Isolation and Structure of Mycobactin T, a Growth Factor from *Mycobacterium tuberculosis*

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1. A growth factor for *Mycobacterium johnei* has been isolated from *Mycobacterium tuberculosis* as an iron complex; it has been named ferric mycobactin T. 2. Ferric mycobactin T as isolated is a mixture of at least four closely related components differing only in their fatty acid side chains. 3. Mycobactin T resembles mycobactin P in structure but differs in the following respects: (a) the aromatic acid residue is salicylic acid in place of 2-hydroxy-6-methylbenzoic acid; (b) the hydroxy acid residue is (+)- β -hydroxybutyric acid in place of (-)-3-hydroxy-2-methylpentanoic acid; (c) its components have fatty acid side chains that are mostly different from the side chains of the components in mycobactin P preparations. One minor component in mycobactin T has probably a *cis*-octadec-2-enoyl side chain. The major side-chain components correspond to acids of chain length C₁₈–C₂₁ that are not *n*-saturated or *n*- Δ^2 -unsaturated acids. 4. The differences in biosynthesis between mycobactins T and P are discussed.

The first evidence that the mycobacteria contained special and characteristic growth factors was the observation by Twort & Ingram (1912) that *Mycobacterium johnei*, which failed to grow on laboratory media, would grow when the dried killed cells of *M. tuberculosis* (human strain) were added to the medium. These workers afterwards found that other species of mycobacteria and extracts from them also acted as growth promoters for *M. johnei*. Francis, Macturk, Madinaveitia & Snow (1953) selected *M. phlei* as a convenient source of growth factor and devised a method of isolation. The structure of this factor, mycobactin P, was established (Snow, 1965); its most notable chemical characteristic was its ability to form very stable binary complexes with iron and with aluminium. Evidence of the biological function of other iron-chelating agents, such as the ferri-chromes and the ferrioxamines, suggested that ferric mycobactin P played a similar biochemical role in *M. phlei*, and was probably concerned in the transfer of iron to enzymes involved in haem biosynthesis, though it stood somewhat apart from the other sideramines both chemically and in its biological specificity (Snow, 1965). Among micro-organisms of many different types, the ability to produce compounds stimulating the growth of *M. johnei* has been found only in the mycobacteria. *M. tuberculosis* is of outstanding importance within this genus and knowledge of the structure of

its specific growth factor could be relevant to the chemotherapy of tuberculosis. The present paper reports the isolation and characterization of this factor, which has been named mycobactin T; its structure in general resembles that of mycobactin P, but differs from it at three points in the molecule.

RESULTS

Fractions referred to by numbers and letters correspond to those described in the Experimental section and Tables.

M. tuberculosis grown on the usual media produces very small amounts of mycobactin T. To obtain workable yields of the factor the organism was grown on a glycerol-beef-infusion medium similar to that used for growing *M. phlei* for mycobactin P production. This medium supported the growth of *M. tuberculosis* only poorly. Recent evidence suggests that stimulation of growth-factor production may depend on the presence of a very limited supply of iron in the medium (see Snow, 1965).

The early stages in the purification of mycobactin T were facilitated by an improved biological assay (D. W. F. Wheeler & G. A. Snow, unpublished work). The extraction method used for mycobactin P failed when applied to *M. tuberculosis* because the growth factor was present entirely as the iron

Scheme 1. Extraction of mycobactin T from *M. tuberculosis*.

complex. A different procedure was therefore used, in which the factor was kept in the ferric form throughout. As purification proceeded the amount of growth factor could be measured spectrophotometrically by its characteristic absorption band at $450\text{m}\mu$, a correction being made where necessary for other coloured substances by measuring their absorption after dissociating the iron complex with strong acid.

The first stages of extraction and concentration of mycobactin T by solvent partition are summarized in Scheme 1. The concentrate (fraction H) (0.7% by wt. of the dried mycobacteria from which it was obtained) contained about 2% (w/w) of mycobactin T. Subsequent purification was by adsorption chromatography on alumina in four main stages by using varying conditions (Scheme 2). Adsorption chromatography on silica gel was also practicable but less useful. Each chromatographic separation of mycobactin T on alumina led to some loss through irreversible adsorption, most noticeably in the final stages. The final product (fraction X) was not crystalline, but showed $E_{1\text{cm}}^{1\%}$ at $450\text{m}\mu$ 42.1, which could not be raised by further chromatography and which slightly exceeded the $E_{1\text{cm}}^{1\%}$ for crystalline ferric mycobactin P (40.9). This fact,

coupled with the behaviour of the product on degradation and the properties of its derivatives, indicated that the compound was substantially free from impurities.

