matters have been discussed in another place.[14] In addition, it now appears that the heat capacity expression of Brandts derived from eq 1 requires revision.

Experimental Section

The experimental details of this work have been described.[2]

Acknowledgments. R. B. was a recipient of the Minnesota Mining and Manufacturing Company Fellowship in Chemistry during part of this work. We also wish to thank Mr. Vincent Madison for his able technical assistance.

Conformations of Cyclic Peptides. III. Cyclopentaglycyltyrosyl and Related Compounds[1]

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Abstract: Proton magnetic resonance studies of cyclopentaglycyl-L-tyrosyl and a partially C-deuterated derivative, using water, dimethyl sulfoxide, and trifluoroacetic acid as solvents, show that the six amide protons of this peptide are divided into two groups. Two protons are shielded from the solvent and four are exposed to it. This observation strongly supports an internally hydrogen-bonded structure, shown in Figure 1, as the stable form of the cyclic hexapeptide backbone. To this stable backbone conformation the single side chain is attached at one of the "corner" positions (1, 3, 4, and 6 in Figure 1), at least in dimethyl sulfoxide or a dimethyl sulfoxide–water mixture. Additional evidence is adduced from the spectra to define more narrowly, although with less certainty, details of the location and conformation of the side chain. The syntheses of the cyclic peptides are described.

Continuing our application of proton magnetic resonance to problems of peptide conformation in solution, we have taken up the study of cyclic hexapeptides, which are the most readily accessible cyclic peptides after diketopiperazines, and, in distinction to the latter, may pose significant questions of peptide backbone conformation. In this report we describe the syntheses and discuss conformational inferences from the proton spectra of cyclopentaglycyl-L-tyrosyl (I) and a partially deuterated derivative, *c*-Gly-Gly-Gly-d_2-Gly-d_2-L-Tyr (II). For these peptides there are two main questions of conformation: the shape of the peptide backbone and the arrangement of the side chain. We have reached definite conclusions about the first, and more tentative ones about the second.

The secondary structure of a cyclic hexapeptide ring undoubtedly depends on its substituents, but the single form that has so far received the most support was originally put forth by Schwyzer.[2] This structure, originally suggested on the basis of the facile cyclodimerization of carboxyl-activated tripeptide derivatives, is illustrated schematically in Figure 1. In it, all of the amide groups are planar and *trans*, and there are two transannular hydrogen bonds. A structure like this does in fact appear in an X-ray crystallographic analysis of cyclohexaglycyl hemihydrate.[3] Although the crystal examined contains four different conformations of the peptide ring, all with planar, *trans* amide groups, and all involved in intermolecular hydrogen bonds, one conformation, the only one containing intramolecular hydrogen bonds, does have the two hydrogen bridges of the Schwyzer structure.

Something close to the Schwyzer structure also appears in the crystal of ferrichrome A, a naturally occurring cyclic hexapeptide that has three hydroxamic acid side-chain groups coordinated to a ferric ion.[4] Here half of the cyclic hexapeptide ring has the Schwyzer structure, *i.e.*, there is one ring of nine heavy atoms closed by a transannular amide–amide hydrogen bond, but the other half is somewhat distorted by the requirements of a hydrogen bond between the peptide backbone and the side-chain hydroxamate complex. Distortion of the backbone by side-chain interactions is likely to be fairly common in heavily substituted cases. In fact, infrared studies of a group of diastereoisomers of *c*-Gly-Phe-Leu-Gly-Phe-Leu have been interpreted to indicate that, if sufficiently bulky substituents are present on the same side of the hexapeptide ring, some of the peptide bonds are constrained to the *cis* configuration.[5] This suggestion of steric interference between side chains is consistent with cyclization yields, which decrease with increasing numbers of *cis* side chains.[6] The molecule reported on in this paper, having only one side chain, should retain the conformational backbone preference of the unsubstituted cyclohexaglycyl backbone.

A priori, if a particular transannularly hydrogen-bonded structure is strongly favored for the cyclic hexapeptide backbone in solution, it should be revealed by studies of the exchange rates and/or chemical shifts of the amide protons. The bridging amide protons, being

(1) (a) This work was supported by research grants from the National Science Foundation, GB 4514, and the National Institute of General Medical Sciences, U. S. Public Health Service, GM 14069. (b) Paper II of this series is K. D. Kopple and M. Ohnishi, *J. Am. Chem. Soc.*, **91**, 692 (1969).

(2) R. Schwyzer, *Record Chem. Progr.*, 20, 147 (1959); R. Schwyzer, P. Sieber, and B. Gorup, *Chimia*, 12, 90 (1958); R. Schwyzer, *et al.*, *Helv. Chim. Acta*, **47**, 441 (1964).

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(6) Y. Chen-su, K. Bláha, and J. Rudinger, *ibid.*, **29**, 2633 (1964).

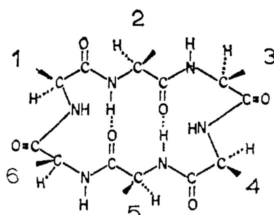

Figure 1. Antiparallel, internally hydrogen-bonded cyclic hexapeptide backbone structure. The numbers refer to positions discussed in the text. Side chains of L-amino acid residues would appear above the paper.

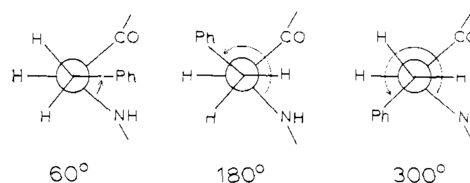

Figure 2. Rotations about the α–β bond of an aromatic peptide residue.

shielded from solvent, might exchange more slowly than the others, and might have significantly different chemical shifts. They might also be identified as belonging to particular residues by decoupling their spins from those of the α-protons, if the molecule is sufficiently simple that the latter can be individually identified. Definitive results of such experiments with cyclic peptides have not before appeared, but Liquori and Conti[7,7a] have examined the proton nmr spectrum of the cyclic decapeptide antibiotic Gramicidin S (for which a crystal structure has not yet been reported). They found that Gramicidin S in dimethyl sulfoxide solution has three groups of amide proton resonances: one, corresponding to four protons, at 8.05 ppm below tetramethylsilane, and the others, corresponding to two protons each, at 8.30 and 8.60 ppm. On addition of deuterium oxide to the dimethyl sulfoxide solution, the two lowest field protons exchange significantly more slowly than the other six, a result possibly consistent with the presence of two stable transannular hydrogen bonds in the peptide molecule.

If several backbone conformations of a peptide are about equally favored, and if the barrier to interchange among them is not high, it is possible that all of the amide protons can appear to exchange at the same rate. In general, one might expect that the less complex (the shorter the backbone and the fewer the side chains) a peptide is, the lower will be the barriers to interchange among conformations, and therefore the less likely will be proton-exchange measurements to afford definitive conformational answers.

We have previously made use of the magnetic effects of an aromatic side chain of one amino acid residue on nearby protons of another residue to determine the preferred conformations of side chains in cyclic dipeptides.[1b,8] The β-methylene protons of aromatic residues themselves appear in an accessible region of the proton spectrum, and their individual couplings to the α-proton, another potential clue to side-chain conformation, can often be determined without undue difficulty. We therefore chose to begin cyclic hexapeptide studies using a single hydroxybenzyl side chain on a cyclohexaglycyl backbone, that is, with cyclopentaglycyl-L-tyrosyl.

