not seem to be much influenced by the dissociation of a proton in the NH group rather distant from the α-carbon atom itself.

Surprisingly, the β-proton coupling constant in radical VIII from succinimide decreases on dissociation of the NH proton suggesting that the spin density on the α-carbon atom decreases from 0.775 to 0.702 using eq 4. However, the observed α-proton coupling constant does not decrease much. In order to explain this discrepancy, nonplanarity around the α-carbon atom and subsequent σ character in the unpaired electron orbital might be introduced again after the NH proton dissociation.

Conclusion

Using the in situ radiolysis–steady state ESR method, the pK_a values for the peptide proton dissociation have been determined to be 7.6 to 13.6 for eight cyclic peptide and one related cyclic radicals. The more delocalized the π-electron system was, the lower the pK_a value became. Significant changes in the ESR parameters of cyclic peptide radicals upon the dissociation were discussed and nonplanarity of the radical structure was introduced to explain the α-proton coupling constant unexpected from simple π-electron spin distribution. To estimate the line-broadening effect in ESR spectra of 2-pyrrolidone-5-carboxylic acid radical, a forward rate constant of 1×10^9 M^{-1} s^{-1} was necessary for $-CONHC(COO^-)- + OH^- \rightarrow -CON^-C(COO^-)- + H_2O$. The deviation of the rate from the value expected under diffusion control might come from the negative charge on the carboxyl group adjacent to the radical center. Structurally related cyclic radicals from 1-methyl-2-pyrrolidinone and γ-butyrolactone were identified and discussed.

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Peptide Strain. Conformation Dependence of the Carbon-13 Nuclear Magnetic Resonance Chemical Shifts in the Ferrichromes[1a]

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Abstract: The flexible, nonrandom conformation of the metal-free ferrichromes in $(CD_3)_2SO$ is reflected in their carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra. The resonances are readily assigned by reference to literature data, previous studies in D_2O, and the comparative spectra of various serine- and alanine-containing analogues. Single-residue substitutions cause resonance shifts in the carbonyl spectral region as a consequence of concomitant conformational drifts. On metal binding the carbonyl and aliphatic ^{13}C resonances spread over wider chemical shift ranges, consistent with a tight chelate structure. Comparison of the spectra of the Al^{3+} analogues of ferrichrome (alumichrome), ferrichrome C (alumichrome C), sake colorant A (alumisake), ferricrocin (alumicrocin), ferrichrysin (alumichrysin), and ferrichrome A (alumichrome A) enables assignment of the peptide aliphatic resonances and identification of those arising from the side chains. The unusual chemical shift span exhibited by the ^{13}C NMR spectra of the alumichromes cannot be accounted for on the basis of theories based solely on electric field and anisotropy effects. Given the strained conformation revealed by the crystallographic model, proved valid for solution conditions by ^1H- and ^{15}N-NMR studies, it is proposed that the aliphatic chemical shifts are likely to be highly dependent on distorted bond geometries causing local orbital rehybridizations which deviate the carbon atoms from a pure sp^3 valence configuration. On going from one analogue to the other the carbonyl resonances shift in good agreement with literature data on model peptides which show the effect of varying the residue at position i on the chemical shifts of carbonyls at locations $i - 1$ and $i + 1$. These observations and the isomorphic nature of the alumichrome suite of analogues lead to a unique solution of the difficult problem of assigning the peptide carbonyl ^{13}C resonances.

The sensitivity of carbon-13 nuclear magnetic resonance (^{13}C NMR)[2] spectroscopy to polypeptide conformations has been amply demonstrated.[3] For ferrichrome and its serine-containing analogues ferricrocin and ferrichrysin (Figure 1) we have shown in a previous report[4] that the conformational change induced by metal binding is revealed dramatically by the aliphatic and carbonyl resonances. These experiments, performed on aqueous solutions at 15 MHz, were consistent

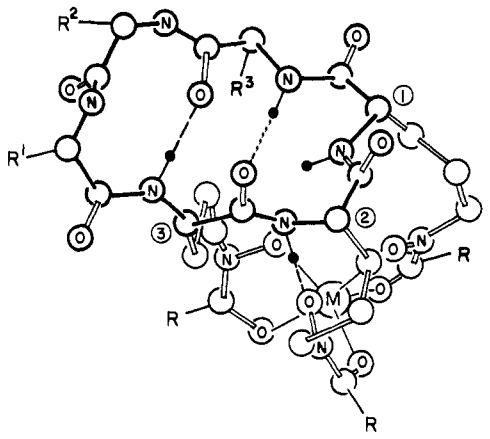
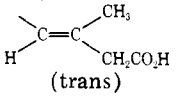


Figure 1. Structure of the ferrichromes.[6] The figure is based on the crystallographic model of ferrichrome A[14] and ^1H NMR studies on the alumichromes.[5,8] R_1, R_2, and R_3 indicate side chains for the residues at sites 1, 2, and 3, respectively. The three ornithyl residues are numbered in circles. The labeling follows the convention initiated by Zalkin et al.[14] and pursued in previous papers.[4-8] The coordinated metal ion (Fe^{3+} in the ferrichromes, Al^{3+} in the alumichromes) is denoted by M. Intramolecular H bonds are indicated by dashed lines (- - -) and locations of H atoms are shown only for the four internal, solvent-protected NH's. The pointed line (···) joining the NH of Gly^3 to the C=O of Orn^3 is intended to represent some intramolecular interaction, weakly stabilizing the amide proton, rather than any significant H bond.[12,14] The various ferrichrome analogues studies in this project differ in the nature of the residues occupying sites 2 and 3, and in the type of hydroxamate acyl substituent R as follows:[8]

