Conformations of Cyclic Peptides. IV. Nuclear Magnetic Resonance Studies of *cyclo*-Pentaglycyl-L-leucyl and *cyclo*-Diglycyl-L-histidyldiglycyl-L-tyrosyl*

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ABSTRACT: Proton magnetic resonance spectra of *cyclo*-Gly-Gly-L-His-Gly-Gly-L-Tyr (I) and a partially C-deuterated derivative, in dimethyl sulfoxide and in water, are interpreted to indicate a peptide backbone containing two short transannularly hydrogen-bonded antiparallel peptide segments joined at their ends by peptide bonds perpendicular to the average ring plane. In this peptide and in *cyclo*-Gly_5-L-Tyr (II) and *cyclo*-Gly_5-L-Leu (III), which have the same backbone, the side chains are shown to be attached at "corner" α-carbons, rather than at the α-carbons in the centers of the extended segments. The structures proposed are based primarily on studies of the peptide proton resonances, which indicate four protons exposed to solvent and two shielded from it, and on spin-decoupling studies used to determine which α-protons and peptide protons correspond to individual residues.

Study of the peptide proton region of the spectrum of a C-deuterated derivative of I, in mixtures of water and dimethyl sulfoxide, demonstrate that the backbone conformation of this peptide does not change in going from one pure solvent to the other. Syntheses of I, of III, and of Gly-L-His-Gly-Gly-Gly-Gly are described.

In a previous paper we described proton magnetic resonance spectra of *cyclo*-pentaglycyl-L-tyrosyl and a deuterated derivative (Kopple *et al.*, 1969). From the data we obtained, we concluded that in dimethyl sulfoxide or water solution, this cyclic hexapeptide possesses a favored conformation in which two antiparallel extended peptide segments are bridged by two transannular hydrogen bonds. From chemical evidence, Schwyzer has suggested this kind of conformation for cyclic hexapeptides and cyclic decapeptides (Schwyzer, 1959; Schwyzer *et al.*, 1958, 1964). We were also able to conclude that in the favored conformation the hydroxybenzyl side chain of *c*-Gly_5-Tyr is attached at a corner of the peptide backbone chain, not to the most extended residues.

We have extended these studies to include *c*-Gly_2-L-His-Gly_2-L-Tyr, a deuterated derivative, and *c*-Gly_5-Leu, which are peptides I, I-d_4, and III of Figure 1; we have also examined some open-chain hexapeptides, also shown in Figure 1, related to these. The structure proposed by Schwyzer appears to be favored in solution for I and III as well as the previously studied II.

Experimental Section

Proton magnetic resonance spectra were obtained with a Varian HA-100 spectrometer, using an internal lock and frequency sweep; homonuclear spin decoupling was done with the same instrumentation plus an audio oscillator (Hewlett Packard 200 CDR). Where necessary (with aqueous solutions), a Varian C-1024 time-averaging computer was used for signal enhancement. Probe temperature was determined using test samples of methanol and ethylene glycol. The ambient temperature of the probe during this work was close to 30°.

Samples in dimethyl sulfoxide (except for proton-exchange studies) and in trifluoroacetic acid were degassed and sealed in vacuum; samples in water usually were not. For replacement of amide protons by deuterium, peptide samples were several times dissolved in trifluoroacetic acid-*d* or dimethyl sulfoxide–deuterium oxide mixtures and evaporated under vacuum to dryness before they were dissolved in the solvent used for the measurement. Samples of linear peptides not sufficiently soluble in water or dimethyl sulfoxide were converted into trifluoroacetate salts by solution in trifluoroacetic acid and removal of the excess acid under 10^{-3}-mm vacuum.

Reference and lock signal for the nonaqueous solutions was internal tetramethylsilane. For the aqueous solutions capillary hexamethyldisiloxane was used as reference and lock. Near 30° 2,2-dimethyl-2-silapentane-5-sulfonate in water is 0.48-ppm downfield from capillary hexamethyldisiloxane in water.

Materials. *c*-GLY-GLY-L-HIS-GLY-GLY-L-TYR (I) was on hand from a previous investigation (Kopple *et al.*, 1963). The syntheses of the partially deuterated peptide *c*-Gly-Gly-L-His-Gly-d_2-Gly-d_2-L-Tyr (I-d_4), of Gly_4-L-Leu-Gly (VI), *c*-Gly-L-Leu (III), and Gly-L-His-Gly_4 (IV) are described below.[1]

c-DIGLYCYL-L-HISTIDYL-DIGLYCYL-d_2-L-TYROSYL (I-d_4). This cyclic peptide was obtained *via* the synthetic scheme shown in Figure 2, utilizing the previously reported tetradeuterated derivative of carbobenzyloxydiglycyl-L-tyrosine (Kopple *et al.*,

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[1] All melting points are corrected. Microanalyses were performed by MicroTech Laboratories, Skokie, Ill.

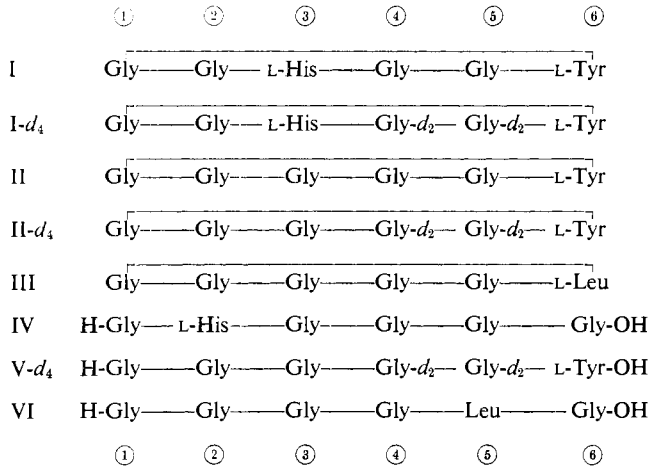

FIGURE 1: Peptides referred to in this paper. Residues, as referred to in Tables I and II, are numbered from the right as depicted here.

