Proton Magnetic Resonance Assignments and Conformational Studies of the Polypeptide Antibiotic [Di-*N*-methylleucine]gramicidin S Dihydrochloride

N. G. Kumar,[1a] N. Izumiya,[1b] M. Miyoshi,[1c] H. Sugano,[1c] and D. W. Urry*[1a]

Contributions from the Laboratory of Molecular Biophysics and the Cardiovascular Research and Training Center, University of Alabama in Birmingham, Birmingham, Alabama 35294, Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka 812, Japan, and the Department of Synthetic Chemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., Kashima, Higashiyodogawa, Osaka 532, Japan.
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Abstract: The 220-MHz proton magnetic resonance spectrum of the cyclic decapeptide antibiotic, [di-*N*-methylleucine]gramicidin S, is reported and all the resonances have been assigned to specific protons of the constituent amino acids. Three methods, temperature dependence and solvent mixture (methanol–trifluoroethanol and dimethyl sulfoxide–trifluoroethanol) dependence of peptide proton chemical shifts and proton deuteron exchange, have been utilized to delineate peptide NH protons. The results of the above methods, taken together with the observed vicinal α-CH–NH coupling constants and chemical shifts, indicate that in methanol the peptide proton of the D-Phe residue is exposed to the solvent and those of L-Orn and L-Val are solvent shielded and transannularly hydrogen bonded; whereas in dimethyl sulfoxide, which is a good proton-accepting solvent, the peptide proton of the L-Val residue is relatively less solvent shielded and that of L-Orn is well shielded from the solvent and transannularly hydrogen bonded as in the case of the L-Leu peptide proton of gramicidin S. As in methanol the amide proton of the D-Phe residue is exposed to the solvent in dimethyl sulfoxide. In both methanol and dimethyl sulfoxide C_2 symmetry is maintained on the NMR time scale. The proposed conformation in methanol has four ten-atom hydrogen-bonded β turns. The hydrogen-bonding pattern forms a partial turn of a right-handed 3_{10} helix related by twofold axis of symmetry. In dimethyl sulfoxide, there are two strongly hydrogen-bonded β turns and two internal protons which may be involved in weak hydrogen-bonded β turns.

Three conformations for gramicidin S in the solid state have been described by Hodgkin and Oughton[2] as consistent with their X-ray diffraction data. One of the three conformations was the antiparallel β-type conformation with four intramolecular hydrogen bonds between the two pairs of L-Val and L-Leu residues, and the β-type conformation was proposed on the basis of ease of cyclization by Schwyzer.[3] The same secondary structure was proposed in solution on the basis of NMR studies by Stern et al.;[4] also, the β-type conformation of Hodgkin and Oughton was independently arrived at in this laboratory[5] for solution using ^{1}H NMR. On the basis of peptide NH exchange[4,6] and of temperature dependence of the circular dichroism pattern[7] the conformation of gramicidin S is unusually stable.

[Di-*N*-methylleucine]gramicidin S dihydrochloride has been synthesized by Sugano et al.[8,9] to elucidate structure–biological activity relationships of gramicidin S and its analogs. They found that the antimicrobial activities of [di-*N*-MeLeu]gramicidin S dihydrochloride were nearly the same as those of gramicidin S demonstrating that the peptide NH protons of the L-Leu residues are not necessary for exhibiting the biological activity. This presents an opportunity to further consider the conformation and to throw some light on the question of the relationships between specific conformations and biological activity.

In the present work a conformational model, in which the peptide NH protons of the L-Leu residues do not participate in the intramolecular hydrogen bondings, is proposed for [di-*N*-methylleucine]gramicidin S dihydrochloride on the basis of NMR data and steric considerations. It is the purpose of this work to present proton magnetic resonance assignments, which is a prerequisite for extracting structural information contained in the ^{1}H NMR spectrum, and also to present ^{1}H NMR studies designed to determine the conformational aspects of [di-*N*-MeLeu]gramicidin S dihydrochloride in solution. The spectral features for distinguishing between solvent-exposed and solvent-shielded peptide protons are discussed as they apply to [di-*N*-MeLeu]gramicidin S dihydrochloride. Detailed discussion of the solution conformation compatible with the experimental NMR data is given.

Experimental Section

Proton magnetic resonance spectra were recorded on a Varian Associates HR-220 spectrometer equipped with an SS-100 computer system. Homonuclear spin-decoupling experiments were performed with a field-tracking decoupling accessory designed in this laboratory; by this accessory a resonance may be irradiated continuously as the remainder of the spectrum or a section is either singly scanned manually or multiscanned on an expanded scale with an SS-100 computer system. This latter technique is extremely useful for decoupling α,β, β,γ, γ,δ proton resonances of a specific amino acid residue from each other in a polypeptide with a number of different amino acid residues. Chemical shifts were measured relative to tetramethylsilane as an internal reference. The chemical shift difference between resonances of ethylene glycol or methanol was used to determine the probe temperature to within $\pm 1°$. The synthesis of [3,3′-di-*N*-methylleucine]gramicidin S dihydrochloride has been reported elsewhere by Sugano et al.[8,9]

Supplementary material describing details of spectral analysis is available (see paragraph at end of paper regarding supplementary material).

Results

Spectral Assignments. The 220-MHz proton magnetic resonance spectrum of [di-*N*-methylleucine]gramicidin S dihydrochloride in DMSO-d_6 at 19° is presented in Figure 1a together with the complete assignment of resonances. The insert in Figure 1b is the α-CH region of [di-*N*-MeLeu]gramicidin S dihydrochloride in DMSO-d_6 containing 10% D_2O at 19°. The assignment of the proton resonances of [di-*N*-MeLeu]gramicidin S in DMSO-d_6 is quite different from gramicidin S[4] in DMSO-d_6. Spectral analysis of a polypeptide with an increasing number of different amino acid residues is often complicated from overlap of α,β,γ proton resonances of different amino acid residues in

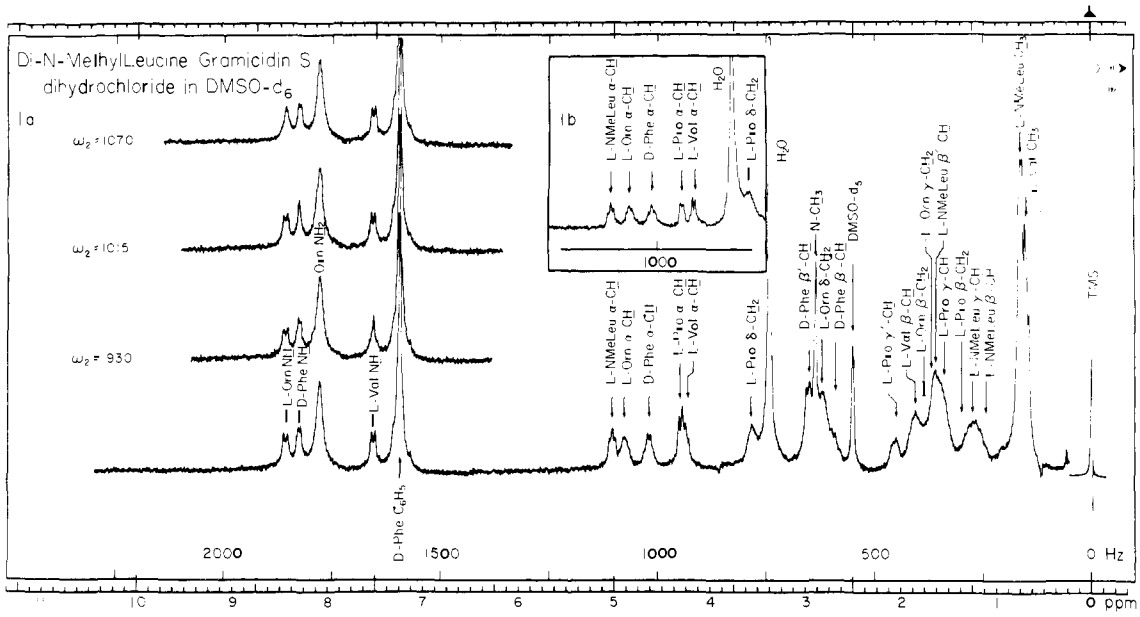

Figure 1. Complete 220-MHz proton magnetic resonance spectrum of 3% (w/v) [di-*N*-methylleucine]gramicidin S dihydrochloride in dimethyl sulfoxide at 19°. Also included in Figure 1 are (a) the peptide NH resonances in DMSO-d_6 with decoupling radiofrequency power applied at f_2, the resonance frequencies of the α-CH resonances, f_2 off; f_2 = 930 Hz, L-Val; f_2 = 1015 Hz, D-Phe; f_2 = 1070 Hz, L-Orn; and (b) 220-MHz ^{1}H NMR spectrum of the α-CH region in dimethyl sulfoxide containing 10% D_2O at 19°.

the α,β,γ region of the spectrum. Individual proton transitions in a particular amino acid are near other transitions of other residues, thereby complicating assignment of the β-CH proton resonances which is the basis for the assignment of the α-CH protons. Hence, the strength of f_2 power used for spin decoupling in the α,β,γ region of the spectrum was less than that utilized for uncomplicated double resonance experiments. One simplifying spectral feature of [di-*N*-methylleucine]gramicidin S dihydrochloride is that the α-CH and NH protons of each of the pair of corresponding amino acids are magnetically equivalent and the pairs of residues are related by a C_2 symmetry axis as in the case of gramicidin S.