The absorption spectrum of ferric mycobactin T (Fig. 1) differed from that of ferric mycobactin P in details at lower wavelengths.

Ferric mycobactin T, like the P compound, dissociated in strong mineral acids giving a metal-free product that had an absorption spectrum similar to that of mycobactin P but shifted to lower wavelengths (Fig. 2). This suggested that mycobactin T resembled mycobactin P in possessing an *o*-hydroxyphenyloxazoline structure but with some difference in substitution. The spectrum corresponded closely to that of an equimolar mixture of 2-*o*-hydroxyphenyloxazoline and *N*-ethyloctadec-2-enohydroxamic acid (cf. structural considerations for mycobactin P; Snow, 1954b). The physical and chemical properties of mycobactin T all suggested a close resemblance to mycobactin P. Both formed aluminium complexes with similar absorption spectra, the peaks of mycobactin T (Fig. 1) being at lower wavelengths than those of mycobactin P. The growth-promoting properties of mycobactins T and P towards *M. johnei* were

Scheme 2. Purification of mycobactin T by chromatography. The values in square brackets show $E_{1\text{cm}}^{1\%}$ at 450m μ due to iron-chelating compounds in the fraction. All chromatography was on alumina; successive fractions emerging from the columns are shown in order from left to right.

quantitatively similar (D. W. F. Wheeler & G. A. Snow, unpublished work).

Degradation studies. Because of the scarcity of material, mycobactin T was not further characterized, but instead the crucial degradations that had been used to establish the structure of mycobactin P were repeated on mycobactin T. The degradative behaviour of the two factors was very similar, showing that the general pattern of their molecular structure was the same. Mild alkaline hydrolysis split mycobactin T into two fragments, which were named by analogy cobactin T and mycobactinic acid T. Cobactin T, a hydroxamic acid, crystallized much less readily than cobactin P and differed from it chromatographically; on acid hydrolysis it gave the same reducing amino acid, 2-amino-6-hydroxyaminohexanoic acid, as cobactin P, but a different hydroxy acid, which was identified as (+)- β -hydroxybutyric acid. This acid has the

same absolute configuration (*S*) (Lemieux & Giguere, 1951) at the β -carbon atom as the (-)-3-hydroxy-2-methylpentanoic acid obtained in the degradation of cobactin P (Snow, 1965). The structure of cobactin T is thus (I).

Mycobactinic acid T, a crude gum like its P analogue, was hydrolysed with boiling acid to give a mixture of amino acids and acidic ether-extractable products. The amino acids were shown by paper chromatography and electrophoresis to be serine and 2-amino-6-hydroxyaminohexanoic acid together with a little lysine, the same products as from mycobactinic acid P. The ether-soluble fraction was separated into two parts by column chromatography. One part was salicylic acid; the corresponding product from mycobactinic acid P was 2-hydroxy-6-methylbenzoic acid, which became decarboxylated to *m*-cresol under the conditions of acid hydrolysis. The second part (fraction III)

Fig. 1. Absorption spectra of metal complexes of mycobactins. —, Ferric mycobactin T; ----, ferric mycobactin P;, aluminium mycobactin T. All were measured as solutions in methanol.

Fig. 2. Absorption spectra. —, Mycobactin T (assuming mol. wt. 856); - · - · - ·, 2-(*o*-hydroxyphenyl)oxazoline; · · · · ·, ethyl-*trans*-octadec-2-enohydroxamic acid; ----, arithmetic sum of curves for 2-(*o*-hydroxyphenyl)oxazoline and ethyl-*trans*-octadec-2-enohydroxamic acid; - - - - -, mycobactin P. All were measured as solutions in methanol.

(I) Cobactin T.

contained a mixture of long-chain fatty acids. Fraction *III* was crystallized to give *IIIA*, still a mixture.