Rotation about the α–β bond of a benzyl side chain, angle χ_1 in peptide configurational nomenclature,[9] gives rise to the three possibilities shown in Figure 2. All of these have been shown to occur in one place or another, and no prediction for cyclopentaglycyltyrosyl can be made. Crystallographic studies of two phenylalanyl peptide derivatives, glycylphenylalanylglycine and threonylphenylalanine nitrobenzyl ester hydrobromide, show that they have the $\chi_1 = 180°$ conformation.[10] The three tyrosyl residues in myoglobin all have values of χ_1 close to 300°.[11] The more crowded conformation with $\chi_1 = 60°$ has been shown to be favored for tyrosyl and phenylalanyl residues on cyclic dipeptides.[1b,8]

Experimental Section[12]

Proton Magnetic Resonance Spectra. Nmr 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 (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 under 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 to 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.

The resonances of glycine α-protons of deuterium-exchanged (N–D) peptides were analyzed, where possible, as AB systems. The assignments shown in Figures 5 and 9 appear to be consistent with intensity ratios, where other possible assignments were not. Analysis of glycine α-proton pairs as ABX systems in unexchanged (N–H) samples was not attempted. The tyrosine β-protons were treated as the AB part of an ABX system.

Thin Layer Chromatography. The course of all reactions and the purity of all products were monitored on thin layer chromatograms using Eastman Chromagram Sheet No. 6060 (silica gel with fluorescent indicator). Spots were visualized using a hypochlorite–iodide spraying sequence for amides[13] or coupling with diazotized *p*-bromoaniline for tyrosyl residues.[14] The solvent systems used

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(7a) NOTE ADDED IN PROOF. The backbone conformation of Gramicidin S has now been satisfyingly established, using nmr, by A. Stern, W. A. Gibbons, and L. C. Craig, *Proc. Natl. Acad. Sci. U. S.*, **61**, 734 (1968).

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(10) A. V. Lakshminarayanan, V. Sasisekharan, and G. M. Ramachandran in "Conformation of Biopolymers," Vol. 1, G. N. Ramachandran, Ed., Academic Press, New York, N. Y., 1967, p 61.

(11) C. Coulter and H. Watson; we thank Dr. Coulter for this information.

(12) All melting points are uncorrected. Microanalyses were performed by MicroTech Laboratories, Skokie, Ill.

(13) D. E. Nitecki and J. W. Goodman, *Biochemistry*, **5**, 665 (1966).

(14) R. J. Block, E. L. Durrum, and G. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed, Academic Press, New York, N. Y., 1958, p 128.

were: 1-butanol–acetic acid–water, 4:1:1 and 4:4:1; 1-butanol–water, 6:1; 2-butanol–water, 4:1; butanol–pyridine–water, 7:1:2; and 2-butanone–acetic acid–water, 4:4:1.

Glycylglycylglycine Benzyl Ester *p*-Toluenesulfonate. In a round-bottomed flask were placed 9.45 g (0.05 mol) of triglycine (Cyclo Chemical Corp.), 10.5 g (0.055 mol) of *p*-toluenesulfonic acid monohydrate, 50 ml of benzene, and 25 ml of benzyl alcohol. The reaction mixture was heated under reflux and a clear solution was soon obtained. Water liberated was removed azeotropically. Heating was continued for 2 hr, when water had ceased to be distilled off. The solution was allowed to cool to room temperature and the crystalline product was collected by filtration and washed with absolute ether.

Addition of absolute ether to the mother liquor afforded a second crop. The combined crops were recrystallized from ethanol, yield, 20 g, 88.7%, mp 168–171°.

An analytical sample was recrystallized twice from ethanol and dried under vacuum for 15 hr. It was chromatographically homogeneous in the five solvent systems used.

Anal. Calcd for $C_{20}H_{23}O_7N_3S$: C, 53.21; H, 5.58; N, 9.31. Found: C, 53.17; H, 5.77; N, 9.38.

Glycylglycylglycine Benzyl Ester. About 10 g (0.022 mol) of triglycine benzyl ester *p*-toluenesulfonate in water was passed through a 2.2 × 19.8 cm column containing 50 g of AG 3-X4 ion exchanger (Bio-Rad) in the hydroxide form. The column was eluted with water until the eluate was no longer basic.

One gram (21.8%) of hexaglycine benzyl ester (see below) was collected from the eluate after it had remained at room temperature for about 1 hr. The aqueous solution was lyophilized. Thin layer chromatography of the residue obtained from lyophilization, using 1-butanol–acetic acid–water, 4:1:1, gave spots corresponding to hexaglycine benzyl ester and free triglycine in addition to the major component, triglycine benzyl ester.

The lyophilized product was used without further purification, yield, 3.7 g, 60%.

Hexaglycine Benzyl Ester from Triglycine Benzyl Ester. Glycylglycylglycine benzyl ester *p*-toluenesulfonate (17 g, 0.038 mol) was dissolved in 75 ml of water; 50 ml of chloroform was added and the aqueous phase was adjusted to pH 9.6 by addition of 21 ml of 2 *N* sodium hydroxide. The organic layer was separated after shaking, and the aqueous phase was immediately extracted further with three 100-ml portions of 1:1 chloroform–ethyl acetate. The combined organic extracts were dried over magnesium sulfate and concentrated at reduced pressure to a noncrystalline residue that gave a single, ninhydrin-positive spot in 4:1:1 1-butanol–acetic acid–water, with R_f value (0.75) identical with that of the starting salt. Only 2.13 g (20%) of the tripeptide ester was obtained.

The aqueous phase, stored overnight at room temperature, deposited a precipitate. The whole was evaporated to dryness under vacuum and washed with 50 ml of water. Recrystallization of the residue from a large volume of hot water gave 3 g (35%) of a product that decomposed above 280° without melting. This material, also ninhydrin positive, had R_f 0.5 in the 4:1:1 solvent system. We were unsuccessful in obtaining satisfactory elementary analyses for this product, but it was identified as hexaglycine benzyl ester by comparison of the R_f values and electrophoretic behavior of its hydrogen bromide in acetic acid cleavage (debenzylation) product with those of commercially obtained samples of diglycine, triglycine, tetraglycine, and hexaglycine. In addition, the elution volumes from a Sephadex G-15 column of the N-dinitrophenyl (DNP) derivatives of the four glycine peptides were compared with that of the unknown. Using a 1.4 × 100 cm column with a void volume of 55 ml, and eluting with 0.1 *N* sodium acetate solution at 9 ml/hr, DNP-diglycine came off at 255 ml, DNP-triglycine at 193 ml, DNP-tetraglycine at 176 ml, and DNP-hexaglycine, as well as the unknown, at 147 ml.

The aqueous phase from which the hexapeptide deposited was shown to contain chiefly triglycine free peptide in significant quantities.

The dimerization process was briefly investigated in the following manner. Solutions of triglycine benzyl ester *p*-toluenesulfonate with added base were allowed to remain at room temperature overnight; the precipitated hexaglycine benzyl ester, if any, was removed by filtration and weighed, and the reaction liquors were titrated to determine their amino and carboxyl content. The results are indicated in Table I. By thin layer chromatography the products of these experiments were shown to be hexapeptide benzyl ester and traces of free hexaglycine, tripeptide benzyl ester, and free triglycine; the last presumably accounts for the carboxyl titer of the reaction liquors.

Table I. Neutralization of Triglycine Benzyl Ester *p*-Toluenesulfonate

Initial ester, *M*	Initial NaOH, *M*	Isolated dimer, %	Decrease in amine, %	Carboxyl yield, %
2.0	2.0	31	30	50
2.0	1.6	28	27	35
1.0	0.9	22	26	52
0.1	0.09	0	0	38
2.0[a]	2.0[a]	40		

[a] This experiment carried out in dimethylformamide with triethylamine as base.