Assignments of individual resonances to specific protons or groups of protons of the constituent amino acids (Figure 1a) were achieved in the majority of cases by performing homonuclear spin-decoupling experiments in different solvents at different temperatures with a field-tracking decoupling accessory. The coupled resonances were then followed as a function of temperature or solvent to determine their positions at the temperature and solvent of interest.

With a completely assigned proton magnetic resonance spectrum of [di-*N*-MeLeu]gramicidin S in DMSO-d_6, the assignment of the amide protons in CH_3OH was confirmed from DMSO-d_6–CH_3OH solvent mixture dependence of the peptide proton chemical shift as a function of the mole percent of CH_3OH. Assignment of the remaining resonances ($\alpha,\beta,\gamma,\delta$, etc.) in CH_3OH was achieved by spin-decoupling experiments in CD_3OD. The resonance assignments and coupling constants of all the amino acid residues in different solvents are listed in Tables I–III.

Temperature Dependence. The temperature dependence of the peptide proton resonances of [di-*N*-methylleucine]-gramicidin S dihydrochloride in CH_3OH and DMSO-d_6 is presented in Figures 2 and 3. The resonances are numbered in the order of increasing field as they appear in DMSO-d_6. Resonances of protons attached to nitrogen broaden out when the rate of proton exchange between the peptide NH moiety, δ-N^+H_3, and residual H_2O becomes comparable to the difference in their respective chemical shifts. The L-Val peptide proton broadens below −12° but sharpens with increasing temperature. The L-Orn and D-Phe amide protons did not exhibit broadening between the temperature range −22 to 50°. The most rapidly exchangeable protons δ-N^+H_3 of L-Orn resonate as a relatively sharp resonance at and below 6° (6 to −22°) but broaden with increasing temperature (20–50°). This indicates that the temperature dependence measurements of L-Orn and D-Phe were performed below their coalescence temperature and that of L-Orn δ-N^+H_3 below and near its coalescence temperature. In DMSO-d_6, the L-Orn amide proton is well resolved between 17 to 65° but becomes broader at 80°. It is possible that the L-Orn amide proton is influenced by the L-Orn δ-N^+H_3 group. At 80°, the L-Orn amide proton resonates at 1815 Hz as a broad resonance, and the D-Phe amide proton exhibits some line broadening, whereas the L-Val peptide proton resonates at 1516 Hz as a well-resolved sharp doublet relative to the L-Orn and D-Phe peptide protons. The origin of this differential broadening may be due to quadrupole relaxation of the ^{14}N nuclei or peptide proton exchange. We have not determined the effect of ^{14}N relaxation on the peptide proton line width by irradiating at the ^{14}N resonance frequency. If ^{14}N nuclei in peptides relax efficiently, then the broadening of peptide proton is not due to the coupling of peptide proton to the relaxing ^{14}N nucleus.

Increasing the temperature of a folded, compact molecule such as [di-*N*-methylleucine]gramicidin S dihydrochloride causes a change in the vibrational states of the molecule, so that the average magnetic environment experienced by the peptide protons could change. Therefore, it is important to conduct temperature-dependence experiments in different solvents in which relative chemical shift temperature dependence has been shown to be different for solvent-exposed and solvent-shielded peptide protons, with linear temperature dependence as the temperature is changed. The temperature coefficients ($d\delta/dT$, ppm/deg) of the peptide NH protons in DMSO-d_6, CH_3OH, CH_3OH–DMSO-d_6 (1:1 v/v), and TFE are listed in Table IV. The temperature dependence plots in DMSO-d_6 and CH_3OH are different.

In DMSO-d_6, the lowest field L-Orn peptide proton exhibited a small temperature coefficient (−0.0034 ppm/deg)

Table I. Chemical Shifts of the Proton Resonances of [Di-*N*-methylleucine]gramicidin S Dihydrochloride[a]

Amino acid residues	Solvent	ν_α	ν_β	$\nu_{\beta'}$	ν_γ	$\nu_{\gamma'}$	ν_δ	Other ν
L-*N*-MeLeu	DMSO-d_6	1102	245	360	275		CH_3 ~165	NCH_3 ~635
	DMSO-d_6 + 10% D_2O	1107	245	365	~275		~163	~650
	CD_3OD	1135	290	380	305		~193	~680
	TFE-d_3	1150		370	~300		~205	~680
L-Orn	DMSO-d_6	1075	385		370		620	$^+NH_3$ ~1780
	DMSO-d_6 + 10% D_2O	1065	390				625	
	CD_3OD	1105					660	
	TFE-d_3	1135					~670	
D-Phe	DMSO-d_6	1017	595	650				Ar ~1595
	DMSO-d_6 + 10% D_2O	1013	590	~650				~1597, 1605
	CD_3OD	1022	~665					~1600, 1605
	TFE-d_3	1055	~595	~650				~1615, 1585
L-Pro	DMSO-d_6	950	300		340	450	785	
	DMSO-d_6 + 10% D_2O	945	305		~340	~445	785	
	CD_3OD	950	350		~390	~575	815	
	TFE-d_3	950	395				815	
L-Val	DMSO-d_6	935	405		CH_3 ~150			
	DMSO-d_6 + 10% D_2O	915	420		~163			
	CD_3OD	875	460		~193			
					~208			
	TFE-d_3	905	450		~205			

[a] Chemical shifts are reported in hertz at 220 MHz; spectra in DMSO-d_6 and DMSO-d_6 containing 10% D_2O were recorded at 19° and in CD_3OD and TFE-d_3 at 17°.

Table II. Coupling Constants of the Proton Resonances of [Di-*N*-methylleucine]gramicidin S Dihydrochloride[a]

Amino acid residues	Solvent	$J_{\alpha,\beta}$	$J_{\alpha,\beta'}$	$J_{\beta,\beta'}$	$J_{\beta,\gamma}$	$J_{\delta,\gamma}$	$J_{\delta,\delta'}$
L-*N*-MeLeu	DMSO-d_6	5.5 ± 0.5	~8.0		4.0 ± 0.5		
	DMSO-d_6 ± 10% D_2O	5.7 ± 0.3	8.1 ± 0.3		3.5 ± 0.5	$J_{CH_3-\gamma\text{-}CH}$ ~ 7.0	
	CD_3OD	5.5 ± 0.5	~8.0		3.5 ± 0.5	~7.0	
	TFE-d_3	7.5 ± 0.5	11.5 ± 0.5			~7.0 ± 0.5	
L-Orn	DMSO-d_6	~8.0	~5.0			6.3 ± 0.3	
	DMSO-d_6 + 10% D_2O	~8.0	~4.5 ± 0.5			6.0 ± 0.5	
	CD_3OD	~8.0	~5.0				
	TFE-d_6[c]						
DPhe	DMSO-d_6	~5.0	8.5 ± 0.5	~10.0			
	DMSO-d_6 + 10% D_2O	4.5 ± 0.5	~8.0	10.0 ± 0.5			
	CD_3OD	~8.0	~8.0				
	TFE-d_3	5.5 ± 0.5	~8.0				
L-Pro	DMSO-d_6	7.5 ± 0.3	~0		~3.6	5.5 ± 0.5	~10.0
	DMSO-d_6 + 10% D_2O[b]	7.6 ± 0.4	~0		~3.0		
	CD_3OD	6.5	~0		~3.0	5.5 ± 0.5	~10.0
	TFE-d_3[b]	6.5	~0				
L-Val	DMSO-d_6	7.5 ± 0.5			$J_{\beta\text{-}CH-CH_3}$ ~ 6.8		
	DMSO-d_6 + 10% D_2O	6.9 ± 0.3			~7.0		
	CD_3OD	8.3 ± 0.2			~6.8 ± 0.3		
	TFE-d_3	7.3 ± 0.2			~7.0 ± 0.5		

