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Design, Synthesis, and Complexing Properties of ([1]Cys-[1']Cys,[4]Cys-[4']Cys)-dithiobis(Ac-L-[1]Cys-L-Pro-D-Val-L-[4]Cys-NH_2). The First Example of a New Family of Ion-Binding Peptides[1,2]

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Abstract: Controlled formation of two interchain disulfide bonds in peptides containing two cysteines affords dimers of well-defined topology. Such an approach has been used in the design and synthesis of the cyclic peptide ([1]Cys-[1']Cys,[4]Cys-[4']Cys)-dithiobis(Ac-L-[1]Cys-L-Pro-D-Val-L-[4]Cys-NH_2) (**1**), inspired in the antibiotic valinomycin. The conformational and ion-binding properties of **1** have been investigated by nuclear magnetic resonance and circular dichroism spectroscopy. The cyclic peptide presents solvent- and temperature-dependent conformations and forms complexes in acetonitrile with barium cations with high affinity (log $K_{Ba^{2+}}$ = 9.09 ± 2.58) and considerable selectivity (log $K_{Sr^{2+}}$ = 4.96 ± 0.10; log $K_{Mg^{2+}}$ = 4.07 ± 0.07; log $K_{Ca^{2+}}$ = 3.21 ± 0.10; log $K_{Li^{+}}$ = 2.60 ± 0.04; log $K_{Na^{+}}$ = 2.11 ± 0.04). Computer-extracted circular dichroism spectra of the 1:1 complexes of **1** with alkali metal and alkali earth cations show that the conformation of the complexed peptide is determined by the size of the metal ion, indicating that there is good balance between flexibility and rigidity in the two strands held by the disulfide bridges.

Introduction

Cyclopeptides form a particular class of compounds with multiple and varied biological functions. Due to the restriction in the conformational space induced by their cyclic nature, it is possible in some cases to relate the activity of these peptides to their conformational states.[5] This is the case of valinomycin. Valinomycin is a depsipeptide with the capability of adopting a C_3 symmetric conformation with a polar cavity and a hydrophobic exterior suitable for ion complexation.[6] While the uncomplexed depsipeptide shows solvent-dependent conformations, the molecular backbone in the ion-binding conformation is made up of alternating type II (-L-Val-D-HyIv-) and type II' (-D-Val-L-Lac-) β-turns. In this bracelet conformation (Figure 1) the ester groups form an octahedral coordination site for the metal ion and the side chains provide a hydrophobic exterior.

Dimerization of peptides containing two cysteines affords cyclic peptides with several characteristics that make them attractive

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(2) A preliminary report of portions of this work was presented in the following: (a) Albericio, F.; García-Echeverría, C.; Giralt, E.; Pérez, J. J.; Pons, M.; Royo, M.; Ruiz-Gayo, M. *Proceedings of the Twenty-First European Peptide Symposium*; Andreu, D., Giralt, E., Eds.; Escom Science: Leiden, The Netherlands, 1991; pp 535–527. (b) García-Echeverría, C.; Albericio, F.; Pons, M.; Giralt, E. *Proceedings of the Twelfth American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; Escom Science: Leiden, The Netherlands, 1992; pp 917–918.

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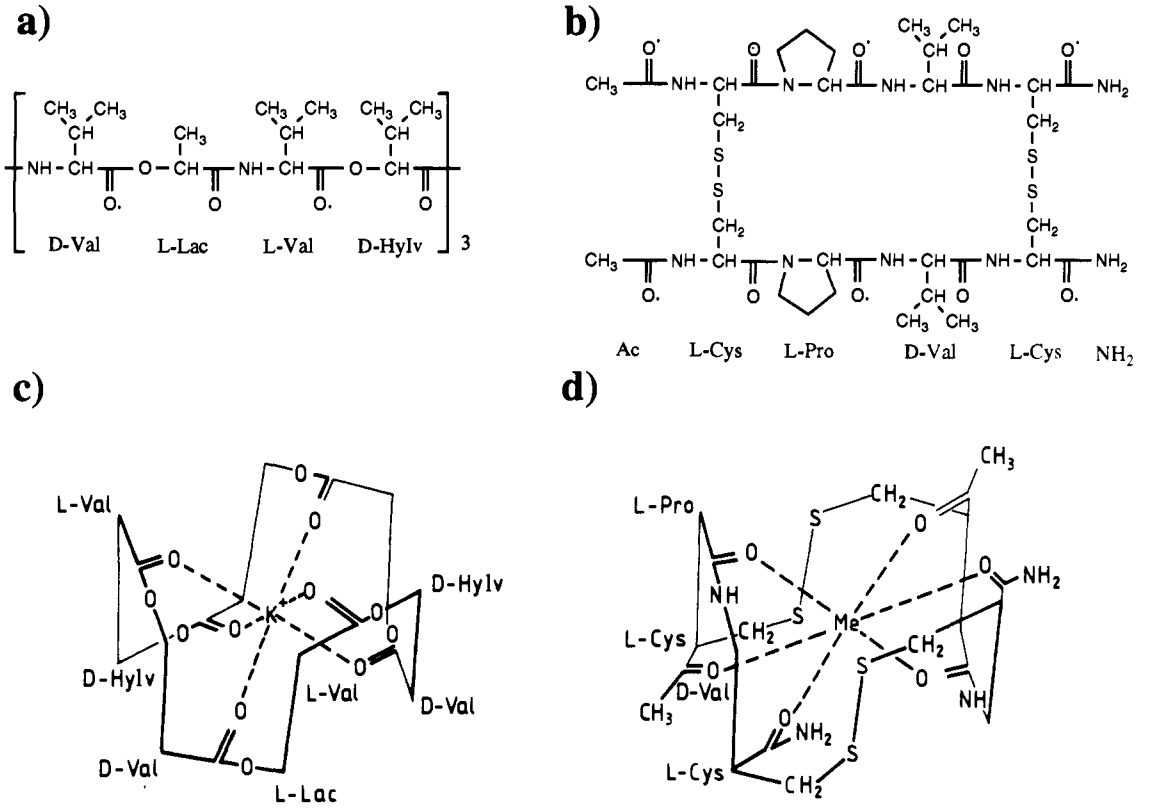

Figure 1. (a) Chemical structure of valinomycin. (b) Chemical structure of peptide **1**. Asterisks mark carbonyl oxygens known or expected to participate in ion binding. (c) Schematic illustration of the structure of the K^+ complex of valinomycin showing the formation of β-turns and the coordination of the metal ion. Vertical segments of the backbone contain amide groups that stabilize the β-turns by hydrogen bonding (not shown). (d) Heuristic representation of the binding of a metal ion by peptide **1**.

building blocks for de novo peptide and protein design:[7] (i) the disulfide bond prefers a gauche conformation with a potential energy minimum at X_{SS} around |90°| and a barrier of 7–14 kcal mol^{-1}, intermediate between those of a typical carbon–carbon single bond and an amide bond;[8] (ii) the dimeric character offers a potential synthetic economy; and (iii) the possibility of controlling the relative direction of the two chains provides a new variable that can be used to achieve maximum simplification in the design.

