Hydrogen–Deuterium Substitution and Solvent Effects on the Nitrogen-15 Nuclear Magnetic Resonance of Gramicidin S: Evaluation of Secondary Structure†

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ABSTRACT: Complete assignments of nitrogen-15 resonances of gramicidin S have been made in dimethyl sulfoxide, trifluoroethanol, and in a solvent mixture of dimethyl sulfoxide (50%) and methanol (50%). The assignments are achieved by utilizing the secondary structure of gramicidin S, by comparing the nitrogen-15 spectrum of gramicidin S with that of di-*N*-methylphenylalanine-gramicidin S and by taking into account the distinguishable value of nitrogen-15 chemical shift for valine in model compounds. Deuterium substitution for labile peptide protons was performed to delineate solvent shielded and deshielded peptide nitrogens and to substantiate further the signal assignments. The solvent titration on going from dimethyl sulfoxide to trifluoroethanol was also performed and shown to have a large deshielding effect on the peptide nitrogen whose corresponding peptide carbonyl, within the peptide moiety, was accessible to the trifluoroethanol solvent.

Recent advancements in nitrogen-15 nuclear magnetic resonance studies (^{15}N NMR) on peptides and proteins (Hawkes et al., 1975b; Gust et al., 1975; Llinas et al., 1976) have shown the potential of ^{15}N NMR as a technique to study biomolecular conformation. In conjunction with well-established proton and carbon-13 NMR, ^{15}N studies should be valuable in providing for a better understanding of the solution conformation(s) of biomolecules. A primary concern in carrying out a structural study of polypeptides and proteins by NMR is the assignment of resonances. Such a study has been made by Llinas et al. (1976) with ^{15}N-enriched alumichrome and ferrichrome by using the ^{13}C–^{15}N decoupling experiments.

One naturally occurring antibiotic, a cyclic decapeptide called gramicidin S (GS[1]) with the primary structure *cyclo*-(-L-Val-L-Orn-L-Leu-D-Phe-L-Pro-)$_2$, has been the subject of extensive study by both ^{1}H and ^{13}C NMR (Stern et al., 1968; Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Gibbons et al., 1970; Pitner and Urry, 1972; Sogn et al., 1974; Urry, 1974; Urry et al., 1975) as a model compound. A recent study of GS by ^{15}N NMR was reported by Hawkes et al. (1975a,b), but the problem of ^{15}N signal assignments remained unresolved. Previous NMR studies have shown that in GS the Phe NH and Orn NH groups are solvent exposed, while the Val NH and Leu NH groups are solvent shielded to form intramolecular H bonds with the Leu and Val C=O groups (see Figure 1), respectively. An intermolecular nuclear Overhauser effect (NOE), obtained by saturating the solvent signal, has recently added support to the proposed solvent exposed and solvent shielded peptide NH groups (Pitner et al., 1975).

Leipert and Noggle (1975) and Giannini et al. (1975) have shown that the ^{15}N NOE is mainly due to the ^{15}N–^{1}H dipole–dipole interaction (DD), and in the absence of exchange effects on the total T_1 (spin–lattice relaxation time) it is given by (Hawkes, 1975b)

$$\mathrm{NOE} = \frac{I_d}{I_c} = 1 + \eta = 1 + \frac{\gamma_{^1H} T_1}{2\gamma_{^{15}N} T_{1DD}} \quad (1)$$

where I_d and I_c are the proton-decoupled and proton-coupled ^{15}N signal intensities and γ is the gyromagnetic ratio. A ^{1}H NMR study (Urry and Ohnishi, 1970) showed the effect of deuterium (D) substitution for the labile peptide protons (H–D exchange), where the Phe N*H* and Orn N*H* proton intensities (being solvent exposed) were completely diminished after a certain period of time with almost no loss of the Val N*H* and Leu N*H* intensities. The results of deuterium exchange for hydrogen on the peptide nitrogen would be less efficient relaxation, since

