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The Solution Conformation of the Ferrichromes, III:

A Comparative Proton Magnetic Resonance Study of Glycine
and Serine Containing Ferrichromes (1a,b,c)

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Abstract: Ferrichrome, ferricrocin, ferrichrysin and ferrichrome A are ferric cyclohexapeptides whose general composition is represented by $[Res^3-Res^2-Gly^1-Orn^3-Orn^2-Orn^1]$ where the $Res^{2,3}$ sites are occupied by glycyl of L-seryl residues and $Orn^{1,2,3}$ 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 Al^{+3} and Ga^{+3} chelates of the four homologous peptides in aqueous and in deuterio-dimethyl sulfoxide solutions are reported and analyzed in terms of the molecular conformations. In both solvents the chemical shifts of the amide proton resonances and their temperature dependences are consistent with a structure containing two transannular hydrogen bonds, as in the Schwyzer model for cyclohexapeptides. The metal ion enforces the structure of the peptide backbone; thus, the reported conformational differences resulting from residue substitutions at sites 2 and 3, as well as from solvation effects, are virtually eliminated upon its binding. The chelates exhibit pronounced chemical shift differences

among the amide NH's with markedly reduced temperature dependences 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 all proton resonances to residues in the absolute sequence. The magnitude of the amide $NH-C_{\alpha}H$ 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.

Introduction

In a previous communication the problem of the solution conformation of ferrichrome was stated and analyzed (Llinás, Klein & Neilands, 1970). It was possible to correlate the proton magnetic resonance (PMR) ⁽²⁾ spectroscopic data for the Al⁺³ analogue (alumichrome) of the ferric peptide with the X-ray structural model for crystalline ferrichrome A tetrahydrate (Zalkin, Forrester & Templeton, 1964 and 1966). 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, viz., by direct comparison of several ferrichrome analogues, is presented in this paper.

Ferrichrome is one member of a group of ferric cyclohexapeptides, of fungal origin, whose amino acid sequence can be generalized as $\overline{[Res^3-Res^2-Res^1-Orn^3-Orn^2-Orn^1]}$ where Ornⁱ 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 (Keller-Schierlein, Prelog & Zähler, 1964; Neilands, 1966 and 1971). The supraindices label the residues following the order established by Zalkin, Forrester and Templeton (1966) in their X-ray study of ferrichrome A. The sites along the peptide backbone occupied by Res¹, Res² and Res³ will henceforth be referred to as sites 1, 2 and 3 respectively and should not be confused with those held by the three

δ -N-acyl- δ -N-hydroxy-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 differ in the residues at sites 1, 2 or 3 and in the acyl group of the hydroxamate function.

We have already investigated the PMR spectra of metal-free ferrichrome, ferricrocin and ferrichrysin to see how the amino acid substitutions affect their solution conformations (Llinás, 1971; Llinás, Klein & Neilands, 1971). Furthermore, the spectra of the chelates might provide comparative data which would enable a more complete assignment of the resonances to absolute positions in the peptide ring.

The data presented in a previous paper for ferrichrome (Llinás, Klein & Neilands, 1970) will be compared with those for the analogous seryl-containing peptides ferricrocin, ferrichrysin and ferrichrome A. As in the case of ferrichrome, aluminum will be substituted for iron in order to obviate the line broadening caused by the paramagnetic ferric ion. The gallic (Ga^{+3}) chelate of ferrichrome, gallichrome, will also be compared with alumichrome.

The primary structure of ferricrocin is $\left[\text{Gly}^3\text{-Ser}^2\text{-Gly}^1\text{-Orn}^3\text{-Orn}^2\text{-Orn}^1 \right]$, where Orn stands for δ -N-acetyl- δ -N-hydroxy-L-ornithyl (Keller-Schierlein & Deer, 1963; Llinás, 1971, Llinás, Myers & Neilands, 1971). The PMR spectrum of its Al^{+3} chelate (henceforth "alumicrocin") is entirely consistent with this sequence. Ferricrocin thus differs from ferrichrome in having L-seryl substituting for the glycyl residue at site 2.

Ferrichrysin and ferrichrome A have the same amino acid sequence (Neilands, 1966; Keller-Schierlein, 1963). In common with ferrichrome and ferricrocin they contain the tetrapeptide $H_2NGly^1-Orn^3-Orn^2-Orn^1COOH$ but close the ring with L,L-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. Figure 1 summarizes the composition of the compounds to be discussed here.

Fig. 1

A comparative conformational study of the particular ferrichromes 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. B rner and Gulyas (1966) 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. 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 (Emery, 1971).

Our main objective will be to analyze the resonance assignment problem and to establish more definite evidence for the conformational model (Fig. 7). The perturbative effects of single residue substitutions on the overall peptide conformation will also be discussed. Thus, we

will deal mainly with the differences in the chemical shifts between the analogues rather than with their particular values. The latter have been discussed in Paper I (Llinás, Klein & Neilands, 1970) for alumichrome and the arguments can be extended readily to the other peptides.

Experimental

The source of ferrichrome, ferricrocin, ferrichrysin and the production of the corresponding metal-free peptides have been described elsewhere (Llinás, 1971; Llinás, Klein & Neilands, 1971).

Ferrichrome A was precipitated from the concentrated growth medium of Ustilago sphaerogena at pH 2.8 and twice recrystallized from water in a yield of 0.45 g/l. The K^+ salt of the metal-free peptides (Emery & Neilands, 1960) was crystallized by addition of three equivalents of alcoholic KOH to a solution of the free acid in warm ethanol.

Gallichrome, alumicrocin, alumichrysin and alumichrome A were obtained by reacting the metal-free peptides with the hydroxides of the respective trivalent metals as previously described for alumichrome (Llinás, Klein & Neilands, 1970). 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 and stored over P_2O_5 under reduced pressure. By reference to alumichrome (Llinás, Klein & Neilands, 1970) the PMR spectra showed that chelation was complete and that the samples were pure (see results).

The PMR instrumentation and spectroscopic methods have been described in previous papers (Llinás, Klein & Neilands, 1970 & 1971).

Results

Fig 2

The 220 MHz PMR spectra for alumicrocin and alumichrysin, in $(\text{CD}_3)_2\text{SO}$ at 45°C , are shown in Figure 2. 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 alumichrome (Llinás, Klein & Neilands, 1970), taken under similar conditions. The latter may serve as a guide 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.

The seryl C_βOH resonances occur at about 4.97 to 5.21 ppm from TMS; due to coupling to the pair of β -hydrogens these resonances are triplets.

Identification of amide 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. It is possible to distinguish between the seryl and ornithyl resonances on the basis of the sequential spin-spin coupling connections: NH (doublet) \leftrightarrow C_αH \leftrightarrow C_βH \leftrightarrow C_βOH for the seryl and NH (doublet) \leftrightarrow C_αH \leftrightarrow C_βH \leftrightarrow C_γH for the ornithyl residues.