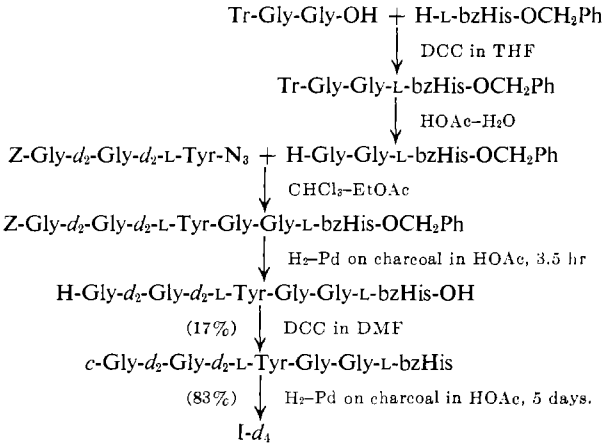

FIGURE 2: Synthetic scheme used in preparing the deuterated peptide I-d_4. Abbreviations used are: DCC, dicyclohexylcarbodiimide; Tr, trityl; THF, tetrahydrofuran; DMF, dimethylformamide; bzHis, N^{im}-benzylhistidine.

1969). None of the intermediate peptide derivatives was recrystallized to analytically pure form, although the course of each step was monitored by thin-layer chromatography. The cyclization step proceeded in 17% yield, from crystalline terminally unblocked hexapeptide to crystalline cyclic *im*-benzyl hexapeptide, the latter being obtained after chromatography of the cyclization reaction mixture on a cellulose powder column, using 1-butanol–water (6:1, v/v). The final product, after hydrogenolysis of the *im*-benzyl group and crystallization from ethanol, was homogeneous and chromatographically identical, on direct comparison in several solvent systems, to the undeuterated analog I. However, proton magnetic resonance examination indicated that it retained a molecule of acetic acid (the hydrogenolysis solvent). This was removed prior to preparation of the proton magnetic resonance samples for detailed study by passing an aqueous solution of the peptide acetate through a carefully washed column of Bio-Rad AG 3 X-4 weak base ion-exchange resin in the free-base form; crystalline free peptide was recovered on evaporation of the eluate.

CARBOBENZYLOXYGLYCYL-L-LEUCYLGLYCINE *p*-NITROBENZYL ESTER. The crude hydrobromide salt of L-leucylglycine nitrobenzyl ester, obtained by treatment of 14.8 g (0.032 mol) of carbobenzyloxyleucylglycine nitrobenzyl ester with 30% hydrogen bromide in acetic acid, was dissolved in 300 ml of equal volumes of chloroform and water. Excess of solid sodium carbonate was added to this mixture, and after equilibration the chloroform layer was separated and dried over anhydrous sodium sulfate. The chloroform was evaporated at reduced pressure, and the residue was mixed with 8.1 g (0.39 mol) of carbobenzyloxyglycine in 130 ml of chloroform. *N,N'*-dicyclohexylcarbodiimide (9.9 g, 0.048 mol) was added to the solution.

After the reaction mixture had been stored overnight, precipitated dicyclohexylurea was collected and washed with chloroform. The combined filtrate and washings were evaporated to dryness, and the residue was taken up in ethyl acetate and washed with water, 0.5 N hydrochloric acid, 5% sodium bicarbonate, and again water. It was then dried over sodium sulfate and concentrated to an oil (19 g) which was used without further purification in the next step. A crystalline sample was also prepared from ethyl acetate solution (mp 99–101°).

Anal. Calcd for $C_{25}H_{30}N_4O_8$: C, 58.36, H, 5.88; N, 10.89. Found: C, 58.60; H, 6.01; N, 10.75.

CARBOBENZYLOXYTETRAGLYCYL-L-LEUCYLGLYCINE *p*-NITROBENZYL ESTER. To 17.5 g (0.034 mol) of carbobenzyloxyglycyl-L-leucylglycine nitrobenzyl ester was added 18.4 g of 30% hydrogen bromide in acetic acid (w/w). After 90 min, 500 ml of anhydrous ether was added; the precipitated oil crystallized on storage in the cold overnight. After further washing with ether the hydrobromide salt was collected and dried over potassium hydroxide pellets.

A solution containing 16.15 g (0.035 mol) of the tripeptide hydrobromide, 13.58 g (0.042 mol) of carbobenzyloxytriglycine, 3.54 g (0.035 mol) of triethylamine, and 10.81 g (0.053 mol) of *N,N'*-dicyclohexylcarbodiimide in 150 ml of dimethylformamide was stirred at room temperature overnight. Dicyclohexylurea precipitated; 2 ml of 50% aqueous acetic acid was added, and after 2 hr further stirring, the urea was removed by filtration. The solvent was removed under vacuum, and ethyl acetate was added to the residue. Blocked hexapeptide crystallized from the ethyl acetate solution (16.4 g, 69%), with some carbobenzyloxytriglycine as impurity. It was recrystallized from methanol–water to provide a chromatographically homogeneous product in over-all 48% yield.

An analytical sample was obtained from methanol (mp 171–172.5°).

Anal. Calcd for $C_{31}H_{39}N_7O_{11}$: C, 54.30; H, 5.73; N, 14.30. Found: C, 54.58; H, 5.71; N, 14.39.

TETRAGLYCYL-L-LEUCYLGLYCINE (VI). The blocked hexapeptide described above (11 g), in 250 ml of glacial acetic acid, was hydrogenated over 10% palladium-on-charcoal catalyst, at atmospheric pressure, for 6 days; 1.1 g of catalyst was added initially, and 1.1 g more was added each day until thin-layer chromatography showed that hydrogenation was complete. After removal and washing of the catalyst by centrifugation, the acetic acid solution plus catalyst washings was concentrated to dryness. The oily residue was taken up in 300 ml of water, extracted with ether, and then treated with decolorizing charcoal. The aqueous solution was concentrated to a volume of about 150 ml and stored in the cold, while the

product free peptide crystallized. A chromatographically pure product crystallized from the aqueous solution, 5.1 g, 78%.

An analytical sample was obtained upon two recrystallizations from water (mp 234–236° dec). It was dried at 100° at 0.05 mm.

Anal. Calcd for $C_{16}H_{28}N_6O_7 \cdot 0.5H_2O$: C, 45.17; H, 6.87; N, 19.75. Found: C, 45.11; H, 6.55; N, 19.68.

c-PENTAGLYCYL-L-LEUCYL (III). Tetraglycyl-L-leucylglycine (500 mg, 0.0012 mol) was dissolved in 50 g of melted phenol, and 350 mg (0.0017 mol) of dicyclohexylcarbodiimide was added to the solution. The reaction mixture was stirred at 60–70°, and after 1 hr phenol was removed by lyophilization. The residue was treated with about 10 ml of ethanol containing a few drops of glacial acetic acid, and precipitated dicyclohexylurea was removed by filtration. The solution was evaporated and the residue was chromatographed on a 1-kg column of Whatman CC-31 microgranular cellulose, using 1-butanol–water (6:1, v/v). The fractions containing the major, ninhydrin-negative, component were combined and evaporated to dryness. The brownish residue was taken up in water, treated with decolorizing charcoal, and recovered by evaporation. It dissolved readily in ethanol, and on evaporation of the ethanol a microcrystalline solid was obtained, 138 mg, 29%, chromatographically homogeneous in several solvent systems.