Previous experience with the degradation of mycobactin P had shown that acid hydrolysis brought about *cis*-to-*trans* isomerization of the $\alpha\beta$ -double bond in the side chain, whereas oxidation with periodate gave the *cis* acids without isomerization. Mycobactin acid T was therefore subjected to periodate oxidation and the fatty acid fraction isolated (fraction *VI*). The mixtures from both degradation procedures were compared with *trans*- and *cis*-octadec-2-enoic acid, the principal products from acid hydrolysis and periodate oxidation respectively of mycobactin P.

The infrared-absorption bands (Table 1) for fractions *VI* and *IIIA* were very similar, and showed many similarities to the bands in *cis*- and *trans*-octadec-2-enoic acid. In particular they both showed a band about 6.1μ , indicating the presence of Δ^2 -unsaturation. However, with fractions *IIIA* and *VI* this band was weak relative to the intense band given by the reference acids. This suggested that only part of the acids in the mixture had Δ^2 -unsaturation. There was also some evidence of a difference of geometrical isomers in the two mixtures: fraction *VI* but not fraction *IIIA* showed bands at 6.90 and 12.13μ , which agreed with strong bands given by the *cis* reference acid but not by the *trans* acid; fraction *IIIA* showed a well-marked band at 10.33μ that was very weak in fraction *VI*; this band is considered to be characteristic of the *trans* structure and was represented in the spectrum of *trans*-octadec-2-enoic acid by a strong band at 10.20μ not found with the *cis* isomer. The infrared evidence thus suggested that some of the side-chain acyl groups had the *cis*- Δ^2 -unsaturated structure and were isomerized by acid treatment, but otherwise the mixture obtained by acid hydrolysis seemed to have a very similar composition to that given by periodate oxidation. The fractions examined by infrared spectroscopy together with fraction *IIIB*, the residue from the mother liquors of the crystallization of fraction *III*, were converted into methyl esters, which were examined by vapour-phase chromatography (Table 2). The main comparison is between fractions *VI* and *IIIB*, since the latter comprised 84% of the total product obtained by

Table 1. *Infrared-absorption bands of fatty acids from mycobactin T*

Fraction *VI* is the product from periodate oxidation; fraction *IIIA* is the crystalline product from acid hydrolysis. Strong bands are shown in **bold type**, weak bands or inflexions in parentheses.

	Wavelength (μ)											
Fraction <i>VI</i>	3.43	3.52	5.90	6.11	6.84	6.90	(6.99)	(7.10)	7.27	7.70	7.77	
Fraction <i>IIIA</i>	3.48	3.55	5.95	6.07	6.84			7.05	(7.31)	7.72	7.82	(7.92)
<i>cis</i> - Δ^2 -C ₁₈ acid	3.43	3.52	5.89	6.04	6.80	6.95		(7.08)	7.25	(7.72)	7.90	7.96
<i>trans</i> - Δ^2 -C ₁₈ acid	3.43	3.51	5.91	6.12	6.81			7.05	7.50		7.80	7.93

	Wavelength (μ)											
Fraction <i>VI</i>	8.03	8.13	8.23	8.35	8.46	(10.33)	10.68			(12.13)	13.75	13.92
Fraction <i>IIIA</i>	8.03	8.14	8.25	8.37	8.47	10.33	10.70				(13.80)	13.93
<i>cis</i> - Δ^2 -C ₁₈ acid	8.07	8.16	(8.26)				10.80	11.30	11.82	12.16	(12.43)	13.43
<i>trans</i> - Δ^2 -C ₁₈ acid	8.05	8.17	8.30				10.20	11.36	11.63	11.93	(13.50)	13.90

Table 2. *Vapour-phase chromatography of methyl esters of fatty acids*

The stationary phase was the succinic ester of diethylene glycol. The temperature was 227°. Fraction *VI* is the periodate-oxidation product of mycobactin acid T; fraction *III* is the acid-hydrolysis product of mycobactin acid T (*A*, crystals; *B*, mother liquors).

