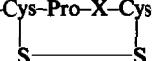


STABILIZATION OF β -TURN CONFORMATIONS IN PRO-X SEQUENCES BY DISULPHIDE BRIDGING. SYNTHESIS AND SOLUTION CONFORMATIONS OF FIVE CYCLIC CYSTINE PEPTIDES

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Abstract—Five cyclic peptide disulphides of the type Boc-Cys-Pro-X-Cys-NHMe have been


synthesized, where X = Gly (1), L-Ala (2), D-Ala (3), Aib (4) and L-Leu (5). ^1H NMR studies at 270 MHz in CDCl_3 and $(\text{CD}_3)_2\text{SO}$ provide evidence of a Pro-X β -turn conformation, stabilized by a transannular 4 \rightarrow 1 hydrogen bond involving the Cys(4) NH, in all the peptides. In addition peptides 2, 4 and 5 also possess a second intramolecular hydrogen bond involving the -NHMe group. The spectroscopic data are consistent with a consecutive type III 8-turn conformation for peptides 2, 4 and 5, a type I(III) β -turn structure for 1 and a type II turn for 3.

β -Turn conformations¹⁻⁴ and disulphide linkages⁵ are important elements of peptide and protein structures.^{6,7} The structural diversity and wide spectrum of biological activity of natural and synthetic peptides has provided a powerful impetus for the development of the experimental^{8,9} and theoretical¹⁰ methods of peptide conformational analysis. The refinement of spectroscopic methods of conformational analysis, necessarily requires the availability of well defined model peptides. Cyclic peptides have proved attractive systems in view of their relatively restricted range of conformations.^{11,12} Acyclic peptides incorporating stereochemically constrained residues like, α -aminoisobutyric acid (Aib)[†] have been developed more recently as conformational models.¹³⁻¹⁶

In this report, we describe the synthesis and spectroscopic analysis of a series of cyclic peptide disulphides (Fig. 1), in which an L-Pro-X sequence separates two Cys residues. The choice of the Pro-X sequence was dictated by the high probability of fl-turn formation.¹⁷ The disulphide bridge provides a means of "covalently locking" the fl-turn structure and additionally allows these peptides to be applied to the development of CD^{18,19} and Raman spectroscopy,²⁰⁻²² in determining the stereochemistry of the -S-S- linkage. The 14-membered disulfide loop is expected to possess only limited structural flexibility, thereby generating conformationally well defined

peptides.²³ The results establish that these cyclic peptide disulphides provide models for type I, II and consecutive type III β -turns.¹

RESULTS

Synthesis and characterization

The peptide disulphides were synthesized using the classical oxidative cyclization of the peptide dithiols, carried out under conditions of high dilution.²⁴ Peptides **1-5** were obtained as homogeneous solids and thus far single crystals, suitable for X-ray diffraction have been obtained in the case of the Pro-Aib (**4**) and Pro-Leu (**5**) disulphides. The monomeric nature of the cyclic disulphides was established by mass spectrometric observation of the molecular ion, using field desorption or fast atom bombardment mass spectrometry.²⁵ Elemental analysis and 270 MHz ^1H NMR spectra were fully consistent with the structures. Further confirmation of the presence of the 14-membered disulphide ring in **4** has been obtained by X-ray diffraction.^{26,27} The yields of the cyclic peptide disulphides were low, ranging from 12 to 25% (Table 1). Side reactions involving cleavage of Cys-Pro bonds during the reductive cleavage of S-benzyl groups by Na/liquid NH₃,²⁸ and the formation of polymeric products during the subsequent oxidation, presumably account for the poor yields. An attempt to establish a correlation between possible Pro-X fl-turn formation in the acyclic precursor and yields of cyclization did not result in a fruitful correlation.

^1H NMR studies

Figure 3 shows a representative 270 MHz ^1H NMR spectrum of the Pro-Gly disulphide **1** in CDCl_3 . ^1H NMR studies on disulphide **4** have been described earlier, in conjunction with X-ray diffraction studies.²⁷ Extremely well resolved spectra were obtained in all cases permitting unambiguous assignment of resonances. In all five peptides the Cys(1)NH could be unequivocally assigned to the high field NH (~ 5.56) in CDCl_3 , in view of the known tendency of urethane NH groups to appear at high field in this solvent.^{29,30} The X-NH groups in **1** (X = Gly) and **4**

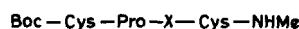
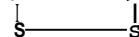


Fig. 1. Cyclic peptide disulphides.

[†]Abbreviations used: Aib, α -aminoisobutyric acid; Boc, t -butyloxycarbonyl; DCC, N, N-dicyclohexylcarbodiimide; Bz, benzyl; DMF, N, N-dimethylformamide; THF, tetrahydrofuran; NMM, N-methylmorpholine; NOE, Nuclear Overhauser Effect. Where configuration is not indicated, L-amino acids are implied.

