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Structure of Mycobactin J

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Abstract. Mycobactin J-1, an iron chelate from *Mycobacterium paratuberculosis,* was characterized by mass spectrum and by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra of the parent molecule and of cobactin J-1. The core structure of mycobactin J-1 contained the phenyloxazoline ring system common to the mycobactins. The benzene ring was disubstituted. The two hydroxamate functions were furnished by 1 linear 6-*N*-hydroxylysine residue and 1 cyclic 6-*N*-hydroxylysine residue as in other members of this class of compounds. The acyl function at the mycobactic acid hydroxamate center was *n-cis*-hexadec-2-enoyl. The hydroxy-acid of the cobactin portion of mycobactin J-1 was 2,4-dimethyl-3-hydroxypentanoic acid. This latter residue differs from those of other known mycobactins by the presence of the isopropyl group.

The structures of mycobactins produced by several species of mycobacteria have been published [6,7,8,10]. The growth-promoting activity of these iron chelates for *Mycobacterium paratuberculosis* has been summarized [8]. Throughout the research on this group of natural products, mycobactin P [6] has served as the reference for both chemical and biological properties. The variation in the structures of the mycobactins from several species of mycobacteria and the concomitant differences in the ^{1}H NMR (nuclear magnetic resonance) spectra of these compounds have been summarized [2].

Mycobactin P has been prepared and supplied by this Center to laboratories throughout the world since 1963 for use as a nutritional factor in the culturing of *M. paratuberculosis*. No compound of this class has been reported from this species.

National Animal Disease Center (NADC) strain 18 *M. paratuberculosis,* used for vaccine and paratuberculin production, has been described [3]. This culture has been induced to synthesize iron-chelating compounds of the mycobactin class. This new group of compounds was designated as mycobactin J. The production, purification, and growth-promoting activity of mycobactin J are described separately [4].

This report describes the chemical structure of the most abundant member of the mycobactin J group, mycobactin J-1. The availability of mycobactin P as a model and of the extensive NMR data extant in the published literature made possible the characterization of mycobactin J-1 with minimal chemical manipulation.

Materials and Methods

Elemental analysis. Elemental analyses were performed by the Galbraith Laboratories, Inc., Knoxville, Tennessee.

Nuclear magnetic resonance (NMR) spectra. The FT-NMR instrument used to produce ^{1}H- and ^{13}C NMR spectra was the FX 90 Q Spectrometer, Joelco Corp., Tokyo, Japan. ^{1}H NMR spectra were produced also with the CW T-60A Spectrometer System, Varian Corp., Palo Alto, California. The latter instrument was used for the proton-proton decoupling experiments. All NMR chemical shift values are reported in parts per million (ppm) downfield from tetramethylsilane (TMS).

Mass spectra. The mass spectra were produced with the Finnegan 4023 Mass Spectrometer and Data System, Finnegan Corp., Sunnyvale, California. Scans were made under conditions ranging from 20 EV to 70 EV and 150°C to 300°C. The molecular ion and first daughter ion from mycobactin J-1 were obtained at 20 EV and 300°C. Extensive fragmentation of the test substance occurred under all operative conditions.

NMR solvents. Deuterated solvents were obtained from Merck & Co., Inc., St. Louis, Missouri

Chelate dissociation. Ferrimycobactin J-1 was converted to the metal-free mycobactin by shaking chloroform solutions of the iron chelate against aqueous 4 N HCl [7].

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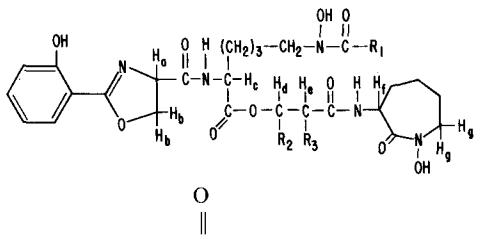


Fig. 1. Mycobactin J. R_1—C— = *n-cis*-hexadec-2-enoyl; R_2 = isopropyl; R_3 = methyl.

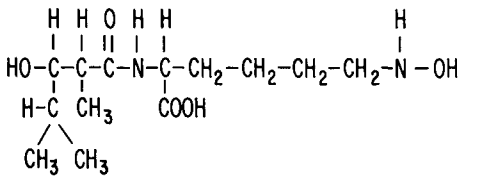


Fig. 2. Structure of 'linear cobactin J-1'. See text.

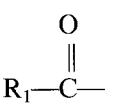
Aluminum mycobactin. The aluminum complex of mycobactin J-1 was prepared by published methods [7].

Chemical degradation. The metal-free mycobactin was hydrolyzed with 1 N NaOH at room temperature, and the process was monitored as described below.