N-Carbobenzyloxyglycylglycyl-L-tyrosine Methyl Ester.[15] A solution of 38.4 g (0.137 mol) of N-carbobenzyloxyglycylglycine hydrazide[16] in 700 ml of water containing 120 ml of glacial acetic acid and 50 ml of 5 *N* hydrochloric acid, was cooled to 0–5°. To this was added 10.43 g (0.151 mol) of sodium nitrite dissolved in a small amount of water. The reaction mixture was stirred at 0° for 10 min and the precipitated azide was extracted with 2.2 l. of ice-cold ether–ethyl acetate mixture. The extract was back-washed with ice water, 3% sodium bicarbonate, and ice water again. It was not dried.

A cooled solution (0°) of L-tyrosine methyl ester (26.8 g, 0.137 mol) in 500 ml of ether plus 1200 ml of ethyl acetate was mixed with the azide solution and the reaction mixture was stirred overnight at room temperature. A precipitate appeared after 1 hr.

The reaction mixture was filtered and the collected precipitate was washed with cold ether–ethyl acetate mixture. A second crop was obtained from the concentrated mother liquor, yield 48.8 g, 80.2%.

Recrystallization from ethyl acetate afforded an analytical sample, mp 164–165°.

Anal. Calcd for $C_{22}H_{25}O_7N_3$: C, 59.59; H, 5.68; N, 9.48. Found: C, 59.55; H, 5.80; N, 9.62.

N-Carbobenzyloxyglycylglycyl-L-tyrosine. Sodium hydroxide (1 *N*, 132.7 ml) was added to a suspension of 30 g (0.068 mol) of N-carbobenzyloxyglycylglycyl-L-tyrosine methyl ester in 260 ml of pyridine, and the resulting clear solution was stored at room temperature for 1 hr and then 2 hr at 0–5°. At 0–5° the solution was adjusted to pH 6 and the solvent was removed under reduced pressure.

The residue was taken up in water and acidified to congo red. The precipitated oil slowly crystallized at room temperature. The crystalline product was collected and the mother liquor was concentrated to give a second crop. Both crops were chromatographically homogeneous in the 1-butanol–pyridine–water and 1-butanol–water solvent systems, yield, 21.1 g, 76%, mp 107–109°.

Two recrystallizations from ethanol–water mixture produced an analytical sample, mp 115°.

Anal. Calcd for $C_{21}H_{23}O_7N_3$: C, 58.74; H, 5.40; N, 9.78. Found: C, 58.80; H, 5.40; N, 9.54.

N-Carbobenzyloxyglycylglycyl-L-tyrosylglycylglycylglycine Benzyl Ester. Crude triglycine benzyl ester free base (3.7 g, 0.0132 mol) was added to a solution of N-carbobenzyloxyglycylglycyl-L-tyrosine (4.73 g, 0.011 mol) in 60 ml of dimethylformamide, followed by 2.26 g (0.011 mol) of N,N′-dicyclohexylcarbodiimide. The reaction mixture was stirred overnight at room temperture.

Precipitated dicyclohexylurea was separated by centrifugation, and the supernatant liquid was concentrated under vacuum to dryness. The residue was taken up in hot ethanol and allowed to crystallize. A second crop was obtained from the concentrated mother liquor.

Recrystallization from ethanol afforded an analytical sample which was chromatographically homogeneous in several solvent systems. The sample was dried under vacuum at 100° for 30 hr, yield, 5.7 g, 75.9%, mp 153–156°.

Anal. Calcd for $C_{34}H_{39}O_{10}N_6$: C, 59.12; H, 5.55; N, 12.17. Found: C, 58.92; H, 5.74; N, 12.11.

Glycylglycyl-L-tyrosylglycylglycylglycine. A suspension of 5.45 g of N-carbobenzyloxyglycylglycyl-L-tyrosylglycylglycylglycine benzyl ester and 0.5 g of 10% palladium on charcoal in 100 ml of glacial acetic acid was purged with nitrogen gas. Hydrogen gas

(15) R. B. Woodward, R. A. Olofson, and H. Mayer, *Tetrahedron Suppl.*, **8**, 321 (1966).

(16) P. Karrer and H. Heynemann, *Helv. Chim. Acta*, **31**, 398 (1948).

was then bubbled through the mixture for about 2 hr. The reaction was stopped and a small volume of water added to dissolve the solid formed. The catalyst was separated by centrifuging and the supernatant liquid was concentrated to dryness under vacuum.

Attempts to obtain a crystalline sample of the hydrogenolysis product were unsuccessful. It was lyophilized from acetic acid to give 2.5 g of a tan powder that showed on thin layer chromatography a single major spot but also a streak of contaminants.

Cyclopentaglycyl-L-tyrosyl (I). To a clear solution of 1.5 g (0.0032 mol) of the crude linear hexapeptide in 230 ml of dimethylformamide was added N,N′-dicyclohexylcarbodiimide (2 g, 0.0096 mol), and the reaction mixture was stirred overnight at room temperature. Another 2 g of N,N′-dicyclohexylcarbodiimide was added and the solution was again stirred overnight.

The reaction mixture was concentrated under reduced pressure to half its initial volume and 4 ml of 50% aqueous acetic acid was added. The precipitated dicyclohexylurea was separated by filtration and the supernatant liquid was concentrated to dryness under vacuum. The residue consisted of a mixture of Pauly-positive, ninhydrin-negative products (thin layer chromatography 4:1 2-butanol–water) with major components at R_f 0.54 and 0.7.

The residue was chromatographed on a 4.7 × 49 cm column containing 500 g of Whatman CC-31 microcrystalline cellulose powder using the 4:1 2-butanol–water solvent system.

Those eluate fractions containing the R_f 0.54 component were combined and concentrated to a small volume. A crystalline product was collected after storage for several days, and was recrystallized from water, yield, 0.165 g, 11.5%, mp 270–273°; optical rotation [α] (5.5 mg/ml in 95% ethanol), at 300 nm −33°, at 400 nm 8.2°, at 500 nm 5.0°, and at 600 nm 3.6°. The analytical sample was dried under vacuum at 100° for 60 hr.

Anal. Calcd for $C_{19}H_{24}O_7N_6 \cdot 0.5H_2O$: C, 49.88; H, 5.51; N, 18.37. Found: C, 49.63; H, 5.50; N, 18.06.

Glycylglycine-d_4. Glycine anhydride (42.75 g, 0.375 mol) was suspended in 75 ml of 99.9% deuterium oxide containing 5.2 g of potassium carbonate. The mixture was stirred and heated under reflux overnight, during which period most of the solid dissolved. The solvent was removed by distillation at atmospheric pressure, and the residue was dried under vacuum over phosphorus pentoxide. To the dried residue another 100 ml of 99.9% deuterium oxide was added, and the mixture again stirred and boiled under reflux overnight. About two-thirds of the solvent was removed by distillation and the remainder by lyophilization. Thin layer chromatography (1-butanol–acetic acid–water 4:4:1) of the brownish residue indicated the presence of both glycine anhydride and glycylglycine.

The exchanged mixture was dissolved in 250 ml of hot concentrated hydrochloric acid; the solution was brought to boiling for 90 sec and then chilled in an ice bath. Deuterated glycylglycine hydrochloride monohydrate precipitated on cooling and was collected by filtration on a sintered glass funnel, washed with ethanol, and dried under vacuum; 39.7 g (57%) of chromatographically pure dipeptide was obtained.

The hydrochloric acid filtrate was evaporated to dryness, and the residue was taken up in the minimum amount of water. Pyridine was added to bring the solution to neutrality. Addition of ethanol precipitated 8.1 g (14%) of deuterated free glycine, which was collected by filtration and washed with ethanol.

Completeness of glycyl α-proton exchange was determined from the proton resonance spectrum of the carbobenzyloxy derivative of the glycylglycine. The integral of this spectrum indicated the product to be 92% deuterated.