Taking the idealized valinomycin structure as a model, we have designed the cyclic peptide (^{1}Cys-$^{1'}$Cys,^{4}Cys-$^{4'}$Cys)-dithiobis-(Ac-L-^{1}Cys-L-Pro-D-Val-L-^{4}Cys-NH_2) (**1**) with a potential for forming two β-turns stabilizing each other through the interactions imposed by the disulfide bonds. Herein we report the conformational analysis of the cyclic peptide **1** in different solvents, along with its ion-binding properties.

Results and Discussion

Design. The sequence Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2 was chosen in light of the theoretical predictions of Venkatachalam[9] and Wilmot and Thorton[10] so that it had a high probability of adopting a type II β-turn conformation. Proline is probably the most preferred amino acid at position $i + 1$ of a type II β-turn.[11] The restriction on Φ to about –60° for L-proline corresponds to the theoretical $\Phi(i+1)$ value of a type II β-turn.[12] The use of valine in the $i + 2$ position introduces a hydrophobic side chain that can shield the ion and the hydrogen bond $4 \rightarrow 1$ of the β-turn. It was further reasoned that the D-configuration of valine might specifically induce a type II β-turn.[9] Cysteine occupies the *N*- and *C*-terminal positions to cross-link the two moieties, and indeed, it has a high probability of occurrence at these positions in β-turns,[10] especially when a prolyl residue occurs at position $i + 2$.[13] ECEPP calculations[14] on the above sequence showed that the folded conformation was only 2 kcal/mol above the extended minimum energy conformer.[15]

In valinomycin, types II and II′ β-turns, corresponding to sequences with L–D- and D–L-configurations of the central residues, are facing each other in pairs across the ring. The two types of β-turns ensure that the crucial carbonyl oxygen of the ester groups point inward of the cavity, providing the necessary complexation points. To obtain the same orientation of the carbonyl groups of the central amide bonds of two identical β-turns, the two peptide chains have to be parallel. This has the additional advantage, from the synthetic point of view, that parallel dimers can be unambiguously obtained from a single protected precursor.[16,17]

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Table I. ^{1}H-NMR Data[a,b] of Peptide 1 in DMSO-d_6 and CD_3CN

	NH	H^α	H^β	others	$^3J(NH,H^\alpha)$	$^3J(H^\alpha,H^\beta)$	$^2J(H^\beta,H^\beta)$	$\Delta\delta/\Delta T$ (ppb/K)
L-Cys[1]	8.35 (7.39)	4.81 (4.87)	2.99 (3.10) (3.00)		8.4 (7.7)	6.9 (4.4) (9.4)	−13.8 (−13.2)	−4.96 (−7.58)
L-Pro[2]		4.41 (4.41)	1.98 (2.10) 1.88 (2.10)	H^γ 1.98 (2.10) H^γ 1.88 (2.10) H^δ 3.70 (3.78) H^δ 3.61 (3.65)				
D-Val[3]	7.94 (7.51)	4.14 (4.07)	2.21 (2.33)	H^γ 0.876 (0.98) H^γ 0.81 (0.95)	8.4 (7.7)	4.8 (4.8)		−2.35 (−6.23)
L-Cys[4]	7.83 (7.70)	4.48 (4.45)	3.16 (3.28) 2.89 (3.24)		8.2 (7.7)	4.8 (5.2) 4.2 (7.4)	−14.0 (−13.7)	−1.63 (−3.47)
NH_2	7.28 (6.89) 7.23 (6.04)							−4.60 (−4.79) −4.56 (−5.70)

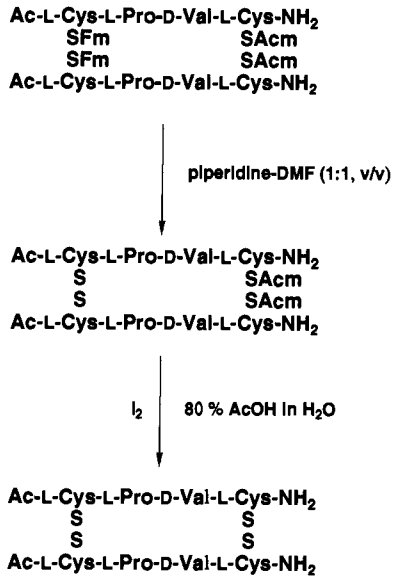
[a] The numbers in parentheses refer to the data recorded in CD_3CN. [b] The chemical shifts (δ) are expressed in ppm and are referenced using tetramethylsilane for DMSO-d_6 or the residual peak of the solvent (1.90 ppm) for CD_3CN as reference. The acetyl group was at 1.83 ppm (1.93 ppm). Coupling constants (J) are expressed in Hz. These data were recorded at 298 K (300 K). For the last column, the temperature coefficient of the amide protons was determined over a range of 295–333 K (293–308 K).

Although valinomycin has been used to inspire the gross features of the designed peptide and the distance between the two C^α carbons of cystine is compatible with the distance between the C^α atoms of residues i and $i + 3$ in a type II or II' β-turn of valinomycin, substitution of the dipeptide units by disulfide bridges is a major change and a different selectivity is expected for **1** and valinomycin. Although the cyclic peptide can assume conformations superimposable to those of valinomycin, it is expected to have more conformational freedom and different binding properties. In this context, energy minimization studies of the cyclic peptide **1** showed that the configuration of the disulfide bonds affects the size of the cavity in the sequence PM < MM < PP < MP,[18] where the first letter refers to the configuration of the disulfide bond of the *N*-terminal cystine. For the combination MM and MP, the cavity defined by the four sulfur atoms increases when the distance between the carbonyl groups of Pro[2] of the two segments decreases. Moreover, the smaller number of bulky side chains in **1** in respect to valinomycin leads to a structure that could be more accessible to bulky or solvent-complexed ions, and the presence of two amide bonds in the two termini of each peptide chain that are not constrained by the formation of the β-turn can provide additional binding sites for the metal ions.

Synthesis. Bis-cysteine cyclic peptides can be synthesized unequivocally by an appropriate selection of protecting groups for the side chains of cysteins. Several strategies to obtain parallel and antiparallel bis-cysteine peptides based on the use of highly orthogonal protecting groups for the side chain of cysteine have been proposed.[16,17] The cyclic peptide **1** was prepared (Scheme I) from a single monomer with the two cysteine residues protected with the *S*-acetamidomethyl[19] and *S*-9-fluorenylmethyl[20,21] groups.