Results and Discussion

The nomenclature proposed for this class of metabolites by White and Snow [9] is used in which the acid and alcohol obtained by hydrolyzing the ester bond of a mycobactin are termed mycobactic acid and cobactin, respectively; the letter designation assigned to the parent molecule is used also with each product.

The structure of the most abundant member of the mycobactin P group of compounds isolated at this laboratory, P-1, was found to be identical to that described as fraction 1 [9] and was used as a model in this study. The structure of mycobactin P-1 may be visualized by appropriate additions to Fig. 1. The reference compound has methyl groups at position 6 of the benzene ring and at R_3, an ethyl group at R_2, and



is *n-cis*-octadec-2-enoyl [9]. The structural characterization of mycobactin J-1 is presented below.

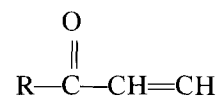
Elemental analysis. The calculations are based on a molecular weight of 841, determined by mass spectroscopy.

Calculated %: C, 64.26; H, 8.50; N, 8.31; O, 19.02.

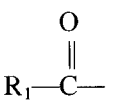
Found %: C, 64.81; H, 8.33; N, 7.82; O, 18.92.

Molecular formula: $C_{45}H_{71}N_5O_{10}$. This formula requires thirteen degrees of unsaturation.

Mass spectrum. Mycobactin J-1 was converted into its aluminum complex for examination by mass spectrometry. The preparation melted at 110°C. The spectrum showed a parent peak at m/e 865, 28 mass units less than the major mycobactin P component at 893 [9]; the first degradation peak was at m/e 682, a finding coincident with the mass of the first daughter ion from P-1, and corresponds to breakage of the side chain immediately beyond the double bond leaving the radical



where R represents the undegraded nucleus. The mass of 183 loss corresponds to the fragment $-(CH_2)_{12}-CH_3$; thus the acyl function labeled



in Fig. 1 is *n-cis*-hexadec-2-enoate. The absence of other parent peaks indicated a high degree of purity of the preparation. Field desorption mass spectrum of the iron chelate of mycobactin J-1 yielded a molecular weight of 894 in confirmation of the m/e data.

^{1}H NMR spectra. The ^{1}H NMR spectrum of mycobactin J-1 dissolved in C^2HCl_3 showed resonance bands for the aromatic protons between 6.6 and 7.6 ppm in an ABCD pattern typical of an ortho disubstituted benzene ring [1]. Within this resonance band pattern, a doublet of doublets at 7.6 ppm was assigned to the proton at position 6 of the benzene ring deshielded by the oxazoline ring. These resonance bands are absent from the mycobactin P-1 spectrum since a methyl group occurs at this position. The protons of the oxazoline ring appeared as a degenerate AB_2 pattern with one H_b yielding 4 lines at 4.9 ppm and the H_b in *cis* relationship with H_a showing a shift identical to that of H_a at 4.6 ppm. H_c appeared at 4.4 ppm. H_d appeared at 5.0 ppm as

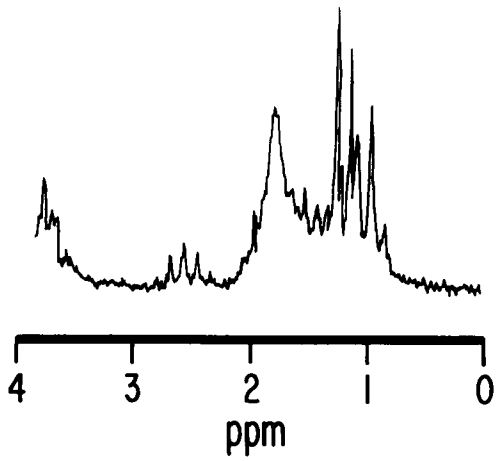


Fig. 3. 60 Mhz ^1H NMR (nuclear magnetic resonance) spectrum of 'linear cobactin J-1', 3% concentration in ^2H_2O. Chemical shift values are downfield from TMS.

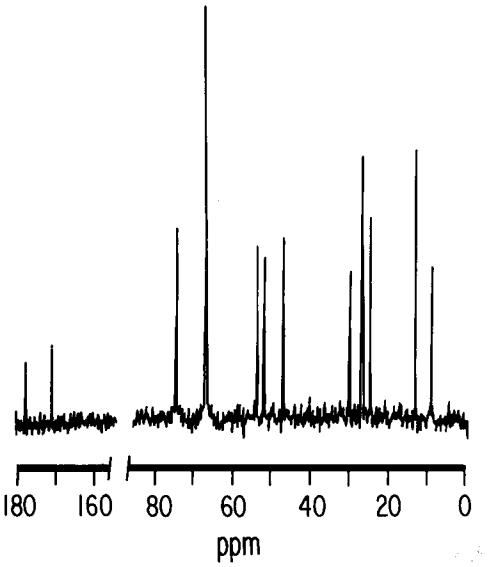


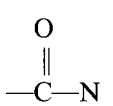
Fig. 4. ^{13}C NMR (nuclear magnetic resonance) spectrum of 'linear cobactin J-1', 3% concentration in ^2H_2O. Dioxane (67.0 ppm) used as internal reference. Pulse, single, 4 μsec (35°). Number of accumulations, 100,000. Decoupling mode, hetero. Chemical shift values are downfield from TMS.

