was extracted several times with an ether–benzene mixture to remove the neutral fraction. The alkaline solution was acidified to yield acid which was recrystallized from benzene–Skellysolve B to yield pure VII, mp 265–267°, in 83% yield.

7,8,8a,9,10,16c-Hexahydro-7-oxohexahelicene (IX). To 1 l. of dry benzene were added 50 g (0.137 mole) of 1,2,3,4-tetrahydro-4-(1-naphthyl)-3-phenanthreneacetic acid (VIII) and 30 g (0.144 mole) of phosphorus pentachloride. The mixture was refluxed for 1 hr followed by removal of benzene and phosphorus oxychloride under reduced pressure. The light yellow solid was taken up in 600 ml of dry *o*-dichlorobenzene followed by the addition of 32 ml (0.275 mole) of stannic chloride while the temperature was maintained near zero. The solution was heated to 60° for 0.5 hr and then poured into dilute hydrochloric acid. The reaction mixture was worked up in the usual manner.

Acidification of the alkaline washes yielded 6.1 g of the starting acid. The neutral extracts, after steam distillation, yielded upon recrystallization from benzene–Skellysolve B 40.4 g (85%) of IX, mp 216–220°.

Hexahelicene (I). A solution of 20.6 g (0.0615 mole) of hexahydrohexahelicene (X) in 300 ml of thiophene-free benzene in the presence of 25 g of 5% rhodium on alumina and under nitrogen was heated to 300° in a Pyrex-lined, high-pressure cylinder for 12 hr. Filtration followed by washing the spent catalyst several times with benzene resulted in about 1 l. of a dark greenish black solution. The benzene was removed under reduced pressure and the solid was recrystallized from benzene–Skellysolve B to give a brown-yellow solid. Purification was achieved by chromatography on alumina to yield 15.9 g (79%) of hexahelicene, mp 225–226°. Further purification over alumina gave a sample that melted at 238–240°.

A picrate of hexahelicene was made by adding a saturated solution of picric acid in chlorobenzene to a solution of hexahelicene in chlorobenzene. The resulting deep red solution was concentrated to two-thirds volume under reduced pressure and allowed to stand. Deep red crystals were obtained which were washed with ethanol and recrystallized three times from chlorobenzene. The picrate melted at 196–197° dec.

Anal. Calcd for $C_{32}H_{19}N_3O_7$: C, 68.9; H, 3.4; N, 7.53. Found: C, 68.9; H, 3.6; N, 7.5.

The resolution was accomplished as before.[3] The sample of (+)-hexahelicene used for the optical measurements had mp 270° and $[\alpha]^{25}D$ +3750 ± 200° (*c* 5.4 × 10^{-3}, chloroform).

The ORD, CD, and absorption spectra were obtained on Cary 60, Jasco, and Cary 14M spectrometer, respectively. The ORD and CD data are presented in Table II.

Table II. ORD and CD Data of (+)-Hexahelicene at 25–26°

ORD				CD	
$CHCl_3$, *c* 1.645 × 10^{-4} *M*		CH_3OH, *c* 1.645 × 10^{-5} *M*		CH_3OH, *c* 1.645 × 10^{-5} *M*	
λ, mμ	$[\phi] \times 10^{-4}$	λ, mμ	$[\phi] \times 10^{-4}$	λ, mμ	$[\theta] \times 10^{-4}$
359	31.36	355.5	28.40	413.5	−0.93[a]
347	18.16	345	17.46	406	0.15[a,b]
339.5	23.91	335	24.14	397	1.24[a]
318.5	−65.67	315	−62.38	395	1.18[a]
312	−51.50	309	−53.04	347	24.08
306	−55.90	302.5	−56.85	343	22.57
296	−41.71	293.5	−43.74	325	64.70
286.3	−46.60	...	...	312	24.08[b]
272	−57.15	270	−59.43	304	17.06[b]
262	−47.29	259.5	−53.92	296	4.01
259.8	−48.64	255.5	−58.46	292	5.52
247.5	17.01			262	−26.08[b]
		232.5	65.68	260	−29.09
		222.5	−4.86	244	−7.12
		212.5	26.77	227	15.05
				216	−22.07
				210	−8.02

[a] In $CHCl_3$, *c* 1.555 × 10^{-4} *M*. [b] Shoulder.

Acknowledgment. The authors are grateful to Dr. Elliot Charney for helpful discussion concerning the circular dichroism features.

Conformations of Cyclic Peptides. The Folding of Cyclic Dipeptides Containing an Aromatic Side Chain

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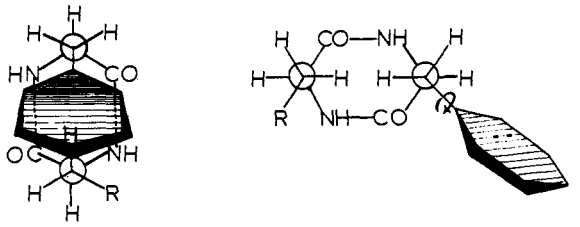
Abstract: Proton magnetic resonances of a number of cyclic dipeptides, with and without an aromatic amino acid residue, were measured; solutions in trifluoroacetic acid, dimethyl sulfoxide-d_6, and deuterium oxide were used. Resonances of the α and β protons of the nonaromatic residue in compounds containing an aromatic side chain were found to be shifted to higher field. This shift suggests that the preferred conformation of the arylmethyl side chain is one in which the aromatic ring faces the dipeptide (diketopiperazine) ring. In three cases the thermodynamic parameters of the aromatic–diketopiperazine interaction that stabilizes this folded conformation were obtained; these were found not to be significantly dependent on solvent. The folded form is favored over other possible conformations of the arylmethyl side chain by an enthalpy change averaging −3 kcal/mole; this results from a direct, rather than solvent-mediated, interaction between the two rings.

The folding of peptide chains is determined by nonbonded interactions among the side chains of amino acid residues and by the geometry of covalent and hydrogen bonds. In principle, peptide folding could be completely determined for each case by X-ray crystallographic analysis, but it is unlikely that this will or can be done for any peptide or protein that may chance to be of interest. It is valuable, then, to explore the nature and magnitude of the forces determining side-chain position in peptides, in order to enhance the probability that an *a priori* estimate of secondary or tertiary structure approximates actuality.