Anal. Calcd for $C_{16}H_{26}N_6O_6$: C, 48.23; H, 6.58; N, 21.10. Found: C, 47.96; H, 6.52; N, 20.57.

CARBOBENZYLOXYGLYCYL-L-HISTIDYLTRIGLYCYLGLYCINE BENZYL ESTER. To a solution of 3.12 g (0.007 mole) of triglycine benzyl ester *p*-toluenesulfonate in 25 ml of freshly distilled dimethylformamide was added 2.5 g (0.0063 mole) of *N*-carbobenzyloxyglycyl-L-histidylglycine. The resulting suspension became clear upon addition with stirring of 1.5 g (0.009 mole) of *N*-ethyl-*N'*-(γ-dimethylaminopropyl)carbodiimide. The yellowish solution was stirred overnight, the solvent was removed under reduced pressure, and the oily residue was washed with water. The washed residue was dried and crystallized from ethanol–water.

Two recrystallizations from ethanol–water afforded an analytical sample, which was dried under vacuum at 100° for 24 hr: yield, 2.1 g, 42%; mp 187–189°.

Anal. Calcd for $C_{31}H_{36}N_8O_9$: C, 56.01; H, 5.46; N, 16.86. Found: C, 55.87; H, 5.69; N, 16.62.

GLYCYL-L-HISTIDYLTRIGLYCYLGLYCINE (IV). To a suspension of 150 mg of 10% palladium on charcoal in 50 ml of glacial acetic acid was added 1.67 g (0.0025 mol) of *N*-carbobenzyloxyglycyl-L-histidylglycylglycylglycylglycine benzyl ester. The reaction mixture was stirred and purged with nitrogen gas, then hydrogen gas was slowly bubbled into the mixture. Hydrogenolysis was allowed to proceed overnight. The catalyst was separated by centrifuging and the clear solution was lyophilized.

The unblocked hexapeptide was recrystallized from ethanol–water mixture. It was chromatographically homogeneous in several solvent systems: yield, 812 mg, 73.4%; mp 204–206°.

An analytical sample was dried under vacuum at 100° for 14 hr.

Anal. Calcd for $C_{16}H_{24}N_8O_7 \cdot 1.5H_2O$: C, 41.11; H, 5.82; N, 23.97. Found: C, 40.78; H, 5.86; N, 23.73.

Results

Syntheses. Both of the new cyclic peptides reported in this paper, I-d_4 and III, were prepared by the standard procedures described in the Experimental Section. Tetraglycyl-L-leucylglycine, the precursor of III, proved to be insoluble in all solvents in which a peptide coupling might normally be carried out, and it did not go into solution on treatment with a variety of coupling agents. However, melted phenol proved a satisfactory solvent, and a 29% cyclization yield was achieved by treating a 0.025 M solution with 1.5 equiv of dicyclohexylcarbodiimide at 60–70° for 1 hr. It seems likely that cyclization in phenol proceeds *via* the phenyl ester.

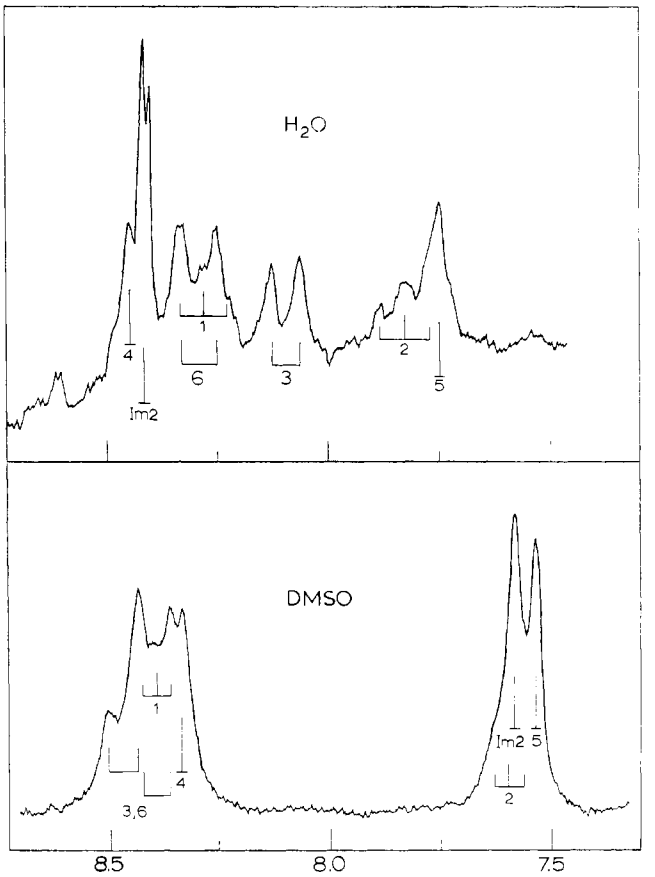

FIGURE 3: Amide proton resonances of *c*-Gly-Gly-L-His-Gly-d_2-Gly-d_2-L-Tyr(I-d_4) in water (trifluoroacetate salt) and dimethyl sulfoxide (free base). The numbered positions of the resonances correspond to the residue numbers used in Figure 1 and Table I. Details of the assignments for the dimethyl sulfoxide solution were established by double-resonance experiments. Reference: tetramethylsilane in dimethyl sulfoxide, sodium 2,2-dimethyl-2-silapentane-5-sulfonate in water.

Nuclear Magnetic Resonance Spectra. Table I presents chemical shift data for the peptide and α-protons of the cyclic peptides and some related linear peptides. Data for II and V, which have been previously reported, are included for comparison. Where possible, α- and peptide protons belonging to the same amino acid residue have been identified using the multiplicity of the peptide proton resonance and the results of double-irradiation (decoupling) studies; these relationships are indicated where the α- and peptide proton chemical shift values are aligned vertically in the table. Assignments of glycyl residue resonances to positions in the peptide sequences have also been made, using the numbering sequence given in Figure 1. Some of these assignments follow directly from

TABLE I: Amide and α-Proton Resonances of Cyclic and Related Linear Peptides, 30°.