a doublet of doublets indicating coupling with a single proton in R_2 and with H_e. H_e yielded a multiplet at 2.6 ppm partially obscured by the $=CH—CH_2—$ group in R_1 at 2.5 ppm. H_f at 4.4–4.6 ppm overlapped bands from H_a and *cis* H_b. The H_g protons and the $CH_2—N$ group in the linear lysine residue gave bands at 3.7 and 3.6 ppm.

The $—CH=CH—$ group in R_1 occurred as a broad multiplet at 5.9 ppm.

The protons of the seven-membered ring produced bands at 1.6–1.7 ppm and the protons of the aliphatic chain appeared as a strong band at 1.25 ppm. Methyl resonance bands appeared at 1.2, 1.1, 0.94, and 0.87 ppm. The abundance of resonance bands in the high field area and the overlapping of resonance bands from the protons of the hydroxy-acid moiety with those of other groups made degradation of mycobactin J-1 necessary to permit definitive observations.

^{13}C NMR spectrum. The ^{13}C NMR spectrum of mycobactin J dissolved in C^2HCl_3 yielded 4

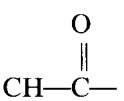


resonance bands at 171.7, 171.0, 168.3, and 167.7 ppm, and 1 ester-carbon band at 160.4 ppm.

Resonance bands at 146.0, 145.9, 129.3, 119.6, 118.2, and 117.4 ppm confirmed that the benzene ring was disubstituted. A weak band at 134.5 ppm was assigned to the quaternary carbon of the oxazoline ring. Resonances at 76.6 and 76.5 ppm were assigned to the CHO group of the hydroxy-acid and to the CH_2O group of the oxazoline ring, respectively.

A band at 52.9 ppm was assigned to the CHN group of the oxazoline ring. The resonances of the lysine residues were assigned as follows: C-2, 51.1 and 51.2 ppm; C-3, 27.6 and 26.3 ppm; C-4, 22.9 and 22.6 ppm; C-5, 31.9 and 31.3 ppm; C-6, 43.9 and 43.6 ppm.

A band at 51.7 ppm was assigned to the



group in the hydroxy-acid. The —CH— of the function at R_2 appeared at 25.7 ppm.

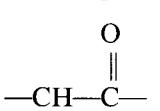
Two weak resonances at 40.6 and 40.4 ppm were assigned to the CH=CH group of R_1. The CH_2 groups in the R_1 side chain appeared as intense bands at 29.6–29.3 ppm. Four methyl group resonance bands at 21.0, 14.0, 12.8, and 10.3 ppm accounted for the remaining carbons. The band at 21.0 ppm was assigned to the terminus of the R_1 side chain. The coincidence in masses of the core structures of mycobactins J-1 and P-1, the absence of a methyl group at position six of the benzene ring in mycobactin J-1, and the identity of the amino acid residues in these compounds, required that the

three remaining methyl groups be assigned to R_2 and R_3 of the hydroxy-acid residue. To determine the structure of the hydroxy-acid, the cobactin portion of mycobactin J-1 was examined separately.

Cobactin J-1. Alkaline hydrolysis was used to cleave the ester bond of mycobactin J-1. Since disruption of the seven-membered ring would yield a product admirably suited for spectroscopic study, the greater lability of the secondary hydroxamate bond versus that of the secondary amide bond to alkaline hydrolysis was exploited. Two hundred mg of mycobactin J-1 was dissolved in 5 ml of 1 N NaOH. The process of hydrolysis was monitored by measuring the reducing activity of the released NHOH function with the triphenyltetrazolium chloride reagent [5]. The reducing activity of the hydrolysate was maximal after 3 h. The solution was adjusted to pH 3.0 by the addition of HCl, then was repeatedly extracted with 2-volume portions of diethyl ether. The ether phase was washed with 3 ml of H_2O and the washings were added to the aqueous fraction. The aqueous solution was adjusted to pH 6.0 with NaOH. Methanol and diethyl ether were added such as to maintain a single phase solution and cause precipitation of the bulk of the NaCl. The solution was decanted and reduced to dryness at 40°C in vacuo. The product was dissolved in 5 ml of H_2O and methanol was added to produce an incipient turbidity. The product crystallized after standing 2–3 h at room temperature and was twice recrystallized from aqueous methanol to yield linear cobactin J. Quantitation of the ^{13}C spectra of this preparation attested to its purity. The structure of this compound is shown in Fig. 2, and the NMR spectra of this compound are summarized below.

