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The Structure of Two Alanine Containing Ferrichromes: Sequence Determination by Proton Magnetic Resonance

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Summary. Metal coordination confers an extraordinary structural stability to the ferrichromes which, independent of their variable amino acid composition, results in a basically unperturbed conformation for all the homologous peptides in the series. The proton magnetic resonance (pmr) characteristics for Al^{3+} analogues (alumichromes) reflect this conformational isomorphism in usual solvents so that single site substitutions are clearly recognized in the pmr spectra. Thus, the substitution of glycine by L-alanine or L-serine introduce new resonances characteristic of the sidechains and alter the pattern of the amide NH pmr region in that doublets substitute for glycyl triplets at the same site. Since for glycine- and L-serine-containing alumichromes the resonances have already been identified, it is possible to unequivocally establish the primary structure of the two L-alanyl homologues ferrichrome C (cyclo-Gly^3–Ala^2–Gly^1–Orn^3–Orn^2–Orn^1–) and sake colorant A (cyclo-Ser^3–Ala^2–Gly^1–Orn^3–Orn^2–Orn^1–) on the basis of the comparative pmr spectra of their Al^{3+} analogues, namely, alumichrome C and alumisake. The resonance assignment, and hence the site occupancy, is substantiated by the temperature coefficients of the NH chemical shifts, rates of ^1H-^2H exchange and homonuclear proton spin decoupling experiments centered on the NH spectral region. Occupancy of site 1 by a glycine residue is observed for all known ferrichromes, which serves to conserve a "hairpin" turn. This method of obtaining sequence information should prove of general use for other systems of homologous polypeptides, provided their conformations are not affected by the residue substitutions.

Key words: Alumichrome – Cyclohexapeptides – Ferrichrome C – Nuclear magnetic resonance – Sake colorant A – Siderophores.

Introduction

The presence of iron in Japanese sake has been recognized to cause coloration and a deterioration of flavor (see, e.g., Kodama, 1970). Silica gel chromatography of the

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colored fraction of sake eluted from active carbon resolved it into a number of bands labelled "sake colorants" A, B, C, D and E. Spectroscopic and chromatographic analysis of these fractions indicated them to be ferrichrome-type compounds (Fig. 1). Tadenuma and Sato (1967) have identified the main fraction, sake colorant C, as

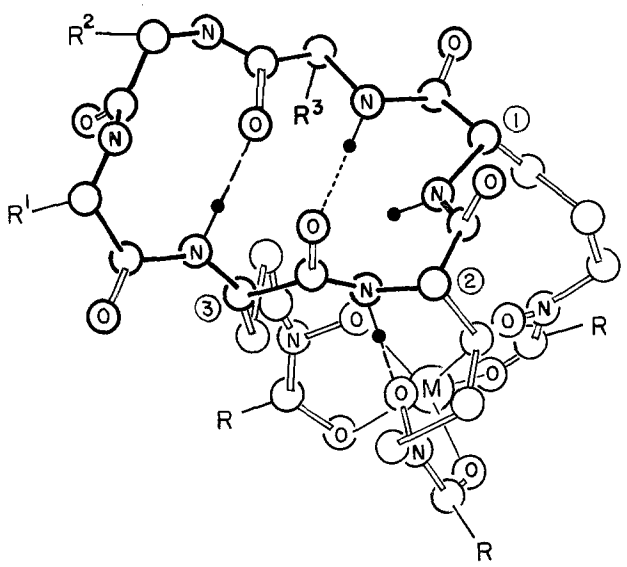
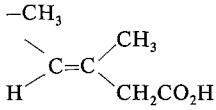
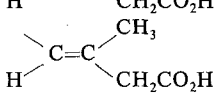
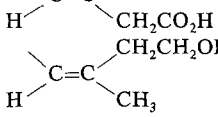


Fig. 1. Structural model for the ferrichromes, cyclohexapeptides involved in microbial high affinity iron transport (Zalkin et al., 1966; Llinás et al., 1970, 1972; Norrestam et al., 1975). R_1, R_2 and R_3 indicate sidechains for the residues occupying sites 1, 2 and 3, respectively. The three ornithyl residues are numbered in circles. Only "internal", protected amide hydrogen atoms are shown. Hydrogen bonds are indicated (- - -), but the one bridging Res^3–NH · · · O = C – Orn^3 is of little significance (Llinás and Klein, 1975). The ornithyl N^δ hydroxamate acyl group is denoted by R. The structures of all the known ferrichromes are given:

Peptide	$-R_1$	$-R_2$	$-R_3$	–R	
Ferrichrome	–H	–H	–H	$-CH_3$	
Ferrichrome C	–H	$-CH_3$	–H	$-CH_3$	
Ferricrocin	–H	$-CH_2OH$	–H	$-CH_3$	
Sake Colorant A	–H	$-CH_3$	$-CH_2OH$	$-CH_3$	
Ferrichrysin	–H	$-CH_2OH$	$-CH_2OH$	$-CH_3$	
Ferrichrome A	–H	$-CH_2OH$	$-CH_2OH$		(trans)
Ferrirubin	–H	$-CH_2OH$	$-CH_2OH$		(trans)
Ferrirhodin	–H	$-CH_2OH$	$-CH_2OH$		(cis)

The amino acid sequence determination of ferrichrome C and sake colorant A are described in this paper; for the other ferrichromes the original references are given in (Neilands, 1966; Llinás and Neilands, 1972). M denotes the metal ion, Fe^{3+} in the natural products and Al^{3+} in the nmr analogues

ferrichrysin. Total hydrolysis of sake colorant A yielded Gly : Ser : Ala : Orn : acetate[1] in the molar ratios 1 : 1 : 1 : 3 : 3 (Tadenuma and Sato, 1971). The finding of alanine was novel as the other ferrichromes known at that time contained only glycyl, L-seryl and tri(δ-N-acetyl-δ-N-hydroxy-L-ornithyl) in various ratios (Neilands, 1966). In a systematic study of siderophores produced by various yeast genera, Atkin et al. (1970) reported that *Cryptococcus melibiosum* (type UCD 52–87) forms a siderophore identical to ferrichrome except for a single alanyl-for-glycyl substitution at an unspecified site. This second alanine-containing ferrichrome was named ferrichrome C.

We have discussed elsewhere the structural unity revealed by the ferrichromes (Neilands, 1966; Llinás, 1973). This uniformity stems from the requirement of octahedral Fe^{3+} coordination by the linear tri-(δ-N-acetyl-δ-N-hydroxy-L-ornithinyl) segment. The hexadentate triacethydroxamate ligand thus defines the "active site" which determines the common spectral (Llinás and Neilands, 1972; Llinás, 1973) and biological (Emery and Emery, 1973; Winkelmann, 1974; Wayne and Neilands, 1975) characteristics of these siderophores. Hence, the structure determination of a new triacethydroxamate ferrichrome essentially reduces to solving the amino acid array of the sites 1-2-3 segment. To-date, a glycyl residue has been found to occupy site 1 in all ferrichromes for which sequences have been determined (Llinás and Neilands, 1972).

Comparative spectral data on homologous peptides (Llinás et al., 1972) and proteins (Stellwagen and Shulman, 1973; Cohen and Hayes, 1974; Packer et al., 1975; Oldfield et al., 1975; Wüthrich et al., 1976) have proved most valuable in the interpretation and assignment of nmr resonances. Essentially, the method assumes constancy of secondary and tertiary structure features as single, non-essential, amino acid residues are substituted at particular sites in the primary structure. The reverse experiment, namely the derivation of primary structure information on the basis of the nmr spectra of homologous, isomorphic polypeptides has, in contrast, not yet been explored. The alumichromes (Al^{3+} analogues of the ferrichrome peptides, Fig. 1) are especially suitable for such experiments. The relative uniqueness of the alumichromes' pmr spectra is a consequence of their underlying conformational uniformity (Llinás, 1973). The excellent resolution of the amide NH resonances of the alumichelates as well as the ease with which these resonances can be assigned to their corresponding residues on the basis of their multiplet structure and proton spin-spin coupling connections (Llinás et al., 1972) suggests that the pmr spectra of similar compounds should be easily rationalized in terms of a sequence provided that their amino acid compositions are known.

In this paper we present a pmr study of the Al^{3+} analogues of ferrichrome C and sake colorant A, "alumichrome C" and "alumisake", respectively. The comparative analysis of the data with that of other alumichromes and consideration of the characteristic ferrichrome conformation (Fig. 1) lead to a complete elucidation of their structure.