Chain assembly was carried out manually using a conventional strategy on a *p*-methylbenzhydrylamine (MBHA) resin[22,53] for establishing the *C*-terminal peptide amide. We used the *tert*-butyloxycarbonyl group for temporary protection of N^α-amino groups and the *S*-acetamidomethyl and the *S*-9-fluorenylmethyl functions for protection of the β-thiols of cysteine of the peptide Ac-L-Cys(Fm)-L-Pro-D-Val-L-Cys(Acm)-NH_2 (**2**). Peptide **2** was cleaved from the resin in 80% yield with anhydrous HF–anisole (9:1, v/v) for 1 h at 0 °C, providing crude material of ≈95% purity. After gel filtration on a Sephadex LH-20 column

Scheme I



(95% yield), the analytically pure peptide was obtained. The first disulfide bond in the *N*-terminal position was formed upon treatment of **2** with piperidine–DMF (1:1), giving the linear precursor (1Cys-1'Cys)-dithiobis(Ac-L-1Cys-L-Pro-D-Val-L-4Cys-(Acm)-NH_2 (**3**) (77% overall yield). The cyclic peptide **1** was obtained upon simultaneous deprotection and oxidation of **3** (56% overall yield). In the formation of the second disulfide bond, iodine[23,24] was preferable to thalium(III) trifluoroacetate,[25] since this last oxidant promotes the formation of an intrachain disulfide bond containing peptide,[26] c(Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2), which was identified by fast atom mass spectroscopy and nuclear magnetic resonance.[27]

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(24) The procedure used in this synthesis is described in the following: Ruiz-Gayo, M.; Albericio, F.; Royo, M.; García-Echeverría, C.; Pedroso, E.; Pons, M.; Giralt, E. *An. Quim.* **1989**, *85C*, 116–118. The excess iodine was removed by extractions with CCl_4. We found that, after purification by MPLC, the peptides obtained via reduction of iodine in an excess of zinc contained an important amount of zinc (5–14% zinc determined by atomic absorption). The presence of this ion excluded the use of this material for binding and conformational analysis studies.

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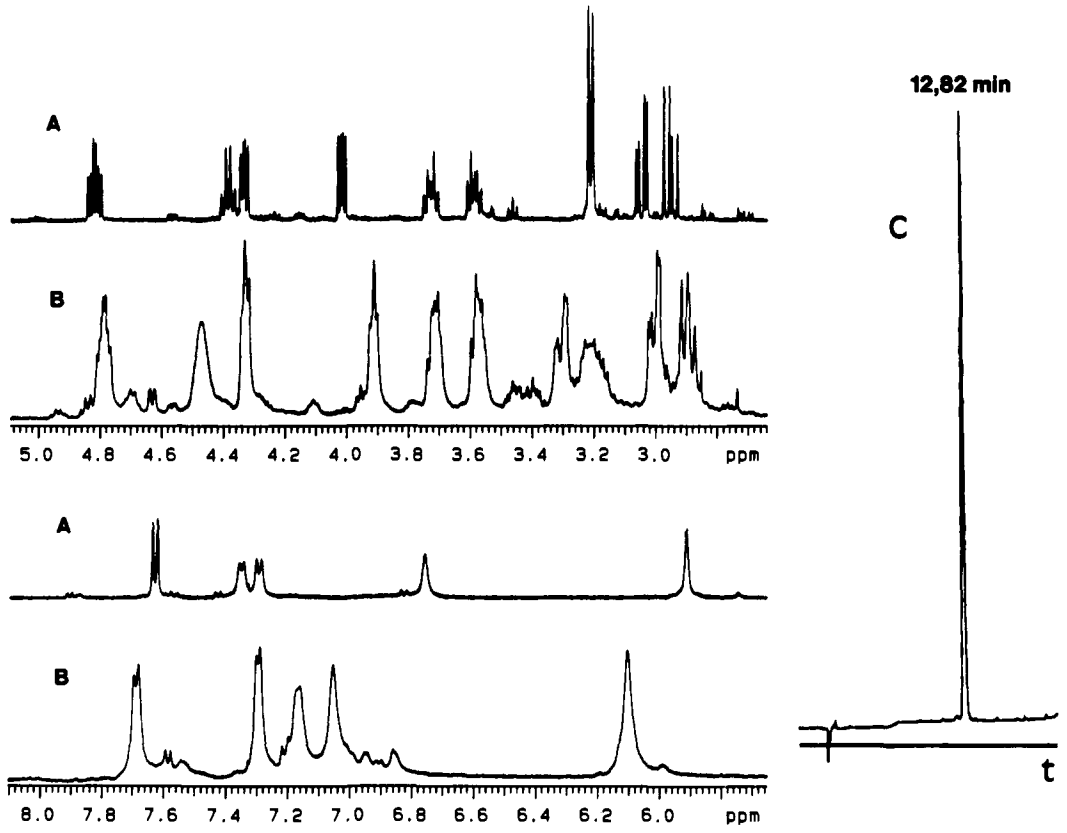

Figure 2. (a) ^{1}H-NMR spectra of **1** in CD_3CN. The minor signals observed correspond to a second conformation with a population of 5.9% of the major one. HPLC analysis (C) indicates that the purity of the peptide was higher than 99.5%. Exchange between the major and minor conformations could be detected in ROESY or NOESY experiments. Spectra marked B are from an equimolar mixture of **1** and $LiClO_4$ that contains ca. 48% complexed peptide. The peptide concentration was 4.56 mM and the temperature 298 K. HPLC conditions of part C are as follows: Vydac C-18 column. Eluents: A, 0.045% TFA in H_2O; B, 0.036% TFA in CH_3CN. Gradient: 5 to 95% of B in 20 min.

Conformational Studies. The conformational analysis of the bis-cysteine peptide **1** in solution was carried out by nuclear magnetic resonance and circular dichroism.

A. Nuclear Magnetic Resonance. ^{1}H-NMR and ^{13}C-NMR data were acquired in DMSO-d_6 and CD_3CN. Signals were assigned by a combination of DQF-COSY[28] and ROESY[29] experiments for the assay in DMSO-d_6 and HOHAHA[30] and NOESY[31] experiments in CD_3CN. Chemical shift assignments, coupling constants, and temperature factors for the amide protons are given in Table I.

The ^{1}H-NMR and ^{13}C-NMR (data not shown) spectra in DMSO-d_6 were found to contain only one set of signals that can be due to the presence of a predominant conformation with C_2 symmetry or to an average conformation in the NMR time scale as a result of a rapid equilibrium between several conformations. The ROE cross peaks between the H^α Cys^1 and both H^δ or Pro^2,[32] and the chemical shifts of C^β (28.4 ppm) and C^γ (24.9 ppm) confirm that the amide bond between Cys^1 and Pro^2 is trans. ROEs were observed between H^α Pro^2/NH D-Val^3, NH D-Val^3/NH Cys^4, and H^α D-Val^3/NH Cys^4. These ROEs and the low-temperature coefficient of the NH of Cys^4 ($\Delta\delta/\Delta T$ = −1.63 ppb·K^{-1}) are in agreement with the short distances and the intramolecular hydrogen bond expected for a type II β-turn structure. The value of the coupling constant $^3J(NH,H^\alpha)$ of D-Val^3 and the absence of a cross peak between H^α Pro^2/NH Cys^4 are indicative of a certain deviation with respect to the theoretical model of a β-turn of type II.[33] On the other hand, the values of the coupling constants $^3J(H^\alpha,H^\beta)$ in both cysteines indicate an average between different rotamers and indicate a considerable flexibility of the disulfide bridge.