Solvent	Peptide[b]	Residue No.[b]	Chemical Shift[a] 1	2	3	4	5	6
Dimethyl sulfoxide	I (50 mg/ml)	α-CH	3.69 ± 0.17[c]	3.78 ± 0.03[d]	4.15 or 4.23	(3.74 ± 0.04, 3.79)		4.23 or 4.15
		NH	8.40	7.59	8.48 or 8.40	8.34	7.53[k]	8.40 ± 8.48
	II (100 mg/ml)	α-CH	3.73 ± 0.20	3.84 ± 0.03	3.70 ± 0.03	(3.75, 3.75)		4.28
		NH	8.29	7.78	8.43	8.17	7.70[k]	8.25
	III[e] (60 mg/ml)	α-CH	(3.78)[j]	3.85	(3.71 ± 0.09, 3.85 ± 0.06)[j]		3.85	4.17
		NH	(8.23)[j]	7.78 or 7.84[k]	(8.30, 8.47)[j]		7.84 or 7.78[k]	8.26
	IV (54 mg/ml)	α-CH	3.64	4.70		(3.77)		
		NH	9.22[f]	8.73		(8.14, 8.18, 8.40, 8.51)		
	V-d_4 (50 mg/ml)	α-CH	3.50	(3.74, 3.79)				4.17
		NH		8.32	(8.07, 8.11, 8.15)			7.57
	VI (66 mg/ml)	α-CH	3.65		(3.75)		4.33	3.85
		NH			(8.10, 8.23, 8.33)		7.95	8.67[g]
H_2O–D_2O	I[h] (50 mg/ml)	α-CH	3.81 ± 0.14	3.66 ± 0.07	4.42	(3.68 ± 0.08, 3.80 ± 0.08)		4.28
		NH	8.30	7.83	8.10	8.45	7.75[k]	8.30
	II (~3 mg/ml)	α-CH	3.92 ± 0.13	(3.74 ± 0.09, 3.78)		(3.81 ± 0.11, 3.84 ± 0.04)		4.29
		NH			(8.3 (4), 7.7 (2))			
	III (40 mg/ml)	NH			(8.3 (3), 8.46, 7.65, 7.69)			
	IV (50 mg/ml)	α-CH	3.68	4.62		(3.83)		
Trifluoroacetic acid	I-d_4 (50 mg/ml)	α-CH	4.21 ± 0.26	4.10 ± 0.10	(5.00)			(4.86)
		NH		(7.70, 7.86, 7.96 (2))[i]		(8.02 (2))		*i*
	II (30 mg/ml)	α-CH		(4.19 ± 0.15, 4.20, 4.31 ± 0.14)		(4.20)		4.77
		NH			(7.85 (larger), 7.7 (smaller))			
	III (50 mg/ml)	NH			(7.74, 7.9 (5), hhw 15 Hz)			

[a] Parts per million below tetramethylsilane in dimethyl sulfoxide and trifluoroacetic acid; below sodium 2,2-dimethyl-2-silapentane-5-sulfonate in water. [b] Peptides and residues numbered as in Figure 1. [c] 3.67 ± 0.16 ppm at 110°. [d] The 0.03-ppm splitting at 30° appears in the 220-MHz spectrum of I-d_4. [e] Data of Kopple *et al.* (1969). [f] Ammonium protons. [g] Assigned on the basis that the low-field amide protons of diglycylglycine and pentaglycylglycine are at the carboxyl end of the chains. [h] Trifluoroacetate salt. [i] The resonances listed under residues 1, 2, and 3 correspond to residues 1, 2, 3, and 6. [j] These three resonances correspond to residues 1, 3, and 4. [k] The assignments of higher field N–H resonances to position 5 rather than 4 are made by assuming the peptide backbone described in the text.

TABLE II: Arylmethylene Resonances in Cyclic Peptides and Related Species.

Peptide[b]	Solvent	Chemical Shift[a] α-Protons ν (ppm)	Chemical Shift[a] β-Protons ν (ppm)	Chemical Shift[a] β-Protons Δν (ppm)	Coupling Constants (Hz) $J_{\alpha,h\beta}$	Coupling Constants (Hz) $J_{\alpha,l\beta}$	Coupling Constants (Hz) $J_{\beta,\beta}$
c-GGHGGT[g]	Dimethyl sulfoxide	4.15	2.91	0.16	~8.3	~6.2	15
		4.23	2.85	0.20	~8.9	~5.6	14
c-GGGGGT[f]	Dimethyl sulfoxide	4.28	2.87	0.27	9.4	5.2	14
GGGGGT[f]	Dimethyl sulfoxide	4.17	2.85	0.16	9.8	3.7	14.5
GHGGGG[c]	Dimethyl sulfoxide	4.70	3.12[e]				
c-GT	Dimethyl sulfoxide	3.97	3.00	0.20	4.5	4.5	13.5
c-GH[c]	Dimethyl sulfoxide	4.19	3.14	~0			
c-GH[d]	Dimethyl sulfoxide	4.01	2.93	~0			
c-GGHGGT[c]	D_2O (T)	4.28	2.82	~0			
	(H)	4.42	3.07	0.12	8.6	5.4	15
c-GGGGGT[f]	D_2O	4.29	2.88	0.11	8.2	7.0	14
GGGGGT[f]	D_2O	4.25	2.82	0.22	8.9	5.1	14
GHGGGG[c]	D_2O	4.62	3.09	0.10	7.9	6.1	15.3
c-GH[c]	D_2O	4.27	3.14	0.08	5.1	4.4	15.5
c-GH[d]	D_2O	4.24	3.04	0.14	5.0	4.3	15
c-GGHGGT	Trifluoroacetic acid	5.00	3.45	0.20	7.2	5.3	16
		4.86	3.13	~0			
c-GGGGGT[f]	Trifluoroacetic acid	4.77	3.16	0.21	7.7	6.8	14
c-GH	Trifluoroacetic acid	4.76	3.59	~0			
c-GT	Trifluoroacetic acid	4.87	3.32	0.10	5.2	4.3	14.5

[a] Ambient probe temperature about 30°. Reference tetramethylsilane for dimethyl sulfoxide and trifluoroacetic acid solutions, sodium 2,2-dimethyl-2-silapentane-5-sulfonate for aqueous solution. [b] Abbreviations used are: G, glycyl; T, tyrosyl; H, histidyl, [c] Trifluoroacetic salt. [d] Free base. [e] Broad, unresolved; apparent width at half-height 13 Hz. [f] Kopple *et al.* (1969). [g] From 220-MHz spectrum, tentative interpretation of β-proton region.

knowledge of the peptide sequence; for example, the resonances assigned to the glycyl residues with sequence numbers 4 and 5 in I and II are identified by their absence in the spectra of I-d_4 and II-d_4. Other assignments have been made on the basis of arguments given below. Where no assignment has been possible, the unplaced groups have been included in parentheses.