$$\frac{\gamma_{^2H}}{\gamma_{^1H}} = 0.15 \quad (2)$$

if broad-band deuterium decoupling were carried out. In the absence of deuterium decoupling, deuterium substitution results both in the loss of the ^{15}N–^{1}H NOE due to proton noise decoupling and a loss of signal height due possibly to deuterium scalar quadrupolar relaxation and/or to N–D J coupling. Thus, one would expect a reduced ^{15}N signal intensity of the exposed Phe NH and Orn NH groups in GS, when an H–D substitution is taking place. The effect of this H–D substitution on the exposed peptide protons in GS has been used in this study to delineate the solvent-exposed and solvent-shielded ^{15}N signals. One analogue of GS, di-*N*-methylphenylalanine-gramicidin S, where the exposed Phe NH protons have been replaced by CH_3 groups, was synthesized in order to delineate the exposed Phe NH and Orn NH peptide nitrogens. The rest of the intramolecularly H-bonded NH groups of Val and Leu residues of GS have been tentatively delineated by comparing them with the ^{15}N chemical shift of valine in several small peptides. After assignment of the ^{15}N signals, a solvent perturbation effect was followed from dimethyl sulfoxide (Me_2SO) to trifluoroethanol (TFE). From the H–D substitution and the solvent-perturbation effects on the ^{15}N signals of GS, the present study demonstrates ^{15}N NMR to be useful in evaluating polypeptide secondary structures in solutions.

Materials and Methods

Gramicidin S (GS) was purchased from Schwarz/Mann. Di-*N*-methylphenylalanine-gramicidin S was synthesized in the research laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Osaka, Japan. The ^{15}N-enriched small linear

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[1] Abbreviations used are: GS, gramicidin S; NOE, nuclear Overhauser effect.

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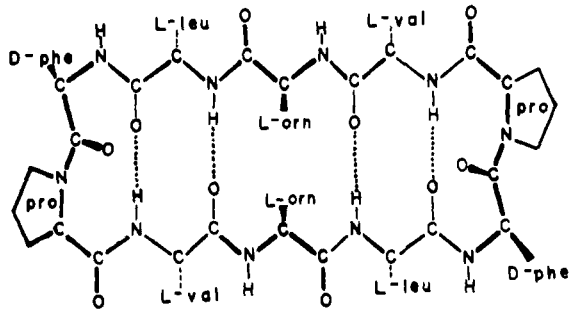


FIGURE 1: The backbone conformation of gramicidin S. The intramolecular H bonds are indicated by dotted lines. This secondary structure has been derived independently by proton magnetic resonance (Stern et al., 1968; Ohnishi and Urry, 1969) and by carbon-13 magnetic resonance (Urry, 1974; Urry et al., 1975).

peptides, Boc-Gly-OH, Boc-L-Val$_1$-Gly$_2$-OMe, Boc-Gly$_1$-L-Val$_2$-Gly$_3$-OMe, and Ac-Gly$_1$-L-Val$_2$-Gly$_3$-OMe were synthesized in this laboratory (Renugopalakrishnan et al., 1977). Trifluoroethanol (TFE) was purchased from Halocarbon Products and spectroanalytical methanol (MeOH) from Mallinckrodt, Inc. The former was redistilled in glass with a Duflon column of glass beads. Me_2SO-d_6 was purchased from the Merck, Sharp and Dohme Co. One-hundred percent deuterium oxide (D_2O, gold band) was purchased from Diaprep, Inc. Since di-*N*-methylphenylalanine gramicidin S was not readily soluble in Me_2SO-d_6, it was dissolved (0.1 M) in a 50% Me_2SO-d_6 and 50% MeOH solvent mixture. Solutions (0.1 M) of GS were made in Me_2SO-d_6, in TFE, and in a 50% Me_2SO-d_6 and 50% MeOH solvent mixture. Diluted solutions of ^{15}N-enriched small peptides were made in Me_2SO-d_6.