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[1] Abbreviations used are: Ala, L-alanine; Gly, glycine; Ser, L-Serine; Orn, L-ornithine; d_6-DMSO, hexadeuterodimethylsulfoxide; nmr, nuclear magnetic resonance; pmr, proton magnetic resonance; ppm parts per million; TMS, tetramethylsilane

Materials and Methods

Ferrichrome C was extracted with benzyl alcohol from a cell-free low iron culture medium of *Cryptococcus melibiosum* after 10 days of growth (Atkin et al., 1970). The dried extract containing the ferric peptide was purified by fractionation through a short silica gel column as described for the sake colorants by Tadenuma and Sato (1967). The clean peptide fraction elutes when the solvent gradient reaches a composition of 20% ethanol in 80% chloroform (organic phase preequilibrated with equal volume of distilled water). The peptide was crystallized once from absolute ethanol. The net yield of crystalline ferrichrome C was 225 mg/l of original cell growth medium.

A sample of pure sake colorant A was kindly made available to us by Drs. S. Sato and M. Tadenuma (Research Institute of Brewing, Tax Administration Agency, Tokyo). The peptide had been obtained from rice koji (ferment of *Aspergillus oryzae* on steamed rice) as described by these investigators (Tadenuma and Sato, 1967).

Iron was removed from ferrichrome C and sake colorant A with 8-hydroxyquinoline, the deferripeptides were then reacted with $Al(OH)_3$, purified by Bio-Gel P2 filtration and dried over P_2O_5 under reduced pressure as described elsewhere for the other alumichromes (Llinás et al., 1972). Given the high affinity of the deferriferrichromes for Fe^{3+}, it is usual that the alumichrome product will exhibit a weak yellow coloration due to some reformation of the ferric complex during the procedure. This residual iron could be easily eliminated from the final product by reextracting the alumichromes with 8-hydroxyquinoline, a process which removes Fe^{3+} but little Al^{3+}. An essentially iron-free substance was thus obtained for the nmr studies.

The pmr spectra was recorded with a Varian HR220 spectrometer. All decoupling frequencies were calibrated by sideband modulation of the TMS line. Other details of the pmr experiments followed the description given previously (Llinás et al., 1972).

Results and Discussion

The 220 MHz pmr spectra of alumisake and alumichrome C dissolved in d_6-DMSO are shown in Figure 2. The well resolved resonances appearing between about 6 and 10 ppm correspond to the six peptidyl amide protons. The glycyl NH lines are characteristic in that they exhibit a non-doublet (a quasi triplet, doublet of doublets) multiplet structure. This criterion identifies the resonances at 8.86 ppm in alumisake and at 8.91 and 6.86 ppm in alumichrome C as arising from glycyl residues. The remaining NH signals appear as doublets. Our problem is to distinguish ornithyl from seryl from alanyl amide resonances. This can be solved by spin-spin decoupling experiments sequentially establishing connections between NH $\leftrightarrow$ $C_\alpha H$, $C_\alpha H \leftrightarrow C_\beta H$, etc. Thus, the ornithyl and alanyl NH's are coupled to single α-protons which themselves are coupled to high field alkyl β-protons. The freely rotating alanyl methyl sidechain yields a characteristic typical sharp doublet because of its spin-spin interaction with the α-proton. In contrast, the ornithyl $C_\alpha H$ is coupled to a pair of magnetically non-equivalent methylene protons easily distinguished from the simple alanyl $C_\beta H_3$ resonance at $\sim$ 1.21 ppm. The complexity of the ornithyl β-γ resonance

