the 19-methyl group from the 4β hydrogen in Δ^5 steroid **1** (2.91 Å).

The O(3) carbonyl group in the acetylenic compound (**3**) sticks up (1.146 Å above the planes of the C and D rings) much more than does O(3) in the Δ^5 compound (**1**) (where it lies 1.00 Å below this plane). However, it is possible that a different orientation prevails in solution, or that the enzyme induces a conformational change in compound **3**, or even that the enzyme is not entirely rigorous in its requirements for the relative orientation of the O(3) carbonyl group. Rotation of the ten-membered ring in compound **3** readily produces a conformation (Figure 4) closely similar (as judged by molecular models) to the Δ^5 ketone (**1**), suggesting that conformational change on the enzyme, or in solution, may indeed occur. An estimate of the energy barrier for such a conformational inversion of the ten-membered ring in compound **3** would be helpful, and variable temperature NMR studies are in progress.

In summary, we suggest that the substrates are positioned in the active site of the enzyme mainly by the C and D rings. Apparently substitution in the A ring has little adverse effect on the binding of substrate. An amino acid side chain in the active site, interacting with O(3), would ensure the correct conformation of the A and B rings for 4β hydrogen abstraction. Formation of the $\Delta^{3,5}$-dienol after proton abstraction is accompanied by a conformational change which now may facilitate addition of a proton at C(6) rather than at C(4). The 19-methyl group may also guide this protonation. Because the acetylenic seco steroid is converted, presumably by an analogous mechanism, to an allenic seco steroid, it is likely that it binds to enzyme in a manner similar to that of the Δ^5-keto steroid. Subsequently alkylation of the enzyme by the electrophilic allenic compound can occur.

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Supplementary Material Available: Anisotropic temperature factors, interatomic distances and angles, conformation angles, and a table of structure factors (36 pages). Ordering information is given on any current masthead page.

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Conformation of Cyclic Peptides. 10. Conformational Averaging in Peptides with the Sequence *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$

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Abstract: NMR studies, supported by circular dichroism measurements, indicate that *cyclo*-(Gly-D-Val-L-Leu)$_2$ in methanol, and *cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn-L-Orn) and *cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn) in water, favor a backbone conformation with extended D-series residues connecting L-Xxx-Gly turns. Evidence for a single backbone conformation is not apparent for *cyclo*(Gly-D-Leu-L-Leu)$_2$ in methanol, nor is it for any of the peptides in dimethyl sulfoxide, and rapid exchange among two or more backbone conformation types is suggested. The temperature coefficient of N-H proton chemical shift is an unreliable guide to solvent exposure in these cases. A conformation is proposed for a related peptide, *cyclo*-(Gly-D-Val-L-Leu-L-Ala-D-His-L-His). Synthesis of the peptides is outlined, and key steps including the cyclizations are described.

Peptides containing proline residues exhibit well-studied conformational heterogeneity resulting from rotation about peptide bonds to the proline ring nitrogen.[1-3] Because of the ~20-kcal barrier to this rotation, coexisting backbone conformers give individual NMR spectra at room temperature. Backbone conformers differing by rotations about the C^{α}–C′ and N–C^{α} bonds are generally connected by lower barriers, and are in fast exchange for NMR at room temperature, although Deber has suggested one case in which this may not be so.[4] In the fast exchange cases it is difficult to come by proofs that differing backbone types, e.g., turns at differing positions in a sequence, contribute to the single spectrum observed.

Recent reports are that in crystals the peptides *cyclo*-(Gly-L-Tyr-Gly)$_2$[5] and *cyclo*-(Gly-L-Leu-Gly)$_2$[6] have nonsymmetric conformations in which the backbone is approximately centrosymmetric, so that the two L residues are not identically situated. However, NMR spectra of these two peptides and others like them[8,9] show only one kind of L residue

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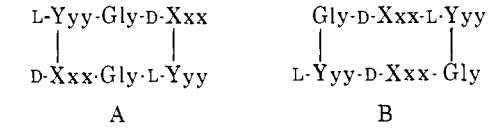
and two kinds of glycine. Therefore, their spectra may be averaged spectra of enantiomeric conformers rather than spectra of single C_2-symmetric structures. This reminder has caused us to reexamine some data on other proline-free cyclic hexapeptides.

Peptides of the types *cyclo*-(Gly-D-Xxx-D-Yyy-Gly-L-Xxx-L-Yyy) and *cyclo*-(Gly-L-Xxx-D-Yyy-Gly-D-Xxx-L-Yyy) can have conformations with exact inversion symmetry. Bláha and Budesinšky prepared examples of these and measured their proton magnetic resonance spectra in dimethyl sulfoxide solution.[10] The spectra are consistent with C_i backbone conformations based on Xxx-Yyy β turns, of type I for the first case and II for the second,[11] linked by extended glycine residues, as they propose. Furthermore, the spectra give evidence that these are stable, dominant conformations. There is a large magnetic nonequivalence of the glycine α protons (0.5–0.6 ppm), and the two glycine α protons also exhibit quite different H–C$^\alpha$–N–H couplings (2–3 Hz and 7–8 Hz). The H–C$^\alpha$–N–H couplings of the L and D residues are also quite different (3–5 Hz and 7–9 Hz).[12]

In contrast, peptides of the type *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$, which they[10] and we[13] both examined, do not exhibit such ranges in coupling constants or such large magnetic nonequivalences. In these cases, the diastereotopic glycine α protons differ by less than 0.15 ppm, and exhibit almost identical H–C$^\alpha$–N–H coupling (4–5 Hz). The H–C$^\alpha$–N–H couplings of the two kinds of substituted residues are also not very different; both are near 7 Hz. Consideration of the literature on β turns suggests that for these peptides two β-turn-containing versions of the backbone are likely: A, with DL turns, and B, with L-Gly turns.



A B

Backbones of type A were proposed in the earlier work,[10,13] largely on the basis of temperature coefficients of N–H chemical shift. We have considered in more detail the peptides I [*cyclo*-(Gly-D-Val-L-Leu)$_2$], II [*cyclo*-(Gly-D-Leu-L-Leu)$_2$], III [*cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn-L-Orn)], and IV [*cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn)], and one related peptide, V [*cyclo*-(Gly-D-Val-L-Leu-L-Ala-D-His-L-His)]. Our data indicate that a backbone of type B, with L-Yyy-Gly turns, predominates in some cases, but that this is in rapid equilibrium with one or more other important conformations. Temperature and solvent dependence of N–H chemical shift proves to be an unreliable guide to solvent exposure in these peptides.

