HIGH FIELD CARBON-13 NMR SPECTROSCOPY. CONFORMATIONAL MOBILITY IN GRAMICIDIN S AND FREQUENCY DEPENDENCE OF ^{13}C SPIN-LATTICE RELAXATION TIMES.

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Summary: The use of superconducting solenoids in ^{13}C NMR can result in a substantial increase in spectral resolution over that obtained at commonly employed magnetic fields. Results are presented here for the cyclic decapeptide gramicidin S in both CD_3OD and DMSO-d_6 solutions. It was possible to monitor the complete motional behavior of the side chains of gramicidin S in CD_3OD using ^{13}C spin lattice relaxation times. Preliminary data are reported which confirm the frequency dependence of spin-lattice relaxation times for carbons in molecules not satisfying the extreme narrowing condition.

The application of natural abundance ^{13}C Fourier transform NMR* to both small[1] and large[2] peptides has increased substantially in the last several years. Particular success has been achieved with peptides of approximately 1000 molecular weight, such as peptide hormones and antibiotics.[1] This has been made possible in part by the fact that the great majority of individual resonances will be resolved at fields normally employed in ^{13}C NMR (14-23 kG). In addition to the availability of chemical shift information, individual spin-lattice relaxation times (T_1) can be measured for all carbons with resolved resonances using the partially-relaxed FT technique.[3]

Quite often, however, a number of resonances will not be resolved in the ^{13}C spectra of small ($\sim$5-15 residues) peptides at typically employed field strengths, resulting in a loss of information. This can arise from the presence of resonances--often several hertz in width for protonated carbons--in chemically similar environments.

*Abbreviations: NMR, nuclear magnetic resonance; T_1, spin-lattice relaxation time; FT, Fourier transform; TMS, tetramethylsilane; T_2, spin-spin relaxation time; NOE, nuclear Overhauser enhancement; N, number of attached hydrogens for a given carbon--therefore $N \cdot T_1$ is the T_1 for a given carbon multiplied by N.

The cyclic decapeptide antibiotic gramicidin S is such a case. It should be pointed out that gramicidin S, which possesses C_2 symmetry, has a ^{13}C spectrum resulting from only five component amino acids. Proton-decoupled ^{13}C spectra of gramicidin S in methanol have been reported at 25[4], 55.3[5], and 15.2[6,7] MHz. At the lower frequencies[4,6,7] the majority of resonances are resolved with the exception of the β and γ carbons of Orn and Pro, the magnetically nonequivalent methyls of Leu and Val, and some carbonyls. In an isolated spectral study at 55.3 MHz, the above resonances are better resolved (the carbonyl region was not shown for this single spectrum).

We report here the 67.9 MHz natural abundance carbon-13 FT NMR spectra of gramicidin S in CD_3OD and DMSO-d_6, as well as ^{13}C T_1's for most protonated carbons in the two solvents. Preliminary results concerning the frequency dependence of ^{13}C T_1's are also presented.

Materials and Methods: Gramicidin S (from bacillus brevis) was purchased from Schwarz-Mann, Orangeburg, N. Y. Solutions of gramicidin S in CD_3OD (150 mg/ml) and in DMSO-d_6 (179 mg/ml) were millipore filtered before use. Proton-decoupled natural abundance ^{13}C FT NMR spectra were recorded at 67.9 MHz on a Bruker HX-270 spectrometer, using 10mm o.d. tubes, with 90° rf pulses of 17 μsec duration, and at 22.6 MHz on a Bruker HFX-90. Chemical shifts were measured with respect to internal TMS. T_1's were determined at 67.9 MHz using the partially relaxed FT technique.[3]

