The Complete Assignment of the ^{13}C Nuclear Magnetic Resonance Spectrum of the Decapeptide Gramicidin S-A by Selective Biosynthetic Enrichment Studies

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Abstract: The complete and unambiguous assignments of the carbon resonances of a naturally occurring peptide of molecular weight >1000 have not yet been reported. Here, by utilizing the technique of selective biosynthetic enrichment, we report the assignment of all the carbon resonances of the decapeptide antibiotic gramicidin S-A. These assignments confirm our previous assignments which were based largely on Grant's rules and comparison with monomeric amino acids and their derivatives, thus showing the essential applicability of these rules to "first-order" assignments of carbon spectra in peptides (and hence proteins). These experiments extend our original assignments by permitting the carbonyl carbons to be identified, a task difficult to achieve by other techniques. The findings in the carbonyl region suggest that the intramolecular hydrogen bonding of Leu and Val carbonyl groups may be reflected in small low-field shifts of their carbonyl carbons in Me_2SO. The advantages and simplicity of the selective enrichment technique both for making ^{13}C chemical shift assignments and for other types of studies such as biosynthetic pathways are discussed.

The field of ^{13}C nmr as applied to peptides and proteins has advanced experimentally a great deal since the first published work of Horsley, *et al.*,[1-3] on amino acids and simple peptides, the first reported protein spectrum[4] and the first report and assignments of the natural abundance spectrum of a naturally occurring peptide, the antibiotic gramicidin S-A.[5] Instrumental and methodological improvements have reduced spectral acquisition time and increased the scope of the studies to include relaxation properties of the nuclei as well as chemical shifts. Our interest in ^{13}C nmr has focused on its use in peptides of moderate molecular weight, from about 5 to 15 residues. For such peptides a complete analysis of the proton nmr spectrum is feasible although in many cases very difficult. To assist in making proton nmr assignments and to add complementary information of backbone rigidity and conformation, studies using the isotopes ^{15}N[6] and ^{13}C can be useful. Gramicidin S-A, a cyclic decapeptide antibiotic of structure *cyclo*(-D-Phe-L-Pro-L-Val-L-Orn-L-Leu)$_2$, is a good model compound for these studies for two reasons. First, the compound is of biological interest as a membrane-active antibiotic, and second, it has been exhaustively studied in the past by a number of physical and chemical techniques as a model for protein studies.[7] Its secondary structure in solution was established by proton nmr[8] and is now accepted by many groups. The full crystal structure has not yet been reported.

For peptides in this size range the natural abundance ^{13}C nmr spectrum with proton noise decoupling is usually well resolved, but since Grant's rules[9] as applied to peptides[2] are only good at best in predicting ^{13}C chemical shifts to about 2 ppm and since in peptides somewhat larger deviations due to specific conformational factors cannot be ruled out, rigorous *à priori* assignments cannot be made for resonances in crowded regions of the spectrum.[5]

A number of experimental methods can be used to make ^{13}C chemical shift assignments in peptides, but each has some serious limitations. Selective ^1H–^{13}C decoupling can be used to assign ^{13}C resonances in cases where the complete proton assignment has been made. This is a severe restriction for peptides of moderate size, since in molecules complex enough for ^{13}C assignments to be questioned, proton assignments are likely to be very difficult to make since the proton spectrum is invariably much more complex than the ^{13}C spectrum. In peptides of ten residues or more it is the relative simplicity of the ^{13}C spectrum that makes ^{13}C nmr such a potentially valuable tool.

For some peptides a number of analogs, each containing one or more amino acid replacements, are available. When a sufficient set of analogs is available, a comparative study by ^{13}C nmr can be used to make ^{13}C chemical shift assignments.[10] Selective ^{13}C enrichment is a modification of this method. It has, however, the substantial advantage that the substitution being made which involves only isotopic changes is not likely to give rise to perturbations of conformation or biological activity, a possibility which must be ruled out with other analogs.