For oligopeptides, in which individual nonbonded interactions can be examined with least ambiguity, it is particularly important to examine conformation directly in solution. The environment of most of a small



IA IB

Figure 1. Conformations of an arylmethyl side chain of a cyclic dipeptide. IA is the folded form that nmr measurements indicate is favored.

molecule changes drastically on going from the crystal to a dispersion in solvent, and whether or not X-ray crystallography determines for globular proteins a structure identical with that in solution, it is not likely to do so consistently for smaller peptides. Further, the ability to vary solvent composition gives the investigator an additional tool with which to study the forces that determine conformation.

Proton magnetic resonance studies of the conformational details of individual amino acids in solution have been reported.[1-3] Conformational studies of peptides, other than homopolymers or homooligomers, however, have been understandably few and more ambiguous. For dipeptides containing an aromatic side chain, proton magnetic resonance studies agree with pK_A measurements in suggesting that a DL-dipeptide is more compactly folded than its LL isomer.[4,5] Dielectric increment investigations also indicate that tri- and tetrapeptides do have conformational preferences in aqueous solution.[6,7] Infrared spectroscopy has been used to determine the ratio of *cis*- to *trans*-peptide bonds in a series of diastereomeric cyclic hexapeptides; the occurrence of *cis*-peptide bonds is related to potential side-chain interferences in the all-*trans* conformer.[8a] Recently hydrogen-bonded secondary structure has been shown in a tetrapeptide.[8b] There is room, however, for much more in the way of systematic studies of conformation of nonhomomeric peptides.

We have undertaken a program to explore conformational preferences of peptides in solution, using as a probe the effects on nearby protons of the magnetic anistropy of an aromatic side chain. We are restricting ourselves to cyclic peptides, where the degrees of rotational freedom are limited, in hopes that the proton magnetic resonance data obtained will be interpretable in terms of side-chain orientations. In this paper we report studies of cyclic peptides in which the peptide backbone is as simple and rigid as possible, *i.e.*, the diketopiperazine ring of cyclic dipeptides.

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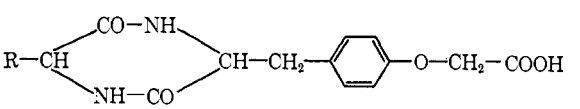
Diketopiperazine itself, cycloglycylglycyl, has been examined in the crystalline state and found to be a planar ring.[9,10] If this planarity also obtains for substituted diketopiperazines and is maintained in solution, then for *c*-glycylphenylalanyl (or *c*-glycyltyrosyl) the only degrees of rotational freedom that need be considered are those about the C_α–C_β and C_β–C_{Ar} bonds (see Figure 1). Use of Dreiding models, with the diketopiperazine ring constrained to be planar, shows that in those staggered conformations of the C_α–C_β bond in which the aromatic ring lies away from the peptide ring (*e.g.*, Figure 1, IB) the effects of the aromatic ring on the shielding of α or β protons of the second amino acid residue must be small (less than +0.1 ppm for any conformation of the C_β–C_{Ar} bond). However, when the C_β–C_{Ar} bond lies over the peptide ring (a folded conformation, *e.g.*, Figure 1, IA), the shielding effects of the aromatic ring on these protons can be considerable. Space-filling models show that with this latter arrangement free rotation of the C_β–C_{Ar} bond is not possible. Table I indicates the shielding effects, taken from the calculations for benzene by Johnson and Bovey,[11] to be expected for two C_β–C_{Ar} rotations in the folded conformation.

Table I. Estimated Shielding[a] by Phenyl Ring in Folded[b] Conformations of 3-Benzyl-2,5-piperazinediones

Proton	Rotation of phenyl Rings face-to-face (Figure 1, IA)	Phenyl eclipsing benzyl C–H
cis-6	1.0	0.6
trans-6	0.3	0.2
cis-6-Methyl[c]		
Individual protons	2.4	1.5
	0.5	0.5
	0.5	0.2
Av	1.2	0.7

[a] Distances taken from Dreiding models, shielding from calculations of Johnson and Bovey.[11] Shielding values all positive and given in parts per million. [b] C_3–C_{benzyl} conformation as in Figure 1, IA. [c] Methyl conformation assumed to be staggered as in Figure 1, R = H.

We have examined the proton magnetic resonance spectra of a series of cyclic tyrosyl-X dipeptides, dissolved in trifluoroacetic acid, dimethyl sulfoxide-d_6, and in water. The solubility of these peptides in water is limited and several of them were converted to O-carboxymethyl derivatives (II) so that they could be dissolved in sodium bicarbonate solution. The observed magnetic shielding effects of the aromatic ring



II

on the α and β protons of the X residue are as large as those indicated in Table I, indicating that the folded

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(11) C. E. Johnson and F. A. Bovey, *J. Chem. Phys.*, 29, 1012 (1958). The calculations are tabulated in J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Pergamon Press, Ltd., Oxford, 1965, pp 595–604.

conformation is the preferred one. In eight cases (three dipeptides and the three solvents) we have made a comparison at varying temperatures of the resonances of these protons with those of cyclic dipeptides containing the X residue but not the aromatic residue. The results of these studies (Table II) indicate that the folded conformer is favored by about 3 kcal in enthalpy, although it has a more negative entropy by about 4 cal/mole deg. This net stabilization of the folded form is, surprisingly, not much affected by the solvent.

Table II. Magnitude of the Aromatic–Diketopiperazine Interaction

Dipeptide cyclo-	Solvent	K_{25}[a]	ΔH, cal/mole	ΔS, eu	$\Delta\delta_{max}$, ppm
Gly-Tyr	D_2O–OD^-	8.0	−5000	−6.4	1.75[b]
	CD_3SOCD_3	3.4	−2450	−2.9	1.52[b]
	CF_3COOH	4.2	−3150	−3.9	1.43[b]
L-Ala-L-Tyr	D_2O–DCO_3^- [e]	2.35	−2400	−3.2	1.45[c]
	CD_3SOCD_3	4.5	−3120	−3.8	0.85[c]
	CF_3COOH	3.8	−3100	−3.9	0.95[c]
L-His-L-Tyr	D_2O–D_3O^+	3.2	−3400	−4.6	1.67[d]
	CF_3COOH	2.1	−2720	−3.8	1.78[d]

[a] [Folded]/[unfolded], calculated as described in text. [b] *cis*-Glycine α-H relative to *c*-Gly_2. [c] Alanine CH_3 relative to *c*-L-Ala_2. [d] Higher field histidine β-H relative to *c*-L-His_2. [e] O-Carboxymethyl derivative.

The assumption, made above, of planar diketopiperazine rings is consistent with the details of the data to be discussed below.