[a] Coupling constants are reported in hertz. [b] In DMSO-d_6 containing 10% D_2O, the L-Pro δ-CH_2 protons overlap with the residual H_2O resonance and in TFE-d_3 with the methylene protons of the solvent. [c] In TFE-d_3, the L-Orn α-CH resonance overlaps with the residual H_2O resonance at 17 and 42° and with the L-*N*-MeLeu α-CH protons at 7°.

with a 0° intercept at 8.53 ppm. The temperature coefficients and 0° intercepts of the D-Phe and the upfield L-Val amide protons are −0.0098 (0° intercept, 8.50 ppm) and −0.007 ppm/deg (0° intercept, 7.63 ppm), respectively. In CH_3OH, the L-Orn and L-Val peptide protons exhibited identical, relatively small temperature coefficients (−0.0048 ppm/deg) with 0° intercepts at 8.45 and 7.47 ppm, respectively, whereas the D-Phe amide proton exhibited a relatively large temperature coefficient (−0.0116 ppm/deg) with a 0° intercept at 8.55 ppm. It is important to be aware that traces of acid or base in the solution may alter the absolute values of the temperature coefficients of the solvent-exposed and partially solvent-shielded peptide protons. This consideration is of particular concern when broadening is observed with change in temperature. Also, delineation depends on the proton-accepting capacity of the solvent. In TFE, which is relatively a poor proton-accepting solvent, differentiation between the temperature coefficients of solvent-exposed and solvent-shielded peptide protons is relatively small as compared to the delineation in DMSO-d_6 and CH_3OH.

Effect of Solvent Variation on the Peptide Proton Chemical Shift and $J_{\alpha\text{-}CH\text{-}NH}$ Coupling Constant. Solvent mixture dependence of the peptide proton chemical shift in dimethyl sulfoxide–trifluoroethanol and methanol–trifluoroethanol is presented in Figures 4 and 5. On addition of TFE to DMSO, the chemical shift of the L-Orn peptide proton is virtually uneffected (<2 Hz) up to 60 mol % of TFE. At this point, there is an abrupt discontinuity in the curve, and on further increasing the mol % of TFE to 100, the peptide proton exhibits a large upfield shift of ~240 Hz (>1 ppm). The D-Phe peptide proton shifts dramatically upfield (233 Hz, >1 ppm) in a more nearly monotonic fashion. From 0 to 60 mol % of TFE, the L-Val peptide proton exhibits an

Table III. Proton Magnetic Resonance Parameters of the Peptide NH Resonances of [Di-*N*-methylleucine]gramicidin S Dihydrochloride

Amino acid residue	Solvent	Coupling constant[a] ($^3J_{\alpha\text{-CH-NH}}$, Hz)	Chemical shift,[b] ν_{NH}, Hz (ppm)
L-Orn	DMSO-d_6	9.0 ± 0.5	1860 (8.45)
	CH_3OH	9.2 ± 0.3	1852 (8.42)
	DMSO-d_6–CH_3OH (1:1 v/v)	8.5 ± 0.5	1860 (8.45)
	TFE[c]		
D-Phe	DMSO-d_6	6.0	1827 (8.30)
	CH_3OH	4.3 ± 0.3	1865 (8.48)
	DMSO-d_6–CH_3OH (1:1 v/v)	4.5	1834 (8.34)
	TFE[c]	4.5 ± 0.5	1566 (7.12)
L-Val	DMSO-d_6	8.3 ± 0.2	1654 (7.52)
	CH_3OH	8.0	1633 (7.42)
	DMSO-d_6–CH_3OH (1:1 v/v)	8.0	1616 (7.35)
	TFE	8.0	1518 (6.90)

[a] Coupling constants within experimental error remained constant between 18 and 65° in DMSO-d_6, −2–50° in CH_3OH, −10–50° in DMSO-d_6–CH_3OH (1:1 v/v), and 19–67° in TFE. In DMSO-d_6, the L-Val peptide proton is well resolved even at 80° and its $J_{\alpha\text{-CH-NH}}$ coupling constant within experimental error remained constant between 18 and 80°. [b] Spectra in DMSO-d_6, CH_3OH, DMSO-d_6–CH_3OH (1:1 v/v), and TFE were recorded at 18, 4, 4, and 41°, respectively. All the chemical shifts are at 220 MHz and are reported at those temperatures where all the peptide proton resonances are well resolved and separated. [c] In TFE, the L-Orn peptide proton overlaps with the aromatic protons between 19 and 67°, but at 19°, in the DMSO–TFE mixture containing 68 mol % TFE, the observed $J_{\alpha\text{-CH-NH}}$ coupling constant is ~8.5 Hz.

upfield shift of ~75 Hz, and from 60 to 100% it shifts upfield by less than ~40 Hz. Above 60 mol % of TFE, the L-Orn and D-Phe peptide protons started broadening as the mol % of TFE was increased, whereas the L-Val peptide proton did not show any broadening even at 100% of TFE. On increasing the mol % of TFE, very little change in the $J_{\alpha\text{-CH-NH}}$ coupling constants of L-Orn and L-Val is observed; however, the $J_{\alpha\text{-CH-NH}}$ of the D-Phe residue decreased from 6 Hz in DMSO to ~4.5 Hz in TFE. A distinctive spectral feature of the changes accompanying the solvent transition was the separation of the aromatic protons of D-Phe from a closely spaced multiplet in DMSO into two multiplets separated by ~30 Hz in TFE.

On addition of TFE to CH_3OH, the D-Phe peptide proton exhibited a large upfield shift (~216 Hz, ~1 ppm) in a monotonic fashion. The L-Val peptide proton showed a relatively small upfield shift (~79 Hz), and the L-Orn peptide proton exhibited a large upfield shift of ~217 Hz (~1 ppm). However, the observed $^3J_{\alpha\text{-CH-NH}}$ coupling constants, within experimental error, remained constant on increasing the mole percent of TFE.

In the DMSO–CH_3OH solvent mixture titration, the peptide proton resonances respond to the increasing mole percent of CH_3OH in a complex manner. The observed curves have a complicated form and show considerable departure from simple monotonic curves exhibited by peptide protons in the absence of conformational change. The conformational changes accompanying the solvent transition result in an abrupt discontinuity in the titration curves. Addition of methanol to dimethyl sulfoxide solution results in a conformational change without cis–trans isomerization of the D-Phe-L-Pro peptide bond. On changing from DMSO to CH_3OH, the observed $J_{\alpha\text{-CH-NH}}$ coupling constants of 8.5 ± 0.5 Hz for the L-Orn and 8.0 Hz for the L-Val residues remained about the same, but the $J_{\alpha\text{-CH-NH}}$ of the

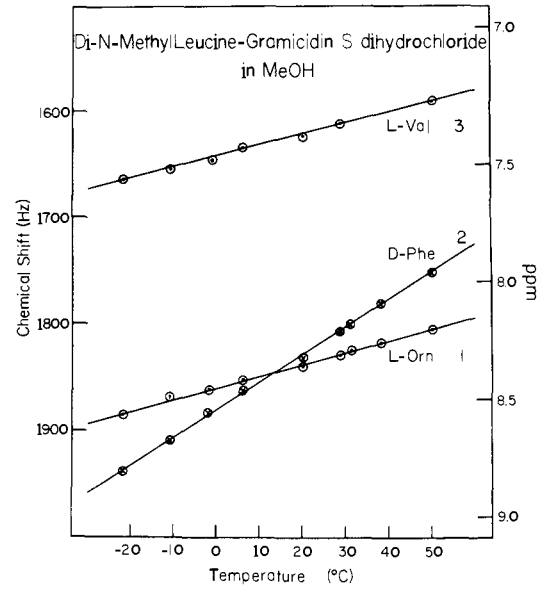

Figure 2. Temperature dependence of the peptide proton resonances in methanol.