All the ^{15}N spectra were recorded on a JEOL FX-100 spectrometer operating at 10.05 MHz and equipped with a TI-980B computer, a ^{1}H decoupler, and a digital quadrature detector. The spectra were collected by using 8K data points, a 12-μs pulse width for 45° pulse angle, and a repetition time of 2.2 s. All samples were run in a microcell and placed within a 10-mm NMR tube. When protonated TFE was used as solvent, D_2O was placed in the 10-mm tube outside the microcell for internal locking on the deuterium signal. To achieve adequate mobility for observation of the ^{15}N signals (Hawkes et al. 1975a), samples of GS were run at 52 °C, while the samples of ^{15}N-enriched peptides were run at a probe temperature of 22 °C. The H–D exchange effect was achieved by adding one small drop of 100% D_2O and running the samples for an average of 35 000 pulses. Due to the negative gyromagnetic ratio of ^{15}N, negative signals are observed, but the spectra were recorded 180° out of phase in order to give the usual positive signals.

Results

^{15}N resonances of the backbone peptide nitrogens of GS in Me_2SO-d_6 (0.1 M) are shown in Figure 2A. The most downfield signal at 112.01 ppm could readily be assigned to Pro *N* by observing its line width and by comparison with the chemical shift of prolines in the other model peptides (Hawkes et al., 1975b; Renugopalakrishnan et al., 1977). The addition of a small drop of 100% D_2O to the Me_2SO-d_6 sample of GS results in the expected effect on the relative signal intensities (vide supra) (see Figure 2B). The relative intensities of two signals at 100.88 and 105.09 ppm have been considerably reduced, because of the less efficient ^{15}N–^{2}H dipole–dipole interaction due to the H–D exchange. Therefore, the two resonances could be assigned to Phe *N*H and Orn *N*H nitrogens

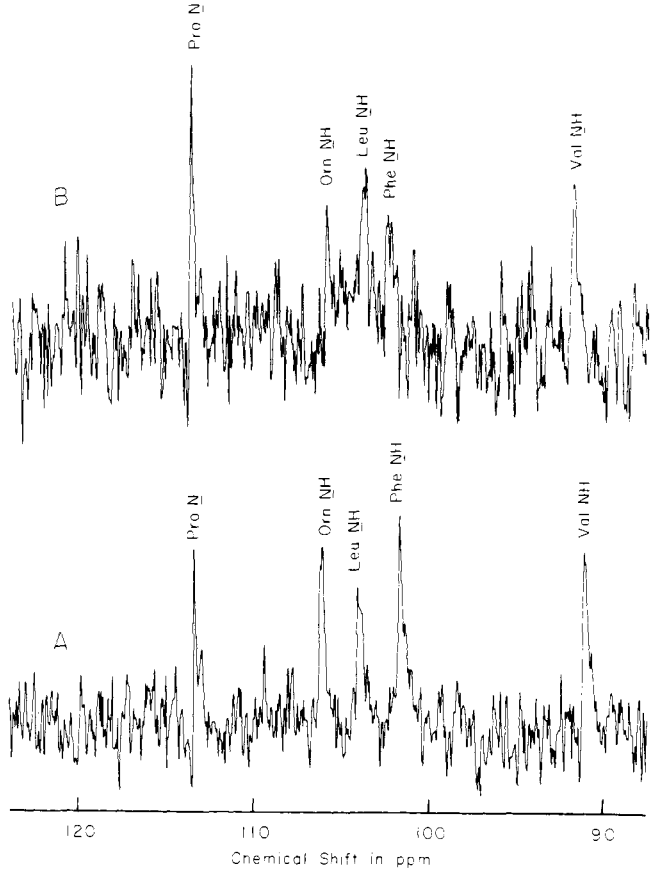


FIGURE 2: The 10.05-MHz ^{15}N spectrum of gramicidin S: (A) in Me_2SO-d_6 and (B) in Me_2SO-d_6 with one drop of 100% D_2O. (Note a slight change in chemical shifts which is due to the added D_2O. Please see the discussion on solvent effects.)