Experimental Section

Proton Magnetic Resonance Spectra. All nmr spectra were recorded with a Varian A-60 spectrometer equipped with a V-6040 variable-temperature accessory. Probe temperatures were determined with Varian samples of methanol and ethylene glycol. Frequency calibration was effected with an audio oscillator, the output of which was continuously monitored with a Hewlett-Packard 5245L electronic counter. A Varian C-1024 time-averaging computer aided in examination of the spectra of dilute solutions.

Samples in trifluoroacetic acid and dimethyl sulfoxide-d_6 were prepared and degassed in the usual manner. Solutions in D_2O were prepared using either sodium bicarbonate (for the carboxylic acids), sodium hydroxide (for *c*-glycyl-L-tryrosyl[12]), or trifluoroacetic acid (for histidyl peptides) to adjust pH. After initial solution of the peptides the solvent was removed by lyophilization and replaced by fresh deuterium oxide. The process was repeated.

Reference for the nonaqueous solutions was internal tetramethylsilane. For the deuterium oxide solutions either capillary benzene or capillary hexamethyldisiloxane was used. In Tables III, IV, and V, the chemical shift values obtained using these capillary reference compounds have been brought more or less into line with those obtained for the nonaqueous solutions by use of the measured differences at 31°: neat hexamethyldisiloxane, 0.47 ppm, and neat benzene, 7.27 ppm below neat tetramethylsilane. The actual measurements in which an aromatic-containing diketopiperazine was compared with a nonaromatic reference peptide at varying temperature used the same reference compound for the pair.

Sample concentrations were close to 0.25 *M* throughout, except for the few cases, noted in Table VI, in which the concentration was about 0.04 *M*.

Cyclic Dipeptides (Diketopiperazines). The cyclic dipeptides used were purchased, prepared according to published procedures, or prepared by fusion of commercially available free dipeptides in

Table III. Temperature Dependence of Glycine α-H Resonances[a] in *c*-Gly-Tyr and *c*-Gly-Gly

T, °C	Solvent	J_{AB}	*c*-Gly-Tyr, ν_B	*c*-Gly-Tyr, ν_A	*c*-Gly_2, ν_α
−33	CF_3COOH	18.2[b]	197.6	254.1	278.3
−11.5			197.3	249.8	274.5
+4			198.0	248.4	272.8
37			200.1	244.9	267
47.5			202.1	243.4	265.5
70			204.6	247.2	263
92			206.9	241.2	260
+37	CD_3SOCD_3	17.0[b]	165.1	200.9	222.5
49.5			167.6	201.3	222
70			171.8	202.0	222.1
92			176.4	203.6	221.5
118			180.7	204.8	221.5
137			184.0	206.0	221.2
+9	D_2O–OD^-	18.2[b]	189.2	250.0	286.6
37			199.0	254.4	289
47.5			207.3	257.8	290.2
70			214.4	260.5	290.4

[a] Hz below Me_4Si at 60 MHz. Capillary benzene reference used for aqueous solutions; values for table converted approximately to capillary Me_4Si assuming $Me_4Si - C_6H_6 = 436$ Hz. [b] No significant trend with temperature.

Table IV. Temperature Dependence of β-CH_3 Resonance[a] in *c*-L-Ala-L-Tyr and *c*-L-Ala_2

T, °C	Solvent	*c*-Ala-Tyr,[b] ν_{CH_3}[c]	*c*-Ala_2, ν_{CH_3}[c]
−32	CF_3COOH	55.5	108.6
−11.5		56.5	106.5
+6		57.8	105.8
31		60.5	104.8
47.5		62.6	103.6
69.5		64.9	102.6
91		67.1	101.0
+37	CD_3SOCD_3	35.4	75.4
54		37.7	75.8
71.5		41.1	75.9
89.5		44.6	76.6
118		48.5	77.5
136		52.0	78.4
+3	D_2O–DCO_3^-	65.1	131.8
37		77.5	133.8
47.5		80.5	135.2
70		85.4	135.7
91.5		91.0	136.5

[a] Hz below Me_4Si at 60 MHz. Capillary benzene reference used for aqueous solutions; values for table converted approximately to capillary Me_4Si assuming $Me_4Si - C_6H_6 = 436$ Hz. [b] *c*-L-CMTyr-L-Ala in D_2O. [c] $J_{\alpha\beta} = 7.0$ Hz in all solvents for both compounds.

Table V. Temperature Dependence of His β-CH_2 Resonances[a] in *c*-L-His-L-Tyr and *c*-L-His_2

T, °C	Solvent	*c*-His-Tyr ν_A[a]	*c*-His-Tyr ν_B[a]	*c*-His-Tyr $J_{\alpha\beta_A}$[b]	*c*-His-Tyr $J_{\alpha\beta_B}$[b]	*c*-His_2, ν_β[a]
−12	CF_3COOH	156.2	128.1	5.8	6.7	215
+3.5	CF_3COOH	158.3	134.1	5.6	6.8	213.5
30	CF_3COOH	161.0	143.2	5.3	6.6	211.5
47.5	CF_3COOH	162.7	146.8	5.6	6.4	210.8
71.5	CF_3COOH	165.4	152.6	6.1	6.9	209.8
91.5	CF_3COOH	167.4	157.9	6.2	6.2	208.8
+5	D_2O–D_3O^+	190.8	141.6	4.3	8.2	229.0[c]
31	D_2O–D_3O^+	198.5	160.9	4.7	7.8	232.2
47.5	D_2O–D_3O^+	201.3	167.7	4.9	7.6	235.7
71	D_2O–D_3O^+	205.7	178.7	5.1	7.4	238.2

[a] Hz below Me_4Si at 60 MHz. Capillary $Me_3SiOSiMe_3$ reference used for aqueous solution; values for table converted approximately to capillary Me_4Si assuming $Me_4Si - Me_3SiOSiMe_3 = 28$ Hz. [b] $J_{AB} = 15.6$ Hz in both solvents. [c] Estimated from the three measured points.

(12) Fresh solutions were made up for each measurement above 30° in this series, because base-catalyzed exchange of all of the α and β hydrogens occurred with increasing rapidity as the temperature was increased.