Peak	Retention time (min.)	Composition (% of total effluent)			Retention times of reference acids (min.)		
		Fraction <i>VI</i>	Fraction <i>IIIB</i>	Fraction <i>IIIA</i>	<i>trans</i> - Δ^2 -Unsaturated acids	<i>cis</i> - Δ^2 -Unsaturated acids	<i>n</i> -Saturated acids
<i>a</i>	4.0	5	1				C ₁₆ 3.9
<i>b</i>	7.3	15	10	5	C ₁₄ 4.5		
	9.8			3	C ₁₆ 7.2	C ₁₈ 7.3	C ₁₈ 7.4
<i>c</i>	11.3		10	5	C ₁₈ 11.2		
<i>d</i>	13.3	70	40	30			
	15.2			2			C ₂₀ 13.8
<i>e</i>	20.3	10	40	55	C ₂₀ 18.3		
					C ₂₂ 28.9		C ₂₂ 24.5

acid hydrolysis. The pattern of peaks was similar in the two mixtures except that peak *c* was only found in the chromatogram from fraction *IIIB*, and the relative sizes of corresponding peaks differed in the two mixtures. Peak *c* showed the same retention time as methyl *trans*-octadec-2-enoate, and there was a substantial peak *b* in the chromatogram from fraction *VI* that had a retention time corresponding to the *cis* ester. The *cis*-octadec-2-enoyl side chain, which occurs in the major component of mycobactin P preparations, was thus apparently present in a minor component of mycobactin T. It was not certain whether this component by itself could account for all the Δ^2 -unsaturation revealed by the infrared spectra, or whether there was a contribution

from other components. The interpretation of the other features of the vapour-phase chromatograms was somewhat doubtful. In the chromatogram from fraction *VI*, peak *d* predominated, but in that from fraction *IIIB* the distribution between peaks *d* and *e* was more nearly equal. This might indicate that here also isomerization occurred during acid hydrolysis, and that part of the material represented by peak *d* was converted into a product running as peak *e*. The retention times of the main peaks *d* and *e* did not correspond to those of the *trans*- Δ^2 -unsaturated series or to the *n*-saturated series of methyl esters, but would be consonant with chain lengths of between C₁₈ and C₂₁. The crystalline product *IIIA* was a mixture somewhat enriched

(II) Mycobactin T (where R is variable, consisting of an alkyl chain of length $C_{14}-C_{21}$. In major components the structure of R is undetermined; in one minor component it is probably *cis*- $CH=CH-[CH_2]_{14}-CH_3$).

(III) Mycobactin P (main component)

in the longer-chain components. Its chromatogram showed two very small components not seen in the other mixtures.

Mycobactin T as isolated thus consisted of a mixture of a number of closely related chemical entities represented by (II). The stereochemistry of asymmetric centres, apart from the one indicated, has not been determined, but is presumably identical with that of mycobactin P (III).

DISCUSSION

The isolation and characterization of the long-sought growth factor from *M. tuberculosis* supports the view that the mycobactins form a distinct family of sideramines peculiar to the mycobacteria. Both *M. tuberculosis* and *M. phlei* produce groups of closely related compounds; the two groups differ substantially from each other but are recognizably of the same chemical type. Other mycobacteria may well produce other compounds of the same general type. The potency of mycobactins P and T in promoting the growth of *M. johnei* is almost identical. The ferrichromes provide a parallel in the occurrence of a number of closely related

sideramines of similar potency, and in this case, too, each species of micro-organism produces its own characteristic group of sideramines (Zähner, Keller-Schierlein, Hütter & Deér, 1963). In the other two main groups of sideramines, naturally occurring competitive inhibitors of related structure have been found: e.g. albomycin, related to ferrichrome (Mikeš, Turková & Šorm, 1963), and ferrimycin A₁, related to the ferrioxamines (Prelog, 1964). So far no corresponding antibiotic related to the mycobactins has been found; if such a compound were discovered it should have a specific action against mycobacteria. Mycobactin T was found in *M. tuberculosis* as its ether-soluble iron complex. This explains the results of Marks (1954), who found that the growth of inocula of *M. tuberculosis* on unfavourable media was stimulated both by mycobactin and by ether extracts of *M. tuberculosis*; these findings were hitherto puzzling because metal-free mycobactin P is not extractable with ether. Marks's (1954) growth factor was evidently ferric mycobactin T.