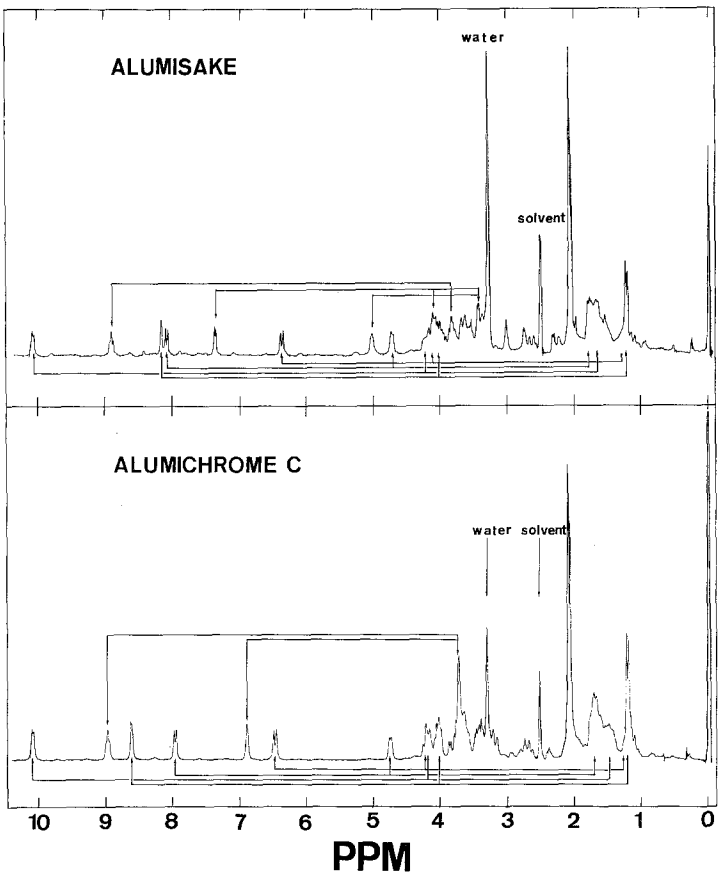


Fig. 2. Pmr spectra of alumisake and alumichrome C. The 220 MHz spectra are for 0.06 *M* d_6-DMSO solutions and were recorded at ~ 43° C. The chemical shift scale (ppm) is referred to internal TMS. Spin-spin coupled resonances are shown connected by arrows. Tables 1 and 2 list chemical shift and $^3J_{NC}$ parameters measured from these spectra. "Water" and "Solvent" indicate resonances arising from residual water and solvent protons

region in the alumichromes arises from the rigid conformation imposed on the side-chains by the metal complex center. Once the glycyl, ornithyl and alanyl amide resonances are identified, the doublet NH peak at 7.33 ppm in alumisake can be assigned by exclusion to the single seryl residue present in this peptide. However, since an unequivocal distinction between the alanyl and seryl resonances in alumisake is crucial for deriving the amino acid sequence, these double resonance experiments are described in more detail below.

The α-protons causing doublet-splitting to non-ornithyl NH resonances in alumisake were assigned, from spin-spin decoupling experiments, to resonances at 4.01 and 4.10 ppm. The identification of these coupled resonance pairs is critically dependent on establishing their spin-spin connections to the β-protons. This permits the differentiation of seryl from alanyl residues since the $C_\beta H$ resonances are unequivocally identified, the first from spin-spin connections to the $C_\beta OH$ triplet at 5.00 ppm

and the second from its relatively sharp doublet at 1.22 ppm. The alanyl $C_\alpha H \longleftrightarrow C_\beta H_3$ decoupling was achieved by irradiating at lower fields while observing collapse of the high field methyl doublet and by the reverse experiment, namely, the observation of the $C_\alpha H$ resonance while irradiating the central methyl region. Both decouplings yielded relative chemical shifts which agree within 1 Hz. Essentially the same procedure was followed with the seryl resonances and excellent agreement was found between the $\alpha \longleftrightarrow \beta$ and the $\beta \longleftrightarrow \alpha$ decoupling experiments. In this case, however, the $C_\beta H_2$ chemical shift established from $C_\beta OH \longleftrightarrow C_\beta H$ decouplings differs by 5 Hz (0.023 ppm) from that determined in the $C_\alpha H \longleftrightarrow C_\beta H$ experiments. The disagreement most likely stems from a lack of magnetic equivalence between the two β protons, which could result in each having different spin-spin coupling with the α and hydroxyl protons and also from the inherent instrumental difficulty of decoupling the impacted α and β resonances. The value given in Table 1 for the seryl $C_\beta H_2$ chemical shift is an average between these two values, the 5 Hz uncertainty being considerably less than the 18 Hz separating the alanyl and seryl resonances being identified. Table 1 also lists chemical shifts for all the resonances decoupled as shown in Figure 2. The approximate $^3J_{NC}$ couplings, directly measured from the NH spectrum, uncorrected for line width effects, and assuming triplet structure for the glycyl lines, are shown in Table 2.

