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The Solution Conformation of the Ferrichromes. II.[1a,b,c]

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Abstract: Ferrichrome, ferricrocin, ferrichysin and ferrichrome A are ferric cyclohexapeptides whose general composition is represented by $\overleftarrow{\text{Res}^3\text{-Res}^2\text{-Gly}^1\text{-Orn}^3\text{-Orn}^2\text{-Orn}^1}$ where the $\text{Res}^{2,3}$ sites are occupied by glycyl or L-seryl residues and $\text{Orn}^{1,2,3}$ stands for δ-N-acyl-δ-N-hydroxy-L-ornithyl. The latter provide the hydroxamate ligands which coordinate the metal ion. The 220 MHz proton magnetic resonance (PMR) spectra of the metal-free and of the chelated peptides in aqueous and in deutero-dimethyl sulfoxide (d_6-DMSO) solutions are reported and analyzed in terms of the molecular conformations. Because of spectral line broadening by the paramagnetic Fe^{+3} ion, the Al^{+3} and Ga^{+3} chelates were used. The chemical shifts of the amide proton resonances and their temperature dependencies are consistent with a structure containing two transannular hydrogen bonds in the metal-free peptides in d_6-DMSO and in the chelates in either solvent. Such hydrogen-bonding results in an antiparallel β-pleated sheet structure as in the Schwyzer model for cyclohexapeptides. In terms of sites paired by hydrogen bonds, however, the β-fold differs among the demetallopeptides but not among the chelates. In water a rather random conformation is suggested for the metal-free peptides. We propose that a fundamental role of the metal is to enforce the structure of the peptide backbone; thus conformational differences resulting from residue substitutions at sites

2 and 3, as well as from solvation effects, are eliminated upon its binding. Metal chelation induces pronounced chemical shifts for all of the amide NH's and markedly reduces the temperature dependencies for four of them. Gross amide hydrogen-deuterium exchange kinetics in D_2O indicates that these four amides have a highly diminished interaction with the solvent. The comparative spectra of the analogous chelates permits unequivocal assignments of proton resonances to residues in the absolute sequence. The magnitude of the amide NH-C_αH spin-spin couplings yields estimates of the conformational ϕ dihedral angles. Aside from slight seryl side-chain solvation pressures, the PMR data for the chelates in solution are in good agreement with the static X-ray crystallographic model for ferrichrome A. Since the alumipeptide conformation is virtually independent of the medium, the chemical shifts of the amide protons may be readily evaluated in terms of the solvation of the peptidyl groups.

Introduction

In a previous communication[1a] the problem of the solution conformation of ferrichrome was stated and analyzed. It was found possible to correlate the proton magnetic resonance (PMR) spectroscopic data for the Al^{+3} analogue (alumichrome) of the ferric peptide with the X-ray structural model for crystalline ferrichrome A tetrahydrate.[2,3] Although the correspondence between these two sets of data proved to be excellent it would be desirable to arrive at a model for the peptide in solution which did not require the assistance of the X-ray data for its justification. In principle, the PMR spectrum of alumichrome should contain all the information necessary to derive such a conformational model. For the time being, however, this task exceeds the theoretical developments. Accordingly, a more naive attempt to achieve such a goal by an experimental approach, _iz._, direct comparison of several ferrichrome analogues, is presented.

Ferrichrome is one member of a group of ferric cyclohexapeptides, of fungal origin, whose amino acid sequence can be generalized as $\overleftarrow{[-Res^3-Res^2-Res^1-Orn^3-Orn^2-Orn^1-]}$ where Orn^i and Res^j (i,j = 1, 2 and 3) denotes δ-_N_-acyl-δ-_N_-hydroxy-_L_-ornithyl and _L_-alanyl, glycyl or _L_-seryl residues respectively.[4,5,6] The supraindices label the residues following the order established by Zalkin, Forrester and Templeton in their X-ray study of ferrichrome A.[3] The sites along the peptide backbone occupied by Res^1, Res^2 and Res^3 will henceforth be referred to as sites 1, 2 and 3 respectively and should not be confused with those held by the δ-_N_-acyl-δ-_N_-hydroxyy-_L_-ornithyl residues. Although "ferrichrome" denotes that particular member of the group for which sites 1, 2 and 3 are occupied by glycyl residues and whose δ-_N_-hydroxy-_L_-ornithyl

δ-N-acyl group is acetic acid, when used in the plural, i.e., "ferrichromes", it is intended to mean all and any of the analogues which may not only differ in the residues at sites 1, 2 or 3 but also in the acyl group of the hydroxamate function.

The opportunity is then afforded to compare the PMR spectra of these peptides (metal-free and their Al^{+3} chelates) to see how these substitutions affect their solution conformations as revealed by changes in the spectrum. Furthermore, such spectra might provide comparative data which could enable us to reach conclusions, independent of the X-ray model, regarding the assignments of the resonances to absolute positions in the peptide ring. Our main concern will be to analyze the resonance assignment problem and to establish more definite evidence for the conformational model (Fig. 12). The perturbative effects of single residue substitutions on the overall peptide conformation will also be discussed.

The data presented in the previous paper[1a] for ferrichrome will be compared with those for the analogous seryl-containing peptides, ferricrocin, ferrichrysin and ferrichrome A. As was done with ferrichrome, the metal-free and Al^{+3} chelate peptides will be used. The gallic (Ga^{+3}) chelate of ferrichrome, gallichrome, will also be compared with alumichrome.

The amino acid composition of ferricrocin is known to be two moles of glycine, one of L-serine and three of δ-N-acetyl-δ-N-hydroxy-L-ornithine.[7] Evidence that its primary structure corresponds to that of ferrichrome, a seryl residue substituting for a glycyl at site 2, is presented separately.[8,9] The PMR spectrum of its Al^{+3} chelate (henceforth "alumicrocin") is entirely consistent with this sequence.

Ferrichrysin and ferrichrome A have the same amino acid sequence.[5,10] In common with ferrichrome and ferricrocin they contain the tetrapeptide H_2NGly^1-Orn^3-Orn^2-Orn^1COOH but close the ring with serylserine. Ferrichrysin differs from ferrichrome A in that its acylating group is acetic acid (as is the case in ferrichrome and ferricrocin), while in ferrichrome A it is trans-β-methyl glutaconic acid. The Al^{+3} complexes of deferriferrichrysin and deferriferrichrome A are henceforth referred to as "alumichrysin" and "alumichrome A," respectively.

The comparative conformational study of the analogous cyclohexapeptides investigated here is justified by their common possession of the tetrapeptide containing the tri(δ-N-acyl-δ-N-hydroxy-L-ornithyl) sequence (see above) responsible for the binding of the metal, and also by the evidence furnished by optical rotation studies in the visible and near ultraviolet. Bürer and Gulyas[11] showed that ferrichrome, ferrichrysin and ferrichrome A have the same configuration around the central metal ion, namely, that of a left-hand propeller as found in crystalline ferrichrome A.[2,3] Furthermore, the value of the Al^{+3} and Ga^{+3} complexes as functional conformational analogues of the Fe^{+3} peptides has been supported recently by biological transport experiments with Ustilago sphaerogena.[12]

In this paper the basic approach will be to consider transitions from one analogue to another as specific perturbations which, by their localized character, will help to interpret the fundamental alumichrome spectrum. Thus, we shall attempt to characterize the alumichrome conformation by perturbing the molecule in a discrete fashion (stepwise compositional changes) and observing an output signal (the PMR spectra). Our main concern will be with the differences in the chemical shifts

between the analogues rather than their particular values. These latter have been discussed in Paper I[1a] for alumichrome and the arguments can be extended readily to the other peptides.

Fig. 1 Figure 1 summarizes the composition of the compounds to be discussed here.

Experimental

The ferricrocin sample was part of a batch, the production, purification and crystallization of which was described elsewhere.[8,9] Ferrichrysin was obtained from low iron cultures of Aspergillus melleus (M2853) in a medium supplemented to 25 mM acetate and 12.5 mM ornithine.[13] The culture was processed in the same way as described for ferricrocin[8,9] and the final powder crystallized twice from cold, anhydrous ethanol with a net yield of the ferric peptide of at least (first crop) 100 mg/l. Purity of the samples could be ascertained by ascending paper chromatography in 4:1:1 n-butanol:acetic acid:water.[14] The PMR spectra of the iron-free and aluminum chelated peptides confirms their purity (vide infra). The iron-free compounds were prepared by extraction of the metal with a 40-fold molar excess of 8-hydroxyquinoline as for the case of ferrichrome.[1a]

Ferrichrome A was a by-product of the Ustilago sphaerogena fermentations that provided ferrichrome.[1a] It was readily crystallized from the crude, unextracted, concentrated cell growth medium as the tricarboxylic acid by adjusting the pH to 2.5-3.0. It was recrystallized twice from water with net yields of 450 mg/l. Iron was extracted from the twice recrystallized compound by the KCN-$Na_2S_2O_4$ method of Emery and Neilands.[15] It was found possible to crystallize

the K^+ salt of the metal-free peptide by dissolving this product in warm ethanol and neutralizing it to "pH 7" (glass electrode reading) with alcoholic KOH.

Gallichrome, alumicrocin, alumichrysin and alumichrome A were made by reacting the free trihydroxamate peptides with the hydroxides of the respective trivalent metals as previously described for alumichrome.[1a] Alumichrome A was readily crystallized from aqueous solution at pH 2.5-3.0. All the other peptides, whether metal-free or coordinated, were desalted by gel filtration through Bio-gel P2. The samples were then evaporated to relative dryness and dehydrated and stored over P_2O_5 under reduced pressure. By reference to alumichrome[1a] the PMR spectra showed that chelation was complete and that the samples were pure (see the results).

The PMR instrumentation and spectroscopic methods are the same as described in Paper I[1a] for the studies on the ferrichrome peptides. A Varian HR220 spectrometer, which operates at 220 MHz, was used. Proton spin-spin decoupling was achieved by double irradiation experiments by sideband modulation. The probe temperature was determined with ethylene glycol. Spectra are referred to internal tetramethylsilane (TMS) or to internal tert. butyl alcohol (TBA) when comparisons between amide NH resonances in H_2O and in d_6-dimethylsulfoxide (d_6-DMSO) were intended. The aqueous solutions were 5 mM d_4-acetic acid, adjusted to pH 5.14 with KOH. Water was quartz distilled. d_6-DMSO (Merck, Sharp & Dohme of Canada, Ltd.) was certified to be 99.5 atom % D.

Results

The PMR spectra for deferriferricrocin and alumicrocin, deferriferrichysin and alumichrysin, and for alumichrome A, in d_6-DMSO at 45°C,

Fig. 2 are shown in Figures 2, 3 and 4 respectively. The regions coupled by proton spin-spin interactions are shown connected by arrows. The overall spectra can be comprehended in a manner similar to that for the spectra of deferriferrichrome and of alumichrome,[1a] taken under similar conditions. These spectra may serve as guides for an approximate identification of the resonances in the analogues. However, the substitution of glycyl residues in ferrichrome by one and two seryl residues in ferricrocin and ferrichrysin, respectively, results in a few new resonances. In alumichrome A the spectrum is even more complex due to the presence of a β-methyl glutaconyl group in the hydroxamic acid.

The seryl $C_\beta\underline{OH}$ resonances occur at about 4.85 to 5.15 ppm from TMS; due to coupling to the pair of β-hydrogens these resonances are triplets which appear relatively broadened in the metal-free peptides. This can

Fig. 3 be attributed to hydrogen exchange between the seryl $C_\beta\underline{OH}$ and the free hydroxamic N$\underline{OH}$, exchange which is absent in the chelates and in deferriferrichrome (compare Figs. 2a and 3a with Figs. 2b and 3b). These resonances are appreciably sharpened upon reducing the temperature to about 20°C.

Identification of amide $\underline{NH}$ resonances as belonging to glycyl or either ornithyl or seryl residues is readily accomplished from their multiplet structure since the first appear as triplets and either of the latter two as doublets. For the Al^{+3} chelates it is possible to distinguish between the seryl and ornithyl resonances on the basis of the sequential spin-spin coupling connections: $\underline{NH}$ (doublet) ↔ $C_\alpha\underline{H}$ ↔ $C_\beta\underline{H}$ ↔ $C_\beta\underline{OH}$ for the seryl and $\underline{NH}$ (doublet) ↔ $C_\alpha\underline{H}$ ↔ $C_\beta\underline{H}$ ↔ $C_\gamma\underline{H}$ for

the ornithyl residues. For the metal-free peptides this could not be accomplished completely. The rather close proximity of the $C_{\alpha}\underline{H}$ resonances established by spin-spin decoupling to amide $\underline{NH}$ doublets made it difficult to ascertain which of these is itself coupled to a seryl-assigned $C_{\beta}\underline{H}$ (established as seryl because of coupling to a $C_{\beta}O\underline{H}$). The experimental difficulty is magnified due to the relative proximity of the ornithyl and seryl $C_{\alpha}\underline{H}$ region to the seryl $C_{\beta}\underline{H}$ resonances which made it cumbersome to directly decouple these last two in the field scanning mode in which the spectrometer operates. In the case of deferriferrichrome this problem did not arise, at least for the assignment of the ornithyl $C_{\alpha}\underline{H}$'s, since these are all well resolved and the $C_{\alpha}\underline{H} \leftrightarrow C_{\beta}\underline{H}$ connections appear less equivocal.[1a] This resulted in a failure to distinguish between seryl and ornithyl $\underline{NH}$ and $C_{\alpha}\underline{H}$ in deferriferricrocin and deferriferrichrysin.

The broadening of the (seryl) $C_{\beta}O\underline{H}$ resonances was not a serious problem in the identification of the corresponding $C_{\beta}\underline{H}$ resonances by spin-spin decoupling. Upon lowering the sample temperature to about 20°C the $C_{\beta}O\underline{H}$ resonance can be sharpened into a neater triplet whose collapse by double irradiation could be detected readily. On raising the temperature back to 45°C we assume that, unlike the coupled $C_{\beta}O\underline{H}$, the position of the seryl $C_{\beta}\underline{H}$ did not shift appreciably.

Chemical shifts and resonance assignments are given in Tables I and II. These assignments were based on the positions of the amide $\underline{NH}$ and (seryl) $C_{\beta}O\underline{H}$ resonances and on the proton spin-spin coupling connections established by homonuclear double resonance experiments. Resonances assigned to amide $\underline{NH}$, hydroxamate $NO\underline{H}$ and seryl $C_{\beta}O\underline{H}$ were confirmed by the

Table I

	Deferriferricrocin						Alumicrocin					
	Gly_1	Gly_2	D_1	D_2	D_3	D_4	Gly_1	Gly_2	Ser	Orn_1	Orn_2	Orn_3
$\underline{NH}$	8.44	8.27	8.02	7.98	7.81	7.82	8.94	6.85	8.50	10.04	7.93	6.46
$C_\alpha\underline{H}$	3.88	3.75	4.21	4.02	4.18	3.97	3.79	3.74	3.99	4.16	4.74	4.21
$C_\beta\underline{H}$				3.83 (Ser)					3.47	1.69	1.75	1.09
$C_\gamma\underline{H}$					1.67 (Orn)							
$C_\delta\underline{H}$					3.28 (Orn)							
$C_\beta C\underline{H}$				5.02 (Ser)					4.97			
$C\underline{H}_3$					2.00 (Orn)					- - - - -2.07 - - - - -		
$NO\underline{H}$					9.63 (Orn)							

Chemical shifts, referred to TMS (ppm), of deferriferricrocin and alumicrocin in d_6-DMSO, at 45°C and 220 MHz. Labeling of residues and their resonances follows the convention given in the text. D_i (i=1, ...,4) denotes an amide doublet and its spin-spin coupled $C_\alpha\underline{H}$ proton. In deferriferricrocin the similar chemical shifts of those $C_\alpha\underline{H}$'s coupled to amide $\underline{NH}$ doublets made the establishment of the corresponding $C_\beta\underline{H}$'s ambiguous by double resonance experiments; hence their assignment to either ornithyl or seryl residues remains undetermined. Those resonances denoted by Ser or Orn were unequivocally assigned by double resonance and/or from their chemical shifts. In all cases, resonances ordered under the same column are assigned to the same residue(s). The $C\underline{H}_3$ resonance for alumicrocin is, as in the case of alumichrome,[1a] the average position of three closely spaced narrow peaks.