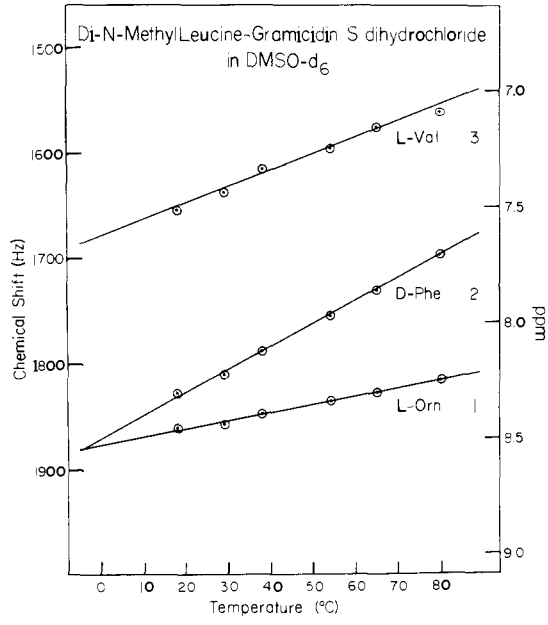

Figure 3. Temperature dependence of the peptide proton resonances in dimethyl sulfoxide.

D-Phe residue decreased from 6.0 Hz in DMSO to ~4.0 Hz in CH_3OH.

H–D Exchange. As H–D exchange rates are subject to general acid–base catalysis, it is important to note that the L-Orn δ-NH_2 groups in [di-*N*-methylleucine]gramicidin S dihydrochloride are present as salts of a strong acid (hydrochloride, $-N^+H_3Cl^-$) which can catalyze H–D exchange. Hence, the relative exchange rates with hydroxylic solvents were very fast and delineation between the exchange rate of the peptide protons in DMSO-d_6 containing 10% D_2O was not achieved. As expected, at 19°, in DMSO-d_6 containing 10% D_2O, all the peptide protons exchanged in less than 15 min. The presence of δ-N^+H_3 groups on the L-Orn residues appears to catalyze the H–D exchange of the L-Orn peptide proton relatively faster than the peptide protons of the other amino acid residues.[10] This H–D exchange result still allows the L-Orn peptide proton in DMSO-d_6 to be largely solvent-shielded. In TFE-d_3, the H–D exchange rates are

Table IV. Temperature Dependence of the Peptide Proton Chemical Shifts in Different Solvents

Solvent	L-Orn		D-Phe		L-Val	
	Temp coeff, ppm/deg	0° intercept, Hz (ppm)	Temp coeff, ppm/deg	0° intercept, Hz (ppm)	Temp coeff, ppm/deg	0° intercept, Hz (ppm)
DMSO-d_6	−0.0034	1875.6 (8.53)	−0.0098	1870.0 (8.50)	−0.007	1678.0 (7.63)
CH_3OH	−0.0048	1859.5 (8.45)	−0.0116	1880.3 (8.55)	−0.0048	1642.3 (7.47)
CH_3OH–DMSO-d_6 (1:1 v/v)	−0.0041	1864.6 (8.48)	−0.0116	1849.2 (8.40)	−0.0047	1625.0 (7.39)
TFE	−0.0075	1654.0 (7.52)	−0.0060	1625.6 (7.39)	−0.0037	1554.6 (7.07)

relatively slow, which is an indication of its low basicity. At 41°, in TFE-d_3, after ~40 min the D-Phe peptide proton had almost completely exchanged, whereas the L-Val amide proton exchanged almost completely after 105 min. Unfortunately, the L-Orn peptide proton is under the aromatic protons, and its exchange rate could not be determined. It appears in TFE-d_3, therefore, that the L-Val peptide proton exchanged slowly relative to the D-Phe NH proton.

Discussion

Three methods for identification of the solvent-exposed and solvent-shielded peptide protons, temperature dependence of peptide proton chemical shifts, effect of solvent variation on the peptide proton chemical shift, and proton–deuterium exchange rates taken together with $J_{\alpha\text{-CH-NH}}$ coupling constants, have been shown to be effective in evaluating conformation when applied to small, relatively rigid, cyclic polypeptides.[4-6,11-21] In gramicidin S, there are no conformational changes and agreement between the three methods is good for the identification of the solvent-exposed and intramolecularly hydrogen-bonded peptide protons. The H–D exchange rates of the solvent-shielded and solvent-exposed peptide protons of gramicidin S are well delineated. It is important to note that there are three structural features present in gramicidin S which contribute to this delineation. Firstly, it is the L-Orn residue with the solvent-exposed peptide proton which has the catalytic δ-N^+H_3 group and not the residues with the solvent-shielded peptide protons. Secondly, the amino groups were present as the free base[5] or as salts of a weak acid (acetate salt)[4], and not a strong acid, and thirdly the peptide backbone is relatively rigid. For [di-*N*-methylleucine]gramicidin S dihydrochloride, it is the L-Orn residues with the solvent-shielded peptide protons, which have the δ-NH_2 groups; moreover, these groups are present as salts of a strong acid (hydrochloride, $-N^+H_3Cl^-$). The above data on [di-*N*-methylleucine]gramicidin S dihydrochloride provide an opportunity to compare the behavior of each amide resonance in the presence of conformational changes, and also allow discussion of possible problems that may be encountered when dealing with solvent-induced conformational changes.

As will be discussed below, NMR experimental data point to a folded conformation for the ring of [di-*N*-MeLeu]gramicidin S dihydrochloride. With such a folded conformation, one must be careful in interpreting chemical shift temperature dependence results; slight rotation about the bonds can alter relative spatial orientation of peptide protons and nearby peptide moieties and can cause significant chemical shift changes due to magnetic anisotropy of the nearby peptide moiety or due to ring currents from the nearby phenyl rings. However, for [di-*N*-MeLeu]gramicidin S dihydrochloride, between the temperature range of 18–80° in DMSO-d_6 and −10 to 50° in MeOH, the $^3J_{\alpha\text{-CH-NH}}$ coupling constants, within experimental error, remained constant and the peptide proton chemical shifts exhibited linear temperature dependences. This indicates that the average peptide backbone conformation, as reflected in the α-CH–NH dihedral angles, remained essentially unchanged in each of the solvents. The temperature dependence and the solvent mixture titrimetric methods have the ability to demonstrate conformational changes accompanying the solvent transition.

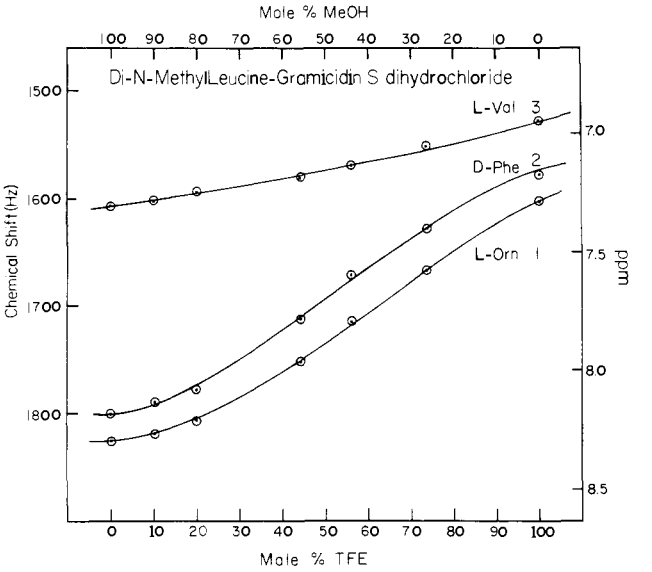

Figure 4. Solvent dependence of the peptide proton chemical shifts in the methanol–trifluoroethanol mixture at 30°.