Table II gives chemical shift and coupling constant data for the α- and β-proton resonances of the histidyl and tyrosyl residues in the cyclic and related peptides.

Spectra in Dimethyl Sulfoxide. The peptide proton regions of the spectra of both I-d_4 and III in dimethyl sulfoxide are like that of II in the same solvent, in that they consist of a group of four protons at lower field (8.2–8.5 ppm) and two protons at higher field (7.5–7.9 ppm) (see Figures 3 and 4). This division has been already rationalized for II as resulting from a preferred backbone conformation containing two transannularly hydrogen-bonded extended peptide segments (Kopple *et al.*, 1969). The temperature dependences of the peptide proton chemical shifts of I-d_4 were determined, and the data are presented in Figure 5. Figure 5 shows the position of the centers of mass of the two groups of resonances for both I-d_4 and II-d_4. In both cyclic peptides the position of the lower field resonances is more temperature dependent, although both higher and lower field resonances move upfield with increasing temperature. For I-d_4 between 30 and 120° the temperature coefficient for the lower field group is 0.61 Hz/deg, which is the same as the measured value for the amide proton of *N*-methylacetamide. The coefficient for the higher field group is only 0.12 Hz/deg, identical with that of the resonance of the imidazole 2-proton of I, which overlaps it (see Figure 3). The 2-proton of the imidazole ring cannot be strongly solvated by, or hydrogen bonded to, dimethyl sulfoxide. The *N*-methylacetamide amide proton, on the other hand, must be strongly associated with the solvent. Therefore the difference in temperature dependence between higher and lower field peptide protons is a clear indication that the four lower field protons are associated with solvent, while the two higher field protons are not. Some additional support for this conclusion is obtained from the actual chemical shifts of the low-field protons. These are in the same range (8.1–8.5 ppm) as those of the peptide protons of the linear hexapeptides IV, V, and VI, which should have predominantly solvated amide protons.

When D_2O is added to dimethyl sulfoxide solutions of I-d_4, the peptide protons are removed by exchange. There appears to be no large difference in rate of exchange between the protons of the high- and low-field groups. This was also the case with *c*-pentaglycyltyrosyl, II-d_4, as reported before.

Spectra of the α- and β-proton resonances of I-d_4 are shown

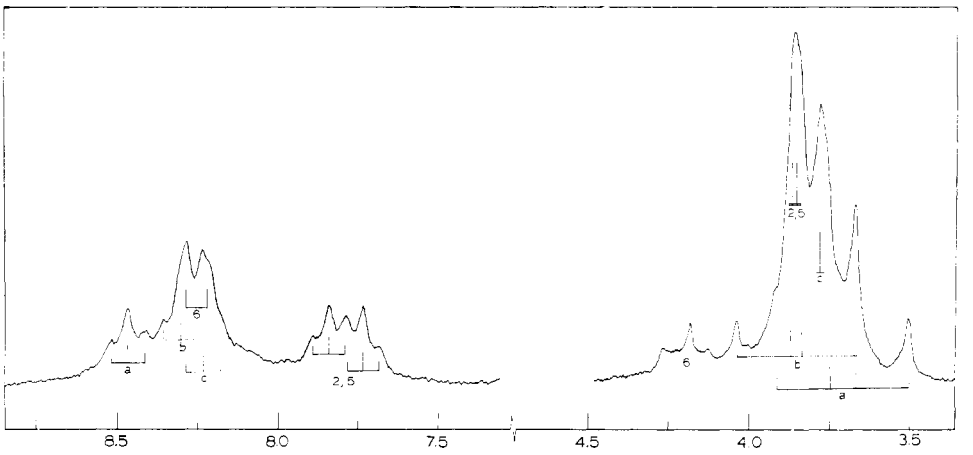

FIGURE 4: Amide (7.5–8.5 ppm) and α-proton (3.5–4.5 ppm) resonances of *c*-Gly$_5$-L-Leu (III) in dimethyl sulfoxide-d_6. The α-proton resonances are shown for a sample in which the amide protons have been replaced by deuterons, but assignments were made on the basis of double-resonance experiments using the unexchanged peptide. Numbers refer to residue numbers used in Figure 1 and Table I; like-lettered resonances are spin coupled, but are not assignable to particular glycyl residues. Reference, tetramethylsilane.

in Figure 6 and a spectrum of III appears as Figure 4. In I, II, and III the α-protons of the substituted amino acid residues, tyrosyl, histidyl, and leucyl, are spin coupled to peptide protons of the low-field group. This was established by experiments in which the α-protons were irradiated while observing the peptide protons, and was confirmed by the reverse experiment.

The tyrosyl and histidyl β-proton resonances of I overlap closely and are not separated even in a 220-MHz spectrum.

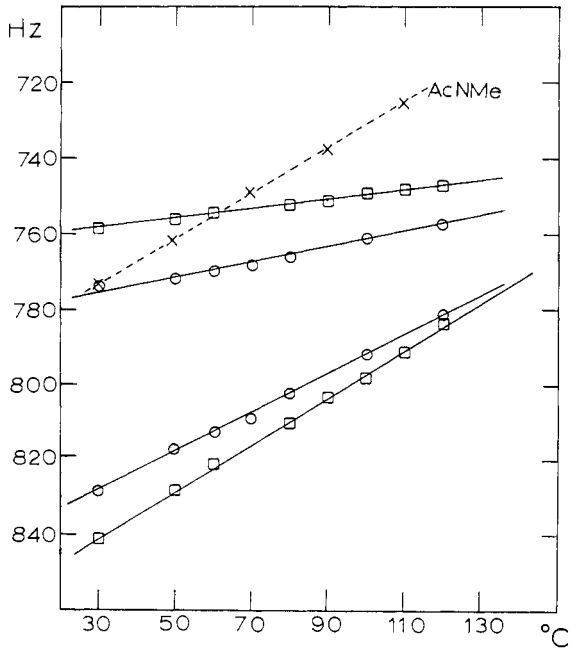

FIGURE 5: Temperature dependence of amide proton resonances of cyclic hexapeptides in dimethyl sulfoxide. Points give positions of centers of mass of the low-field group of four proton resonances (lower lines) and the high-field group of two resonances (upper lines), relative to internal tetramethylsilane. Circles, *c*-Gly$_5$-Tyr; squares, *c*-Gly-Gly-L-His-Gly-Gly-L-Tyr; crosses, amide proton of *N*-methylacetamide.