Table II

	Deferriferricrocin						Alumicrocin					
	Gly_1	Gly_2	D_1	D_2	D_3	D_4	Gly_1	Gly_2	Ser	Orn_1	Orn_2	Orn_3
H_2O	∿6.5			∿5.6	∿6.3	∿4.9	∿5.2	∿3.7		5.3	6.9	9.0
	±0.6			±0.2	±0.5	±0.5	±0.1	±0.1		±0.2	±0.2	±0.1
DMSO	5.1	5.1	7.3	7.1	5.2	7.5	5.5	∿3.6	∿2.9	5.4	7.1	9.1
	±0.1	±0.1	±0.1	±0.1	±0.4	±0.2	±0.1	±0.2	±0.2	±0.1	±0.1	±0.1

Spin-spin coupling constants (J_{NC}) in Hz between amide N$\underline{H}$ and C$_\alpha\underline{H}$ protons for deferriferricrocin and alumicrocin in water, at pH 5.14, and in d_6-DMSO. Residues are labeled as in Table I. Values are averages derived from determinations at different temperatures. The uncertainties are their standard deviations. Poorly resolved splittings are indicated by ∿. Gly_2 and D_1 amide N$\underline{H}$'s could not be resolved for deferriferricrocin in water even at 220 MHz so their J_{NC} are not reported (see Fig. 3a). In general, the amide resonances for deferriferricrocin in water appear quite broadened and the values for the spin-spin splittings are less accurate. The J_{NC} value for the alumicrocin seryl N$\underline{H}$ in water is also not reported because this resonance is too broad. D_i's denote amides that appear as doublets but whose assignment to seryl or ornithyl residues is uncertain.

disappearance of these peaks upon dissolution in D_2O because of isotope exchange. This exchange resulted in turn in a collapse of the corresponding spin-spin coupled resonances.

As in the previous communication,[1a] we resort here to the convention of using subindices to denote the ordering of the amino acid residues in accord with their order of appearance in the PMR spectrum and superindices when referring to the absolute peptide sequence.[16]

Comparison of Figures 2a and 3a with 2b and 3b shows that profound spectral changes are induced upon chelation of Al^{+3}. The changes are qualitatively similar to those observed in the ferrichrome peptides[1a] and, as in that case, are more extensive in the amide NH region.

Fig. 4 The PMR spectrum of alumichrome A in d_6-DMSO is shown in Figure 4. The resonances appear relatively broadened, particularly so in the amide NH region. We interpret this to result from hydrogen exchange. Since alumichrysin and alumichrome A have the same amino acid sequence, their spectra should be compared (Figs. 3b and 4). It is then evident that the seryl $C_\beta OH$ peaks do not appear in the alumichrome A spectrum, reinforcing the suspicion of exchange broadening. The reason for the higher rates of hydrogen exchange in this peptide should perhaps be sought in the catalytic effect of H^+ introduced into the solution by the three free carboxylic acid group present in the β-methyl glutaconic acid acyl groups. Although the amide NH broadening did not allow a determination of the J_{NC} splittings for this peptide in d_6-DMSO, their chemical shifts could be determined with sufficient accuracy to permit useful comparisons with the other analogues. In aqueous solution, at pH 5.14, the amide NH splittings were, however, easily resolved. Another consequence of the

Table III

	Deferriferrichrysin							Alumichrysin				
	Gly	D_1	D_2	D_3	D_4	D_5	Gly	Ser_1	Ser_2	Orn_1	Orn_2	Orn_3
$N\underline{H}$	8.35	8.14	7.79	7.76	7.66	7.53	8.93	8.07	7.28	10.03	8.00	6.33
$C_\alpha\underline{H}$	3.77	4.11	4.03	4.06	4.17	4.14	3.83	4.02	4.06	4.23	4.72	4.11
$C_\beta\underline{H}$		3.81 (Ser)		3.79 (Ser)				3.56	3.29	1.66	1.79	1.08
$C_\gamma\underline{H}$					1.57 (Orn)							
$C_\delta\underline{H}$					3.49 (Orn)							
$C_\beta O\underline{H}$		5.12 (Ser)		4.97 (Ser)				5.07	5.21			
$C\underline{H}_3$					1.99 (Orn)					- - - - 2.07 - - - - -		
$NO\underline{H}$					9.45 (Orn)							

Chemical shifts, referred to TMS (ppm), of deferriferrichrysin and alumichrysin in d_6-DMSO, at 45°C and 220 MHz. Labeling of residues and their resonances follows the same convention as in Table I. In deferriferrichrysin D_i denotes amide $N\underline{H}$ doublets and their spin-spin coupled $C_\alpha\underline{H}$'s which are unassigned either to seryl or ornithyl residues. Those resonances denoted by (Ser) and (Orn) were unequivocally assigned by double resonance, by their chemical shifts, or both. In all cases, resonances ordered under the same column are assigned to the same residue(s). The $C\underline{H}_3$ resonance for alumichrysin is, as for the cases of alumichrome and alumicrocin, the average position of three closely spaced narrow peaks.

amide broadening was that homonuclear double resonance experiments were rather unreliable since the amide multiplet collapse was detected poorly, thus making it equivocal to establish direct resonance assignments to the different protons in the molecule. This need not be a matter of major concern as the alumichrysin spectrum should be a reliable guide to the identification of the resonances. Additional resonances arising from the β-methyl glutaconyl group were easily inferred from the known chemical shifts of analogue compounds. Thus the peaks around 6 ppm, slightly to higher field with respect to the 6.33 ppm Orn_3 amide $\underline{NH}$ resonance, were attributed to the three vinyl hydrogens. Also some extra resonances appear in alumichrome A overlapping the broad ornithyl $C_{\beta}\underline{H}$ and $C_{\gamma}\underline{H}$ band at ~1.7 ppm. These were attributed to the β-methyl glutaconyl ethylene hydrogens. Two other perturbatory effects to be expected from the different nature of the hydroxamate acyl groups are also evident. One is the relatively enhanced resolution of the three sharp methyl peaks shown by the alumichrome A spectrum. This could perhaps be rationalized by noting that because of the rather extensive conjugation of the β-methyl glutaconyl group, its backbone structure should be quite rigid. Hence the methyl groups in alumichrome A may be restricted to relatively less symmetric environments and more subtly reflect their individual sidechain conformations. The other effect is on the $C_{\delta}\underline{H}$ resonances, since these groups are immediate neighbors of the hydroxyamate $NO\underline{H}$ group. These peaks, although never directly assigned because of decoupling difficulties in the alumipeptides, are known from the spectra of the metal-free analogues to lie around 3.5 ppm. Indeed, a comparison of the alumichrysin and the alumichrome A spectra shows the resonances in this region are somewhat different.

Table IV

	Deferriferrichrysin						Alumichrysin					
	Gly	D_1	D_2	D_3	D_4	D_5	Gly	Ser_1	Ser_2	Orn_1	Orn_2	Orn_3
H_2O	∿5.8 ±0.3	∿6.5 ±0.1	∿5.7 ±0.3	∿6.9 ±0.5	5.3 ±0.3	4.5 ±0.2	∿5.6 ±0.2		4.8 ±0.1	5.5 ±0.1	7.3 ±0.2	7.8 ±0.2
DMSO	4.7 ±0.2	7.5 ±0.1	∿6.7 ±0.4	∿4.6 ±0.6	8.2 ±0.2	5.9 ±0.1	5.5 ±0.1	∿2.1 ±0.3	3.7 ±0.2	5.6 ±0.2	6.9 ±0.1	8.5 ±0.1

Spin-spin coupling constants (J_{NC}) in Hz between amide $\underline{NH}$ and $C_{\alpha}\underline{H}$ protons for deferriferrichrysin and alumichrysin in water at pH 5.14 and in d_6-DMSO. Residues are labeled as in Table III. Values are averages derived from determinations at different temperatures. The uncertainties are their standard deviations. For alumichrysin in water the J_{NC} splitting for the Ser_1 $\underline{NH}$ could not be resolved and is not reported. D_i's denote amides that appear as doublets and that are of uncertain assignment as to whether they belong to seryl or ornithyl residues. Poorly resolved splittings are indicated by ∿. Broadening is a relatively major source of uncertainty for the deferriferrichrysin amide splittings in water.

Fig. 5

In Figures 5 and 7 the amide NH resonances of deferriferricrocin and deferriferrichrysin are shown in water (pH 5.14) and in d_6-DMSO at three different temperatures. The width of the overall amide region at 23.3°C is 0.64 ppm in DMSO vs 0.37 ppm in water for deferriferricrocin and 1.02 ppm in DMSO vs 0.732 ppm in water for deferriferrichrysin. Thus for these peptides the same solvent effect found in deferriferrichrome[1a] appears, i.e., a wider spread of the amide NH region in DMSO than in water, suggesting once again a less random environment (or more rigid conformation) for the deferripeptides in the less polar solvent. In this regard it is relevant to mention here that addition of $DCCl_3$ to deferriferrichrysin in DMSO results in an enhanced resolution of the amide NH resonances with further increase in the total spread of this region. Thus at room temperature a doublet shifts to lower fields out of the complex band composed of three resonances at about 6.8 ppm (see Fig. 7, spectrum at 23.3°C in DMSO).

Another feature shown by Figures 5 and 7 and already found in the case of deferriferrichrome[1a] is a more uniform temperature dependence of the amide NH chemical shifts in water than in d_6-DMSO. Furthermore, while in water the widths of the resonances are affected by tempera-

Fig. 6

ture (hydrogen exchange broadening), this is not the case in d_6-DMSO, where temperature increase results in (motional?) narrowing. As a result, it was found possible to resolve satisfactorily overlapping amide NH resonances in DMSO, but not in water, just by varying the sample temperature.

The temperature dependence of the amide NH chemical shifts, in both water and in d_6-DMSO, for deferriferricrocin and alumicrocin, and

for deferriferrichrysin and alumichrysin are shown in Figures 6 and 8, respectively. The lines are least squares fits of the experimental points and the slopes are indicated in parentheses. As discussed for deferriferrichrome and alumichrome[1a] the slopes of these linear plots serve as useful indicators of the extent of protection (whether by steric shielding, intramolecular hydrogen-bonding or both) of the particular amides within the peptide structure. The wider range in slopes shown by the alumi- versus the deferripeptides supports this contention. As Figure 5a shows for deferriferricrocin in water, the complex band, which at 23.3°C occurs at about 7.3 ppm, does not allow complete resolution of the three overlapping resonances within any measurable temperature interval. At most a single triplet appeared to shift out towards relatively lower fields as the temperature was increased, so that a remaining triplet plus a doublet could not be resolved. Hence, only the temperature shift of the center of these last two resonances, indicated by $G_2 + D_1$, is indicated in Figure 6a. In the case of deferriferrichrysin in d_6-DMSO (Fig. 8b), the set of points for doublet 5 (D_5) obviously does not satisfy a linear plot over the entire temperature range and the linear trend is manifested at only lower temperatures. As for the Orn_3 NH resonance in the alumi-peptides, the positive sign of the initial slope for the deferriferrichrysin D_5 NH chemical shift might imply that the predominant thermally activated process is different for these amides than for the others.

Fig. 7

Fig. 8

While the amide NH chemical shift temperature dependence for alumicrocin and for alumichrysin in water are shown in Figures 6c and 8c, the complete spectra of these peptides neither in this solvent nor in D_2O are presented; aside from the relative shifts of the amide NH resonances the rest of the spectra differed little from that in d_6-DMSO. The 60 MHz

PMR spectra of deferriferricrocin and deferriferrichrysin in D_2O have been published.[7,14]

Proton spin-spin coupling constants for amide NH-C_αH interactions (J_{NC}) are given in Tables II and IV for the free and chelated ferricrocin and ferrichrysin peptides in water and in d_6-DMSO. As in the case of the ferrichrome peptides,[1a] the values are averages of determinations at different temperatures within the range of the chemical shift temperature dependence studies, this treatment was again justified by an apparent independence of the J_{NC} on temperature within the experimental errors. As mentioned above, the exchange broadening of the amide NH resonances of the deferri-peptides resulted in poorer resolution of their splittings. In these cases and/or when the number of averaged data points was low, the uncertainties were found to be rather large (e.g., deferriferricrocin in H_2O, Fig. 5a and Table II). Only those amide NH resonances whose multiplet structure could be resolved satisfactorily or estimated from line shape are reported. In general, and as was the case for the ferrichrome peptides,[1a] glycyl amide NH triplets were rather poorly resolved. Thus, for Gly_2 in alumicrocin the reported J_{NC} was estimated from the line shape. The small splitting of Ser at site 2 was not resolved in water at pH 5.14, probably because of exchange broadening, and hence it is reported neither for alumicrocin nor for alumichrysin in this solvent. The degree of reliability of the reported J_{NC}'s is in each case reflected in the standard deviations and in most cases these uncertainties were small enough to allow useful conformational conclusions to be drawn.

Fig. 9 In Figure 9 the temperature dependence of the alumichrome A amide NH chemical shifts is represented for comparison with the corresponding

alumichrysin plots (Fig. 8). The purpose is to estimate the effect on the overall stability of these peptides produced by a change in the hydroxamate acyl substituent, as inferred from these linear plots. The J_{NC} values, averaged from these data in water at pH 5.1 (in d_6DMSO the amide NH resonances are too broad), are given in Table V.

Because the ionic radius of the diamagnetic Ga^{+3} ion (r_o = 0.62 Å) is closer to that of Fe^{+3} (r_o = 0.64 Å) than is that of Al^{+3} (r_o = 0.53 Å), some of the PMR spectral properties of gallichrome were examined. The complete spectrum of gallichrome is not reported here; it did not differ appreciably from that of alumichrome,[1a] suggesting that the conformation of the two chelates is very similar, in agreement with the cell transport experiments by Emery.[12] Because our analysis will be focused on the amide NH resonances as conformational probes for the whole peptide in solution, the amide J_{NC} splittings and the temperature dependence of the chemical shifts for gallichrome in water are reported in Table VI and Figure 10, respectively.

Fig. 10

Discussion

In Paper I,[1a] it was shown that the chemical shift temperature dependence plots for the deferriferrichrome NH's exhibited a narrower range of chemical shifts and of slopes in H_2O than in DMSO. These features, together with the corresponding J_{NC} data, suggested that intramolecular hydrogen bonding could be present in the less polar solvent. The data were consistent with an antiparallel β-pleated sheet structure for the demetallopeptide in d_6-DMSO, but not in water. For alumichrome, however, the evidence pointed to a more rigid structure, which was little affected by the solvent composition. Along these lines, i.e., pointing towards a relatively more flexible structure of the metal-free peptides,

Table V

Alumichrome A					
Gly	Ser_1	Ser_2	Orn_1	Orn_2	Orn_3
∿5.5		4.8	5.3	7.3	7.5
±0.2		±0.1	±0.1	±0.1	±0.1

Spin-spin coupling constants (J_{NC}) in Hz between amide NH and $C_\alpha H$ protons for alumichrome A in water, pH 5.14. Residues are labeled conventionally in the order the NH resonances occur in scanning from low to high field strength. The splitting for the Ser_1 NH resonance is not given because of lack of resolution. The tricarboxylic acid alumipeptide, dissolved in d_6-DMSO, shows excessively broadened amide resonances; hence it is also not reported here. The more poorly resolved splitting of the glycyl NH triplet is indicated by ∿.

