Conformational Analysis of Cyclic Partially Modified Retro-Inverso Enkephalin Analogues by Proton NMR†

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ABSTRACT: The conformations of five cyclic retro-inverso enkephalin analogues have been probed by proton NMR. After assignment of peaks, intramolecularly hydrogen-bonded amide protons were detected by temperature perturbation. Carbonyl hydrogen-bond acceptors were surmised from the computer simulations of minimum energy conformations of Hassan and Goodman [Hassan, M., & Goodman, M. (1986) *Biochemistry* (preceding paper in this issue)]. Hydrogen bonds were identified in dimethyl-d_6 sulfoxide solutions and monitored as H_2O was added. One hydrogen bond was observed in each of the retro-inverso-modified enkephalin analogues although in the parent analogue H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu) two were detected. The change in solvent altered the conformations of two of the analogues.

Enkephalins are endogenous opiate peptides that function as neurotransmitters and neuromodulators. Modifications of the native pentapeptide enkephalin structure have led to more potent and long-lived analogues. Comparisons of structure and activity have aided the identification of the functional groups necessary for biological activity (Morley, 1980; Beddell et al., 1977).

To probe the shape of the opiate receptors, the spatial array of the important functional groups must be determined. The flexibility of linear enkephalins makes the interpretation of conformational data difficult because these peptides may assume a broad range of secondary structures. To incorporate constraint, several cyclic enkephalin analogues have been synthesized that show high opiate potency (DiMaio & Schiller, 1980; DiMaio et al., 1982; Schiller et al., 1981, 1985a,b; Mosberg et al., 1982). Among these is a 14-membered ring analogue with the structure H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu), where A_2bu refers to α,γ-diaminobutyric acid (DiMaio & Schiller, 1980). This analogue shows 17 times the potency of native Leu-enkephalin in the guinea pig ileum (GPI) assay and selectivity for the μ-receptor. We recently reported the conformational analysis of this cyclic enkephalin by proton NMR and computer simulations (Mammi et al., 1985). Our results showed a doubly hydrogen-bonded structure with two C_7 rings within the cycle for the enkephalin analogue in dimethyl-d_6 sulfoxide (Me_2SO-d_6) solution. In solutions of increasing H_2O concentration, one hydrogen bond is disrupted, but a large charge in the backbone conformation does not occur as evidenced by very small changes in $^3J_{NH-C^{\alpha}H}$ coupling constants. We now report the proton NMR study of five partially modified retro-inverso analogues of this 14-membered ring compound. The computer simulations of molecular dynamics and energy minimization are presented separately (Hassan & Goodman).

The partial retro-inverso modification involves the reversal of the sense of a portion of a peptide backbone without changing the orientations of the side chains (Shemyakin et al., 1969; Chorev et al., 1979; Goodman & Chorev, 1979, 1980). Reversal of a single amide bond yields a *gem*-diaminoalkane derivative (g) followed by a 2-alkyl malonic acid (m) residue in place of two sequential amino acids. The purpose of retro-inverso modification is 2-fold: (1) to determine the relative importance of the backbone vs. the side chains for a particular segment of the peptide and (2) to incorporate resistance to enzymatic degradation.

Materials and Methods

The partially modified retro-inverso enkephalin analogues were prepared in our laboratories (Berman & Goodman, 1984; Richman et al., 1985) and tested for biological potency at a Clinical Research Institute of Montreal (Berman et al., 1983; Richman et al., 1985). These compounds are listed below;

I TFA·H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu)

II TFA·H-Tyr-*c*-(D-A_2bu-Gly-gPhe-LD-mLeu)

III TFA·H-Tyr-*c*-(D-A_2bu-gGly-LD-mPhe-Leu)

their structures are shown in Figure 1. During the synthesis, two diastereomers result from the racemic structure of the 2-alkylmalonic acids. For this reason there are two diastereomers of compounds II and III. The biological activities of the analogues are tabulated in the paper of Hassan and Goodman (1986).

Proton NMR spectra were obtained on a 360-MHz NMR spectrometer built in-house from a Varian instrument equipped with an Oxford magnet and a Nicolet 1280 computer. Assignments were made on the basis of two-dimensional shift correlation spectra (Bax, 1981), two-dimensional relayed coherence transfer spectra (Eich et al., 1982), and one-dimensional difference NOE spectra. Samples for NOE spectra were prepared in mixed (1:1 by volume) Me_2SO-d_6 and tetramethylene-d_8 sulfone (sulfolane) and degassed by repeated freeze–thaw cycles (Kartha et al., 1984).

Solutions of 5–15 mM were prepared in Me_2SO-d_6. The temperature/titration studies were carried out by adding H_2O to the Me_2SO-d_6 solutions and obtaining spectra at five to six temperatures over a range of 20–65 °C for several solvent compositions. A symmetric 1331 pulse sequence was used to suppress the H_2O signal (Hore, 1983).

Results

The conformational preferences of the cyclic enkephalin analogues were determined in the following manner: (1) the resonance peaks were assigned, (2) intramolecularly hydrogen-bonded amide protons were identified by temperature studies of the analogues in mixed Me_2SO-d_6/H_2O solutions, and (3) the NMR results were compared to computer-simu-

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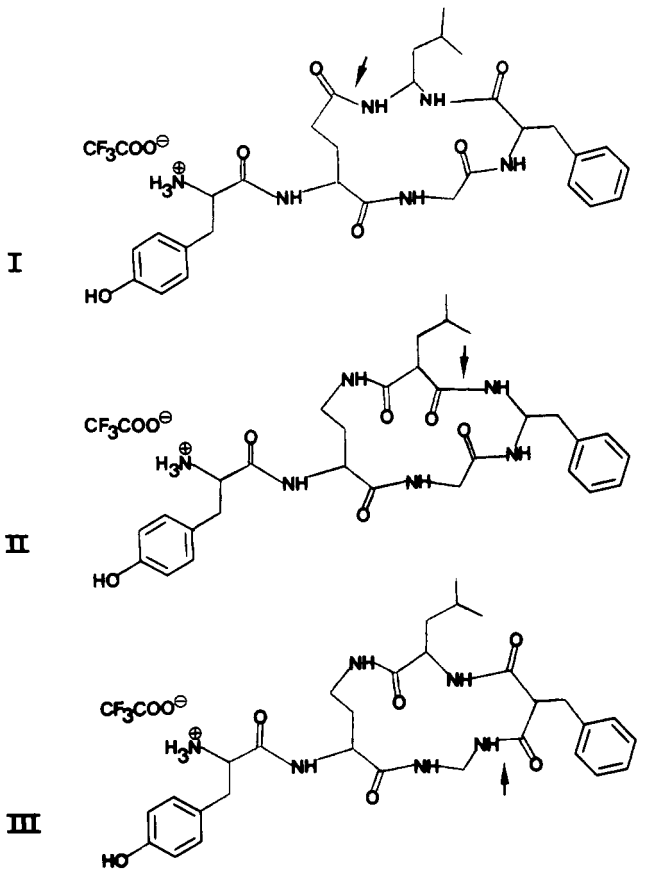