Peptide	R_1	R_2	R_3	R
Ferrichrome	H	H	H	CH_3
Ferrichrome C	H	CH_3	H	CH_3
Ferricrocin	H	CH_2OH	H	CH_3
Sake colorant A	H	CH_3	CH_2OH	CH_3
Ferrichrysin	H	CH_3OH	CH_2OH	CH_3
Ferrichrome A	H	CH_2OH	CH_2OH	 (trans)

with ^1H NMR studies at 220 MHz[5] in showing that the Al^{3+} peptides ("alumichromes", diamagnetic analogues of the ferrichromes) have essentially identical spectra, reflecting a basically uniform conformation, quite independent of the amino acid composition. The minor ^{13}C resonance shifts were attributed to strain effects rather than to any significant conformational differences among the analogues.[4] The structural strain arises from metal coordination and is subtly dependent on the primary structure.[6] Indeed, the uniform conformation of the alumipeptides disappears upon metal release, in which state the peptides assume the free conformational dictated by the amino acid sequence and the solvent. Thus, on going from water to DMSO the ^1H NMR spectra of the amide NH region, as well as the temperature dependence of these resonances, indicate a more differentiated structure for deferriferrichrome in DMSO than in water ("random" conformation).[7]

The following questions remain unanswered: (1) Is the solvent-dependent conformation of the metal-free peptide also detected by the ^{13}C NMR spectrum? (2) What are the assignments of the aliphatic and carbonyl ^{13}C resonances? (3) To what extent can the alumichrome ^{13}C NMR spectrum be correlated with effects arising from a strained molecular geometry? In this paper we will attempt to provide some answers to these questions by: (1) examination of spectra taken in DMSO; (2) comparison of ^{13}C NMR spectra of various analogues to determine the influence of substituent effects on the aliphatic and carbonyl spectral region; (3) analysis of the crystallographic coordinates, which are known to give excellent agreement with the NMR data[6,8] to detect geometrically distorted valence configurations.

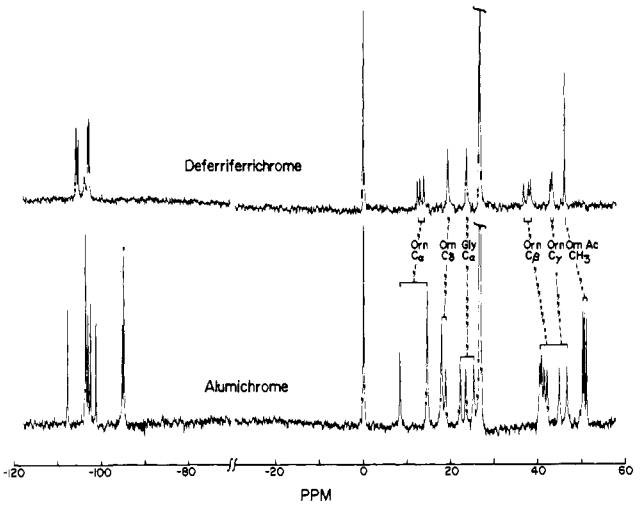


Figure 2. Proton noise-decoupled carbon-13 NMR spectra of metal-free ferrichrome and its derived Al^{3+} complex, alumichrome, dissolved in DMSO-d_6 at ~50 °C (with deuterium decoupling of the solvent resonance). The spectra were recorded at 25.1 MHz and represent Fourier transformed averages of 7000 times domain transients. The chemical shifts, referred to internal dioxane, are listed in Tables I-IV together with those of other analogues.

Experimental Section

The biological sources of the ferrichrome peptides studies in this project as well as the methods for extraction, purification, and metal substitution have been described in previous publications.[5,8] Deuterated DMSO was purchased from a commercial source and was used without further purification. Except for the data related to alumichrome A, measured in a custom-fabricated instrument which operates at 15.1 MHz,[4] all the spectra were recorded at 25.1 MHz by use of a Varian XL-100 spectrometer modified for quadrature detection and double (^1H, ^2H) decoupling performance.[9] The peptide concentration varied from about 100 to 150 mg dissolved in 1.6 mL of solvent and the samples were contained in 10 mm NMR tubes. The spectra were obtained under the following conditions: 80° ^{13}C nutation pulses at 5-s intervals were followed by dual phase data sampling at a rate of 5.55 kHz per channel for 1.47 s; 2000-4000 such accumulations were filtered with a 0.6 Hz pretransform filter function and Fourier transformed. Broadband ^1H noise decoupling was used in all experiments.

Results

The spectrum of deferriferrichrome in DMSO-d_6 is shown in Figure 2. The assignment of the aliphatic resonances can be achieved by reference to our earlier work[4] and is independently substantiated by comparison with the data for other metal-free analogues examined in the same solvent.