Table VI

Gallichrome					
Gly_1	Gly_2	Gly_3	Orn_1	Orn_2	Orn_3
5.9	∿4.6	∿4.2	5.6	7.6	8.8
±0.1	±0.1	±0.2	±0.2	±0.1	±0.1

Spin-spin coupling constants (J_{NC}) in Hz between amide NH and $C_\alpha H$ protons for gallichrome in water, pH 5.14. The labeling of residues follows the convention given in the text and used for alumichrome in Paper I.[1a] Values are averages, and their standard deviations, derived from determinations at different temperatures. Poorly resolved splittings are indicated by ∿.

it is found that successive substitutions for Gly^2 and Gly^3 in ferrichrome by seryl residues appears to have a smaller influence upon the PMR spectral properties of the alumichelates as contrasted to the metal-free forms. Comparison of Figures 6a, 6b, 8a and 8b with the corresponding plots for deferriferrichrome[1a] reveals that these substitutions result in the amide NH resonances of each demetallopeptide showing wider ranges both in their chemical shifts and in the slopes of the linear temperature dependence plots. These data are summarized below:

	Slope Range $(ppm/°K)x10^3$		Chem. Shift Range (ppm at 23°C)	
	water	d_6-DMSO	water	d_6-DMSO
deferriferrichrome	1.21	2.81	0.37	0.57
deferriferricrocin	1.94	4.10	0.37	0.64
deferriferrichrysin	5.14	7.93	0.73	1.02

Although the rather serious steric restriction imposed by the cyclic nature of the peptide is a major conformational determinant, the comparative evidence between the different analogues indicates clearly that minor differences in the composition, arising from single residue substitutions, also generate significant pressures in establishing the backbone conformation. As was observed for deferriferrichrome,[1a] the chemical shift and linear slope ranges for deferriferricrocin and deferriferrichrysin are wider in d_6-DMSO than in water, suggesting again a more constant environment for the amide hydrogens, or a more rigid conformation for the backbone of the peptides, in the less polar solvent. Water and DMSO are solvents which amplify different effects; the first, solvation of the hydroxyl side-chain, and the second, protection of the

intramolecular hydrogen bonds. Since ranges in the chemical shifts and in their temperature dependence are both narrower for deferriferrichrome than for the seryl demetallopeptides in either solvent, it is suggested that the solvent conformational effects are larger in these latter peptides.

The trend in the ranges tabulated above indicates the seryl-for-glycyl substitution at site 3 is the one that results in the greatest conformational effects. In Paper I[1a] it was shown that the solution conformation of derriferrichrome was consistent with a Schwyzer-type structure[17] and that it is largely maintained in the chelate. If such a structure is accepted for the metal-free seryl peptides, the position of Ser^2 would be such that its side-chain hydroxyl would be quite exposed to the solvent. This would not be the case, however, for Ser^3, whose side-chain, lying below the plane of the β-folded sheet, would find itself relatively more shielded to interaction with the solvent, as an inspection of a CPK space filling model clearly shows. The more hydrophilic seryl side-chain will tend to offer maximum exposure of its hydroxyl group for hydrogen bonding to the solvent and in so doing will perturb the basic deferriferrichrome backbone conformation. The conformational pressure from the hydration energy of Ser at site 3 might hence be larger than at site 2.

Conformational inferences based on the temperature dependence plots are less evident for the seryl deferripeptides than for deferriferrichrome. This is because of their wider and more continuous range in slopes, which makes it less obvious to classify them neatly as more and less temperature dependent. Steric hindrances due to bulk spatial interferences between

the side-chains would be expected to result in different conformational stabilities for these cyclohexapeptides. With synthetic cyclic peptides, there is evidence suggesting that the number of cis peptide bonds increases, with concomitant decreases in the cyclization yields, as a result of increased steric interference between side-chains.[18a,b]

In water, where the temperature dependence of the different deferriferrichrome amide NH's is quite parallel,[1a] the resonances labeled D_4 in deferriferricrocin and D_4 and D_5 in deferriferrichrysin show temperature dependences that are significantly weaker than any of the NH's in deferriferrichrome. Steric and hydration energy conformational pressures could be such that even in water some amide hydrogens now become internal in a conformation different from that of deferriferrichrome. In d_6-DMSO D_1 and D_3 in deferriferricrocin (Fig. 6b) and D_2 and D_3 in deferriferrichrysin (Fig. 8b) show reduced temperature dependencies, while D_5 in deferriferrichrysin is rather unique among the demetallopeptides in that it exhibits a positive slope at lower temperatures (linear region) and plateaus as the temperature is raised. As will be seen below, in good agreement with the PMR and X-ray correlations previously reported for ferrichrome,[1a] the comparative evidence between the alumi-analogues proves that Orn_3 corresponds to Orn^1. Thus, as already conjectured for the case of alumichrome, the evidence supports the view that a non hydrogen-bonded, sterically buried amide NH can exhibit positive slope in the chemical shift vs temperature plot. The positive slope shown in DMSO by the deferriferrichrysin D_5 amide NH (Fig. 8b) hence reinforces the idea that the conformation arising from the introduction of a second seryl at site 3 results in steric hindrance for this amide hydrogen (compare with equivalent plot for deferriferricrocin, Fig. 6b).

Since both seryl demetallopeptides show at least two amide NH resonances with a decreased temperature dependence in DMSO, it is possible that a Schwyzer conformation might be present in either of them. However, in the case of deferriferricrocin the particular conformation does not correspond to that for deferriferrichrome in DMSO. This would require antiparallel pairing of the site 3 residue with Orn^3 so that in deferriferricrocin at least one of the glycyl NH's, a triplet, should be in a transannular H-bond and hence manifest a reduced temperature dependence. This was not observed. Once again this points to the conformational influence of the seryl-for-glycyl substitution even in the less polar solvent.

The $C_\alpha H$ resonances also appear to be quite dependent upon conformation, as a comparison between the spectra of the deferriferrichromes and the alumichromes indicates. In the metal-free peptides, however, conformational dependent environmental effects on the chemical shifts should be diminished since the overall peptide backbone conformation would be more flexible relative to the alumi-chelates. It is interesting to notice that, in d_6DMSO, Gly_1 $C_\alpha H$ resonates at 3.74 ppm in deferriferrichrome,[1a] Gly_2 at 3.75 ppm in deferriferricrocin, and Gly at 3.77 ppm in deferriferrichrysin (Tables I and III). Since the glycyl residue in deferriferrichrysin is necessarily at site 1, this correspondence implies that these resonances be assignable to Gly^1, as was proposed from inspection of the NH temperature dependence.[1a] Also, Gly_3 $C_\alpha H$ is at 3.90 ppm in deferriferrichrome while Gly_1 $C_\alpha H$ is at 3.88 ppm in deferriferricrocin in agreement with the belief[1a] that Gly_3 corresponds to Gly^3. Because of the uncertainties in the assignment of the doublet NH resonances in

the deferripeptides, no correlation of this type is possible between Gly_2 in deferriferrichrome and the corresponding seryl residue in deferriferricrocin or deferriferrichrysin. Furthermore, while the three ornithyl $C_{\alpha}\underline{H}$ resonances were neatly resolved as individual peaks in the deferriferrichrome spectrum,[1a] this is not the case for either seryl deferripeptide where the $C_{\alpha}\underline{H}$ resonances assigned to amide $\underline{NH}$ doublets by proton spin-spin decoupling experiments are more clustered together (Figs. 2a and 3a and Tables I and III). The bulky and hydrophilic character of the ornithyl hydroxamate side-chain apparently causes these residues to be sterically more sensitive to the seryl-for-glycyl substitutions. Small rotations along the peptide backbone bonds at the ornithyl sites, in new conformations arising from single residue substitutions, could result in changes in the extent of anisotropic shielding of the $C_{\alpha}\underline{H}$ from neighbor peptidyl π bonds.

Constancy of position of the aliphatic proton resonances of equivalent residues at corresponding sites is more noticeable in the alumipeptides (compare Figs. 2b and 3b, and Table I and III, and the chemical shift data for alumichrome in Paper I[1a]). An outstanding example is the Orn_2 $C_{\alpha}\underline{H}$, which appears as an isolated band at 4.75 ppm in alumichrome, 4.74 ppm in alumicrocin and 4.72 ppm in alumichrysin. The amide $\underline{NH}$ resonance for this same residue does not show such a constancy, reflecting its more subtle sensitivity to the environment, degree of exposure to the solvent and extent of hydrogen bonding. For these reasons, the amide $\underline{NH}$ resonances prove to be excellent conformational probes and most of the discussion that follows will be centered on them. In particular, in the alumichromes the pattern of the amide $\underline{NH}$ resonances is surprisingly clear. Because of the unequivocal assignment of the

glycyl and ornithyl amides in alumichrome itself--unequivocal because the first are triplets while the second are doublets--it is relatively unambiguous to recognize in the seryl alumi-peptides, because of their similar chemical shifts and J_{NC} splittings, those amide NH resonances corresponding to Orn_1, Orn_2 and Orn_3. Before discussing the alumichromes, it is pertinent to reexamine the interpretation given to the temperature dependence of the amide NH chemical shift.

It can be thought that thermal activation results in breakage of any structure in which the amide hydrogen participates. If it is hydrogen-bonded it may break the bond, and in so doing will make the particular amide available for other interactions which are themselves temperature dependent. If it is buried within the peptide structure, H-bonded or not, thermal activation will tend to unfold the secondary and tertiary structures so that the buried amide will become more exposed to the solvent and hence susceptible to H-bonding with the solvent. For an intramolecularly H-bonded NH the activation energy of unfolding might be larger than the activation energy of H-bonding and thus dominate the observed chemical shift *vs* temperature slope. The net temperature dependent equilibrium in the extent of intra- *vs* inter-molecular hydrogen bonding will depend on the hydrogen-bond length, polarity of the solvent, and the relative polarizability of the donor and acceptor groups. Thus, although because of its lower polarity DMSO as a solvent is better than water in protecting internal H-bonds, NH hydrogen bonding to the solvent will be stronger in DMSO than in water because the former is a better electron donor than is the latter. The analysis of this kind of plot is further complicated because it cannot be assumed (*vide infra*) that the absolute

strength of an intramolecular hydrogen bond is solvent independent. These plots are, however, extremely useful in determining internal vs external (i.e., exposed to the solvent) amide NH's even if the complexity of its governing parameters makes it difficult to distinguish between the extents of the H-bonding and steric contributions. An attempt can be made, however, to clarify this aspect of the problem in the case of the complexed peptides. This is possible because the basic alumichrome conformation is controlled by the coordination to the metal rather than by solvation effects, and hence solvent-induced chemical shifts of the amide NH resonances may be interpreted solely on the basis of solvent stabilization of the peptidyl dipole. The temperature dependencies will be considered first, the absolute assignment of the resonances to the peptide sequence will then be made, and, finally, the solvent-induced shifts of the NH resonances will be rationalized in terms of the conformational model.

Figures 6c and 8c show that in water, pH 5.14, the amide NH resonances of Gly_1 and Ser in alumicrocin and Gly and Ser_1 in alumichrysin show larger temperature dependencies than do the other four amides. Similarly, these two pairs of amides exchange their hydrogen for deuterium much faster than any other in the same peptides upon dilution in D_2O. This is similar to the behavior of the Gly_1 and Gly_2 amide NH's in alumichrome under similar conditions,[1a] and is entirely consistent with a peptide backbone conformation where the amide hydrogens of residues at sites 1 and 2 are exposed to the solvent. It is then interesting to note that although for alumichrome and alumicrocin in d_6-DMSO the residues at sites 1 and 2 also show higher temperature dependencies, such does not occur with alumichrysin here, Gly still shows a higher slope but Ser_1 gives a reduced slope as if

this amide had become relatively more protected in d_6-DMSO. Inspection of a space filling model constructed on the basis of the structure depicted in Figure 12 suggests contact interaction of the amide hydrogen at site 2 with either its own or the site 3 (i.e., Ser^3) seryl hydroxyl oxygen. Since the effect is absent in alumicrocin, it appears likely that in solvents of low polarity the amide of Ser_1^2 (site 2) could be hydrogen-bonded to the seryl hydroxyl of the residue side-chain at site 3 only when solvation effects on these hydrogen-bonding groups are reduced, i.e., in DMSO but not in water. There is an alternative possibility to be considered, however, i.e., that the protection of the Ser_1 amide NH be a consequence of minor conformational differences between alumicrocin and alumichrysin and/or between alumichrysin in water and in d_6-DMSO, which could result in improved steric shielding by the seryl side-chains without intramolecular H-bonding. For those amide hydrogens which are freely exposed and hence hydrogen-bonded to the solvent, the trend to higher slopes in water relative to DMSO suggests weaker hydrogen-bonding in the former relative to the latter solvent, in agreement with their different electron-donor abilities.

The remaining four amides show, in all the analogues, smaller temperature dependencies as judged from the absolute values of the slopes. They may be classified as "internal", whether H-bonded or not. The Orn_3 NH was assigned primarily to the buried amide hydrogen of Orn^1 which, in the steric model (Fig. 12) is not hydrogen-bonded.[1a] The thermally activated unfolding of the peptide should result in exposure of this amide for hydrogen-bonding to the solvent, hence in an increased deshielding in agreement with its positive slope in all the analogues (Figs. 6c,d;

8c,d; 9a,b; 10; and Paper I[1a]). The reduced slope in DMSO relative to water might be a manifestation of a tighter structure in the less polar solvent because of reduced ionic dissociation of the complex. Thus the amide NH of the residues at sites 1 or 2 (freely exposed, intermolecularly H-bonded, i.e., large negative slopes) and Orn_3^1 (deeply buried, not hydrogen-bonded, i.e., small positive slope) exemplify two extremes. Intermediate cases are the Orn_2^3, the Gly or Ser at site 3, and Orn_1. Orn_2 is assigned to Orn^3 paired to the site 3 residue in a type of β-fold structure. As discussed previously,[1a] these two amides are conformationally quite equivalent. The temperature dependence of the amide chemical shifts tends to suggest a more protected location for the site 3 NH than for the Orn_2 NH within the molecule, as the positive slope for the Orn_3^1 resonance indicates. By contrast, hydrogen-deuterium exchange experiments in D_2O,[8,19] show that the Orn_2 NH exchanges more slowly than does the residue at site 3, suggesting that the former is more stable with regards to interaction with the solvent than is the latter. The enhanced kinetic stability of the Orn_2 NH against hydrogen exchange might be attributable to a stronger transannular hydrogen bond relative to the residue at site 3. Finally, the reduced temperature dependence of the Orn_1 NH resonance (attributed to Orn^2) can be rationalized according to the conformational model in Figures 1 and 12, which shows this amide is attached to its own side-chain in a relatively short H-bond.

Figures 9a and b reveal similar plots for the amide NH's of alumichrome A. Comparison with the equivalent plots for alumichrysin (Figs. 8c and d) shows that the overall pattern of these plots is very similar for both analogues. Like alumichrysin, alumichrome A also shows the

drastic change in the Ser_1 NH slope when going from water to DMSO. Although a direct comparison of the temperature dependence plots for alumichrysin and alumichrome A in DMSO might be risky, given the acidic character of the solution of the latter peptide, the smaller differences in slope of the corresponding amides in both compounds indicate minor conformational stability differences. These can be attributed to different ornithyl side-chain acylating groups in the two compounds. The larger absolute values of the alumichrome A slopes relative to alumichrysin suggest the β-methyl glutaconate-containing peptide to be conformationally less stable than the acetyl analogue. These differences, which are not apparent from the pattern of the amide NH resonance region of the spectra, are highly magnified in the hydrogen-deuterium exchange behavior, which is in complete agreement with this view.[8,19]

Similar comparisons can be established between gallichrome and alumichrome, whose amide NH temperature dependence plots for their aqueous solutions are given in Figure 10 and in Paper I.[1a] Thus, while Gly_1, Gly_2 and Orn_2 show similar slopes, those amides that are closer to the metal, namely, Orn_1^2, Gly_3^3 and Orn_3^1, are those that are more affected. This might be a reflection of a different conformational stability and/or degree of exposure around the coordination center resulting from the different ionic radii and binding affinity for the two metal ions. This interpretation is again confirmed by the hydrogen-deuterium exchange behavior in D_2O, which clearly shows that the slowly exchanging amides exchange faster in gallichrome than in alumichrome.[8,19]

Previously the assignemnt of the PMR resonances was based on the assumption of the validity of the X-ray model.[1a] This was justified since

the PMR data for alumichrome in solution showed excellent agreement with such a model. Throughout the previous discussion that model was tacitly assumed to be adequate for all the analogous alumichromes. It would be desirable, however, to assign the different resonances to the primary structure of the peptides on the sole basis of the PMR evidence so that the X-ray model could be tested independently.

Fig. 11

An attempt will be presented here to reach a definite assignment of amide NH resonances from the comparative evidence provided by the different analogues and perturbations arising from the solvent and metal substitution. The requisite information is contained diagrammatically in Figure 11, where the chemical shifts are all referred to the methyl peak in TBA and for spectra recorded at 56.5°C. For the sake of unity in the exposition some of the data already discussed will be reexamined briefly and the information that is useful for the comparative analysis will be stressed.

The amide NH region of gallichrome in water (Fig. 11a) allows one to distinguish ornithyl from glycyl resonances since the first are doublets and the second triplets. Barring complications, inter- or intra-molecular hydrogen-bonded amides will experience positive increments (i.e., to lower field) in their chemical shifts. However, and as discussed earlier,[1a] other factors may cause the amide NH resonances to exhibit shifts of either sign, depending on their particular environment. Thus the inference of a conformation on the basis of a single spectrum is unfeasible.