Experimental Section

Peptide Synthesis. The routes and final steps in the synthesis of the cyclic peptides are outlined below. Unless otherwise stated, the intermediates were not purified for elementary analysis, but were homogeneous on thin-layer chromatography (TLC), gave appropriate functional group tests (hypochlorite/iodide for amides, ninhydrin for amino groups, ferric chloride/ferricyanide for hydrazides, *p*-bromobenzenediazonium ion for imidazoles) on the TLC plates, and had proton NMR spectra and spectrum integrals fully consistent with their expected structures. None of the final cyclic products showed any indication of minor components in any of their high-field NMR spectra, which may be taken as an indication that any single diastereomeric impurity is below the 5% level.

Dimethylformamide (DMF) was purified by reaction with ethylene–maleic anhydride copolymer and distillation in vacuo. *N*-Methylmorpholine was purified by distillation from phenyl isocyanate.

Abbreviations used in the peptide notation are: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; -ONSu, hydroxysuccinimide ester.

Elemental analyses were carried out by Micro-Tech Laboratories, Skokie, Ill.

Z-(D-Val-L-Leu-Gly)$_2$-OEt. Z-D-Val-ONSu[14] was coupled in chloroform with the free base of H-L-Leu-Gly-OEt, which had been obtained by hydrogen bromide in acetic acid cleavage of Z-L-Leu-Gly-OEt.[15] The coupling and workup followed standard procedures,[14] and the tripeptide was isolated in 59% yield after recrystallization from ethanol–water, mp 171–173 °C. Part of the Z-D-Val-L-Leu-Gly-OEt was converted to the hydrazide by treatment with 95% hydrazine in absolute ethanol (88% yield after recrystallization from ethanol), and part was converted to the free amino ester by hydrogenolysis in ethanol using 10% palladium on charcoal. The two tripeptide components were coupled via the azide procedure in DMF[16] to give 80% of Z-(D-Val-L-Leu-Gly)$_2$-OEt after recrystallization from ethanol, mp 239–241 °C dec. Anal. ($C_{36}H_{58}N_6O_9$) C, H, N.

***cyclo*-(Gly-D-Val-L-Leu)$_2$.** Treatment of Z-(D-Val-L-Leu-Gly)$_2$-OEt with hydrazine in ethanol gave the carbobenzyloxy hexapeptide hydrazide, recrystallized from ethanol before deblocking with hydrogen bromide in trifluoroacetic acid.[17] The unblocked hexapeptide hydrazide was cyclized by the azide procedure described in detail for *cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn(Z)-L-Orn(Z)).

cyclo-(Gly-D-Val-L-Leu)$_2$, mp >330 °C dec, was obtained in 56% yield after recrystallization from ethanol–water. Its mass spectrum (direct inlet, 155 °C) showed a strong parent ion at *m/e* 538 plus fragments corresponding to the parent minus C_3H_6, C_4H_8, amino acid residues, and the corresponding imines. Anal. ($C_{26}H_{46}N_6O_6$) C, H, N.

Z-D-Leu-L-Leu-Gly-OEt. This peptide was prepared in the same manner as the D-valine analogue above, and was obtained in 91% yield, mp 105–106 °C after recrystallization from ethyl acetate–hexane. Anal. ($C_{26}H_{37}N_3O_6$) C, H, N.

***cyclo*-(D-Leu-L-Leu-Gly)$_2$.** The synthesis exactly paralleled that for *cyclo*-(D-Val-L-Leu-Gly)$_2$, and each step proceeded in comparable yield. The cyclic hexapeptide was crystallized from ethanol–water, mp 338–340 °C dec, and was dried in vacuo at 100 °C overnight. The mass spectrum (direct inlet, 235 °C) exhibited the parent ion at *m/e* 566. This and an ion at M − C_4H_8 gave the most intense peaks. Anal. ($C_{28}H_{50}N_6O_6 \cdot 1.5H_2O$) C, H, N.

Boc-L-Orn(Z)-Gly-OEt. Boc-L-Orn(Z)-ONSu[18] was coupled at 0.3 M in dimethoxyethane with glycine ethyl ester hydrochloride plus 1 equiv of *N*-methylmorpholine. The dimethoxyethane was concentrated to half-volume and diluted with 5 vol of water. The precipitated oil crystallized on trituration. Recrystallization from ethanol–water gave 80% of the dipeptide, mp 94–95 °C. Anal. ($C_{22}H_{33}O_7N_3$) C, H, N.

Boc-D-Orn(Z)-L-Orn(Z)-Gly-OEt. Boc-L-Orn(Z)-Gly-OEt was stored for 30 min in anhydrous trifluoroacetic acid before the solution was evaporated at reduced pressure. The residue was rubbed with several changes of anhydrous ether, stored overnight in vacuo over potassium hydroxide pellets, and used directly for coupling with Boc-D-Orn(Z)-ONSu[18] in dimethoxyethane, at 0.2 M, in the presence of 1 equiv of *N*-methylmorpholine. The reaction mixture was poured into 5 vol of cold water, and the tripeptide was collected by filtration. It was recrystallized from ethanol–water to yield 90%, mp 146–148 °C. Anal. ($C_{35}H_{49}O_{10}N_5$) C, H, N.

***cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn(Z)-L-Orn(Z)).** H-D-Orn(Z)-L-Orn(Z)-Gly-D-Val-L-Leu-Gly-NHNH$_2$·2CF$_3$COOH was prepared by converting Boc-D-Orn(Z)-L-Orn(Z)-Gly-OEt to the corresponding hydrazide by treatment with hydrazine in ethanol, and coupling this by the azide in DMF procedure[16] with D-Val-L-Leu-Gly-OEt obtained from the corresponding carbobenzyloxy derivative. The hexapeptide product was converted to the hydrazide, and finally the butyloxycarbonyl group was removed by treatment with anhydrous trifluoroacetic acid. From the coupling step the overall yield was 57%.