However, at 220 MHz they and the corresponding α-proton resonances are resolved sufficiently to indicate that each residue has one α–β proton–proton coupling of 8–9 Hz and another of 5–6 Hz. This assymmetry indicates that side-chain rotamers with a *trans* relationship of vicinal protons are favored, and that one of these is more stable than the other.

Of the glycine residues in I, only one exhibits a large degree of magnetic nonequivalence between its α-protons. This splitting, 0.35 ppm, is unchanged within experimental accuracy over the temperature range 30–110°. II in dimethyl sulfoxide also shows a single glycyl residue split by about this amount, also with a negligible temperature dependence. In both of these peptides the split residue is coupled to one of the amide protons in the low-field group, and it is one of those glycyls remaining undeuterated in the deuterated peptides, I-d_4 and II-d_4. In II this residue must be that numbered 1, 2, or 3 (Figure 1). The observations on peptide I narrow the possibilities to residues 1 or 2, one of the two residues on the carboxyl side of the tyrosyl residue. The residue assignments of Table I have been made on the unproven assumption that the much split glycyl residue is the one closer to the tyrosyl side chain, that numbered 1 in Figure 1. This assumption rests on the argument that, whatever the source of the large splitting, much of it is related to proximity of the aromatic side chain.

c-Pentaglycylleucyl (III) was prepared to test the hypothesis that the major splitting of glycyl methylenes in I and II results from the magnetic anisotropy of the aromatic ring. It does turn out that in III no glycyl residue shows so large a difference between its α-protons as the one in the tyrosine-containing peptides. However, one glycyl residue of III is split by 0.18 ppm, and so at least some of the nonequivalence must arise from factors other than ring current, such as the magnetic anisotropy of peptide bonds.

Spectra in Water. The peptide proton region of the spectrum of I-d_4, as its trifluoroacetate salt in water (Figure 3) superficially differs from the four at lower field and two at higher field arrangement seen in spectra of dimethyl sulfoxide solutions. There is a doublet resonance belonging to one of

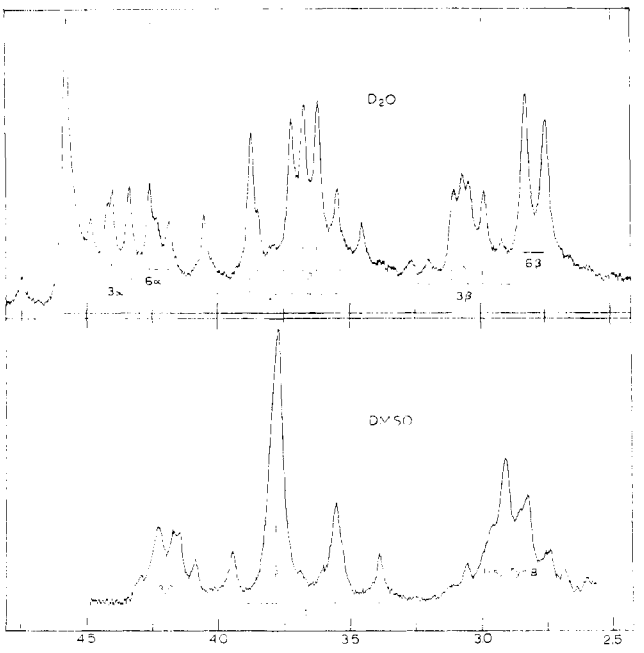

FIGURE 6: α- and β-proton resonances of *c*-Gly-Gly-L-His-Gly-d_2-Gly-d_2-L-Tyr, in which amide protons have been replaced by deuterons, in deuterium oxide (trifluoroacetate salt) and dimethyl-d_6 sulfoxide (free base). The numbered positions of the resonances correspond to the residue numbers used in Figure 1 and Table I. Reference: tetramethylsilane for dimethyl sulfoxide solution, sodium 2,2-dimethyl-2-silapentane-5-sulfonate for aqueous solution. The absorption near 4.6 ppm in the deuterium oxide spectrum arises from HDO.

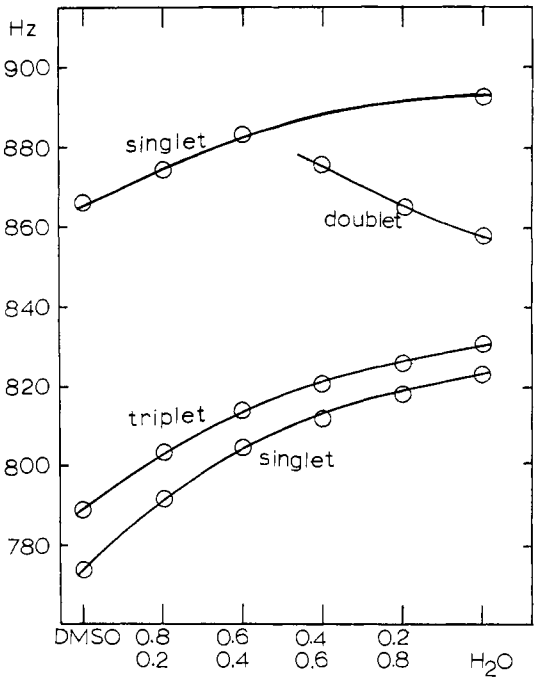

FIGURE 7: Chemical shifts of "landmark" amide proton resonances of *c*-Gly-Gly-L-His-Gly-Gly-L-Tyr in dimethyl sulfoxide–water mixtures, in the presence of about 3 equiv of trifluoroacetic acid. The singlet resonances are from residues 4 and 5 in Figure 1, the triplet is from residue 2, and the doublet is from residue 3. The reference is *external* (capillary) hexamethyldisiloxane.