Results and Discussion: Figure 1 gives the structure of gramicidin S as well as $N \cdot T_1$ (normalizing for the number of attached hydrogens) values for all protonated carbons in CD_3OD at 42°C. Figures 2 and 3 compare the aliphatic region of the ^{13}C spectra of gramicidin S at 22.6 and 67.9 MHz in CD_3OD and DMSO-d_6, respectively. A considerable increase in spectral resolution is apparent upon tripling the resonance frequency. (In each spectrum shown, ca. 0.7 Hz line broadening is present due to exponential filtering). ^{13}C chemical shifts and T_1's are listed in Table 1. In methanol, eight additional resonances, which appear as four unresolved or partially resolved peaks at 22.6 MHz, are observed at 67.9 MHz. A considerable increase

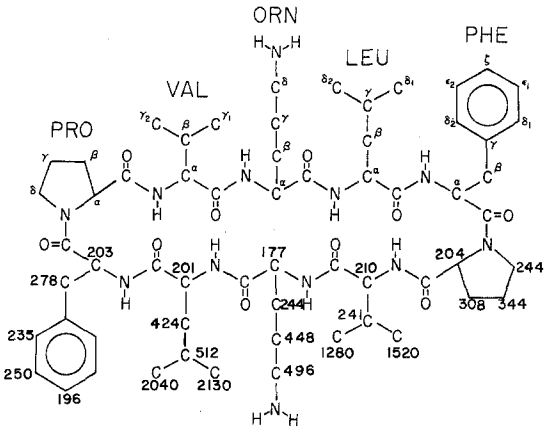

Figure 1. Schematic diagram of gramicidin S. Standard IUPAC-IUB carbon designations are shown in the top portion of the structure. In the lower portion are $N \cdot T_1$ values of protonated carbons in CD_3OD at 42°C and 67.9 MHz, where N is the number of directly bonded protons and T_1 is taken from Table 1.

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in spectral resolution also occurs for gramicidin-S in DMSO-d_6. Several resonances are obscured at low field by resonances arising from deuterated solvent; overlap between solute and solvent resonances is minimal at 67.9 MHz.

The increased spectral resolution obtained at 67.9 MHz made it possible to monitor the complete motional behavior of the side chains of gramicidin S in CD_3OD using ^{13}C T_1's, something that was not possible at 15.2 MHz.[6,7] Overlap of the β and γ carbon resonances of the Orn and Pro side chains precluded measurement of individual T_1's at the lower field. The data at high field (see $N \cdot T_1$ values in Figure 1) indicate considerable flexibility of the pyrrolidine rings of the proline residues at the β and γ carbons, a phenomenon which has been observed in poly-L-proline[8,2] and several small proline-containing peptides.[1] Qualitatively, $N \cdot T_1$ values along the ornithine side chain indicate that a substantially larger increase in motion occurs about the C_β-C_γ bond than about the C_α-C_β or C_γ-C_δ bonds. Similar behavior has been observed for arginine side chains in small peptides and in luteinizing-hormone releasing hormone.[9] It should be pointed out that, since the overall rotation of the backbone of gramicidin S does