In considering the chemical divergence between mycobactins T and P, the difference in the fatty acid side chain may be compared with the differences

found in the aliphatic side chains of other pairs of chemically related compounds isolated from *M. tuberculosis* and from *M. phlei*, such as the two different members of the vitamin K group (Noll, Rüegg, Gloor, Ryser & Isler, 1960; Gale *et al.* 1963) and the two types of mycolic acid (Lederer, 1960). Analogy with other micro-organisms suggests that the β -hydroxybutyric acid fragment in mycobactin T is derived from the condensation of two acetyl units. Probably, as suggested by Asselineau (1962), the corresponding acid, 3-hydroxy-2-methylvaleric acid, from mycobactin P comes from the condensation of two propionic acid units. Salicylic acid is rarely found in micro-organisms. It has been reported in *Penicillium griseofulvum* (Simonart & Wiaux, 1959), but otherwise only in mycobacteria. Its occurrence in *M. tuberculosis* (Stendal, 1934; Ratledge & Winder, 1962) therefore suggests that it may be a precursor in the biosynthesis of mycobactin T. The corresponding precursor for mycobactin P is presumably 2-hydroxy-6-methylbenzoic acid. The biosynthesis of this acid in *P. urticae* has been shown to involve the condensation of one acetate and three malonate units (Lynen & Tada, 1961; Birch, Cassera & Rickards, 1961; Bu'Lock, Smalley & Smith, 1962), and this mechanism is probably common to other micro-organisms. In isolated plant tissues salicylic acid has been shown to be formed from phenylalanine and from benzoic acid (Klämbt, 1962; El-Basyouni, Chen, Ibrahim, Neish & Towers, 1964). This type of biosynthesis from phenylalanine, probably involving cinnamic acid as an intermediate, does not seem to be characteristic of micro-organisms, though it cannot be excluded as a possibility. Another route to salicylic acid would be by elimination of the methyl group from 2-hydroxy-6-methylbenzoic acid. Evidence of oxidation of this methyl group, first to the aldehyde and then to the carboxylic acid, has been found in *P. urticae* (Bassett & Tanenbaum, 1958; Tanenbaum & Bassett, 1958). The product, 3-hydroxyphthalic acid, is synthesized from acetate by *P. islandicum* (Gatenbeck, 1958), though evidence suggests that it is formed in this organism not from 2-hydroxy-6-methylbenzoic acid but by oxidation of islandicin (Gatenbeck, 1960). However, the enzymic decarboxylation of 3-hydroxyphthalic acid to salicylic acid has not been demonstrated experimentally; chemical decarboxylation yields *m*-hydroxybenzoic acid. Ratledge (1964) suggested that salicylic acid is formed from shikimic acid by reactions analogous to those leading to anthranilic acid. He showed that when *M. smegmatis* was grown on media in which quinic acid or shikimic acid formed a considerable part of the carbon source the production of salicylic acid was enhanced, and at the same time anthranilic acid, 3,4-dihydroxybenzoic acid,

p-hydroxybenzoic acid and *p*-hydroxyphenylacetic acid were formed. He also suggested that the pathway to salicylic acid in *M. smegmatis* may be closely related to that which leads to 2,3-dihydroxybenzoic acid in other micro-organisms. The argument in favour of such a relationship was that iron deficiency increases the output of both salicylic acid by *M. smegmatis* and 2,3-dihydroxybenzoic acid by *Aerobacter aerogenes*. However, Ito & Neilands (1958), who observed an increased production of 2,3-dihydroxybenzoylglycine by *Bacillus subtilis* during iron deficiency, attributed the effect to the need for the organism to produce a chelating agent that would trap and make available any iron in the vicinity. Lack of iron frequently causes an increase in formation of iron-chelating agents by micro-organisms; production of ferriochrome, ferrioxamines and mycobactin is stimulated in this way. The response is thus not related to any single biosynthetic pathway. The origin of salicylic acid in *M. tuberculosis* still remains to be established with certainty. If the aromatic moiety in mycobactin T is shown to be derived from shikimic acid whereas that in mycobactin P comes from acetate and malonate, it will provide an interesting example of a common biological need being met in different ways by two species within the same genus.

EXPERIMENTAL

Determination of the ferric mycobactin content of partly purified concentrates by extinction measurements. The extinction of the solution of the concentrate in CHCl_3 at 450 μ was measured directly and again after it had been shaken with an equal volume of 5.5N-HCl and clarified by centrifugation. The difference between the two extinction measurements was used to calculate the $E_{450}^{1\%}$ of the sample attributable to iron-chelating compounds. In the more purified preparations (fraction N onwards) the whole extinction at 450 μ was due to ferric mycobactin and the acid-extraction step could be omitted.