Carbobenzyloxyglycylglycine-d_4. The free deuterated dipeptide (33.6 g, 0.18 mol) was treated with carbobenzyloxy chloride under the Schotten–Baumann conditions usual[17] for benzyloxycarbonylation of amino acids (0°, pH controlled at 11, monitored by pH meter). The product was recrystallized from hot water; 32.2 g (67%) was obtained, mp 176–178°, reported for the undeuterated material 178°.[18]

Carbobenzyloxyglycyl-d_2-glycyl-d_2-L-tyrosine Methyl Ester. To a solution of benzyloxycarbonylglycylglycine-d_4 (26.6 g, 0.1 mol) in 120 ml of freshly distilled dimethylformamide was added 21.5 g (0.11 mol) of L-tyrosine methyl ester[19] and 22.7 g (0.11 mol) of dicyclohexylcarbodiimide. Precipitation of dicyclohexylurea began in a few minutes, but the reaction mixture was stirred at room temperature overnight before the urea was collected by filtration and washed with ethyl acetate. The dimethylformamide solution and the ethyl acetate washings were combined and concentrated under vacuum to an oil that crystallized from ethyl acetate. Recrystallization from ethyl acetate yielded 25.87 g (58%) of blocked tripeptide, mp 155–157°.

An analytical sample was obtained upon two recrystallizations from chloroform, mp 157–158°. It was indistinguishable on thin layer chromatography from the undeuterated compound, mp 164–165°.

Anal. Calcd for $C_{22}H_{21}D_4N_3O_7$: C, 59.05; H + D, 5.63; N, 9.39. Found: C, 59.54; H + D, 5.67; N, 9.66.

Glycyl-d_2-glycyl-d_2-L-tyrosine Methyl Ester Hydrobromide. The carbobenzyloxy tripeptide (25.9 g, 0.058 mol) was suspended in 31 g of 30% anhydrous hydrogen bromide in acetic acid. Reaction was complete in 1 hr, and the product precipitated on addition of 500 ml of anhydrous ether. It was collected by filtration and washed thoroughly with anhydrous ether before drying and storage over phosphorus pentoxide, yield, 21.6 g (95%), mp 189–191°. This material was not further purified before use.

Carbobenzyloxytriglycylglycyl-d_2-glycyl-d_2-L-tyrosine Methyl Ester. To a suspension of benzyloxycarbonyltriglycine[20] (16.2 g, 0.05 mol) and deuterated diglycyl-L-tyrosine methyl ester hydrobromide (19.7 g, 0.05 mole) in 80 ml of freshly distilled dimethylformamide was added 7 ml (0.05 mol) of triethylamine. To the resulting clear solution was added 12.4 g (0.06 mol) of dicyclohexylcarbodiimide and the reaction mixture was stirred overnight at room temperature. The precipitated dicyclohexylurea was collected and washed by stirring several hours with fresh dimethylformamide. The washings and reaction solution were combined and concentrated under vacuum to a small volume, to which water was added to precipitate the hexapeptide ester. Recrystallization from methanol yielded 16.7 g (54%) of chromatographically clean (1-butanol–water, 6:1) product, mp 195–198°.

Anal. Calcd for $C_{28}H_{34}D_4N_6O_{10} \cdot 0.5H_2O$: C, 53.89; H + D, 5.73; N, 13.47. Found: C, 53.47, 53.87; H + D, 5.54, 5.65; N, 13.57, 13.72.

Carbobenzyloxytriglycylglycyl-d_2-glycyl-d_2-L-tyrosine. The blocked hexapeptide ester (14.5 g, 0.027 mol) was dissolved in 160 ml of pyridine and mixed with 34 ml of 1 *N* sodium hydroxide (1.5 equiv). The solution was allowed to remain at room temperature for 1 hr and at 0–5° for 2.5 hr more. At 0–5°, 6 *N* hydrochloric acid was added to bring the acidity to pH 6, measured by indicator paper, and the solvent was stripped off under vacuum. The residue was taken up in water and lyophilized.

The lyophilized residue was stirred with two 100-ml portions of saturated aqueous sodium bicarbonate, 10–12 hr each portion. The bicarbonate-insoluble material remaining proved to be 1.33 g (9%) of starting material. From the bicarbonate solution, on acidification to congo red and prolonged storage at 0–5°, there precipitated hexapeptide acid, which was collected by filtration and washed thoroughly with water. This product (5.6 g 44%) was chromatographically homogeneous in 7:1:2 butanol–pyridine–water and had mp 161–166°.

Recrystallization from ethanol–water afforded an analytical sample, mp 164–166°.

Anal. Calcd for $C_{27}H_{28}D_4O_{10}N_6 \cdot H_2O$: C, 52.08; H + D, 5.57; N, 13.50. Found: C, 52.00; H + D, 5.18; N, 13.44.

Triglycylglycyl-d_2-glycyl-d_2-tyrosine. The carbobenzyloxy deuterated hexapeptide (5.3 g) was mixed with 4 ml of 30% hydrogen bromide in acetic acid. After 1 hr 100 ml of anhydrous ether was added to the clear solution. The precipitated solid was collected by filtration and washed thoroughly with more anhydrous ether before it was dissolved in 50 ml of 95% ethanol. Pyridine (4 ml) was added to the solution, which was stored at 0–5° until precipitation was complete. The product (2.23 g) was collected by filtration; it was chromatographically homogeneous (1-butanol–acetic acid–water, 4:4:1), but still gave a positive halide test. It was freed of residual halide by solution in a minimum amount of water and reprecipitation with two volumes of ethanol. Only 1.86 g of free hexapeptide was thus obtained. An analytical sample was dried at 100° (0.05 Torr) for 2 days.

Anal. Calcd for $C_{19}H_{22}D_4N_6O_8 \cdot 0.5H_2O$: C, 47.60; H + D, 5.77; N, 17.53. Found: C, 47.95; H + D, 5.68; N, 17.42.

(17) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961, p 891.

(18) S. Goldschmidt and M. Wick, *Ann.*, **575**, 217 (1952).

(19) The free base, mp 131–133°, is obtained by treatment of a chloroform (10 ml/g) suspension of the hydrochloride with triethylamine (0.75 ml/g). A solution results, which soon deposits crystalline free ester.

(20) M. Rothe, H. Brunig, and G. Eppert, *J. Prakt. Chem.*, [4] **8**, 323 (1959).

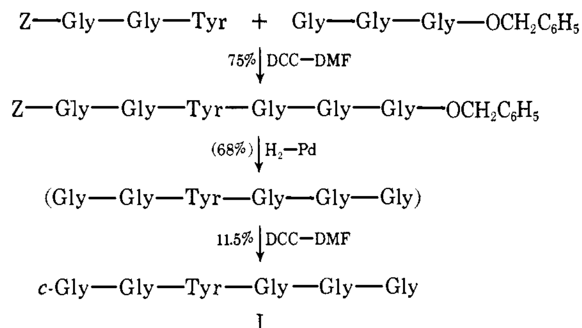


Figure 3. Synthesis of cyclopentaglycyl-L-tyrosyl. The linear hexapeptide shown in parentheses was not obtained in pure form. Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide.

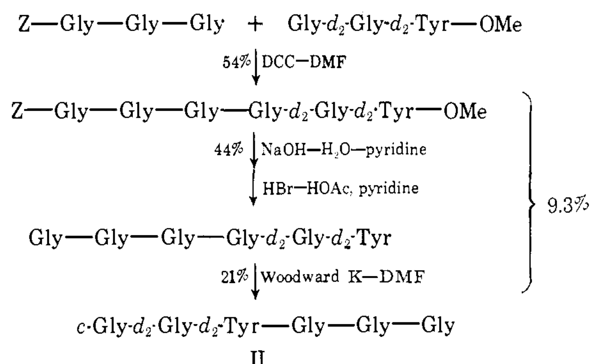


Figure 4. Synthesis of deuterated derivative of cyclopentaglycyl-L-tyrosyl. Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; Woodward K, N-ethyl-5-phenylisoxazolium-3′-sulfonate.