Chemical shifts and resonance assignments are given in Table I.

Table I

These assignments are based on the positions of the amide NH and (seryl) C_βOH resonances and on the proton spin-spin coupling connections established by homonuclear double resonance experiments. Resonances assigned to amide NH and seryl C_βOH were confirmed by the disappearance of these peaks upon dissolution in D_2O . This exchange resulted, in turn, in a collapse of the corresponding spin-spin coupled resonances.

As in the previous communications (Llinás, Klein & Neilands, 1970 and 1971) 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. ⁽³⁾

By reference to our preceding paper (Llinás, Klein & Neilands, 1971) it can be observed that profound spectral changes are induced upon chelation of Al^{+3} . The changes are qualitatively similar to those observed in the ferrichrome peptides (Llinás, Klein & Neilands, 1970) and, as in that case, are more extensive in the amide NH region.

Constancy of position of the aliphatic proton resonances of equivalent residues at corresponding sites is more noticeable in the aluminum than in the metal-free peptides [compare data in Table I with that in (Llinás, Klein & Neilands, 1971)]. An outstanding example is the Orn_2 $C_{\alpha}H$, which appears as an isolated band at 4.75 ppm in alumichrome (Llinás, Klein & Neilands, 1970), 4.74 ppm in alumicrocin and 4.72 ppm in alumichrysin. The amide NH resonance for this residue does not show such a constancy and thus reflects its more subtle sensitivity to the environment, degree of exposure to the solvent and extent of hydrogen bonding. For these reasons, the amide NH resonances prove to be excellent conformational probes and the discussion that follows will be centered on them.

The temperature dependence of the amide NH chemical shifts, in both H_2O and in $(CD_3)_2SO$, for alumicrocin and alumichrysin are shown in Figure 3. The lines are least squares fits of the experimental points and the slopes are indicated in parentheses. As discussed for deferriferrichrome and alumichrome, the slopes of these linear plots serve as

Fig 3

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 (Llinás, Klein & Neilands, 1970 and 1971).

The amide NH chemical shift temperature dependence for alumicrocin and for alumichrysin in water are shown in Figures 3, a and c. The spectra of these peptides in this solvent or in D_2O are not presented; aside from the relative shifts of the amide NH resonances the rest of the spectra differed little from that in $(\text{CD}_3)_2\text{SO}$ (Fig. 2b).

Proton spin-spin coupling constants for amide $\text{NH}-\text{C}_\alpha\text{H}$ interactions (J_{NC}) for alumicrocin and alumichrysin in water and in $(\text{CD}_3)_2\text{SO}$ are given in Table II. The values are averages of determinations at different temperatures within the range of the chemical shift temperature dependence studies, this treatment being justified by an apparent independence of the J_{NC} on temperature within the experimental errors. 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 alumichrome, glycyI 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 is not reported for alumicrocin or alumichrysin in this solvent. The degree of reliability of the reported J_{NC} 's is reflected in the standard deviations and in most cases these uncertainties were small enough to allow useful conformational conclusions to be drawn.

Fig 4
Table III

The PMR spectrum of alumichrome A is not reported here since, apart from resonances ascribed to the different acyl groups, it is analogous to that of alumichrysin (Fig. 2b). In Figure 4 the temperature dependences of the alumichrome A amide NH chemical shifts are represented for comparison with the corresponding alumichrysin plots (Fig. 3). These linear plots enable an estimation of the influence of the acyl group substituent of the hydroxamate functions on the overall stability of the peptides. The J_{NC} values, averaged from these data in water at pH 5.14 [in $(CD_3)_2SO$ the amide NH resonances are too broad], are given in Table III(a).

Fig 5

Since the ionic radius of the diamagnetic Ga^{+3} ion ($r_0 = 0.62 \text{ \AA}$) is closer to that of Fe^{+3} ($r_0 = 0.64 \text{ \AA}$) than is Al^{+3} ($r_0 = 0.53 \text{ \AA}$), 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, suggesting, in agreement with the cell transport experiments by Emery (1971), that the conformation of the two chelates is very similar. 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 III(b) and Figure 5, respectively.

Discussion

An attempt will now be made to reach a definite assignment of amide NH resonances from the comparative evidence provided by the use of the different analogues. The requisite information is contained diagrammatically in Figure 6, where the chemical shifts for spectra recorded at $56.5^\circ C$ are all referred to the methyl peak in tert butyl alcohol (TBA).

Fig 6
 The amide NH region of alumichrome in $(\text{CD}_3)_2\text{SO}$ (Fig. 6a) allows one to distinguish ornithyl from glycyll resonances, since the first are doublets and the second triplets. On substitution of Gly² in alumichrome by Ser² in alumicrocin, the spectra show that the triplet at 1674.5 Hz disappears and is replaced by a doublet at 1611 Hz (compare a and b in Fig. 6). This enables assignment of these peaks to the residues at site 2. A further substitution of the glycyll 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 b and c, Fig. 6). Thus these resonances are assigned to the residue at site 3. Since there is only one glycyll residue in alumichrysin, the assignment of any NH triplet in this peptide is unambiguous. The triplet at 1706 Hz is then assignable to the glycyll 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 7) indicates that seryl-for-glycyll substitutions at sites 2 and/or 3 should leave the Orn² amide NH resonance relatively unperturbed, since this residue is hydrogen-bonded to its own side-chain and always sandwiched between Orn¹ and Orn³. 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 to alumichrysin. Indeed, as can be seen in Figure 6 a, b and c, there is only one ornithyl amide NH resonance showing an invariant position in the three analogues, namely, the doublet for Orn₁. Hence this peak can be assigned to Orn². This assignment is

confirmed by comparing the spectra of alumichrysin and alumichrome A. As shown in Figure 6, c and d, 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 7).

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 7, 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 the latter site. Furthermore, 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 6 (compare a and b), 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 NH resonance on going from alumicrocin to alumichrysin (Fig. 6, b and c). However, this substitution also affects the chemical shift of the Orn_3 NH resonance. Since the Orn_1 and Orn_2 ornithyl doublets have already been assigned, by elimination, the

Orn₃ NH resonance can be assigned to Orn¹. As shown in the steric model (Fig. 7), 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 Orn¹ amide hydrogen. Such increase in the steric shielding should result in a shift of its resonance to higher fields, which is observed.

It should also be noted in comparing the spectra of all the alumi-peptides in $(\text{CD}_3)_2\text{SO}$ that the Gly₁¹ 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 \rightarrow alumichrysin \rightarrow alumichrome A the position of the Gly₁¹ 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 which does not interact with variant and distant parts of the molecule.