Upon substitution of Ga^{+3} by Al^{+3} (compare a and b in Fig. 11) Gly_1 (1653.5 Hz), Gly_2 (1580.7 Hz) and Orn_2 (1506.5 Hz) are little affected, while the other three NH resonances appear to suffer a more significant

perturbation. We interpret this to mean that Orn_1, Gly_3 and Orn_3 experience a more pronounced environmental change induced by the metal substitution than do the other three residues; hence they may be spatially located closer to the metal ion. This was also apparent from analysis of the temperature dependence of the NH chemical shifts (see above).

On substitution of Gly^2 in alumichrome by Ser^2 in alumicrocin the spectra show that the triplet at 1674.5 Hz disappears and is replaced by a doublet at 1611 Hz (compare c and d in Fig. 11). This enables the assignment of these peaks to the residues at site 2. A further substitution of the glycyl residue at site 3 in alumicrocin by a seryl in alumichrysin eliminates the triplet at 1259.3 Hz and results in the appearance of a doublet at 1356 Hz (compare d and e, Fig. 11). Thus these resonances are assigned to the residue at site 3. Since there is only one glycyl residue in alumichrysin, the assignment of any NH triplet in this peptide is unambiguous. The triplet at 1706 Hz is then assignable to the glycyl at site 1, invariant in all the analogues. All the non-ornithyl amide NH resonances and those spin-spin coupled to them are thus unequivocally assigned.

Inspection of the X-ray model of ferrichrome A (Figs. 1 and 12) indicates that seryl-for-glycyl substitutions at sites 2 and/or 3 should leave the Orn^2 amide NH resonance relatively unperturbed, since this residue is both sandwiched between the unsubstituted Orn^1 and Orn^3, and also hydrogen-bonded to its own side-chain. Any perturbation originating at sites 2 or 3 is thus buffered, insuring a rather invariant environment for this proton which should result in constancy of its chemical shift on going from alumichrome to alumicrocin and to alumichrysin. Indeed, as

can be seen in Figure 11c, 11d and 11e, there is only one ornithyl amide NH whose (doublet) resonance shows an invariant position in the three analogues, namely, the doublet for Orn_1. Hence this peak can be assigned to Orn^2. This assignment is confirmed by comparing the spectra of alumichrysin and alumichrome A. As shown in Figure 11 e and f, the Orn_1 NH resonance is most affected by the substitution of the ornithyl side-chain acyl group. The amide hydrogen best situated to sense this particular perturbation should be the one that is hydrogen-bonded to the hydroxamate group, namely, Orn^2 (Figs. 1 and 12). Furthermore, and as discussed above, the Orn_1 NH resonance is found sensitive to the metal substitution (Fig. 11 a and b) as would be expected for an amide hydrogen-bonded to the metal coordination center in a stable fashion; according to the X-ray data this is a short H-bond: 2.80 Å.

The substitution of the glycyl residue at site 2 in the transition from alumichrome to alumicrocin also permits assignment of the Orn_2 NH resonance to Orn^3. As is shown in Figures 1 and 12, and even better in space filling models, the particular location of the Orn^3 amide hydrogen, lying immediately adjacent to the π electron cloud of the peptide bond between the amino of Gly^1 and the carboxyl of the residue at site 2, should make its chemical shift relatively sensitive to the substitution at site 2. Furthermore, the entire electron cloud of the peptide bond between residues at sites 2 and 3 will be sensitive to the residue substitution at either site or both. According to the X-ray model (Fig. 1) Orn^3 is transannularly hydrogen-bonded to the carbonyl of the residue at site 3, and hence it should sense the substitution at site 2 and reflect it in a resonance shift. As can be seen in Figure 11 (compare c and d) of the three ornithyl resonances, Orn_2 is the most affected by the substitution.

Similarly, it may be argued that the seryl-for-glycyl substitution at site 3 should also affect the local susceptibility felt by the Orn_2^3 amide hydrogen since this substitution now directly affects the carbonyl at site 3 to which this amide is hydrogen-bonded. This is consistent with the observed shift of the Orn_2 $\underline{NH}$ resonance on going from alumicrocin to alumichrysin (d and e in Fig. 11). However, this substitution also affects the chemical shift of the Orn_3 $\underline{NH}$ resonance. Since the Orn_1 and Orn_2 ornithyl doublets have already been assigned, by elimination, the Orn_3 $\underline{NH}$ resonance can be assigned to Orn^1. As shown in the steric model (Fig. 12), this particular amide hydrogen lies buried within the pouch formed by the three coordinated ornithyl side-chains and the peptide backbone plane. Hence substitution of the glycyl at site 3 in alumicrocin by seryl in alumichrysin is operationally equivalent to a substitution of a single α-hydrogen by the bulkier seryl side-chain, which results in further covering the Orn^1 amide hydrogen. Such increase in the steric shielding should result in a shift of its resonance to higher fields, which is observed in Figure 11 d and e.

It should also be noted in comparing the spectra of all the alumi-peptides in DMSO, that the Gly_1^1 $\underline{NH}$ resonance shifts 16 Hz towards lower fields in the transition from alumichrome to alumicrocin, and this can be attributed to a direct perturbation of its local susceptibility by the seryl-for-glycyl substitution at site 2: it is hence a nearest neighbor effect. In the progression alumicrocin → alumichrysin → alumichrome A the position of the Gly_1^1 $\underline{NH}$ resonance remains practically unaffected. Since the compositional replacements are rather remote from site 1, this lack of effect is entirely consistent with a proton at this

site that is fully exposed and does not interact with distant parts of the molecule.

Another effect of interest is the shift of the Ser^2 amide NH resonance from 1611 Hz to 1522.5 Hz on going from alumicrocin to alumichrysin in DMSO. The shift is concomitant with a rather drastic change in the temperature dependence of its chemical shift from a slope of -5.23×10^{-3} ppm/deg in alumicrocin (Fig. 6d) to -2.69×10^{-3} ppm in alumichrysin (Fig. 8d). When discussing the relatively reduced temperature dependence in DMSO it was argued that in this solvent the overall peptide conformation could be such that the Ser^2 NH became either buried between or hydrogen-bonded with the seryl hydroxyl side-chains or both. Since on going from alumicrocin to alumichrysin the shift of the Ser_1^2 NH resonance is towards higher fields, it is suggested that steric shielding rather than intramolecular hydrogen bonding is the cause of its hindrance to interaction with the solvent in DMSO.

Although the seryl-for-glycyl substitution should be expected to affect the chemical shift of the Orn_2^3 amide NH resonance, its direction is difficult to predict since the steric modifications brought about by the substitutions could result in minor displacements of this hydrogen atom such that its net anisotropic electronic shielding would be affected. With this reservation, it can be stated, however, that the shift towards lower fields observed for the Orn_2^3 amide NH resonance on going from alumichrome to alumicrocin and then to alumichrysin is consistent with a strengthening of the transannular hydrogen bond. It should be noted that the bulkier seryl side-chains by themselves should result in increased

steric shielding and hence in shifts towards higher fields, which are not observed. A similar effect seems to operate on the amide NH of the residue at site 3. The amide NH of the glycyl residue occupying this site in alumichrome appears little affected by the substitution at site 2 in going from alumichrome to alumicrocin (Fig. 11 c and d). However, on going from alumicrocin to alumichrysin the NH resonance of Ser_2^3 appears shifted to lower fields with respect to Gly_2^3 in alumicrocin and Gly_3^3 in alumichrome (Fig. 11 d and e). The effect of the seryl-for-glycyl substitution at site 2, alumichrome → alumicrocin, is to shift the NH resonance at this site to higher fields. this probably reflects additional steric shielding by the bulkier seryl side-chains. By contrast, in going from alumicrocin to alumichrysin the shift of the amide NH resonance of the residue at site 3 is towards lower fields. We interpret this as a strengthening of the $Res^3NH\cdots O{=}C\text{-}Orn^3$ transannular hydrogen bond. The relative rates of hydrogen-deuterium exchange in D_2O[8,19] suggest an increased stability in the peptide conformation as the number of seryl residues increases, which further supports this interpretation.

Solvent effects on peptidyl bonds and on amide hydrogen-bonding are clearly evident in the amide NH spectral region of the alumichromes. These peptides are especially well suited to study such effects since the backbone conformation is severely constrained by chelation of the metal and the solvent, per se, seems to have only minor conformational effects.

DMSO is a better electron donor than H_2O and hence is a stronger hydrogen-bonding solvent for exposed NH's. On the other hand, water, although a weaker electron-donor, is a better electron acceptor than

DMSO to H-bond to any free, exposed carbonyl oxygen atom. This interaction between water and a peptidyl C=O will stabilize the net negative charge on the carbonyl oxygen thus resulting in an enhancement of the $-\overset{O^-}{\overset{\|}{C}}-\underset{|}{NH}^+$ dipole. Such local charge transfer can be readily detected by its effect on the Orn^1_3 amide NH chemical shift (Fig. 11 b and c). As the conformational model shows (Fig. 12) this hydrogen atom is buried within a hydrophobic environment and does not undergo appreciable direct interaction with the solvent. It is, however, part of a peptide bond with the Orn^2 residue whose C=O oxygen atom is directly exposed to the solvent. The negative charge on this carbonyl oxygen is more stabilized in water than in DMSO so that the net electron density on the NH proton will increase in going from the first to the second solvent. As Fig. 11 shows, there is a shift of 19 Hz for the alumichrome Orn^1_3 NH resonance in a direction that suggests increased diamagnetic shielding in going from water to DMSO, in agreement with the expected solvation effect. An example of the opposite type of effect, i.e., solvation of the NH without affecting the C=O, is provided by the NH of the residue at site 2. This amide is directly exposed to the solvent for alumichrome and alumicrocin in either solvent and for alumichrysin and alumichrome A in H_2O, while its peptidyl-bonded carbonyl neighbor (namely, that of residue at site 3, see Fig. 12) is internal and involved in a transannular H-bond. Hence any solvent-induced chemical shift should primarily be due to solvation (H-bonding) of the site 3 NH itself, effects through the C=O being sterically impaired. According to the expected solvent effect, a shift towards lower fields should result from stronger H-bonding in the transition from water to DMSO: indeed, Fig. 11 shows

that such a solvent change shifts the alumichrome Gly_2^2 NH resonance by ∿96 Hz towards lower fields. Furthermore, the alumichrome Gly_1^1 NH group is also exposed to the solvent but peptidyl-bonded to a fully exposed C=O (Gly^2) group (Fig. 12). As Figure 11 shows, the water to DMSO transition shifts this Gly_1^1 resonance by 41 Hz, i.e., it undergoes the same kind of solvation effect that the Gly_2^2 NH exhibits but to a lesser extent. The 55 Hz difference between the solvent-induced chemical shifts on the Gly^1 and the Gly^2 NH resonances may thus be attributed to the Gly^2 C=O solvation which affects more significantly the Gly^2-Gly^1 than the Gly^3-Gly^2 peptide bonds. That is, in the transition from water to DMSO the net deshielding gained by the Gly_1^1 NH should be less than that gained by the Gly_2^2 NH because of the extra deshielding lost by the former due to the greater $C=O^-$ charge stabilization in water relative to DMSO. Further insight into the effect of C=O solvation on intramolecular H-bonds can be gained by observing the effect of solvent change on those two amides which are internal, pointing inwards from the ring, and presumably involved in an antiparallel β-pleated sheet. As shown in Figure 12, the site 3 and Orn^3 NH's are peptidyl bonded, respectively, to the Orn^1 and Gly^1 C=O's which are external. The Gly_3^3 and Orn_2^3 NH show ∿38 Hz shift in the transition from water to DMSO, shift which is in a direction suggesting further shielding in DMSO than in water. The Orn_3^1 NH resonance, as previously discussed, shows a similar effect but only half as extensive as for those NH protons involved in the β-structure pair. This could be a manifestation of some extra deshielding of the Gly_1^3 and Orn_1^3 amide NH's resulting from a strengthening of the transannular H-bonds when the peptidyl dipoles are stabilized in water. On the other hand,

the chemical shift difference between the alumichrome Gly_1^1 and Gly_2^2 NH resonances in water (~73 Hz) suggests that the NH charge stabilization due to C=O solvation is stronger than that due to the intramolecular C=O H-bonding to the Orn_2^3 NH. The same comparison in DMSO shows a chemical shift difference of ~18 Hz indeed suggesting that the intramolecular H-bond is not strong, as would have been predicted from the 2.99 Å H-bond distance found in crystalline ferrichrome A.[3] The analysis is completed by considering the alumichrome Orn_1^2 NH resonance, which exemplifies a case of solvent-independent chemical shift (Fig. 11 b and c). As the model shows (Figs. 1 and 12), this particular NH is involved in an intramolecular H-bond to its own side-chain hydroxamate NO^-. Since it is peptidyl-bonded to an internal C=O (that of Orn^3), neither the NH nor the C=O should sense solvent perturbations; hence its chemical shift should not be affected significantly by the transition from water to DMSO, as is observed.

The selection of alumichrome on which to base the discussion of solvent-induced NH chemical shifts was founded on the absence, in this peptide, of any seryl residue which might complicate the analysis because of side-chain solvation effects. Qualitatively, similar effects are revealed by the seryl alumipeptides as well as by gallichrome. The gain in conformational stability in the alumipeptides upon successive seryl-for-glycyl substitutions has already been discussed. It has been noted (Fig. 11) that on going from alumichrome → alumicrocin → alumichrysin in DMSO, negligible shifts occur in the Orn_1^2 NH resonance although the transannular Orn_2^3 NH does show a resonance shift from 1471.5 Hz → 1493 Hz → 1511.5 Hz in agreement with the interpretation of H-bond strengthening. In water, however,

where the solvent stabilizing effect is more noticeable because of stronger seryl side-chain solvation, the shift of the Orn^3_2 NH resonance is found to be more pronounced: from 1509.2 Hz → 1534 Hz → 1597 Hz. Indeed, in water, even the Orn^2_1 NH shows a chemical shift in going from one analogue to the other (1953.5 Hz → 1962.7 Hz → 1597 Hz), which is also in a direction that suggests a stronger H-bonding. Consistent with this trend, a shift of 60.8 Hz toward lower fields is found for the site 3 seryl NH on going from alumicrocin to alumichrysin in water. In summary: the amide NH chemical shifts of the different ferrichrome analogues in aqueous solution also indicate strengthening of intramolecular H-bonds upon successive seryl-for-glycyl substitution and allows prediction of the overall trend in the relative peptide conformational stability shown by the amide H-D exchange studies and discussed elsewhere.[8,19]

The comparative analysis presented above on the amide chemical shifts has made possible not only the achievement of a direct assignment of the resonances, based exclusively on PMR data, but also has rendered it possible to reach a finer rationalization of the relative chemical shifts of the amide NH resonances resulting from different perturbations. The perturbations considered have been: (1) metal substitution (gallichrome vs alumichrome in water), (2) solvent composition (alumichrome in water vs alumichrome in DMSO), (3) amino acid substitutions (alumichrome vs alumicrocin vs alumichrysin in DMSO), and (4) hydroxamate substitution (alumichrysin vs alumichrome A). It could be questioned that since the chemical shifts of the amide NH resonances are temperature dependent, the analysis presented on the basis of spectroscopic data at 56.5°C might collapse at other temperatures, thus invalidating the conclusions. A

response to this possible objection is that the slope of the corresponding resonances are different but sufficiently close to render the above analysis valid at any temperature within the range studied. As can be seen from the convergence trend which all the amide NH resonances exhibit in the metal-free and in the chelated peptides, increasing the temperature results in further randomization of the conformation. Hence a comparative analysis at the lowest possible temperature would have probably been more significant and certainly desirable. However, the temperature (56.5°C) at which the spectra in Figure 11 were recorded was selected not only because it is at about middle range between the extremes at which the temperature dependence studies were performed, but also because at this temperature line resolution is about optimal, both in terms of line width and line overlap in either solvent. In summary, the temperature will affect the magnitude of the relative chemical shifts for the resonances diagrammed in Figure 11, but the comparative trends shown in the analysis above are, for our purposes, temperature independent.