Table VI. Proton Resonances in Cyclic Dipeptides Containing an Aromatic Side Chain

Dipeptide[b,d] cyclo-	Solvent	Chemical shift, ppm, Me_4Si reference,[a] 31–37° — Other residue: *trans*-α-H	*cis*-α-H	β-H	CH_3	Aromatic residue: α-H[e]	βH[e]	Misc
Gly-Phe	CF_3COOH	4.12	3.23			4.92	3.47	
Gly-Tyr[g]	CF_3COOH	4.08	3.35			4.73	3.32	
c	CF_3COOH	4.08	3.34			4.73	3.32	
	CD_3SOCD_3	3.35	2.75			3.98	2.89	
c	CD_3SOCD_3	~3.38	*h*			3.96	2.88	
	D_2O–OD^-	4.24	3.32			5.01	3.73	
L-Ala-L-Phe	CF_3COOH	4.44		(0.89)	0.89	4.92	3.43	
L-Ala-L-Tyr[g]	CF_3COOH	4.40		(1.01)	1.01	4.77	3.33	
	CD_3SOCD_3	3.78		(0.58)	0.58	4.08	2.90	
L-Ala-L-CMTyr[g]	CD_3SOCD_3	3.65		(0.59)	0.59	4.12	2.94	
	D_2O–DCO_3^-	4.61		(1.17)	1.17	5.11	3.85	
c	D_2O–DCO_3^-	4.68		(1.18)	1.18	5.13	3.86	
L-Val-L-Tyr	CF_3COOH	4.23		2.17	0.82, 1.10	4.67	3.33	
	CD_3SOCD_3	3.55		1.90	0.39, 0.71	4.13	2.92	
L-Val-L-CMTyr	CD_3SOCD_3	3.53		1.87	0.36, 0.68	4.15	2.96	
	D_2O–DCO_3^-	4.55		2.30	1.33, 1.68	5.18	3.94	
L-Leu-L-Tyr	CF_3COOH	4.23		*f*	0.88	4.78	3.32	
	CD_3SOCD_3	3.46		*f*	0.67	4.07	2.88	Leu γ-H 1.42
L-Leu-L-CMTyr	CD_3SOCD_3	3.50		*f*	0.67	4.10	2.94	Leu γ-H 1.43
	D_2O–DCO_3^- [k]	4.48		0.82, 1.81	1.55, 1.58	5.20	3.94	Leu γ-H 1.95
D-Leu-L-Tyr	CF_3COOH		3.42	1.79	0.88, 0.98	4.73	3.31	γ-H obscured
D-Leu-L-CMTyr	CD_3SOCD_3		3.07	1.47	0.77, 0.80	4.10	2.96	Leu γ-H 1.67
	D_2O–DCO_3^-		3.60	2.34	1.59, 1.64	5.20	3.94	Leu γ-H 2.28
L-His-L-Tyr[g]	CF_3COOH	4.65		2.39, 2.68		4.88	3.42	
	D_2O–D_3O^+	4.92		2.69, 3.33		5.17	3.75	
c	D_2O–D_3O^+	~4.9		~2.74, ~3.34		~5.2	3.75	
DL-His-LD-Tyr[i]	CF_3COOH		~3.4	~3.4		~4.6	~3.4	
L-Tyr-L-Tyr	CF_3COOH	4.55		2.42, 3.08		4.55	2.42, 3.08	
	D_2O–OD^- [i]	4.97		2.99, 3.75		4.97	2.99, 2.85	
DL-Tyr-LD-Tyr[i]	CF_3COOH		~4.1	~3.2		~4.1	~3.2	

[a] Internal reference for nonaqueous solutions; referred to capillary Me_4Si for aqueous solutions. [b] Peptide concentration about 0.25 *M* except where noted. [c] Peptide concentration about 0.04 *M*. [d] CMTyr, O-carboxymethyltyrosyl. [e] Center of multiplet. [f] Straddles CH_3 resonances; see Figure 5. [g] See Tables III–V for further details. [h] Obscured. [i] 73°. [j] Racemic *trans* isomer. [k] See Figure 5.

phenol, a method to be described in detail in a separate report.[13] They were all analytically pure and chromatographically homogeneous. Where presence of an aromatic ring produced large changes in the proton magnetic resonance spectra of diastereomers, the dipeptides were shown to be homogeneous by the nmr criterion as well.

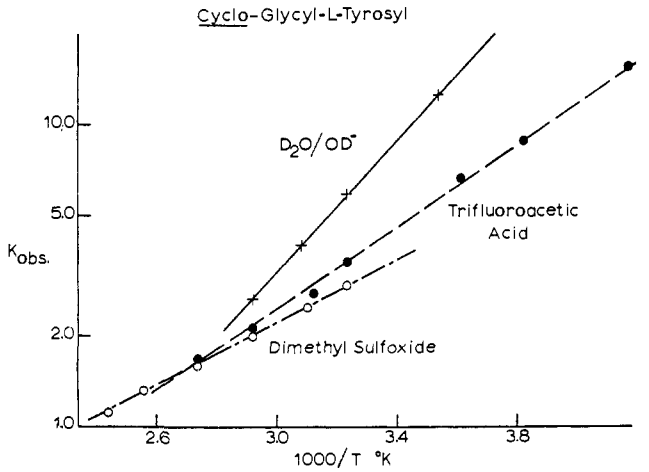

Figure 2. Plot of the logarithm of the ratio of folded to unfolded forms *vs.* $1/T$ for *c*-glycyl-L-tyrosyl in dimethyl sulfoxide, trifluoroacetic acid, and, as its anion, in D_2O (*c*, cyclo).

O-Carboxymethyltyrosyl peptides were obtained by alkylation of the corresponding tyrosyl cyclic peptides (converted to the phenoxide form in a separate step) with methyl bromoacetate in dimethylformamide, followed by basic hydrolysis of the methyl ester. This procedure will be fully reported in a separate communication.[13]

Calculation of Thermodynamic Parameters. The principal assumption made in obtaining values of ΔH and ΔS for the equilibrium between folded and unfolded forms of the tyrosine-containing diketopiperazines was that the chemical shifts of the α- and/or β-proton resonances of the second residue would be the same, in all of the unfolded conformations, as the values observed in the tyrosine-free cyclic dipeptides that were used as reference compounds. This assumption was expected to hold only when the two substances were in the same solvent, at the same temperature, and at approximately the same molar concentration. If it holds, the fraction of folded conformer is then given by

$$(\delta - \delta_{\text{ref compd}})/(\delta_{\max} - \delta_{\text{ref compd}}) = \Delta\delta/\Delta\delta_{\max}$$

and the equilibrium constant by

$$K_{\text{obsd}} = [\text{folded}]/[\text{all unfolded}] = \Delta\delta/(\Delta\delta_{\max} - \Delta\delta)$$

Values for $\Delta\delta_{\max}$ could not be obtained experimentally because of the high freezing points of the solvents used. (Dimethyl sulfoxide freezes at about +19°, trifluoroacetic acid at about −15°, although some supercooling was possible.) $\Delta\delta_{\max}$ was therefore chosen to give linear plots of log K_{obsd} *vs.* $1/T$. That this could be done with the success shown in Figures 2, 3, and 4 argues that the procedure and its underlying assumption are usable and the resultant thermodynamic parameters meaningful.