H-D-Orn(Z)-L-Orn(Z)-Gly-D-Val-L-Leu-Gly-NHNH$_2$·2CF$_3$COOH (1.6 g, 1.48 mmol) was dissolved in 5 mL of purified DMF and cooled to −35 °C before addition of 7.4 mmol of hydrogen chloride as a freshly prepared 2.97 N solution in tetrahydrofuran, followed by 0.26 mL (1.92 mmol) of isoamyl nitrite. After 30 min at −35 °C a test for residual hydrazide was negative, and the reaction mixture was cooled to −60 °C and diluted with 850 mL of purified DMF at −60 °C; *N*-methylmorpholine (1.05 g, 10.4 mmol) was added. The solution was stored at −10 °C for 2 days. The solvent was then removed in vacuo and the residue triturated with 250 mL of water to yield about 1 g of crystalline solid. This was dissolved in 95% ethanol, freed of insoluble material, and caused to crystallize by addition of

water. Chromatographically homogeneous, ninhydrin-negative product was obtained (650 mg; 53%). Its proton magnetic resonance spectrum (270 MHz) exhibited all of those resonances, and only those resonances anticipated for the desired product. An analytical sample, mp 271–274 °C dec, was prepared by recrystallization from ethanol–water. Anal. ($C_{41}H_{58}O_{10}N_8$) C, H, N.

***cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn-L-Orn)·2HCl.** Hydrogen was bubbled overnight through 450 mg of the dicarbobenzyloxy cyclic peptide in 150 mL of ethanol containing 4 equiv of hydrochloric acid. The catalyst was 90 mg of 10% palladium on charcoal. An equal amount of fresh catalyst was then added, and hydrogenation continued 24 h longer. After removal of catalyst and evaporation of solvent, the product was crystallized from 95% ethanol to give 274 mg (79%). The high-field proton magnetic resonance spectra of this product agreed with the expected structure and indicated no impurities. An analytical sample from ethanol decomposed without melting above 280 °C. Anal. ($C_{25}H_{46}O_6N_8$·2HCl) H, N, Cl; C: calcd, 47.84; found, 48.41.

Titration of this peptide at 30 °C, 0.0011 M ionic strength, revealed only one apparent ionization, $pK_A = 10.3 \pm 0.1$.

Z-D-Phe-L-Phe-Gly-OEt. Z-D-Phe-ONSu[14] was coupled in dimethylformamide with the hydrobromide of H-L-Phe-Gly-OEt[19] in the presence of 1 equiv of *N*-methylmorpholine. After the usual workup,[14] the product was crystallized from ethyl acetate–hexane and obtained in 54% yield, mp 173–175 °C. Anal. ($C_{30}H_{33}N_3O_6$) C, H, N.

***cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn(Z)-L-Orn(Z)).** H-D-Orn(Z)-L-Orn(Z)-Gly-D-Phe-L-Phe-Gly-$NHNH_2$·$2CF_3COOH$ was prepared from Boc-D-Orn-(Z)-L-Orn(Z)-Gly-OEt and Z-D-Phe-L-Phe-Gly-OEt in a manner analogous to the case described above. The unblocked hexapeptide hydrazide (2.03 g, 1.74 mmol) was cyclized as described above for H-D-Orn(Z)-L-Orn(Z)-Gly-D-Val-L-Leu-Gly-$NHNH_2$. After evaporation of the DMF and trituration with water, the residual solid was crystallized from ethanol–water. Beyond an initial 868 mg of product, another 200 mg was obtained from the mother liquors after treatment with a mixed bed ion exchange resin, total 68%. An analytical sample, mp 264–266 °C, was obtained from ethanol. The proton NMR spectrum of the homogeneous, ninhydrin-negative product was entirely consistent with the expected structure. Anal. ($C_{48}H_{56}O_{10}N_8$) C, H, N.

***cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn)·2HCl.** Hydrogen was bubbled overnight through a solution of 300 mg of the dicarbobenzyloxy derivative in 300 mL of 95% ethanol containing 0.5 mL of 3 N aqueous hydrochloric acid. Thin-layer chromatography showed complete hydrogenolysis. After removal of catalyst, the solvent was evaporated, and the crystalline residue recrystallized from ethanol. An analytical sample decomposed without melting above 280 °C. The NMR spectrum was fully consistent with the expected product. Anal. ($C_{32}H_{44}O_6N_8$) C, H, N, Cl.

Titration of this peptide at 30 °C, 0.001 M ionic strength, revealed only one apparent ionization, $pK_A = 10.0 \pm 0.1$.

Z-D-His-L-His-Gly-NHNH-Boc. An approximately 1:1 mixture of Z-D-His-OH and Z-D-His(Z)-OH (7 g, 19 mmol total), obtained by Schotten–Baumann acylation of D-histidine, and H-L-His-Gly-NHNH-Boc (6.9 g, 21 mmol), obtained by hydrogenation of Z-L-His-Gly-NHNH-Boc, were combined in 35 mL of DMF. At −10 °C 5.35 g (19 mmol) of diphenyl phosphoryl azide (DPPA)[20] in 5 mL of DMF was added, followed by 4.66 g (46 mmol) of *N*-methylmorpholine. The reaction mixture was stirred several hours at −10 °C and allowed to remain overnight at room temperature, before evaporation at reduced pressure. The residue was distributed between ethyl acetate and 5% sodium bicarbonate. On standing the aqueous layer precipitated a crystalline product, shown to be Z-D-His-L-His-Gly-NHNH-Boc. More of the same was obtained by concentration, to give a total of 2.4 g. The ethyl acetate extract also precipitated 2.6 g of the product, after which the organic mother liquor was evaporated to an oil that yielded a further 3.6 g on sonication with bicarbonate. The total yield was 75%.

An analytical sample, mp 210–213 °C dec was prepared by several recrystallizations from ethanol. A difficult to remove impurity was identified as Z-His-Gly-NHNHBoc. Since the dipeptide amino component was free of this substance, it was probably formed by transacylation from Z-His(Z) units. Anal. ($C_{27}H_{35}O_7N_9$) C, H, N.