FIGURE 1: Structures of cyclic retro-inverso enkephalin analogues. Arrows indicate reversed bonds.

lated minimum energy conformations to determine hydrogen-bond acceptors.

Peptide aggregation can lead to erroneous conclusions about conformations. Therefore, the presence or absence of aggregates under our experimental NMR conditions was established prior to conformational interpretation of the data. There was no evidence for intermolecular association in Me_2SO-d_6 solutions. The enkephalin analogues dissolved easily, and on several occasions, samples of varying concentration (3–30 mM) were prepared. These samples showed no detectable changes in the NMR spectra obtained.

Since enkephalin analogues are not soluble in H_2O except at low pH, the problem was more critical in the mixed H_2O and Me_2SO-d_6 solutions. To be certain that aggregation did not occur, a concentration study was carried out. No change was observed in the spectrum of compound I, the analogue containing D-Glu[2] and gLeu[5], for solutions of 5–15 mM in 50% (v/v) H_2O in Me_2SO-d_6.

(*I*) *H-Tyr-c-(*D*-Glu-Gly-Phe-gLeu)*. The assigned one-dimensional spectrum of compound I is shown in Figure 2. To distinguish gLeu NH resonance peaks from one another, we sought nuclear Overhauser effects (NOE). An NOE was detected (not shown) between the Phe NH and the most upfield-shifted gLeu NH, which completed the assignment of the resonances.

The temperature dependence of the amide chemical shifts at various concentrations of H_2O in Me_2SO-d_6 is shown in Figure 3. Chemical shifts are plotted vs. solvent composition in Figure 3a for three temperatures, as calculated from the least-squares fit analysis. Error bars indicate 95% confidence intervals. The spread between the curves for each resonance reflects temperature coefficients. Temperature coefficients vs. solvent composition are shown in Figure 3b. Two types

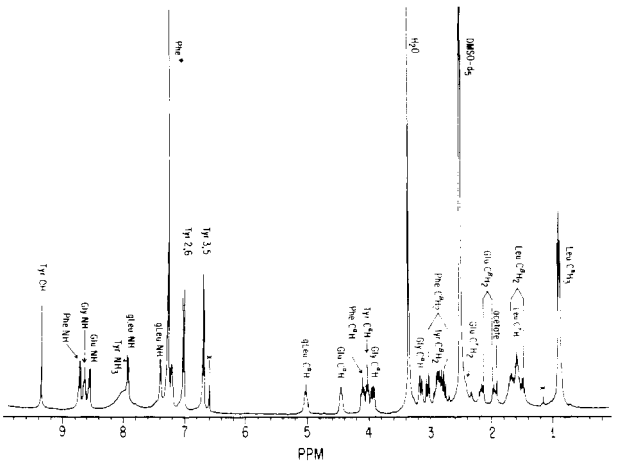

FIGURE 2: ^1H NMR spectrum of compound I [H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu)] in Me_2SO-d_6. The upfield-shifted gLeu NH is adjacent to Phe, and the downfield-shifted gLeu is adjacent to the side chain of D-Glu.

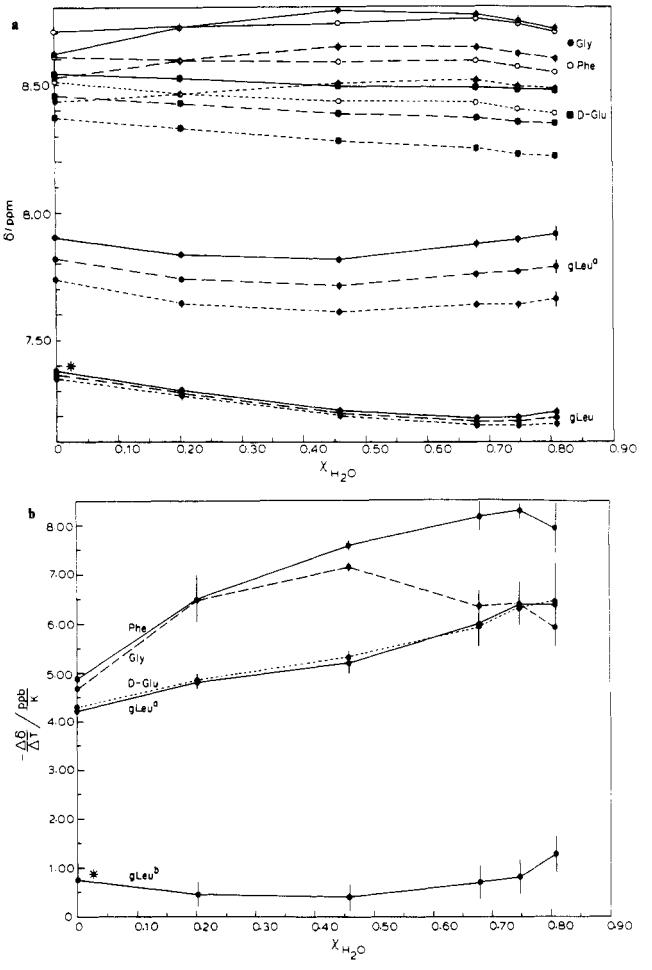

FIGURE 3: (a) Chemical shifts of amide resonances of compound I [H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu)] vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies. The results shown are at three interpolated temperatures: 20 (solid lines), 40 (dashed lines), and 60 °C (dotted lines). (b) Temperature coefficients of amide resonances of compound I vs. mole fraction of H_2O in Me_2SO-d_6. (Footnote a) The gLeu NH adjacent to the side chain of D-Glu. (Footnote b) The gLeu NH adjacent to Phe. (*) Two points were used to calculate this point because of overlapping peaks.