Although the seryl-for-glycyl substitution should be expected to affect the chemical shift of the Orn₂³ 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 on going from alumichrome to alumicrocin (Fig. 6 a and b). 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. 6 b and c). The effect of the seryl-for-glycyl substitution at site 2, alumichrome \rightarrow 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 $\text{Res}_3^3\text{NH}\dots\text{O}=\text{C}-\text{Orn}_3^3$ transannular hydrogen bond. The relative rates of hydrogen-deuterium exchange in D_2O suggest an increased stability in the peptide conformation as the number of seryl residues increases, which further supports this interpretation. A detailed study of the comparative amide hydrogen exchange will be reported elsewhere; see Llinás, 1971.

It has been noted above (see Fig. 6) that on going from alumichrome \rightarrow alumicrocin \rightarrow alumichrysin in $(\text{CD}_3)_2\text{SO}$, negligible shifts occur in the Orn_1^2 NH resonance although, in agreement with the interpretation of H-bond strengthening, the transannular Orn_2^3 NH does show a resonance shift from 1471.5 Hz \rightarrow 1493 Hz \rightarrow 1511.5 Hz. In water, however, where the seryl side-

chain solvation is stronger, the shift of the Orn₂³ NH resonance is found to be more pronounced: from 1509 Hz → 1534 Hz → 1597 Hz. Indeed, in water, even the Orn₁² NH shows a chemical shift in going from one analogue to the other (1953 Hz → 1963 Hz → 1597 Hz), which is also in a direction that suggests a stronger H bonding. Consistent with this trend, a shift of 61 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 (Llinás, 1971).

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. It could be objected 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. As can be seen below, the temperature will affect the magnitude of the relative chemical shifts for the resonances diagrammed in Figure 6, but the comparative trends shown in the analysis above are, for our purposes, temperature independent.

Figure 3 a and c show that in water, pH 5.14, the amide NH resonances of Gly₁ and Ser in alumicrocin and Gly and Ser₁ in alumichrysin show

larger temperature dependences 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, 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 $(CD_3)_2SO$ the residues at sites 1 and 2 also show higher temperature dependences, 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 $(CD_3)_2SO$. Inspection of a space filling model constructed on the basis of the structure depicted in Figure 7 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 dimethylsulfoxide 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 dimethylsulfoxide, which could result in improved steric shielding by the seryl side-chains without intramolecular H-bonding. Indeed, since on going from alumicrocin to alumichrysin the shift of the $Ser_1^2 NH$ resonance is towards higher fields (Fig. 6), it is suggested that steric

shielding rather than intramolecular hydrogen bonding is the cause of its hindrance to interaction with the solvent in dimethylsulfoxide.

The remaining four amides show, in all the analogues, smaller temperature dependences as judged from the absolute values of the slopes. They may be classified as "internal", whether H-bonded or not. The $\text{Orn}_3 \text{NH}$ was assigned primarily to the buried amide hydrogen of Orn^1 which, in the steric model (Fig. 7) is not hydrogen-bonded. 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. 3a,b; 3c,d; 4a,b; 5; and Paper I). The reduced slope in $(\text{CD}_3)_2\text{SO}$ 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 (Llinás, Klein & Neilands, 1970), 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 $\text{Orn}_2 \text{NH}$ within the molecule, as the positive slope for the Orn_3^1 resonance indicates. By contrast, hydrogen-deuterium exchange experiments in D_2O show that the $\text{Orn}_2 \text{NH}$ 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 Orn₂ NH against hydrogen exchange might be attributable to a stronger trans-annular hydrogen bond relative to the residue at site 3. Finally, the reduced temperature dependence of the Orn₁ NH resonance (attributed to Orn²) can be rationalized according to the conformational model in Figures 1 and 7, which shows this amide is attached to its own side-chain in a relatively short H-bond.

Figure 4 a and b reveal similar plots for the amide NH's of alumichrome A. Comparison with the equivalent plots for alumichrysin (Fig. 3 c 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 Ser₁ NH slope when going from water to dimethylsulfoxide. Although a direct comparison of the temperature dependence plots for alumichrysin and alumichrome A in (CD₃)₂SO might be tenuous, 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 (Llinás, 1971).

Similar comparisons can be established between gallichrome and alumichrome, whose amide NH temperature dependence plots for their aqueous solutions are given in Figure 5 and in Paper I. 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.

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 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 to allow 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 the formula of Bystrov, Portnova, Tsetlin, Ivanov and Ovchinnikov (1969) can be applied. The ϕ dihedral angles, calculated from amide NH resonance doublet splittings measured in water and in $(\text{CD}_3)_2\text{SO}$ (Tables II, III, and data in Paper I) are shown in Table IV. (4)

Given the rather large uncertainties inherent in the formula used, 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 IV(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 $\text{NH-C}_\alpha\text{H}$ 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 $\text{NH-C}_\alpha\text{H}$ 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 arising from the successive seryl-for-glycyl substitutions. In $(\text{CD}_3)_2\text{SO}$, Table IV(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. (1966) for crystalline ferrichrome A $\cdot (\text{H}_2\text{O})_4$ are included in Table IV(c). The agreement between the X-ray and the PMR values is excellent 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 implies 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 with which 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 IV 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 the equation of Bystrov *et al.* (1969) 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 (Llinás, Klein & Neilands, 1971). The poorer correspondence between the X-ray and PMR ϕ angle for Ser₂³ mentioned above, might thus reflect the latter effect. 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 Orn₂³ amide NH and carbonyl groups and hence modify the relative strength of the paired transannular hydrogen bonds. This would slightly refine the X-ray model and suggests the possibility of a modified β -fold structure for the metallo-peptide backbone, as shown in the model (Fig. 7).

3ng?

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 alumi-peptides' PMR spectra and that conformation unique? Gibbons, Némethy, Stern and Craig (1970) have recently reviewed critically the general problem of conformational analysis of peptides in solution on the basis of PMR data. As in the Russian school (see, e.g., the PMR conformational analysis for enniatin B, Ovchinnikov et al., 1969) 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 ψ - ϕ conformational map. The problem is 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 (Momany, Vanderkooi, Tuttle & Scheraga, 1969) have shown that when the iterative calculation is started from a Hodgkin-Oughton-Schwyzler model, for which the PMR evidence is excellent (Stern, Gibbons & Craig, 1968; Ovchinnikov et al., 1969), the resulting structure has an energy lower than for any other structure previously computed. Similarly, the conformational analysis of cyclohexapeptides by Ramakrishnan and Sarathy (1969) shows relatively good agreement between the crystalline ferrichrome A structure and the minimum energy (Schwyzler-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 (Llinás, 1971) and through measurements now in progress of the relaxation rates of the nuclear resonances.

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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) Taken in part from the Ph.D. dissertation of M.Ll. at the University of California, Berkeley, 1971.