Other methods of making assignments have still more limited applicability. Titration effects can be used to assign carbon resonances of ionizable side chains or the N and C terminal carbons of linear peptides, soluble and stable in water over a wide pH range.[11] Off-

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resonance decoupling and relaxation time measurements can differentiate between members of broad classes of carbon atoms and can occasionally be used. In many cases some combination of the above methods is sufficient to provide a reasonable degree of certainty in chemical shift assignments,[12] but in others selective ^{13}C enrichment *via* biosynthetic or chemical means offers significant advantages in speed and clarity. It is the single most unambiguous method of making assignments, frequently is a very simple technique, and provides as a by-product a set of ^{13}C-enriched peptides which are useful for a variety of other studies.

Selective biosynthetic incorporation of ^{13}C-labeled precursors into compounds other than peptides has been reported before[13] although in these cases the emphasis was the opposite of that here, in that assignments were already known and the studies were intended to elucidate the pathway of biosynthesis.

Experimental Section

For production of labeled gramicidin S-A, *Bacillus brevis* (ATCC 9999) was grown from a spore suspension on the minimal medium of Eikhom, *et al.*[14] This medium contains salts, vitamins, glutamic acid as the major carbon source, ammonium sulfate as the principal nitrogen source, and a 1 m*M* supplement of each of the amino acids found in gramicidin S-A. Without the constituent amino acids growth proceeds well but the culture does not sporulate or produce a significant amount of antibiotic. With batches of 1 l. of medium, which yielded about 75 mg of purified gramicidin S-A, a shaking incubator maintained at 32° was used. Higher temperatures increased the yield of a yellow material which was difficult to separate from the peptide. The spores grew out in 18 hr and growth then proceeded logarithmicly for about 12 hr, after which it slowed down. Harvesting was done after 48 hr or longer, at which time none of the amino acid nutrients remained. To isolate the gramicidin, four volumes of ethanol were added per volume of culture and the cells were disrupted with eight 45-sec bursts from a Branson sonifier. After sonication the suspension was left overnight and then centrifuged. The supernatant was evaporated to dryness and extracted five times with 20 ml of absolute ethanol. Evaporating this ethanol extract to dryness gave a crude gramicidin S-A which could be adequately quantitated by comparison with a known quantity of pure material after thin-layer chromatography and ninhydrin spray detection. The chromatography was best accomplished with the system ethyl acetate–glacial acetic acid–pyridine–water (60:6:20:11), in which the gramicidin has an R_f of 0.35. Purification was completed by countercurrent distribution in the system chloroform–methanol–0.1 *N* HCl (1:1:1) with 5 ml upper phase to 3 ml lower phase. The partition ratio of the peptide varied between 0.05 and 0.15, being lower with a higher load of material. The sample was loaded in a single tube. In all cases 200 transfers was enough to resolve the peptide from the main impurity, a yellow pigment which was strongly retained at the beginning of the CCD train. The gramicidin S-A cut taken after such a CCD was identical by proton nmr (except for the effects of isotopic substitution) with more fully purified gramicidin and it was used as such.

Specific enrichment of the ^{13}C resonances originating from a single amino acid residue could be accomplished by replacing the normal 1 m*M* supplement of the amino acid of interest with the same amount of the amino acid 10% enriched in ^{13}C. These amino acids were isolated[15] from a 10% ^{13}C protein hydrolysate purchased from Merck of Canada. The ^{13}C spectra were consistent with the idea that no enrichment occurred in resonances other than those expected from the single enriched amino acid. In addition, control syntheses using radioactive (^{3}H or ^{14}C) amino acids, which were located in the peptide by hydrolysis and counting of the amino acid peaks off the amino acid analyzer, showed no detectable nonspecific labeling.

The ^{13}C nmr spectra were taken at 22.63 MHz on the Bruker HFX-90 of Bruker Scientific at Elmsford, N. Y., and on the Bruker HX-90 at the Rockefeller University. Spectra were taken in the pulsed Fourier transform mode. From 1000 to 4000 scans were accumulated for each spectrum at a cycle time of 0.8 sec. Samples contained 75 mg of peptide in 1 ml. Sample temperature was 50°.