based on the secondary structure of Figure 1. To delineate between the two resonances for Phe *N*H and Orn *N*H, the ^{15}N spectrum of di-*N*-methylphenylalanine-gramicidin S in a 50% Me_2SO-d_6 and 50% MeOH solvent mixture was obtained (see Figure 3A). By comparing line width and signal intensity with the Pro *N* signal at 114.59 ppm, the signal at 99.17 ppm could be assigned to the Phe *N*-methyl nitrogen. The H–D exchange effect on ^{15}N resonances of di-*N*-methylphenylalanine-gramicidin S is shown in Figure 3B. It could be seen in this figure that the signal at 104.73 ppm exhibits a dramatic reduction in the signal intensity on H–D exchange. This signal is, therefore, assigned to the Orn *N*H nitrogen. Similar experiments were performed with GS in the same solvent mixture, and a similar effect was found as shown in Figure 2. Previously assigned Orn *N*H and Phe *N*H nitrogens at 105.24 and 103.69 ppm, respectively, in 50% Me_2SO-d_6 and 50% MeOH were found at 100.88 and 105.09 ppm in Me_2SO-d_6 by following a Me_2SO-d_6–MeOH solvent titration (results are not shown here). The two signals at 103.16 and 90.87 ppm in Figure 2 and at 103.58 and 96.44 ppm in Figure 3, which were less affected by H–D exchange, could thus be assigned to Leu *N*H and Val *N*H nitrogens, again utilizing the secondary structure of Figure 1.

Assignment of the Val *N*H nitrogen resonance was made by comparing the chemical shifts of valine nitrogen in some small ^{15}N-enriched peptides in Me_2SO-d_6 in the following manner. The ^{15}N resonances of Boc-Gly-OH, Ac-Gly$_1$-Val$_2$-Gly$_3$-OMe, and Boc-Gly$_1$-Val$_2$-Gly$_3$-OMe in Me_2SO-d_6 are shown in Figure 4. It could be seen here that the Boc-Gly-*N*H nitrogen in both Boc-Gly-OH and Boc-Gly$_1$-Val$_2$-Gly$_3$-OMe

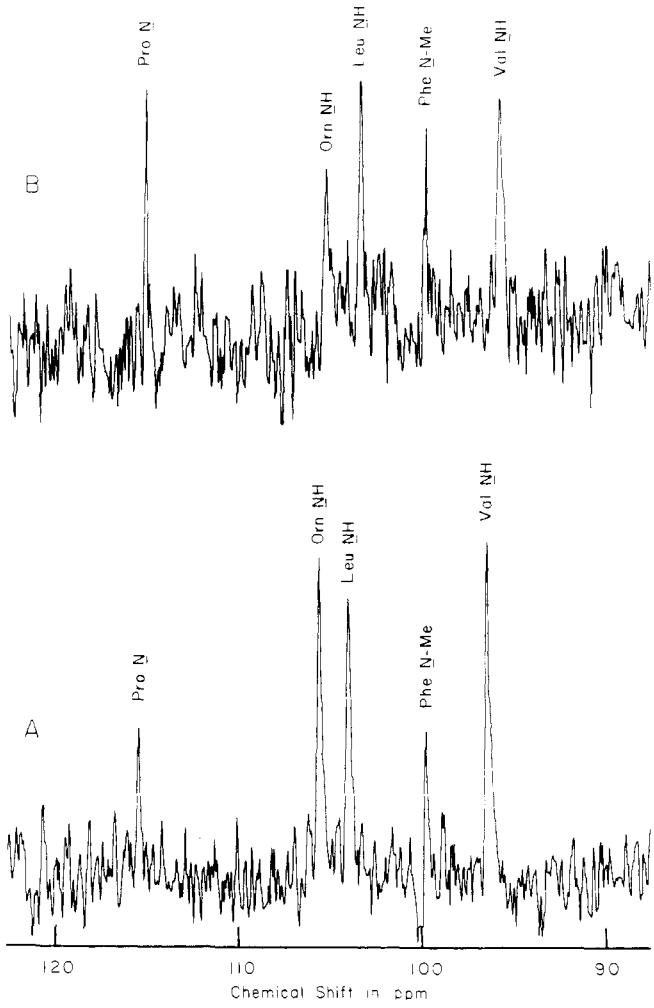


FIGURE 3: The 10.05-MHz ^{15}N spectrum of di-*N*-methylphenylalanine-gramicidin S: (A) in Me_2SO-d_6 (50%) and MeOH (50%) solvent mixture and (B) in the same solvent mixture with one drop of 100% D_2O.