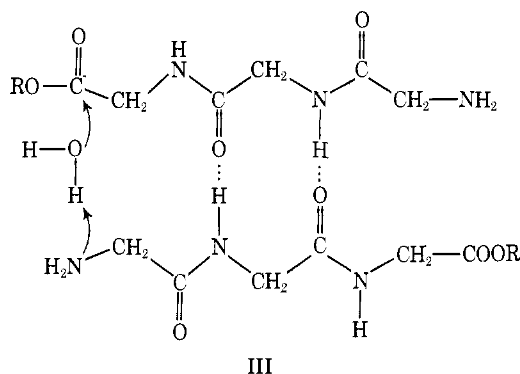
Cyclotriglycylglycyl-d_2-glycyl-d_2-L-tyrosyl (II). The unblocked linear hexapeptide (1.39 g, 0.003 mol) was suspended in 300 ml of freshly distilled dimethylformamide. At 0° 1.5 g (0.0045 mol) of N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) was added, and the mixture was stirred at 0° for 2 hr, during which period most of the peptide dissolved. Triethylamine (0.63 ml, 0.0045 mol) was added, and stirring was continued for another hour at 0° and overnight at room temperature.

The reaction mixture was centrifuged free of a trace of insoluble material that was identified as starting peptide by thin layer chromatography, then it was concentrated to dryness under vacuum. Upon trituration with about 10 ml of ethanol the residue solidified. The solid product was recrystallized from hot water (212 mg obtained), and proved to be chromatographically identical with undeuterated c-Gly$_5$-L-Tyr in several solvent systems (1-butanol–acetic acid–water, 4:4:1 and 4:1:1, 2-butanol–water, 4:1).

The ethanolic and aqueous mother liquors were combined diluted to 500 ml with water, and passed through a 1.4 × 33 cm column of Bio-Rad AG 3X-4 (weak base) resin, chloride form. The column was rinsed with more water, and everything eluted was concentrated to a small volume from which, on seeding, there crystallized another 70 mg of clean cyclic hexapeptide. The total yield was 282 mg, 21%. A plot of the specific rotation of this material *vs.* wavelength was everywhere within 10% of that of the undeuterated material (I).

Results

Syntheses. The cyclic hexapeptides used in this study were prepared according to the schemes outlined in Figures 3 and 4. The initial choice of route was determined by the existence of a convenient route to glycylglycine-d_4. This C-deuterated dipeptide was readily obtained by treatment of diketopiperazine (glycine anhydride) with deuterium oxide in the presence of potassium carbonate, followed by acid hydrolysis[21] of the exchanged diketopiperazine. Glycylglycine could readily be converted to its N-carbobenzyloxy derivative and coupled with L-tyrosine methyl ester. Our initial intention was to couple carbobenzyloxydiglycyltyrosine, *via* the azide, with triglycine benzyl ester. Triglycine benzyl ester p-toluenesulfonate was prepared in good yield by direct esterification of the commerically available free tripeptide, in the manner commonly applied to free amino acids.[22] We found, however, that neutralization of the salt, dissolved at preparative concentration in water or dimethylformamide, or suspended in chloroform, resulted in formation of what we identified as hexaglycine benzyl ester, in 20–40% yield, at a rate comparable to that observed[23] for triglycine methyl ester. If the neutralization was done in water, extensive hydrolysis of the benzyl ester also resulted, even when less than stoichiometric base was used. (Details are discussed in the Experimental Section, and some yields are given in Table I there). The dimerization of triglycine derivatives is probably assisted by hydrogen-bonded association between antiparallel chains.[23] Perhaps the hydrolysis observed is at least partially the result of amine-catalyzed attack of water on the ester function in an antiparallel complex of two peptides as in III.



It did not seem advisable to use triglycine benzyl ester as the amine component in an azide coupling because of the rapidity of its dimerization. We did obtain the desired tyrosine-containing hexapeptide in the undeuterated series, with moderate success, by dicyclohexylcarbodiimide coupling of carbobenzyloxydiglycyltyrosine and triglycine benzyl ester in dimethylformamide. To minimize racemization of the L-tyrosyl residue a crude form of triglycine benzyl ester free base was used in the coupling; this was obtained by washing an aqueous solution of the tosylate salt through a column of strong base ion exchange resin in the hydroxide form and lyophilizing the basic eluate. The synthesis of I was completed then as shown in Figure 3.

The synthesis of the deuterated peptide was carried out in an alternative manner (Figure 4). Carbobenzyloxytriglycine, prepared by direct acylation of triglycine, was coupled to deuterated glycylglycyl-L-tyrosine methyl ester. The methyl ester was used because it was on hand, although its use led to complications

(21) H. F. Schott, J. B. Larkin, L. B. Rockland, and M. S. Dunn, *J. Org. Chem.*, 12, 490 (1947).
(22) Reference 17, p 942.
(23) P. S. Rees, D. P. Tong, and G. T. Young, *J. Chem. Soc.*, 662 (1954).

Table II. Amide and α-Proton Resonances of Cyclopentaglycyltyrosyl, 30°

Solvent		Chemical shifts[a] Gly, Gly[b]	Tyr[h]	Gly, Gly, Gly[c]
DMSO[d]	CH	3.75 (D, F)	4.28 (C)[e]	3.73 ± 0.20 (B),[f] 3.70 ± 0.30 (A), 3.84 ± 0.03 (E)
(100 mg/ml)	NH (30°)	7.70 (F), 8.17 (D)	8.25 (C)	8.29 (B), 8.43 (A), 7.78 (E)
	NH (80°)	7.63 (F), 7.95 (D)	~7.95 (C)	~8.00 (B), 8.19 (A), 7.70 (E)
DMSO–20% H_2O[d]	NH (30°)	8.45 (D), 8.05 (F)	8.67 (C)	8.56 (B), 8.79 (A), 8.14 (E)
	NH (80°)	8.27 (D), 7.96 (F)	8.36 (C)	8.32 (B), 8.53 (A), 8.02 (E)
TFA-*d* (30 mg/ml)	CH[i]	4.20	4.77	4.19 ± 0.15, 4.20 (s), 4.31 ± 0.14
TFA	NH	Overlapping bands (25 Hz wide[g]) at 7.7 (lesser) and 7.85 (greater)		
D_2O (3 mg/ml)	CH[i,j]	4.29 ± 0.11, 4.32 ± 0.04	4.77 (m)	4.22 ± 0.09, 4.26 (s), 4.40 ± 0.13
H_2O	NH	8.20 (two protons), 8.75 (four protons)		(No structure visible)

[a] In parts per million (ppm) below tetramethylsilane in dimethyl-d_6 sulfoxide and trifluoroacetic acid, below capillary hexamethyldisiloxane for aqueous solutions. To convert data for water at 30° to internal 2,2-dimethyl-2-silapentane-5-sulfonate substract 0.48 ppm. [b] Residues on amino side of tyrosyl. [c] Residues on carboxyl side of tyrosyl. [d] Capital letters in parentheses are assignments of coupled α- and amide protons. [e] $J_{\alpha,NH}$ = 6.5 Hz. [f] $J_{\alpha,\alpha}$ = 17 Hz, $J_{\alpha,NH}$ = 5 Hz (high-field proton), 6.5 Hz (low-field proton). Linear least-squares fit of 11 points (peptide I only) at varying temperatures gives $\Delta\nu$ (Hz) = 41.1 − 0.044T (°C) between 10 and 110°. [g] Width at half-height. [h] Center of multiplet. [i] $J_{\alpha\alpha}$ = 17 Hz for all glycine residues where measurable. [j] From ~150-scan accumulations.

during the subsequent saponification step.[24] To minimize racemization in the cyclization step the free dipolar hexapeptide was used for cyclization. Pentaglycyl-L-tyrosine, in contrast to diglycyl-L-tyrosyldiglycylglycine, would not go into solution in dimethylformamide. It did not go into solution under the influence of dicyclohexylcarbodiimide, of dicyclohexylcarbodiimide plus N-hydroxysuccinimide,[25] or of the two reagents plus an equivalent of *p*-toluenesulfonic acid. A suspension of the peptide did react with N-ethyl-5-phenylisoxazolium-3′-sulfonate, and cyclization was thereby achieved.