the substituted residues (His or Tyr) at 8.1 ppm, between the two major groups. This resonance is absent in the corresponding region of the spectrum of II-d_4, which lacks histidine, and therefore it probably corresponds to the peptide proton of the histidyl residue. To determine whether a fundamental change in peptide backbone conformation occurs on going from dimethyl sulfoxide to aqueous solution, the peptide proton resonances of I-d_4 were examined in water–dimethyl sulfoxide mixtures of varying composition. (The solutions used contained several equivalents of trifluoroacetic acid, since neutral I is quite insoluble in water.) Landmark peptide proton resonances could be identified at all compositions; these were the two singlets from the deuterated glycine residues, one at high field and one at low field, and the high-field triplet from a third glycine residue. As Figure 7 shows, these all moved smoothly with change in composition, and did not leave their respective clusters of resonances. (The apparent chemical shift changes shown in Figure 7 are large because an external standard was used; most of the change with composition is the effect of bulk magnetic susceptibility changes.) At high water concentrations one doublet resonance moves out of the low-field cluster in a direction opposite to the general trend (*i.e.*, upfield). As already suggested, this proton probably belongs to the histidyl residue, the side chain of which is becoming more completely protonated and/or better solvated as the solvent becomes more aqueous. At all compositions the amide proton of the other substituted residue, tyrosyl, must be in the low-field group of peptide protons, since the composition of the high-field group is completely accounted for by two glycine residues.

The amide proton spectrum of the leucyl cyclic peptide, III, exhibits the same four-and-two pattern in water that it does in dimethyl sulfoxide solution, although the individual proton resonances are not so well resolved. The appearance of this pattern can be taken as an indication that the backbone conformation is the same in both solvents, and like that of the other two cyclic peptides.

The aqueous solution spectrum of I-d_4 trifluoroacetate shows separated α- and β-proton resonances for the histidyl and tyrosyl residues, and the spin-coupled sets can be identified by double resonance: assignment of the coupled resonances at 3.07 and 4.42 ppm to the histidyl residue is made by comparison with the model compounds for which data are also listed in Table II. For the histidyl side chain the α–β coupling constants show that one of the conformations with a *trans* arrangement of α- and β-protons is again slightly preferred. The tyrosyl β-protons of I in water, in contrast to those of II, do not show observable magnetic nonequivalence, so that an indication of α–β rotational preferences cannot be obtained from the β-proton lines.

A time-averaged spectrum of the dilute aqueous solution of the peptide free base shows that the β-protons of histidyl and tyrosyl residues overlap, as they do in dimethyl sulfoxide solutions of the free base.

Table I indicates that all of the glycine methylenes of I trifluoroacetate in water are observably magnetically nonequivalent, although one is somewhat more so than the others (0.28 *vs.* ~0.15 ppm). In the time-averaged spectrum of the free base of I-d_4, only one of the two undeuterated methylenes shows any splitting; the observed nonequivalence is 0.26 ppm. The more split residue in either case is again tentatively as-

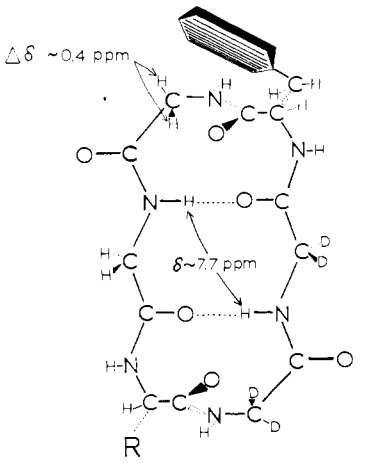

FIGURE 8: Proposed conformation of cyclic hexapeptides bearing a benzyl side chain, I and II, in dimethyl sulfoxide and aqueous solutions, as deduced from proton magnetic resonance spectra. A "corner" position is also taken by the isobutyl side chain of III.

signed to the position adjacent to the tyrosyl residue, on the carboxyl side.

Spectra in Trifluoroacetic Acid. In trifluoroacetic acid all of the peptide proton resonances of I, II, and III lie in the 7.7–8.0-ppm region. This is consistent with reduced involvement of solvent-exposed protons in hydrogen bonds to the less basic solvent. The protons that were at lower field in the other solvents have moved upfield, but the chemical shifts of those that were in the higher field group remains approximately the same as in water or dimethyl sulfoxide.

Discussion

The amide proton resonances of water and dimethyl sulfoxide solutions of *c*-pentaglycyl-L-leucyl (III) and *c*-diglycyl-L-histidyl-diglycyl-L-tyrosyl (I) support the peptide backbone conformation that we have previously (Kopple *et al.*, 1969) deduced from the nmr spectra of *c*-pentaglycyl-L-tyrosyl (II), a conformation of the type originally proposed by Schwyzer (Schwyzer, 1959; Schwyzer *et al.*, 1958, 1964). In this structure, a version of which appears in Figure 8, there are two antiparallel extended peptide segments connected by two interpeptide hydrogen bonds, constituting a recognizable fragment of a β-polypeptide structure. The extended segments are joined by two *trans* peptide bonds lying in planes perpendicular to the average plane of the hexapeptide ring. The central observation indicating this backbone conformation is the division of the amide protons into a group of four protons that behave as if exposed to solvent and a group of two that appear to be shielded from solvent, as described in the Results section, above.

In the proposed conformation, the solvent-shielded protons belong to amino acid residues that are in a 1,4 relationship, counting residues around the ring. The spectra of the peptides we have studied are consistent with such a 1,4 relationship, and they do rigorously exclude a 1,2 arrangement, but they do not rule out the 1,3 case, which is also inconsistent with the proposed backbone. However, Schwyzer (Schwyzer *et al.*, 1964) has published a 60-MHz spectrum of *c*-diglycyl-L-phenylalanyldiglycyl-L-phenylalanyl in trifluoroacetic acid-*d*; from this spectrum it appears that this peptide has only one kind of phenylalanyl residue and only two kinds of glycyl residues.[2] This seems to rule out conformations that possess a 1,3 relationship between the solvent-shielded amide protons.