A number of alanine- and serine-containing deferriferrichromes were studied and their aliphatic ^{13}C resonances are listed in Table I. Substitution of a glycine at site 2 for L-alanine (deferriferrichrome → deferriferrichrome C) or for L-serine (deferriferrichrome → deferriferricrocin) separated the two remaining glycyl C_α resonances, which are unresolved in deferriferrichrome, into distinct peaks at ~23.4 and ~23.6 ppm. A second seryl-for-glycyl substitution at site 3 (deferriferrichrome → deferriferrichrysin) leaves the peak at 23.2 ppm as the only remaining glycyl C_α resonance. The comparative data hence allow assignment of sites 1, 2, and 3 glycyl, alanyl, and seryl resonances as indicated in Table I. However, little effect of these substitutions is discerned on the triornithyl C_α resonances. This does not imply lack of conformational effects since the ornithyl C_β resonances, which span from 36.8 to 38.4 ppm in deferriferrichrome, converge to a single, unresolved peak at ~38.0 ppm in deferriferrichrysin and in its isopeptide, deferriferrichrome A. The shift of the methyl peak from 46.2 to

Table I. Chemical Shifts[a] of the Aliphatic Carbon-13 Resonances (Deferriferrichromes[b,c])

	Glycyl	Alanyl		Seryl		Ornithyl				Hydroxamate[d]
	C_α	C_α	C_β	C_α	C_β	C_α	C_β	C_γ	C_δ	CH_3
Deferriferrichrome	23.8 (3)					12.4 13.0 13.9	36.8 38.0 38.4	43.0	43.5 (2)	19.4 (3)	46.2 (3)
Deferriferrichrome C	23.5[1] 23.6[3]	17.8[2]	49.3[2]			12.7 12.9 13.5	37.1 37.8 38.1	43.1 (2)	43.2	19.3 (3)	46.2 (3)
Deferriferricrocin	23.3[1] 23.7[3]			11.0[2]	5.5[2]	12.6 12.9 13.4	37.1 37.8 38.2	43.3 (2)	43.5	19.3 (3)	46.2 (3)
Deferriferrichrysin	23.2[1]			10.7[2] 10.0[3]	5.5 (2)[2,3]	12.3 12.9 13.2	38.0 (3)	42.9 43.1	43.4	19.3 (3)	46.2 (3)
Deferriferrichrome A	23.1[1]			10.4[2] 10.0[3]	5.6 (2)[2,3]	12.8 (3)	38.0 (3)	43.0 (3)		23.1 (3)	41.9 (3)

[a] The resonance positions are given in ppm, upfield from dioxane (internal reference), for the peptides dissolved in DMSO-d_6 at ~50 °C. [b] When more than one carbon contributes to a peak, the estimated number of unresolved resonances is indicated in parentheses. [c] Superindeces assign resonances to specific residues as labeled in Figure 1 (e.g., 10.0^3 refers to the chemical shift of the Ser^3 C_α resonance in deferriferrichrysin). [d] The hydroxamate acyl group is acetic acid for all peptides except in the case of deferriferrichrome A which has *trans*-β-methylglutaconic acid (non-methyl chemical shifts are: $-CH=$, -14.4 ppm; $>CH_2$, 23.1 ppm; the quaternary $=C<$ resonance was not detected under the pulse conditions used in these experiments).

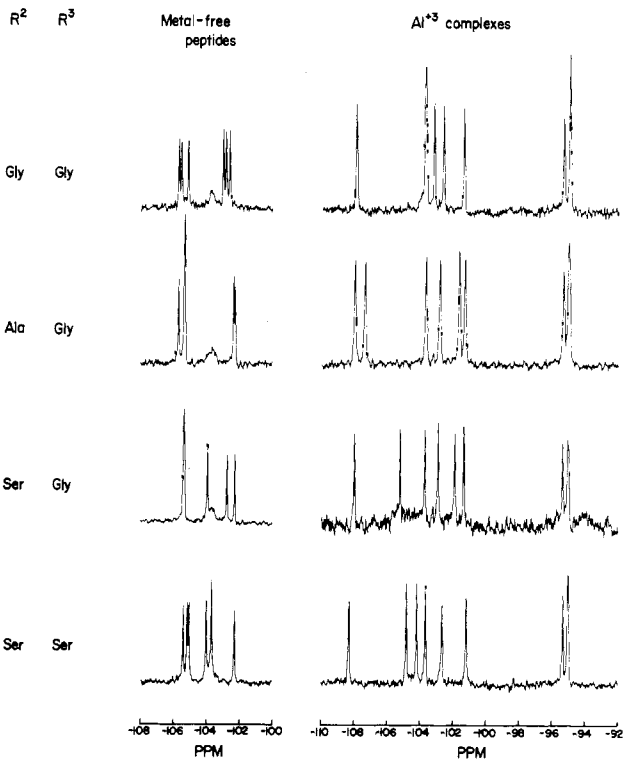


Figure 3. Expanded carbonyl ^{13}C NMR spectra of the metal-free and Al^{3+} coordination derivatives of ferrichrome, ferrichrome C, ferricrocin, and ferrichrysin. The four analogues differ in the nature of the sites 2 and 3 substituents, as indicated on the left margin (Figure 1). The hydroxamic acid carbonyls appear as a broad, unresolved peak at ca. −103.7 ppm in the deferriferrichromes, and as sharp resonances at ca. −95 ppm in the ionic alumichrome complexes. The experimental conditions are the same as for Figure 2. The chemical shift and resonance assignments are listed in Tables II and IV.

41.9 ppm is consistent with the change in acyl group on going from deferriferrichrysin to deferriferrichrome A (Figure 1).

In contrast to the NH ^1H NMR spectrum of deferriferrichrome in DMSO,[5a] the amide carbonyl ^{13}C resonances are scarcely resolved and appear grouped in two sets of three lines each, one clustered at ca. −105 ppm, arising from ornithyl residues, and the other at ca. −102.5 ppm, due to glycyl residues (Figure 2). The broad peak between these two resonance groups is assigned to the hydroxamic carbonyls.