Growth of M. tuberculosis and extraction of ferric mycobactin T. A beef-infusion broth was prepared containing 2% (w/v) of peptone and 5% (w/v) of glycerol. Portions (600 ml.) were transferred to a series of plugged Winchester bottles and sterilized. The bottles were stacked on their sides and pellicles from an established colony of *M. tuberculosis* H37_{av} were floated on to the surface with a wire loop. The cultures were incubated for 4–6 weeks at 37° and then autoclaved to kill the organisms; growth was often slow and sparse. The pellicles were filtered off, well washed with water, sucked as dry as possible and transferred to methanol (750 ml./kg. of moist filter cake). The suspension was well stirred, and stored until it was convenient to carry out further extraction. The extraction procedure is outlined in Scheme 1, which shows yields at the different stages. The methanol suspension [containing about 50% (v/v) of water in methanol] was spun on a basket centrifuge; the supernatant solution was evaporated under reduced pressure to a thick brown syrup (fraction A). The moist residue was again stirred with methanol (1.6 l./kg.) for 1 hr. and centrifuged.

The supernatant [containing about 16% (v/v) of water in methanol] was evaporated under reduced pressure to a brown gum (fraction B). The residue was spread on trays, dried in a stream of air and ground to a fine powder in a vibration energy mill packed with porcelain pellets. The powder (fraction C) was washed out of the mill with ether, and the suspension (4.5 l. of ether/kg. of fraction C) was stirred for $\frac{1}{2}$ hr. and allowed to settle. The supernatant was siphoned off and the residue was extracted twice more with similar volumes of ether. The final residue was dried in air (fraction D). The combined ether extract, which was sometimes turbid with very fine suspended matter, was concentrated to one-twentieth of the original volume, and the concentrate was mixed with acetone (4 vol.). Some waxy material was precipitated and any suspended matter still present in the ether concentrate was carried down. The precipitate was removed by centrifuging and was washed by resuspension in acetone and dried (fraction E). The combined supernatants were evaporated to give a brown wax (fraction F).

Fraction F (50 g.) was dissolved in light petroleum (b.p. 40–60°) (2.5 l.) and repeatedly extracted with ethanol containing 30% (v/v) of water (3 × 2.5 l.). The light-petroleum layer on evaporation gave a golden-brown wax (fraction G). The aqueous ethanolic extracts were combined and evaporated under reduced pressure. The residue was dissolved in light petroleum–CHCl₃ (1:1, v/v) and washed with water to remove a little syrupy material and the solution was again evaporated giving a deep-red-brown pasty residue (fraction H).

Purification of ferric mycobactin T by chromatography. The description gives weights and volumes for single columns at each stage. Separations were each carried out twice or more and the combined yields for several columns are given in Scheme 2.

Stage 1. Fraction H (8 g.) was dissolved in benzene (100 ml.) and applied to a column (4.5 cm. × 18 cm.) of alumina (Spence grade 0) (280 g.) suspended in benzene, and the chromatogram was developed with benzene containing 2% (v/v) of methanol. Several bands separated; the required product was in the most prominent deep-red-brown band. The eluate emerging before this band (880 ml.) on evaporation gave fraction I. The eluate containing the red-brown band (420 ml.) gave fraction J and the column was then eluted with 500 ml. of methanol–CHCl₃ (3:2, v/v) to give fraction K.

Stage 2. Fraction J (1 g.) was dissolved in benzene–CHCl₃ (9:1, v/v) (10 ml.) and applied to a thin layer of alumina (2 g.) in a sintered funnel; a dark rubbery material was left on the surface of the adsorbent. The alumina was washed with the same solvent and the combined filtrate was evaporated, giving fraction L. Alumina (acid-washed, grade 1; Woehlm, Eschwege, Germany) was suspended in benzene–CHCl₃ (9:1, v/v) to give a column 0.35 cm. × 30 cm. Fraction L (270 mg.) dissolved in solvent (1 ml.) was applied and development was continued with the same solvent; 1 ml. fractions were collected and evaporated, and $E_{1\text{cm}}^{1\%}$ at 450 mμ was determined for each; fractions of similar purity were combined and evaporated. Successive combined fractions and corresponding volumes of eluate were: fraction M, yellow oil (4.5 ml.); fraction N, deep-red-brown gum (7 ml.); fraction O, red-brown gum (8 ml.); fraction P, red-brown gum (9 ml.). The column was finally washed with benzene–CHCl₃–methanol mixture (7:1:2, by vol.)