Results and Discussion

Spectra of Cyclic Dipeptides at Ambient Temperature. Tables VI and VII report the positions of the proton resonances, excluding those of the aromatic and amide protons, for a series of cyclic dipeptides. Table VI deals with peptides containing phenylalanine or tyrosine and Table VII with reference cyclic peptides lacking

(13) K. D. Kopple and H. G. Ghazarian, *J. Org. Chem.*, in press. See also N. Lichtenstein, *J. Am. Chem. Soc.*, 60, 560 (1938).

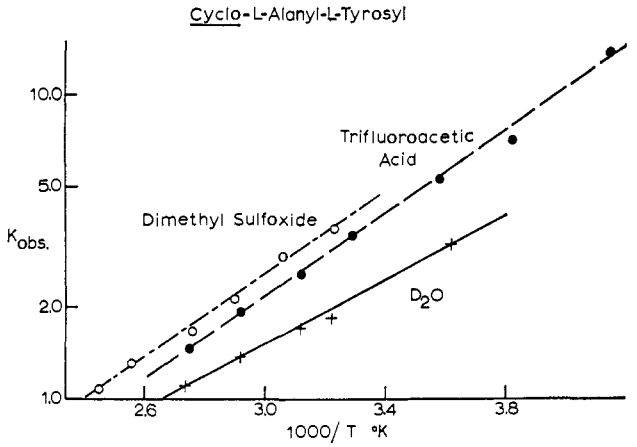

Figure 3. Plot of the logarithm of the ratio of folded to unfolded forms *vs.* $1/T$ for *c*-L-alanyl-L-tyrosyl in dimethyl sulfoxide and trifluoroacetic acid and for the anion of *c*-L-alanyl-O-carboxymethyl-L-tyrosyl in D_2O.

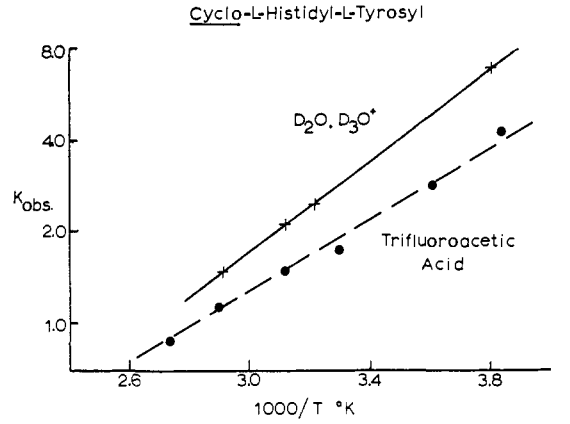

Figure 4. Plot of the logarithm of the ratio of folded to unfolded forms *vs.* $1/T$ for *c*-L-histidyl-L-tyrosyl in trifluoroacetic acid and in D_2O.

an aromatic residue. The data given in Table VI for *c*-L-alanyl-L-tyrosyl, *c*-L-valyl-L-tyrosyl, and *c*-D- and *c*-L-leucyl-L-tyrosyl show clearly that the presence of the O-carboxymethyl solubilizing group does not significantly affect the resonances of the other protons in dimethyl sulfoxide; it seems a reasonable assumption that this group has no major perturbing effect in water either.

Table VII. Proton Resonances in Nonaromatic Cyclic Dipeptides

Dipeptide[b] cyclo-	Solvent	Chemical shift, ppm, Me_4Si reference,[a] 31–37° α-H	β-H	CH_3	Gly-α-H
Gly-Gly	CF_3COOH	4.45			
	CD_3SOCD_3	3.80			
	D_2O	4.82			
L-Ala-L-Ala	CF_3COOH	4.58	(1.75)	1.75	
	CD_3SOCD_3	3.90	(1.25)	1.25	
	D_2O	4.95	(2.23)	2.23	
L-His-L-His	CF_3COOH	4.75	3.52		
	D_2O–D_3O^+	5.23	3.87		
Gly-Val	CF_3COOH	~4.33	2.54	1.10, 1.19	4.42
	CD_3SOCD_3	3.54	2.08	0.86, 0.93	3.73
	D_2O	5.02	3.31	2.00, 2.09	5.13
Gly-Leu	CF_3COOH	~4.44	1.92	1.06	4.44
	CD_3SOCD_3	~3.7	1.58	0.88	3.75

[a] Internal reference for nonaqueous solutions; referred to capillary Me_4Si for aqueous solutions. [b] Peptide concentration about 0.25 *M*.

Comparison of the chemical shift values recorded in Table VI for tyrosine- and phenylalanine-containing cyclic dipeptides with those in Table VII for the aromatic-free reference compounds reveals the following points.

(1) A benzyl or *p*-hydroxybenzyl side chain at the 3 position of a diketopiperazine ring affects the *cis* hydrogen at position 6 of that ring by shifting the resonance of the latter 1–1.5 ppm upfield. This shift is seen in the cyclic peptides glycylphenylalanyl, glycyltyrosyl, D-leucyl-L-tyrosyl, and DL-histidyl-LD-tyrosyl.

(2) The effect of a 3-benzyl or hydroxybenzyl side chain on the β-hydrogens of a *cis* side chain at position 6 is shielding averaging 1 ppm. This shift is seen in the

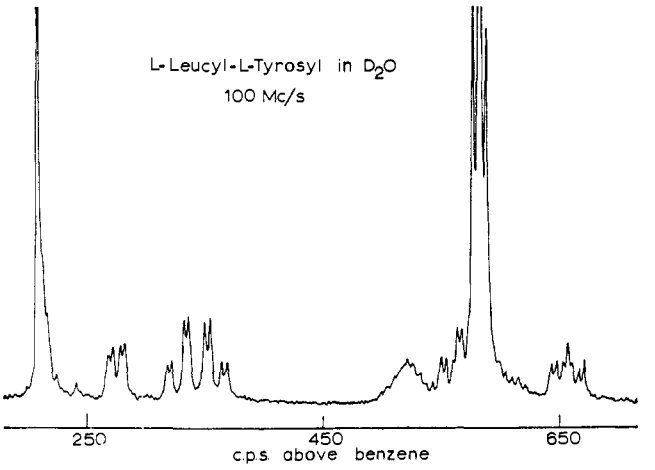

Figure 5. 100-MHz proton spectrum of 0.25 *M* *c*-L-leucyl-O-carboxymethyl-L-tyrosyl in bicarbonate-buffered D_2O. The leucyl β protons referred to in the text exhibit the resonances centered at about 550 and 650 Hz above the capillary benzene reference. We are indebted to Dr. J. J. Katz and Miss Gail D. Norman of the Argonne National Laboratory for this spectrum.

cyclic peptides L-alanyl-L-phenylalanyl, L-alanyl-L-tyrosyl, L-leucyl-L-tyrosyl, and L-histidyl-L-tyrosyl. (An apparent exception, discussed later, is *c*-L-valyl-L-tyrosyl in dimethyl sulfoxide and in trifluoroacetic acid.)