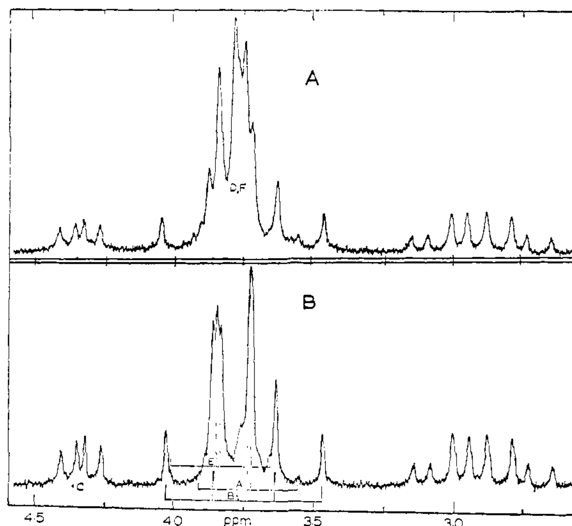


Figure 5. 100-MHz proton magnetic resonance spectra of the α- and β-protons of cyclopentaglycyltyrosyl (A) and cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl (B) in dimethyl-d_6 sulfoxide, tetramethylsilane reference. The amide protons have been replaced by deuterium. Concentration is about 100 mg/ml. The lettered lines indicate the analysis made of the spectra; the letters pair the α-proton resonances with the amide proton resonances as indicated in Table II. α- and amide proton resonances with the same letter are coupled, and therefore on the same amino acid residue.

It is not established that racemization was avoided in the preparations of I and II. However, the peptides I and II are optically active and yield congruent optical rotatory dispersion curves ($[\alpha]_{300}$ −33° (*c* 1.1 mg/ml, ethanol)).

Proton Magnetic Resonance Spectra. Spectra of cyclopentaglycyltyrosyl (I) and cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl (II) were measured at 100 MHz in dimethyl-d_6 sulfoxide, trifluoroacetic acid, trifluoroacetic acid-*d*, deuterium oxide, and water. The spectra of several related open-chain peptides were also examined. Spectra in dimethyl sulfoxide and dimethyl sulfoxide–water were determined for the N–H and N–D forms of the cyclic peptides, and spectra of II were examined at varying temperatures. Illustrative portions of the spectra appear in Figures 5, 6, 7, and 9.

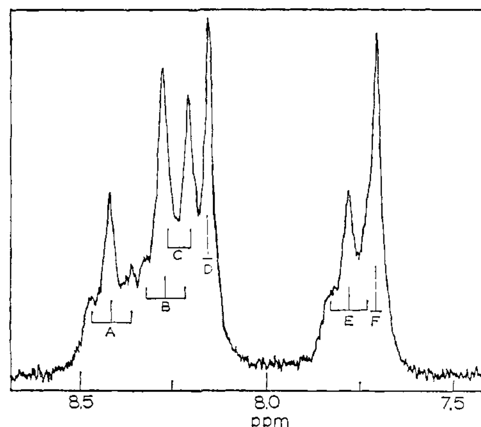


Figure 6. 100-MHz proton magnetic resonance spectrum of the amide protons of cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl in dimethyl-d_6 sulfoxide. Reference is internal tetramethylsilane; the letters refer to amino acid residue assignments indicated in Table II.

The amide proton resonances of II in dimethyl sulfoxide and dimethyl sulfoxide–water were assigned to particular α-proton resonances on the basis of their apparent multiplicities and with the aid of homonuclear spin decoupling experiments; the assignments are indicated in Table II and the figures by letters A–F. Table II gives numerical values of the chemical shifts for the α- and amide protons of the cyclic peptide. For comparison, Table III gives analogous data for the related open-chain compounds. The assignments indicated in Table III were made on the basis of spin decoupling

(24) N-Carbobenzyloxy peptides sometimes form hydantoins or ureas under conditions of alkaline ester hydrolysis: J. A. MacLaren, *Autstralian J. Chem.*, 11, 360 (1958); S. Shankman, S. Higa, S. Makineni, and W. Noll, *J. Med. Chem.*, 6, 746 (1963).
(25) J. E. Zimmerman and G. W. Anderson, *J. Am. Chem. Soc.*, 89, 7151 (1967).

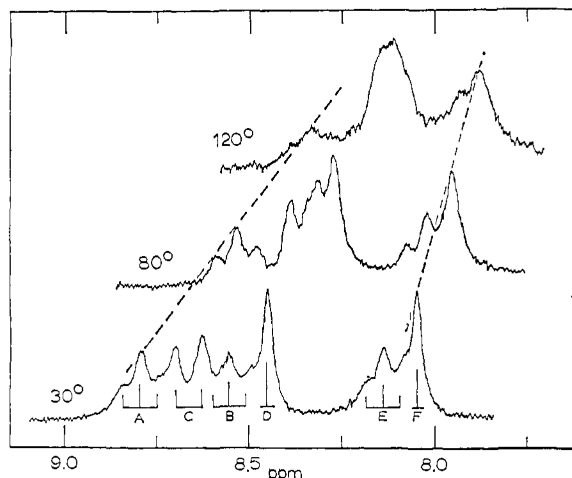


Figure 7. 100-MHz proton magnetic resonance spectra of cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl, amide proton region, in dimethyl-d_6 sulfoxide–20% water mixture, at 30, 80, and 120°, reference capillary hexamethyldisiloxane. The lettered lines refer to pairing with the α-protons.

wherever possible, and on the basis of logical consistency otherwise.

Data referring to the β-protons of the tyrosyl residues in all peptides are presented in Table IV.

Table III. Amide and α-Proton Resonances of Some Glycine-Containing Peptides

Peptide[b]	Residue[c]	Chemical Shift, ppm[a] DMSO NH	DMSO CH	TFA NH	TFA CH	Water NH	Water CH
$GGGG_dG_dT$	1		3.50[e]				4.21[e]
	2	8.32	3.74				4.32
	3	8.11[f]	3.79				4.37
	4	8.07[g]					
	5	8.15[g]					
	6	7.57	4.17				4.73
GGGGGG[d]	1		3.63	7.43			4.21
	2			7.67			
	3						
	4	8.12	3.76	7.93	4.28	8.65[h]	4.31
	5						
	6	8.64	3.86	8.02			4.37
GGG[d]	1		3.63	7.46	4.23		4.22
	2	8.23	3.78	7.62	4.30	8.75[h]	4.27
	3	8.69	3.84	8.03	4.37		4.36
APGL	1						
	2	8.67					
	3	8.09	3.73[i]		4.21		3.82[i]
	4	8.23					
Z-GGT	1	7.44	3.61				
	2	8.02	3.70				
	3	8.06	4.37				
Z-GGTG-OEt	1	7.45	3.63				
	2	7.98	3.68[j]				
	3	8.04	4.45				
	4	8.38	3.82				

[a] Ambient probe temperatures, about 30°. Reference tetramethylsilane for dimethyl sulfoxide and trifluoroacetic acid solutions, capillary hexamethyldisiloxane for aqueous solutions. [b] Abbreviations: G, glycyl; G_d, α,α-dideuterioglycyl; T, tyrosyl; P, phenylalanyl; L, leucyl; A, alanyl; Z, carbobenzyloxy. [c] Numbered from N-terminus. [d] Hexaglycine and triglycine were studied in water and dimethyl sulfoxide as trifluoroacetate salts for reasons of solubility. [e] Uncertain assignment, made only on the basis that in GGG and GGGGGG the highest field methylene is N-terminal. [f] Under 8.07 and 8.15 peaks; detected by integration. [g] Singlets, assigned to deuterated glycyls. [h] Width at half-height about 0.35 ppm. [i] AB pattern, J_{AB} = 17 Hz, $\Delta\nu$ = 0.16 ppm. [j] AB pattern, J_{AB} = 17 Hz, $\Delta\nu$ = 0.17 ppm.