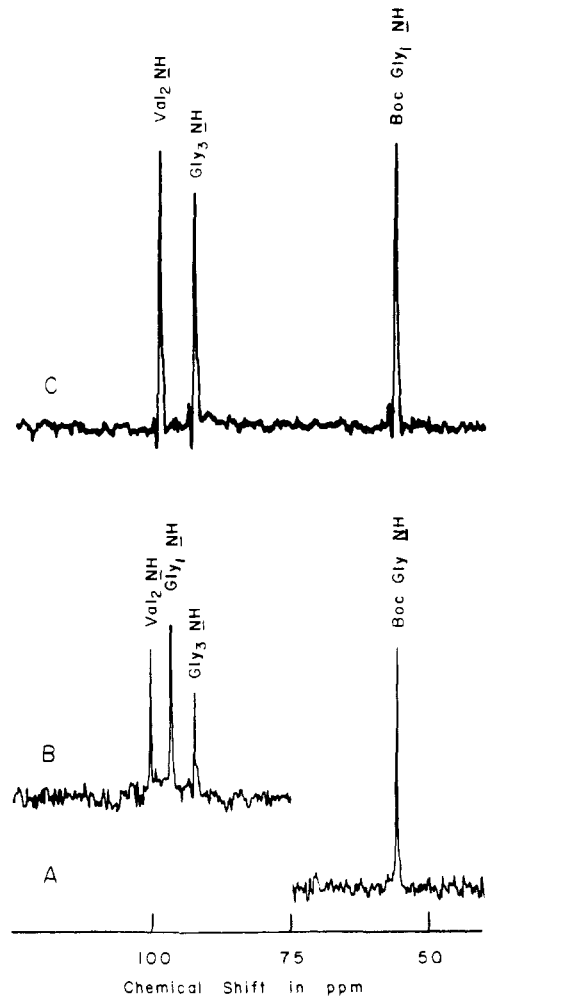


FIGURE 4: The 10.05-MHz ^{15}N spectra of some small peptides in Me_2SO-d_6. (A) Boc-Gly-OH, (B) Ac-Gly_1-Val_2-Gly_3-OMe, and (C) Boc-Gly_1-Val_2-Gly_3-OMe.

appears almost exactly at 56.46 ppm (see Table II). The resonances at 90.60 and 85.17 ppm in Boc-Gly_1-Val_2-Gly_3-OMe are, therefore, due to either the Val_2 *N*H or to the Gly_3 *N*H nitrogen. The chemical-shift values of the Val_1 *N*H and Gly_2 *N*H nitrogens in Boc-Val_1-Gly_2-OMe show that Gly *N*H nitrogen preceded by valine appears at 84.35 ppm (see Table II). Similarly, a Gly_3 *N*H nitrogen preceded by Val_2 in Ac-Gly_1-Val_2-Gly_3-OMe and in Boc-Gly_1-Val_2-Gly_3-OMe (Figure 4B,C) appears at 85.22 and 85.17 ppm, respectively (see Table II). Obviously, resonances near 85 ppm should be assigned to the Gly *N*H and those around 91 ppm should be assigned to the Val *N*H nitrogen. A similar chemical-shift value of 92.6 ppm (downfield from $^{15}NH_4NO_2$) for Val *N*H nitrogen was found by Hawkes et al. (1975a) in their model systems. The resonance at 90.87 ppm in Me_2SO was, therefore, tentatively assigned to the Val *N*H nitrogen in GS. By elimination, the signal at 103.16 ppm would be due to the Leu *N*H nitrogen. The ^{15}N chemical shifts of all peptide nitrogens of GS and di-*N*-methylphenylalanine-gramicidin S, thus obtained, are listed in Table I. Studies are being considered which will provide a firmer basis for the Val *N*H and Leu *N*H delineation, which is not essential to our present effort.