(3) In aqueous solution, where the effect is largest, the β-hydrogens of the histidyl residue of *c*-L-histidyl-L-tyrosyl exhibit magnetic nonequivalence of 0.7 ppm and the β-hydrogens of the leucine residue in *c*-L-leucyl-L-tyrosyl are nonequivalent by about 1 ppm. In the isomers in which the two side chains are *trans* on the diketopiperazine ring, these large nonequivalences are absent. Magnetic nonequivalence of β-hydrogens is observed in a number of amino acids, but not to the extent of more than 0.3 ppm.[1-3] The two methyl groups of the valine residue in *c*-L-valyl-L-tyrosyl differ by 0.3 ppm or more, but in *c*-valylglycyl by less than 0.1 ppm.

These observations are most simply accommodated by the assumption that a conformation approximating that shown in Figure 1 (IA) is generally favored over those in which the aromatic ring takes a position away from the diketopiperazine ring, as in Figure 1 (IB). However,

it is conceivable that intermolecular association is a source of the observed shielding effects. Most of the measurements in this work were made using 0.25 *M* peptide solutions. In three cases spectra of 0.04 *M* solutions were also recorded. The data obtained are included in Table VI; the effects of this dilution on the chemical shifts are seen to be negligible. It is not likely that this invariance arises from very strong association, for in other work we have determined the association constant for benzene and unsubstituted diketopiperazine in water and found it to be between 1.0 and 0.5 in the temperature range 1–40°.[14] In water, therefore, the extent of intermolecular association of the aromatic peptides can be expected to be small at 0.25 *M* and negligible at 0.04 *M*. In addition, association in aqueous solution is inhibited because the peptides used in water all carry charges. Intermolecular association of the peptides will probably be even less favorable in trifluoroacetic acid than in water. We believe that we are looking only at intramolecular effects.

The assumption of a planar diketopiperazine ring may be examined here. Two boat conformations preserve the planarity of the amide groups and are alternatives to a planar conformation for the diketopiperazine ring. If diketopiperazine itself were to exist as a boat, the two α protons of each glycine residue would not be equivalent. At the lowest temperatures accessible in the solvents used there is, in fact, no evidence of nonequivalence. In D_2O solution the line for these protons is quite narrow; in dimethyl sulfoxide it is a 2-Hz doublet, the result of coupling with the adjacent N–H proton. (This 2-Hz coupling also appears, for example, superimposed on the 7-Hz quartet for the 3 and 6 protons of *cis*-3,6-dimethyl-2,5-piperazinedione, *i.e.*, *c*-L-alanyl-L-alanyl, in dimethyl sulfoxide.) In trifluoroacetic acid coupling with the N–H proton manifests itself as line broadening at lower temperatures, but above room temperature exchange with solvent is sufficiently rapid to eliminate evidence of it.

Admittedly, the absence of detectable nonequivalence of geminal protons in the 3 and 6 positions might be the result of rapid interconversion between the two boat forms. However, in a *cis*-3,6-disubstituted 2,5-piperazinedione, only one of the two boat forms should be stable, that one with the two substituents in the "bowsprit" positions. In this conformation, the aromatic ring of a *c*-phenylalanyl-X (or tyrosyl-X) peptides is too far removed from the α and β protons of the X residue to exert shielding effects of the magnitude observed. It seems safe to conclude that the diketopiperazine ring is planar, or close to planar, in solution.

The Aromatic–Diketopiperazine Interaction. Three of the cyclic dipeptides were studied at varying temperatures to determine the degree of stabilization of the folded form. Tables III–V list chemical shift data collected for *c*-glycyl-L-tyrosyl, *c*-L-alanyl-L-tyrosyl, and *c*-L-histidyl-L-tyrosyl, respectively, together with comparable data for the corresponding reference nonaromatic diketopeperazines. The van't Hoff plots of Figures 2, 3, and 4 were obtained from these data, according to the procedure described in the Experimental Section. Table II summarizes the enthalpy and entropy changes for the equilibrium

$$\underset{\text{IB}}{\text{all unfolded forms}} \rightleftharpoons \underset{\text{IA}}{\text{folded form}}$$

Table II also indicates, in each case, the maximum upfield shifts of the proton most affected by the aromatic ring in the folded conformation. These maximum shift values do not directly reflect the geometry of the folded conformation, because they include not only the effect of the aromatic ring but also the effect of solvent displacement from the vicinity of the proton in question. They may be roughly compared, however, with the values given in Table I, which were obtained from models and the Johnson–Bovey calculation.

The most striking thing about the data given in Table II is the constancy of the ΔH and ΔS values. The cases reported include three different cyclic peptides, studied in three widely differing solvents: a strongly acidic, relatively nonpolarizable solvent, trifluoroacetic acid; a polar, polarizable but neutral solvent, dimethyl sulfoxide; and water, which is *sui generis*. In water there are measurements made on a positively charged species, *c*-L-histidyl-L-tyrosyl, and two negatively charged species, the anions of *c*-glycyl-L-tyrosyl and *c*-L-alanyl-O-carboxymethyl-L-tyrosyl. In all cases save one the folded configuration is favored by between 2.4 and 3.4 kcal/mole in enthalpy and the entropy change is 3–4 cal/mole deg unfavorable to the folded form. The exception is *c*-glycyl-L-tyrosyl anion in water, the only case in which a phenolate ion is involved.