Table 1. Characterisation of cyclic peptide disulphides Boc-Cys-Pro-X-Cys-NHMe



X	Yield (%)	Mass Spectrum		MP (°C)	R _f		[α] _D ^{25°C}	Elemental Analysis					
		M ⁺	MH ⁺		A	B		C			H		N
		Calc	Obs		Calc	Obs		Calc	Obs	Calc	Calc	Obs	Calc
Gly 1	25.4	-	490 ^c	148-149	0.85	0.89	-22.5	46.6	46.3	6.3	6.5	14.3	
LAla 2	13.6	503 ^e	504 ^{c,d}	227-228	0.77	0.93	-65	47.7	47.4	6.6	7.0	13.9	
DAla 3	15.9	503 ^e	504 ^{c,d}	198-199	0.74	0.93	-90	47.7	47.1	6.6	6.9	13.9	
Aib ^b 4	12.2	517 ^e	518 ^{c,d}	188-190	0.74	0.86	-97.5	46.9	47.0	5.8	6.3	11.6	
Leu 5	15.3	545 ^e	-	155-157	0.89	0.95	+25	50.6	50.8	7.1	7.3	12.8	

a) A = 15% Methanol in chloroform; B = Ethylacetate-pyridine-acetic acid-water (5:5:1:1).

b) Analytical Data computed with a molecule of $(\text{CD}_3)_2\text{SO}$

c) Field Desorption

d) Fast Atom Bombardment

e) Electron impact

f) C = 0.2, CH_3OH

(X = Aib) are unambiguously recognized as triplet and singlet resonances, respectively. In peptides **2**, **3** and **5** both the Cys(4) and X-NH groups are doublets. Unequivocal assignments were made by double resonance experiments, which establish the connectivities between C^2H_2 , C^4H and **NH** resonances; the C^2H_2 Cys resonances being clearly distinct from the Ala C^2H_2 or Leu C^2H_2 peaks. The methylamide NH resonances are readily identified as broad quartets. Assignments in $(\text{CD}_3)_2\text{SO}$ were made on the basis of spin decoupling and by monitoring NH chemical shifts in mixtures of CDCl_3 and $(\text{CD}_3)_2\text{SO}$. The chemical shifts and $J_{\text{HNC}_2\text{H}}$ values for the NH groups in peptides **1-5** are listed in Table 2.

Delineation of hydrogen bonded NH groups

The presence of solvent shielded or intramolecularly hydrogen bonded NH groups was established using three criteria: (i) temperature dependence of NH chemical shifts in $(\text{CD}_3)_2\text{SO}$,³¹⁻³³ (ii)

solvent dependence of NH chemical shifts $\text{CDCl}_3-(\text{CD}_3)_2\text{SO}$ mixtures³⁴ and (iii) rates hydrogen-deuterium (H-D) exchange in CDCl_3-D mixtures.³⁵ A typical H-D exchange experiment is shown in Fig. 3 (inset) for peptide **1**. It is clear that the exchange rates follow the order Gly(3)NH > Cys(1)NH > NHMe > Cys(4)NH. The Cys(4) and methylamide NH protons exchange significantly slower than the other two, indicative of their relative inaccessibility to solvent. A similar order of H-D exchange rates is observed in the other peptide disulphides (**2-5**) although the precise values of the exchange half lives ($t_{1/2}$) vary. The use of heterogeneous solvent system precludes comparison between different sets of peptides. No attempt has been made to quantitate the H-D exchange data since a qualitative ordering of NH groups is sufficient for delineating solvent shielded NH groups.

The results of experiments to determine the solvent and temperature dependences of NH chemical shifts

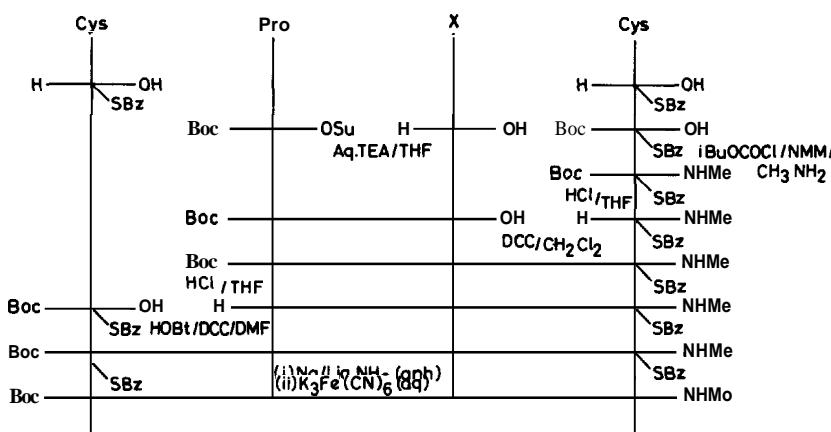


Table 2. ^1H NMR parameters of NH group in the peptides Boc-Cys-Pro-X-Cys-NHMe

X	δ_{CDCl_3} (ppm)			$\Delta\delta^a$ (ppm)				
	Cys(1)	Cys(4)	NHMe	X	Cys(1)	Cys(4)	NHMe	X
Gly 1	5.61	7.55	6.82	7.15	1.79	0.11	0.87	1.57
L-Ala 2	5.44	7.26	6.55	6.35	2.11	0.26	0.76	1.72
D-Ala 3	5.72	7.67	6.97	7.13	1.55	0.25	0.96	1.70
Aib 4	5.35	7.43	6.78	6.42	2.13	0.01	0.86	1.91
Leu 5	5.4	7.22	6.54	6.2	2.05	0.16	1.1	1.59