We have established the assignment of the resonances to the absolute amino acid sequence in the alumipeptides, and demonstrated its consistency with the X-ray model on the basis of the gross hydrogen exchange behavior of the amides and the temperature dependence of their chemical shifts. It is now of interest to calculate the ϕ dihedral angles for all the analogues, as was done in Paper I[1a] for alumichrome, from the measured splittings for each NH doublet. For alumichrome, the correspondence between the X-ray and the PMR data was sufficiently close that it allowed a correct prediction of the ornithyl resonance assignments. The analogues examined here introduce one and two seryl residues in alumicrocin and in

alumichrysin respectively and thus provide two new doublets to which Bystrov's formula[20] can be applied. The ϕ dihedral angles, calculated from amide NH resonance doublet splittings measured in water and in DMSO (Tables II, IV, V, VI, and data in Paper I[1a]) are shown in Table VII. Given the rather large uncertainties inherent in the formula, the differences between the calculated angles for corresponding residues in the analogues might be more significant than the absolute values of the angles. These differences reflect both solvent effects and inherent minor conformational differences among the analogues. Small conformational differences between gallichrome and alumichrome and between alumichrysin and alumichrome A are indicated in Table VII(a) for aqueous solutions of these peptides. More significant, although still small, are the differences among alumichrome, alumicrocin and alumichrysin, as suggested by the Orn_2 and Orn_3 NH-C_αH bond rotations. It is also interesting to note here the constancy of the Orn_1 ϕ angle which is also a reflection of the invariant conformation of this residue (see above). By contrast, the rotation of the Orn_2^3 NH-C_αH bond on going from alumichrome to alumicrocin and from alumicrocin to alumichrysin might once again be a manifestation of minor rotations induced by different side-chain solvation effects. In DMSO (Table VII(b)), the trend repeats: the variations in ϕ are larger for Orn_2^3 and Orn_3^1 than for Orn_1^2. It is interesting to see that ϕ for both Ser_2^3 and Orn_3^2 appears to be sensitive to the solvent change, another expression of the marked conformational effect of solvation on the Ser_2^3 side-chain. The ϕ angles reported by Zalkin et al.[3] for crystalline ferrichrome A $\cdot$ $(H_2O)_4$ are included in Table VII(c). The agreement between the X-ray and the PMR values is excellent

Table VII

	Ser_1 (±6)	Ser_2 (±7)	Orn_1 (±7)	Orn_2 (±12)	Orn_3 (±22)
a) H_2O					
Gallichrome			105	30	80
Alumichrome			105	30	83
Alumicrocin	unres		107	24	79
Alumichrysin	unres	10	105	27	89
Alumichrome A	unres	10	107	27	91
b) DMSO					
Alumichrome			102	28	82
Alumicrocin	124		106	26	76
Alumichrysin	130	3	105	24	83
c) X-ray					
Ferrichrome A	123	17	103	35	76
	Ser^2	Ser^3	Orn^2	Orn^3	Orn^1

The ϕ dihedral angle between the $\underline{HNC_\alpha}$ and the $NC_\alpha\underline{H}$ planes. Values in a) and b) are calculated from the amide $\underline{NH}$ - $C_\alpha\underline{H}$ doublet spin-spin splittings (J_{NC}) in water and in deuterodimethylsulfoxide, respectively, and on the basis of the semi-empirical relationship of Bystrov et al.[20] Numbers in parentheses, below the PMR labeling of the residues at the top of the table, are uncertainties arising from Bystrov's expression rather than from experimental errors in the J_{NC} determinations. In c) the ϕ angles with their corresponding amides for crystalline ferrichrome A are the values reported by Zalkin et al.[3] on the basis of X-ray studies. The labeling of the particular residues according to an arbitary PMR classification (subindices) and the absolute sequence (superindices) follows the convention given in the text.

for Ser_1^2 and Orn_1^2, good to fair (depending on the particular peptide) for Orn_2^3 and Orn_3^1 and relatively poor for Ser_2^3. The correlation between the X-ray and the PMR data thus appears to deteriorate as the value of ϕ decreases. This insinuates a variable accuracy of Bystrov's formula to relate J_{NC} and ϕ over the entire range of values. In fact, the alumichromes provide an excellent set of J_{NC} values to adjust the parameters appearing in the semi-empirical formula on the basis of the X-ray angles. We have such work in progress.

In view of the extent of agreement between the X-ray and PMR angles, the values in Table VII suggest that alumichrome (and gallichrome) have solution conformations closer to that of crystalline ferrichrome A than any of the analogues, alumichrome A included. This paradox might be explained by assuming that Bystrov's equation is insensitive to such minor conformational distinctions because of the uncertainty in the coefficients. However, solvation effects on the seryl side-chains, absent in alumichrome and maximum in alumichrysin and alumichrome A, could be crucial determinants of conformation. The poorer correspondence between the X-ray and PMR ϕ angle for Ser_2^3 mentioned above, might thus reflect the latter effect. It is hence implied that the crystallographic model might better apply to alumichrome than to the seryl analogues in solution because of the relative absence in that peptide of solvation pressures on its backbone.

The PMR data for the alumichromes is thus entirely consistent with the X-ray model. The solvation effects that perturb this conformation are rather small and involve rotations of the bonds along the peptide backbone. Such minor conformational differences would affect the distances between the site 3 and Orn_2^3 amide NH and carbonyl groups and hence modify the relative strength of the paired transannular hydrogen bonds. This would

Fig. 12

slightly refine the X-ray model and suggest the possibility of a modified β-fold structure for the metallo-peptide backbone, as shown in the model (Fig. 12).

However, is the X-ray model the one most consistent with the PMR data? Or, in other words, to what extent is the mapping between the measured parameters of the alumipeptides' PMR spectra and that conformation unique? Gibbons, Némethy, Stern and Craig have recently reviewed critically the general problem of conformational analysis of peptides in solution on the basis of PMR data.[21] As in the Russian school (see, e.g., the PMR conformational analysis for enniatin B,[22]) these authors have emphasized the value of a set of ϕ angles, determined from a Karplus-Bystrov type relationship, for evaluation of the energetically accessible regions of the ϕ-ψ conformational map. The problem is then directed towards a computational search for the minimal conformational energies consistent with the set of derived ϕ angles. It is curious that for the case of gramicidin S, energy minimization calculations[23] have shown that when the iterative calculation is started from a Hodgkin-Oughton-Schwyzer model (for which the PMR evidence is excellent[24a,b]) the resulting structure has an energy lower than for any other structure previously computed. Similarly, the conformational analysis of cyclohexapeptides by Ramakrishnan and Sarathy[25] shows relatively good agreement between the crystalline ferrichrome A structure and the minimum energy (Schwyzer-type) conformation derived under the constraints of twofold symmetry with intramolecular hydrogen bonds. Since the latter calculation did not restrict itself either to a fixed set of ϕ angles or to the steric requirement of (optically active) metal coordination by the ornithyl side-chains, these results again

indicate that the X-ray model, slightly perturbed in each case by the particular primary structure of each analogue, is most likely the only one compatible with the variety of PMR data reported here for the alumichromes in solution. It should be stressed, however, that it is the comparative PMR evidence provided by the different analogues that has allowed us to justify such a model with absolute independence from the X-ray data except, of course, as a most useful working hypothesis.

The above discussion has been based on the view that the observed PMR spectra correspond to the fundamental, ground state, and statistically most significant, conformation of each peptide. Although the role of the metal in stabilizing a certain conformation is obvious, the picture reached is static: it provides no major clues regarding the extent of the conformational stability. This aspect of the problem, namely, the dynamics of the conformation of the ferrichromes, has been approached through study of the kinetics of the amide NH hydrogen-deuterium exchange[8,19] and from measurements of the relaxation rates of the nuclear resonances now in progress.

Footnotes

(1)(a) Paper I: M. Llinás, M. P. Klein, and J. B. Neilands, J. Mol. Biol., 52, 399 (1970). (b) A preliminary report was presented at the Joint Western Regional Meeting of the American Chemical Society and the Society for Applied Spectroscopy, San Francisco, California, October 6-9, 1970. (c) Supported by grants from the National Science Foundation (GB 5276) and the Public Health Service (AI-04156), and the U. S. Atomic Energy Commission. Taken in part from the PH.D. dissertation of M.Ll. at the University of California, Berkeley, 1971.

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(16) To minimize the possibility of confusion in the comparison of spectra of the analogues, a given non-ornithyl residue is labeled with a superindex according to the site it occupies in the absolute sequence. This follows the order used by Zalkin et al.[3] but differs in that it stresses the site occupancy rather than the ordinal number of appearance of a given type of residue in the sequence. Thus, Gly^1 in ferrichrysin (and in ferrichrome A) is the only glycyl residue occurring in the peptide which would not, otherwise, require any superindex. Similarly, Ser^2 denotes the seryl residues at site 2 irrespective of whether it is the only one (as is the case of ferricrocin) or the first one (as is the case of ferrichrysin and ferrichrome A). We summarize here (refer also to Fig. 1) the convention followed throughout the text:

	site 1	site 2	site 3
ferrichrome	Gly^1	Gly^2	Gly^3
ferricrocin	Gly^1	Ser^2	Gly^3
ferrichrysin	Gly^1	Ser^2	Ser^3
ferrichrome A	Gly^1	Ser^2	Ser^3

With the ornithyl residues no ambiguity is possible since the labeling is the same for all the ferrichromes and is identical to that used by Zalkin et al.[3] The use of subindexes follows the convention established previously.[1a] Thus, Gly_j denotes that glycyl residue whose amide is the

j-th glycyl NH to resonate in scanning from low to high fields. The subindex thus refers to a particular spectrum. Occasionally double labeling, such as Orn^{3}_{2}, has been used in order to stress the fact that we are referring to the second ornithyl NH resonance already assigned to the third ornithine in the amino acid sequence.

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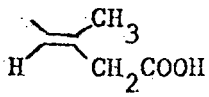
FIGURE LEGENDS

Figure 1. Structure of the ferrichromes investigated in this paper. The numbers in circles label the sites and the δ-N-acyl-δ-N-hydroxy-L-ornithyl residues consistent with the convention used by Zalkin et al. for crystalline ferrichrome A·$(H_2O)_4$.[3] R^2 and R^3 represent side-chains of those glycyl or seryl residues at sites 2 and 3 respectively, site 1 being occupied by a glycyl residue (R^1=H). R represents the acyl group in the hydroxyamate moiety (acetic acid or trans-β-methyl-glutaconic acid) and M represents the octahedrally coordinated trivalent metal ion. H-bonds found in crystalline ferrichrome A are shown here by dashed lines. The compositional differences among the ferrichrome analogues studied here are:

	R^2	R^3	R	M^{+3}
gallichrome	H	H	CH_3	Ga^{+3}
alumichrome	H	H	CH_3	Al^{+3}
alumicrocin	CH_2OH	H	CH_3	Al^{+3}
alumichrysin	CH_2OH	CH_2OH	CH_3	Al^{+3}
alumichrome A	CH_2OH	CH_2OH		

All these peptides contain the common tetrapeptide sequence H_2NGly^1-Orn^3-Orn^2-Orn^1-COOH bridging between sites 2 and 3.

Figure 2. The 220 MHz PMR spectra of (a) deferriferricrocin and (b) alumicrocin at 45°C dissolved in d_6-DMSO. The peak marked "solvent" arises from the residual H in d_6-DMSO, and that marked H_2O results from water residual after low pressure dessication over P_2O_5. In (a)

FIGURE LEGENDS (Cont.)

the resonance at lowest field arises from the NOH protons of the three δ-N-acetyl-δ-N-hydroxy-L-ornithyl residues; the group centered at ∿8.0 ppm is the amide NH protons of the six residues. In (b) the NOH resonances have disappeared as these protons are replaced by the metal and the NH resonances extend from 10 to 6.4 ppm. The peaks connected by light arrows are coupled by proton-proton spin-spin interactions and were determined by double resonance. In (a) and (b) the resonance at ∿5 ppm arises from the seryl hydroxyl proton. In (a), due to exchange, this peak is broadened, as is the hydroxamic NOH. Those peaks arising from exchangeable protons sharpen upon reducing the temperature to ∿20°C; the triplet nature of the seryl C_βOH resonance then becomes clearly apparent. The spectra are referred to internal TMS.

Figure 3. The 220 MHz PMR spectra of (a) deferriferrichrysin and (b) alumichrysin at 45°C dissolved in d_6-DMSO. The peak marked "solvent" arises from the residual H in d_6-DMSO and that marked H_2O results from water residual after low pressure dessication over P_2O_5. In (a) the resonance at lowest field arises from the NOH protons of the three δ-N-acetyl-δ-N-hydroxy-L-ornithyl residues; the group centered at 8.0 ppm is the amide NH protons of the six residues. In (b) the NOH resonances have disappeared as these protons are replaced by the metal and the NH resonances extend from 10 to 6.3 ppm. The peaks connected by light arrows are coupled by proton-proton spin-spin interactions and were determined by double resonance. In (a) and (b) the resonances at ∿5.0 and ∿5.15 ppm arise from the two seryl hydroxyl protons. In (a), due to exchange, these peaks are broadened, as is the hydroxamic NOH peak. The peaks broadened by exchange sharpen upon reducing the temperature to ∿20°C; the triplet nature of the seryl C_βOH resonances then becomes clearly apparent. The spectra are referred to internal TMS.

FIGURE LEGENDS (Cont.)

Figure 4. The 220 MHz PMR spectrum of alumichrome A (tricarboxylic acid) at 45°C dissolved in d_6-DMSO. The peak marked "solvent" arises from the residual H in d_6-DMSO. Due to the identical primary sequence between alumichrome A and alumichrysin, the assignments for the latter compound (Figure 3b, Table III) apply to the former. A few differences to be noticed here are: (1) the seryl $C_\beta\underline{OH}$ resonances are absent in the alumichrome A spectrum because of exchange broadening, (2) a set of peaks are present in the alumichrome A spectrum at ∿6 ppm which do not appear in the alumichrysin spectrum, these being assignable to the three vinyl protons of the trans-β-methylglutaconic acid group, (3) the $C_\delta\underline{H}$ resonances, which occur at ∿3.5 ppm, and are sensitive to the different hydroxamate groups in both peptides, and (4) a neater resolution of the methyl groups, ∿2 ppm, in alumichrome A relative to alumichrysin. The spectrum is referred to internal TMS.

Figure 5. The amide $\underline{NH}$ PMR region of deferriferricrocin in water, pH 5.14, and in d_6-DMSO is shown in (a) and (b) respetively. Dotted lines indicate temperature shifts of characteristic resonances. In DMSO the peaks are further separated than in H_2O, where the cluster of one doublet and two triplets at lower fields allows only partial resolution of a triplet at intermediate temperatures, leaving the other two unresolved over the whole temperature range. The spectra are referred to internal TBA.

Figure 6. The temperature dependence of the chemical shifts of the amide $\underline{NH}$ protons of deferriferricrocin and of alumicrocin in water at

FIGURE LEGENDS (Cont.)

pH 5.14 and in DMSO. G, S and O denote glycyl, seryl and ornithyl amide protons respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. In deferriferricrocin, D denotes an amide NH doublet assigned neither to seryl nor to ornithyl residues. The numbers in parentheses are 10^3 times the slope of the corresponding lines expxpressed in the graph units, i.e., -5.24 = -5.24×10^{-3} ppm/°C. The chemical shifts in both solvents were measured with respect to internal TBA. In aqueous solution of deferriferricrocin Gly_2 and the first doublet (D_1) were never resolved and the line labeled $G_2 + D_1$ refers to the center of the complex band (see Fig. 5).

Figure 7. The amide NH PMR region of deferriferrichrysin in water, pH 5.14, and in d_6-DMSO is shown in (a) and (b) respectively. Dotted lines indicate temperature shifts of characteristic resonances. Even though the peaks are less separated in H_2O than in DMSO, the different temperature dependence of the resonances and certain spectral details permit the peak drifts to be followed unequivocally even in that solvent. The spectra are referred to internal TBA.

Figure 8. The temperature dependence of the chemical shifts of the amide NH protons of deferriferrichrysin and of alumichrysin in water at pH 5.14 and in DMSO. G, S and O denote glycyl, seryl and ornithyl amide protons, respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. In deferriferrichrysin D denotes an amide NH doublet assigned neither to seryl

FIGURE LEGENDS (Cont.)

nor to ornithyl residues. The numbers in parentheses are 10^3 times the slope of the corresponding lines expressed in the graph units, i.e., $-8.09 = -8.09 \times 10^{-3}$ ppm/°C. The chemical shifts in both solvents were measured with respect to internal TBA.