The carbonyl resonance region of the first four metal-free analogues is shown in Figure 3, and the corresponding chemical shifts are listed in Table II. To simplify, the spectrum of deferriferrichrome A has been excluded since this peptide has three extra free carboxylate groups which resonate in this region with substantial broadening. The substitution affording deferriferrichrome C removes one of the high-field glycyl C=O resonances (at ca. −102.8 ppm) while introducing an alanyl peak at lower field (at −105.3 ppm). As suggested by the data of Grathwohl and Wüthrich,[10] the substitution also results in a closer equivalence of the remaining pair of glycyl resonances and shifts one of the corresponding ornithyl resonances. Consistently,[10] if a serine substitutes for Gly^2 (deferriferrichrome → deferriferricrocin) a new resonance appears at −103.9 ppm while the remaining two glycyl carbonyls are still well resolved. Concomitantly, the three ornithyl resonances converge to a single peak at −105.3 ppm. Finally, a further serine-for-glycine substitution at site 3 (deferriferricrocin → deferriferrichrysin) introduces a second seryl resonance at −103.6 ppm leaving the peak at −102.3 ppm as the only glycyl resonance (hence, assigned to Gly^1).

Metal binding shifts resonances everywhere in the spectrum

Table II. Chemical Shifts[a] of the Carbonyl Carbon-13 Resonances (Deferriferrichromes[b-e])

	Glycyl		Alanyl	Seryl		Ornithyl			Hydroxamate
Deferriferrichrome	-103.0^{2}	-102.8^{3}*	-102.6^{1}*			-105.7^{3}	-105.5^{2}*	-105.1^{1}*	−103.7 (3)
Deferriferrichrome C		-102.4^{3}*	-102.3^{1}*	-105.3^{2}		-105.7^{3}	$-105.3^{1,2}$ (2)		−103.7 (3)
Deferriferricrocin		-102.7^{3}	-102.3^{1}		-103.9^{2}		$-105.3^{1,2,3}$ (3)		−103.7 (3)
Deferriferrichrysin			-102.3^{1}		-104.0^{2} -103.6^{3}	-105.4^{3}	-105.2^{2}*	-105.1^{1}*	−103.7 (3)

[a] The resonance positions are given in ppm, upfield from dioxane (internal reference), for the peptides dissolved in DMSO-d_6 at ~50 °C. [b] When more than one carbon contributes to a peak, the estimated number of unresolved resonances is indicated in parentheses. [c] Superindeces assign resonances to specific residues as labeled in Figure 1 (e.g., -105.3^{2} ppm refers to the chemical shift of the Ala^{2} carbonyl resonance in deferriferrichrome C). [d] Tentative assignments are indicated by an asterisk. [e] In deferriferrichrome A, the 12 carbonyl resonances appeared broadened and with extensive overlap: −105.2 (6) ppm, −104.1 (2) ppm, −102.7 ppm, −101.9 (3) ppm.

(Figure 2). Table III lists chemical shifts of the aliphatic region for a number of Al^{3+}-coordination analogues including alumichrome A and all the acetyl hydroxamate alumichromes known to date.[8] In contrast to the metal-free peptides, the conformation of the alumichromes is maintained among the analogues. That is, the tertiary structure is highly independent of primary structure modifications at sites 2 and 3.[8] It is hence possible to achieve assignment of most $^{13}C_{\alpha}$ resonances on the basis of the comparative data. Thus, on going from alumichrome to alumichrome C, a glycyl residue is substituted by alanine at site 2, which is consistent with loss of the 22.1 ppm resonance (hence, due to the Gly^{2} C_{α}) and the appearance of two resonances at 16.1 and 50.4 ppm arising from the alanyl α and β carbons, respectively.[10,11] A serine-for-glycine substitution at site 3 (alumichrome C → alumisake) leaves the peak at 23.7 ppm as the single glycyl C_{α} resonance (hence, assigned to Gly^{1}) and results in the appearance of the corresponding Ser^{3} resonance at 13.2 ppm (C_{α}) and 5.4 ppm (C_{β}).[10,11] The assignments thus obtained from the comparative data of alumichrome and the two alanine-containing alumichromes are in full agreement with those previously derived by comparing alumichrome with alumicrocin and alumichrysin[4] in $^{2}H_2O$ solutions (the conformation of the alumichromes is highly solvent independent, which, as mentioned in the introductory section, is not the case for the deferriferrichromes[7,12]). Allowing for the alanine-by-serine substitution at site 2, the spectra within each pair of analogues, alumichrome C and alumicrocin, and alumisake and alumichrysin, are essentially identical, which further reinforces the assignments given in Table II.