Z-D-Val-L-Leu-L-Ala-OMe. Z-D-Val-ONSu[14] was coupled in dimethoxyethane with the hydrobromide of H-L-Leu-L-Ala-OMe (from Z-L-Leu-L-Ala-OMe[21]) in the presence of 1 equiv of *N*-methylmorpholine. After 2 days the reaction mixture was mixed with 10 vol of water. The product was collected by filtration and recrystallized from ethanol; an 81% yield was obtained. An analytical sample was obtained from alcohol, mp 183–185 °C. Anal. ($C_{23}H_{35}N_3O_5$) C, H, N.

Z-L-His-Gly-NHNH-Boc. This dipeptide was obtained by reaction of Z-L-His-OH[22] with a 10% excess of H-Gly-NHNH-Boc[23] at 0.6 M in DMF, −10 °C, under the influence of diphenylphosphoryl azide and 2 equiv of *N*-methylmorpholine. After evaporation of solvent at reduced pressure, the residue was taken up in ethyl acetate and washed thoroughly with water and aqueous bicarbonate. The product precipitated from the dried ethyl acetate solution on concentration; yield 84%. An analytical sample was obtained from ethanol, mp 174.5–176 °C dec. Anal. ($C_{21}H_{28}N_6O_6$) C, H, N.

***cyclo*-(Gly-D-Val-L-Leu-L-Ala-D-His-L-His).** H-D-Val-L-Leu-L-Ala-D-His-L-His-Gly-$NHNH_2$·$4CF_3COOH$ was prepared by converting Z-D-Val-L-Leu-L-Ala-OMe to the corresponding hydrazide by treatment with hydrazine in ethanol, and coupling this via the azide in DMF procedure with H-D-His-L-His-Gly-NHNH-Boc, obtained by hydrogenolysis of the corresponding carbobenzyloxy derivative. The hexapeptide was unblocked by treatment with trifluoroacetic acid to remove the butyloxycarbonyl, followed by hydrogenation to remove the benzyloxycarbonyl group. From the coupling step, the overall yield was 50%.

The unblocked hexapeptide hydrazide (1.86 g, 2.88 mmol) was dissolved in 10 mL of purified DMF and cooled to −30 °C. Hydrogen chloride (22 mmol as a 0.65 N solution in THF) and 0.5 mL (3.75 mmol) of isoamyl nitrite were added. After 30 min at −30 °C a test for residual hydrazide was negative; the reaction mixture was cooled to −60 °C, diluted with DMF to 1.6 L, and 2.2 g (22 mmol) of *N*-methylmorpholine was added. The solution was allowed to remain at −10 °C for 2 days.

The solvent was evaporated in vacuo and the residue was taken up in water. The pH was adjusted to 10, and the aqueous solution was evaporated to dryness. The residue was taken up in ethanol and separated from insoluble sodium chloride, and the ethanol was evaporated. The residue was stored at room temperature under a pumped vacuum to remove residual *N*-methylmorpholine.

Purification of the cyclic peptide was achieved by chromatography on Sephadex G-15 in 2% acetic acid. The fractions containing a single component, ninhydrin active and diazo coupling, were combined and evaporated. Crystalline product was obtained by ether precipitation from isopropyl alcohol containing sufficient concentrated hydrochloric acid just to convert the peptide to its dihydrochloride. A total of 600 mg (30%) was obtained, homogeneous in variety of TLC systems. An analytical sample was obtained from isopropyl alcohol–ether. The proton NMR spectra of the product in dimethyl sulfoxide and in water agreed with the expected structure. Anal. ($C_{28}H_{42}O_6N_{10}$·2HCl·$3H_2O$) C, H; N: calcd, 18.89; found, 18.04; Cl: calcd, 9.56; found, 8.85.

Potentiometric titration indicated a single ionization at $pK_a = 5.9$, 25 °C, $\mu = 0.1$. For NMR titration see Figure 6.

Measurements. Proton magnetic resonance spectra of the aqueous and methanolic solutions were obtained using the 250-MHz instrument of the Carnegie-Mellon University NMR Facility for Biomedical Research, operated usually in the correlation spectroscopy mode. Spectra in other solvents were obtained using the CW mode of the Bruker HX-270 spectrometer of the Department of Chemistry, University of Chicago. The spectrometers were operated with resolution and filtering such that the individual coupling constants reported are probably known to ±0.2 Hz. Details of solvent, reference, and temperature are given in the tables and figures as they apply.

Circular dichroism measurements were made with a Cary 60 spectropolarimeter operated with a Model 6002 circular dichroism accessory. Mass spectra were obtained using a Varian MAT CH-7 instrument.

Results and Discussion

We begin with a discussion of the spectroscopic evidence for conformation in *cyclo*-(Gly-D-Val-L-Leu)$_2$. N-H proton resonance data for this peptide in several solvents are given in Table I. Resonances appear for only one kind of leucine, one kind of valine, and one kind of glycine residue, and the assignments are completely unambiguous. The peptide proton spectra of *cyclo*-(Gly-D-Leu-L-Leu)$_2$ so closely parallel those of the D-valine analogue that it is possible with confidence to

Table I. Peptide Proton Resonance Data for *cyclo*-(Gly-D-Val-L-Leu)$_2$ and *cyclo*-(Gly-D-Leu-L-Leu)$_2$[a]

	Gly				D-Val				L-Leu			
	δ	J_{HNCH}	$\Delta\nu/\Delta[R\cdot]$[b]	$\Delta\delta/\Delta T$[c]	δ	J_{HNCH}	$\Delta\nu/\Delta[R\cdot]$[b]	$\Delta\delta/\Delta T$[c]	δ	J_{HNCH}	$\Delta\nu/\Delta[R\cdot]$[b]	$\Delta\delta/\Delta T$[c]
MeOH, 30 °C[d]	8.15	5.9, 4.8	160		7.86	8.2	90		8.43	5.7	220	
MeOH–water, 2:1, 30 °C[d]	7.94	Σ = 11	150		7.54	8.4	60		8.34	5.4	190	
Me_2SO, 20 °C[e,f]	7.72	5.4, 3.8	60	0.0005	8.06	7.3	80	0.0058	8.68	6.6	120	0.0065
TFE, 30 °C[a]	7.72	Σ = 11		0.0051	7.48	8.7		0.0040	7.42	5.8		0.0100
HFP, 20 °C[e]	7.41	4.8, 3.7	80	0.0032	7.10	7.7	150	0.0058	6.80	6.2	150	0.0073
	Gly				D-Leu				L-Leu			
MeOH, 30 °C	8.08	Σ = 10			8.04	7.0			8.54	6.7		
MeOH–water, 4:1, 30 °C	7.90	Σ = 10			7.81	7.3			8.44	6.3		
Me_2SO, 20 °C	7.78	5.4, 4.2	50	0.001	7.95	7.0	70	0.006	8.57	7.0	90	0.008
HFP, 20 °C	7.36	5.0, 4.6	90		7.09	7.3	90		6.79	6.5	90	
1-BuOH, 20 °C	8.22	5.8			8.02	7.8			8.43	6.0		
2-BuOH, 20 °C	8.25	5.7, 4.2			7.99	7.8			8.41	6.0		