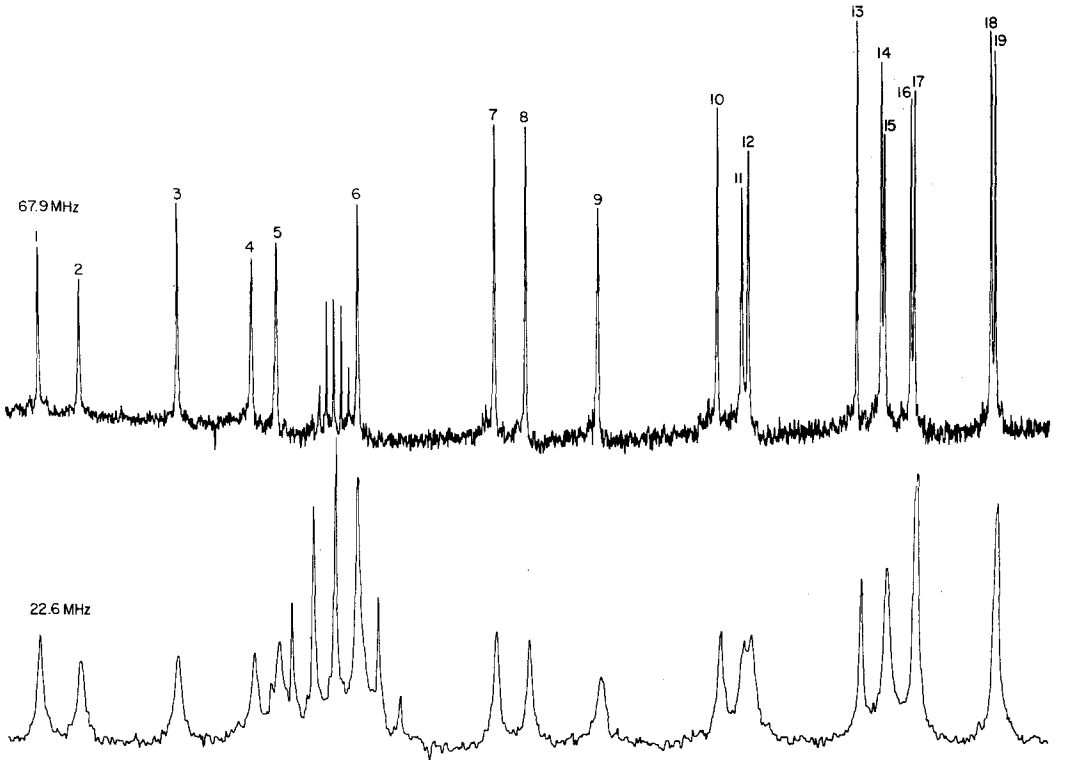

Figure 2. Comparison of the aliphatic regions of the ^{13}C spectra of gramicidin S (in CD_3OD at 42°C) at 22.6 and 67.9 MHz. 22.6 MHz, 4096 scans, pulse delay 2 sec; 67.9 MHz, 2300 scans, pulse delay 1.8 sec. Numbering refers to assignments in Table 1.

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not satisfy the extreme narrowing condition (see below), $N \cdot T_1$ values at 67.9 MHz are not direct indicators of molecular motion.[10] The full, frequency dependent equation that relates T_1 to τ_C, the correlation time for isotropic rotation, must be used to extract values of τ_C.[10] For gramicidin S in CD_3OD the deviations from the extreme narrowing values are relatively small, especially for side chain carbons, and semi-quantitative conclusions on motion can be reached using $N \cdot T_1$ values directly.

At high field it was also possible to resolve resonances due to magnetically non-equivalent methyl groups of the valine and leucine side chains. No difference was detected in the rates of internal spinning of the leucine methyl groups,