^1H NMR spectra. 'Linear cobactin J' was dissolved in deuterated dimethyl sulfoxide at a concentration of 3% and the ^1H NMR spectrum was produced. The NOH and NH functions yielded resonance bands at 7.9 and 7.7 ppm, respectively. The alcoholic proton was not visualized. This solvent compressed the spectral area of the methyl resonance bands to obscure the band shapes.

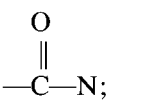
The ^1H NMR resonance spectrum of 'linear cobactin J-1' in ^2H_2O is shown in Fig. 3. The chemical shift of the —OCH— group (H_d, Fig. 1) was moved upfield to 3.6 ppm as a result of ester bond cleavage. The multiplet for



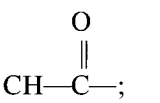
(H_e, Fig. 1) appeared at 2.5 ppm. A 100 Hz-width scan through this spectral area showed this multiplet to be a quartet. The CHN resonance at 4.4–4.6 ppm was obscured by the water band (not shown). The CH_2N group appeared at 3.7 ppm with the remaining lysine methylene groups at 1.7–1.8 ppm. The methine proton of the isopropyl group gave a multiplet at about 1.5 ppm. The exchangeable protons of the NH, NOH, and OH groups were deuterated in this solvent. Three methyl doublets were recorded at 1.3, 1.1, and 0.9 ppm. Proton-proton decoupling was used to assign the sources of the latter three bands.

Proton-proton decoupling. Irradiation of the multiplet at 2.5 ppm collapsed the band at 1.1 ppm to a singlet and moved the doublet at 3.6 ppm slightly downfield. Irradiation at 1.1 ppm left a doublet at 2.5 ppm. The 1.1 ppm band was assigned to the single methyl (R_3, Fig. 1). Irradiation of each of the doublets at 1.3 and 0.9 ppm yielded alteration in the CH multiplet at 1.5 ppm; irradiation at 1.5 ppm collapsed the bands at 1.3 and 0.9 ppm to singlets and moved the doublet at 3.6 ppm upfield to 3.5 ppm. The methyl resonance bands were assigned to the nonequivalent isopropyl group (R_2, Fig. 1).

^{13}C NMR spectrum. The ^{13}C NMR spectrum of 'linear cobactin J-1' is shown in Fig. 4. The lines at 27 and 12.8 ppm each represent two carbons. The resonance bands were assigned as follows: 177.7 ppm, COOH; 171.2 ppm,



74.6 ppm, —C—O; 53.6 ppm, C-2 lysine; 51.9 ppm,



46.2 ppm, C-6 lysine; 29.8 ppm, C-5 lysine; 27.3 ppm, C-3 lysine; 27.2 ppm, CH of isopropyl; 25.4 ppm, C-4 lysine; 12.8 ppm, isopropyl methyls; 9.8 ppm, methyl (R_3, Fig. 1).

The data presented show that mycobactin J-1 differs from mycobactin P-1 by having a shorter acyl group at the main hydroxamate center (R_1), in the absence of a methyl group in position 6 of the benzene ring, and in the presence of an isopropyl group at R_2 (Fig. 1). This latter group distinguishes mycobactin J-1 from all known mycobactins. The ^{13}C NMR spectrum of mycobactin J-1 in $^2HCCl_3$

differentiated the isopropyl group methyls as did the ^{1}H NMR spectrum of 'linear cobactin J-1'. The ^{13}C NMR spectrum of the cobactin in $^{2}H_2O$ yielded the same shielding value for these methyls.

The ^{1}H NMR spectrum of cobactin J-1, supplemented by the decoupling experiments, supports an erythro configuration for the hydroxy-acid agreeing with the spectral pattern observed [6] for the erythro hydroxy acid fragment derived from mycobactin P. The absolute configuration of the hydroxy-acid is not known.

The ^{1}H NMR spectrum supports a *cis*-configuration in the acyl side chain as is the case in mycobactin P-1.

The structure shown in Fig. 1 satisfies the molecular formula requirement of 13 degrees of unsaturation.

Knowledge of the absolute configuration of the iron chelate of mycobactin J-1 and of related compounds would be useful in explaining the basis for the enhanced biological activity of mycobactin J versus mycobactin P [4]. This information would also be of value in studies of binding of these compounds by microbial membranes and in serological investigations.

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