The ^{1}H-NMR of **1** in CD_3CN (Figure 2) presents a major[34] set of signals characteristic of a symmetrical species in the NMR time scale. The NOE cross peak observed between H^α Cys^1 and the two H^δ of Pro^2 indicates that the amide bond between Cys^1 and Pro^2 is trans.[32] The proximity of the chemical shifts of the diastereotopic β-protons of Cys^4 and the coupling constants $^3J(H^\alpha,H^\beta)$ of these protons suggests the existence of a rapid equilibrium between several conformations. The coupling con-

(26) The relative concentration of dimer/monomer was 1–5. This value was ascertained by comparison of the HPLC peak areas with those from an authentic standard of pure peptide of known concentration, which was determined by amino acid analysis. Co-injection of the pure peptide with crude material was used to assure the identity and integrity of the two major peaks.

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(33) The expected spin–spin constant $^3J(NH_{i+2},H^\alpha_{i+2})$ for a type II β-turn is 5 Hz. Wütrich, K. *NMR of Protein and Nucleic Acid*; John Willey & Sons: New York, 1986; p 166.

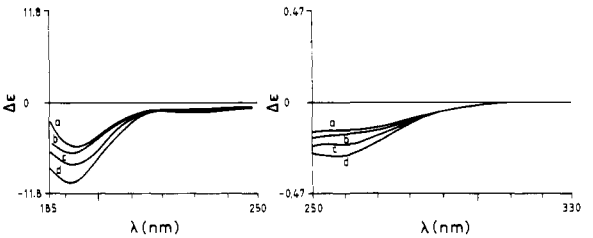

Figure 3. Circular dichroism spectra of **1** recorded in MeOH at (a) 293 K; (b) 288 K (c = 2.74 mM); (c) 296 K (c = 2.89 mM); and (d) 200 K (c = 3.06 mM).

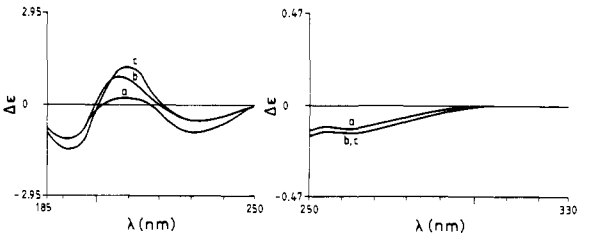

Figure 4. Circular dichroism spectra of **1** recorded in (a) TFE (293 K; c = 2.48 mM); (b) CH_3CN (293 K; c = 2.11 mM); and (c) CH_3CN (243 K; c = 2.26 mM).

stants $^3J(H^{\alpha},H^{\beta}_{h,l})$[35] of Cys[1] are near the limits of pure J_{trans} (12 Hz) and pure J_{gauche} (3.2 Hz), indicating a single predominant classical rotamer about the corresponding α–β bond. Analysis of the 2D-NOESY (400 and 200 ms)[36] spectra of **1** revealed a short distance between the H^{α} of Pro[2] and the NH of D-Val[3] characteristic of a type II β-turn structure. The NOEs observed between H^{α} Pro[2]/NH Cys[4], NH D-Val[3]/NH Cys[4], and H^{α} D-Val[3]/NH Cys[4] are also in agreement with the short distances expected for a type II β-turn.[37] In this solvent also, the amide proton of Cys[4] shows the lowest temperature coefficient in agreement with the expected hydrogen bond in a type II β-turn.

B. Circular Dichroism. Figures 3 and 4 show representative CD spectra of the cyclic peptide **1** recorded in MeOH, TFE, and CH_3CN. The CD spectra of **1** in MeOH (Figure 3) or H_2O (data not shown) are very similar. The intense negative band close to 195 nm and the weak negative band at 230 nm are characteristic of unordered peptides.[38] The lack of a discernible regularity in the conformation of **1** in these solvents is corroborated by the insensitivity of the CD spectra to the addition to the water solution of sodium dodecyl sulfate or urea (data not shown). Furthermore, the changes in the intensities of the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ amide transitions and the $n \rightarrow \sigma^*$ disulfide transitions upon temperature variations from 295 to 200 K in MeOH are indicative of the mobility of the peptide backbone and the disulfide bridges in this solvent. The CD spectra of **1** in less polar solvents such as CH_3CN or TFE (Figure 4) show weak negative bands at ≈230 and 208 nm. The shapes of the CD spectra in these solvents resemble those of Class C′ spectra,[39] which was predicted for type II β-turn conformers with Ψ_{Pro} in the 70° to 90° range. Some discrepancies with respect to the theoretical Class C′ spectra are due to the ratio of intensities between the first $n \rightarrow \pi^*$ transition and the other two, $\pi \rightarrow \pi^*$ and the second $n \rightarrow \pi^*$, amide transitions. This difference with respect to the model spectra is particularly notable in the CD spectra in TFE, where the first $n \rightarrow \pi^*$ transition is the dominant band. The small differences observed in the CD spectra of **1** in CH_3CN from 293 to 243 K (Figure 4) are indicative of a dominant conformer or a family of conformations although with a certain amount of local mobility.

One common feature of the CD spectra of **1** in all the solvents employed is the absence of a discrete maximum in the 250–300-nm region attributable to the disulfide bridge. This could be due to a mutual cancellation of the disulfide contribution by opposite chirality of each disulfide bond, or it may be originated by disulfide bond angles X_{SS} close to |90°|, which, according to the quadrant rule,[40,41] should also give zero rotational strength. The remaining possibility, i.e., that each disulfide bridge has identical population of the P and M conformers, is unlikely given the insensitivity to temperature of the $n \rightarrow \sigma^*$ transitions of **1** in CH_3CN.

Ion-Binding Properties. Addition of barium, magnesium, or lithium perchlorate results in large shifts and broadening of the NMR spectrum of **1** in acetonitrile. The broadening is probably the result of slow exchange between different species including free peptide and complexes of different stoichiometry and precludes the detailed study of complex formation by NMR. The presence of at least two types of complexes is evident from the CD data (vide infra) in the presence of barium, strontium, and calcium perchlorates.

Figure 2 shows the spectrum of **1** in the presence of 1 equiv of $LiClO_4$. The fact that **1** forms only one type of complex with lithium ions and the low binding constant with this ion allow the observation of changes in the spectra while retaining reasonable line widths. The spectrum was assigned using COSY and NOESY spectra. The largest shifts with respect to the free peptide correspond to the C-terminal carboxamide protons, NH of Cys[1] and H^{α} of D-Val[3]. Changes are also observed in the β protons of Cys[1], where the intense signal becomes a broader multiplet as the changes in chemical shift destroy the near degeneration of the diastereotopic protons. Analysis of NOESY spectra obtained at three different mixing times (200, 300, and 400 ms) gives a pattern of short distances typical of a type II β-turn. According to the binding constant measured by CD and the concentration of the NMR sample (4.56 mM), the amount of peptide complexed to the metal in these conditions is 48%; however, while the free peptide gives positive NOEs typical of a rapid tumbling molecule, the observed NOESY cross peaks in the presence of lithium perchlorate are negative, indicating a considerable decrease in the mobility of the lithium-bound peptide. This change of sign allows the assignment of the observed NOEs to the lithium–**1** complex and demonstrates that the β-turn conformation is retained on binding to the metal ion.