Results and Discussion

The first and second columns of Table I show the assignments made previously on an *à priori* basis for gramicidin S-A.[5] Some peaks are sufficiently well resolved to have been satisfactorily assigned by this method but the bracketed groups were considered at the time to be too close for any assignment to be made. All of these ambiguities were easily resolved by selective incorporation of the enriched amino acids. Valine and leucine were each put in alone, but since in no case was there any group of unassigned residues containing lines from both phenylalanine and proline, these two amino acids were put in together in a single experiment. The potential assignment ambiguity of Phe and Pro carbonyls in Me_2SO (where they are separated) was not a problem since Phe was incorporated with a consistent 20% higher enrichment, allowing assignment by integration to be made in this case. Ornithine was of course not available in the ^{13}C enriched protein hydrolysate we had, so the resonances of this amino acid were assigned by difference. The three labeled peptides, ([^{13}C]Val) gramicidin S-A, ([^{13}C]Leu) gramicidin

Table I. ^{13}C Peak Assignments for Gramicidin S-A[a]

Carbon	Tentative Assignments in[b] Methanol	Tentative Assignments in[b] Me_2SO	Final assignments in Methanol	Final assignments in Me_2SO
Val CH_3	172.5	174.5	172.5	174.5
Val CH_3	172.5	173.6	172.5	173.6
Leu CH_3	168.9	170.0	168.9	170.0
Leu CH_3	168.9	170.0	168.9	170.0
Pro γ	(166.4)	(168.5)	167.5	169.5
Orn γ	(167.5)	(169.5)	167.5	169.5
Leu γ	(167.5)	(169.5)	166.4	168.5
Pro β	(161.6)	(163.4)	161.6	163.4
Orn β	(160.2)	(161.3)	161.2	162.8
Val β	(161.2)	(162.8)	160.2	161.3
Phe β	154.9	156.8	154.9	156.8
Orn δ	(150.4)	(Solvent)	151.5	Solvent
Leu β	(151.5)	(154.1)	150.4	154.1
Pro δ	144.3	146.1	144.3	146.1
Leu α	140.7	142.8	140.7	142.8
Orn α	(136.4)	(138.6)	139.6	141.4
Phe α	(239.6)	(141.4)	136.4	138.6
Val α	(131.9)	(135.7)	131.9	135.7
Pro α	(130.3)	(132.6)	130.3	132.6
Phe aromatics				
Para	64.9	67.1	64.9	67.1
Meta	62.7	65.7	62.7	65.7
Ortho	62.1	64.6	62.1	64.6
Bridge	55.6	57.8	55.6	57.8
Pro C_0	Unassigned		19.1	24.7
Orn C_0	Unassigned		20.0	24.4
Val C_0	Unassigned		20.0	23.8
Phe C_0	Unassigned		19.1	23.5
Leu C_0	Unassigned		19.1	22.9

[a] In ppm upfield from external $^{13}CS_2$. [b] Tentative assignments taken from ref 5.

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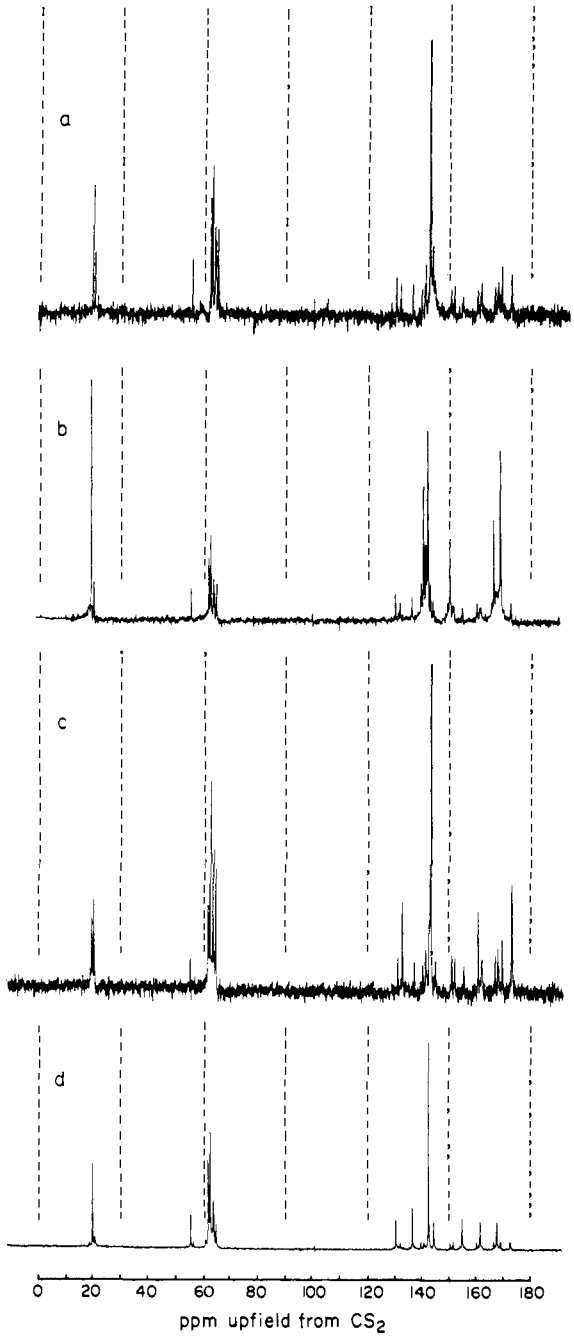