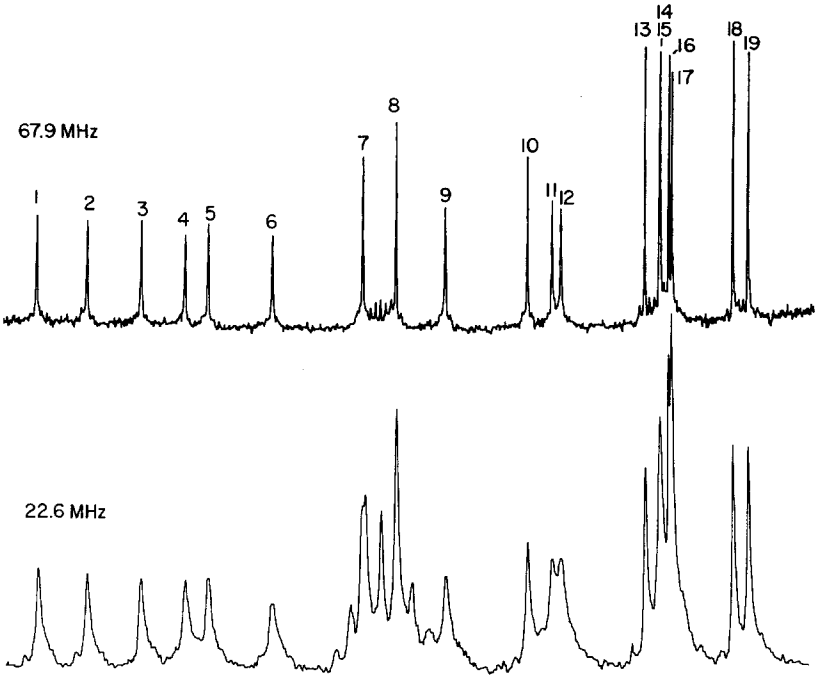

Figure 3. Comparison of the aliphatic regions of the ^{13}C spectra of gramicidin S (in DMSO-d_6 at 50°C) at 22.6 and 67.9 MHz. 22.6 MHz, 15, 860 scans, pulse delay 2 sec; 67.9 MHz, 1400 scans, pulse delay 1.6 sec. Numbering refers to assignments in Table 1.

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three bonds away from the backbone. The valine methyls, two bonds from the backbone, do rotate at measurably different rates (Fig. 1) in CD_3OD at 42°C.

In DMSO-d_6 at 50°C, roughly the same behavior was observed as in CD_3OD (Table 1). In this solvent no evidence of different spinning rates was found for either the valine or leucine methyls. T_1's of carbons well removed from the backbone are quite comparable in the two solvents, even though the backbone is reorienting at considerably different rates in the two cases (Fig. 4). Unfortunately, the motional behavior of the Pro and Orn side chains could not be followed entirely, due to overlap of their γ carbon resonances, even at 67.9 MHz.

For molecular systems where the extreme narrowing condition $[(\omega_H + \omega_C)\tau_C \ll 1]$

Table 1. ^{13}C Chemical Shifts and Spin-Lattice Relaxation Times of Gramicidin S at 67.9 MHz.

	in DMSO-d_6		in CD_3OD		67.9 MHz T_1[e]	T_1(msec)[f]
Assignment	δ(ppm)[a]	T_1(msec)[b]	δ(ppm)[c]	T_1(msec)[d]	(Predicted)	at 15.2 MHz
Phe-γ	136.24		136.92			
Phe-δ	129.21		130.36	235	241	213
Phe-ε	128.15		129.60	250	238	211
Phe-ζ	126.76		128.34	196	207	174
Pro-α(1)	59.89	162	61.95	204	185	150
Val-α(2)	56.92	153	60.18	210	180	144
Phe-α(3)	53.88	150	55.85	203	177	141
Orn-α(4)	51.04	154	52.58	177	174	135
Leu-α(5)	49.68	150	51.46	201	183	147
Pro-δ(6)	46.00	90	47.88	122	92	75
Leu-β(7)	40.77	175	41.88	212	178	170
Orn-δ(8)	38.60	230	40.49	248	201	194
Phe-β(9)	35.68	110	37.30	139	113	99
Val-β(10)	30.94	208	31.92	241	211	180
Orn-β(11)	29.58	124	30.89	122		h
Pro-β(12)	29.01	134	30.57	154		h
Leu-γ(13)	23.97	460	25.67	512	435	421
Orn-γ(14)	23.07	g	24.57	224		h
Pro-γ(15)	23.07	g	24.43	172		h
Leu-δ_1(16)	22.63	610[i]	23.29	679	638	638[h]
Leu-δ_2(17)	22.47	685[i]	23.14	710		
Val-γ_1(18)	18.90	480	19.64	428[j]	395	~395[h]
Val-γ_2(19)	17.98	530	19.49	506[j]		

a) at 42°C. Estimated accuracy ±0.04 ppm.

b) at 50°C. Estimated accuracy ±10% except as noted. Relative accuracy is better than ±10%.

c) at 45°C. Estimated accuracy ±0.03 ppm.

d) at 42°C. Estimated accuracy ±10%. Relative accuracy is considerably better than ±10%.