In DMSO-d_6, addition of NaCl caused shifts in the amide proton resonances which could be partially reversed by the addition of 18-crown-6. However, when the experiment was repeated using $NaNO_3$, the chemical shifts of **1** remained constant and no shifts could be detected by addition of $Ba(ClO_4)_2$. This indicates that

(34) A second set of signals that integrate 5.9% of the major ones could be detected in CD_3CN. These are not detectable in a DMSO-d_6 solution of the same sample and exchange cross peaks between the two sets of signals could be detected in a ROESY experiment with a mixing time of 500 ms, confirming that they arise from a small population of a second conformation in slow exchange, probably involving cis–trans isomerization of the Cys–Pro amide bond. This conformation has not been explored further.

(35) h and l refer to the high- and low-field β-protons of cysteine.

(36) Interproton distances were evaluated (INTRA method) using the ratio between the cross peak and the diagonal peak: Esposito, G.; Pastore, A. *J. Magn. Reson.* **1988**, *76*, 331–336.

(37) Average values in Å of some of the distances obtained with the INTRA method are as follows: 2.1 H^{α} Pro[2]/NH Val[3]; 2.5 NH Val[3]/H^{α} Val[3]; 3.1 H^{α} Val[3]/NH Cys[4]. Calculated distances in Å between the same protons in a theoretical type II β-turn model peptide are as follows: 2.2 $H^{\alpha}_{i+1}/NH_{i+2}$; 2.4 $NH_{i+2}/H^{\alpha}_{i+2}$; 3.2 $H^{\alpha}_{i+2}/NH_{i+3}$; values taken from ref 33.

(38) Woody, R. W. In *The Peptides: Analysis, Synthesis and Biology*; Hruby, V. J., Ed.; New York, 1985; Vol. 7, pp 15–114.

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(40) (a) Linderberg, J.; Michl, J. *J. Am. Chem. Soc.* **1970**, *92*, 2619–2625. (b) Ludescher, U.; Schwyzer, R. *Helv. Chim. Acta* **1971**, *54*, 1637–1644. (c) Webb, J.; Strickland, R. W.; Richardson, F. S. *J. Am. Chem. Soc.* **1973**, *95*, 4775–4783. (d) Woody, R. W. *Tetrahedron* **1973**, *29*, 1273–1283.

(41) Although the theoretical methods reported in ref 40 agree on a change in sign near $X_{SS} \approx |90°|$, the exact crossover point is not certain. Extended Hückel molecular orbital calculations of dimethyl disulfide, a model for cystine, showed that the crossover point depends on the CSS bond angle with a X_{SS} value close to ≈|107°|. (a) Kahn, P. C. Ph.D. Thesis, Columbia University, New York, 1972. (b) Kahn, P. C. In *Methods in Enzymology*; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1979; Vol. 61, pp 339–378.

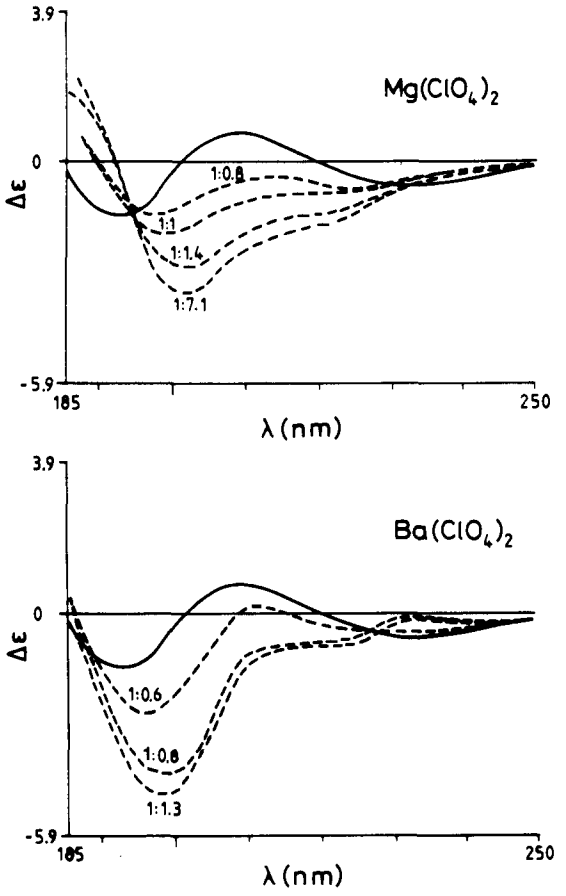

Figure 5. Representative circular dichroism spectra of **1** obtained during the titration with magnesium and barium perchlorates. The numbers by each curve indicate the peptide/metal ion ratio. The solid curve corresponds to free peptide. $\Delta\epsilon$ values at 200 nm change monotonically and reach a plateau at the largest ratio shown in each case. The solvent is CH_3CN and the temperature 293 K.

in DMSO peptide **1** interacts with chloride ions but does not form complexes with metal ions. A similar finding has been reported by Chang and Marzilli[42] for the changes in chemical shift of the NH protons of guanosine induced by the addition of alkali metal chlorides.

The binding of metal ions by peptide **1** in CH_3CN can be followed by the changes induced by the complexation process in the CD spectra (Figure 5). On the basis of concentrations of free peptide and peptide complex determined from the CD spectra, equilibrium constants were defined for the formation of the complexes from their free constituents. The following equations for the two equilibria were used for evaluating the binding constants of the PC and P_2C complexes:

$$P + C = PC \qquad K_1 = [PC]/[P][C]$$

$$2P + C = P_2C \qquad K_{1/2} = [P_2C]/[P]^2[C]$$

where [P], [C], [PC], and [P_2C] are respectively the molar concentrations of the free cyclic peptide **1**, the uncomplexed cation, the 1:1 complex, and the 2:1 sandwich complex; K_1 and $K_{1/2}$ are the binding constants for the two equilibria. If $\Delta\epsilon_P$, $\Delta\epsilon_{PC}$, and $\Delta\epsilon_{P_2C}$ represent the intensity of the CD spectrum of the pure species P, PC, and P_2C, respectively, at the wavelength n, then the observed intensity at this wavelength ($\Delta\epsilon^n$) may be calculated with the relation[6b]

$$\Delta\epsilon^n = [PC]\Delta\epsilon_{PC}/[P_0] + 2[P_2C]\Delta\epsilon_{P_2C}/[P_0] + [P]\Delta\epsilon_P/[P_0]$$

where [P_0] is the total peptide concentration in molar units. The

(42) Chang, C.; Marzilli, L. G. *J. Am. Chem. Soc.* **1974**, *96*, 3656–3657.