(2) Abbreviations used: PMR, proton magnetic resonance; p.p.m., parts per million; TMS, tetramethylsilane; TBA, tert. butyl alcohol; d₆-DMSO, deuterio dimethylsulfoxide.

(3) 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. (1966) 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, Gly¹ in ferrichrysin (and in ferrichrome A) is the only glycyl residue occurring in the peptide which would not, otherwise, require any superindex. Similarly, Ser² 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. (1966). The use of subindexes follows the convention established previously. 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 Orn₂³, 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.

(4) The relation is $J_{NC}(\text{hz}) = (8.9 \pm 0.9) \cos^2 \theta + (0.9 \pm 0.9) \sin^2 \theta - (0.9 \pm 0.9) \cos \theta$. $\phi (= 240 - \theta)$ is defined according to the convention of J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Némethy, G. N. Ramachandran, and H. A. Scheraga, J. Mol. Biol., 15, 399 (1966).

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Table I

	Alumicrocin						Alumichrysin				
	Gly ₁	Gly ₂	Ser	Orn ₁	Orn ₂	Orn ₃	Ser ₁	Ser ₂	Orn ₁	Orn ₂	Orn ₃
NH	8.94	6.85	8.50	10.04	7.93	6.46	8.07	7.28	10.03	8.00	6.33
C _α H	3.79	3.74	3.99	4.16	4.74	4.21	4.02	4.06	4.23	4.72	4.11
C _β H			3.47	1.69	1.75	1.09	3.56	3.29	1.66	1.79	1.08
C _γ H											
C _δ H											
C _β OH			4.97				5.07	5.21			
CH ₃					2.07					2.07	
NOH											

Chemical shifts, referred to TMS (ppm), of alumicrocin and alumichrysin in (CD₃)₂SO, at 45°C and 220 MHz. Labeling of residues and their resonances follows the convention given in the text. Resonances ordered under the same column are assigned to the same residue(s). The CH₃ resonance is, as for the cases of alumichrome, the average position of three closely spaced narrow peaks.

Table II

	Alumicrocin						Alumichrysin					
	Gly ₁	Gly ₂	Ser	Orn ₁	Orn ₂	Orn ₃	Gly	Ser ₁	Ser ₂	Orn ₁	Orn ₂	Orn ₃
H ₂ O	~5.2	~3.7		5.3	6.9	9.0	~5.6		4.8	5.5	7.3	7.8
	±0.1	±0.1		±0.2	±0.2	±0.1	±0.2		±0.1	±0.1	±0.2	±0.2
(CD ₃) ₂ SO	5.5	~3.6	~2.9	5.4	7.1	9.1	5.5	~2.1	3.7	5.6	6.9	8.5
	±0.1	±0.2	±0.2	±0.1	±0.1	±0.1	±0.1	±0.3	±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 alumicrocin and alumichrysin in water at pH 5.14 and in $(CD_3)_2SO$. Residues are labeled as in Table I. Values are averages derived from determinations at different temperatures. The uncertainties are their standard deviations. In water the J_{NC} splitting for certain seryl amides could not be resolved and are not reported. Poorly resolved splittings are indicated by ~.

Table III

Alumichrome A						Gallichrome					
Gly	Ser ₁	Ser ₂	Orn ₁	Orn ₂	Orn ₃	Gly ₁	Gly ₂	Gly ₃	Orn ₁	Orn ₂	Orn ₃
~5.5		4.8	5.3	7.3	7.5	5.9	~4.6	~4.2	5.6	7.6	8.8
±0.2		±0.1	±0.1	±0.1	±0.1	±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 alumichrome A and gallichrome 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 Ser₁ NH resonance in alumichrome A is not given because of lack of resolution. Alumichrome A tricarboxylic acid, dissolved in $(CD_3)_2SO$, shows excessively broadened amide resonances; hence it is also not reported here. The more poorly resolved splitting of the glycyl NH triplets are indicated by ~.

Table IV

	Ser ₁ ([±] 6)	Ser ₂ ([±] 7)	Orn ₁ ([±] 7)	Orn ₂ ([±] 12)	Orn ₃ ([±] 22)
<u>a) H₂O</u>					
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
<u>b) DMSO</u>					
Alumichrome			102	28	82
Alumicrocin	124		106	26	76
Alumichrysin	130	3	105	24	83
<u>c) X-ray</u>					
Ferrichrome A	123	17	103	35	76
	Ser ²	Ser ³	Orn ²	Orn ³	Orn ¹

The ϕ dihedral angle between the HNC_α and the NC_αH planes. Values in a) and b) are calculated from the amide $\text{NH} - \text{C}_\alpha\text{H}$ 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.* (1969). 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.* (1966) on the basis of X-ray studies. The labeling of the particular residues according to an arbitrary PMR classification (subindices) and the absolute sequence (superindices) follows the convention given in the text.

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. (1966) for crystalline ferrichrome A $\cdot (H_2O)_4$. 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 ₂ OH	H	CH ₃	Al ⁺³
alumichrysin	CH ₂ OH	CH ₂ OH	CH ₃	Al ⁺³
alumichrome A	CH ₂ OH	CH ₂ OH	$\begin{array}{l} \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{C} \\ \diagdown \quad \diagup \\ \text{H} \quad \text{CH}_2\text{COOH} \end{array}$	Al ⁺³

All these peptides contain the common tetrapeptide sequence $H_2N\text{Gly}^1\text{-Orn}^3\text{-Orn}^2\text{-Orn}^1\text{-COOH}$ bridging between sites 2 and 3.

FIGURE LEGENDS (Cont.)

Figure 2. The 220 MHz PMR spectra of (a) alumicrocin and (b) alumichrysin at 45°C dissolved in $(\text{CD}_3)_2\text{SO}$. The peak marked "solvent" arises from the residual H in $(\text{CD}_3)_2\text{SO}$ and that marked " H_2O " results from water residual after low pressure dessication over P_2O_5 . 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. The resonances at ~ 5.0 and ~ 5.15 ppm arise from the seryl hydroxyl protons. The peaks broadened by exchange sharpen upon reducing the temperature to $\sim 20^\circ\text{C}$; the triplet nature of the seryl C_βOH resonances then becomes clearly apparent. The spectra are referred to internal TMS.

Figure 3. The temperature dependence of the chemical shifts of the amide NH protons of alumicrocin and alumichrysin in water at pH 5.14 and in $(\text{CD}_3)_2\text{SO}$. G, S and O denote glycylyl, 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., $-8.09 = -8.09 \times 10^{-3}$ ppm/ $^\circ\text{C}$. The chemical shifts in both solvents were measured with respect to internal TBA.

FIGURE LEGENDS (Cont.)

Figure 4. The temperature dependence of the chemical shifts of the amide NH protons of alumichrome A in water at pH 5.14, and in $(\text{CD}_3)_2\text{SO}$. 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.