of behavior are apparent: four amide protons maintain high temperature coefficients, indicating solvent exposure; one proton maintains a low temperature coefficient, indicating involvement in intramolecular hydrogen bonding. The gLeu

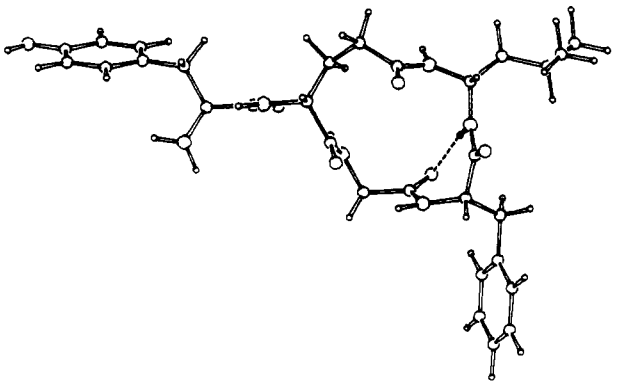

FIGURE 4: Preferred conformation of H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu).

Table I: $^3J_{NH-C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions for H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu) in Me_2SO-d_6 Solution and 0.81 Mole Fraction H_2O in Me_2SO-d_6 Solution

	X_{H_2O} = 0.00		X_{H_2O} = 0.81	
residue[c]	J	ϕ	J	ϕ
D-Glu	7.9	−77	7.9	−77
		−43		−43
		87		87
		153		153
Gly	10.6[b]	−138	10.8[b]	−136
		−52		−53
		52		53
		138		136
Phe	7.6	−155	7.2	−157
		−85		−83
		39		36
		81		84
gLeu[1]	6.5	−162	6.8	−160
		−78		−80
		30		33
		90		87
gLeu[2]	7.2	−81	7.9	−77
		−36		−43
		83		87
		157		153

[a] Torsions were calculated after Bystrov (1976). [b] The Gly coupling constants are $\sum(^3J_{NH-C^\alpha H})$. [c] gLeu[1] is adjacent to Phe, and gLeu[2] is adjacent to Glu side chain.

NH adjacent to Phe is the amide proton that exhibits a low temperature coefficient throughout the H_2O titration. The intramolecular hydrogen bond appears to be very stable up to very high concentrations of H_2O. One hydrogen bond acceptor for this amide proton was seen in computer-simulated conformations (Hassan & Goodman), the carboxyl oxygen of glycine. Figure 4 shows the C_7 structure formed by this hydrogen bond.

The ϕ torsions calculated from coupling constants are shown in Table I. There is negligible change in torsion angles with increasing H_2O concentration, which suggests no change in backbone conformation.

(*II*) *H-Tyr-c-*(D-*A_2bu-Gly-gPhe-*DL-*mLeu*). Two diastereomers were obtained in the synthesis of this retro-inverso compound and were separated by reversed-phase HPLC. Gradient elution from a preparative C-18 column using 20–40% CH_3CN in H_2O with 0.1% TFA gave 4-min separation of the isomers. The configuration of the mLeu is defined here as D and L with respect to the parent analogue, instead of *R* and *S*, because it is simplest to think of a modified peptide in terms of its parent peptide. The direction of the backbone is assumed to be from Tyr to Leu in spite of the reversal of an amide bond. Thus, the L-mLeu diastereomer has the same side-chain array as the parent peptide while the D-mLeu diastereomer has a different side-chain array.

Table II: Temperature Coefficients (ppb/K) of D-A_2bu NH of the Diastereomers of Compound II at Different Mole Fractions of H_2O in Me_2SO-d_6

L-mLeu		D-mLeu	
X_{H_2O}	$\Delta\delta/\Delta T$	X_{H_2O}	$\Delta\delta/\Delta T$
0.00	0.3[a]	0.00	2.1 ± 0.3
0.22	1.4 ± 1.4	0.18	2.1 ± 0.1
0.42	1.4 ± 0.2	0.36	2.2 ± 0.2
0.59	1.1 ± 0.2	0.52	2.0 ± 0.3
0.74	1.4 ± 0.2	0.73	1.7 ± 0.4
0.83	1.8 ± 0.2	0.83	2.0 ± 0.5

[a] Only two points were used to calculate this point because of overlapping peaks.

The absolute configuration of mLeu in each diastereomer was determined by two different methods: analysis of enzymatic degradation data and spectroscopic data. Proteolytic enzymes are very specific for normal peptides or proteins. Resistance to enzymatic degradation implies that the analogue does not bind to the enzyme or that it cannot be cleaved once it is bound. It was found that one diastereomer was completely resistant to degradation in rat brain homogenate over 3 days while the other diastereomer degraded to 48% of its initial concentration in the same time (Berman & Goodman, 1984). The most resistant diastereomer was presumed to be the D-mLeu isomer.

The NMR spectra of the diastereomers support the assignment of the resistant compound as the D-mLeu diastereomer. There is a ring current shift experienced by the alkyl side chains (especially at the γ-position) of amino acids adjacent to aromatic side chains that is very pronounced when the two residues are of opposite configuration (Deber & Joshua, 1972). The spectrum of the enzymatically resistant compound shows a 0.33 ppm upfield shift of the mLeu γ-proton resonance (1.04 ppm) relative to the chemical shift of the same resonance in the other diastereomer (1.37 ppm).

A complete assignment of the one-dimensional spectra was accomplished (not shown). NOE spectra were obtained to distinguish gPhe resonance peaks from one another, which allowed complete spectral assignment. For both diastereomers, the most upfield-shifted gPhe NH peak decreased in intensity when the Gly $C^\alpha H$ was irradiated, and the downfield-shifted gPhe NH peak decreased when Leu $C^\delta H_3$ was irradiated.