Table II. Binding Constants of the Complexes of the Cyclic Peptide **1** with Perchlorate Salts in Acetonitrile[a]

ion	diameter (Å)	$\log(K_1) \pm \sigma_{\log(K_1)}$	$\log(K_1K_{1/2}) \pm \sigma_{\log(K_1K_{1/2})}$
Li^+	1.20	2.60 ± 0.04	
Na^+	1.96	2.11 ± 0.04	
Mg^{2+}	1.30	4.07 ± 0.07	
Ca^{2+}	1.98	3.21 ± 0.10	6.86 ± 0.11
Sr^{2+}	2.24	4.96 ± 0.10	9.02 ± 0.22
Ba^{2+}	2.70	9.09 ± 2.58	16.21 ± 5.07

[a] Temperature = 20 °C.

binding constants, K_1 and $K_{1/2}$ in molar units, and the limiting values $\Delta\epsilon_{PC}$ and $\Delta\epsilon_{P_2C}$ were obtained by minimization of the error function U:

$$U = \sum_{i=1}^{I}\sum_{k=1}^{Nw}(\epsilon_{i,k}^{calc} - \epsilon_{i,k}^{exp})^2$$

with the indices running over the number of spectra per titration and the number of wavelengths digitized per spectra. The minimization was carried out with the program SQUAD[43] conveniently modified to use the CD data as input. Table II shows the set of binding constants obtained by using the above method. Inclusion of P_2C sandwich complexes in the calculation yielded better results for Ca^{2+}, Sr^{2+}, and Ba^{2+}. It was found that the P_2C species were not necessary to fit correctly the titration curves of Li^+, Na^+, and Mg^{2+}, which is in agreement with the isodichroic points observed during the titration with those ions. As expected, the charge of the cation has a significant effect on the strength of binding in the complexes. Although the ionic radii of Ba^{2+} and K^+ are similar (2.66 Å versus 2.70 Å, respectively), no measurable affinity was observed for K^+.[44]

The binding constant for **1**–barium is larger than the one observed for K^+–valinomycin (log K = 5.47)[45] or Ca^{2+}–antamanide (log K = 5.0)[46] in acetonitrile. A preference for binding large divalent cations had already been found in homodetic cyclooctapeptides containing Pro and Gly/Sar (that can adopt the conformation of a D-amino acid) but with lower selectivity: For example[47,48] cyclo[Gly-L-Lys(Z)-Sar-L-Pro]$_2$ has log K values of 4.43, 3.86, and 3.74, respectively for Ba^{2+}, Ca^{2+}, and Mg^{2+} as perchlorates in acetonitrile, and the corresponding values for cyclo-[Pro-Gly]$_4$ are 6.27, 5.53, and 5.08. These figures can be compared to the values of log K of the same ions with **1**: 9.09, 3.21, and 4.07, which also show that the selectivity between Ca^{2+} and Mg^{2+} has been reversed. This is probably due to the higher charge density of the smaller metal ion. The same effect explains the fact that Li^+ binds better than Na^+ or K^+. This result and the observed binding constants of Table II suggest that there may be different binding modes[49] for the 1:1 complexes: for large or intermediate cations, the ion resides in the center of the cavity and interacts with an increased number of carbonyls; for small cations with high charge density (e.g., Li^+ and Mg^{2+}), the cation interacts strongly with a reduced number of carbonyls. The formation of peptide-sandwich complexes is more favorable

(43) (a) Leggett, D. J.; McBryde, W. A. E. *Anal. Chem.* **1975**, *47*, 1065–1070. (b) Leggett, D. J. *Anal. Chem.* **1977**, *49*, 276–280. (c) Leggett, D. J. *Anal. Chem.* **1978**, *50*, 718. (d) Leggett, D. J.; Kelly, S. L.; Shiue, L. R.; Chang, Wu. D.; Kadish, K. M. *Talanta* **1986**, 579–586.

(44) Measured up to a 12-fold molar excess of $KClO_4$. The limit was set by the low solubility of potassium perchlorate in CH_3CN (c_{max} = 0.4 μM).

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(49) Similar structures for the PC complexes of the cyclic dodecapeptide cyclo-(Val-Gly-Gly-Pro)$_3$ were proposed in the following: Baron, D.; Pease, L. G.; Blout, E. R. *J. Am. Chem. Soc.* **1977**, *99*, 8299–8306.

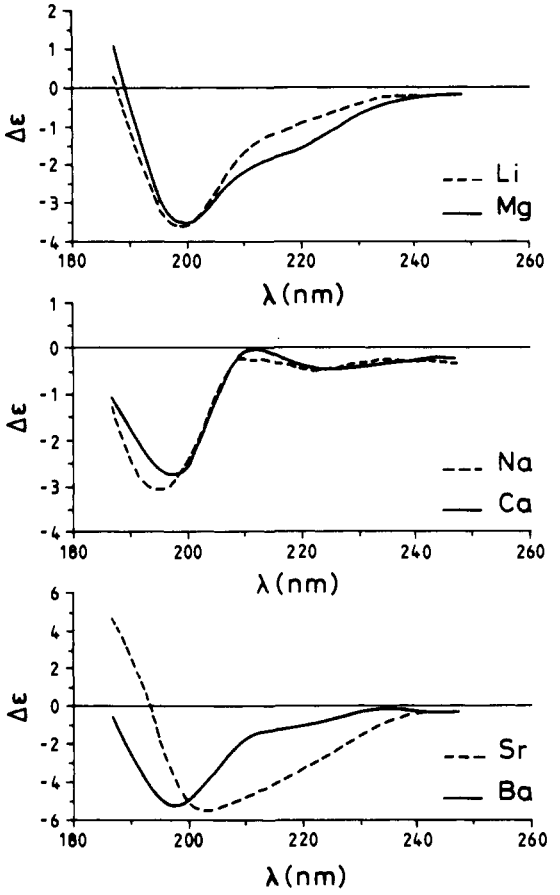

Figure 6. Computer-extracted CD spectra corresponding to pure 1:1 complexes of **1** with cations of different ionic radii.

for Ba^{2+}, Ca^{2+}, and Sr^{2+}, as the two peptides can approach the cation closely enough for a stable interaction without steric hindrance from the peptide chains. The association constants for Ca^{2+} and Sr^{2+} suggest that for cations of this size the binding arrangement in the peptide-sandwich complex is as favorable as that in the 1:1 complex.

The different nature of the CD spectra observed for the free peptide compared to that for the complexed peptide indicates the different conformations for peptide **1**. The calculated CD spectra for the 1:1 complexes obtained from the best fitting of the titration data are presented in Figure 6. Clearly the conformation of the peptide, as reflected in the CD spectra, shows a good correlation with the ionic radii of the cation: Li^+/Mg^{2+} and Na^+/Ca^{2+}. This correlation suggests the formation of inclusion complexes and indicates that the peptide has the appropriate flexibility to optimize ion binding by adapting the size of its cavity to that of its guest.