Table 1. PMR chemical shifts

	Alumisake						Alumichrome C					
	Gly	Ala	Ser	Orn_1	Orn_2	Orn_3	Gly_1	Ala	Gly_2	Orn_1	Orn_2	Orn_3
NH	8.86	8.12	7.33	10.01	8.02	6.33	8.91	8.56	6.86	10.02	7.93	6.45
$C_\alpha H$	3.83	4.01	4.10	4.21	4.71	4.11	3.72	4.01	3.72	4.18	4.73	4.22
$C_\beta H$		1.22	3.42	1.64	1.77	1.24		1.20		1.47	1.69	1.26
$C_\beta OH$			5.00									
CH_3				- - - -	2.06	- - -				- - -	2.06	- - -

The chemical shifts are for d_6-DMSO solutions at about 45° C and are referred to internal TMS. The residues are labelled with a subindex to indicate the order in which the corresponding amide NH resonances occur in scanning from low to high magnetic field: absolute assignments are given in the text. The acetyl methyl resonance is, like in the other alumichrome-type peptides, composed of three closely spaced narrow peaks

Table 2. Amide vicinal proton-proton spin couplings

Alumisake						Alumichrome C					
Gly	Ala	Ser	Orn_1	Orn_2	Orn_3	Gly_1	Ala	Gly_2	Orn_1	Orn_2	Orn_3
~ 5.2 ± 0.2	~ 2.8 ± 0.3	3.4 ± 0.1	4.9 ± 0.3	6.5 ± 0.3	7.9 ± 0.1	~ 5.4 ± 0.2	3.1 ± 0.3	~ 3.0 ± 0.2	5.9 ± 0.2	6.0 ± 0.2	9.2 ± 0.2

The spin-spin coupling constants ($^3J_{NC}$) given in Hz, are between amide NH and $C_\alpha H$ protons, for the peptides dissolved in d_6-DMSO. The labelling of residues follows the same convention as in Table 1. Values are averages and their standard deviations are derived from determinations at different temperatures. Poorly resolved multiplets are indicated by ~

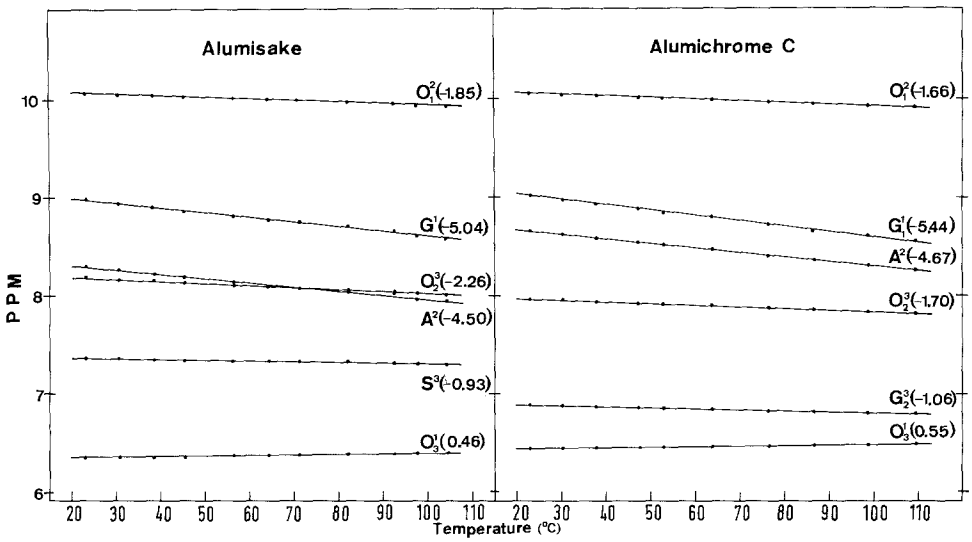


Fig. 3. Plots of the alumisake and alumichrome C amide NH chemical shifts (ppm) versus temperature. The experimental data points were linearly least-squares fitted and the corresponding slopes $\times 10^3$ are given in parenthesis (i.e., $-4.50 = -4.50 \times 10^{-3}$ ppm/° C). The amino acid residues are denoted by A (alanyl), G (glycyl), O (ornithyl) and S (seryl) according to the sub- and super-index convention explained in the text. The chemical shifts are given by reference to internal TMS