[a] Chemical-shift reference is internal tetramethylsilane except for solutions containing water, where it is defined as 0.64 ppm upfield of capillary hexamethyldisiloxane. Abbreviations used are: Me_2SO, dimethyl sulfoxide; MeOH, methanol; TFE, trifluoroethanol; HFP, hexafluoro-2-propanol; BuOH, butanol. [b] Line broadening by nitroxyl: R· is 2,2,6,6-tetramethylpiperidin-1-oxyl in methanol and methanol–water studies, and 2,2,5,5-tetramethyloxazolidin-3-oxyl in others; radical concentration range is 0–0.2 M. Slopes given are change in width at half-height with nitroxyl concentration, ±20%. [c] Temperature coefficient, parts per million upfield per degree. [d] Concentrations about 10 mg/mL (0.02 M). [e] Concentrations about 40 mg/mL (0.07 M). [f] Chemical shifts in Me_2SO essentially unchanged at 0.16 mg/mL (0.0003 M).

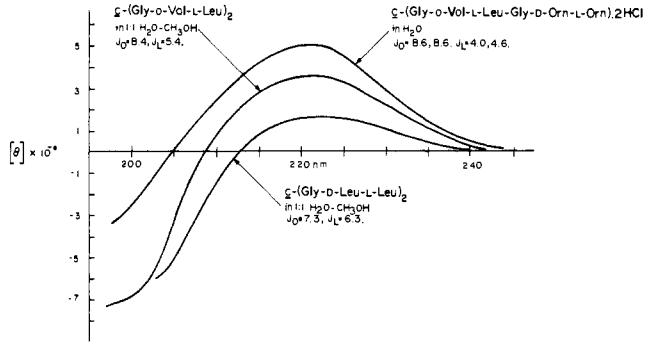


Figure 1. Circular dishroism of *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$ peptides in aqueous solvents. Note the parallelism between ellipticity at the 222-nm maximum and the range of H–N–C$^\alpha$–H coupling constants.

assign by analogy the lower field doublet to the L-leucine residues and the doublet at higher field to the D-leucine residues. Data for this peptide are also given in Table I. Both of these peptides are almost completely insoluble in water, chloroform, or acetonitrile.

For *cyclo*-(Gly-D-Val-L-Leu)$_2$, the various measures of solvent exposure, nitroxyl-induced line broadening,[24] temperature coefficient of chemical shift,[7] and shift variation between basic solvents (dimethyl sulfoxide, methanol, and water) and nonbasic solvents (fluorinated alcohols)[24b,25] all agree in identifying the N–H of L-leucine as solvent exposed. This resonance is most affected by the nitroxyl paramagnetism, has a high temperature coefficient (0.006–0.01 ppm upfield/deg), and moves upfield 1–2 ppm on changing from basic to nonbasic solvents.

On the other hand, these measures are solvent dependent for the glycine and D-valine N–H units, and where they are not equivocal they tend to disagree in their indications. In dimethyl sulfoxide (Me_2SO) the temperature coefficients suggest that the D-valine N–H is exposed to solvent, since its resonance has almost the same temperature sensitivity as the N–H resonance of *N*-methylacetamide in Me_2SO. The glycine N–H resonance shows almost no temperature dependence, which would argue that the glycine N–H is shielded from solvent. However, the valine and glycine N–H lines are broadened comparably by nitroxyl, which is not consistent with the clear-cut distinction in temperature dependence. In contrast with the Me_2SO case, in methanol or methanol–water the D-valine N–H is distinctly more buried than the glycine N–H by the nitroxyl line-broadening criterion. (Temperature studies were not carried out in methanol.) In trifluoroethanol (TFE) the glycine N–H temperature coefficient is greater than that of the valine N–H; in hexafluoro-2-propanol (HFP) the situation is reversed; in neither solvent can either the glycine or the valine N–H units be called buried by the temperature-dependence criterion.

This kind of ambiguous behavior is not shown by the N–H resonances of cyclic peptides that give other indications of a stable backbone arrangement.[12] It suggests that in the present case there is a solvent-dependent distribution among conformations. The H–N–C$^\alpha$–H coupling constants listed in Table I provide support for this view. In methanol, methanol–water, or TFE the coupling for the D-valine residue is near 8.5 Hz, and for the L-leucine residue it is near 5.5 Hz. In Me_2SO both couplings approach more average values, near 7 Hz. Arguing that wide ranges of coupling constants are more likely to indicate a single conformation, we suggest that *cyclo*-(Gly-D-Val-L-Leu)$_2$ may have a dominant conformation in methanol and methanol–water, and a more even distribution of conformations in Me_2SO. For *cyclo*-(Gly-D-Leu-L-Leu)$_2$ the fact that the couplings in methanol, Me_2SO, or HFP are near 7 Hz suggests that there is no single dominant conformation in these values. However, in the more hydrocarbon-like environment of 1-butanol or 2-butanol the coupling constants of the two kinds of leucine residue in *cyclo*-(Gly-D-Leu-L-Leu)$_2$ differ by almost 2 Hz.[13] (Data from reference 13 are included in Table I.)