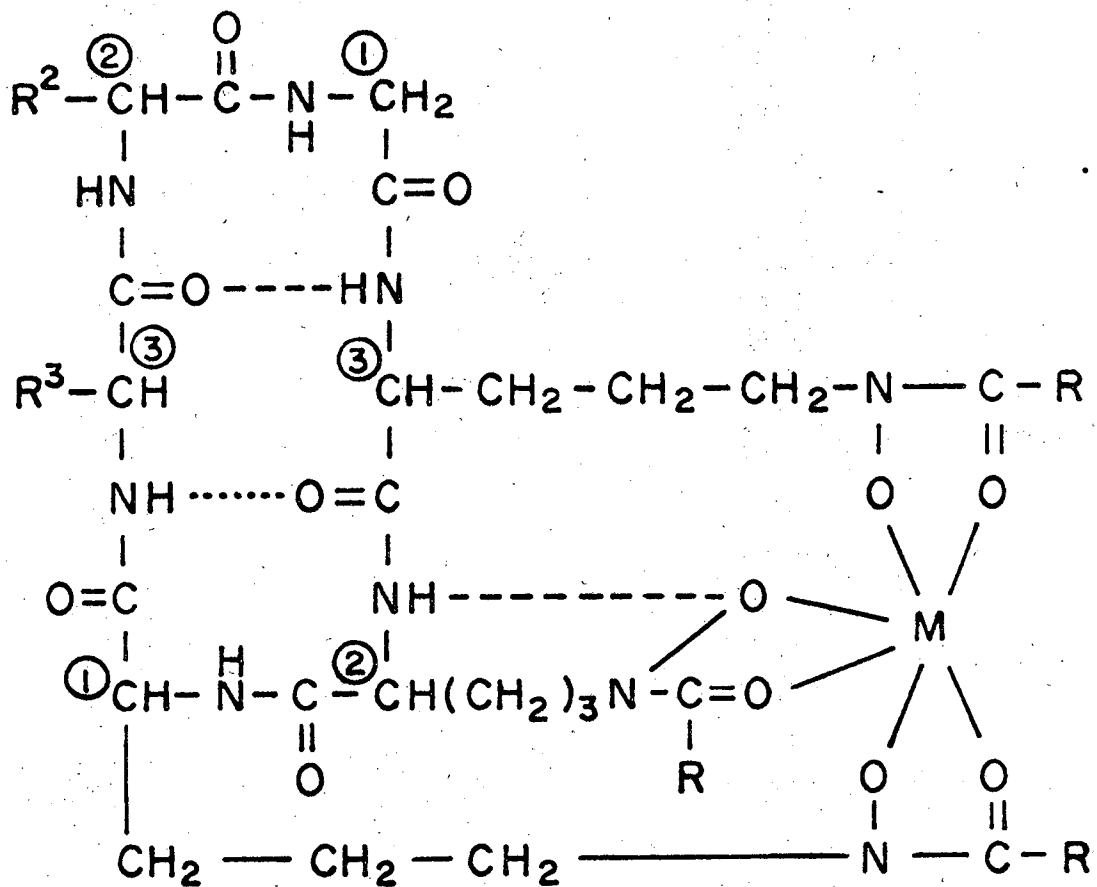
Figure 5. The temperature dependence of the chemical shifts of the amide NH protons of gallichrome in water at pH 5.14. O and G denote ornithyl and glycyl amide protons respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. The number in parentheses 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 6. 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. Triplets arise from glycyl NH protons and doublets derive from either seryl or ornithyl amides. The seryl and ornithyl resonances may be distinguished by comparison of 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 alumichrysin (the correspondence

FIGURES LEGENDS (Cont.)

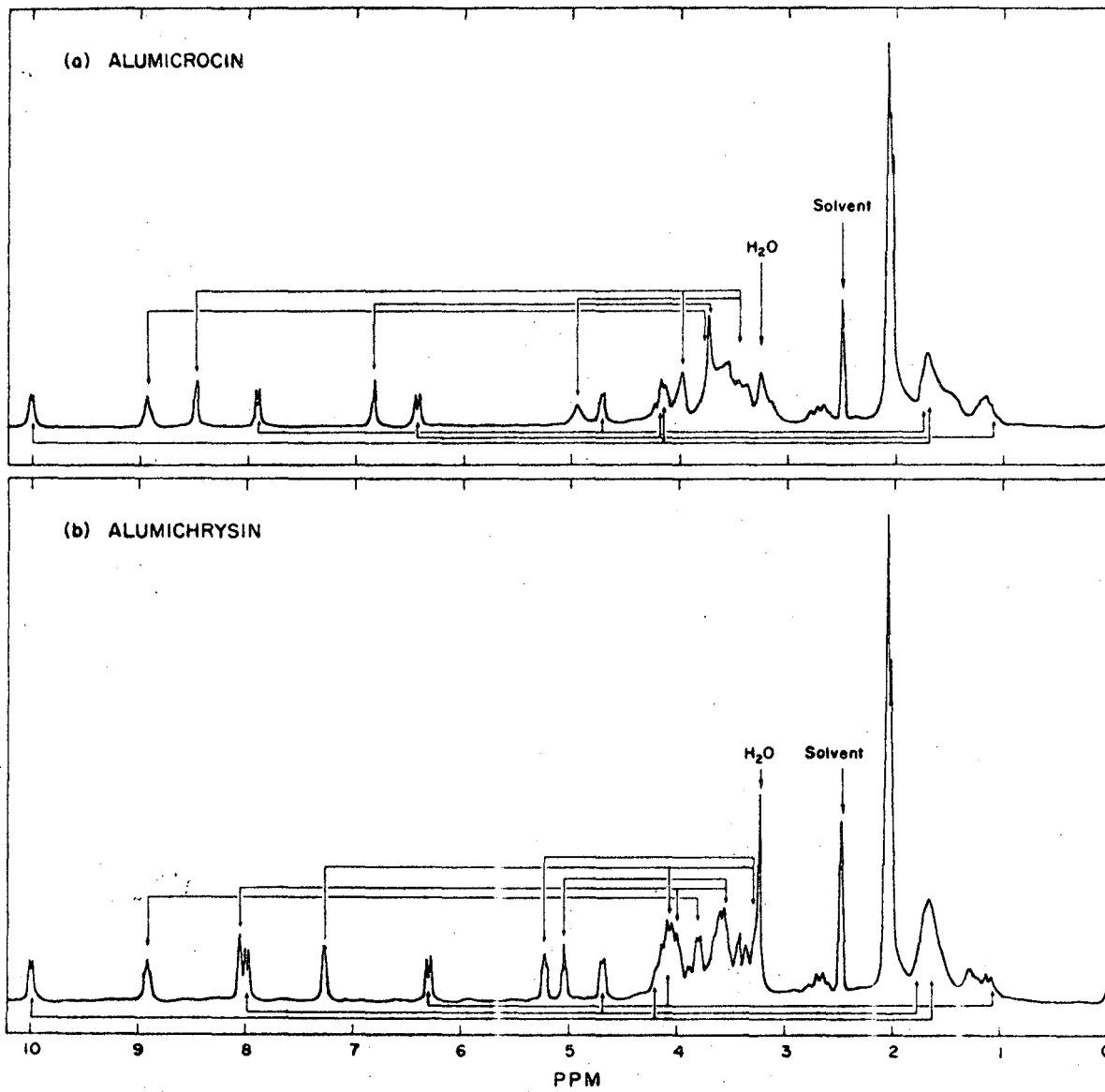
being ascertained by their similar doublet splittings), while a second seryl-for-glycyl substitution (alumicrocin \rightarrow alumichrysin) results in the doublet at 1356 Hz and is accordingly assigned to a seryl residue common to alumichrysin and alumichrome A.