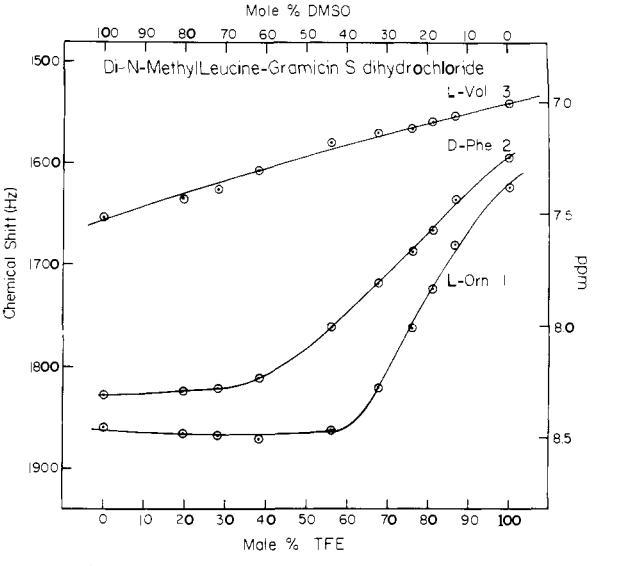

Figure 5. Solvent dependence of the peptide proton chemical shifts in the dimethyl sulfoxide–trifluoroethanol mixture at 19°.

Delineation of the Peptide NH Moieties in Dimethyl Sulfoxide. In DMSO-d_6, the temperature coefficient of the L-Orn peptide proton is small ($d\delta/dT$ = −0.0034 ppm/deg) and is of the same magnitude as the internally hydro-

gen-bonded L-Leu peptide proton of gramicidin S. On going from DMSO to ~60 mol % TFE (Figure 5), the peptide proton chemical shift is insensitive to this large solvent variation, but on further increasing the mole percent of TFE, there is an abrupt discontinuity in the curve indicative of a solvent-induced conformational change. There is a change in the solvent structure at ~60 mol % of TFE in DMSO, suggested by perturbation of the solvent resonances themselves. This change in the solvent structure may supply the free energy for conformational change in [di-*N*-MeLeu]-gramicidin S. In DMSO-d_6 at 18°, the L-Orn peptide proton resonates at the lowest field position (8.45 ppm). The chemical shift of the L-Leu peptide proton of gramicidin S in DMSO-d_6 is 8.35 ppm. By the temperature coefficient criteria and solvent dependence of the peptide proton chemical shift, the L-Orn peptide proton is delineated as solvent-shielded, possibly through the formation of a strong intramolecular hydrogen bond, as reflected in the chemical shift (8.45 ppm).[22]

In DMSO-d_6 at 18°, the D-Phe peptide proton resonates at 8.3 ppm; it exhibited a large negative temperature coefficient (−0.0098 ppm/deg) and a dramatic upfield shift (~1 ppm) on addition of TFE to DMSO, as did the solvent-exposed D-Phe peptide protons of gramicidin S. The magnitude of this temperature coefficient is slightly larger than the solvent-exposed D-Phe peptide protons of gramicidin S. The chemical shift in DMSO (8.3 ppm), the temperature coefficient, and the DMSO → TFE upfield shift indicate that the D-Phe peptide proton is solvent-exposed.

In DMSO-d_6, a good proton-accepting solvent, the L-Val peptide proton exhibited a temperature coefficient (−0.007 ppm/deg) intermediate between a solvent-exposed (D-Phe) and a solvent-shielded peptide proton (L-Orn). On addition of TFE to DMSO, it exhibited lesser upfield shift (~113 Hz) than D-Phe. The chemical shift of the L-Val peptide proton is 7.5 ppm as compared to 7.2 ppm for the L-Val NH of gramicidin S. The temperature coefficient criteria suggest that the Val NH is not completely hidden from the solvent. The DMSO–TFE solvent shift indicates that the L-Val peptide proton is more shielded from the solvent than the D-Phe peptide proton. These spectral features allow that the L-Val peptide proton is partially shielded and perhaps involved in a weak intramolecular hydrogen bond. Presumably, the 1 ← 4 hydrogen bond of L-Val is relatively less stable than the strong and stable intramolecular hydrogen bond.

Delineation of the Peptide NH Moieties in Methanol and Trifluoroethanol. In MeOH, the L-Orn peptide proton exhibited a relatively small temperature coefficient ($d\delta/dT$ = −0.0048 ppm/deg) and in TFE a relatively large coefficient (−0.0075 ppm/deg). This indicates that there is a solvent-induced conformational change. The temperature coefficient in CH_3OH of the Orn NH is less than one-half that of the solvent-exposed D-Phe peptide proton; this comparison is the same as observed in gramicidin S for the L-Leu peptide proton (involved in strong intramolecular hydrogen bond) relative to the corresponding solvent-exposed D-Phe peptide proton. On addition of TFE to MeOH, the L-Orn peptide proton exhibited a large upfield shift of ~217 Hz. Since the β-conformational feature of the peptide proton changes from solvent-shielded to solvent-exposed, as the mole percent of TFE increases, the MeOH–TFE titration curve reflects an averaging of the chemical shift of the two conformational states populated to different extents. As the mole percent of TFE is increased, the solvent-exposed conformational state becomes more populated. The average $J_{\alpha\text{-CH-NH}}$ coupling constant is unchanged as the mole percent of TFE in MeOH is increased. At 18°, the chemical shift of the L-Orn peptide proton in MeOH is 8.36 ppm and is not considerably different from its position in DMSO (8.45 ppm). The constancy of the chemical shift in both the solvents indicates that the L-Orn peptide proton is in an internal environment and unexposed to hydrogen bonding with the solvents; whereas in TFE, the chemical shift (7.38 ppm) is very different from its position in DMSO and MeOH. The difference is as large as ~1 ppm. The temperature coefficient criteria, MeOH–TFE solvent shift, and the chemical shift delineate the peptide proton of the L-Orn residue as being exposed to TFE. In DMSO-d_6 and in methanol, the above spectral features suggest that the L-Orn peptide proton is well shielded from the solvent, as when intramolecularly hydrogen bonded.

The D-Phe peptide proton exhibited a large, negative temperature coefficient (−0.0116 ppm/deg) in MeOH, and on going from MeOH to TFE, it exhibited a large upfield shift of ~1 ppm, as did the solvent-exposed peptide protons of gramicidin S. At 18°, the chemical shift of the amide proton in MeOH (8.3 ppm) is the same as in DMSO (8.3 ppm). The temperature coefficient criteria, MeOH–TFE upfield shift, and the chemical shift delineate the D-Phe peptide proton as being solvent-exposed. On changing from MeOH to TFE, the $J_{\alpha\text{-CH-NH}}$ coupling constant (~4.3 Hz), within experimental error, remained unchanged, indicating no change in the α-CH–NH dihedral angle (θ) of the D-Phe residue. At 19° in TFE, the peptide proton resonates at 7.25 ppm and exhibited a temperature coefficient of −0.006 ppm/deg. It is important to note that delineation between the solvent-exposed and solvent-shielded peptide protons is not as clear-cut in TFE as in DMSO-d_6 and CH_3OH, presumably due to the poor proton-accepting ability of TFE. Hence, the solvent-exposed D-Phe peptide proton exhibited a coefficient of −0.006 ppm/deg in TFE as compared to 0.01 and 0.012 ppm/deg in DMSO-d_6 and CH_3OH, respectively. By the temperature coefficient criteria and MeOH–TFE upfield shift (~1 ppm), the D-Phe peptide proton is delineated as being solvent-exposed. However, it is not well solvated by TFE, as reflected in its temperature coefficient and chemical shift.

In MeOH, the L-Val peptide proton exhibited a small temperature coefficient ($d\delta/dT$ = −0.0048 ppm/deg) and a relatively small upfield shift in going from MeOH to TFE (~79 Hz). The temperature coefficient of the L-Val peptide proton is less than one-half that of the solvent-exposed D-Phe amide proton. This relative ratio is approximately the same as observed in the case of gramicidin S for the intramolecularly hydrogen-bonded L-Leu or L-Val peptide protons (−0.0030 ppm/deg) relative to the corresponding solvent-exposed D-Phe proton (−0.0077 ppm/deg). The L-Val peptide proton resonates at 7.36 ppm in MeOH, as compared to 7.7 ppm for the L-Val proton of gramicidin S. It appears from the resonance positions and temperature coefficients that the L-Val amide proton of gramicidin S is involved in a relatively stronger transannular hydrogen bond than the L-Val proton of [di-*N*-MeLeu]gramicidin S. The chemical shift of the L-Val peptide proton is not considerably different in MeOH (7.36 ppm) and DMSO (7.52 ppm). The constancy of the resonance position in both the solvents indicates that the peptide proton is in an internal environment. The above spectral features delineate the L-Val peptide proton as being shielded from the solvent, possibly by an intramolecular hydrogen bond. In TFE, the L-Val peptide proton resonates at 7.0; it exhibits a relatively small temperature coefficient (−0.0037 ppm/deg), and in TFE-d_3, it exchanges slowly relative to the D-Phe proton. The internal environment of the peptide proton is not appreciably altered on changing from MeOH (7.36 ppm) to TFE (7.0 ppm), as is indicated by the chemical shift in both the solvents. By the above criteria, the L-Val peptide proton is