The temperature dependence of the amide chemical shifts allows the NH groups to be classified as internal or external according to the magnitude of their linear slopes (Kopple et al., 1969; Ohnishi and Urry, 1969). As shown in Figure 3, the low field glycyl and alanyl amide resonances exhibit relatively larger temperature coefficients in both peptides which indicates they should be less protected by the overall tertiary structure. This was confirmed by their faster ^1H-^2H exchange upon addition of ^2H_2O to the d_6-DMSO solutions and agrees with our previous pmr studies on other alumichromes in that two NH's, occupying sites 1 and 2 in the model (Fig. 1) are exposed to the solvent (Llinás et al., 1970, 1972). The amides which exhibit diminished slopes are the four protected amide NH's belonging to the residue at site 3 and the three ornithines. This means that the NH resonances labelled Gly_2 in alumichrome C and Ser in alumisake occupy site 3 in each peptide[2]. It should be noted that an absolute assignment of the ornithyl resonances is not required to establish the amino acid sequence since a tri-ornithyl sequence bridges the gap between sites 3 and 1 in all ferrichromes (Llinás and Neilands, 1972). It is nevertheless possible to reach an assignment on the basis of the model (Fig. 1) and what is known about the pmr spectra of other alumichromes (Llinás et al., 1970, 1972). Thus, the model points to a very strong intramolecular H bond between the Orn[2] amide and its own sidechain hydroxamate. This causes a strong deshielding so that the resonance

[2] Consistent with former papers in this series (e.g. Llinás et al., 1972), subindexes are used to label resonances in the order they occur *in scanning the spectrum from low to high field* and are independent of an absolute structural assignment. Superindexes, in contrast, label residues according to the absolute amino acid sequence following the convention used in Figure 1

should appear at low field, as is the case for Orn_1 (Fig. 2, Table 1). In contrast, the Orn^1 NH points towards the inside of the molecule, the H atom being completely surrounded by the peptide backbone sheet from "above" and the three ornithyl side-chains "laterally" and from "below". This buried proton is effectively protected from H bonding to, e.g., the solvent and should not experience significant deshielding. We thus attribute the high field Orn_3 resonance to Orn^1. Finally, Orn^3 structures a β loop and the model indicates an extent of H bonding intermediate between Orn^2 (strong H bond) and Orn^1 (no H bond). It follows that the Orn_2 NH resonance should be assigned to Orn^3. Such assignment of the ornithyl NH resonances totally agrees with the relative trend of their chemical shift temperature coefficients (Fig. 3) in that the more positive the slope the more "protected" the amide hydrogen atom.

The spectra shown in Figure 2 were recorded under the same conditions reported elsewhere for the other alumichromes and are hence directly comparable (Llinás et al., 1970, 1972). The similarity in all these spectra is most striking and points towards a virtual conformational isomorphism among the ferrichromes. Some of the salient common features are an isolated Orn^3 $C_\alpha H$ resonance at $\sim$ 4.72 ppm, a typical group of three sharp acyl methyl lines centered at $\sim$ 2.06 ppm, and a characteristic pattern of $Orn_{\beta,\gamma}$ aliphatic resonances spread between 1 and 2 ppm. Thus, the alumichrome C spectrum can be derived from that of alumicrocin by replacing the set of $-CH_2OH$ resonances in alumicrocin by the characteristic alanyl $-CH_3$ doublet at 1.20 ppm (residue substitution at site 2, see Fig. 1). However, in order to derive the alumisake spectrum from, e.g., that of alumichrysin, the occupancy of sites 1 and 2 should first be specified.

We assign a glycyl and an alanyl residue to the alumisake sites 1 and 2, respectively. The assignment is based on two main criteria. First, the chemical shift of the glycyl $C_\alpha H$ resonance is identical in alumisake and in alumichrysin ($\delta = 3.83$ ppm). Such agreement is also manifest for the Gly^1 α-proton in alumichrome C ($\delta = 3.72$ ppm) and in alumicrocin ($\delta = 3.79$ ppm) (Llinás et al., 1972). Second, the magnitude of the NH "triplet" splitting ($^3J_{NC}$) is closer to that of the glycyl NH at site 1 than to that at the two other sites as the following data shows:

	Gly_1^1	Gly_2^2	$Gly_{2,3}^3$
Alumichrome	5.4 ± 0.1 Hz	4.8 ± 0.2 Hz	3.7 ± 0.2 Hz
Alumicrocin	5.5 ± 0.1		3.6 ± 0.2 Hz
Alumichrome C	5.4 ± 0.2		3.0 ± 0.2 Hz
Alumichrysin	5.5 ± 0.1		
Alumisake	5.2 ± 0.2		