The observed entropy changes are readily understood. Excluding the folded form, the possible rotational conformations for the benzyl group are 12: there are two rotational minima about the C_α–C_β bond and six about the C_β–C_{aryl} bond. Probably all of these conformations are of about equal energy and the entropy loss on going to the folded form is R ln 12, or about 5 cal/mole deg, if all motion of the benzyl side chain is frozen out in the folded form. There is thus no important contribution from binding or release of solvent molecules on going between folded and unfolded forms. The somewhat greater negative entropy of folding for the *c*-glycyl-L-tyrosyl anion in water is reasonably ascribed to changes in solvation of the phenolate ion; such changes are less significant for the other two ions studied in water, because the charges on those molecules are farther removed from the diketopiperazine ring. Thus, the folding of the aromatic ring against the diketopiperazine ring in these cyclic peptides is not the result of a hydrophobic or solvophobic interaction, if by this is meant an entropy-driven[15,16] association of the two ring moieties resulting in the release of bound or ordered solvent molecules.

In connection with the question of hydrophobic bonding, the observations made on *c*-L-leucyl-O-carboxymethyl-L-tyrosyl in water are of interest. Thermodynamic parameters for the folding of this molecule have not been obtained, but the ambient temperature spectrum shown in Figure 5 demonstrates that the upfield chemical shift and the degree of nonequivalence of the two leucyl β-hydrogens are at least as large as those of the histidyl β-hydrogens in *c*-L-histidyl-L-tyrosyl. Therefore, it can be concluded that the hydrophobic leucyl side chain does not compete effectively,

(14) K. D. Kopple and V. R. Sreenivasan, in progress.

(15) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).
(16) G. Nemethy, *Angew. Chem. Intern. Ed. Engl.*, **6**, 195 (1967).

even in water, for the space next to the diketopiperazine ring. (Tanford[17] gives for transfer of a leucine side chain from ethanol to water at 25° ΔF = +2420 cal/mole, and for a tyrosyl side chain +2870 cal/mole.)

It is also clear that the stabilization of the folded form of tyrosyl cyclic dipeptides does not arise from the work required to create in the solvent a larger cavity for the extended forms.[18] This work should be considerably less in trifluoroacetic acid (ΔH_{vap}/cc = 95 cal,[19] surface tension (25°) = 20 dynes/cm[20]) than in dimethyl sulfoxide (ΔH_{vap}/cc = 178 cal,[21] surface tension (25°) = 43 dynes/cm[22]), and much less in either one than in water (ΔH_{vap}/cc = 582 cal, surface tension (25°) = 72 dynes/cm).

There is thus a direct interaction between the aromatic ring and the diketopiperazine ring or the amide bonds in it. If this were of the π–π donor–acceptor type one might anticipate that the hydroxyphenyl group would serve as the donor moiety and would be distinctly more effective in this role than the phenyl group in a phenylalanyl residue. No such difference is apparent in the limited data at present available; these are the proton resonances, measured at ambient temperature in trifluoroacetic acid, of the *cis*-glycyl α-proton in *c*-phenylalanylglycyl (upfield shift, 1.2 ppm) and the alanyl methyl in *c*-L-phenylalanyl-L-alanyl (upfield shift, 0.85 ppm). We also have measured[23] the ultraviolet absorption spectra in methanol of *c*-glycyl-L-tyrosyl and *c*-glycyl-L-phenylalanyl and compared them with the spectra of glycyl-L-tyrosine and L-phenylalanylglycine, respectively. Within the two pairs the spectra are essentially identical, there being nothing indicative of a charge-transfer absorption band in the spectra of the cyclic peptides. The donor–acceptor possibility should not be ruled out entirely, however, because the increased preference of *c*-L-tyrosylglycyl anion for the folded form (ΔH = −5 kcal/mole) is consistent with a contribution from donor–acceptor interaction, the phenolate ion acting as donor.

At this point, it seems likely that the folded form of the cyclic dipeptides is largely stabilized by interaction of the two amide dipoles with the polarizable π electron system of the aromatic side chain. Proton magnetic resonance studies of dipolar solutes, including amides, in aromatic solvents have consistently shown that there is an attraction between solute and solvent.[24–29] This appears to result from dipole–induced dipole interactions. Aromatic molecules are more polarizable in the molecular plane than perpendicular to that plane; the chemical shifts observed for a variety of dipolar solutes show that the solute dipole tends to lie in a plane parallel to the aromatic molecular plane. With amides, it appears that the permanent dipole is oriented with the negative end (oxygen) toward the periphery of the aromatic ring and the positive end (nitrogen) toward the center.[27,28] ΔH for the association of dimethylformamide and toluene is estimated at between −1 and −2 kcal/mole.[28] Although the desirable parallel–planar arrangement of phenyl and diketopiperazine rings is not attainable in the present case, the projections of the amide dipoles[30] on the aromatic plane do have a component in the favored, positive to center, direction.

In addition to the dipole–induced dipole interaction, the folded form of the aromatic-containing peptides may be further stabilized by dispersion forces acting between the polarizable π system of the aromatic ring and the polarizable π systems of the two amide groups.

If the folded form of the aromatic-containing cyclic dipeptides is, in fact, stabilized by dipole–induced dipole and dispersion forces, it would seem that there should be solvents that would tend to unfold the molecules because of favorable solute–solvent interactions of the same type. On the basis of the dipole–induced dipole mechanism, dimethyl sulfoxide (μ = 3.9[31]) should compete (with the diketopiperazine ring) for the aromatic ring more successfully than water (μ = 1.84) or trifluoroacetic acid (μ = 2.28[32]). Also, if dispersion forces are important, because of its greater polarizability, dimethyl sulfoxide should again tend to destabilize the folded form more than would trifluoroacetic acid. Any such unfolding tendency in dimethyl sulfoxide is not obvious. Whatever the mechanism producing the folded form, an amide solvent ought to favor the unfolded forms. However, one preliminary measurement of *c*-glycyl-L-phenylalanyl in formamide at 50° indicates that the α protons of the glycine residue are nonequivalent by 0.65 ppm, a separation comparable, for that temperature, to that observed in any of the other solvents used. It seems, then, that there must be some short range, highly directional effects favoring intramolecular interactions.

It will be of interest to examine this intramolecular amide–aromatic interaction in systems of different geometry than the one discussed here.

Comments on Specific Cyclic Peptides. ***c*-L-Histidyl-L-tyrosyl.** Some years ago, in a study of the reaction of *p*-nitrophenyl acetate with this cyclic dipeptide, we observed facile acetyl transfer from histidylimidazole to tyrosyl hydroxyl; this transacetylation competed very favorably with hydrolysis of the acetylimidazole function.[33] In the light of current observations facile intramolecular acyl transfer is understandable, since the necessary rotation of the tyrosyl C_α–C_β bond is already stabilized by the aromatic–diketopiperazine interaction.