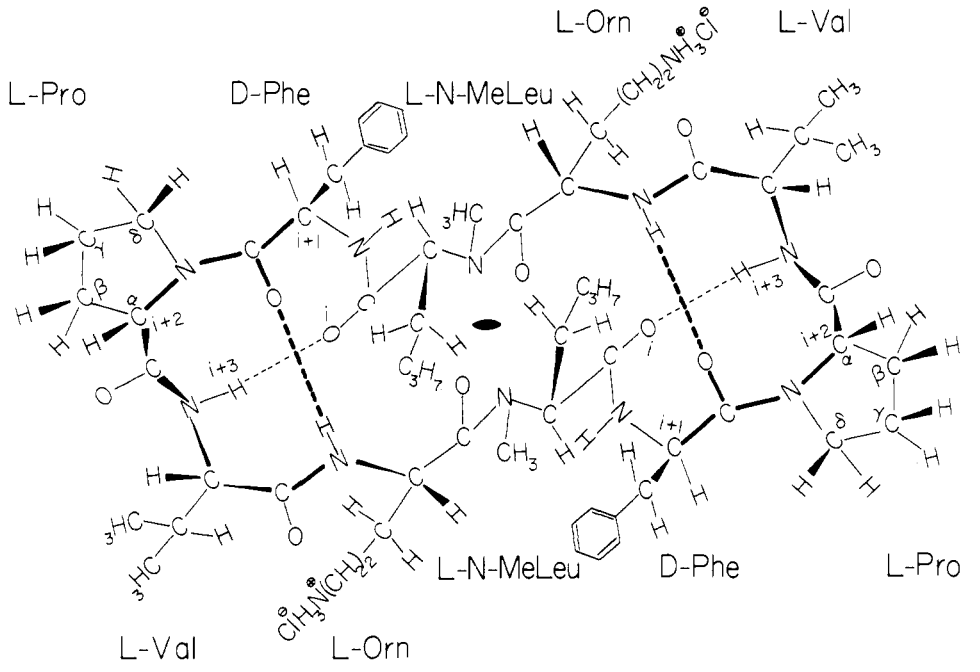

Figure 6. One possible conformation of [3,3′-di-*N*-methylleucine]gramicidin S, viewed down the twofold axis. The ring is partially unfolded to reveal β-conformational features.

delineated as being shielded in TFE, as when intramolecularly hydrogen bonded.

Probability of Occurrence of DL and LL Hydrogen-Bonded β Turns in Dimethyl Sulfoxide and Methanol with Reference to Gramicidin S. It is informative to calculate probabilities or mole fractions, χ_i, of solution molecules present as solvent-shielded peptide protons by using an experimentally observable property such as temperature coefficients or solvent shifts.[23] This does not imply the presence of a locked conformation, but rather the frequency of occurrence of the conformation with solvent-shielded peptide protons in solution. The magnitude of the mole fraction is a relative measure of the solvent-shielded peptide protons with reference to a model system and in a qualitative sense is a measure of the relative stability of an intramolecular hydrogen bond.

The general mole fraction expression calculated from an experimental observable, a, is

$$\chi_i = \frac{a_{obsd} - \sum_{j \neq i}\left(1 + \sum_{k \neq i,j} \chi_k\right) a_j}{a_i - \sum_{j \neq i} a_j} \quad (1)$$

Assuming two states solvent-exposed, e, and solvent-shielded, s, this reduces to eq 2. For gramicidin S as a reference state, the temperature coefficients in methanol are a_s^T = −0.0030 ppm/deg (L-Val and L-Leu) and a_e^T = −0.0077 ppm/deg (D-Phe). Using the value of a_{obsd}^T = −0.0048 for the L-Val and L-Orn in eq 2, one obtains a value of 0.62 for χ_s or 62% of the time L-Val and L-Orn are intramolecularly hydrogen bonded in methanol solution. Using the D-Phe NH value of a_e^T = −0.0116, one obtains for L-Val and L-Orn relative to the solvent-exposed D-Phe a χ_s of 0.80, i.e., 80% of the time the peptide protons are solvent-shielded, as when intramolecularly hydrogen bonded.

$$\chi_s = (a_{obsd} - a_e)/(a_s - a_e) \quad (2)$$

In dimethyl sulfoxide, the temperature coefficients of the reference state (gramicidin S) are a_s^T = −0.0034, a_e^T = 0.0081 ppm/deg. Using the value of a_{obsd}^T = −0.0034 ppm/deg for the L-Orn, the mole fraction of the solvent-shielded peptide protons for [di-*N*-MeLeu]gramicidin S in DMSO calculates to be $\chi_s^T \simeq 1$ or 100% of the time L-Orn peptide protons are solvent-shielded, as when intramolecularly hydrogen bonded.

A major limitation of the temperature coefficient method is that a_{obsd}^T is itself a function of temperature, i.e.

$$a_{obsd}^T = \sum_i a_i^T \chi_i \quad (3)$$

For the two states, solvent-exposed and solvent-shielded

$$\chi_s = \chi_e e^{-\Delta F/RT} \quad (4)$$

With rapidly interconverting conformational states ($\Delta F <$ 12 kcal/mol), the relative population of the conformational states can change with temperature. This can result in a nonlinear temperature dependence at higher temperatures, because different conformational states are appreciably populated and it can also give fortiutously large or small temperature coefficients.

Solution Conformation in Methanol and Dimethyl Sulfoxide. In proposing conformational models, one must be aware of the fact that there may be more than one conformation which agrees with experimental data. Usually the term "conformation" stands for an "ensemble average on the NMR time scale", i.e., one is dealing with an ensemble of conformations which may be in rapid equilibrium so that the resulting ^{1}H NMR spectrum is determined by a weighted time average of spectral parameters of the conformations. With these considerations in mind, a possible conformation will be discussed which satisfies the steric requirements and experimental NMR data. CPK space-filling models were used in constructing the proposed conformational model of [di-*N*-methylleucine]gramicidin S dihydrochloride.

The primary structure of [di-*N*-MeLeu]gramicidin S dihydrochloride has ten amino acid residues containing twice the D-Phe-L-Pro sequence, as in gramicidin S. Being cyclic with the DL sequence, the ring system has the distinct possibility of being folded using DL and LL sites for the bend and ten-atom hydrogen-bonded β turns.[6,24,25] As reported by Ramachandran et al.[26] for the LL β turn, the conformational angle ϕ_{i+1} at α-C_{i+1} in the minimum energy conformation ($\phi_{i+1} = -50°$) closely corresponds to that favorable

for the occurrence of a proline residue ($\phi_{i+2} = -60°$), so that such a bend could be readily formed by the sequence -L-Pro$_{i+1}$-L-Val$_{i+2}$-, utilizing L-proline as the first residue in the LL β turn.

Proposed Conformation in Methanol. In the proposed conformational model (Figure 6), one type of β turn has L-Pro and L-Val at the corners of the LL bend with the carbonyl and peptide NH of the end peptide moiety in the most sterically favored orientation.[6,24] This type of β turn with Pro as residue $i + 1$ was first proposed in solution for the tail of oxytocin[27] and was subsequently confirmed by the X-ray data of Rudko et al.[28] Since then, β turns utilizing proline as residue $i + 1$ have been proposed for several polypeptides such as telomycin,[18] elastin peptides,[23,29] and [mono-*N*-methylleucine]gramicidin S dihydrochloride.[30] The L-Orn peptide proton is internally hydrogen bonded to the carbonyl oxygen of D-Phe, resulting in the formation of a ten-atom hydrogen-bonded β turn with L-Pro$_{i+1}$-L-Val$_{i+2}$ at the corners. This β-conformational feature is supported by the relatively low-temperature coefficient and lowest field position. The low-field position implies a strong intramolecular hydrogen bonding and neutralization of shielding by the magnetic anisotropy of an end peptide moiety. These results can be accommodated in the proposed conformational model, in which the carbonyl of the end peptide moiety is actually pointing away from the L-Orn peptide proton. The 9.2-Hz $J_{\alpha\text{-CH-NH}}$ coupling constant ($\theta \sim 170°$) is consistent with a near-trans orientation of the two protons.[31,32]