In circular dichroism, as in NMR, greater extremes are more likely to be associated with single conformations. In accord with this generalization, the CD spectra of the peptides under study do show effects that parallel the variation in coupling constant range. For *cyclo*-(Gly-D-Val-L-Leu)$_2$ in TFE, $J_{D\text{-Val}} - J_{L\text{-Leu}}$ is about 3 Hz, and there is a molar ellipticity maximum at 220 nm of $5.1 \pm 0.3 \times 10^4$ deg cm^2/dmol; in HFP the coupling constant range is smaller, about 1.5 Hz, and the maximum at 222 is also smaller, $3.2 \pm 0.2 \times 10^4$. (The average deviation of three separate samples is given.) For *cyclo*-(Gly-D-Leu-L-Leu)$_2$ in methanol–water, $J_{D\text{-Leu}} - J_{L\text{-Leu}}$ is about 1 Hz, and its ellipticity at 222 nm is about one-half that for *cyclo*-(Gly-D-Val-L-Leu)$_2$, which has $J_{D\text{-Val}} - J_{L\text{-Leu}} =$ 3 Hz in the same solvent. This is illustrated in Figure 1. Figure 1 also shows that the water-soluble unsymmetrical analogue, *cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn-L-Orn) (III), which has still larger values of $J_D - J_L$, 4–4.5 Hz, has an even larger

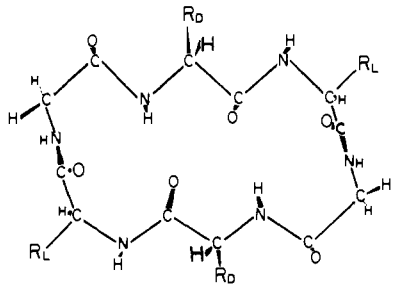


Figure 2. Proposed dominant conformation of the *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$ peptides in methanol and water. Backbone parameters are given in Table II.

Table II. Backbone Dihedral Angles for Proposed Dominant Conformation of *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$

Residue	Possible H-N-C$^\alpha$-H angles,[a] deg	Proposed conformation[b] ϕ, deg	ψ, deg	*cyclo*-(L-Ala-L-Pro-D-Phe)$_2$[d] ϕ, deg	ψ, deg
Gly	10 and 130 ($\phi = \pm 70$)	70[c]	30 °[c] (or 60)	78	9 (D-Phe)
D-Xxx	160 (ϕ = 100 or 140)	140	180	−157	172 (L-Ala)
L-Yyy	30 or 130 (ϕ = 30, 90, −70, or −170)	−70	150 (or 120	−60	122 (L-Pro)

[a] See ref 26 for correlation used; ±10° is an appropriate error estimate. [b] Estimated values of ψ are rounded to the nearest multiple of 30°. [c] A choice of $\phi = 70°$ and a small value for ψ is based on the large values (about 17.5 Hz) observed for J_{H-C-H} in the glycine residues in the light of the correlation proposed by M. Barfield, V. J. Hruby, and J. P. Meraldi [*J. Am. Chem. Soc.*, **98**, 1308-1314 (1976)] between this geminal coupling and ϕ and ψ. [d] Reference 27.

value of molar ellipticity at the maximum near 220 nm.

If it is correct that several conformations are in equilibrium in these peptides, then solvent exposure of peptide protons is probably best estimated by methods that minimize the environmental perturbation. We rely, then, on the nitroxyl-induced line broadening, because it seems likely that less than 3% of, say, 2,2,6,6-tetramethylpiperidinoxyl in a polar solvent like Me_2SO or methanol will affect the population distribution less than a temperature variation of 40–60 °C or a complete change in solvent. Thus, if a single conformation of *cyclo*-(Gly-D-Val-L-Leu)$_2$ dominates in methanol, methanol-water, or TFE, we consider that it is one in which the D-valine N-H is more shielded from the solvent than is the glycine or L-leucine N-H. As it happens, satisfactory models subject to this constraint and matching the four observed backbone coupling constants[26] can be constructed. Table II gives the H-N-C$^\alpha$-H dihedral angles matching the coupling constants, and the backbone dihedral angles for one possible range of conformations.

The proposed backbone, which is illustrated in Figure 2, contains two L-Leu-Gly turns of type II (L–D) connected by extended D-valine residues. Although other sets of ψ values can be chosen while retaining the two turns, the values given in Table II give a ring very much analogous to the backbone found in crystalline *cyclo*-(L-Ala-L-Pro-D-Phe)$_2$.[27] The proposal and the crystal differ in that the side chain of the residue with the internally directed N-H is equatorial in the present case rather than axial. The dihedral angles of the two conformations are compared in Table II.

We turn to the two water-soluble analogues in this series, the hydrochlorides of *cyclo*-(Gly-D-Val-L-Leu-Gly-D-Orn-L-Orn) (III) and *cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn) (IV). To emphasize analogies, the N-H proton resonance data are shown schematically in Figure 3, rather than in a table. In water these peptides each show two upfield N-H doublets with larger coupling constants; in peptide III one of these is the D-valine N-H. They also show two downfield doublets with smaller H-N-C$^\alpha$-H couplings; in III one of these is the L-leucine N-H. This pairing is the basis for assignment of resonances to L- or D-ornithine and L- or D-phenylaline by analogy. Other necessary distinctions were made by homonuclear decoupling experiments. (Although the chemical-shift spread is less than in the water-soluble peptides, the L-residues downfield, D-residues upfield pattern is also observed for *cyclo*-(Gly-D-Val-L-Leu)$_2$ in methanol or methanol-water.)

