The Structure of Mycobactin P, a Growth Factor for *Mycobacterium johnei*, and the Significance of its Iron Complex

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1. The growth factor from *Mycobacterium phlei* is now named mycobactin P to distinguish it from related but chemically distinct growth factors from other species of mycobacteria. It is shown to comprise four closely similar chemical entities differing only in the fatty side chain. 2. The structure of the main component (85%) of mycobactin P has been established and the absolute configuration of all asymmetric centres determined. 3. Ferric mycobactin P has been crystallized; its structure is discussed. 4. Evidence is given for regarding ferric mycobactin P as a member of the class of compounds known as sideramines, though the mycobactins are more specialized than other sideramines.

Iron-containing growth factors occur in many micro-organisms, and have been named sideramines (Bickel *et al.* 1960). The structures of two groups of sideramines, the ferrichrome type (Rogers, Warren & Neilands, 1963) and the ferrioxamine type (Prelog & Walser, 1962), have been established; they differ substantially, but resemble each other in possessing three hydroxamic acid groups within a single molecule. The hydroxamic acid groups are so linked that they can engage all six co-ordination positions of a ferric iron atom. The involvement of ferrichrome in the utilization of iron for haem biosynthesis has been demonstrated (Burnham, 1962, 1963), and this seems likely to be a widespread biochemical mechanism in micro-organisms (Zähner, Bachmann, Hütter & Nüesch, 1962). There is no evidence of any parallel requirement for sideramines in haem biosynthesis in mammalian cells.

A growth factor for *Mycobacterium johnei* was isolated from *M. phlei* (Francis, Macturk, Madina-veitia & Snow, 1953) and its structure was determined (Snow, 1954*a,b*); it has now been named mycobactin P, to distinguish it from a related growth factor, mycobactin T (Snow, 1961), from *M. tuberculosis*. The mycobactins are very different in structure from the sideramines already mentioned but have sufficient similarities to suggest strongly that they serve the same biochemical function; therefore, as tentatively discussed by Bickel *et al.* (1960), they should be considered to be another type of sideramine. They contain only two hydroxamic acid groups, but have a third metal-chelating centre consisting of a phenolic hydroxyl group situated in an appropriate position relative to

the nitrogen atom of an oxazoline ring. They therefore form exceptionally stable complexes with iron or aluminium. In the mycobactins the hydroxyamino unit which gives rise to each of the hydroxamic acid groups is 2-amino-6-hydroxyaminohexanoic acid. There is thus a close parallel to the three 2-amino-5-hydroxyaminopentanoic acid units which are present in compounds of the ferrichrome type and the three 1-amino-5-hydroxyaminopentane units present in members of the ferrioxamine group. In the original isolation of mycobactin P good yields were obtained only when the organisms were grown on beef infusion broth. It now seems probable that the high yield and the occurrence of the factor in an iron-free form were due to the low content of ionized iron in the medium. Dr N. E. Morrison (personal communication) at the Johns Hopkins-Leonard Wood Memorial Laboratory, Baltimore, Md., U.S.A., has recently found that large amounts of mycobactin are produced by *M. phlei* growing on a synthetic medium containing less than 0.2 µg. of iron/ml. This experiment resembles observations of the influence of iron deficiency on the production of ferrichrome A by *Ustilago sphaerogena* (Garibaldi & Neilands, 1955). In both cases the increase in the production of iron-chelating compounds may be advantageous to the organism in trapping all the limited supply of iron in the vicinity. For demonstration of the growth-promoting action it is immaterial whether the mycobactin is added as the iron complex or in the metal-free form, since there are always sufficient traces of iron in the medium to convert the factor into the iron complex.

Since the iron complex is probably the active form of mycobactin P, its properties have been examined more closely. It has now been obtained crystalline and provides an alternative route for the purification of mycobactin. The structure proposed for mycobactin P (Snow, 1954b) contained some ambiguities. These have been resolved and the full stereochemistry is now known. This enables some speculations to be made about the structure of the iron complex.