Figure 9. The temperature dependence of the chemical shifts of the amide NH protons of alumichrome A in water at pH 5.14, and in d_6-DMSO. G, S and O denote glycyl, seryl and ornithyl amide protons respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. The numbers in parentheses are 10^3 times the slope of the corresponding lines expressed in the graph units, i.e., $-2.14 = -2.14 \times 10^{-3}$ ppm/°C. The chemical shifts in both solvents were measured with respect to internal TBA.

Figure 10. The temperature dependence of the chemical shifts of the amide NH protons of gallichrome in water at pH 5.14. O and G denote ornithyl and glycyl amide protons respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. The number in parentheses are 10^3 times the slope of the corresponding lines expressed in the graph units, i.e., $-2.13 = -2.13 \times 10^{-3}$ ppm/°C. The chemical shifts were measured with respect to internal TBA.

Figure 11. Diagramatic representation of the amide NH resonance region of the ferrichrome analogues under various conditions. The spectra were all obtained at 56.5°C and referred to internal TBA. Each spectrum is labeled at the left according to the compound and solvent. Triplets

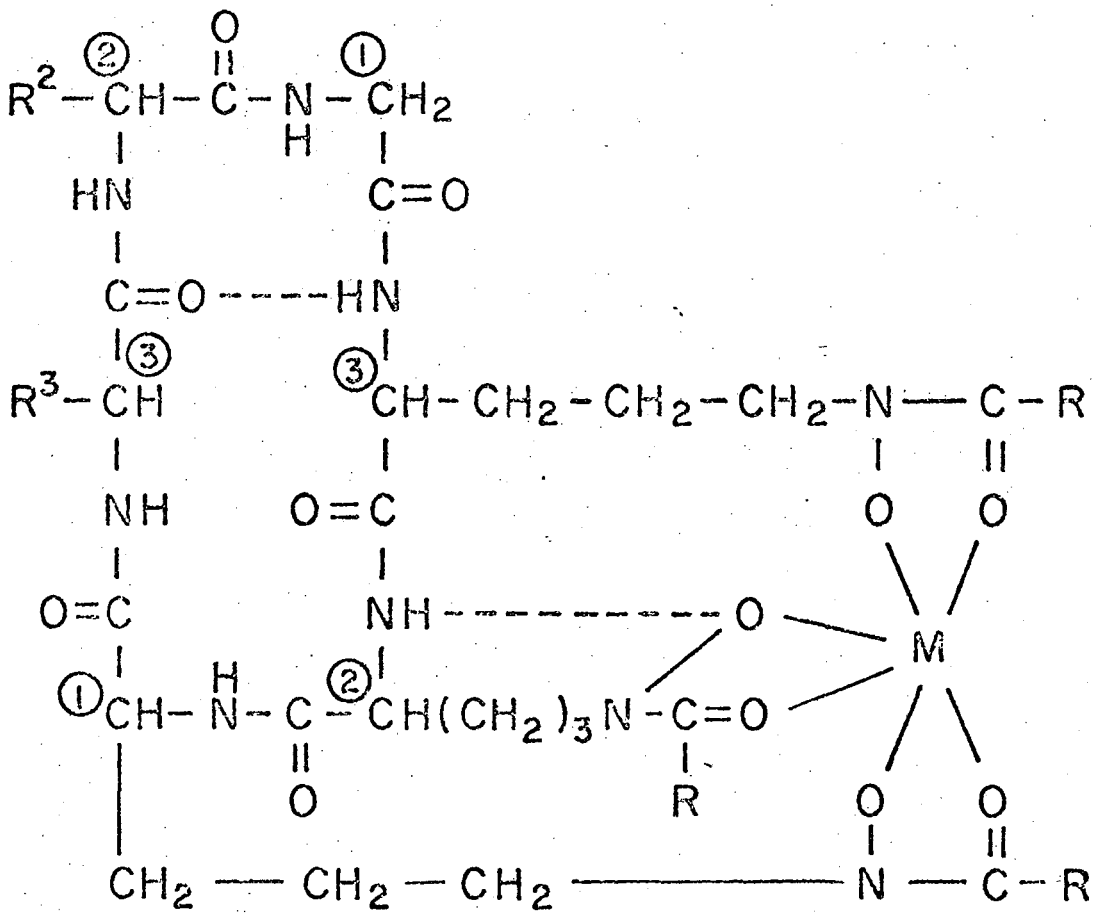
FIGURE LEGENDS (Cont.)

arise from glycyl NH protons and doublets derive from either seryl or ornithyl amides. It is possible to distinguish the seryl from the ornithyl resonances by comparing the spectra of alumicrocin vs alumichrome, and of alumichrysin vs alumicrocin, since a single seryl-for-glycyl substitution occurs between each pair. Thus in alumicrocin the doublet which occurs at 1611 Hz corresponds to its single seryl residue. This resonance shifts to 1522.5 Hz in alumicrysin (the correspondence being ascertained by their similar doublet splittings), while a second seryl-for-glycyl substitution (alumicrocin → alumichrysin) results in the doublet at 1356 Hz which is hence assigned to a seryl residue in both alumichrysin and alumichrome A.

Figure 12. The solution conformation of the ferrichromes. Bonds along the peptide backbone are drawn with heavier lines. H atoms are not shown with the exception of the four amide hydrogens that manifest reduced interaction with the solvent; of these, the one belonging to Orn^1 is buried between the peptide backbone ring and the chelated side-chains and the other three are intramolecularly H-bonded. The proposed H-bonds are: 1) between the amide proton of Orn^3 and the carbonyl oxygen of Res^3 (residue at site 3); 2) between the amide proton of Res^3 and the carbonyl oxygen of Orn^3, and 3) between the amide proton of Orn^2 and the δ-N-hydroxyl oxygen atom on the same residue. The first and third were predicted from X-ray data and the second revealed by this work. A distinction is made between more (---) and less (···) stable H-bonds. For all the ferrichromes studied, the residue at site 1 is always glycine (R^1 = H), while R^2 and R^3 may be H or CH_2OH (glycyl or seryl).

FIGURE LEGENDS (Cont.)

R denotes methyl for all the peptides except for ferrichrome A where it represents the trans-β-methyl glutaconyl group (see legend to Fig. 1). The conformation depicted here is basic for all the alumichromes and for gallichrome; however, for each compound minor solvent-dependent perturbations arise which are apparent both in the PMR spectral parameters and in the amide H-D exchange kinetics. Hence the relative strength of the intramolecular H-bonds, as well as the degree of amide hydrogen steric shielding, varies among the different analogues and from solvent to solvent.



XBL 717-5282

Figure 1

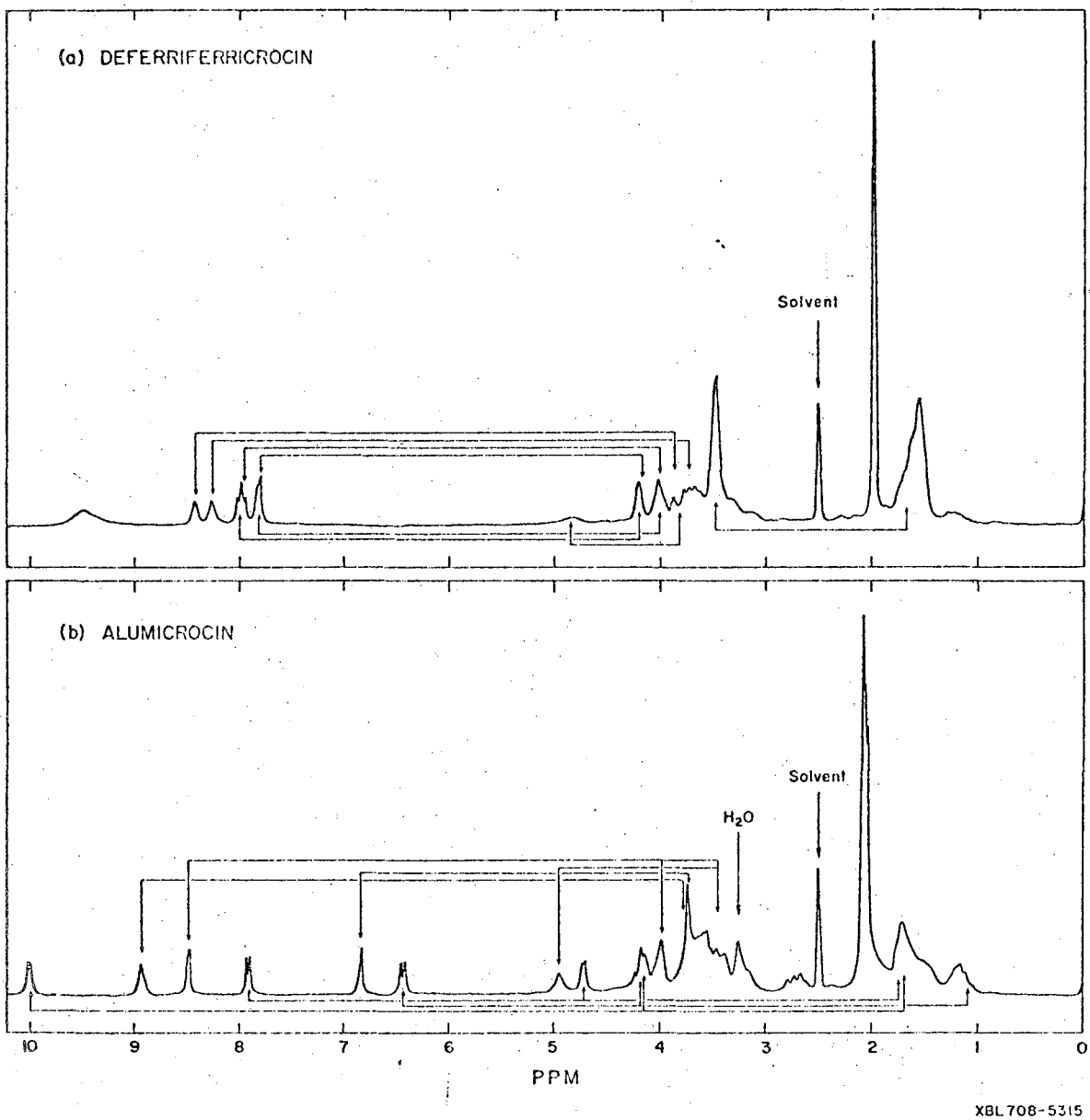

(a) DEFERRIFERRICROCIN
Solvent
(b) ALUMICROCIN
Solvent
H_2O
10
9
8
7
6
5
4
3
2
1
0
PPM
XBL708-5315

Figure 2

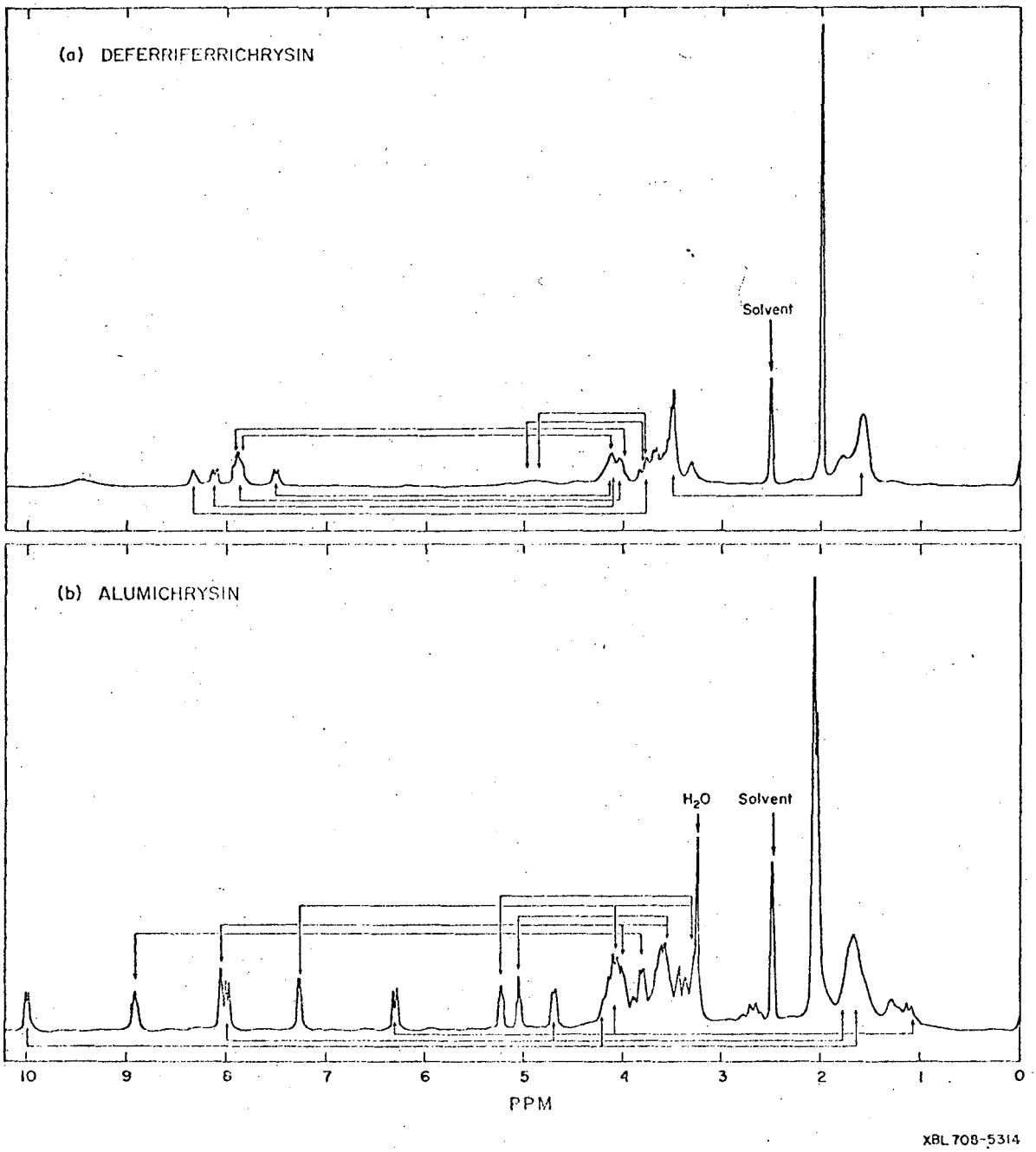

(a) DEFERRIFERRICHRYSIN
Solvent
(b) ALUMICHRYSIN
H_2O
Solvent
10
9
8
7
6
5
4
3
2
1
0
PPM
XBL 708-5314