The results of H_2O titration/temperature studies for the ring amide protons are shown in Figures 5 and 6. The results for the D-A_2bu protons of both diastereomers are tabulated (Table II) to ease the analysis of the data for the ring protons. A hydrogen bond involving the γ-NH of D-A_2bu is apparent in both isomers and persists with the addition of H_2O. From the computer-simulated minimum energy conformations (Hassan & Goodman, 1986), we find two possible hydrogen-bond acceptors for this amide proton in each diastereomer. A C_6 ring may be formed by a hydrogen bond involving the mLeu carbonyl adjacent to gPhe or a C_7 ring with D-A_2bu carbonyl as acceptor. Conformations with these features are shown in Figure 7.

Some small changes in $^3J_{NH-C^\alpha H}$ coupling constants are observed with the introduction of 50% (v/v) H_2O, especially in the amide protons of Gly and the gPhe adjacent to Gly in both diastereomers (Tables III and IV). These data indicate that large conformational changes do not occur since the transannular hydrogen bonds remain intact.

(*III*) *H-Tyr-c-*(D-*A_2bu-gGly-*LD-*mPhe-Leu*). Two diastereomers resulted from the introduction of the 2-benzylmalonic acid residue in compound III. The isomers were separated

Table III: $^3J_{NH-C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions for H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu) in Me_2SO-d_6 Solution and 0.83 Mole Fraction H_2O in Me_2SO-d_6 Solution

	X_{H_2O} = 0.00		X_{H_2O} = 0.83	
residue[d]	*J*	ϕ	*J*	ϕ
D-A_2bu	8.7	−64	*c*	
		−56		
		92		
		148		
Gly	12.6[b]	−124	10.8[b]	−136
		−64		−53
		64		53
		124		136
gPhe^1	8.3	−151	7.9	−153
		−89		−87
		48		43
		72		77
gPhe^2	6.1	−93	6.8	−87
		−27		−33
		76		80
		164		160

[a] Torsions were calculated after Bystrov (1976). [b] The Gly coupling constants are $\sum(^3J_{NH-C^\alpha H})$. [c] D-$A_2$bu broadened extensively in solutions of high H_2O concentration. [d] gPhe^1 is adjacent to Gly, and gPhe^2 is adjacent to Leu.

Table IV: $^3J_{NH-C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions for H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu) in Me_2SO-d_6 Solution and 0.83 Mole Fraction H_2O in Me_2SO-d_6 Solution

	X_{H_2O} = 0.00		X_{H_2O} = 0.83	
residue[c]	*J*	ϕ	*J*	ϕ
D-A_2bu	7.9	−77	6.8	−87
		−43		−33
		87		80
		153		160
Gly	10.1[b]	−141	11.2[b]	−134
		−49		−55
		49		55
		141		134
gPhe^1	7.2	−157	6.8	−160
		−83		−80
		36		33
		84		87
gPhe^2	8.6	−64	8.3	−72
		−56		−48
		92		89
		148		151

[a] Torsions were calculated after Bystrov (1976). [b] The Gly coupling constants are $\sum(^3J_{NH-C^\alpha H})$. [c] gPhe^1 is adjacent to Gly, and gPhe^2 is adjacent to Leu.

by HPLC, and the absolute configuration of the mPhe residue in each diastereomer was deduced from biological and spectroscopic data (Richman et al., 1985). A D-Phe residue is not well tolerated in enkephalins (Morley, 1980); thus, the D-mPhe diastereomer should show the least activity. The one-dimensional spectrum of the less potent isomer shows the Leu γ-proton (1.23 ppm) at 0.17 ppm upfield of the same proton in the more potent isomer (1.40 ppm), which confirms that the less potent diastereomer contains D-mPhe. A complete assignment of the one-dimensional spectra was accomplished (not shown). In both isomers, an NOE was detected between the downfield-shifted Gly NH and the D-A_2bu C^α proton. Another was detected in the L-mPhe isomer between the upfield-shifted gGly NH and the mPhe C^α proton.

Temperature studies revealed a hydrogen bond involving the γ-NH protons of D-A_2bu for both isomers in Me_2SO-d_6. The H_2O titration/temperature results are shown in Figures 8 and 9. The hydrogen bonds for each isomer are disrupted with the addition of H_2O. Computer-simulated conformations of

Table V: $^3J_{NH-C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions for H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu) in Me_2SO-d_6 Solution and 0.86 Mole Fraction H_2O in Me_2SO-d_6 Solution

	X_{H_2O} = 0.00		X_{H_2O} = 0.86	
residue[d]	*J*	ϕ	*J*	ϕ
D-A_2bu	7.9	−77	*c*	
		−43		
		87		
		153		
gGly^1	11.4[b]	−132	9.3[b]	−146
		−56		−44
		56		44
		132		146
gGly^2	13.0[b]	−121	12.2[b]	−127
		−67		−61
		67		61
		121		127
Leu	7.6	−155	6.1	−164
		−85		−76
		39		27
		81		93

[a] Torsions were calculated after Bystrov (1976). [b] The Gly coupling constants are $\sum(^3J_{NH-C^\alpha H})$. [c] D-$A_2$bu broadened extensively at high H_2O concentrations. [d] gGly^1 is adjacent to D-A_2bu, and gPhe^2 is adjacent to mPhe.

Table VI: $^3J_{NH-C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions for H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu) in Me_2SO-d_6 Solution and 0.81 Mole Fraction H_2O in Me_2SO-d_6 Solution

	X_{H_2O} = 0.00		X_{H_2O} = 0.81	
residue[d]	*J*	ϕ	*J*	ϕ
D-A_2bu	7.6	−81	*c*	
		−39		
		85		
		155		
gGly^1	12.4[b]	−125	10.1[b]	−141
		−63		−49
		63		49
		125		141
gGly^2	*c*		*c*	
Leu	7.6	−155	7.6	−155
		−85		−85
		39		39
		81		81

[a] Torsions were calculated after Bystrov (1976). [b] The Gly coupling constants are $\sum(^3J_{NH-C^\alpha H})$. [c] D-$A_2$bu and gGly^2 peaks overlapped. [d] gGly^1 is adjacent to D-A_2bu, and gGly^2 is adjacent to mPhe.

the L-mPhe diastereomer show a hydrogen bond between D-A_2bu γ-NH and the mPhe CO adjacent to Leu (Hassan & Goodman, 1986). This hydrogen bond is also present in the minimum energy conformations of the D-mLeu isomer in addition to a conformation with the D-A_2bu CO as the hydrogen-bond acceptor. These conformations, shown in Figure 10, include C_7 structures.