The above identification of the sites 1 (Gly), 2 (Gly, Ala or Ser), and 3 (Gly or Ser) C_{α} resonances indicates that their positions follow the relative order of the respective H_{α} ^{1}H NMR lines.[4,5] The Orn C_{α} region also exhibits a distribution of signals closely resembling the characteristic pattern shown by the proton spectra in that a low-field resonance (~8.4 ppm in the ^{13}C spectra, ~4.72 ppm from TMS in the ^{1}H spectra) is separated from a pair of compacted resonances, positioned at higher fields (~14 ppm in the ^{13}C spectra, ~4.18 ppm from TMS in the ^{1}H spectra). This suggests that from a knowledge of the proton assignments, the corresponding Orn C_{α} resonances can be identified, which enables assignment of the ~8.4 ppm resonance to the Orn^{3} C_{α} and the two at ~14 ppm to Orn^{2} and Orn^{1}. Since Orn^{1} is the only ornithyl residue that would sense the serine-for-glycine substitution at site 3, the resonances at ~14.5–14.7 ppm in alumichrome, alumichrome C, and alumicrocin and at ~13.6–13.7 ppm in alumisake, alumichrysin, and alumichrome A can be assigned to the Orn^{1} C_{α}. In contrast, the positional invariance of Orn^{2} and Orn^{3}, sandwiched in all the analogues between Orn^{3} and Orn^{1} (Orn^{2}) and between Gly^{1} and Orn^{2} (Orn^{3}), is consistent with the chemical shift uniformity they exhibit within the series of analogues (Table III). Finally, the relative invariance of the C_{β}–C_{γ}, C_{δ}, and acyl methyl chemical shifts is in agreement with the conformational isomorphism of these peptides.[8] In particular, the constancy of the three high-field carbonyl resonances due to the hydroxamate ligand (δ = −95.4 to −95.0 ppm, Table IV) agrees with an invariant distorted octahedral complex configuration through the suite of analogues; the relative shifts detected for these carbonyls on going from alumichrysin to alumichrome A arise from the different nature of the acyl group in these two peptides.

An assignment of the low-field peptide carbonyl resonances can be achieved by a similar comparative analysis of the spectra of the alumichrome analogues (Figure 3, Table IV). The two lines at ca. −108 and at −101.2 ppm, which limit the chemical shift range within which the six amide C=O's resonate, are assigned to Orn^{2} and Gly^{1}, respectively. Orn^{2} is in an absolute invariant position through the series of analogues, namely, sandwiched between Orn^{1} and Orn^{3}. Furthermore, the Orn^{2} carbonyl itself should suffer minimal perturbation since by being exposed to the solvent, it does not interact with other peptide units and remote residue substitutions cannot affect its chemical shift by, e.g., perturbing the extent of intramolecular H bonding (Figure 1). Gly^{1}, common to all the analogues, is potentially susceptible to direct (inductive) perturbation when the site 2 residue (glycine in alumichrome) is substituted by alanine (alumichrome C and alumisake) or serine (alumicrocin, alumichrysin, and alumichrome A). However, as shown in Figure 4 of the paper by Grathwohl and Wüthrich,[10] the substitution of a glycine by an alanine shifts a linked glycyl carbonyl (Gly^{3} in our case) to a higher field position while leaving the carbonyl of the NH-linked residue (Gly^{1} in our case) essentially unaffected. Such an analysis assigns the resonance at −101.7 ppm in alumichrome C to Gly^{3} and that at −101.3 ppm to Gly^{1}, the new site 2 alanine carbonyl giving rise to the new resonance at −107.4 ppm. The effect of a Ser^{2} for Gly^{2} substitution can be detected on going from alumichrome to alumicrocin. In agreement with the spectra compilation of Grathwohl and Wüthrich,[10] the consequences of such a substitution are less pronounced than when alanine replaces GLY^{2}/ On this basis, the alumicrocin resonances at −105.2 and at −101.9 ppm are assigned to Ser^{2} and Gly^{3}, respectively. Since the resonances at ca. −103.7 and −102.9 ppm remain practically unperturbed by the site 2 substitutions, these must arise from the remaining two ornithyl residues.

On going from alumichrome C to alumisake, the Gly^{1} and Ala^{2} carbonyl resonances at −101.2 and −107.1 ppm remain, but the Gly^{3} resonance at −101.7 ppm disappears because of its substitution by Ser^{3} (Table IV). A similar situation exists on going from alumicrocin to alumichrysin. This evidence suffices for assignment of the −107.1-ppm resonance in alumisake and the −104.8-ppm resonance in alumichrysin to Ala^{2} and Ser^{2}, respectively. The fact that the site 2 residues are about +0.3 ppm (alumisake) or +0.4 ppm (alumichrysin) from their positions in alumichrome C and alumicrocin, where the site 3 is occupied by Gly^{3}, reflects identical effects arising from the Ser^{3} substituent, whose carbonyl resonates at −103.3,

Table III. Chemical Shifts[a] of the Aliphatic Carbon-13 Resonances (Alumichromes[b,c])

	Glycyl	Alanyl		Seryl		Ornithyl			Hydroxamate[d,e]
	C_α	C_α	C_β	C_α	C_β	C_α	C_β and C_γ (unidentified)	C_δ	CH_3
Alumichrome	22.1[2] 23.3[1] 25.2[3]					8.3[3] 14.5 (2)	40.3 40.7 41.3 42.0 44.8 46.6	17.8 (2) 18.8	50.2 50.6 51.1
Alumichrome C	23.3[1] 25.1[3]	16.1[2]	50.4[2]			8.5[3] 14.5[2] 14.7[1]	40.2 40.8 41.5 42.0 45.3 46.8	17.8 (2) 18.9	50.2 50.6 51.1
Alumisake	23.7[1]	15.9[2]	50.2[2]	13.2[3]	5.4[3]	8.5[3] 13.7[1] 14.4[2]	39.3 40.3 41.8 41.9 44.8 45.8	17.9 (2) 18.9	50.2 50.5 51.1
Alumicrocin	23.2[1] 25.0[3]			8.8[2]	6.0[2]	8.4[3] 14.5[2] 14.7[1]	40.2 40.8 41.6 42.0 45.3 46.6	17.8 (2) 18.9	50.2 50.6 51.1
Alumichrysin	23.5[1]			8.6[2] 13.2[3]	5.7[3] 6.0[2]	8.5[3] 13.6[1] 14.4[2]	39.1 40.2 41.7 42.0 44.8 45.8	17.9 (2) 18.9	50.3 50.6 51.1
Alumichrome A	23.5[1]			8.6[2] 13.1[3]	5.9 (2)	8.3[3] 13.6[1] 14.3[2]	39.2 39.9 41.9 (2) 44.9 45.8	17.8 (2) 19.0	43.4 47.9 48.6