X	$d\delta/dT \times 10^3$ (ppm/ $^{\circ}\text{C}$) ^b			$J_{\text{HN}-\text{C}^{\alpha}\text{H}}$ (Hz) ^c			No. of H Bonds	
	Cys(1)	Cys(4)	NHMe	X	Cys(1)	Cys(4)	X	
Gly 1	8.7	1.4	4.1	4.6	8.0	7.0	d	1
L-Ala 2	7.3	2.1	1.9	5.1	9.0	5.5	9.0	2
D-Ala 3	8.8	0.4	5.8	5.2	9.0	9.0	4.5	1
Aib 4	7.4	1.5	3.4	4.5	8.5	7.5	-	2
Leu 5	3.8	1.2	2.0	4.0	8.5	8.0	9.5	2

a) $\Delta\delta = \delta_{(\text{CD}_3)_2\text{SO}} - \delta_{\text{CDCl}_3}$ b) Solvent, $(\text{CD}_3)_2\text{SO}$ c) Errors in J value ≈ 0.5 Hzd) J not determined due to poorresolution of triplet and accidental degeneracy of $\text{C}^{\alpha}\text{H}_2$ protons.

in peptides **2(L-Ala)** and **3(D-Ala)** are shown as illustrative examples in Fig. 4. The values of the temperature coefficients ($d\delta/dT$) and solvent shift values ($\Delta\delta$) for the NH groups in the various peptides are summarized in Table 2. In the Pro-Gly (**1**) and Pro-D-Ala (**3**) disulphides only the Cys(4)NH group

appears to be shielded from the solvent ($d\delta/dT < 0.004$ ppm/ $^{\circ}\text{C}$),³¹⁻³³ presumably by involvement in an intramolecular hydrogen bond. In the Pro-L-Ala (**2**) and Pro-L-Leu (**5**) disulphides two NH groups, Cys(4) and NHMe, show evidence for being hydrogen bonded. In the Pro-Aib (**4**) disulphide, Cys(4)NH is strongly solvent shielded, while NHMe has an intermediate $d\delta/dT$ value in $(\text{CD}_3)_2\text{SO}$ (0.0034 ppm/ $^{\circ}\text{C}$). In all peptides the A6 values for the Cys(1) and X-NH groups are large (1.5–2.0 ppm), whereas the Cys(4)NH exhibits very little solvent dependence ($\Delta\delta < 0.3$ ppm). The NHMe group has an intermediate $\Delta\delta$ value for all five peptides (0.8–1.1 ppm), suggesting that the solvent titration experiments may be less reliable in differentiating free and hydrogen bonded terminal methylamide groups.

Further evidence for the presence of intramolecular hydrogen bonded conformations was obtained from IR studies in CHCl_3 solution, at peptide concentrations of $\sim 3 \times 10^{-3}$ M (Fig. 5). The presence of the broad intense band at $< 3350\text{ cm}^{-1}$ is indicative of the presence of intramolecularly hydrogen bonded NH groups.³⁶⁻³⁸ In both ^1H NMR and IR studies, effects due to peptide association³⁹⁻⁴² have been disregarded for the following reasons: (i) non-specific aggregation effects tend to equalize the $d\delta/dT$ values for a set of NH groups in a peptide; (ii) in both NMR and IR studies, clear distinctions were obtained between the spectroscopic parameters ($d\delta/dT$, $\Delta\delta$ and area of the ν_{NH} (bonded) band) of the acyclic di-S-benzyl precursors and the cyclic peptide disulphides.

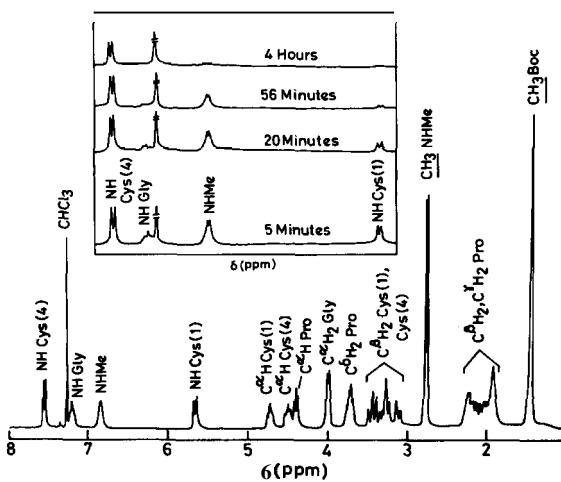


Fig. 3. 270 MHz ^1H NMR spectrum of **1** in CDCl_3 (inset) NH resonances monitored as a function of time after addition of $\sim 10\%$ D_2O . Note that Cys(4) and methylamide NH groups exchange at a significantly slower rate than Cys(1) and Gly NH groups.