Conclusions

The cyclic peptide **1** is the first member of a new family of ion-binding molecules containing two β-turn-forming peptide chains linked by two disulfide bonds. Synthesis of **1** has been carried out univocally using an orthogonal protection scheme for the side chains of cysteine that include the use of the *S*-9-fluorenylmethyl and the *S*-acetamidomethyl protecting groups. The easy synthetic access to peptide **1** should be an incentive for the preparation of other members of this new class of ion-binding peptides in the quest for different selectivity.

(1Cys-1'Cys,4Cys-4'Cys)-dithiobis(Ac-L-1Cys-L-Pro-D-Val-L-4Cys-NH_2) shows solvent- and temperature-dependent conformations and forms very stable complexes in acetonitrile solution with alkali metal and alkaline earth metal cations. Circular dichroism spectra obtained at different peptide–metal ion ratios have been interpreted in terms of PC and P_2C sandwich complexes. The ionic radii and the charge density of the cation affect the stability, expressed as binding constants, and the stoichiometry of the complexes. Sandwich complexes are only favorable for the larger cations. Although the binding mode cannot be determined unequivocally because of the difficulties in obtaining NMR spectra of the complexes, the CD data on the PC complexes, derived from the analysis of the titration experiments, indicate that the conformation of the peptide in the complex depends on the size of the complexed ion.

Experimental Section

Synthesis. A. General Methods. All resins used in this work were copoly(styrene–1% divinylbenzene). The 4-methylbenzhydrylamine (MBHA) resin[22] was obtained from USB (Cleveland, OH). All solvents and materials were reagent or HPLC grade quality purchased commercially and used without any further purification. N^{α}-Boc-Cys(Fm)-OH was synthesized by a previously described procedure.[50] Linear assembly of chains by Boc chemistry was carried out manually on an ≈5.0-g scale (50 mL wash volumes), with Boc removal by TFA–CH_2Cl_2 (3:7, v/v; 2 × 30 min) and CH_2Cl_2 (3 × 30 s), followed by neutralization with DIEA–CH_2Cl_2 (1:19, v/v; 3 × 1 min) and CH_2Cl_2 (5 × 30 s). Coupling (1.5 h) was performed in CH_2Cl_2, using the Boc-amino acid and DCC (3.0 equiv each). In all cases, the ninhydrin[51] or chloranil[52] tests were negative after a single coupling. Final acetylations were carried out by adding HOAc and DCC (3.0 equiv each) in CH_2Cl_2.

Peptides and peptide resins were hydrolyzed at 110 or 130 °C with 6 N HCl and 12 N HCl–propionic acid (1:1) for 24 h, respectively, in vacuum-degassed tubes. Amino acid analyses were carried out on a Biotronik Model LC 7000 with a thermostated polystyrene sulfonated BT 2710 column (20 × 0.4 cm). High-performance liquid chromatography was carried out in a Waters Associates apparatus with two solvent delivery systems and a variable-wavelength UV monitor; a Nucleosyl C_{18} (25 × 0.4 cm; 5 μm) or a Vydac C_{18} column (25 × 0.6 cm; 10 μm) was used. Preparative medium-pressure liquid chromatography was achieved on a Michel-Miller Vydac C_{18} column (15–20 μm; 1.5 × 14 cm), using a Milton Roy pump to achieve a flow rate of ≈2 mL/min and a LKB 2158 Uvicord Sd single-path monitor connected to a Servoscribe 1S recorder for detection of peaks. Gel permeation chromatography was performed with a column (95 × 2 cm) using Sephadex LH-20 in MeOH. The absorbance of each fraction was determined at 254 nm. Fast atom bombardment mass spectrometry (FABMS) to characterize synthetic peptides was carried out on a VG 707E-HF instrument, with glycerol or thioglycerol matrices being used to obtain both positive- and negative-ion spectra. Positive-ion spectra often include not only $[M + H^+]$ ions but also $[M + Na^+]$.

B. Ac-L-Cys(Fm)-L-Pro-D-Val-L-Cys(Acm)-NH_2 (2). The solid-phase synthesis of **2** was performed on 5.02 g (3.36 mmol; 0.67 mmol/g) of MBHA resin using the Boc-chemistry outlined above. After completion of the synthesis, a portion of the peptide resin was hydrolyzed and subjected to amino acid analysis (Pro 0.95, Val 1.05, Cys 1.65) with a substitution level of 0.44 mmol of NH_2/g of resin. A portion of this peptide resin (0.23 g, 103 μmol) was cleaved in 5 mL of HF–anisole (9:1, v/v) for 1 h at 0 °C to provide 82 μmol (80%) of crude peptide, which was ≈95%

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(53) Abbreviations used are as follows: AA, amino acid residue (free or protected, depending on context. Symbols denote the L-configuration unless indicated otherwise.); Acm, acetamidomethyl; Boc, *tert*- butyloxycarbonyl; CD, circular dichroism; COSY, correlation spectroscopy; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; DQF, double quantum filtered; Δδ, difference in chemical shifts; $\Delta\delta/\Delta T$, temperature factors of the chemical shifts; 2D-NMR, two-dimensional nuclear magnetic resonance spectrum; DMF, *N,N*-dimethylformamide; FABMS, fast atom bombardment mass spectrometry; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HOAc, acetic acid; HOHAHA, homonuclear Hartman Hahn spectroscopy; HPLC, high-performance liquid chromatography; HyIv, α-hydroxyisovaleric acid; Lac, lactic acid; MBHA, *p*-methylbenzhydrylamine (resin); *p*-MeBzl, *p*-methylbenzyl; MPLC, medium-pressure liquid chromatography; NMM, *N*-methylmorpholine; Npys, 3-nitro-2-pyridinylsulfenyl; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ROE, rotating frame nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TFA, trifluoroacetic acid; TFE, trifluoroethanol; t_m, mixing time; TMS, tetramethylsilane; t_R, retention time.

pure by analytical HPLC. This last material was dissolved in 1 mL of MeOH and applied to a Sephadex LH-20 column (95 × 2 cm). Elution was with MeOH at a flow rate of 28 mL/h and UV detection at 254 nm. The major peak corresponded to pure peptide **2** (78 μmol; 95% yield), which was characterized by analytical HPLC and FABMS: 711 [M + H⁺], 733 [M + Na⁺], 745 [M + Cl⁻]. Amino acid analysis: Pro 0.92, Val 1.08, Cys 1.76.