Since the solvent-perturbation method, especially from Me_2SO to TFE, in proton and carbon-13 NMR (Urry and Long, 1976; Urry et al., 1974, 1975) and in ^{15}N NMR (Llinas et al., 1976) has been shown as a useful technique in elucidating the secondary structure of peptides, such a study was performed on the ^{15}N resonances of GS. The solvent titration results in the solvent pair Me_2SO-d_6 and TFE are shown in Figures 5 and 6. To confirm the solvent exposed and solvent shielded *N*H nitrogens of GS in TFE, the H–D exchange was followed and shown in Figure 6. It could be seen here that the intensities of the Orn *N*H and Phe *N*H resonances at 104.32 and 104.07 ppm (Figure 6B and Table I) relative to Pro *N* have significantly diminished on the addition of a drop of 100% D_2O (see Figure 6B,C).

TABLE I: Nitrogen-15 Chemical Shifts of Gramicidin S (GS) and its Analogue Di-*N*-methylphenylalanine-gramicidin S [Expressed in Parts per Million (±0.05) Downfield from $^{15}NH_4Cl$ (0.1 M) in 2 N HCl Used as an External Standard].

Solvents	Amino acid residues of GS				
	L-Val	L-Pro	D-Phe	L-Leu	L-Orn
Me_2SO	90.87	112.01	100.88	103.16	105.90
TFE	97.85	116.22	104.07	108.11	104.32
50% Me_2SO, 50% MeOH	94.69	113.95	103.69	104.42	105.24
	Di-*N*-MePhe-GS				
50% Me_2SO, 50% MeOH	96.44	114.59	99.17	103.58	104.73

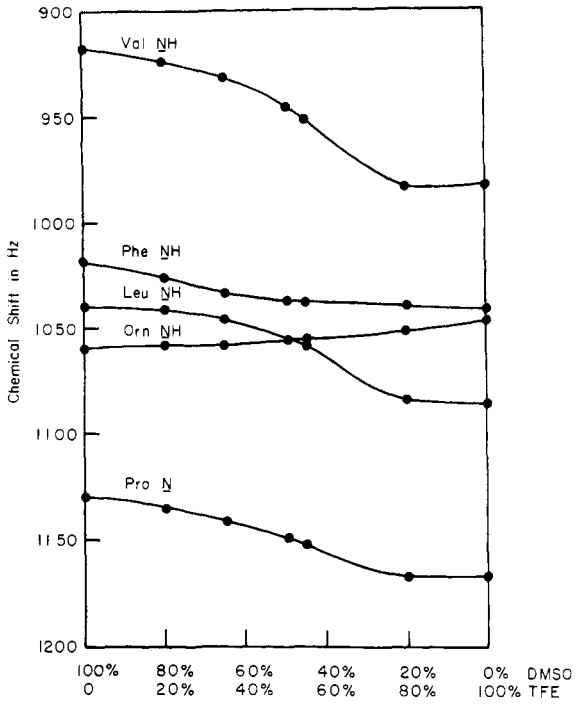


FIGURE 5: Solvent titration of ^{15}N resonances of gramicidin S on going from Me_2SO-d_6 to TFE.