At first thought, observation of resonances at higher field for the solvent-shielded protons may seem to argue against participation of these protons in transannular hydrogen bond. However, the external amide protons must also be involved in strong hydrogen bonds, since the solvents used are good hydrogen-bond acceptors. The upfield shift of the internal peptide protons should, in fact, be taken as support for the proposed backbone. If this conformation is correct, the internal amide protons lie directly above and close to the planes of the respective end amide groups; they are therefore in a region of diamagnetic shielding by the amide π-electron system and should come into resonance at abnormally high field. The suggestion of shielding by amide groups in an analogous situation has already been made by Stern *et al.* (1968) to explain the unusually high-field position (7.2 ppm in dimethyl sulfoxide containing 5% water) of the amide protons of the valine residues in the cyclodecapeptide gramicidin S; these protons undergo deuterium (tritium) exchange only slowly and, in the excellently reasonable model of these authors, which also is one of the Schwyzer type, are involved in transannular hydrogen bonds.

In our peptides both internal amide protons belong to the same kind of residue, glycyl; if identically situated relative to the end amide groups, they should be identically affected by the amide anisotropy, and should have the same chemical shifts. They come very close to doing so (within 0.08 ppm). In Figure 8, C_2 symmetry is shown for the peptide backbone; both end carbonyl groups are made to point to the same side of the ring, but it is an open question on which side the carbonyl oxygens actually are. The internally hydrogen-bonded form of cyclohexaglycyl in the crystal of its hemihydrate is centrosymmetric, *i.e.*, the carbonyl groups of the end carbonyls point to opposite side of the peptide ring (Karle and Karle, 1963). On the other hand, X-ray (Hodgkin and Oughton, 1957) and nuclear magnetic resonance (Stern *et al.*, 1968) studies of gramicidin S strongly indicate that this decapeptide has C_2 symmetry.

Spin-decoupling studies of the dimethyl sulfoxide solutions of I, II, and III establish that the nonglycyl residues of these peptides bear external amide protons, and that (in I and II) the glycyl methylene with the ~0.4-ppm nonequivalence is also coupled to an external amide proton. Corresponding decoupling experiments could not be carried out using aqueous solutions because of proximity of the intense water absorption in the α-proton resonances. However, since we established that I undergoes no major change in backbone conformation on going from dimethyl sulfoxide to water (see Results section), the histidyl and tyrosyl residues of that peptide must have external amide protons in both solvents. We suggest that the most probable attachment of the hydroxybenzyl

[2] Schwyzer *et al.* (1964) also reported that *c*-diglycyl-L-prolyldiglycyl-L-prolyl shows two two-proton amide resonances separated by 0.3 ppm. Their spectrum was of a trifluoroacetic acid solution, so that our conclusions based on aqueous and dimethyl sulfoxide solutions may not be unequivocally applicable. However their observation, as they tentatively suggested, is consistent with a transannularly hydrogen bonded structure for the proline-containing hexapeptide.

group on the peptide backbone of I and II is one of the two positions shown as substituted in Figure 8. We also suggest that, at least in dimethyl sulfoxide, the tyrosyl side chain favors the $\chi_1 = 180°$ rotamer shown in the Figure. These conformational assignments follow if the magnetically nonequivalent glycine residue is adjacent to the tyrosyl residue. The alternative possibility, that the split glycyl is 1,3 to the tyrosyl, requires a different position of side-chain attachment. Two arguments can be used to support the 1,2 relationship. First, the maximum splitting of a glycyl methylene is halved (0.18 *vs.* 0.35–0.4 ppm) when tyrosyl is replaced by the nonaromatic leucyl, indicating that the magnetic anisotropy of the aromatic ring makes some contribution to the methylene splitting. For this contribution to be significant, the split methylene must be near the aromatic side chain. Model building shows that a 1,2 residue relationship with the $\chi_1 = 300°$ tyrosyl side-chain rotamer, or any version of the 1,3 relationship, is unlikely on this basis. Second, if the split residue were 1,3 to the tyrosyl, the principal source of the splitting would have to be the magnetic anisotropy of amide groups, which is known to give rise to substantial effects (Paulsen and Todt, 1967). However, there will then be two corner glycyl residues similarly situated relative to their flanking amide groups, and there should be *two* methylene resonances with large splittings. This is not observed, so the 1,3 relationship seems unlikely on this count also. Preparation and examination of a differently deuterated version of I and II would, of course, resolve the question unequivocally. In connection with the effects of magnetic anisotropy of peptide bonds, it should be remarked that a model of the conformation of Figure 8 predicts no magnetic nonequivalence for the glycyl methylenes coupled to the internal amide protons, *i.e.*, those in the middle of the extended peptide segment. These protons are symmetrically disposed above and below the σ plane of the adjacent peptide bonds and should be identically affected by their anisotropy. The other glycyl proton pairs of the model are not so symmetrically arranged relative to the flanking peptide bonds. This is in accord with observation: in dimethyl sulfoxide solution magnetic nonequivalence of the "central" methylenes is small or undetected.

The relationship of the hydroxyphenyl group to the nearby end peptide bond in the conformation of Figure 8 is similar, although not congruent, to the relationship that nmr studies have shown for cyclic depeptides containing aromatic side chains, in that the aromatic ring lies over and facing the plane of a peptide bond. The cyclic dipeptide work (Kopple and Marr, 1967; Kopple and Ohnishi, 1969) has shown that this interaction, which is perhaps a combination of dispersion forces and dipole (amide)-induced dipole (aromatic π system) forces, can amount to 3–4 kcal/mole. The hexapeptide model we have arrived at in Figure 8 suggests that it may also be a conformational determinant in larger cyclic peptides that contain aromatic residues.

All of the amide protons of I or II appear to exchange at about the same rate in dimethyl sulfoxide–water. Since the internal and external amide protons should exchange at different intrinsic rates, the simplest explanation for an equality of exchange rates is that conformational interchanges exposing the internal protons are rapid relative to proton exchange, and that the nuclear magnetic resonance spectra show a weighted average of conformations, one of which is heavily dominant. This would mean that the favored conformation, which we think is that of Figure 8, is not in a very deep potential well. (In passing, it should be noted that studies of *c*-L-Ala-L-Ala-Gly-L-Ala-Gly-Gly show that it also has no slowly exchanging amide hydrogens; Emery, 1967.) For gramicidin S, which does have slowly exchanging hydrogens that are probably the internally bonded protons of a Schwyzer type of structure, Laiken *et al.* (1969) have suggested that the conformation change necessary to permit exchange of the slow protons cannot be large, because the resulting energy barrier would be too high to account for the observed rates. This may also be true in the smaller molecules with which we are dealing, and absence of slowly exchanging amide protons in the cyclic hexapeptides may still be consistent with high stability of the Schywzer hexapeptide backbone.

Acknowledgments

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