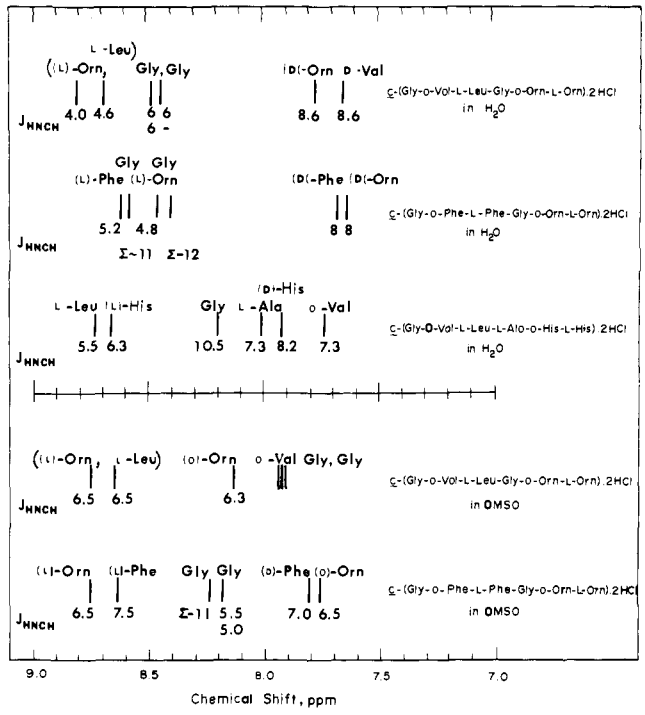


Figure 3. Peptide (N-H) proton resonance chemical shifts and H-N-C$^\alpha$-H coupling constants for water-soluble peptides III–V. Spectra are measured at about 20 mg/mL (0.03 M). Reference is internal Me_4Si for Me_2SO and 0.64 ppm upfield of capillary hexamethyldisiloxane for water. Assignments of configuration by analogy are indicated by parentheses about the configuration symbol. For III, L-Orn and L-Leu cannot be distinguished because the H$^\alpha$ resonances overlap exactly.

The CD spectrum of III in water, already referred to, is shown in Figure 1; that of IV is affected by the aromatic chromophores and cannot be directly compared.[28] The spectrum of III is closely similar to that of the more rigid peptide, *cyclo*-(D-Ala-L-Pro-Gly)$_2$ (in water $[\theta]_{215} = 6 \times 10^4$, $[\theta]_{187} = 8.4 \times 10^4$). For *cyclo*-(D-Ala-L-Pro-Gly)$_2$ conformation like that proposed in Table II and Figure 2 (substituting L-Pro for L-Xxx) fits the NMR data well.[29]

For solutions of III and IV in Me_2SO the assignments are made as they are for the aqueous solutions. The L-residues downfield, D-residues upfield pattern persists, but the coupling constants of the doublets tend to more toward 6–7 Hz, suggesting that a single dominant conformation in water may be replaced by a mixture in Me_2SO, just as in the nonpolar cases. Because the D-residue resonances are superimposed on the $-NH_3^+$ band, precise comparison of the line broadening they and the other N-H lines experience in the presence of nitroxyl is not possible. Figure 4 shows the nitroxyl experiment for peptide IV in water.

In the peptides I–IV one other correlation is apparent. When the coupling constant range is small, the glycine N-H reso-

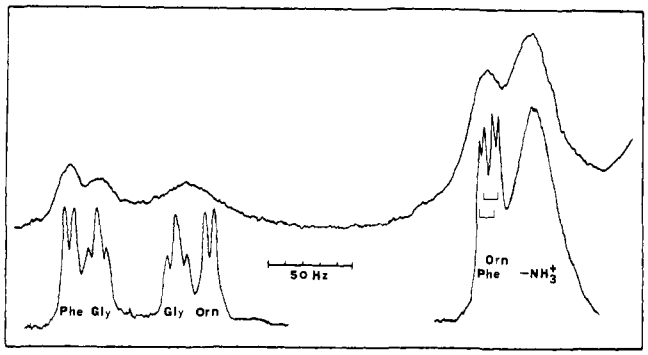


Figure 4. Low-field 250-MHz proton resonances of *cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn)·2HCl, 0.1 M in water acidified with trifluoroacetic acid, alone (below) and in the presence of 0.14 M 2,2,5,5-tetramethyloxazolidin-3-oxyl (above). See Figure 3 for chemical-shift data.

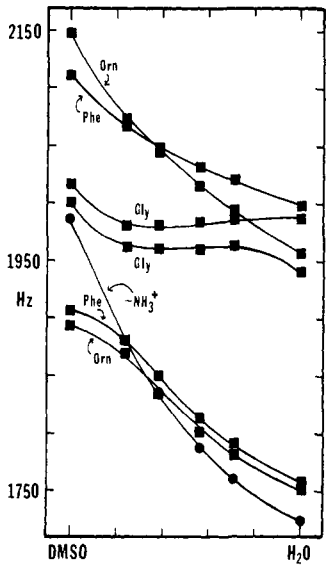


Figure 5. Solvent dependence of the chemical shifts of the peptide protons of *cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn)·2HCl in Me_2SO-water mixtures (250 MHz). The reference is capillary hexamethyldisiloxane, and there is a continually varying bulk susceptibility correction which has not been applied. With reference to an internal standard, the resonances of the nonglycine residues move only slightly (see Figure 3).

nances are at the high-field end of the N-H pattern in the basic solvents, and when the range is large, these resonances move downfield. In the extreme cases, III and IV in water, the pattern of N-H resonances is reminiscent of that for peptides of the *cyclo*-(Gly-Xxx-Gly)$_2$ type in water, methanol, or Me_2SO, where the internally directed N-H of the extended glycine residue provides the highest field peptide proton resonance.[7-9] The solvent dependence of the change in N-H pattern in *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$ is illustrated in Figure 5 for *cyclo*-(Gly-D-Phe-L-Phe-Gly-D-Orn-L-Orn) in Me_2SO-water mixtures. If this dependence indicates that the position of the glycine resonance is a function of the conformational distribution, then the temperature coefficient does not have a clear meaning. The zero temperature dependence observed for Me_2SO solutions of I and II may be the sum of the upfield shift with increasing temperature, usually exhibited by a solvated peptide proton, and a downfield contribution resulting from an increasing fraction of the conformation proposed in Table II. This is a potential pitfall in applying the temperature-dependence criterion when there is no additional information to indicate the stability of a single conformation. It has been recognized in principle by Urry[30] and also by Glickson.[31]