Figure 1. The ^{13}C spectra of gramicidin S-A at 22.63 MHz: (a) normal unenriched gramicidin S-A; gramicidin S-A enriched by biosynthesis in the presence of (b) 10% [^{13}C]Leu; (c) 10% [^{13}C]Val; (d) 10% [^{13}C]Phe and 10% [^{13}C]Pro.

S-A, and ([^{13}C]Phe + Pro) gramicidin S-A, were examined both in methanol and in dimethyl sulfoxide. The collected spectra in methanol are shown in Figure 1. These spectra allowed the complete assignments shown in columns 3 and 4 of Table I to be made quite unambiguously when expansions of the spectra in Figure 1 were carefully superimposed. New carbonyl carbon chemical shift assignments, which are commonly ignored because they are impossible to make with any confidence on an *à priori* basis, can be made in this way as easily as any other assignments. It will be necessary to study a number of peptides before deciding what factors affect carbonyl carbon chemical shifts but it may be noted tentatively that in Me_2SO the carbons of the carbonyl groups involved in intramolecular hydrogen bonding (Leu and Val) occur slightly to lower field than would otherwise be expected. One can predict approximate carbonyl chemical shifts on the same basis as carboxyl carbon shifts in the free amino acids. The carboxyl carbon shifts[1] may be rationalized using a set of empirical substituent parameters. Ignoring the α-carbon and the α-amino group, which are common to all of the amino acids, a β-carbon substituent parameter of -3.2 ppm and a γ-carbon parameter of $+1.0$ ppm give good results. In a peptide it would be reasonable to expect these parameters to continue to be approximately correct, although of course in a peptide the carbonyl carbon senses substituents through the peptide bond from the next side chain, as well as from its own side chain. On this basis one predicts an order of carbonyl chemical shifts for gramicidin S-A of (low field to high field): Phe, (Orn, Leu), (Pro, Val). This order is preserved in Me_2SO, where there is no solvent hydrogen bond donating character to complicate matters, except for Leu and Val, which are shifted to low field. Downfield movement is what would be predicted if, as might be expected, the hydrogen bonding involved some small net donation of electrons from the carbonyl carbon to the oxygen. Whether the observed effect can be proven to be related to hydrogen bonding is a question that depends on studies of more peptides containing well-established intramolecular hydrogen bonds.

Proton nmr spectra of the peptides in dimethyl-d_6 sulfoxide revealed the expected 5% ^{13}C satellites in those cases where other peaks did not interfere. These satellites confirmed in all respects previous proton assignments of gramicidin S-A.[8] This provides the first totally unambiguous assignment of all ^{13}C resonances, including carbonyl carbons, of a peptide of this complexity. Rigorous assignments in a number of larger peptides will be necessary before useful correlations can be established between ^{13}C chemical shifts and structural or conformational features of the peptide. To date such correlations in the peptide field, in contrast to the steroid field, for example, have been almost nonexistent.

One of the few which has been attempted is the assignment of proline lines in peptides to either the cis or trans configuration. It has proved possible[16] in several instances to distinguish cis from trans when both isomers were present in the same sample. Unfortunately most peptides give only one set of lines for proline and the correlation is not yet good enough to allow one to decide whether the proline is cis or trans in these cases. The variation in chemical shift from peptide to peptide and the lack of good model studies with proline peptides of size large enough for the proline residue to be insulated from electrical effects due to the charged groups have been the principal reasons for this.