It is also interesting to examine the observed spin–spin coupling constants between the β-methylene and α protons of the histidyl residue. These are reported for *c*-L-histidyl-L-tyrosyl in Table V. Were rotation

(17) C. Tanford, *J. Am. Chem. Soc.*, 84, 4240 (1962).
(18) O. Sinanoglu and S. Abdulnur, *Federation Proc. Suppl.*, 24, 512 (1965).
(19) From data of E. A. Kauck and A. R. Diesslin, *Ind. Eng. Chem.*, 43, 2332 (1951).
(20) H. M. Scholberg, R. A. Guenthner, and R. I. Coon, *J. Phys. Chem.*, 57, 923 (1953).
(21) T. B. Douglas, *J. Am. Chem. Soc.*, 70, 2001 (1948).
(22) C. Marsden and S. Mann, "Solvents Guide," 2nd ed, Interscience Publishers, Inc., New York, N. Y., 1962.
(23) A. Cary Model 14 recording ultraviolet spectrophotometer was used.
(24) T. L. Brown and K. Stark, *J. Phys. Chem.*, 69, 2679 (1965).
(25) P. Diehl, *J. Chim. Phys.*, 61, 199 (1964).
(26) J. V. Hatton and R. E. Richards, *Mol. Phys.*, 5, 139, 153 (1962).
(27) W. G. Schneider, *J. Phys. Chem.*, 66, 2653 (1962).
(28) J. V. Hatton and W. G. Schneider, *Can. J. Chem.*, 40, 1285 (1962).
(29) Gurudata, R. E. Klinck, and J. B. Stothers, *ibid.*, 45, 213 (1967).
(30) 2-Pyrrolidone has a dipole moment of 3.5 D.: R. Huisgen and H. Walz, *Ber.*, 89, 2616 (1956). This is probably also a reasonable value for the moment of an individual amide in diketopiperazine; the whole ring, if planar, would have no dipole.
(31) F. A. Cotton and R. Francis, *J. Am. Chem. Soc.*, 82, 2986 (1960).
(32) J. H. Gibbs and C. P. Smyth, *ibid.*, 73, 5115 (1951).
(33) K. D. Kopple, R. R. Jarabak, and P. L. Bhatia, *Biochemistry*, 2, 958 (1963).

about the histidyl C_α–C_β bond sufficiently rapid, only an average of *trans* and *gauche* couplings would appear. If the tyrosyl side chain occupies the space over the diketopiperazine ring, preventing rotation about a full circle, oscillation between the two remaining conformations might still result in time-averaging the observed couplings. It is noteworthy that completely averaged values are only observed in trifluoroacetic acid at the higher temperatures; oscillation of the imidazolymethyl side chain must therefore be considerably inhibited, and more inhibited in water, which seems reasonable, than in trifluoroacetic acid.

c-L-Valyl-L-tyrosyl. Only single temperature measurements of the spectra of this peptide are available at present, but it appears, from data in Tables VI and VII, that the valyl β-hydrogen is shifted to higher field by a noticeably smaller amount than one would estimate if conformation IA were as favored as in the other peptides. This is especially so in dimethyl sulfoxide and trifluoroacetic acid. In its version of conformation IA, the valyl peptide must have all rotation about the C_α–C_β bond removed, since a γ-methyl and the aromatic ring cannot simultaneously occupy the space over the diketopiperazine ring. In contrast, leucyl and histidyl residues have two C_α–C_β rotamers allowed to them in the IA conformation. The additional restriction in the valyl case may account for reduced stability of the folded form.

L-Tyrosyl-L-tyrosyl. The four β and two α protons of this peptide give rise to a single ABX pattern with the chemical shifts indicated in Table VI. This spectrum does not suggest major contributions from conformers of type IA. The two kinds of β protons differ by 0.7 ppm; the more shielded has an apparent coupling to the α proton of 8 Hz; the less shielded, one of 4 Hz. If conformations of type IA were important and persisted long enough to prevent averaging of this nonequivalence, there would be separate resonance patterns for the two kinds of methylene. As just indicated, this is not observed. Therefore, it seems that a preferred conformation is likely to be one in which each hydroxyphenyl tends to associate with one amide group, sharing the space over the diketopiperazine ring in such a fashion that the two β-methylenes have identical environments.

The same conclusion may be drawn for *c*-L-phenylalanyl-L-phenylalanyl, which has a similar nmr spectrum.

Acknowledgment. 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. The initial observations were made by K. K. during a stay in the laboratory of Professor Melvin Calvin, University of California, Berkeley, in 1965; we wish to express gratitude for the facilities extended to us at that time.

Steric Control of Geometrical Isomerism in Cytosine Cations. A Nuclear Magnetic Resonance Study

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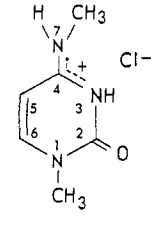
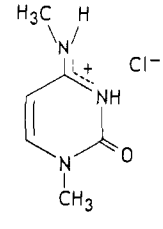
Abstract: Proton nmr spectra are reported for several methyl derivatives of cytosine hydrochloride, with emphasis on the geometrical isomerism resulting from hindered rotation about the bond joining the amino group to the ring. A definitive assignment is made of the spectra of the two geometrical isomers of 1,7-dimethylcytosine hydrochloride. Regularities are pointed out in the spectral changes resulting from introduction of methyl substituents and from temperature variation.

In a recent detailed nmr study of some derivatives of 1-methylcytosine, Becker, Miles, and Bradley[1] demonstrated that the rotation of the amino group in various species is restricted, and that this restriction leads to the observation of two isomers of 1,7-dimethylcytosine hydrochloride (or hydriodide) (I).

The spectrum of 1,7-dimethylcytosine hydrohalide showed a long-range spin coupling of 0.7 Hz[2] between the protons attached to atoms in the 6 and 7 positions in more abundant isomer. The spectra of the two isomers were assigned to IA and IB on the assumption that the all-*trans* arrangement of the protons in IB would lead to an observable long-range coupling $J_{6H,7H}$, while in IA $J_{6H,7H}$ would be less than 0.1 Hz.



IA



IB

We present here independent experimental evidence that this assignment is correct.

(1) E. D. Becker, H. T. Miles, and R. B. Bradley, *J. Am. Chem. Soc.*, 87, 5575 (1965).
(2) 1 Hz = 1 cps.