[a] The resonance positions are given in ppm, upfield from dioxane (internal reference), for the peptides dissolved in DMSO-d_6 at ~50 °C. [b] When more than one carbon contributes to a peak, the estimated number of unresolved resonances is indicated in parentheses. [c] Superindeces assign resonances to specific residues as labeled in Figure 1 (e.g., 16.1[2] refers to the chemical shift of the Ala[2] C_α resonance in alumichrome C). [d] The hydroxamate acyl group is acetic acid for all peptides except in the case of alumichrome A which has *trans*-β-methylglutaconic acid (non-methyl chemical shifts are: —CH=, ~48.0 (2) and ~52.6 ppm; CH_2, 21.4 (3) ppm). [e] Although extensive peak overlap occurs between the alumichrome A ornithyl C_β, C_γ, and acyl-CH_3 resonances, it is possible to tentatively identify the methyl resonances on the basis of closest fit among the $\beta-\gamma$ resonances in alumichrome A and its homologue, alumichrysin.

−103.6, and −103.7 ppm in alumisake, alumichrysin, and alumichrome A, respectively.

The site 3 substitution enables a decision regarding the identity of the Orn^1 and, by exclusion, the Orn^3 carbonyl resonances. Thus, the ~0.4 ppm low-field shift (Table IV) experienced by the −103.7-ppm resonance on going from alumichrome C to alumisake or from alumicrocin to alumichrysin assigns this signal to Orn^1, covalently linked to the site 3 residue (Figure 1).

The assignment of the Ser^3 carbonyl resonances in alumichrome C and alumichrysin can be reached on the basis of two considerations: (1) the shift between Ser^2 and Ser^3 (in alumichrysin) should be of about the same magnitude as the shift between Gly^2 and Gly^3 in alumichrome; (2) the move of the site 2 resonance on going from alumichrome to alumicrocin ($Gly^2 \rightarrow Ser^2$, all the other residues remaining constant) should be of about the same magnitude as the move of the site 3 resonance on going from alumichrome C to alumisake or from alumicrocin to alumichrysin (in both cases $Gly^3 \rightarrow Ser^3$, all the other residues remaining constant). The carbonyl resonances at −103.6 ppm in alumichrysin and at −103.3 ppm in alumisake agree with those calculated from the above considerations in better than ±0.1 ppm (Table IV).

Discussion

The spectrum of deferriferrichrome exhibits better resolution in DMSO than in aqueous solution[5a] (Figure 2). Thus, in contrast to ^2H_2O, the three ornithyl C_α resonances are well separated and even the C_γ resonances are somewhat resolved in DMSO. The C_β resonances, resolved in D_2O, also show little overlap in the less polar solvent. The resolution of the ornithyl resonances is larger for the α and β carbons, smaller for C_γ, and disappears for the distal C_δ and acyl methyl groups. This trend suggests a nonrandom conformation in the region of the triornithine sequence, involving steric interaction among the residues which is maximal near the peptide backbone and decreasing along the side chains. This is consistent with our earlier ^1H NMR data,[5a] which indicated a more defined structure for the deferripeptide in DMSO than in D_2O. The proton data suggested a conformation in DMSO involving transannular H bond pairing between a glycyl and an ornithyl residue as in the Schwyzer-type β-sheet model for cyclohexapeptides.[5a] It is apparent that in aqueous solution the serine-for-glycine substitutions gave smaller carbonyl resonance shifts than in the DMSO solutions (Figure 3). This is consistent with the more defined conformations of the metal-free peptides in this solvent[7] where intramolecular H bonds can affect the chemical shift of the $^{13}C{=}O$ resonance. Since inductive effects on the triornithyl carbonyl resonances should be negligible when residue substitutions occur at site 2 (buffered by Gly^1 and Gly^3 from direct interaction with the ornithyl residues), the observed ornithyl carbonyl resonance shifts on going from deferriferrichrome to deferriferrichrome C or deferriferricrocin indicate conformational effects which are also revealed by the ^1H spectra.[7]

Upon metal binding the ferrichromes experience a drastic change in their conformational state.[6] Figure 2 shows how this effect is reflected in the ^{13}C NMR spectrum of the peptide dissolved in DMSO. Although significant resonance shifts occur everywhere in the spectrum, we shall concentrate the discussion of the aliphatic region on the $C_\alpha H$ resonances since these have been assigned in the proton[5b] and carbon-13 spectra. The ^1H and ^{13}C NMR data show a uniform trend for the $C_\alpha H$ chemical shifts of amino acids kept at constant positions throughout the isomorphic series of analogues. For example, the ornithyl C_α chemical shifts vary, in alumichrome, from 14.5 to 8.3 ppm upfield from dioxane (Table II). This trend is paralleled by the α-proton spectrum[7,10] which, downfield from

Table IV. Chemical Shifts[a] of the Carbonyl Carbon-13 Resonances (Alumichromes[b,c])