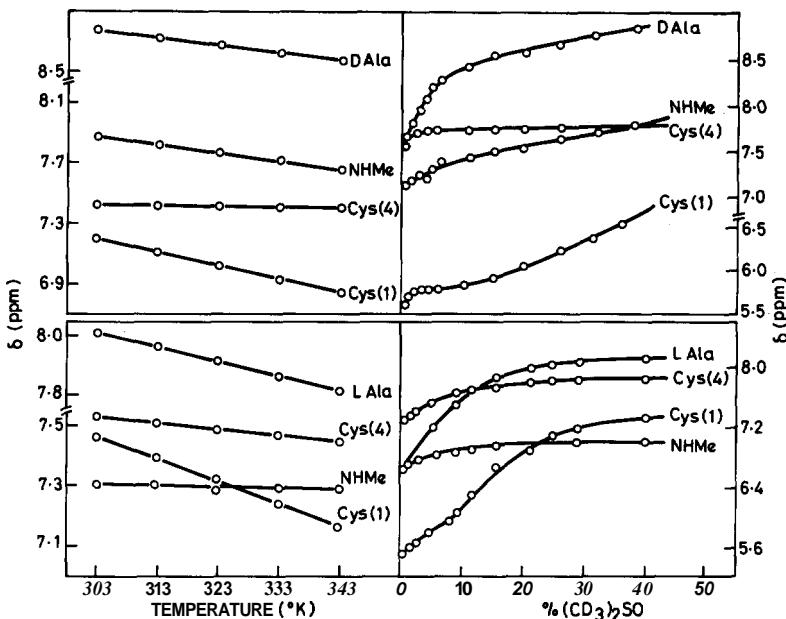


Fig. 4. Temperature and solvent dependence of NH chemical shifts in peptide disulphides (bottom) 2 and (top) 3.

The acyclic peptides, with the exception of **Boc-Cys(SBz)-Pro-Aib-Cys(SBz)-NHMe**, did not show evidence for the presence of intramolecularly hydrogen bonded NH groups. Aggregation effects, particularly via intermolecular β -sheet formation in organic solvents,¹³ would be expected to be more facile for the acyclic peptides. In the case of the Pro-Aib acyclic peptide, the presence of two stereochemically-constrained amino acids,¹³⁻¹⁶ would favour consecutive β -turn formation for the -Pro-Aib-Cys-sequence in solution, a feature demonstrated in several cases in related peptides.^{44,45}

DISCUSSION

The number of intramolecular hydrogen bonds and the conformations consistent with the NMR data for peptides **1-5** are listed in Table 2. The NMR

results suggest that in all five peptide disulphides, the Cys(4) NH is inaccessible to solvent. This observation is consistent with the involvement of the Cys(4)NH in a transannular $4 \rightarrow 1$ intramolecular hydrogen bond with the Cys(1)CO group, stabilizing a Pro-X β -turn (Fig. 6). The conformational angles in the ideal Pro-X- β -turns are type I $\phi_{\text{Pro}} = -60^\circ$, $\psi_{\text{Pro}} = -30^\circ$, $\phi_X = -90^\circ$, $\psi_X = 0^\circ$; type II $\phi_{\text{Pro}} = -60^\circ$, $\psi_{\text{Pro}} = 120^\circ$, $\phi_X = 80^\circ$, $\psi_X = 0^\circ$; type III $\phi_{\text{Pro}} = \phi_X = -60^\circ$, $\psi_{\text{Pro}} = \psi_X = -30^\circ$. Note that type III turns are only a slight variant of the Type I conformation. This conformation is in accord with the established propensity of Pro-X sequences to adopt β -turn structures.

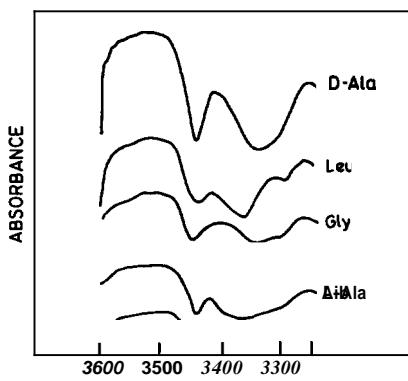


Fig. 5. IR spectra of -Pro-X-disulphides ($\sim 3 \times 10^{-3}$ M) in CHCl_3 . Only ν_{NH} stretching bands are shown.