Table IV. Arylmethylene Resonances in Cyclopentaglycyltyrosyl and Related Peptides

Solvent	Peptide[a]	T, °C[b]	Chemical shift,[c] ppm ν	$\Delta\nu$	Coupling constants, Hz $J_{\alpha,h\beta}$	$J_{\alpha,h\beta}$	$J_{\beta,\beta}$
DMSO	c-GGGGGT[d]	10	2.87	0.273	9.6	5.1	
		30	2.87	0.264	9.4	5.2	14
		100	2.90	0.234	8.9	5.6	
	GGGGGT[e]		2.85	0.16	9.8	3.7	14.5
	Z-GGTG-OEt		2.80	0.27	9.8	4.2	14
	Z-GGT		2.85	0.15	8.6	4.4	14
	APGL		3.02	0.19	10.7	3.8	14
	c-GT		3.00	0.22	4.5	4.5	13.5
TFA	c-GGGGGT		3.16	0.21	7.7	6.8	14
	c-GT		3.32	0.10	5.2	4.3	14.5
D_2O	c-GGGGGT		3.36	0.11	8.2	7.0	14
	GGGGGT[e]		3.30	0.22	8.9	5.1	14
	APGL		3.37	0.09	9.3	6.0	14
	c-LT[f]		3.41	0.15	4.9	3.6	14.1

[a] Abbreviations: G, glycyl; T, tyrosyl; A, alanyl; P, phenylalanyl; L, leucyl; Z, carbobenzyloxy. [b] Unless specified, ambient probe temperature, near 30°. [c] Tetramethylsilane reference for dimethyl sulfoxide and trifluoroacetic acid solutions; capillary hexamethyldisiloxane for aqueous solutions. [d] Values taken from linear least-squares fit of 15 measurements between 10 and 110°. Standard deviation of chemical shifts is near 0.2 Hz and of coupling constants near 0.1 Hz. [e] From 150-scan accumulations; concentration about 5 mg/ml. [f] O-Carboxymethyltyrosyl derivative. Reference 1b.

The amide protons of cyclopentaglycyltyrosyl can be divided into two groups, one coming into resonance at higher field than the other. In dimethyl sulfoxide and in water the groups are clearly separated (see Table II and Figures 6 and 7). The higher field group corresponds to two protons, the lower field group to four. In trifluoroacetic acid the two groups overlap, and separate integration is not possible, but it is clear that the higher field group is the smaller. From Tables II and III it is seen that the mean chemical shift of the lower field set, in each solvent, corresponds more closely to the chemical shift of the observed internal amide protons of linear hexaglycine. The chemical shift of the high-field pair of amide protons is virtually independent of solvent. At about 30°, as Table II indicates, it is close to 7.7 ppm (below tetramethylsilane) in dimethyl sulfoxide and trifluoroacetic acid. If the measurements in water are referred to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate, it is about 7.7 ppm in that solvent also.

Figures 7 and 8 show the temperature dependence of the amide proton resonances in dimethyl sulfoxide containing 20% water. The protons in the lower field group are about twice as sensitive to temperature-induced changes than are the higher field pair.

In dimethyl sulfoxide containing 10% heavy water the amide protons of the cyclic peptide all exchange at the same rate, *i.e.*, there is no change in the N–H proton pattern, save a decrease in intensity, as exchange proceeds. Complete exchange at room temperature requires less than 3 min when the added heavy water contains about 10% of trifluoroacetic acid, about an hour when the water is at pH 4, and several hours when no acidic catalyst has been added.

The data of Table II and Figures 5 and 9 show that several of the glycyl residues in cyclopentaglycyltyrosyl have methylene proton pairs that experience sufficiently different average environments to show discernible AB

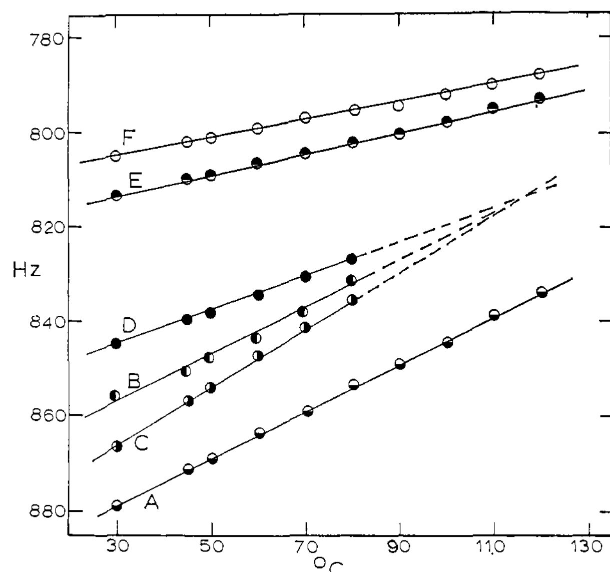


Figure 8. Temperature dependence of the chemical shift, referred to capillary hexamethyldisiloxane, of the amide protons of cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl in dimethyl-d_6 sulfoxide–20% water mixture. The letters refer to the amino acid residue assignments indicated in Table II, and the resonances in Figure 7.

spectral patterns, adjacent amide protons having been replaced by deuterium. (Because of overlapping resonances, chemical-shift differences of the order of 0.1 ppm between geminal glycyl protons are not readily seen, and pairs listed as unsplit in Table II may not be completely so.) In dimethyl sulfoxide and trifluoroacetic acid solutions these residues are among those remaining undeuterated in peptide II. In water observably nonequivalent methylenes appear on both sides of the tyrosyl residue, four of the five glycyls showing AB patterns; the overlapping patterns were sorted by comparison of the spectrum of II (Figure 9) with that of I. In contrast to the cyclic peptide, the open-chain peptide, triglycylglycyl-d_2-glycyl-d_2-tyrosyl, does not show split methylene groups.

The mean chemical shifts for the glycyl methylenes of cyclopentaglycyltyrosyl do not deviate, in any solvent, very far from the glycyl methylene resonances of open-chain hexaglycine in that solvent. In each of the three solvents, the geminal coupling constant for those glycyl methylenes for which it can be determined is (presumably minus) 17 Hz.