From the indications presented, we conclude that one

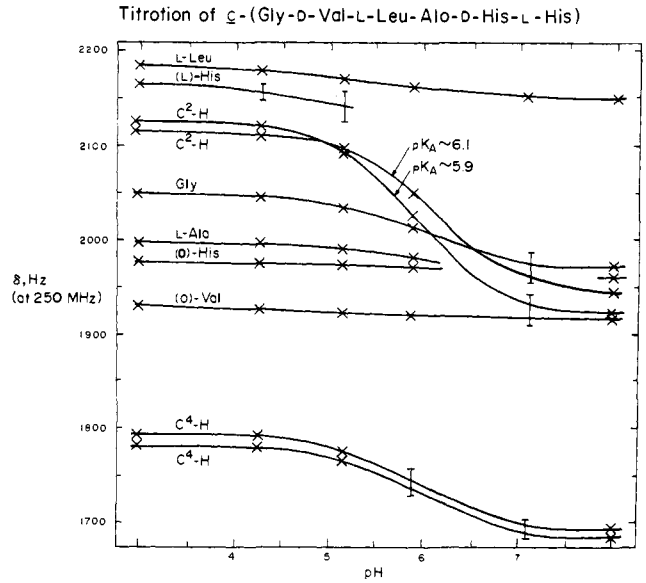


Figure 6. Effect of titration of *cyclo*-(Gly-D-Val-L-Leu-L-Ala-D-His-L-His)·2HCl, ~0.05 M in water, on the peptide and imidazole ring proton resonances. The curves drawn through the experimental points for the C^2-H resonances are calculated for the indicated pK_as. The error bars for the peptide proton of the downfield (presumably L) histidine indicate the observed width of this resonance at half-height. By pH 6 this line is broadened to invisibility.

identifiable minimum energy conformation of the *cyclo*-(Gly-D-Xxx-L-Yyy)$_2$ peptides contains L-Yyy-Gly turns and extended D-Xxx units and that this conformation is more stable in methanol and in water than it is in dimethyl sulfoxide. From the comparison of I and II it appears that this conformation is more stable when D-Xxx is valine than when D-Xxx is leucine. Without knowledge of the competing conformation or conformations it is not possible to be definitive about the factors involved. As stated earlier, it may be that a major competing form is the frame-shifted version of the backbone with turns at D-Xxx-L-Yyy, type A of the introductory section. There is an empirical reason for this conformation to be less stable than the one we identify. In cyclic hexapeptides that exhibit the β turn based backbone in the crystal (*cyclo*-(Gly)$_6$,[32] *cyclo*-(Gly$_4$-D-Ala$_2$),[33] *cyclo*-(L-Ala-L-Pro-D-Phe)$_2$,[27] and *cyclo*-(Gly$_2$-L-Leu)[6]) the N-C$^\alpha$-C'-N angle (ψ) takes a value near zero in the residue preceding the extended residue. Whatever the forces causing this, $\psi = 0°$ is less favorable for a substituted residue, because of C=O···C$^\beta$ crowding, than it is for glycine. A preference for L-Xxx-Gly turns over L-Xxx-D-Yyy turns on this basis need not extend to less constrained peptides.

Beyond this, the observations may be variously rationalized. The enhanced stability in methanol and water may be related to solvation of the carbonyl oxygens. In the porposed conformation they all can be hydrogen bonded to protic solvents. Looked at the other way, the decreased stability in Me_2SO may have to do with stronger solvation of peptide protons by a better hydrogen bond acceptor.[34] The enhanced stability when the D residue is valine is likely to be related to those factors (in turn related to branching at the β carbon) that cause valine residues to form or to stabilize extended β secondary structures in polymers[35] and proteins.[36,37] The indication that the conformation may become important for *cyclo*-(Gly-D-Leu-L-Leu)$_2$ in higher alcohols may point to solvophobic effects as well.

In the possibly competing type A conformation, the H-N-C$^\alpha$-H coupling of the D residues would be small (ϕ near 60°, H-N-C$^\alpha$-H angle (θ) near 120°); the coupling in the L-residue could be large or small (ϕ between −60 and −150°, θ between 120 and 180°).[38] The glycine N-H resonance would be at the upfield end of the N-H pattern and least affected by

nitroxyl, and the valine resonance would tend to be downfield. The observed spectra of Me_2SO solutions may be considered an average of such a spectrum with that of the conformation in Table II, but we did not find conditions in which the type A backbone was clearly expressed.

When this work was underway, we were anticipating that the β turns would occur preferentially at the D-L sequences. Therefore we prepared *cyclo*-(Gly-D-Val-L-Leu-L-Ala-D-His-L-His) (V) to investigate the interaction of two adjacent imidazoles in a β turn. The N-H spectrum of V in water at low pH is included schematically in Figure 3. Titration curves of the low-field resonances are given in Figure 6.

The N-H resonance pattern is much like that of the other peptides, except that there is less evidence for the dominant single backbone conformation. The L-leucine peptide N-H resonance appears at low field with a small coupling to the proton, and the D-valine N-H is at high field with a larger coupling; one histidine N-H line, by analogy the L, is at low field with a smaller coupling, and the other, by analogy the D, is at high field with a larger coupling. However, the range of coupling constants is not so great as for aqueous solutions of III and IV, and the glycine and alanine N-H resonances are at higher field than the glycine resonances of III and IV in water. Also, there is no distinguishable difference in sensitivity to nitroxyl between the N-H resonances of the glycine and alanine on the one hand and the resonances of the D residues on the other. Only the leucine N-H is demonstrably more exposed to nitroxyl than the other N-H resonances, although the L-histidine N-H should be similarly affected if the peptide backbone is analogous to that of the other four peptides. The resonance of the L-histidine N-H does show imidazole-catalyzed exchange broadening (see Figure 6), so that it may be close enough to an imidazole side chain to be somewhat shielded from nitroxyl. (Note that a similar effect is apparent in Figure 4 for peptide IV; the L-Phe N-H, between two aromatic side chains, is less sensitive to nitroxyl than the L-ornithine and glycine N-H lines.)

The titration data indicate that there is no strong acid-base interaction between the two imidazole side chains; from the chemical shifts of the C^2 protons the estimated pK_as are 5.9 and 6.1, close and not unusual. The N-H resonance most affected by titration (other than the exchange-broadened one) is that of the glycine residue, which moves about 0.25 ppm upfield in the free base form; this may suggest hydrogen bonding between protonated imidazole and carbonyl of the L-histidine residue.

A conformation like the one proposed for I-IV, except that the corresponding turn at L-His-L-Ala is of type I, can be accommodated by the observations. That it is less clearly expressed in the aqueous solution of V than in the solutions of III and IV is probably more due to a difference in stability of type I (L-L) and type II (D-L) turns than to differences in side chains.

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