Figure 7. 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 Orn¹ 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 Orn³ and the carbonyl oxygen of Res³ (residue at site 3); 2) between the amide proton of Res³ and the carbonyl oxygen of Orn³, and 3) between the amide proton of Orn² 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 glycine ($R^1 = H$), while R^2 and R^3 may be H or CH₂OH (glycyl or seryl). 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.



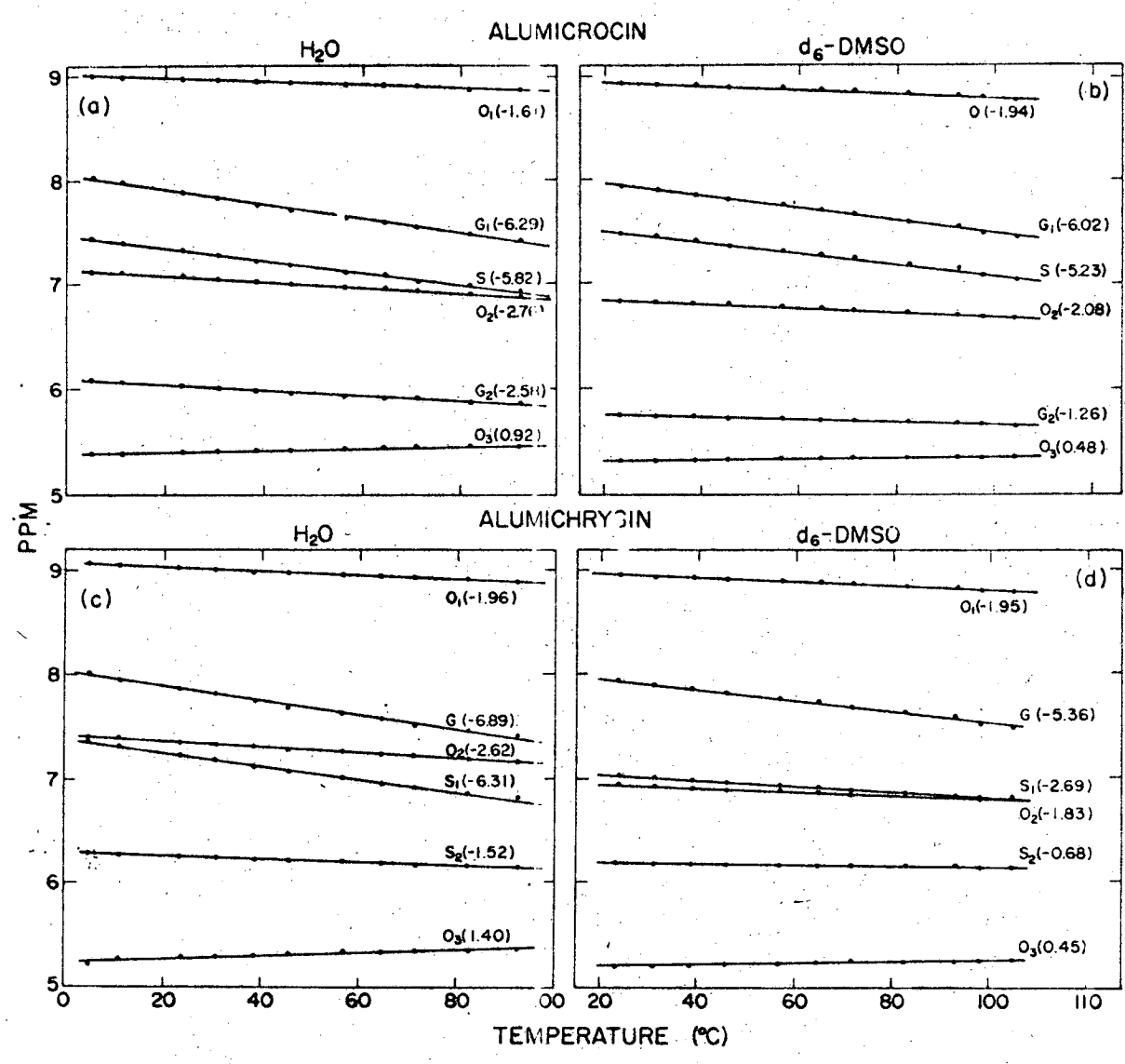
XBL 717-5282 A

Fig. 1



XBL 708-5314 A

Fig. 2



XBL 709-5435 A

Fig. 3

ALUMICHROME A

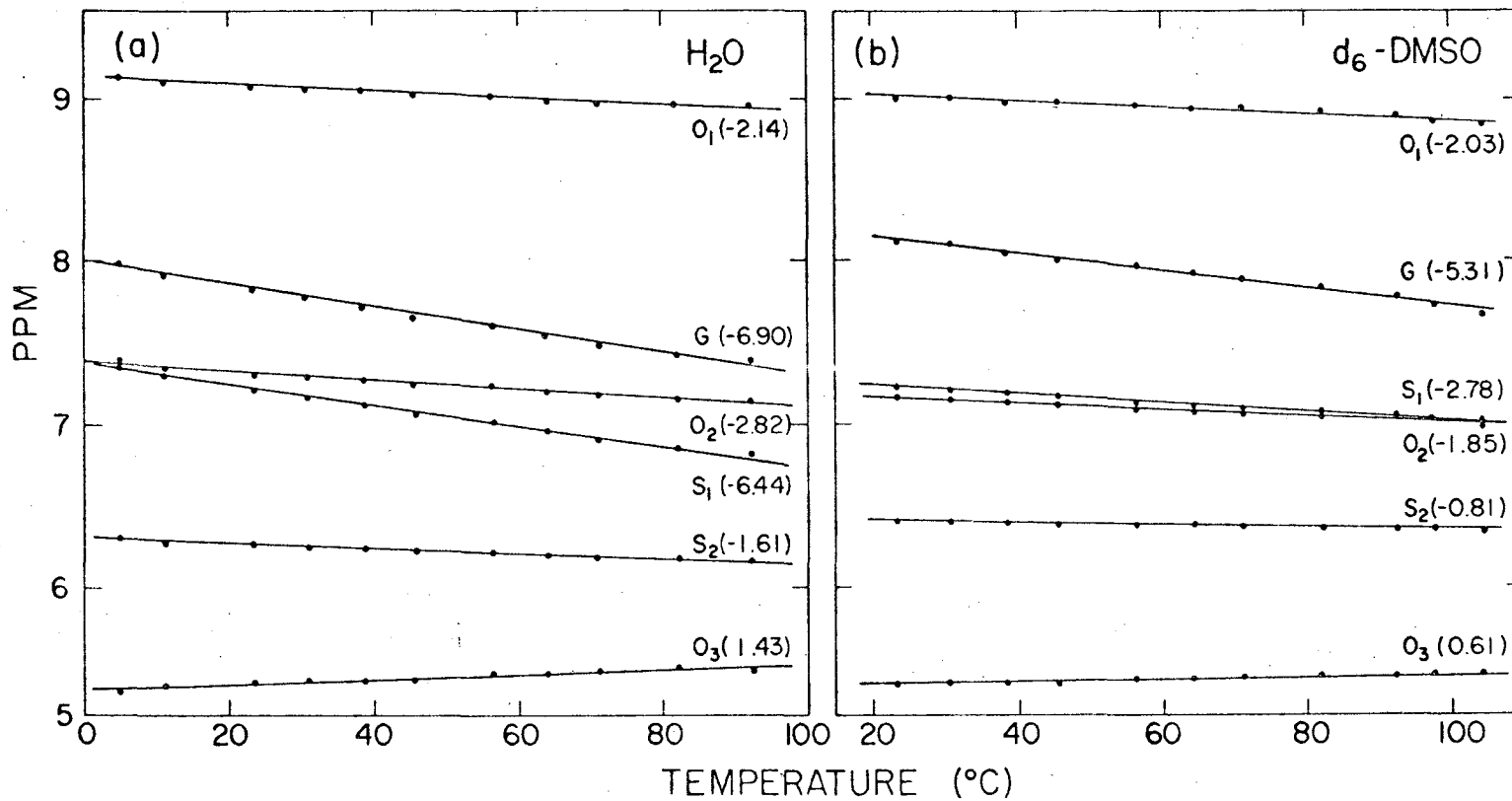
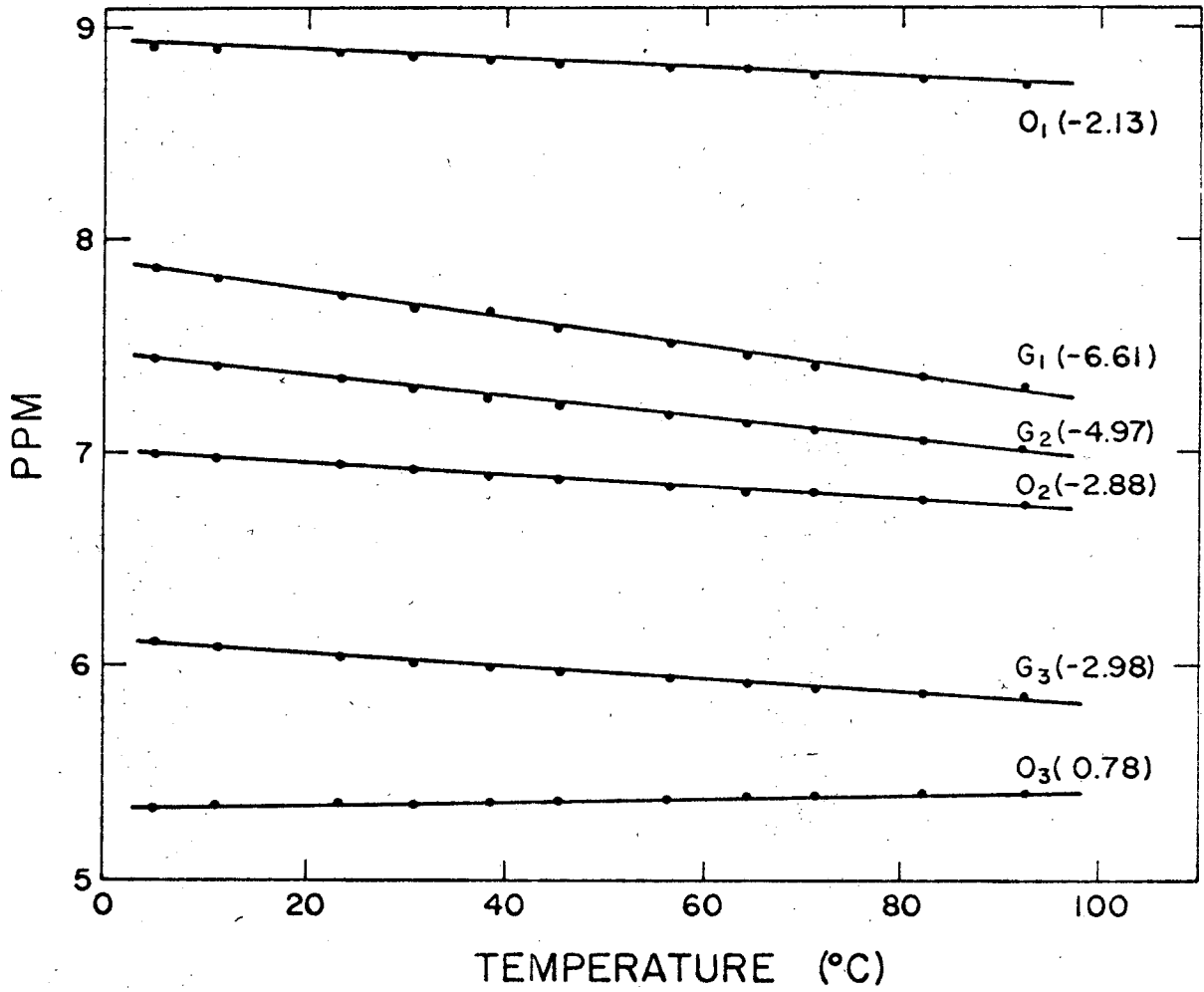