The $^3J_{NH-C^\alpha H}$ coupling constants for the two diastereomers show moderate changes between 0% and 50% (v/v) H_2O in Me_2SO-d_6 solutions (Tables V and VI). The $^3J_{NH-C^\alpha H}$ of the gGly NH adjacent to D-A_2bu exhibited the largest change, more than 2 Hz for each isomer, which corresponds to a change in torsion of up to 16°. The ring conformations of these analogues are somewhat altered with the change in solvent.

Discussion

The computer simulations of molecular dynamics showed the extent of the motion available to the functional groups in the enkephalin analogues and the types of motion they undergo (Mammi et al., 1985; Hassan & Goodman, 1986). On the time scale of NMR chemical shift measurements, a con-

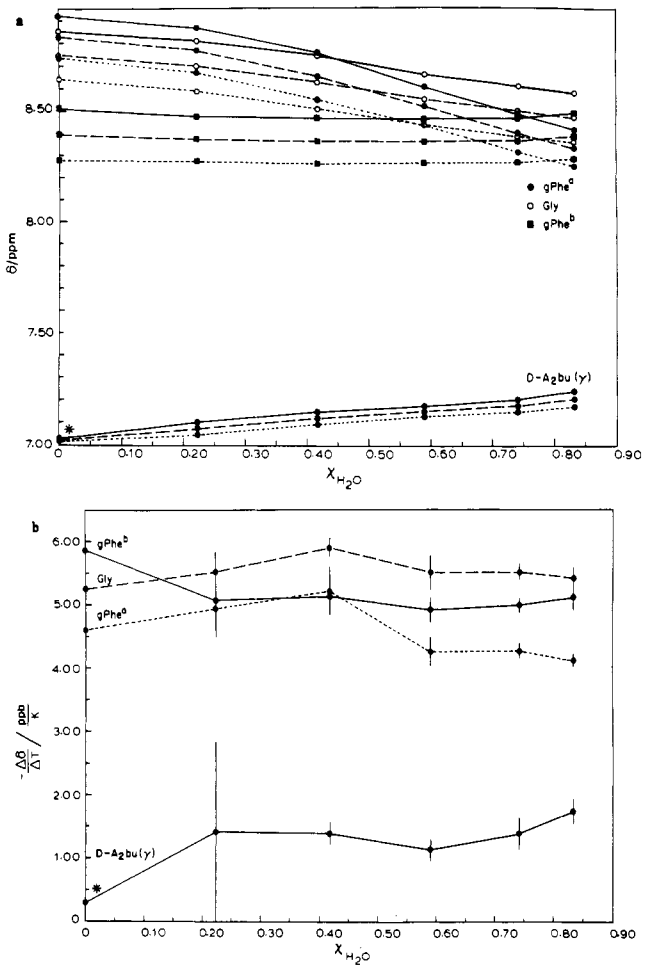

FIGURE 5: (a) Chemical shifts of amide resonances of L-mLeu diastereomer of compound II [H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu)] vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies. The results shown are at three interpolated temperatures: 20 (solid lines), 40 (dashed lines), and 60 °C (dotted lines). (b) Temperature coefficients of amide resonances of L-mLeu diastereomer of compound II vs. mole fraction of H_2O in Me_2SO-d_6. (Footnote a) The gPhe NH adjacent to mLeu. (Footnote b) The gPhe NH adjacent to Gly. (*) Two points were used to calculate this point because of overlapping peaks.

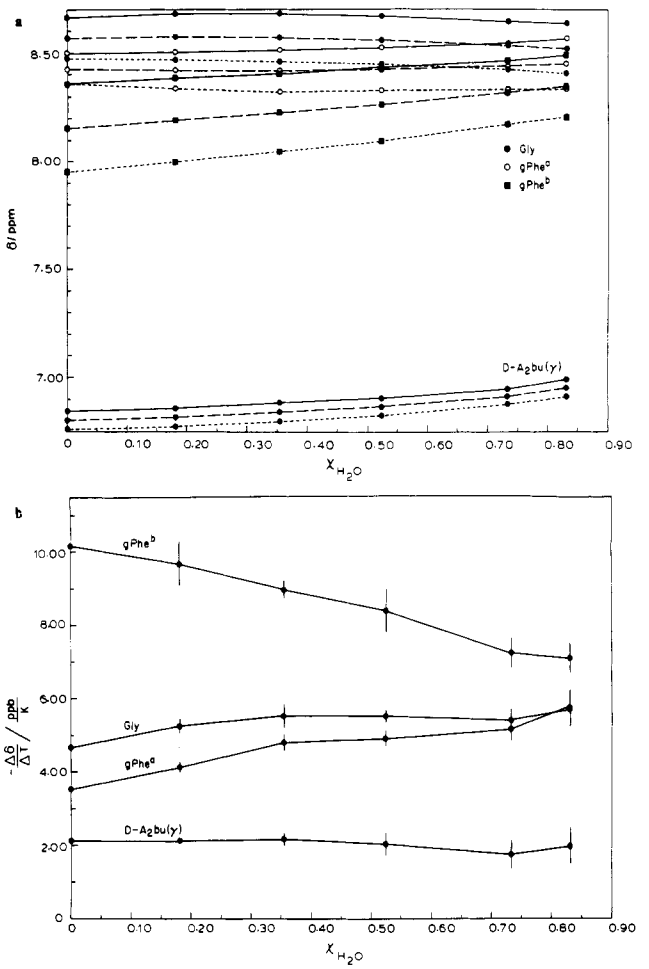

FIGURE 6: (a) Chemical shifts of amide resonances of D-mLeu diastereomer of compound II [H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu)] vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies. The results shown are at three interpolated temperatures: 20 (solid lines), 40 (dashed lines), and 60 °C (dotted lines). Gly (filled circles), gPhe NH adjacent to mLeu (open circles), gPhe NH adjacent to Gly (filled squares), and D-A_2bu(γ) (fill circles). (b) Temperature coefficients of amide resonances of D-mLeu diastereomer of compound II vs. mole fraction of H_2O in Me_2SO-d_6. (Footnote a) The gPhe NH adjacent to mLeu. (Footnote b) The gPhe NH adjacent to Gly.

strained enkepahlin analogue may fluctuate significantly, making a precise description of the conformation impossible. Here, we have described the backbone ring conformations of the enkephalin analogues in terms of transannular hydrogen bonds. The appearance of the analogues may be visualized as dynamic structures constrained by these hydrogen bonds.