Only relatively small changes in the nonequivalence of the most split α-proton pair (glycyl residue B) occurred on varying the temperature of dimethyl sulfoxide solutions; this amounted to a decrease of about 4.5 Hz in going from the lowest to the highest temperature studied. (It is of interest to note that the glycyl nonequivalence in II, the deuterated peptide, is 2–3 Hz less than that of I at the three temperatures (12, 30, and 83°) at which it was measured.) Similarly, the observed couplings between the α- and β-protons of the tyrosyl residue approached each other slowly as the temperature increased. Presumably these changes represent leveling of conformational populations, but no attempt was made to analyze them. The data from 11 measurements on I were merely fitted by linear least-squares analysis to give the results cited in Tables II and IV. Variable-temperature work was not carried out in water,

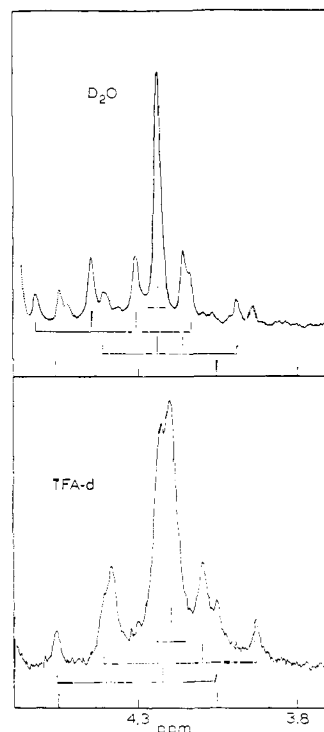


Figure 9. 100-MHz proton resonance spectra of the glycyl α-protons of cycloglycyl-d_2-glycyl-d_2-tyrosyltriglycyl in trifluoroacetic acid-d (30 mg/ml), tetramethylsilane reference, and in heavy water (3 mg/ml) capillary, hexamethyldisiloxane reference. The spectrum in heavy water is the averaged result of 138 scans, and the dashed peaks in it are side bands.

in which the peptide was sparingly soluble (3 mg/ml), or in trifluoroacetic acid.

Discussion

The firmest conclusion from our measurements is that the behavior of amide protons of cyclopentaglycyltyrosyl supports a peptide backbone arrangement of the type proposed by Schwyzer[2] and shown in Figure 1. The four-to-two division of amide protons is consistent with this internally hydrogen-bonded arrangement, provided that the internally bonded protons are taken to be the two at higher field, and the external amide protons, exposed to and associated with solvent, to be those at lower field. There is no reason to insist, *a priori*, that protons transannularly associated with an amide carbonyl be either more or less shielded than those externally associated with water, dimethyl sulfoxide, or trifluoroacetic acid. However, the assignment we suggest is confirmed by the solvent independence of the lower field four. It is also supported by the agreement between the chemical shifts of the lower field set and the values for internal glycine residues of open-chain glycine peptides, which are presumably less structured. Since downfield shifts are associated with hydrogen bonding, it is satisfying also to note that the lower field set of protons is most shielded in the least basic solvent, trifluoroacetic acid. That the internal amide protons are the more highly shielded even in trifluoroacetic acid does seem to suggest that the transannular hydrogen bonds are not strong, and that the backbone conformation is not so much determined by this hydrogen bonding as by the geometry of the covalent bonds of the peptide backbone. However, such a conclusion can

only be very tentative, because contributions of magnetic anisotropies of the surrounding bonds, especially the amide groups, to the chemical shifts of the internal protons are not known, and they may be large.

Stronger confirmation of the internally hydrogen-bonded structure of Figure 1 would have been obtained had it been possible to demonstrate a significant difference between the chemical exchange rates of the two sets of amide protons. However, observed equality of exchange rates is not inconsistent with longevity of a particular backbone structure on the nmr time scale. Interconversions between the preferred conformation and other conformations result in internal (presumably slowly exchanging) amide hydrogens becoming external (presumably more rapidly exchanging) hydrogens. To produce an equality of observed exchange rates it is only necessary that these interconversions occur rapidly relative to the exchange rate of either kind of hydrogen. Since equal exchange rates are observed, it appears that the preferred backbone conformation of the cyclic peptide in dimethyl sulfoxide is not surrounded by prohibitive barriers to conformational change.

Because of the detail visible in the amide proton spectra of dimethyl sulfoxide and dimethyl sulfoxide–water solutions of II (Figures 6 and 7), it is possible to eliminate two of the six possible positions for attachment of the hydrobenzyl side chain, given the rigid backbone of Figure 1. Homonuclear spin decoupling experiments convincingly demonstrate that the α-proton of the tyrosyl residue is vicinal to an amide proton in the lower field group (labeled C in the figures and Table II). Therefore the tyrosyl side chain must be attached at one of the corner positions, numbered 1, 3, 4, or 6, in Figure 1 but not in position 2 or 5. The preference of the tyrosyl side chain for a corner position on the cyclic peptide backbone is noteworthy. Space filling models (CPK) do indicate that the side chain is somewhat less crowded at a corner residue than on one of the extended residues (2 or 5). The preference for a corner might not be exhibited by residues, such as alanyl, with smaller side chains, or by residues, such as seryl, whose homopolymers take the β-polypeptide secondary structure.

Unfortunately a similar narrowing of possibilities is not possible for solutions in water or trifluoroacetic acid, because the N–H lines are sufficiently broadened, probably by exchange with solvent, to prevent their identification in decoupling experiments.

Turning to the question of side chain orientation: Table IV shows that for cyclopentaglycyltyrosyl in dimethyl sulfoxide the tyrosyl α–β proton–proton coupling constants are unequal, about 9.5 and 5 Hz. Values close to these are also observed for dimethyl sulfoxide solutions of open chain peptides with internal aromatic residues, and some cases are included in Table IV. Judged by these coupling constants, one of the side chain conformations with a *trans* relationship of α- and β-protons is favored, *i.e.*, $\chi_1 = 180$ or $300°$ (Figure 2). Which is favored in the cyclic peptide is uncertain, although a possible indication is discussed below. Assuming limiting coupling constants of 13.6 and 2.6 Hz for *trans* and *gauche* vicinal protons,[26] one calculates a fractional population of 0.6+ for the favored rotamer and 0.2+ for the other rotamer with *trans* vicinal protons. The observed coupling constants are in marked contrast to the α–β couplings of aromatic residues in cyclic dipeptides, 4.5–5 Hz, where the $\chi_1 = = 60°$ rotamer is known to be favored.[1b,8]

In water and in trifluoroacetic acid, the tyrosyl α–β couplings of cyclopentaglycyltyrosyl are close to each other, near 7 and 8 Hz, indicating that the $\chi_1 = 180$ and $300°$ states are about evenly populated and account for 0.9 of the total, again in contrast to aromatic cyclic dipeptides. In the latter the $\chi_1 = 60°$ rotamer is the favored one also in these two solvents.[1b,8]

The observed splittings of the glycyl methylene protons are not so informative as had been hoped. To begin with, they cannot be solely the result of the magnetic effects of the aromatic ring. Models indicate that if the α–β rotamer $\chi_1 = 60°$ is not important, as appears from coupling constants to be the case, then only the two residues immediately adjacent to the tyrosyl (along the peptide chain) should be detectably affected by ring current. The results in water and trifluoroacetic acid cannot thus be accounted for. In water three glycyl residues have significant splittings, and in trifluoroacetic acid two, one of which cannot be adjacent to the tyrosyl, do. Rather, it is likely that the observed glycyl splittings result in large part from the magnetic anisotropy of the amide groups that flank each methylene.

In dimethyl sulfoxide solution there is only one glycyl residue with a strongly split (0.4 ppm) methylene, and it is on the carboxyl side of the tyrosyl. One is sorely tempted to say that this indicates that the preferred rotation about the tyrosyl α–β bond is $\chi_1 = 180°$. Since the strongly split residue is, like the tyrosyl, not one of those providing an internal bridging amide proton, *i.e.*, either 2 or 5 of Figure 1, the position of the tyrosyl residue could then be narrowed to locations 3 or 6. However, the mean chemical shift for the split methylene has the value (~3.7 ppm) that seems to be characteristic of internal glycyl residues of all of the peptides we examined; this is difficult to accommodate on the basis of a ring-current effect. Enhanced rigidity of the particular glycyl residue, on the other hand, could enhance splitting resulting from amide anisotropy without changing the mean value of the chemical shift. Although enhanced rigidity would result from the presence of the hydroxybenzyl side chain, it does not necessarily require close proximity of the side chain, and so the more specific conformational assignment, though likely, is not certain. We hope that the data from later peptides will settle this question.

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