RESULTS AND DISCUSSION

Structure of mycobactin P. Two possible structures were originally given for mycobactin P (Snow, 1954b). One was preferred on grounds of the relative rates of hydrolysis of the hydroxamic acid and amide links, and this structure also agreed better with the ultraviolet-absorption data, but conclusive proof was lacking. Proof has now been supplied by oxidation of mycobactin P with periodate, a reagent which specifically attacks the hydroxamic acid linkage (Emery & Neilands, 1960) by a reaction similar to that occurring with hydroxyamino compounds (Snow, 1954a). From this oxidation a nitrogen-free fatty acid was isolated and was shown to be mainly *cis*-octadec-2-enoic acid. Thus the fatty acid side chain must be attached by a hydroxamic acid link and not by an amide link. The fatty acid unit previously isolated from mycobactin by acid hydrolysis was *trans*-octadec-2-enoic acid. Hot mineral acid under the conditions used for hydrolysis has now been shown to isomerize the *cis* acid to an approximately equal mixture of *cis* and

trans acids. Presumably the isomerization is more complete when the fatty acid is liberated by hydrolysis of a hydroxamic acid linkage since no *cis* acid could be detected in unpurified hydrolysates of mycobactin P. It thus appears that the configuration of the side chain in the undegraded molecule is *cis*.

Examination of the methyl esters of the fatty acid-degradation products by vapour-phase chromatography showed that the main product was accompanied by three minor products (Table 1). The retention times of several reference compounds were measured under the same conditions. The C₁₂ to C₂₀ normal saturated acid methyl esters showed the usual rectilinear relationship between the number of carbon atoms and log retention time, and a similar relationship, though with a different slope, was shown for the C₁₄ to C₂₂ *trans*- Δ^2 -acid methyl esters. A comparison of the retention times of the esters of the minor components of the acid-hydrolysis product shows that one of the components could be the C₁₆ Δ^2 -acid, but the remaining components may not belong either to this series or to the normal saturated series. The product of periodate oxidation also showed three minor components. In a comparison of the groups of acids obtained by the two degradation methods, the ratios of the retention times of the methyl esters of corresponding members of the two series were approximately the same. It is thus likely that the products of periodate oxidation are all *cis* acids and that acid hydrolysis gives the corresponding *trans* acids. The mycobactins with these different side chains have closely similar physical properties, and no method has so far been found of separating them.

One acid-hydrolysis product of mycobactin P was (–)-3-hydroxy-2-methylpentanoic acid (Snow, 1954b). The absolute configuration of this compound has now been determined. Its conversion into the crystalline laevorotatory anilide of 2-methylpentanoic acid has already been described. This anilide has been compared with that prepared from (+)-2-methylpentanoic acid, which is known to have the S configuration (Stenhagen & Stållberg, 1947). Although the rotations do not agree exactly, there is no doubt that the product from mycobactin is the enantiomer and thus has the R configuration. Racemates of the two diastereoisomeric forms of 3-hydroxy-2-methylpentanoic acid were prepared by oxidation (method of Lemieux & Rudloff, 1955) of the two racemic forms of 3-methylhex-1-en-4-ol, the absolute configuration of which has been established by infrared spectroscopy (Sicher, Cherest, Gault & Felkin, 1963); these authors name the alcohols *erythro* and *threo* according to a defined structural analogy, and for convenience of description these terms have been extended here to the derived acids. The two acids were compared with

Table 1. Vapour-phase chromatography of methyl esters of fatty acids

The stationary phase was the succinic ester of diethylene glycol. The temperature was 227°. The values show retention times in minutes. The values in parentheses give approximate percentages of total effluent in each peak.

Parent compound ...	C ₁₂	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂
Saturated acids	1.1	2.2	3.9	7.4	13.8	
Δ^2 -Unsaturated acids		4.5	7.2	11.1	18.3	28.9
(<i>trans</i>)						
Δ^2 -Unsaturated acid (<i>cis</i>)				7.3		
Fatty acid from acid hydrolysis of mycobactin P		5.9	7.2	11.1	18.7	
		(2)	(4)	(84)	(10)	
Fatty acid from periodate oxidation of mycobactin P	2.8	3.8	7.1	10.1		
	(3)	(6)	(87)	(5)		
Acid-isomerization product of <i>cis</i> -octadec-2-enoic acid				7.2	11.1	
				(55)	(45)	

the hydroxy acid from mycobactin by infrared and nuclear-magnetic-resonance spectroscopy. The infrared spectrum of the degradation acid agreed with that of the *erythro* acid in showing strong peaks at 9.15 and 9.75 μ , which were absent from the spectrum of the *threo* acid; the latter showed a peak at 8.97 μ not found in the other spectra. In discussing the nuclear-magnetic-resonance spectra the assignation of protons to the different peaks is shown by letters:

The spectra for the degradation acid and the *erythro* acid were identical, showing a singlet at 2.5 τ (a), a multiplet at 6.15 τ (b), two quartets at 7.48 τ (c) and a multiplet from 8.5 to 9.2 τ (d, e) containing a doublet at 8.74 τ (f). The spectrum of the *threo* acid showed a singlet at 2.5 τ (a), a doublet of triplets at 6.35 τ (b), a sextet at 7.50 τ (c) and a multiplet from 8.5 to 9.2 τ (d, e) containing a doublet at 8.74 τ (f). In all three nuclear-magnetic-resonance spectra the integrations of the peaks agreed quantitatively with the numbers of protons assigned to them. The group of peaks associated with proton b occurs at lower field with the *erythro* acid than with the *threo*. Considering the population of rotational isomers, the *threo* acid would be expected to have the greatest proportion of the isomer in which the β -hydrogen atom is most shielded. Thus the mean shielding in the *threo* is probably greater than the *erythro*, in agreement with

the observed result. [3-Hydroxy-2-methylpentanoic acid prepared by the Reformatsky reaction has now been shown by infrared and nuclear-magnetic-resonance spectroscopy to contain equal proportions of the two diastereoisomers. When this mixture was used for preparing a *p*-bromophenacyl ester (Snow, 1954b) the derivative of the *threo* form crystallized out preferentially.] Since the relative configuration of the two asymmetric centres is thus established and the absolute configuration at the α -carbon atom is known, the configuration at the β -carbon atom must be S, giving the structure (I).

The absolute stereochemistry of the main component of mycobactin P is now fully known and is represented by (II).

Ferric mycobactin P. Mycobactin P dissolved in chloroform and treated with ethanolic ferric chloride gives a deep-purple solution containing the hydrochloride of the iron complex. Repeated washing with water hydrolyses the salt and leaves the reddish-brown iron complex in a chloride-free form. This complex can be purified by chromatography on alumina, and then crystallized from ethanolic solvents. The iron is removed by shaking a chloroform solution with 5 N-hydrochloric acid. This procedure forms a more convenient method of purifying mycobactin P than those previously available; it gives a product with a slightly higher extinction coefficient and optical rotation than material purified by other methods.

It now becomes possible to discuss the structure of ferric mycobactin P; for this purpose two assumptions have been made. First, in the iron chelation at the hydroxyphenyloxazoline centre the bonding to the iron atom is supposed to involve the phenolic

(I)

(II)

hydroxyl group and the nitrogen atom of the oxazoline ring; the possibility that the oxygen atom of the oxazoline ring could be engaged is regarded as much less likely since this would imply that the oxygen is more basic than the nitrogen. Secondly, the configuration of the seven-membered ring is assumed to be the staggered form in which the amide group occupies an equatorial position; the other staggered form and the two boat forms seem unlikely. Since all three chelating groups in mycobactin P are different and unsymmetrical, there are 16 different ways of arranging the chelating groups around the metal atom. However, the fact that the chelating groups are connected by a bifurcated chain limits the number of structures which are stereochemically possible. The structures have been investigated by the use of Leybold models. These models cannot exactly represent all the interatomic angles and distances but provide a useful guide; where a model cannot be made because of the awkward disposition of the groups the structure may reasonably be regarded as unlikely. Out of the 16 conceivable structures only eight can be made as models. These are indicated diagrammatically in Fig. 1, where the letters represent the chelating atoms as shown in (II). The models vary considerably in overall shape and in the closeness of packing of the atoms. All are roughly globular and have the rigidity that would be expected from a structure containing nine interlocking rings. The fatty side chain is flexible and can be packed into the irregularities of the core of the structure. The models in which compactness is combined most favourably with lack of strain and convenient disposition of groups are nos. 5, 6 and 7. In all the models which can be made the iron atom lies well to one side, so that three of the oxygen atoms which form part of its co-ordination shell are unobstructed by any other atoms in the core of the structure. Fig. 2 is a photograph of a model of

structure no. 7 without the side chain; the exposed oxygen atoms are clearly seen. Models of possible structures of ferrichrome show the iron atom to be in a similar situation. This molecular arrangement may be significant in relation to the mechanism of enzymic release of the iron atom from these firmly chelated compounds.