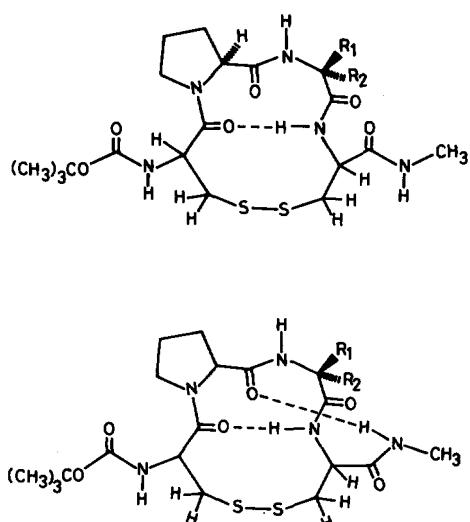


Fig. 6. (top) Pro-X β -turn conformation proposed for peptides 1 and 3 (bottom consecutive β -turn conformation proposed for peptides 2, 4 and 5).

tures.^{3,4,17} An interesting feature in peptides **2**, **4** and **5** is the low $d\delta/dT$ value for the C-terminal NHMe group, suggestive of its participation in an intramolecular hydrogen bond. A consecutive β -turn structure involving a Pro-X turn followed by an X-Cys turn (Fig. 6) is consistent with the data. In this conformation two 4→1 hydrogen bonds (Cys(1)CO---HN Cys(4) and Pro(2)CO---HNMe) stabilize the successive β -turns. A structure involving a repetitive type III β -turn (incipient 3_{10} helix), with a disulphide bridge linking Cys(1) and Cys(4) has been established in the solid state for **4**.^{26,27} There are only two stereochemically acceptable ways of generating repetitive β -turns in the L-Pro-L-X-L-Cys sequence. These are (i) type III-III, with all residues having $\phi \sim -60^\circ$, $\psi \sim -30^\circ$. (ii) Type II-type I'(III') with $\phi_{\text{Pro}} \sim -60^\circ$, $\psi_{\text{Pro}} \sim +120^\circ$, $\phi_x \sim +70^\circ \pm 20^\circ$, $\psi_x \sim -20^\circ \pm 20^\circ$, $\phi_{\text{Cys}} \sim +80^\circ$, $\psi_{\text{Cys}} \sim 0^\circ$. Of these, the type II-I' structure is expected to be energetically less favourable since it forces the L-X and L-Cys residues to adopt positive ϕ , ψ values.²⁸ It is, therefore, likely that peptides **2** and **5** adopt the incipient 3_{10} helical structure, crystallographically observed for **4**.

For the Pro-Gly (**1**) and Pro-D-Ala (**3**) disulphides, conformations involving a Pro-X β -turn need to be considered. Type I(III) and II β -turns are stereochemically allowed, with the type II conformation being energetically favourable for the Pro-D-Ala sequence. Crystallographic studies of N-isobutyl-L-proyl-D-alanyl isopropylamide have established a type II 8-turn in the solid state.⁴⁶ Analyses of Pro-Gly β -turns in protein crystal structures have yielded a slight preponderance of type II conformation.²⁹ A direct way of distinguishing the type I and II structures is by nuclear Overhauser effect (NOE) techniques.⁴⁷ A detectable NOE between Pro C^αH and the X-NH resonances is expected for Pro-X type II 8-turns! Difference NOE studies establish an intensity enhancement of 6% for the Pro C^αH resonance on saturating the D-Ala NH of **3**, in CDCl₃. In the case of **1** NOE's were not detectable.⁴⁸ The results suggest that peptide **3** favours a type II 8-turn, while **1** adopts a type I (III) conformation. This latter conclusion is, however, not based on substantive experimental evidence, since non-observation of an NOE could, presumably, also be an experimental artifact. Analysis of the ABX spectrum due to the Gly C^αH₂ and NH protons can yield vicinal and geminal coupling constants, which in turn can be used to derive information about ϕ_{Gly} and ψ_{Gly} .⁴⁹ Such an approach has in fact been used to establish a type II Pro-Gly 8-turn in a synthetic oxytocin analogue.⁵⁰ Unfortunately, in the present case, the accidental degeneracy of the Gly C^αH₂ chemical shifts (at 270 MHz) precluded such an analysis. Magnetic non-equivalence of Gly C^αH₂ protons in Pro-Gly sequences has often been used as a diagnostic for folded conformations and absence of dynamic averaging over multiple conformational states.^{50,51} The results on the Pro-Gly disulphide **1**, suggest that this may not be true in all cases. A type I- β -turn, within a 14-membered disulphide loop has been proposed for the -Cys-Pro-Val-Cys- sequence from NMR and conformational energy calculations.²³ In that sequence the bulky 8-branched Val residue, lowers the probability of consecutive β -turn formation.