The second β turn of the gramicidin S type β turn utilizes D-Phe$_{i+1}$-L-Pro$_{i+2}$ at the corners of the DL bend with the carbonyl and nitrogen of the end peptide moiety in the most sterically favored orientation.[6,24] The L-Val peptide proton is intramolecularly hydrogen bonded to the carbonyl oxygen of L-*N*-MeLeu resulting in the formation of a ten-atom hydrogen-bonded β turn. The occurrence of this β turn is supported by the relatively low-temperature coefficient in methanol and small upfield shift on addition of TFE. The relatively high-field position of the L-Val peptide protons (7.36 ppm) implies either shielding as by the magnetic anisotropy of an end peptide moiety or weak intramolecular hydrogen bond (as suggested by Kopple)[22] or both. The $J_{\alpha\text{-CH-NH}}$ coupling constant is large (8.0 Hz, Table III) corresponding to a dihedral angle (θ) near 155°, as would be expected from the trans orientation of these two protons.[31,32]

In the proposed conformation in methanol, the [di-*N*-methylleucine]gramicidin S dihydrochloride ring is folded utilizing two DL and two LL bends, resulting in the formation of four ten-atom hydrogen-bonded β turns. The hydrogen-bonding pattern in Figure 6 forms a partial turn of a right-handed 3_{10} helix related by a twofold axis of symmetry with the helix axis perpendicular to the twofold axis of symmetry. The skewing of the ring, which results from the above proposed β turns, places the phenyl moiety of D-Phe and L-Pro in close proximity, thereby explaining the relatively large ring-current upfield shifts exhibited by the γ-CH relative to the γ'-CH protons of L-Pro.

Conformation in Dimethyl Sulfoxide. The first β turn, utilizing L-Pro and L-Val in positions $i + 1$ and $i + 2$ of the LL β turn, respectively, is also present in DMSO. The L-Orn peptide proton is internally hydrogen bonded to the carbonyl oxygen of D-Phe, resulting in the formation of a very stable ten-atom hydrogen-bonded β turn. The occurrence of this β turn in DMSO is supported by the low-temperature coefficient, a near zero shift on addition of TFE to DMSO, and the very low-field position in the peptide region (8.45 ppm). This low-field position implies a strong intramolecular hydrogen bond and neutralization of the shielding by the magnetic anisotropy of an end peptide moiety. The observed $J_{\alpha\text{-CH-NH}}$ coupling constant is large (9.0 Hz, Table III) with a corresponding α-CH-NH dihedral angle (θ) near 170°. By the above criteria, the strength of the 1,4-intramolecular hydrogen bond (C-O$_i$- - -H-N$_{i+4}$) involving the L-Orn peptide proton is comparable to the intramolecular hydrogen bond of the L-Leu peptide proton of gramicidin S.

The gramicidin S type β turn with the D-Phe$_{i+1}$-L-Pro$_{i+2}$ residues at the corners of the DL bend appears less stable in DMSO than in methanol, as suggested by the intermediate temperature coefficient, the upfield shift on addition of TFE, and the resonance position of the L-Val peptide proton. The $J_{\alpha\text{-CH-NH}}$ coupling constant of the L-Val is large (8.2 Hz, Table III) corresponding to a dihedral angle (θ) near 155°, which is consistent with a near-trans orientation of these two protons. By the above criteria, the L-Val peptide proton is in an environment partially shielded from the solvent. The L-Val peptide proton resonates at an upfield position (7.5 ppm) as compared to the L-Orn (8.45 ppm). This difference, as large as 1 ppm, implies that either the internal hydrogen bonding to the carbonyl oxygen of the L-*N*-MeLeu is very weak or the peptide proton is shielded by the magnetic anisotropy of the end peptide moiety or both. The resonance position of this peptide proton is near the L-Val peptide proton of gramicidin S (7.2 ppm).

The solvent-exposed D-Phe peptide proton is well solvated by both the solvents (MeOH and DMSO), as suggested by its large temperature coefficient, the large upfield shift on addition of TFE, and the low-field position in the peptide region. The observed $^3J_{\alpha\text{-CH-NH}}$ coupling constants decreased from 6.0 Hz in DMSO to 4.5 Hz in TFE, and to 4.3 Hz in MeOH, corresponding to an average change of ~20° in the phenylalanine H-N-C$_\alpha$-H dihedral angle (θ).[31,32]

Conformation in Trifluoroethanol. The NMR data indicate that, in TFE, the ring is folded using two DL bends, resulting in the formation of two ten-atom hydrogen-bonded β turns. In TFE, the presence of this β turn in which D-Phe$_{i+1}$-L-Pro$_{i+2}$ residues are in the corners of the DL bend is supported by the relatively low temperature coefficient of the L-Val peptide proton, a small upfield solvent shift, and relatively slow H-D exchange, in TFE-d_3. The upfield position of the L-Val peptide proton (7.0 ppm) again implies either the peptide proton is shielded by the magnetic anisotropy of the end peptide moiety or the intramolecular hydrogen bond to the carbonyl oxygen of the L-*N*-MeLeu is weak or both. The 8.0-Hz $J_{\alpha\text{-CH-NH}}$ coupling constant ($\theta \sim 155°$) is consistent with a near-trans orientation of the two protons.

In all solvents the chemical shift of the aromatic protons exhibits near zero temperature dependence, indicating that there is no intramolecular stacking or intermolecular association of the phenyl moieties of the D-Phe residue at 3% (w/v) concentration.

Side Chains.[33] The chemical shift difference between the β-CH$_2$ protons of D-Phe is ~60 Hz. The $^3J_{\alpha\text{-CH-}\beta\text{-CH}}$ coupling constants of the D-Phe, in DMSO-d_6, are near 9 and 5 Hz, indicating major population of a conformer in which the relative orientation of the α and β' protons is trans ($\chi_1 \sim 180$ or 300°) and the α and β protons is approximately gauche. This fits well with the aromatic ring current effects of the D-Phe residue on the γ protons of L-Pro. The γ protons shift upfield by ~110 Hz relative to the γ' protons of L-Pro, which appear at the usual position.

The observed vicinal coupling constants between the α and β protons of L-*N*-MeLeu are near 8 and 5 Hz. This suggests major population of an α,β conformer in which relative orientation of the α and β' protons is approximately trans ($\chi_1 \sim 180°$) and the α and β is approximately gauche. This is consistent with our proposed conformational model (Figure 6), in which shieldings of the β protons by ~115 Hz

relative to the β' protons, and the γ protons by ~85 Hz from its usual position, may be due to the magnetic anisotropy of the peptide carbonyl moiety of L-*N*-MeLeu residue itself and aromatic ring current effects of the D-Phe residue. Also, the 8.0-Hz $J_{\alpha,\beta}$ vicinal coupling constant suggests predominently trans orientation for the Val α-CH-β-CH bond. Space-filling models indicate no overcrowding of the L-Val methyl groups with the side chains of the vicinal residues. The L-Val α proton appears at the highest field position in the α region. In our proposed model, it is shielded by its own peptide carbonyl moiety and also by the L-Pro carbonyl moiety.

Conformation of the D-Phe-L-Pro Peptide Bond. [Di-*N*-methylleucine]gramicidin S dihydrochloride has C_2 symmetry on the NMR time scale as is indicated by the 220-MHz ^{1}H NMR spectrum in Figure 1a. The ^{13}C NMR assignments were made in DMSO-d_6 at 22° by comparison of the [di-*N*-MeLeu]gramicidin S spectra with gramicidin S spectra. In the proton-decoupled carbon-13 magnetic resonance spectrum of [di-*N*-methylleucine]gramicidin S dihydrochloride, the α,β,γ, and δ carbon resonances of L-Pro showed no doubling due to cis-trans isomerism of the D-Phe-L-Pro peptide bond.[16,17] For unhindered amides, the energy barrier to cis-trans peptide bond isomerization is ~20 kcal/mol.[34] This requires that only the cis or trans isomer is present.

According to Patel,[35] the proline α-CH proton chemical shift along with its splitting pattern differs for cis and for trans X-Pro peptide bonds. For the cis X-Pro bond, the proline α-CH proton resonates at a lower field and exhibits a doublet with one large and one near-zero $J_{\alpha,\beta}$ coupling constant, and for the trans X-Pro bond a more complex multiplet is observed at a higher field. It has been reported[35] for several polypeptides containing proline residues that, in DMSO-d_6, the chemical shift for the Pro α-CH proton of the trans conformer was within the range 4.2-4.4 ppm and for the cis isomer was 4.26-4.7 ppm.