Indeed, if an alanine occupied site 1 its conformational ϕ angle[3], as determined by crystallography in ferrichrome A and in ferrichrysin ($\phi \sim 86°$, $\theta \sim 26°$ [Zalkin et al., 1967; Norrestam et al., 1975]) would result in doublet-splitting the NH resonance by $\sim$ 9 Hz (Bystrov et al., 1973). This readily measurable value would be

[3] The definition of ϕ follows the IUPAC-IUB recommendation (J. molec. Biol. **52**, 1–17, 1970), and $\theta = |60 - \phi|$

significantly larger than the poorly resolved ~ 3 Hz splitting shown by the alanyl amide, (Fig. 2, Table 2). Our conclusions are consistent with the Gly_1^1 and Orn_2^3 amide chemical shifts in the same set of compounds (T ~ 45° C):

	Gly_1^1	Orn_2^3
Alumicrocin	8.94 ppm	7.93 ppm
Alumichrome C	8.91	7.93
Alumichrysin	8.93	8.00
Alumisake	8.86	8.01

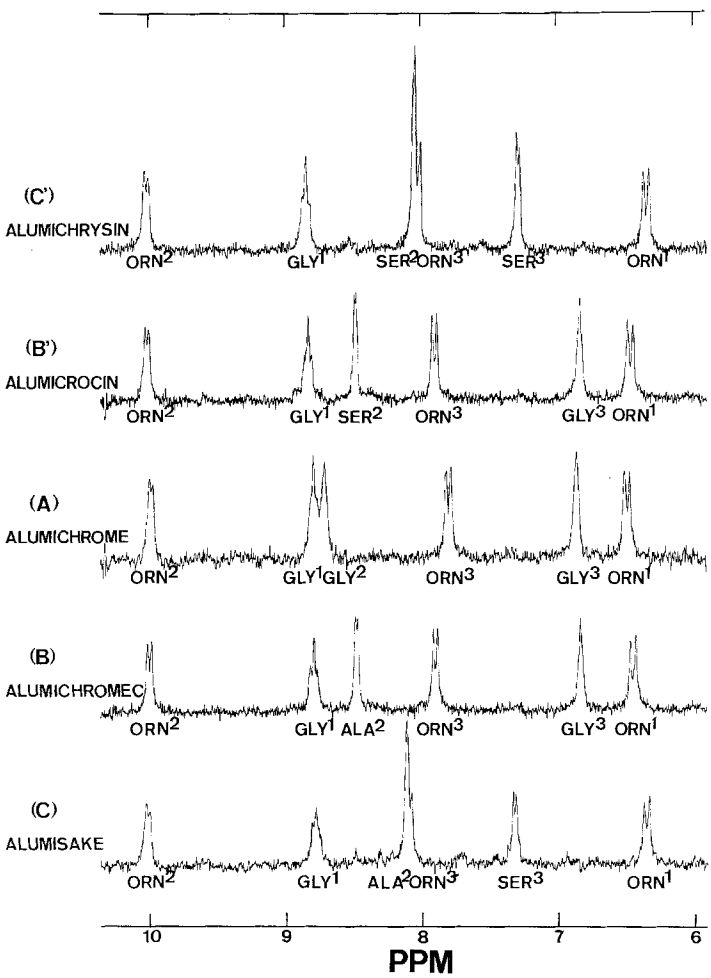


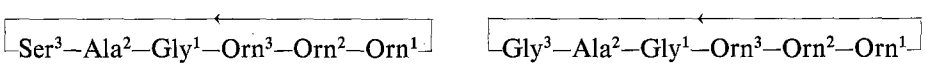
Fig. 4. Expanded pmr spectra of the amide NH region of alumichrome (A), alumichrome C (B), alumicrocin (B'), alumisake (C) and alumichrysin (C') for the peptides dissolved in d_6-DMSO. These spectra were recorded at ~ 50° C which explains why the resonances are somewhat shifted from the positions exhibited in Figure 2 (see Fig. 3). The chemical shifts (ppm) are referred to internal TMS. Each spectrum represents a single scan of a ~ 0.06 *M* solution

We note the relative constancy for the Gly_1^1 resonance. The particularly good agreement shown by the Orn_2^3 NH chemical shifts between each pair of analogues provides excellent support for the proposed assignments. By H bonding to the site 3 residue (Gly in alumicrocin and in alumichrome C, Ser in alumichrysin and in alumisake) and by lying between Orn^2 and an invariant residue at site 1 (Gly), its pmr senses conservancy of the microscopic milieu about the bridging H atom, within each pair of analogues (see model, Fig. 1).