Figure 3

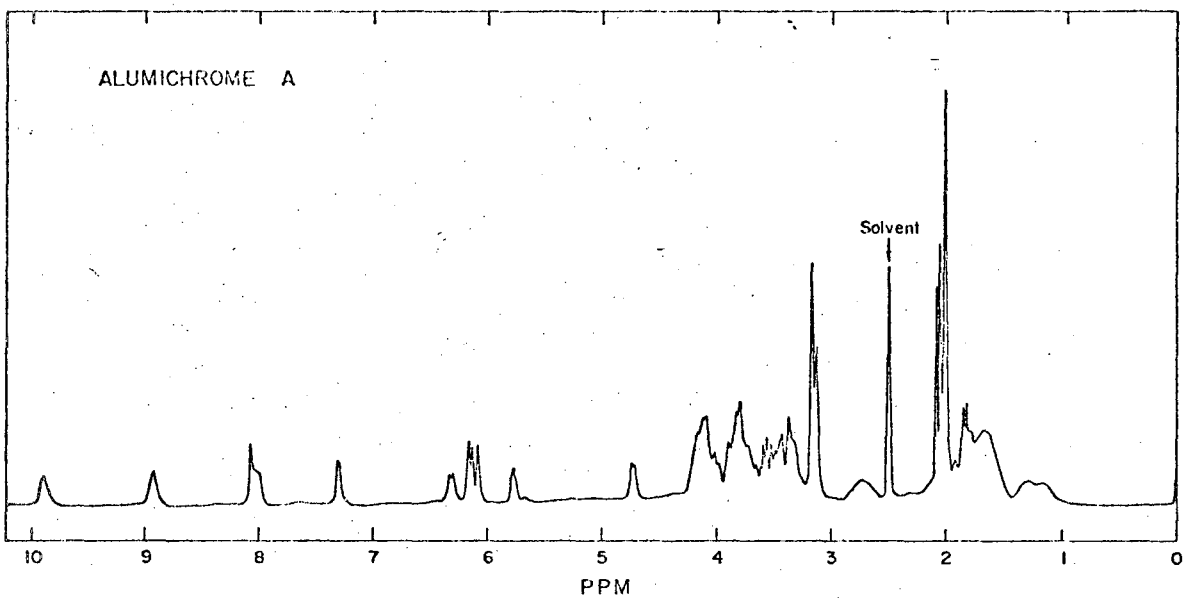
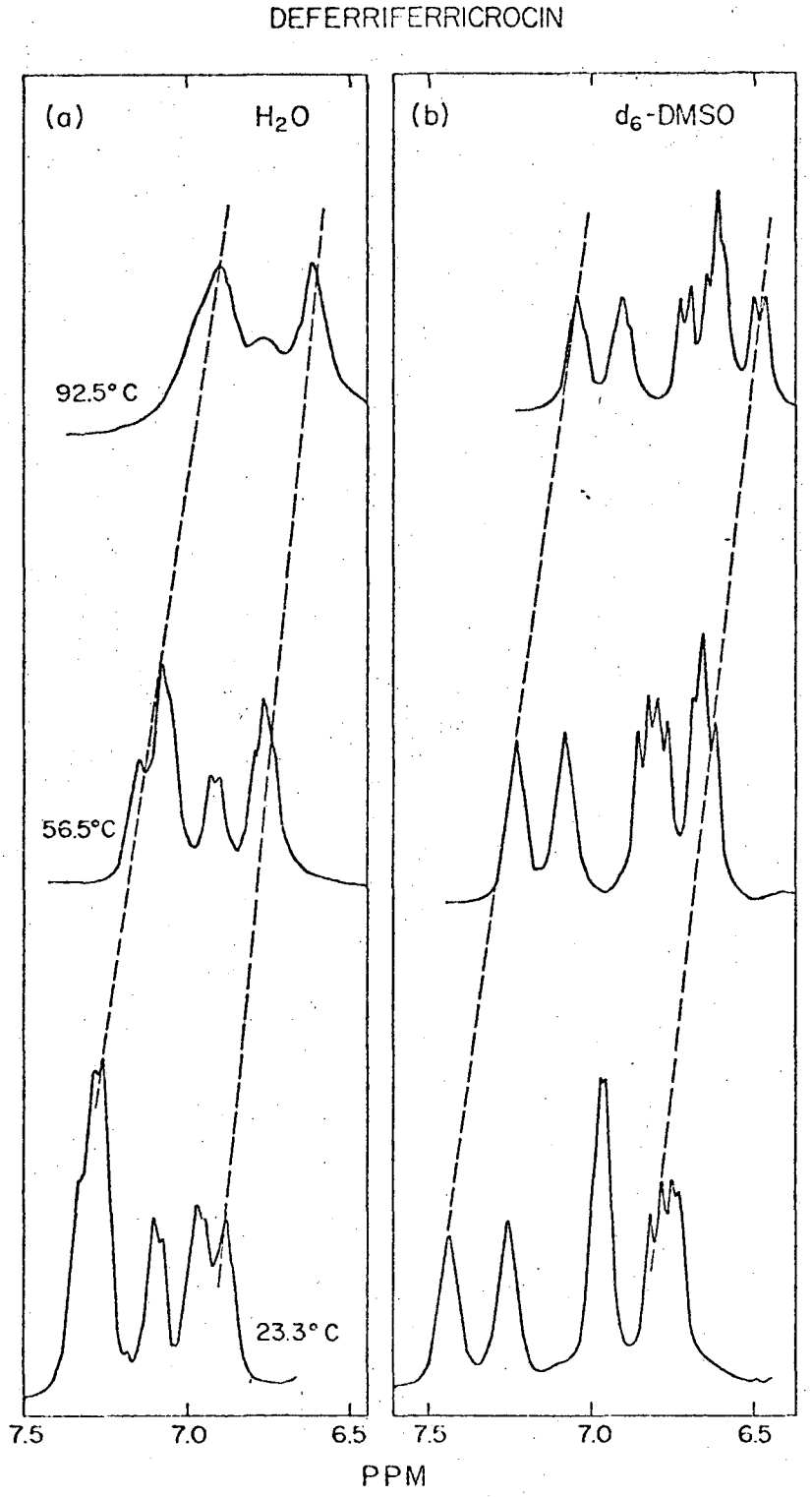


Figure 4



XBL709-5437

Figure 5

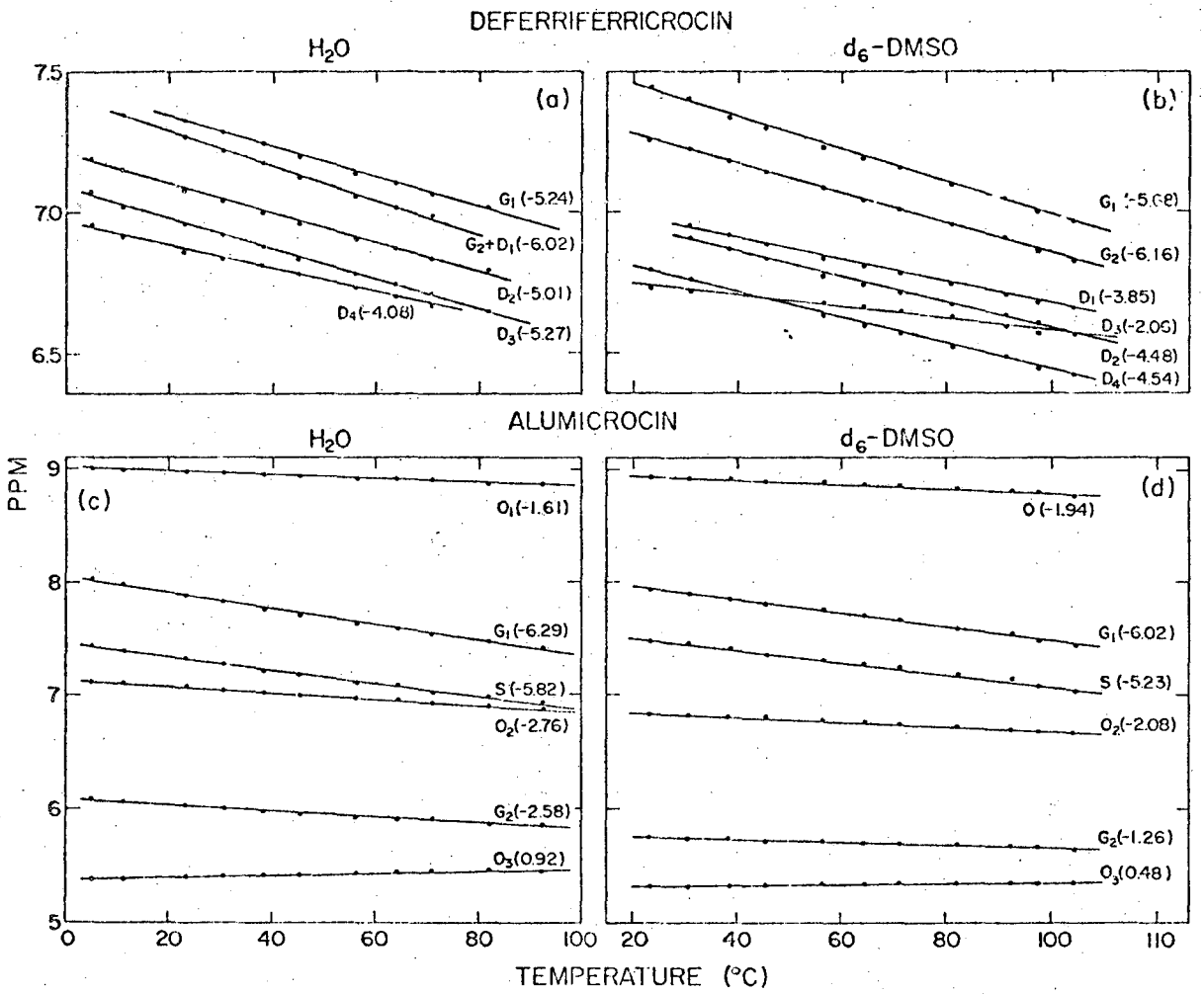
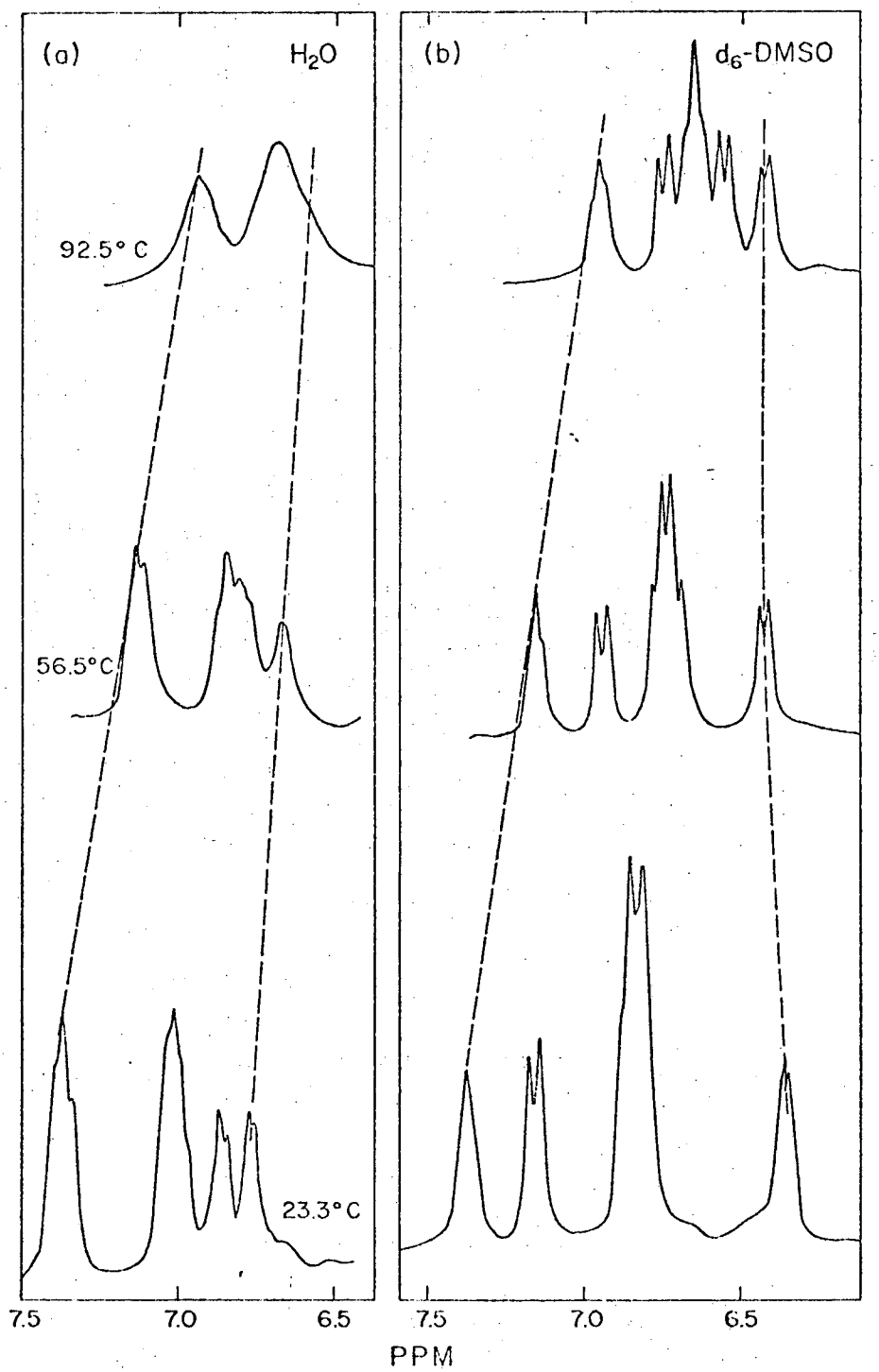

DEFERRIFERRICROCIN
H2O
d6-DMSO
(a)
(b)
G1 (-5.24)
G2+D1(-6.02)
D2(-5.01)
D4(-4.08)
D3(-5.27)
G1 (-5.08)
G2(-6.16)
D1(-3.85)
D3(-2.05)
D2(-4.48)
D4(-4.54)
ALUMICROCIN
H2O
d6-DMSO
(c)
(d)
O1(-1.61)
G1(-6.29)
S(-5.82)
O2(-2.76)
G2(-2.58)
O3(0.92)
O (-1.94)
G1(-6.02)
S (-5.23)
O2(-2.08)
G2(-1.26)
O3(0.48)
PPM
TEMPERATURE (°C)
XBL 709-5434