(40 ml.), and the eluate evaporated to give fraction Q, as a light-brown wax.

Stage 3. Another column was prepared exactly as in stage 2 except that the solvent was benzene–CHCl₃ (39:1, v/v). Fraction N (89 mg.) was dissolved in the solvent (1 ml.) and applied to the column. Development was begun by running through 17.5 ml. of the same solvent. The front of the main red-brown band was then 2–3 cm. from the lower end of the column; the eluate on evaporation yielded fraction R as a yellow oil. Gradient elution was then started. The column was fed from a stirred cylindrical vessel containing 70 ml. of the same solvent; attached to this was a parallel cylindrical feeder vessel of half the cross-sectional area containing 35 ml. of benzene–CHCl₃–methanol (191:5:4, by vol.); 1 ml. fractions were collected and combined on the same principle as before; successive combined fractions came from the following volumes of eluate (some evaporation of solvent occurred during collection): fraction S, 2 ml.; fraction T, 3 ml.; fraction U, 10 ml.; fraction V, 11 ml. All gave red-brown resinous gums on evaporation. The column was washed with 15 ml. of benzene–CHCl₃–methanol (35:1:4, by vol.); this eluate on evaporation gave fraction W (red-brown gum).

Stage 4. Fraction U (41 mg.) was again submitted to gradient chromatography under conditions identical with stage 3 except that the column dimensions were 0.25 cm. × 28 cm. and the volumes of the gradient solutions were 50 ml. and 25 ml. Two combined fractions X and Y were obtained, both deep-red-brown gums.

Fraction X represented the purest *ferric mycobactin T* that could be obtained. It had λ_{max} 450 mμ, with inflexions at 276 and 245 mμ ($E_{1\text{cm}}^{1\%}$ 42.1, 137 and 250 respectively), and the iron content was 5.8%. The full absorption spectrum is shown in Fig. 1.

Preparation of mycobactin T from its ferric complex. Ferric mycobactin T (48 mg.) was dissolved in CHCl₃ (25 ml.) and shaken with 5 N-HCl (2 × 25 ml.); the CHCl₃ solution, now colourless, was washed repeatedly with water until the washings were not acidic, then clarified and evaporated. The residue was treated with dry ether (2.5 ml.); it dissolved, but slowly a white gelatinous precipitate separated from solution. The suspension was kept for 20 hr. at 0° and then centrifuged; the residue was washed twice by resuspension in cold ether and was finally dried in a vacuum desiccator, giving *mycobactin T* (36 mg.) as an almost white powder, m.p. 128–129.5°, $[\alpha]_D^{25}$ –13.1 ± 1° (c 2.1 in CHCl₃), λ_{max} 242, 249 and 304 mμ ($E_{1\text{cm}}^{1\%}$ 156, 153 and 56 respectively) and an inflexion at 258 mμ (full absorption spectrum in Fig. 2). The solid showed an apple-green fluorescence in ultraviolet light.

Preparation of aluminium mycobactin T. Mycobactin T (1.26 mg.) was dissolved in butanol (0.2 ml.), and AlCl₃ solution (0.02 ml., 2% in ethanol) was added. After dilution with 0.5 ml. of butanol the solution was washed with water (4 × 0.5 ml.) until the washings were not acidic. The butanol was evaporated under reduced pressure and the residue dried at 100° *in vacuo*, to give *aluminium mycobactin T* (1.28 mg.). The solid and its methanolic solutions showed an intense purple fluorescence. The ultraviolet spectrum is shown in Fig. 1.

Scission of mycobactin T in alkaline solution. Mycobactin T (28.0 mg.) was dissolved in methanol (0.2 ml.), and N-NaOH (1 ml.) was added. After 1 hr. at 20° the solution was neutralized with N-HCl (1 ml.). The yellowish flocculent

Table 3. *Paper chromatography and paper electrophoresis of water-soluble products from acid hydrolysis of mycobactic acid T and cobactin T*

The results show R_F values for paper chromatograms and distance travelled (in cm.) for paper electrophoretograms of spots revealed by ninhydrin. Values in *italics* indicate spots also reacting with triphenyltetrazolium chloride and alkali.

