeates, S. B. Kent, *Annu. Rev. Biophys.* **2012**, *41*, 41–61.
- [25] S. W. Wukovitz, T. O. Yeates, *Nat. Struct. Biol.* **1995**, *2*, 1062–1067.
- [26] B. L. Pentelute, K. Mandal, Z. P. Gates, M. R. Sawaya, T. O. Yeates, S. B. Kent, *Chem. Commun.* **2010**, *46*, 8174–8176.
- [27] L. E. Zawadzke, J. M. Berg, *Proteins Struct. Funct. Genet.* **1993**, *16*, 301–305.
- [28] M. Doi, A. Ishibe, H. Shinozaki, H. Urata, M. Inoue, T. Ishida, *Int. J. Pept. Protein Res.* **1994**, *43*, 325–331.
- [29] C. Toniolo, C. Peggion, M. Crisma, F. Formaggio, X. Shui, D. S. Eggleston, *Nat. Struct. Biol.* **1994**, *1*, 908–914.
- [30] W. R. Patterson, D. H. Anderson, W. F. DeGrado, D. Cascio, D. Eisenberg, *Protein Sci.* **1999**, *8*, 1410–1422.
- [31] L. W. Hung, M. Kohmura, Y. Ariyoshi, S. H. Kim, *J. Mol. Biol.* **1999**, *285*, 311–321.
- [32] R. Okamoto, K. Mandal, M. R. Sawaya, Y. Kajihara, T. O. Yeates, S. B. Kent, *Angew. Chem. Int. Ed.* **2014**, *53*, 5194–5198; *Angew. Chem.* **2014**, *126*, 5294–5298.
- [33] B. Dang, T. Kubota, K. Mandal, F. Bezanilla, S. B. Kent, *J. Am. Chem. Soc.* **2013**, *135*, 11911–11919.
- [34] K. Mandal, B. L. Pentelute, V. Tereshko, A. A. Kossiakoff, S. B. Kent, *J. Am. Chem. Soc.* **2009**, *131*, 1362–1363.
- [35] E. Zabłotna, K. Kaźmierczak, A. Jaśkiewicz, M. Stawikowski, G. Kupryszewski, K. Rolka, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 855–859.
- [36] E. L. Millard, N. L. Daly, D. J. Craik, *Eur. J. Biochem.* **2004**, *271*, 2320–2326.
- [37] L. Skjeldal, L. Gran, K. Sletten, B. F. Volkman, *Arch. Biochem. Biophys.* **2002**, *399*, 142–148.
- [38] O. Saether, D. J. Craik, I. D. Campbell, K. Sletten, J. Juul, D. G. Norman, *Biochemistry* **1995**, *34*, 4147–4158.
- [39] K. J. Rosengren, N. L. Daly, M. R. Plan, C. Waine, D. J. Craik, *J. Biol. Chem.* **2003**, *278*, 8606–8616.
- [40] U. Goransson, D. J. Craik, *J. Biol. Chem.* **2003**, *278*, 48188–48196.
- [41] M. L. Colgrave, D. J. Craik, *Biochemistry* **2004**, *43*, 5965–5975.
- [42] S. M. Simonsen, L. Sando, K. J. Rosengren, C. K. Wang, M. L. Colgrave, N. L. Daly, D. J. Craik, *J. Biol. Chem.* **2008**, *283*, 9805–9813.