	Glycyl		Alanyl	Seryl		Ornithyl			Hydroxamate	
Alumichrome	−103.8[2]	−102.7[3]	−101.4[1]			−107.9[2]	−103.8[1]	−103.2[3]	−95.4	−95.0 (2)
Alumichrome C		−101.7[3]	−101.3[1]	−107.4[2]		−108.0[2]	−103.7[1]	−102.9[3]	−95.3	−95.0 (2)
Alumisake			−101.2[1]	−107.1[2]	−103.3[3]	−108.3[2]	−104.1[1]	−102.7[3]	−95.3	−95.0 (2)
Alumicrocin		−101.9[3]	−101.4[1]	−105.2[2]		−108.0[2]	−103.7[1]	−102.9[3]	−95.4	−95.1 (2)
Alumichrysin			−101.2[1]	−104.8[2]	−103.6[3]	−108.3[2]	−104.2[1]	−102.6[3]	−95.3	−95.0 (2)
Alumichrome A[d]			−101.1[1]	−104.8[2]	−103.7[3]	−108.3[2]	−104.1[1]	−102.8[3]	−95.1	−92.8 −92.7

[a] The resonance positions are given in ppm, upfield from dioxane (internal reference), for the peptides dissolved in DMSO-d_6 at ~50 °C. [b] When more than one carbon contributes to a peak, the estimated number of unresolved resonances is indicated in parentheses. [c] Superindeces assign resonances to specific residues as labeled in Figure 1 (e.g., −103.3[3] refers to the chemical shift of the Ser[3] carbonyl resonance in alumisake). [d] The three *trans*-β-methyl glutaconate carboxylate resonances in alumichrome A appear at −105.0 (2) and −100.6 ppm.

Table V. $\langle p \rangle^2/\langle s \rangle^2$ for the α-Carbon Valence Orbitals in Ferrichrome A[a]

Orn[1]	2.65	2.36	3.05	4.50
Orn[2]	2.13	2.98	3.08	4.43
Orn[3]	4.58	2.89	1.65	4.37
Gly[1]	2.39	2.39	3.87	3.87
Site 2[b]	2.25	2.61	3.84	3.79
Site 3[b]	4.23	4.10	1.49	3.73

[a] LCAO orbital hybridizations required to reproduce the crystallographic C_α bond geometries in ferrichrome A.[14] [b] Sites 2 and 3 are occupied by glycyl residues in ferrichrome and by L-seryl residues in ferrichrome A.

TMS, extends from ~4.17 ppm (Orn[1], Orn[2]) to 4.74 ppm (Orn[3]). That is, for the same amino acid residue in different primary locations within the cyclic peptide, one observes a chemical shift variation of 6.2 and 0.6 ppm in the carbon and proton spectra, respectively. A fit of the proton data with magnetic anisotropy (Flygare anisotropic center, Flygare C=O and Zürcher C=O) models and the electric field models of the peptide group monopole distributions ($\epsilon = 4$) was attempted using the crystallographic coordinates for ferrichrome A[14] and assuming tetrahedral symmetry and 1.07 Å $C_\alpha H$ bond lengths. *No combination of magnetic anisotropy and electric field models predicted the observed shifts.*[13] It is known that except in the case of strong acids such as trifluoroacetic acid, solvents do not significantly affect the aliphatic resonances of these peptides[12] so that solvation poses no problem in regard to the above calculations.

A calculation of the bond angles from the x-ray data[14] reveals the Orn[3] C_α moiety to be the most distorted from the nearly tetrahedral angles found in poly(L-alanine)[15] (shown in parentheses): $\angle C_\beta C_\alpha N$, 112.4° (109.5°); $\angle C_\beta C_\alpha C'$, 119.1° (109.4°); $\angle N C_\alpha C'$, 100.4° (109.7°). A naive LCAO orbital rehybridization calculation fitting the crystallographic C_α bond geometries yields the results listed in Table V which shows analogous electronic configurations for the Orn[1] and Orn[2] α-carbons, significantly different from that calculated for the Orn[3] C_α whose ^1H and ^{13}C resonances are appreciably low field shifted (Table II). Likewise, the glycyl α-carbons, which exhibit an extended ^{13}C NMR chemical shift span (Table II), also have distinct hybridizations. Although it is premature to attempt to predict from quantum mechanical first principles[16a,b] chemical shifts for complex molecules such as those we are studying here, we propose that *microscopic bond distortions, affecting the normal carbon valence configuration, can play a crucial role in determining directly or through space the spread in chemical shift we observe in the aliphatic region ^1H and ^{13}C spectra of the alumichromes.* Such strained situations ought to be expected in rigid polypeptides where a significant portion of the conformational free energy is contributed by structural features other than the primary sequence (-S-S- bridges, metal binding, etc.).[17]

The nonequivalence of the three ornithyl and three glycyl carbonyl resonances in alumichrome (Figures 2 and 3) must be due to inter- and intramolecular chemical shift mechanisms. In the Results section, use was made of intramolecular substituent effects to quantitatively, albeit empirically, account for shifts induced on given resonances by neighbor residue substitutions. Calculations of the electric field contribution to the carbonyl chemical shifts in D_2O show only small effects from electric monopoles at the peptide backbone. The ligand field at the hydroxamate center has a negligible effect as far away as the peptide backbone because of the octahedral symmetry of the ligand structure. The results of these calculations are presented in Table VI; neither the resonance positions nor the chemical shift span (calculated, from −108.6 to −104.8 ppm, $|\Delta\delta| = 3.8$ ppm; measured, from −110.2 to −103.9 ppm, $|\Delta\delta| = 6.7$ ppm) agree with the experimental spectrum.[4] The anisotropy effects are, at most, ~0.25 ppm.[13] Corrections for out-of-plane distortions[20] of the peptide backbone do not improve the fit in that they fail to correlate with the *relative* carbonyl chemical shifts[13] (based on LCAO-MO calculations, Yan et al.[19] have postulated a reduction in electron density at the carbonyl carbons participating in out-of-plane rotations which would correspond to low-field shifts). In a future communication[21] we will report on solvation effects on peptidyl amides and present evidence that H bonding dominates the conformation dependence of the backbone carbonyl chemical shift.