In those cases, such as gramicidin S-A, where minimal media can be used to produce molecules with suitably selective ^{13}C enrichment, biosynthesis offers a simple, relatively rapid, and powerful technique not only for the assignment of ^{13}C resonances, but other

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nuclei such as ^{1}H and ^{15}N as well. Where these conditions are not met chemical synthesis of labeled peptides can be used. In either case, no method of assignment offers the substantial side benefits of ^{13}C enrichment. The set of ^{13}C-enriched peptides produced can be used in a variety of further studies. First, as mentioned above for gramicidin S-A, the substantial ^{13}C satellites made visible in the proton spectrum can be used to confirm proton assignments. A proton difference spectrum between unenriched peptide and peptide labeled in one residue could be used in complex cases to bring out these satellites more clearly, allowing one in effect to study the proton spectrum of one residue at a time. Second, proton assignments could also be investigated by selective ^{1}H–^{13}C decoupling. This and all other studies involving measurement of the ^{13}C spectrum are facilitated by the increased ^{13}C signal intensity resulting from the enrichment. Third, relaxation time studies of poorly resolved carbons can be done separately using the enriched compounds. Fourth, binding studies involving interaction of the peptide with a high molecular weight species can be done with greater resolution and in higher dilution, as suggested in the preparation of a specifically active enriched fragment of the ribonuclease S-peptide which could be studied in its interaction with the S-protein.[17]

After completion of this manuscript, an article[18] appeared on the use of ^{13}C T_1 values as indicators of intramolecular motion in gramicidin S. This article used the previous[5] nonrigorous ^{13}C assignments in its discussion, which limited the certainty of some of the conclusions. Specifically enriched ^{13}C compounds would have aided the interpretation in two ways. First, unambiguous assignments would have been possible for peaks whose T_1 values could be measured. Tentative specific assignments made in Figure 2 of the Allerhand and Komoroski article[18] are confirmed by our results except for Leu α and Orn α, which should be reversed. Second, accurate T_1 values could have been obtained for unresolved peaks containing two resonances from different residues (as in Pro β + Orn β, and Pro γ + Orn γ). The individual components of these unresolved peaks could have been studied in separate experiments.

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Conclusion

For peptides that can be produced chemically or biosynthetically from defined precursors available in ^{13}C-enriched form, selective ^{13}C incorporation provides a method for making unambiguous ^{13}C chemical shift assignments and gives as a by-product a set of labeled compounds useful for a variety of other studies. This method, applied to the antibiotic gramicidin S-A, has allowed the establishment of all ^{13}C chemical shift assignments, including those of the difficult to resolve carbonyl region.

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Communications to the Editor

Magnetic Circular Dichroism Studies. XXVIII.[1] Solvent Dependent Absorption and Magnetic Circular Dichroism Spectral Studies of Triphenylcarbenium Ion

Sir:

Triphenylcarbenium ion (**1**)[2] is the most-investigated carbocation and its propeller-shaped structure is well established.[3] The absorption spectrum of ion **1** has been previously studied by Gold,[4] Deno,[5] Evans,[6] Harmon,[7] Olah,[8] and others.[9] The results of these investigations (first eight entries in Table I) show that the absorption spectral parameters (both λ_{max} and ϵ) appear to be independent of medium or ion precursor. We would now like to report, for the first time, medium dependent absorption and magnetic circular dichroism (MCD)[10] spectroscopic studies of this ion.

Triphenylcarbinol (**2**) was ionized in four superacid systems (100% H_2SO_4, FSO_3H, 20% oleum, and FSO_3H–SbF_5 (1:1 mole:mole)) at room temperature to give ion **1**.[11] The absorption and MCD spectra of the resulting solutions are shown in Figure 1 while the

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(11) One milligram of **2** was dissolved in approximately 1 ml of each of the superacid solutions. In general, concentrations of the solutions were approximately 10^{-3} *M*. All spectra were run in short path length (0.01 cm) quartz absorption cells. The absorption spectrum of FSO_3H–SbF_5 (blank run) indicates there is essentially no absorption above 215 nm (a weak absorption tail begins at 205 nm). MCD and absorption spectra were immediately recorded in all cases and shown to be reproducible.