The hydrogen-bonding patterns varied among the cyclic enkephalin analogues; the reversals of amide bonds altered the relative positions of the functional groups available to participate in hydrogen bonds. It was observed that conformers of the parent compound exhibited two hydrogen bonds while conformers of the retro-inverso-modified analogues formed only one hydrogen bond each in Me_2SO-d_6 solutions (Mammi et al., 1985). The C_7 structure dominates the conformations of the backbone rings throughout the series. Six-membered (C_6) rings were suggested by the computer simulations of Hassan and Goodman as possible structures in two of the analogues, but there was no evidence for β-turns in any minimum energy conformation. The constraint imposed by the 14-membered ring makes the formation of a 1–4 hydrogen bond energetically unfavorable.

The reversal of the amide bond between Leu and the side chain of the second residue resulted in an analogue (compound I) with very similar biological activity to that of the parent compound. There is a moderate decrease in the binding affinity that reduces the potency of compound I relative to the parent analogue.

The D-mLeu diastereomer of compound II has similar biological activity ot those of the parent analogue and compound I (Berman & Goodman, 1984). The L-mLeu diastereomer, however, exhibits very high potency, 17 times the potency of the D-mLeu diastereomer and over 9 times the activity of the parent compound. The reversal of the amide bond between Phe and Leu has an effect on biological activity. This suggests a difference in the efficiency of the signal transduction between the two diastereomers because the binding affinity of the L-mLeu isomer toward receptors is only twice that of the D-mLeu isomer and the latter is more stable toward enzymatic degradation (Berman et al., 1983). There is very little difference in the backbone ring conformations of the two diastereomers, so the side-chain configuration of the mLeu probably plays an important role in biological activity.

Both diastereomers of compound III have very little potency in MVD and GPI assays, and the D-mPhe isomer exhibits much less activity than the L-mPhe isomer. Richman et al. (1984) have attributed the low potency of the isomers of

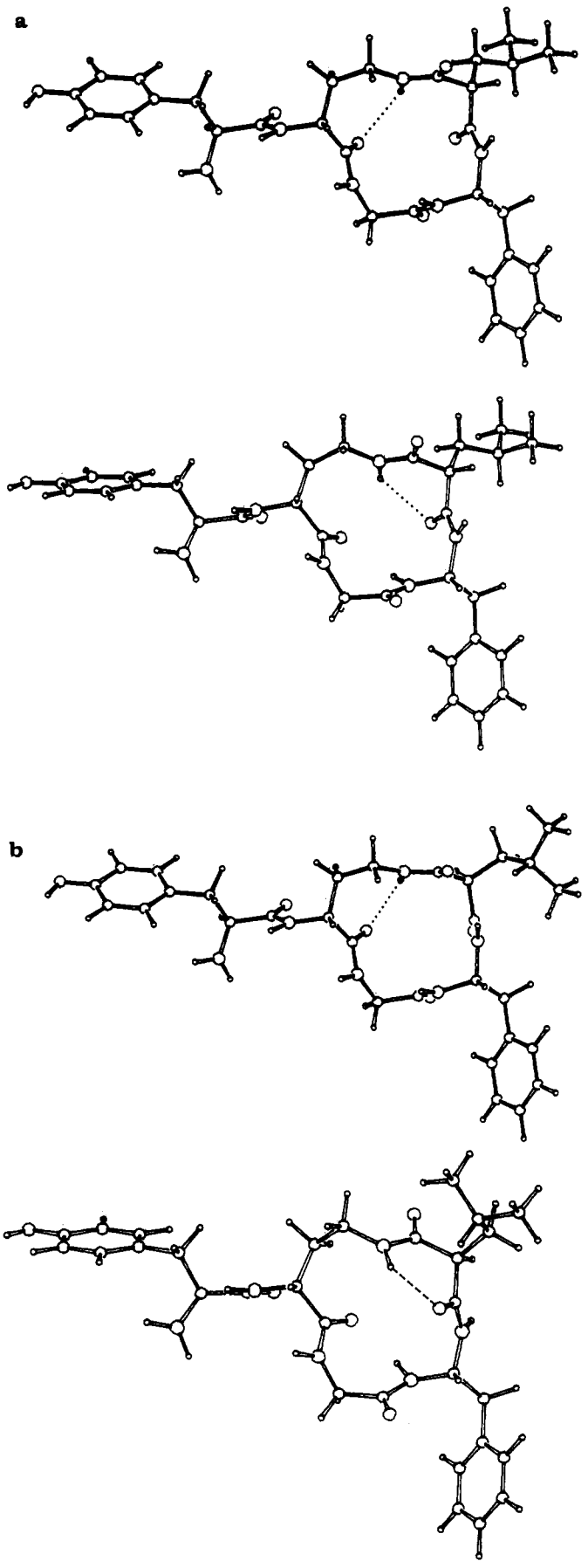

FIGURE 7: (a) Preferred conformations of H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu). (b) Preferred conformations of H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu).