The J_{HNOH} values for the X residue listed in Table 2 suggest that the ϕ_X values in the X = L-Ala (**2**) and L-Leu (**5**) peptides (3 = 9.0 – 9.5 Hz) are similar, while the D-Ala residue in **3** (3 = 4.5 Hz) adopts a different conformation (ϕ_X). This is consistent with the conclusion reached above that type III β -turns are favoured in peptides **2** and **5**, while **3** adopts a type II structure. In view of the known uncertainties in dihedral angle-coupling constant relationships? no attempt has been made to derive precise ϕ_X values.

An intriguing observation in Table 2 is that the $d\delta/dT$ value of the NHMe group in the Pro-D-Ala peptide (**3**) is significantly higher than in the other disulphides. While the $d\delta/dT$ value of 0.0041 ppm/°C observed in **1** is not conclusive evidence for a hydrogen bonded NH group, it may reflect a tendency of the -Pro-Gly-Cys- sequence towards consecutive 8-turn formation, with the second Gly-Cys bend being destabilized in polar media, like (CD₃)₂SO. In such a situation the initial Pro-Gly turn may in fact prefer a type I(III) conformation.

The preceding discussion suggests that varied 8-turn conformations are observed in the peptide disulphides **1–5**. A more complete elaboration of the stereochemistry of the backbone and disulphide linkage in these peptides must await the results of future X-ray diffraction studies, conformational energy calculations, Raman and CD spectroscopic analysis of these peptides. The presently available data establishes these systems as useful "cyclized 8-bend" mode 1 ~The peptide disulphides described in this study constitute relatively rigid models for type I, II and consecutive type III 8-turn structures and should prove useful in the spectroscopic characterization of these conformations. The application of these peptides and related acyclic systems in analysis of β -turn structures by NOE and CD methods is described elsewhere.⁴⁸

EXPERIMENTAL

Synthesis of peptides

The general scheme adopted is summarized in Fig. 2. All peptides were characterized by ¹H NMR and checked for purity by TLC on silica gel. Elemental analyses were also carried out on the acyclic and cyclic tetrapeptides. The detailed protocol is described below for **1**.

Synthesis of disulphide **1**

Boc-Cys(SBz)-NHMe. Boc-Cys(SBz)-OH⁵³ (3.1 g; 10 mmol) dissolved in anhyd THF (20 ml) was cooled in a freezing mixture (–15°C). N-Methylmorpholine (1.1 ml) and isobutylchloroformate (1.3 ml) were added successively, with stirring. After 15 min, dry THF saturated with CH₃NH₂ gas (25 ml) was added and the mixture stirred for 2 hr. Insoluble salts were filtered off, the THF was evaporated and the residue was dissolved in EtOAc and washed successively with 1N HCl, 1N NaHCO₃ and sat aq NaCl. The organic layer was dried over Na₂SO₄ and evaporated to yield a white solid (3.1 g; 95%), m.p. 123°C.

Boc-Pro-Gly-Cys(SBz)-NHMe. Boc-Cys(SBz)-NHMe (1.8 g; 5.5 mmol) was treated with dry THF saturated with HCl gas (10 ml). After Boc group removal was complete, as evidenced by TLC, the mixture was carefully neutralized with aqueous Na₂CO₃ and extracted with CHCl₃. The organic layer was dried and evaporated to yield Cys(SBz)-NHMe as a viscous oil (1.1 g; 88%) which was directly used. The oil was dissolved in CH₂Cl₂ (15 ml) and cooled in an icebath. Boc-Pro-Gly-OH⁵⁴ (1.4 g, 5.1 mmol) and 1 g (5 mmol) DCC were successively added and the mixture stirred overnight at room temperature. The precip-

itated dicyclohexylurea was filtered off and the filtrate was washed with 1N HCl, 1N NaHCO₃, and H₂O. Drying and evaporation yielded the tripeptide as a white solid (2.1 g; 87%), m.p. 137°. The 270 MHz ¹H NMR spectrum was fully consistent.

Boc-Cys(SBz)-Pro-Gly-Cys(SBz)-NHMe. Boc-Pro-Gly-Cys(SBz)-NHMe (2.0 g, 4.1 mmol) was deprotected with HCl/THF as described above to give Pro-Gly-Cys(SBz)-NHMe as a colorless oil (yield 1.5 g, 97%). This was dissolved in 10 ml DMF, cooled to 0° and 1.2 g (4 mmol) Boc-Cys(SBz)-OH was added followed by 500 mg 1-hydroxybenzotriazole and 800 mg (4 mmol) DCC. The mixture was stirred overnight, the precipitated dicyclohexylurea filtered off, 30 ml EtOAc added and the product obtained after workup, as described above, as a white, hygroscopic solid, which slowly turns yellow on standing. Yield 2.5 g (93%). Column chromatography on silica gel with (3% CH₃OH-CHCl₃) as the eluent yielded the acyclic tetrapeptide as a fine white solid. M.p. 95-96°. $[\alpha]_D^{25} = -94^\circ$ (c = 0.2, CH₃OH). (Found C, 58.9; H, 7.0; N, 10.4. Calc. for C₁₉H₃₁O₆H₂S₂C, 59.0; H, 6.7; N, 10.4%). The 270 MHz ¹H NMR spectrum was fully consistent.