Table VI. Predicted ^{13}C Chemical Shifts of Alumichrome Carbonyls in D_2O[a,b]

	Carbonyl group					
	Gly[3]	Gly[2]	Gly[1]	Orn[3]	Orn[2]	Orn[1]
$\Delta\sigma_1$, ^{13}C shift generated by ligand field	−0.04	0	0.03	0.04	0.14	0.03
$\Delta\sigma_2$, ^{13}C shift generated by peptide group monopoles	−2.25	0.20	−2.20	0.12	−0.01	−1.49
σ_0, Component peptide carbonyl ^{13}C shifts in D_2O[4]	−105.0	−105.0	−105.0	−107.1	−107.1	−107.1
Predicted resonance positions in D_2O (sum of σ_0, $\Delta\sigma_1$, $\Delta\sigma_2$)	−107.3	−104.8	−107.2	−106.9	−107.0	−108.6

[a] Based upon x-ray coordinates of ferrichrome A and the electric field model for the carbonyl group. [b] The chemical shifts are given in ppm upfield from dioxane.

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References and Notes

(1) (a) This paper is the 10th in a series of studies on the solution conformation of the ferrichromes. The data have been partially extracted from the doctoral dissertation of D. M. Wilson at the University of California, Berkeley, Calif., 1974. (b) Address correspondence to this author at the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pa. 15213.
(2) Abbreviations: ^{13}C NMR, ^{13}C nuclear magnetic resonance; NMR, nuclear magnetic resonance; ^{1}H NMR, ^{1}H nuclear magnetic resonance; DMSO, dimethyl sulfoxide or dimethyl-d_6 sulfoxide; TMS, tetramethylsilane; ppm, parts per million.
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Nitrogen-15 Nuclear Magnetic Resonance Studies of Porphyrins[1a]

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Abstract: The ^{15}N NMR spectra of *meso*-tetraphenylporphyrin, its dication, and zinc tetraphenylporphyrin have been investigated both at the natural-abundance level and with isotopic enrichment. The resonances of tetraphenylporphyrin at ambient temperatures are broadened by chemical exchange of the central hydrogen atoms. At lower temperatures, the ^{15}N spectra provide structural information concerning the bonding of these hydrogens. The variable-temperature ^{15}N spectra of mixtures of tetraphenylporphyrin and its dication also reveal chemical-exchange phenomena. Spectra for tetraphenylporphyrin dication and zinc tetraphenylporphyrin at the natural-abundance level are reported. Spin-lattice relaxation times and nuclear Overhauser effect data have been determined for ^{15}N enriched samples of these two compounds.

Nitrogen-15 nuclear magnetic resonance spectroscopy offers several advantages over other forms of nmr spectroscopy for the study of problems of biological interest. Some of these are a greater chemical shift range than either ^{13}C or ^{1}H, narrow natural line widths, and simplicity (there are usually fewer nitrogen atoms than protons or carbon atoms in a molecule of biological origin). In addition, nitrogen is usually present in molecules from living organisms and is often intimately involved in biochemical processes of interest (e.g., enzyme active sites). These advantages are offset by the lack of sensitivity of ^{15}N NMR spectroscopy, which is a result of low natural abundance and magnetogyric ratio. However, new instruments and techniques are making ^{15}N NMR studies of large molecules more practical.

Porphyrins and related ring systems are important components of several biological macromolecules, and many of the biologically significant properties of these molecules are directly related to the interaction among the chelating nitrogen atoms and the central metal, and among these nitrogen atoms and the hydrocarbon moiety. Although such interactions would appear to be ideally suited for investigation by ^{15}N NMR spectroscopy, few studies have been reported.

Isotopically enriched chlorophyll a (95% ^{15}N) has been prepared but no ^{15}N NMR spectrum could be observed directly even after two days of signal averaging.[3] In contrast, the ^{15}N spectrum of the corresponding ^{15}N-enriched pheophytin a was readily obtained.[3] The ^{15}N NMR spectrum of isotopically enriched carbomonoxide FLC hemoglobin (50% ^{15}N enriched at the heme nitrogen atoms) has also been investigated,[4] but no heme nitrogen resonances were observed. Although the amide nitrogens of cyanocobalamin gave excellent spectra at the natural abundance level, the signals for the ring nitrogens were not observed.[2] In the light of these somewhat disappointing findings and the results of our preliminary studies of porphyrins,[2] we report here the results of an investigation of the ^{15}N NMR spectra of simple porphyrins. The models chosen were the well-characterized *meso*-tetraphenylporphyrin (**1**) and its derivatives. The ^{15}N NMR spectra of these compounds were investigated from the point of view of chemical shift, coupling, relaxation behavior, and nuclear Overhauser effects.

Results and Discussion

Tetraphenylporphyrin. The observation of the natural-abundance ^{15}N NMR spectrum of 0.04 M **1** in chloroform was attempted using a 25-mm diameter sample tube and the