Biological activity of mycobactin and other sideramines. Some results are now available on the cross-activity between mycobactin and ferrichrome. Both substances promote the growth of *Arthrobacter* JG-9; for growth at half the maximum rate the concentrations required were: ferrichrome 0.3 $\mu\text{g./ml.}$, mycobactin 8 $\mu\text{g./ml.}$ (Burnham & Neilands, 1961). In these laboratories ferrichrome and ferrichrome A at concentrations up to 10 $\mu\text{g./ml.}$ and haemin at concentrations up to 100 $\mu\text{g./ml.}$ failed to promote the growth of *M. johnei* under conditions where mycobactin P produces an easily observable stimulation at 4 $\mu\text{g./ml.}$ The sideramines ferrichrome, ferrioxamine B and coprogen show considerable cross-over of activity between different organisms (Hesseltine *et al.* 1952; Burton, Sowden & Lochhead, 1954; Neilands, 1957; Bickel *et al.* 1960), and several organisms which require sideramines for growth will also grow if presented with a much higher concentration of haemin (Hesseltine *et al.* 1952; Burton *et al.* 1954; Demain & Hendlin, 1959). Thus, although the mycobactins may be reasonably classed as sideramines, they are much more specialized and are probably the only type of compounds that will act as iron-transport cofactors within the genus *Mycobacterium*.

Fig. 1. Structures of ferric mycobactin P for which models can be made. The iron atom lies at the centre of the plane and the co-ordinating atoms are lettered as in structure (II).

Fig. 2. Photograph of model of one possible structure of ferric mycobactin P without the side chain (corresponding to structure no. 7, Fig. 1) showing exposed oxygen atoms. Points of linkage to parts of the molecule invisible in the photograph are shown by broken lines.

EXPERIMENTAL

Ferric mycobactin P. Mycobactin P (1.00 g.) prepared as by Francis *et al.* (1953) was dissolved in CHCl_3 (50 ml.) and FeCl_3 (2.5 ml.; 10%, w/v, in methanol) was added. The dark-purple solution was washed with water (6×80 ml.) until the washings were not acidic, and evaporated to give a reddish-brown gum. This was stirred with methanol (4×30 ml.) and filtered, leaving brown insoluble gum (167 mg.). The filtrate was evaporated. The residue (934 mg.) was dissolved in benzene- CHCl_3 (9:1, v/v) and applied to a 2 cm. $\times$ 30 cm. column of neutral grade 1 alumina (M. Woehlm, Eschwege) suspended in the same solvent mixture. Development was carried out with a gradient device. A stirred cylindrical vessel feeding the column contained 400 ml. of the solvent mixture as above, and this was connected to a parallel cylindrical vessel, of half its cross-sectional area, containing 200 ml. of CHCl_3 -ethanol-benzene (1:1:8, by vol.). Several coloured bands separated, but the required product moved as an intensely red-brown band. The band reached the lower end of the column after passage of about 200 ml. of solution and was collected in the succeeding 50 ml. of effluent. After evaporation of solvent a red-brown gum (0.49 g.) remained. This was dissolved in methanol (12 ml.) and allowed to crystallize slowly at 0° , giving first crop (0.26 g.) and second crop (0.16 g.). The first-crop material was crystallized twice more from methanol, giving *ferric mycobactin P* of constant m.p. $199\text{--}201^\circ$ in brown hexagonal plates, λ_{max} 254 and $445 \text{ m}\mu$ ($10^{-3}\epsilon$ 21.8, 3.78 respectively) (Found: C, 60.9; H, 7.8; N, 7.5; Fe, 6.0. $\text{C}_{47}\text{H}_{72}\text{N}_5\text{O}_{10}\text{Fe}$ requires C, 61.1; H, 7.9; N, 7.6; Fe, 6.0%).

Conversion of crystalline ferric mycobactin P into mycobactin P. Crystalline ferric mycobactin P (693 mg.) was dissolved in CHCl_3 (250 ml.) and repeatedly washed with 5 N-HCl (6×100 ml.) until all colour was discharged from the CHCl_3 layer, which was then washed with water (3×300 ml.) and evaporated. The residue (665 mg.) was dissolved in warm CHCl_3 (10 ml.) and ether (70 ml.) was added with vigorous shaking. After 2 hr. the supernatant liquid was poured from the white gel, which was twice resuspended in ether (80 ml.) and decanted. The product was filtered off, washed with ether and dried, giving mycobactin P as an almost white powder (586 mg.), m.p. $165.5\text{--}166.5^\circ$, $[\alpha]_{\text{D}}^{25} -19.8^\circ$ (c 4.9 in CHCl_3), λ_{max} 250 and $311 \text{ m}\mu$ ($10^{-3}\epsilon$ 14.4 and 3.9 respectively).