For glycyl-L-prolinediketopiperazine, in DMSO-d_6, the L-Pro α-CH proton resonates at 905 Hz (4.1 ppm) as an unsymmetrical triplet ($J_{\alpha,\beta} \sim 5.0$ Hz, $J_{\alpha,\beta'} \sim 8.0$ Hz). Also, the L-Pro α-CH proton of prolyl-L-prolinediketopiperazine resonates at 943 Hz (4.28 ppm) as a triplet ($J_{\alpha,\beta} \sim J_{\alpha,\beta'} \sim 8.0$ Hz) in DMSO-d_6. In gramicidin S in CD_3OD at 19°, and in DMSO-d_6 at 75°, the L-Pro α-CH proton resonates at 955 (4.34 ppm) and 950 Hz (4.32 ppm), respectively, as a doublet exhibiting 6.5 Hz and near-zero $J_{\alpha,\beta}$ coupling constants. According to the correlation suggested by Patel,[35] the D-Phe-L-Pro peptide bond of gramicidin S would be assigned to the trans configuration on the basis of the chemical shift and to the cis conformation on the basis of the $J_{\alpha,\beta}$ coupling constants. Clearly, in ^{1}H NMR, the chemical shift and multiplicity of the Pro α-CH protons are not a good basis for determining the cis-trans isomerism about the X-Pro bond.[36]

However, in ^{13}C NMR, Bovey[36] and other workers[37] have reported that the chemical shifts of the γ-carbon resonances could be used to determine the conformation about the X-Pro bond. In many proline derivatives, the reported[36] chemical shift of the γ-carbon resonance for the trans conformer is 168.5 ± 0.5 ppm and for the cis isomer is 170.3 ± 0.3 ppm, relative to external CS_2. The carbon-13 spectra of gramicidin S in CD_3OD and DMSO-d_6 are very similar,[36,38] and in DMSO-d_6, the α, β, γ, and δ carbon resonances of L-Pro appear at 132.6, 163.4, 168.5, and 146.1 ppm, respectively, with reference to external CS_2.[38,40] Therefore, the proline spectrum of gramicidin S in DMSO-d_6 is entirely consistent with that of a typical trans X-Pro bond.[36]

In DMSO-d_6 at 22°, we have observed that the L-Pro α, β, γ, and δ carbon resonances of gramicidin S appear at 59.85, 28.93, 23.05, and 46 ppm, respectively, with reference to internal TMS at 25 MHz. For [di-*N*-methylleucine]gramicidin S dihydrochloride, in CD_3OD, the L-Pro α-CH proton resonates at 950 Hz (4.32 ppm) as a doublet exhibiting 6.5 Hz and near-zero $J_{\alpha,\beta}$ coupling constants, and also in DMSO-d_6 at 950 Hz as a doublet exhibiting 7.5 Hz and near-zero coupling constants. The chemical shifts of the δ-CH_2 protons of gramicidin S and [di-*N*-MeLeu]gramicidin S dihydrochloride are the same (815 Hz). The L-Pro α, β, γ, and δ carbon resonances of [di-*N*-methylleucine]gramicidin S dihydrochloride appear at 59.9, 28.0,[39] 23.2, and 46.06 ppm, respectively, with reference to internal TMS. From the ^{13}C NMR spectra, in DMSO-d_6, it is seen that the chemical shifts of the L-Pro α, γ, and δ carbon resonances of gramicidin S and [di-*N*-MeLeu]gramicidin S dihydrochloride are very similar and therefore the trans isomer is most reasonable in [di-*N*-methylleucine]gramicidin S. The hydrogen-bonding pattern in Figure 6, based on CPK models, requires the trans isomer.

Conformation and Biological Activity. Because of the conformational stability of gramicidin S it has generally been assumed that the biologically active conformation could reasonably be taken to be the solution conformation. As noted above two of the four intramolecular hydrogen bonds involved in the proposed solution conformation of gramicidin S utilize the peptide NH moieties of the two leucine residues. For this conformation there is considerable agreement. The recent synthesis and bioassay of [di-*N*-methylleucine]gramicidin S by Sugano et al.[8,9] surprisingly demonstrated this derivative to have biological activities similar to those of gramicidin S, that is, to be just as effective against *Staphylococcus aureus*, *Bacillus subtilis*, and *Shigella flexneri* and to be about one-half as effective against *Escherichia coli* and *Candida albicans*.

If the biologically active conformation were the proposed solution conformation, then N-methylation of the leucine residues would disrupt the secondary structure and as a consequence dramatically alter the biological activity. The absence of a dramatic change in biological activity implies either that the proposed solution conformation is incorrect or that the rigidity of secondary structure was not the fundamental factor relating to biological activity.

The demonstration in the present report that the secondary structure of [di-*N*-methylleucine]gramicidin S in DMSO-d_6 and methanol is very different from that of gramicidin S suggests either that the biologically active conformation is different from the solution conformation or that parameters such as the spacing of γ-N^+H_3 moieties of ornithine and/or the phenyl moieties of phenylalanine could be the critical factors. That the latter is a distinct possibility comes from the recent observations that the antimicrobial action of tyrocidines (a structural class of antibiotics to which gramicidin S belongs) may be at the stage of RNA transcription.[40,41] One way of assessing whether the secondary structure may be different at the site of action would be in effect to lock the secondary structure by N-methylation of the ornithine and phenylalanine residues. Knowledge of the biological activity of this derivative would be of considerable interest.

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Supplementary Material Available. Details of spectral analyses will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the

supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JACS-75-4105.

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Chemically Induced Phase Separation in Mixed Vesicles Containing Phosphatidic Acid. An Optical Study

H.-J. Galla and E. Sackmann*[1]

Contribution from the Max-Planck-Institut für Biophysikalische Chemie, D-34 Göttingen, Germany, and Abteilung Experimentelle Physik III, Universität Ulm, D-79 Ulm, Germany.

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Abstract: The possibility of chemically induced phase separation in lipid lamellae containing charged lipids is demonstrated. In mixed membranes of synthetic dipalmitoyllecithin (DPL) and dipalmitoylphosphatidic acid (DPA), a lipid domain structure may be triggered both by the addition of Ca^{2+} and of polylysine in random coil configuration. A new optical (excimer forming) probe, pyrenedecanoic acid, is introduced. The rate of excited complex formation is the crucial physical parameter yielding quantitative information on both the coefficient of lateral diffusion and on the lipid segregation. At all pH values bivalent ions bind strongly to DPA or mixed DPA–DPL lamellae. The number of ions bound per lipid molecule is one at pH 9 and about one-half at pH 5–7. The Ca^{2+} bound phosphatidic acid segregates into regions characterized by a rigid (or crystalline) structure. A quantitative analysis of the experiments shows that at pH 9 the number of DPA molecules segregated is about equal to the number of Ca^{2+} ions. At pH 9 polylysine attaches strongly to DPA membranes and triggers the formation of lipid domains that differ in their microviscosity from the rest of the membrane. At this pH the transition temperature T_t of the lysine-bound DPA is shifted from $T_t = 47°$ to $T_t = 61°$, corresponding to a shift from pH 9 to 2. Strong evidence is provided that the polylysine assumes such a (random coil) configuration that the charged lysine groups point toward one direction and that one lysine group may bind one DPA molecule. The coefficient of lateral diffusion of pyrenedecanoic acid in DPA at pH 9 and at 60° has been determined as $D_L = 1.7 \times 10^{-7}$ cm²/sec. The corresponding value for DPL at 60° is $D_L = 0.8 \times 10^{-7}$ cm²/sec.

Many properties of biological membranes may be understood on the basis of a two-dimensional fluid model. Direct evidence for this model was provided by the detection of the rapid lateral mobility of lipoids[2a] and phospholipids[2b] in lipid lamellae and of surface antigens in cells or cell hybrids.[3] The fluid membrane model also accounts for the observation of a rapid rotational mobility of membrane-bound proteins.[4] Both the rotational and lateral mobility of membrane-bound macromolecules may be directly related to the lipid lateral diffusion coefficients.[5,6] Ample evidence has