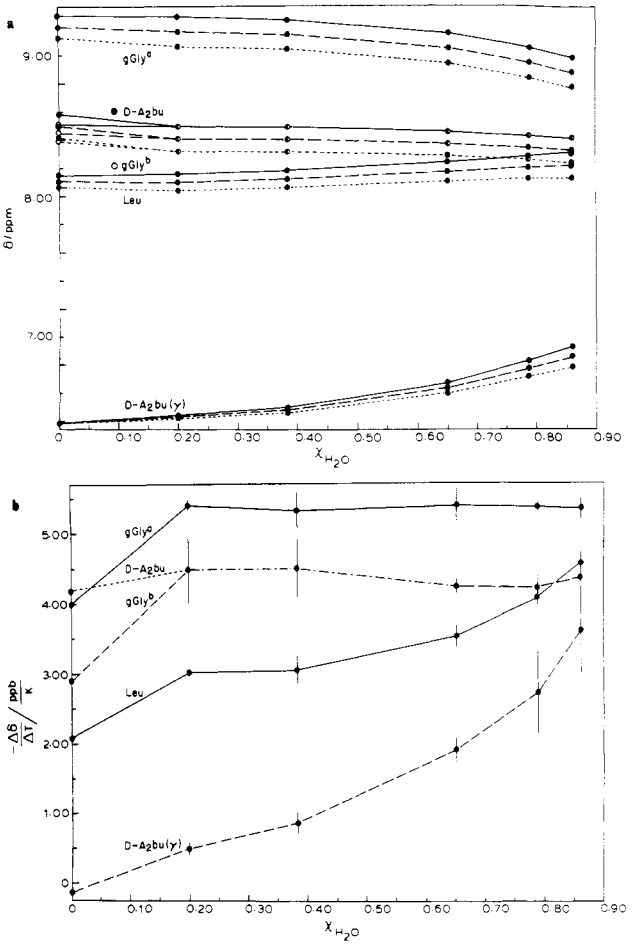

FIGURE 8: (a) Chemical shifts of amide resonances of L-mPhe diastereomer of compound III [H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu)] vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies. The results shown are at three interpolated temperatures: 20 (solid lines), 40 (dashed lines), and 60 °C (dotted lines). (b) Temperature coefficients of amide resonances of L-mPhe diastereomer of compound III vs. mole fraction of H_2O in Me_2SO-d_6. (Footnote a) The gGly NH adjacent to D-A_2bu. (Footnote b) The gGly NH adjacent to mPhe.

compound III to the displacement of the Gly carbonyl, which may be crucial for binding. The D-mPhe isomer is lower in potency because a D-configuration is not well tolerated at the fourth position (Morley, 1980). A comparison of a and b of Figure 10 reveals the conformational differences between the L-mPhe and D-mPhe diastereomers owing to the configurational change at that residue. The position of the aromatic ring relative to the rest of the molecule is very different between the two isomers.

Changing solvent environment may affect side-chain conformational preferences in addition to the possible variations in backbone ring conformations. Hydrophobic interactions between side chains and the backbone or among side chains change with solvent polarity. The effects from side chains were not investigated in this study because the motional freedom of the side chains limits the definitive information we can obtain from the conformational studies.

Temperature coefficients for the amide protons of the enkephalin analogues in Me_2SO-d_6 fell into two ranges. The intramolecularly hydrogen-bonded amide protons exhibited temperature coefficients of 0–2 (absolute values) ppb/K. The remaining temperature coefficients were typically 3–5 (absolute values) ppb/K. These were indicative of amide protons hydrogen bonded to solvent molecules. The gPhe amide proton adjacent to Gly in compound II (D-mLeu diastereomer)

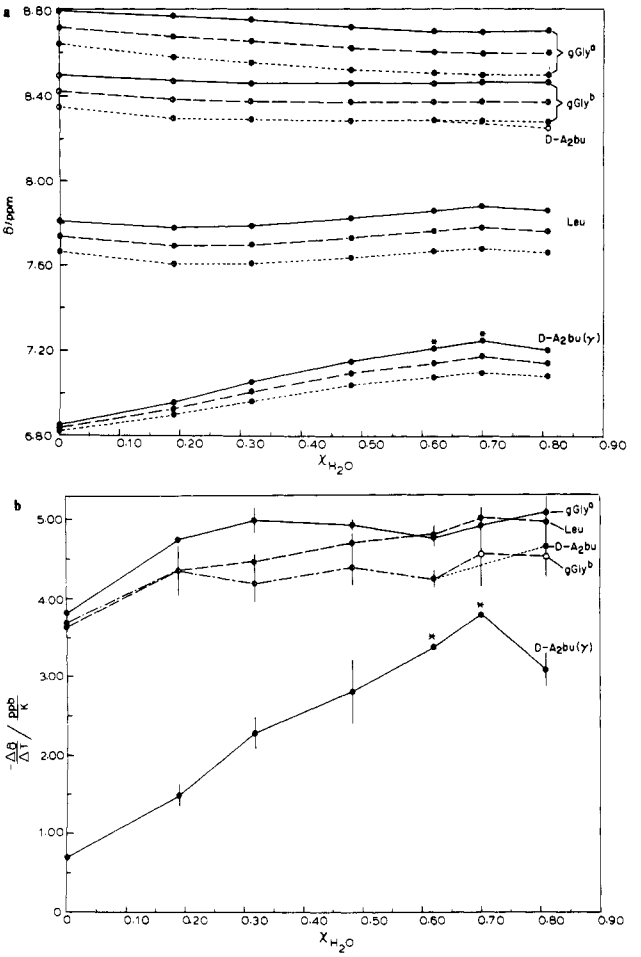

FIGURE 9: (a) Chemical shifts of amide resonances of D-mPhe diastereomer of compound III [H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu)] vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies: 20 (solid lines), 40 (dashed lines), and 60 °C (dotted lines). (b) Temperature coefficients of amide resonances of D-mPhe diastereomer of compound III vs. mole fraction of H_2O in Me_2SO-d_6. (Footnote a) The gGly NH adjacent to D-A_2bu. (Footnote b) The gGly NH adjacent to mPhe. (*) Two points were used to calculate these points.