Cyclic disulphide 1

2.0 g of acyclic tetrapeptide was dissolved in 400 ml of dry, distilled liquid NH₃. Small pieces of metallic sodium were added, while stirring vigorously until the blue color persisted. The excess of sodium was carefully destroyed by addition of a few drops of glacial acetic acid. The ammonia was slowly evaporated under a stream of nitrogen and the residue dissolved in 1800 ml H₂O (0.073% solution). The pH was adjusted to 6.8 and small amounts of 0.02 M K₃Fe(CN)₆ was added to ensure complete oxidation.²⁴ The solution was lyophilized and the residue extracted with 4 x 25 ml EtOAc. The organic layer was washed with 1N HCl, 1N NaHCO₃, and H₂O and dried. Evaporation yielded 600 mg of a solid which was chromatographed on a silica gel column. Elution with 3% CH₃OH-CHCl₃ yielded 365 mg (25.4%) of disulphide 1. The physical characteristics are summarized in Table 1. The 270 MHz ¹H NMR spectrum is shown in Fig. 3.

Synthesis of disulphides 2, 3, 4, 5

The disulphides 2-5 were synthesized using a protocol similar to that described for 1. The dipeptide acids Boc-Pro-X-OH were synthesized by the succinimide ester procedure,²⁵ except for X = Aib which was prepared by saponification of Boc-Pro-Aib-OMe synthesized by the DCC/CH₂Cl₂ procedure. Except for X = Gly and Aib, 1-hydroxybenzotriazole was used as a racemization suppressing additive²⁶ in the coupling of Boc-Pro-X-OH to Cys(SBz)-NHMe. In all cases the acyclic tetrapeptides were purified by silica gel column chromatography and fully characterized by elemental analysis and 270 MHz ¹H NMR.

The yields of cyclization and various parameters characterizing the cyclic peptide disulphides are summarized in Table 1. All five disulphides gave extremely well resolved 270 MHz ¹H NMR spectra. A representative spectrum is illustrated in Fig. 3.

Acknowledgements— We thank Dr S. Prasanna, Oregon Graduate Centre, for the mass spectral measurements. This research was supported by a grant from the Department of Science and Technology, Government of India. NMR studies were carried out at the Sophisticated Instruments Facility, Indian Institute of Science, Bangalore.