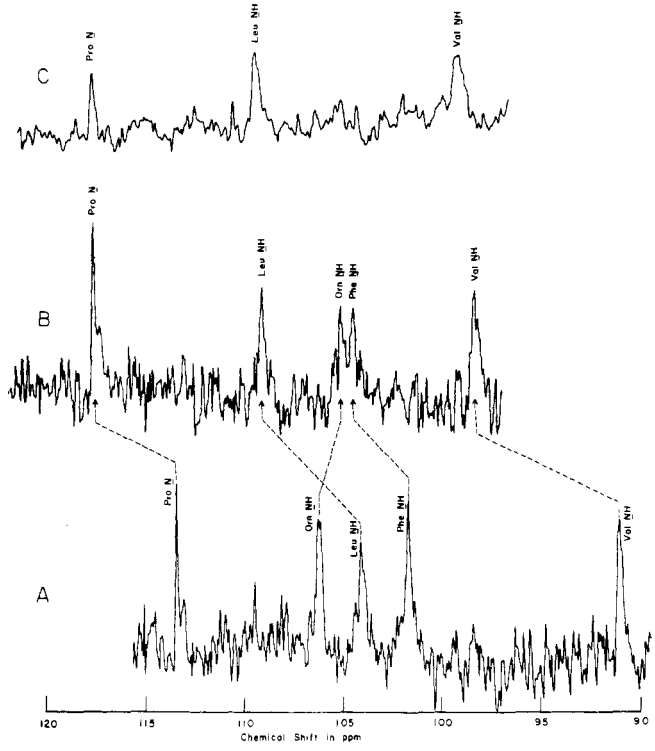


FIGURE 6: The 10.05-MHz ^{15}N spectrum of gramicidin S: (A) in Me_2SO-d_6, (B) in TFE, and (C) in TFE with one drop of 100% D_2O.

TABLE II: Nitrogen-15 Chemical-Shift Parameters of Some Small Peptides in Me_2SO [Expressed in Parts Per Million Downfield from $^{15}NH_4Cl$ (0.1 M) in 2 N HCl Used as an External Standard].

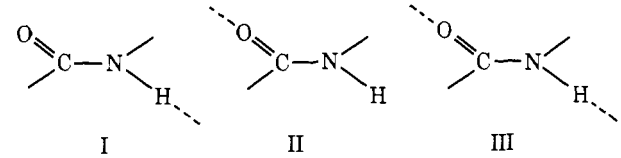
Peptides	Amino acid residues	Chemical shifts in ppm (±0.05)
Boc-Gly-OH	Gly *N*H	56.46
Boc-Val_1-Gly_2-OMe	Val_1 *N*H	63.89
	Gly_2 *N*H	84.35
Boc-Gly_1-Val_2-Gly_3-OMe	Gly_1 *N*H	56.45
	Val_2 *N*H	90.60
	Gly_3 *N*H	85.17
Ac-Gly_1-Val_2-Gly_3-OMe	Gly_1 *N*H	88.64
	Val_2 *N*H	91.59
	Gly_3 *N*H	85.22

Discussion

The present experiments, utilizing complete proton decoupling, demonstrate that the NOE of the nitrogen is greatly reduced by H–D substitution. The signal-to-noise ratio in the direct detection of ^{15}N resonances is significantly decreased as expected, since the ^{15}N–^{1}H dipolar interaction is a dominant factor of the ^{15}N spin–lattice relaxation. A general prediction for decreased ^{13}C (in the case of C=O) and ^{15}N NOE was made earlier (Giannini et al., 1975) for samples containing D_2O. The present study shows that a trace of D_2O has a large effect on solvent-exposed nitrogen nuclei. Therefore, careful addition of D_2O in the ^{15}N experiments becomes useful in delineating the most solvent exposed peptide NH groups. Figures 2, 3, and 5 show the NOE with the signal-to-noise ratio of the exposed Phe *N*H and Orn *N*H nitrogen signals decreased. To obtain information on the mechanism from the line width of the D-substituted ^{15}N nuclei, the correct line shape of the signal with a high signal-to-noise ratio is required. The present study of natural-abundance ^{15}N resonances in GS under H–D substitution conditions makes difficult deduction of the mechanism for decreased intensity (see Figures 2B and 3B).

The serious problem of ^{15}N signal assignments for GS, which was previously faced by Hawkes et al. (1975b), was overcome in this study: first, by H–D substitution of the solvent-exposed Phe *N*H and Orn *N*H nitrogen nuclei; next, by comparing the ^{15}N spectrum of GS with that of di-*N*-methylphenylalanine-gramicidin S with a view to delineate between the Phe *N*H and Orn *N*H nitrogen resonances; and, finally, by taking account of a common shift position of the valine nitrogen in several peptides in order to distinguish between the Leu *N*H and Val *N*H nitrogen resonances (see Results).