Oxidation of mycobactin acid P with periodate. Mycobactin acid P (1.00 g.) was dissolved in ether (10 ml.). Sodium hydroxide soln. (12 ml.; 0.1 N) was added to give a turbid solution about pH 9. Air was blown over the shaken solution to remove most of the ether; 0.1 N- NaIO_4 (30 ml.) was added. After 1 hr. at 25° , 0.1 N-HCl (20 ml.) was added. The flocculent precipitate was extracted with CHCl_3 , the extract was washed, dried over Na_2SO_4 and evaporated to 5 ml., and light petroleum (b.p. $40\text{--}60^\circ$) was added. A flocculent brown precipitate (544 mg.) was filtered off and the filtrate was evaporated leaving a white waxy solid (166 mg.), which was mainly *cis*-octadec-2-enoic acid; the infrared spectrum was identical with that of the synthetic *cis* acid but differed from that of the *trans* acid. The main distinguishing peaks present in one spectrum and absent in the other were: *cis*, 12.16, 13.43 μ ; *trans*, 10.18 μ .

Isomerization of *cis*-octadec-2-enoic acid with mineral acid. *cis*-Octadec-2-enoic acid (48 mg.) was refluxed for 8 hr. with 5.5 N-HCl (5 ml.). The solution was cooled and extracted

with ether. The extract was dried over Na_2SO_4 and evaporated. The waxy residue gave an infrared spectrum showing features of both the parent *cis* acid and the corresponding *trans* acid. The nuclear-magnetic-resonance spectrum also indicated a mixture of approximately equal amounts of the two isomers. This was confirmed by vapour-phase chromatography of the methyl ester (Table 1).

***trans*- Δ^2 -Unsaturated fatty acids.** The C_{16} and C_{18} acids were made by standard methods via the α -bromo and α -iodo compounds. The palmitic acid used in preparation of the C_{16} acid contained some myristic acid which gave rise to the corresponding C_{14} Δ^2 -acid in the product. Arachidic acid containing 2% of stearic acid and 23% of behenic acid treated similarly gave a product, m.p. 67° , equiv.wt. 315, which was mainly *trans*-eicos-2-enoic acid but contained the expected proportions of the corresponding C_{18} and C_{22} acids.

Vapour-phase chromatography of long-chain acids. The fatty acid fractions were converted into methyl esters by treatment with a slight excess of ethereal diazomethane; 1–2 μg . of ester dissolved in 2–4 μl . of ether was applied to column P (stationary phase, succinic ester of diethylene glycol) on a Perkin-Elmer Fractometer; conditions were: temperature 227° , carrier gas hydrogen 60 ml./min., thermal conductivity detector.

(+)-2-Methylpentanoanilide. (+)-2-Methylpentanoic acid (having $[\alpha]_{\text{D}}^{16} +17.0^\circ$, homogeneous) was converted into the *anilide*, which was distilled (0.03 mm., bath temp. 120°) and crystallized from hexane; m.p. $92.5\text{--}93.5^\circ$. Optical rotations of methanolic solutions at 20° were measured at different wavelengths by Professor W. Klyne: (+)-anilide (2.3 mg./ml.), λ 435, 400, 333: $\phi +54^\circ$, $+66^\circ$, $+110^\circ$; (–)-anilide from mycobactin degradation (1.7 mg./ml.), $\phi -55^\circ$, -77° , -184° .

Oxidation of erythro- and threo-3-methylhex-1-en-4-ol to the corresponding 3-hydroxy-2-methylpentanoic acids. Each 3-methylhex-1-en-4-ol (61 mg.) was dissolved in 1.71% (w/v) NaIO_4 sol. (55 ml.), 5% (w/v) K_2CO_3 solution (2 ml.) was added and then 10 N-NaOH (0.25 ml.) to bring it to pH 7.5; 2% (w/v) KMnO_4 solution (0.55 ml.) was added. After 1 hr. at 25° conc. H_2SO_4 (3 ml.) was added and the solution was continuously extracted with ether for 20 hr. The ether solution (150 ml.) was shaken with 0.1 N-NaOH solution (10.4 ml.), giving an aqueous layer of pH 8.5. The ether layer was further washed with water (3×2 ml.). The combined aqueous extract was concentrated to a syrup (1.2 ml.), acidified with conc. HCl (0.25 ml.) and extracted with ether (5×5 ml.). The ether extract was dried over Na_2SO_4 , evaporated and distilled (0.02 mm., bath temp. 105°). Each product (*erythro* 53 mg., *threo* 48 mg.; viscous oils) was compared by infrared and nuclear-magnetic-resonance spectroscopy with (–)-3-hydroxy-2-methylpentanoic acid obtained by degradation of mycobactin P.

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