REFERENCES

- C. M. Venkatachalam, *Biopolymers* 6, 1425 (1968).
- P. N. Lewis, F. A. Momany and H. A. Scheraga, *Biochim. Biophys. Acta* 303, 211 (1973).
- P. Y. Chou and G. D. Fasman, *J. Mol. Biol.* 115, 135 (1977).
- J. A. Smith and L. G. Pease, *CRC Crit. Rev. Biochem.* 8, 315 (1980).
- J. M. Thornton, *J. Mol. Biol.* 151, 261 (1981).
- J. Richardson, *Adv. Protein Chem.* 34, 167 (1981).
- C. B. Anfinsen and H. A. Scheraga, *Ibid.* 29, 205 (1975).
- H. R. Wyssbrod and W. A. Gibbons, *Surv. Prog. Chem.* 6, 207 (1973).
- L. C. Craig, D. Cowburn and H. Bleich, *Ann. Rev. Biochem.* 44, 477 (1975).
- G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.* 23, 283 (1968).
- C. M. Deber, V. Madison and E. R. Blout, *Acc. Chem. Res.* 9, 106 (1976).
- E. R. Blout, *Biopolymers* 20, 1901 (1981).
- Y. V. Venkatachalam, C. M. K. Nair, M. Vijayan and P. Balaram, *Ibid.* 20, 1123 (1981).
- Y. V. Venkatachalam and P. Balaram, *Ibid.* 20, 1137 (1981).
- B. V. V. Prasad, H. Balaram and P. Balaram, *Ibid.* 21, 1261 (1982).
- B. V. V. Prasad and P. Balaram, *Conformation in Biology* (Edited by R. Srinivasan and R. H. Sarma), pp. 133-140. Adenine Press, New York (1982).
- S. S. Zimmennan and H. A. Scheraga, *Biopolymers* 16, 811 (1977).
- J. Linderberg and J. Michl, *J. Am. Chem. Soc.* 92, 2619 (1970).
- R. W. Woody, *Tetrahedron* 29, 1273 (1973).
- H. E. Van Wart and H. A. Scheraga, *J. Phys. Chem.* 80, 1812 (1976).
- H. E. Van Wart and H. A. Scheraga, *Ibid.* 80, 1823 (1976).
- H. Sugeta, A. Go and T. Miyazawa, *Bull. Chem. Soc. Japan* 46, 3407 (1973).
- Y. V. Venkatachalam, B. V. V. Prasad and P. Balaram, *Biochemistry* 21, 5502 (1982).
- D. B. Hope, V. V. S. Murti and V. du Vigneaud, *J. Biol. Chem.* 237, 1563 (1962).
- M. Barber, R. S. Bordoli, R. D. Sedgwick and A. N. Tayler, *J. Chem. Soc. Chem. Commun.* 325 (1981).
- B. V. V. Prasad, A. Ravi and P. Balaram, *Biochem. Biophys. Res. Commun.* 103, 1138 (1981).
- A. Ravi, B. V. V. Prasad and P. Balaram, *J. Am. Chem. Soc.* 105, 105 (1983).
- P. G. Katsoyanis, C. Zalut, A. Tometsko, M. Tilak, S. Johnson and A. C. Trakatellis, *Ibid.* 93, 5871 (1971).
- V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov and Yu. A. Ovchinnikov, *Tetrahedron* 25, 493 (1965).
- R. Nagaraj and P. Balaram, *Biochemistry* 20, 2828 (1981).
- V. J. Hruby, *Chemistry and Biochemistry of Aminoacids, Peptides and Proteins* (Edited by B. Weinstein), pp. 1-188. Dekker, New York (1974).
- K. D. Kopple, M. Ohnishi and A. Go, *J. Am. Chem. Soc.* 91, 4264 (1969).
- M. Ohnishi and D. W. Urry, *Ibid.* 36, 194 (1969).
- T. P. Pitner and D. W. Urry, *Ibid.* 94, 1399 (1972).
- A. Stem, W. A. Gibbons and L. C. Craig, *Proc. Natl. Acad. Soc. U.S.A.* 61, 734 (1968).
- M. Tsuboi, T. Shimanouchi and S. Mizushima, *J. Am. Chem. Soc.* 80, 1406 (1958).
- M. Avignon, P. V. Huong, J. Lascombe, M. Marraud and J. Neel, *Biopolymers* 8, 69 (1969).
- Ch. P. Rao, R. Nagaraj, C. N. R. Rao and P. Balaram, *Biochemistry* 19, 425 (1980).
- F. R. Maxfield, S. J. Leach, E. R. Stimson, S. P. Powers and H. A. Scheraga, *Biopolymers* 18, 2507 (1979).
- N. Sugawara, E. S. Stevens, G. M. Bonora and C. Toniolo, *J. Am. Chem. Soc.* 102, 7044 (1980).
- E. S. Stevens, N. Sugawara, G. M. Bonora and C. Toniolo, *J. Am. Chem. Soc.* 102, 7048 (1980).
- M. Iqbal and P. Balaram, *Biopolymers* 21, 1427 (1982).
- E. S. Pysh and C. Toniolo, *J. Am. Chem. Soc.* 99, 6211 (1977).

⁴⁴R. Nagaraj, N. Shamala and P. Balaram, *J. Am. Chem. Soc.* **101**, 16 (1979).

⁴⁵G. D. Smith, V. A. Pletnev, W. L. Duax, T. M. Balasubramanian, H. E. Bosshard, E. W. Czerwinski, N. C. E. Kendrick, F. S. Mathews and G. R. Marshall, *Ibid.* **103**, 1493 (1981).

⁴⁶A. Aubry, J. Protas, G. Boussard and M. Marraud, *Acta Crystallogr. Sect. B* **32**, 2399 (1977).

⁴⁷A. A. Bothner-By, *Magnetic Resonance in Biology* (Edited by R. G. Shulman), pp. 177-219. Academic Press, New York (1979).

⁴⁸B. N. N. Rao, A. Kumar, H. Balaram, A. Ravi and P. Balaram, *J. Am. Chem. Soc.* **105**, 7423 (1983).

⁴⁹V. F. Bystrov, *Prog. Nucl. Magn. Reson. Spectrosc.* **10**, 41 (1976).

⁵⁰A. Ballardin, A. J. Fischman, W. A. Gibbons, J. Roy, I. L. Schwartz, C. W. Smith, R. Walter and H. R. Wyssbrod, *Biochemistry* **17**, 4443 (1978).

⁵¹M. A. Khaled, V. Renugopalakrishnan and D. W. Urry, *J. Am. Chem. Soc.* **98**, 7547 (1976).

⁵²R. Deslauriers, S. J. Leach, F. R. Maxfield, E. Minosian, J. R. McQuie, Y. C. Meinwald, G. Nemethy, M. S. Pottle, I. D. Rae, H. A. Scheraga, E. R. Stimson and J. W. Van Nispen, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2512 (1979).

⁵³G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.* **79**, 6180 (1957).

⁵⁴C. M. Deber and E. R. Blout, *Israel. J. Chem.* **12**, 15 (1974).

⁵⁵G. W. Anderson, J. E. Zimmerman and F. Callahan, *J. Am. Chem. Soc.* **86**, 1839 (1964).

⁵⁶W. Konig and R. Geiger, *Chem. Ber.* **103**, 788 (1970).