In addition to assignment of all nitrogen-15 nuclei in GS, there has been observed the interesting effect of solvent titration on-going from Me_2SO-d_6 to trifluoroethanol (see Figures 5 and 6). It is seen in Figure 1 that both the Val NH and Leu NH groups are intramolecularly H bonded with their C=O groups (solvent shielded), while the Phe NH and Orn NH groups are exposed to the solvent. A basic solvent, Me_2SO, and a protic acidic solvent, TFE, could interact with the peptide NH and C=O groups, respectively, depending on the degree of their exposure to the solvents (Schwyzer and Ludescher, 1969; Pitner and Urry, 1972; Urry et al., 1974; Llinas and Klein, 1975). A peptide bond under such conditions could be considered with the following H-bonded states:



State I is possible when the peptide NH is either intermolecularly H bonded with a solvent (such as with Me_2SO) or intramolecularly H bonded with a C=O group or other nega-

tively charged functional groups in the same molecule. The protonation effect of an acidic protic solvent such as TFE could be seen in H-bonded state II. This structure is also applicable when the peptide C=O is intramolecularly H bonded. The H-bonded state III is for a peptide bond when both the functional groups (NH and C=O) are inter- and/or intramolecularly H bonded.

Figure 1 shows that the Val NH is intramolecularly H bonded, while the C=O group (Pro C=O) of the same peptide bond is exposed to the solvents, and the same is true for the intramolecularly H-bonded Leu NH group and for the covalently bonded Pro *N*. On the other hand, the Phe *N*H and Orn *N*H groups are exposed to the solvent, while their peptide C=O groups (Leu C=O and Val C=O) are involved in the intramolecularly H-bonded interaction (Figure 1). Since the exposed C=O groups could easily be partially protonated by trifluoroethanol, a charge delocalization could take place between the peptide functional groups, rendering the peptide NH groups more electron deficient. Under such circumstances, a deshielding would be expected for the ^{15}N resonance of the peptide NH, irrespective of whether it is involved in the H-bonded interaction or not. Such phenomena were observed by Urry (1974) and Urry et al. (1975) in the ^{1}H and ^{13}C experiments on GS and also by Llinas et al. (1976) in a ^{15}N study of alumichrome.

In GS, on going from Me_2SO to trifluoroethanol, the Val *N*H moved 7.02-ppm downfield, while Leu *N*H and Pro *N* nitrogen shifted 4.99- and 4.21-ppm downfield, respectively (see Table I). These downfield shifts correspond very well with the protonation effect of trifluoroethanol on the exposed peptide C=O groups of the same peptide moiety. On the other hand, the Phe *N*H moved comparatively less downfield (3.19 ppm) while Orn *N*H moved 1.58-ppm upfield (see Table I and Figures 5 and 6), indicating the solvent-shielded situation of their corresponding peptide C=O group within the same peptide moiety. Llinas et al. (1976) also observed an upfield shift of Gly_2 *N*H in alumichrome, when the corresponding peptide C=O was solvent shielded, and this phenomenon was explained on the basis of the loss of the deshielding effect on *N*H on going from Me_2SO to trifluoroethanol, while the *N* of a solvent-shielded peptide linked C=O group was less affected by trifluoroethanol. This explanation seems also plausible in the case of the Orn *N*H chemical-shift changes in GS; however, the Phe *N*H does not follow (downfield shift of 3.19 ppm) the same reasoning, since the NH group is solvent exposed and the corresponding C=O group is solvent shielded (see Figure 1). In addition to the above explanation, two more possible causes could be responsible for such differential chemical-shift movements of ^{15}N signals on going from Me_2SO to trifluoroethanol. One such reason could be the relative exposure of the peptide functional groups (NH and C=O) due to either the differing bulkiness of side chains or the relative outward/inward orientation of the peptide groups. A second reason could be the relative distortion at the peptide bond, since the electronic shift from NH to C=O could be effected by a distorted peptide bond. More experimental work with model compounds is needed in order to clarify these effects. Such solvent effects do, in general, follow direct and indirect H-bonding deshielding effects on the ^{14}N resonances of amides as observed by Saito et al. (1971). It can be argued, qualitatively, therefore, that solvent effects on the nitrogen resonances can be a useful tool with which to delineate the solvent-exposed and solvent-shielded (H-bonded) peptide C=O groups for the purpose of evaluating peptide secondary structure in solution.

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