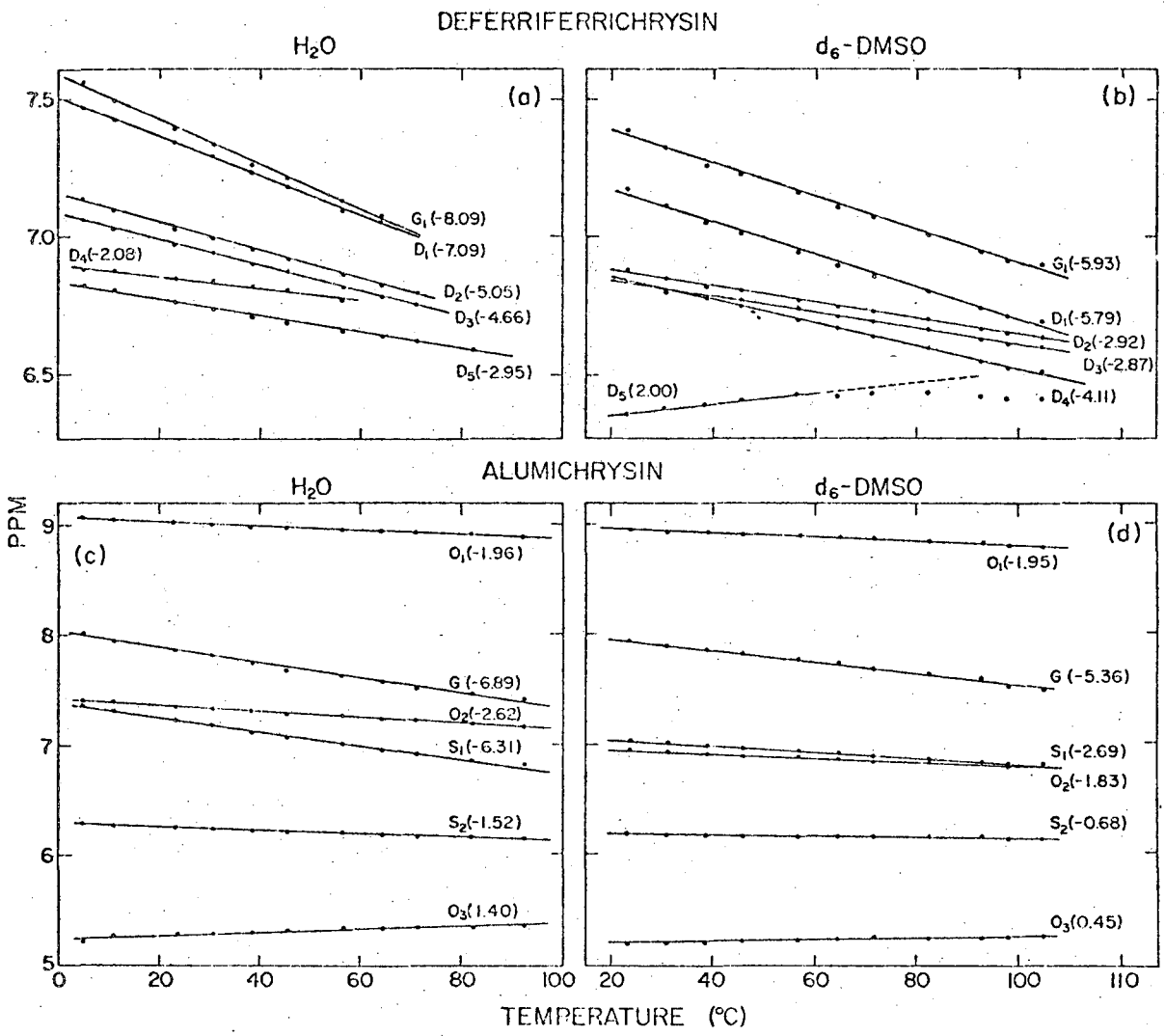
Figure 6

DEFERRIFERRICHRYSIN



XBL 709-5438

Figure 7



XBL 709-5435

Figure 8

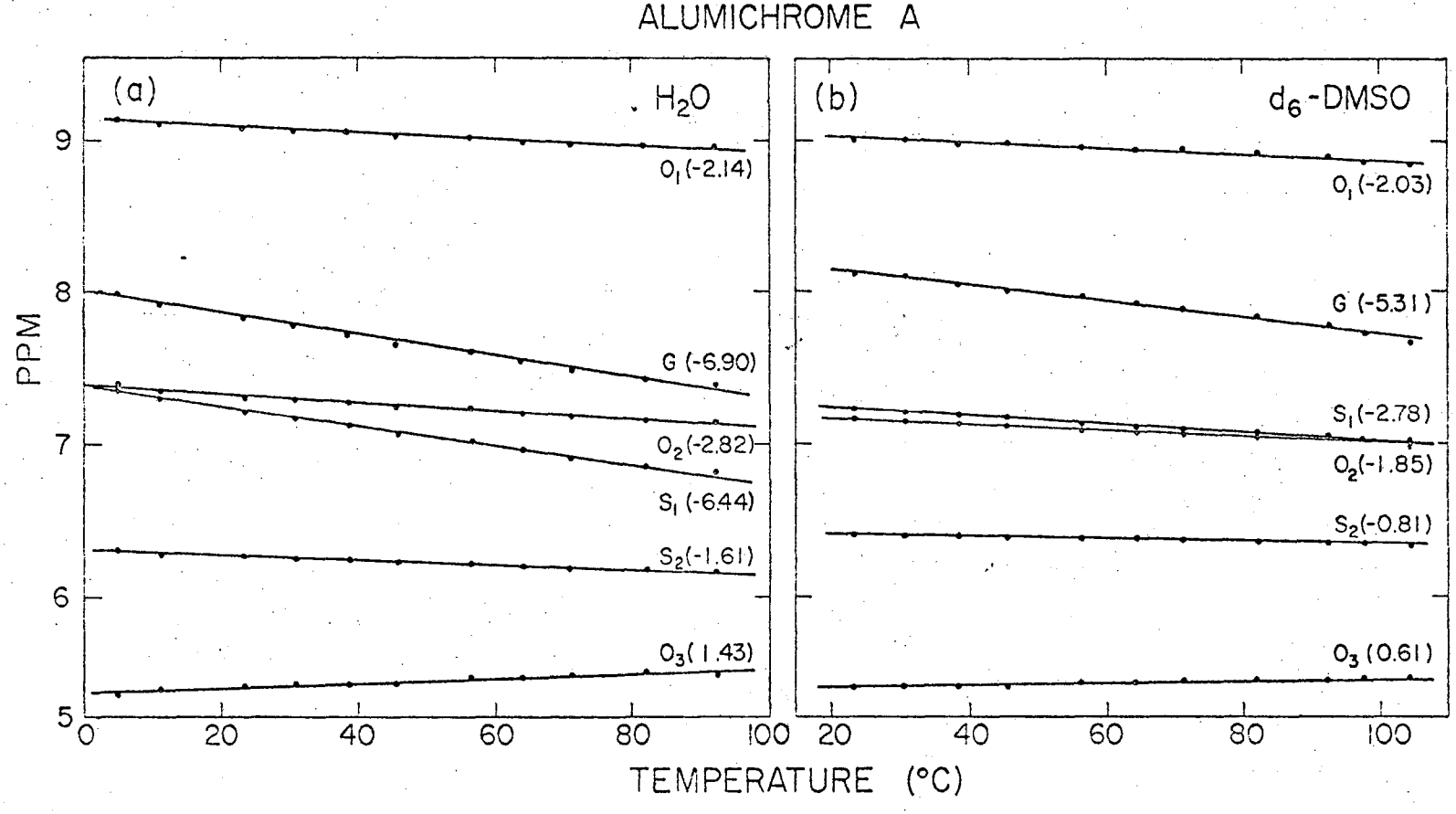
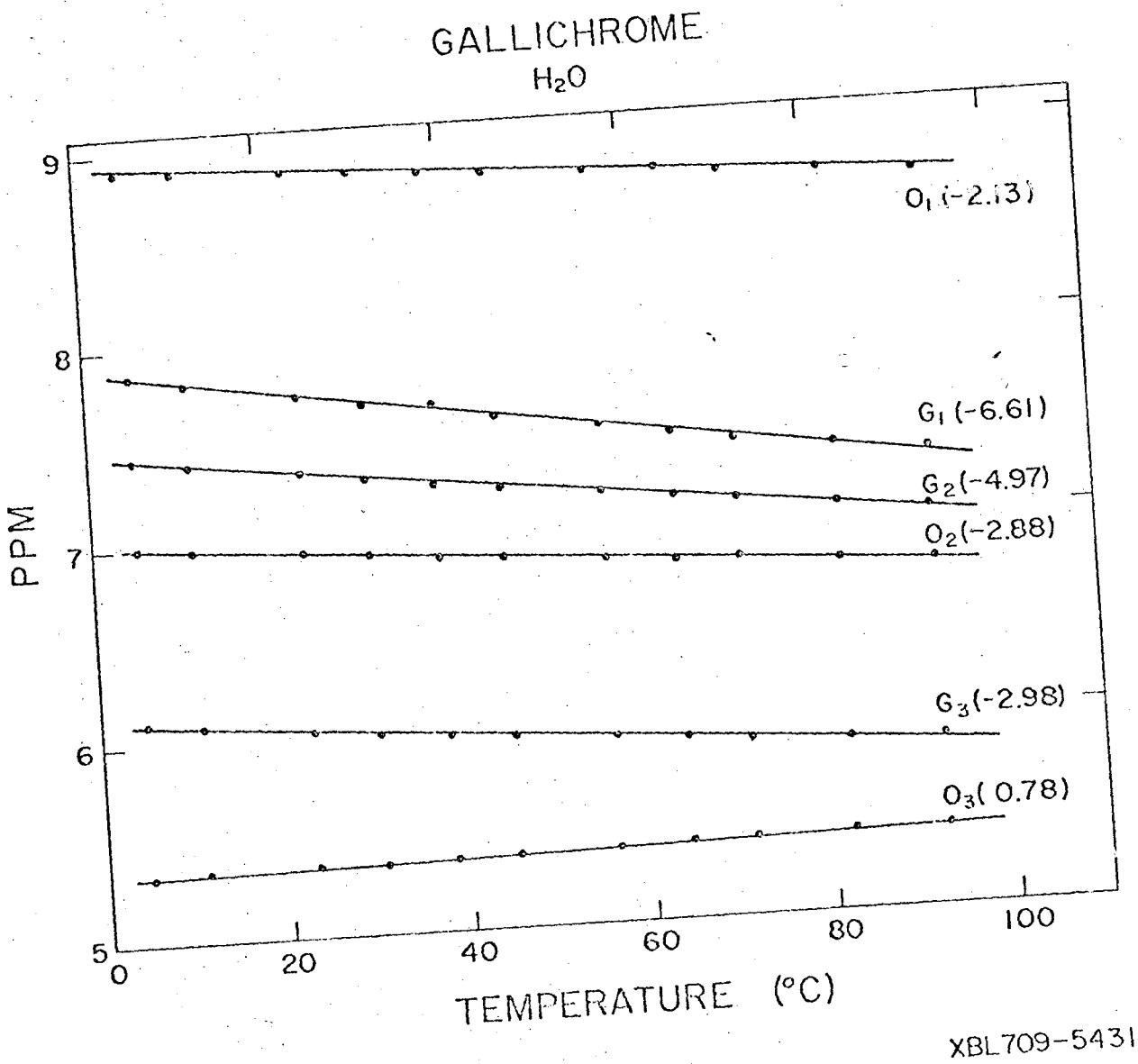

ALUMICHROME A
(a)
H_2O
(b)
d_6-DMSO
O_1(-2.14)
G (-6.90)
O_2(-2.82)
S_1 (-6.44)
S_2(-1.61)
O_3(1.43)
O_1(-2.03)
G (-5.31)
S_1(-2.78)
O_2(-1.85)
S_2(-0.81)
O_3 (0.61)
PPM
9
8
7
6
5
0
20
40
60
80
100
TEMPERATURE (°C)
XBL709-5433

Figure 9



XBL709-5431

Figure 10

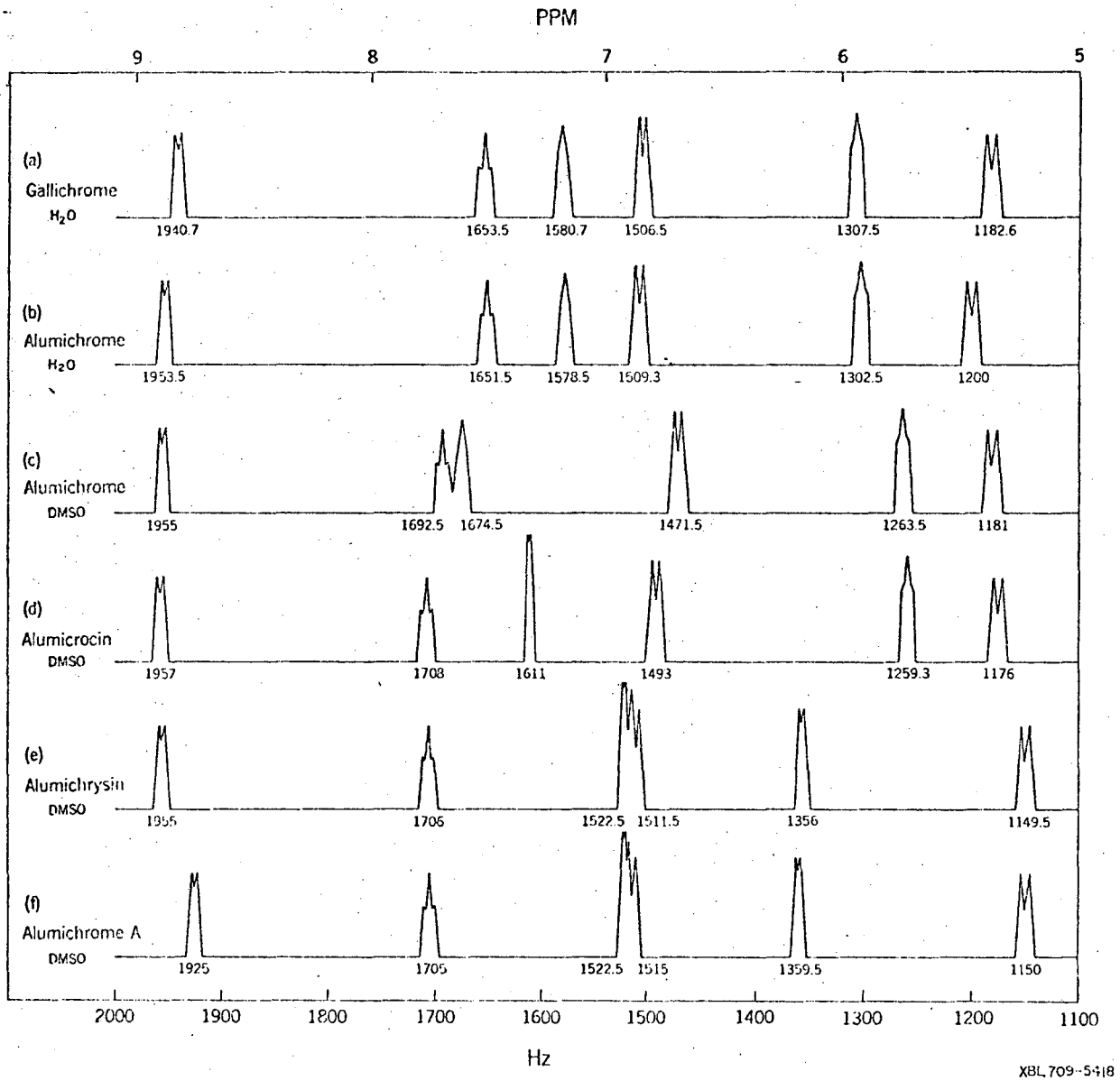
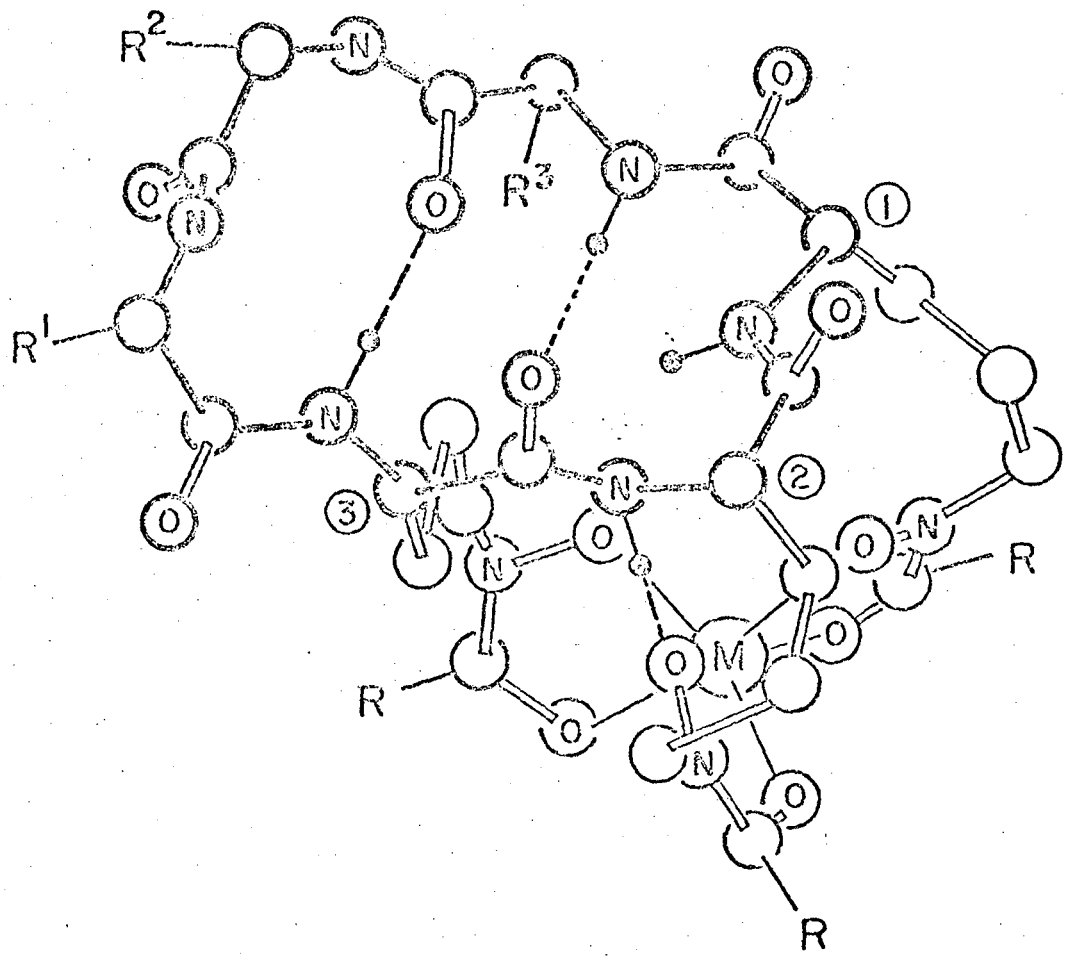

PPM
9
8
7
6
5
(a)
Gallichrome
H_2O
1940.7
1653.5
1580.7
1506.5
1307.5
1182.6
(b)
Alumichrome
H_2O
1953.5
1651.5
1578.5
1509.3
1302.5
1200
(c)
Alumichrome
DMSO
1955
1692.5
1674.5
1471.5
1263.5
1181
(d)
Alumicrocin
DMSO
1957
1708
1611
1493
1259.3
1176
(e)
Alumichrysin
DMSO
1956
1706
1522.5
1511.5
1356
1149.5
(f)
Alumichrome A
DMSO
1925
1705
1522.5
1515
1359.5
1150
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
Hz
XBL 709-5418

Figure 11



XBL 709-5412

Figure 12

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