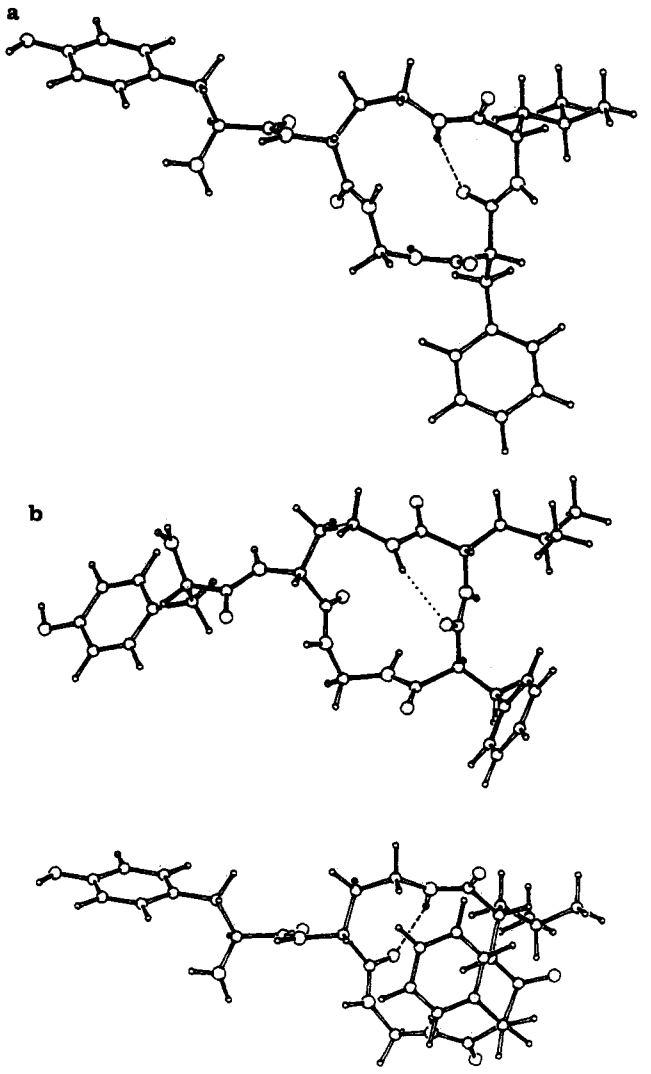

FIGURE 10: (a) Preferred conformation of H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu) in Me_2SO-d_6. (b) Preferred conformations of H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu) in Me_2SO-d_6.

showed an exceptionally high temperature coefficient of −10 ppb/K (Figure 7b). This result implies that a degree of solvent shielding occurs in all of the other amide protons and that this proton is freer to hydrogen bond to Me_2SO-d_6 molecules. High temperature coefficients have been reported for more flexible peptides in Me_2SO-d_6 solution, up to −11.5 ppb/K for a Phe amide proton in somatostatin (Deleuze & Hull, 1982). With the addition of H_2O, the absolute value of the temperature coefficient of this proton (−10 ppb/K) decreased to a value comparable to those of other exposed amide protons. We do not understand the basis of this effect.

The temperature dependence of chemical shift of the amide protons in the enkephalin series generally increased as H_2O was added to Me_2SO-d_6 solutions. In a similar study, high temperature dependences of chemical shift in pure H_2O vs. pure Me_2SO-d_6 were observed by Llinas and Klein (1975). An amide proton is sensitive to hydrogen bonds involving its neighboring carbonyl. It is deshielded by the hydrogen bond to the neighboring carbonyl as well as by the hydrogen bond in which it is acting as donor. Llinas and Klein attributed this sensitivity to charge relay through the π-system of the peptide bond. Because H_2O is a hydrogen-bond donor, two hydrogen bonds per solvent-exposed peptide are perturbed with increasing temperature in H_2O solutions. As molecular motion increases, disrupting peptide–solvent hydrogen bonds, the chemical shifts of the protons decrease. In solutions with an abundance of hydrogen-bond donors, there will be a larger effect than in solutions lacking proton donors.

Hydrogen bonds formed between H_2O molecules and carbonyl groups on peptides explain the disruption of intramolecular hydrogen bonds in the diastereomers of compound III with the addition of H_2O. The intramolecularly hydrogen-bonded carbonyls in these cases form more stable hydrogen bonds to the solvent than to amide protons within the molecule.

When hydrogen bonds were disrupted by the addition of H_2O, the resonances of the amide protons involved in these hydrogen bonds shifted substantially downfield. The diastereomers of compound III exhibited this behavior (Figures 8a and 9a) as did the parent analogue (Mammi et al., 1985). This downfield shift indicates that the intramolecular hydrogen bond to a carbonyl is less deshielding of the amide proton than the hydrogen bond to Me_2SO-d_6 or H_2O.

For the compounds that did not experience conformational changes, the resonances of intramolecularly hydrogen-bonded amide protons shifted downfield as H_2O was added, and the resonances of solvent-exposed amide protons generally shifted upfield. Alumichrome in H_2O and Me_2SO-d_6 solutions exhibited similar behavior (Llinas & Klein, 1975). According to Llinas and Klein, hydrogen bonds between amide protons and Me_2SO-d_6 cause more deshielding of the proton than hydrogen bonds to H_2O because Me_2SO-d_6 is a stronger base,

thus a stronger hydrogen-bond acceptor, than H_2O. The downfield shift of the resonance peaks of amide protons involved in intramolecular hydrogen bonds occurs because their neighboring carbonyls become involved in hydrogen bonds to H_2O. We expect little change for the resonances of solvent-exposed amides whose neighboring carbonyls are intramolecularly hydrogen bonded. In fact, for some of the amide protons whose neighboring carbonyls participate in intramolecular hydrogen bonds, this proved to be the case. For the Phe NH of compound I, very little change in chemical shift was observed with the addition of H_2O. The gPhe NH adjacent to mLeu and the Gly NH of the D-mLeu diastereomer of compound II also showed little change. However, the gPhe NH adjacent to mLeu and the Gly NH of the L-mLeu diastereomer of compound II did not behave as predicted, because of the small conformational changes that occurred with the addition of H_2O.

The conformations of five cyclic retro-inverso enkephalin analogues have been investigated by proton NMR. Interpretation of the NMR data has been developed with the aid of computer calculations of minimum energy conformations.

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Registry No. I, 87619-78-1; II (L-MLeu), 104372-38-5; II (D-mLeu), 104372-37-4; III (L-mPhe), 101854-38-0; III (D-mPhe), 101854-39-1.

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