Fig. 4

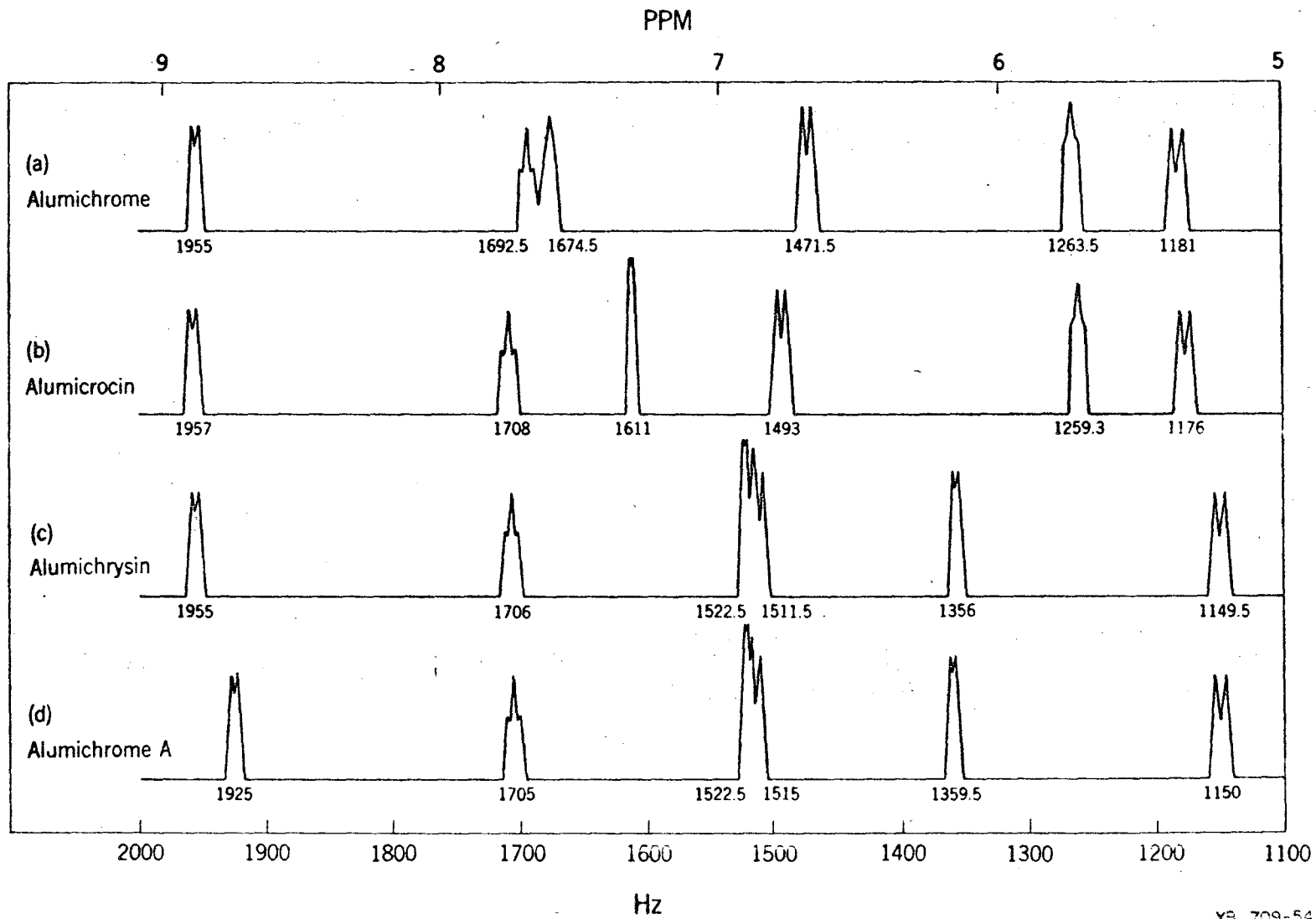
XBL709-5433

GALLICHROME H_2O



XBL709-5431

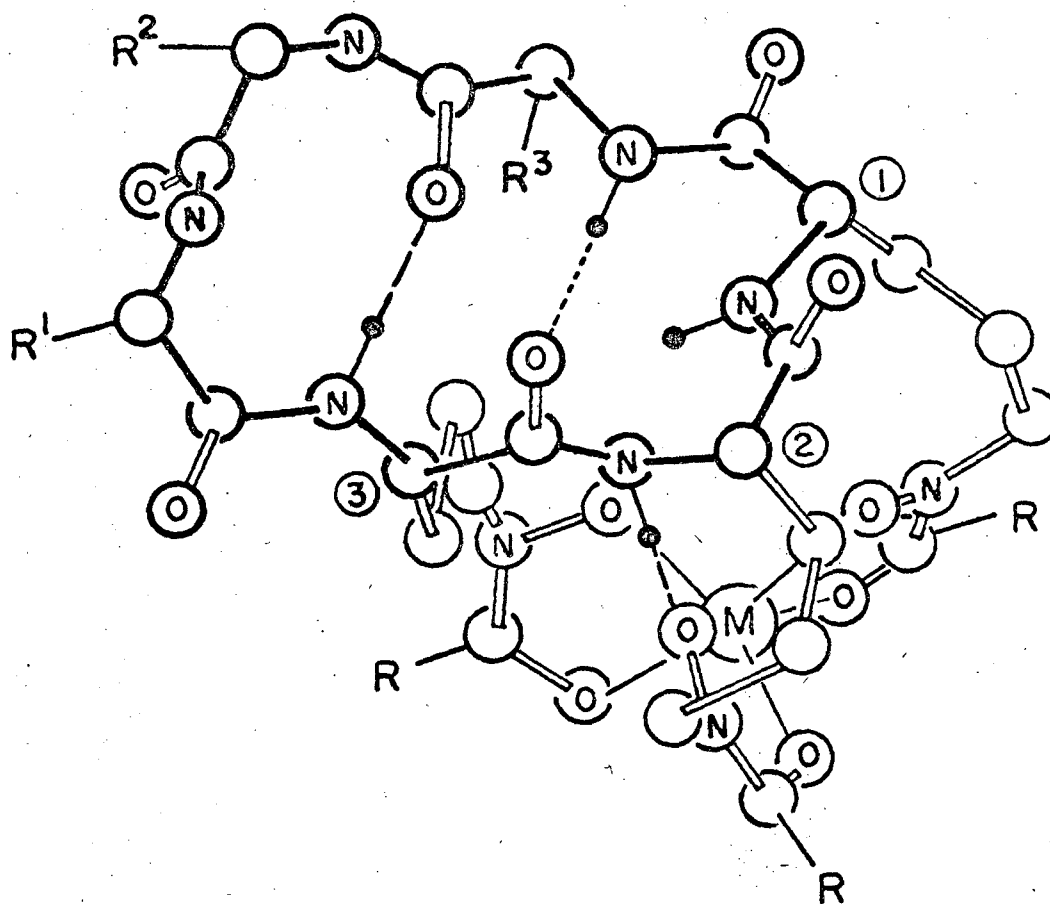
Fig. 5



XP-709-5419 A

Fig. 6

00005704979



XBL 709-5412

Fig. 7

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