The subtle dependence of the peptidyl NH pmr on the chemical environment points to the usefulness of the amide proton as an intrinsic reporter group to probe into fine details of the polypeptide structure. Figure 4 includes expanded amide region pmr spectra of all the alumichrome analogues possessing acetyl hydroxamic acid groups. This figure dramatically shows the spectral similarity of alumichrome C (B) and alumisake (C) with allumicrocin (B′) and alumichrysin (C′), respectively. It is most interesting to note that the overall resonance shifts induced on the alumichrome spectrum (A) by single substitutions at sites 2 and 3, is basically independent of whether Gly^2 is substituted by alanine (B, C) or by serine (B′, C′). From inspection of molecular models and considering that for alanine $pKa_1 = 9.91$ and $pKa_2 = 2.36$ while for serine $pKa_1 = 9.12$ and $pKa_2 = 2.29$ (pKa values at 25° C [Sillén and Martell, 1971]), Figure 4 indicates that the NH pmr chemical shifts are more dependent on the bulkiness of the side chain (steric hindrances, extent of proton exposure for solvent H bonding, etc.) than on inductive effects resulting from that substituent. In this regard, it is interesting to note that while the site 2 alanyl NH resonance exhibits a temperature coefficient of −0.00450 ppm/° C in alumisake (Fig. 3), a seryl residue identically located shows a corresponding slope of −0.00269 ppm/° C in alumichrysin (Llinás et al., 1972). This effect is consistent with a further steric protection conferred by the bulkier seryl side chain and should be taken into consideration when the temperature dependence of the amide chemical shift is used for conformational analyses.

Conclusions

The pmr spectra of alumisake and alumichrome C indicate the following amino acid sequences for the cyclohexapeptides:



Sake Colorant A Ferrichrome C

Hence, sake colorant A and ferrichrome C differ from ferrichrysin and ferricrocin, respectively, by an alanine-for-serine substitution at site 2. Given the conformational stabilization conferred by a single serine-for-glycine substitution on going from ferricrocin to ferrichrysin (Llinás et al., 1973), it is predictable that the structural stability ought to be greater for sake colorant A than for ferrichrome C. It is interesting to note the constancy of a glycyl residue at site 1 through all the ferrichromes. The residue at site 1 structures a β-turn (3_{10} helix) and steric consideration would dictate the

requirement that in this conformation it be occupied by either a D-amino acid or glycine (Venkatachalan, 1968; Urry and Ohnishi, 1970), which is also indicated by statistical sampling of protein structures (Dickerson et al., 1971; Crawford et al., 1973). The sequence proposed for ferrichrome C is totally consistent with solvent perturbation effects detected on the NH pmr, which have been the subject of a separate communication (Llinás and Klein, 1975).

To our knowledge, the present study of alumisake and alumichrome C represents a first attempt in the use of structural isomorphism to derive a complete peptide structure purely on the basis of nmr data. A similar approach, making use of chemical modification, has allowed Meyer and coworkers (1975) to derive the structure of tentoxin, a cyclic tetrapeptide. It is recognized that sequence determinations are more difficult for cyclic than for linear structures, for which acidity (Sheinblatt, 1967) and lanthanide ion (Bradbury et al., 1974) pmr titration effects have been found extremely useful. Compared with classical chemical methods, the nmr approach is simpler, unambigous and non-destructive. Furthermore, the approach is, in principle at least, not limited to simple compounds: it should also be of use for protein-sized molecules, where the nmr spectrum of the native structure can be used as a fingerprint of the primary structure. The only requirement is constancy of the secondary and tertiary structures for the homologous polypeptides. Recent conformational calculations have shown that structural similarity is energetically favored for homologous cytochrome *c*'s derived from different eukaryotic species (Warme, 1975). It is hence very likely that evolutionary or mutagenically related proteins will exhibit this property so that single amino acid substitutions may be assigned to particular sites solely from their effect on the pmr spectrum.

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