e) Assuming isotropic reorientation, and using data in reference 6.

f) from reference 6.

g) Unresolved at 67.9 MHz.

h) Unresolved or partially resolved at 15.2 MHz.

i) Estimated accuracy ±15%.

j) These two resonances null at slightly different τ values in the partially relaxed FT experiment.

is not fulfilled, it is predicted theoretically that T_1 (as well as T_2 and the NOE) can be frequency dependent.[11,12] Figure 4 shows the variation of the ^{13}C T_1 for a methine carbon with rotational correlation time at two magnetic fields assuming isotropic reorientation. The vertical lines roughly indicate the positions of the backbone carbons of gramicidin S in CD_3OD and DMSO-d_6 under the

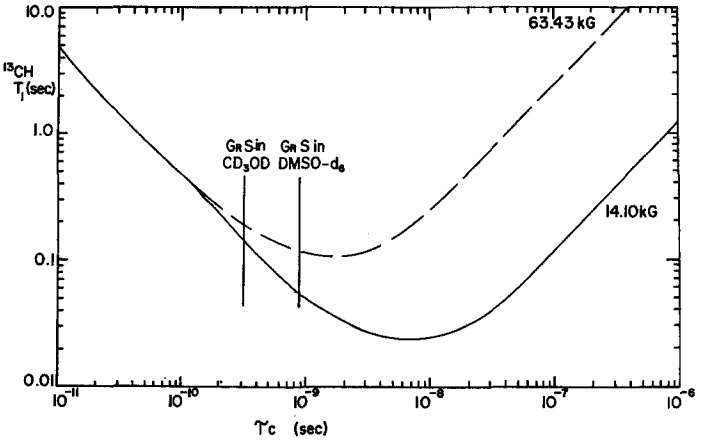

Figure 4. Variation of $^{13}C-\{^1H\}$ T_1 vs. τ_c, the correlation time for isotropic overall reorientation, for a methine carbon (r_{CH}=1.09Å) at 2 magnetic field strengths.

conditions reported previously[6,7] and in this paper.

The last three columns of Table 1 present a comparison of ^{13}C T_1's of carbons in gramicidin S at 67.9 MHz, the values predicted for 67.9 MHz from those at 15.2 MHz, and the previously reported[6] values at 15.2 MHz. For carbons on or relatively close to the backbone, T_1 at 67.9 MHz is considerably higher than at 15.2 MHz on a percentage basis. Considering the experimental errors in the T_1's reported at both fields, the values at the high field agree reasonably well with the predicted values based on the low field data, although measurements made at 67.9 MHz are consistently higher than the predicted values.* For gramicidin S in DMSO-d_6 at 50°C, approximate T_1's have been reported[7] for some carbons, mainly on the backbone, at 15.2 MHz. While the α-carbon T_1's at high field are roughly comparable to the predicted values, more accurate data is needed at the low field.

Both T_2 and the NOE can also be frequency dependent outside the region of extreme narrowing.[11] In particular, these parameters will exhibit frequency de-

*The cause of this small discrepancy is under investigation.

pendence in the "intermediate" correlation time region (10^{-10} to 10^{-8} sec), where many systems such as gramicidin S will lie. Assuming isotropic reorientation, the natural linewidth ($1/(\pi T_2)$) is predicted to decrease slightly with increasing frequency in this intermediate region, resulting in an additional increase in spectral resolution over low field. In addition, the NOE will decrease with increasing frequency for correlation times roughly in the region 10^{-10}-10^{-8} sec. Hence some sensitivity loss (in addition to that resulting from longer T_1 values) may be encountered when studying small to intermediate-size peptides at high field, unless sufficient segmental motions are present to result in a full NOE.[11,12] However, such losses may be offset by the basic increase in NMR sensitivity at high field, given careful spectrometer design.[13]

High field ^{13}C NMR should be particularly valuable in the study of peptide hormones and antibiotics[1,9] of greater complexity and molecular weight (MW≤2000).

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