C. (¹Cys-¹'Cys)-dithiobis(Ac-L-¹Cys-L-Pro-D-Val-L-⁴Cys(Acm)-NH₂) (3). A solution of **2** (78 μmol) in piperidine–DMF (1:1, v/v; 2 mL) was stirred for 2 h at 20 °C. The evolution of the reaction was monitored by HPLC using the following conditions: linear gradient over 20 min on a Nucleosyl C_{18} column of 0.036% CH_3CN and 0.045% H_2O from 1:4 to 9:1, flow rate 1.0 mL/min; UV detection at 220 nm; t_R = 6.4 min (**3**), 13.0 min (**2**), 17.8 min (fluorenylmethylpiperidone). After completion of the reaction, the mixture was evaporated in vacuo. After addition of dry ether to remove the fluorenylmethylpiperidone, the resulting powder was collected by centrifugation. Crude peptide from the oxidation was purified by gel filtration using conditions identical to those used in the purification of **2** but detecting at 206 nm. Yield: 30 μmol (77%) of **3**; pure by analytical HPLC. FABMS: 1063 [M + H⁺], 1085 [M + Na⁺], 1097 [M + Cl⁻]. Amino acid analysis: Pro 2.06, Val 1.94, Cys 3.42.

D. (¹Cys-¹'Cys,⁴Cys-⁴'Cys)-dithiobis(Ac-L-¹Cys-L-Pro-D-Val-L-⁴Cys-NH₂) (1). **(i) Oxidation with Iodine.** Iodine (39 mg; 150 μmol) was added in one portion to a solution of 30 μmol of **3** in 30 mL of HOAc–H_2O (8:2, v/v). Aliquots of the solution (60 μL) were removed at different times, quenched with a saturated solution of ascorbic acid (60 μL), and then applied onto the HPLC using the following conditions: linear gradient over 20 min on a Nucleosyl C_{18} column of 0.036% CH_3CN in TFA and 0.045% H_2O in TFA from 1:19 to 7:3, flow rate 1.0 mL/min; UV detection at 220 nm; t_R = 13.2 min (**3**), 13.8 min (**1**). After 2 h all the starting material had been deprotected. The solution was diluted with 30 mL of H_2O, extracted with CCl_4 (5 × 50 mL), and concentrated to remove the remaining CCl_4. The crude peptide product (26 μmol) included 77% (20 μmol) of the desired cyclic product. Crude peptide from the oxidation (20 μmol) was purified by MPLC on a Vydac C-18 column, using a convex gradient of 0.3% propionic acid in CH_3CN–H_2O (5:95, v/v; 500 mL) to 0.3% propionic acid in CH_3CN–H_2O (4:6, v/v; 500 mL) at 2.6 mL/min; UV detection at 206 nm. Yield: 13 μmol (65% purification) of cyclic peptide **1**; pure by analytical HPLC. FABMS: 919 [M + H⁺], 941 [M + Na⁺], 953 [M + Cl⁻]. Amino acid analysis: Pro 1.96, Val 2.04, Cys 3.50.

(ii) Oxidation with Thallium(III) Trifluoroacetate. A solution of **3** (4.4 μmol) and anisole (140 μL) in TFA (2.9 mL) was cooled in an ice bath. $Tl(TFA)_3$ (5.5 μmol) was added, and the mixture was stirred at 0 °C for 1 h. The reaction mixture was evaporated in vacuo to remove the TFA, including chasing with ether; the resulting powder was collected by centrifugation. The crude peptide product (8.4 μmol) included 1.2 μmol of the desired cyclic product **1** and 5.6 μmol of Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2.

Molecular Graphics. Energy minimizations were performed with the CHARMM program on an Iris system (Silicon Graphics) using the molecular graphics QUANTA (Polygen Corp.) program and parameter set. Energy minimizations were performed with a combination of steepest descents and conjugate gradient methods. Starting conformations were produced by incorporating experimental distances (H^α Pro²/NH D-Val³ 2.1 ± 0.2; NH D-Val³/H^α D-Val³ 2.5 ± 0.2; and H^α D-Val³/NH Cys⁴ 3.1 ± 0.2) and angular constraints and by changing the configurations of both disulfide bridges. Changes in the size of the cavity were determined by measuring the distances between the corresponding carbonyl groups of the two segments and between all sulfur atoms.

NMR Measurements. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **1** in DMSO-d_6 or CD_3CN were recorded on a Bruker AM or a Varian VXR-500 spectrometer. The sample concentration was ≈5.4 × 10^{-3} M, and the sample temperature was 298 K. Chemical shift values are quoted in parts per million (ppm) downfield from TMS. The residual peak of CD_3CN was used as the internal reference in this solvent. The temperature dependence of the chemical shift for each amide proton was calculated by recording one-dimensional spectra at different temperatures and fitting the chemical shifts to a linear least-squares fit. Pure sequences and parameters used to collect and process the 2D-NMR data are described below.

The ROESY and HOHAHA spectra were recorded at 500 MHz with 64 scans, a relaxation delay of 2 s, and 2048 data points in t_2. An MLEV mixing scheme with a 11.2-kHz spin-locking field strength was used for the HOHAHA, while a continuous wave mixing of 100 or 200 ms was used for the ROESY.

The NOESY spectra were recorded at 500 MHz with 1024 points in t_1, 32 scans, a relaxation delay of 2 s, and 2048 points in the t_2 dimension. The mixing time was varied randomly (±20 ms) to suppress zero-quantum J-cross peaks. The solvent signal was reduced by presaturation during the recycle delay period. Interproton distances were evaluated (INTRA method) from the ratio between the cross peak and the diagonal peak. No baseline correction was employed. The correlation time used in the calculation was computed using a known distance (1.77 Å) between the two geminal δ protons of proline. The DQF-COSY spectra were recorded at 500 MHz with 2048 or 4056 points in t_2 and 1024 points in the t_1 dimension, 32 scans.

CD Measurements. Circular dichroism (CD) spectra were recorded on a Jasco-600 spectrometer connected to an IBM 50 Z computer, using a 0.02- or 1-cm cell path length. Both the optics and the chamber were flushed continuously with dry N_2 throughout each experiment. All data are corrected for solvent contraction at low temperature, are reversible, and are independent of concentration (the peptide concentrations were 2.11–3.08 mM). CD spectra are reported in $\Delta\epsilon/\alpha$-amino acid residue. Low-temperature measurements were carried out with a system equipped with an Electronic Thermometer Comark 1603. Spectrophotometric grade TFE and CH_3CN (Spectrosol BDH) or redistilled water were used as the solvents. A blank run of solvent and cell was subtracted from the measured solution.

For the CD titration experiments a single stock solution of known cyclic peptide **1** concentration was prepared using acetonitrile previously dried over molecular sieves. The dilution of this stock solution was made depending upon the sensitivity of the instrument using 0.02- or 1-cm path length cells. Another stock solution of **1**, of concentration equal to the first, was prepared to which a weighted quantity of the dry perchlorate salt was added. When this was mixed, in calculated quantities, with the free cyclic peptide **1** solution, solutions of different molar ratios were prepared. After thorough mixing over the rotor mixer, the solutions were left for 10 min of incubation before use. The extent of ion binding was determined from CD spectra, assuming a superposition of spectra from the free peptide and each complex present. Equilibrium binding constants were defined in terms of concentration.

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