Separation method	Product I from mycobactic acid T	Product V from cobactin T	Lysine	Serine	2-Amino- 6-hydroxyamino- hexanoic acid
Paper chromatography (ethyl methyl ketone-propionic acid-water)	0.20 (small) 0.27 0.43	(0.21, trace) 0.43	0.20	0.27	0.43
Paper electrophoresis at pH 2 (3.4 kv for 30 min.)	10.9 17.7 18.9 (small)	17.5 (19.0, trace)	19.3	11.0	17.4
Paper electrophoresis at pH 5 (6.4 kv for 30 min.)	8.3 29.0 34.0 (small)	28.8 (34.6, trace)	34.5	8.5	28.7

precipitate was extracted out with ether (3 × 3 ml.). The ether extract was washed, dried over $MgSO_4$ and evaporated, giving crude *mycobactic acid T* (21.2 mg.) as a yellowish gum, λ_{max} , 243, 249 and 304 $m\mu$ ($E_{1\%}^{1cm}$, 191, 192 and 72 respectively).

The aqueous layer from the ether extraction was evaporated and the residue was powdered and extracted repeatedly with boiling acetone (3 × 8 ml.). The extracts were filtered and evaporated to give crude *cobactin T* (9.6 mg.), which slowly crystallized. It was compared with cobactin P (the cobactin from mycobactin P) by thin-layer chromatography on silica-gel plates by using water for development (R_F values: cobactin T, 0.42; cobactin P, 0.35), and by paper electrophoresis in aq. *m*-diethylamine buffer for 90 min. at 3.5 kv (distance of migration: cobactin T, 9.7 cm.; cobactin P, 8.0 cm.; in a mixture there was a clear separation of the spots). For these tests the spots were revealed by spraying with ethanolic $FeCl_3$.

Acid hydrolysis of mycobactic acid T. Crude mycobactic acid T (21.2 mg.) was refluxed for 6 hr. with 5*N*-HCl (3 ml.); the solution was cooled and extracted with ether (3 × 4 ml.). The aqueous layer was evaporated to give a pale-brownish gum (fraction I) (16.1 mg.). The ether extract was washed, dried and evaporated to a crystalline solid (fraction II) (15.8 mg.). Silica gel (Mallinckrodt) was ground with water (0.4 ml./g.), suspended in cyclohexane saturated with water and used to prepare a column 26 cm. × 0.5 cm.

Product II was dissolved in ether (0.2 ml.) and the solution was adsorbed on dry silica gel (0.2 g.). After evaporation of the ether the dry solid was allowed to settle on the top of the prepared column. For gradient elution the column was fed from a stirred cylindrical vessel containing cyclohexane (50 ml.); attached to this was a smaller parallel cylindrical feeder vessel containing ether-cyclohexane (1:1, v/v) (30 ml.). Fractions were collected and combined to give two clearly separated products: fraction III (9.0 mg.), a waxy solid, and fraction IV (2.3 mg.), a white crystalline solid (λ_{max} in methanol 236 and 305 $m\mu$) that gave a violet colour with $FeCl_3$; its infrared spectrum in a KBr disk showed it to be salicylic acid.

Product III was crystallized from light petroleum

(b.p. 40–60°) at –15° and then from methanol at –10° to give a white crystalline solid (fraction IIIA) (1.5 mg.), m.p. 61.5–62°; the combined mother liquors were evaporated, giving a waxy residue (fraction IIIB). The infrared spectrum of fraction IIIA was recorded; fractions IIIA and IIIB were examined by vapour-phase chromatography (see below).

Acid hydrolysis of cobactin T. Crude cobactin T (9.6 mg.) was refluxed for 6 hr. with 5*N*-HCl (1 ml.); the solution was cooled and extracted with ether (5 × 2 ml.). The ether extract was washed with 5*N*-HCl (0.3 ml.) and dried over $MgSO_4$. The ether was gently evaporated and the residue distilled in a small retort (0.01 mm. Hg; bath temp. 90°), giving a colourless oil (approx. 2 mg.). The infrared spectrum as a liquid film was identical with that of β -hydroxybutyric acid. The product recovered from spectroscopy showed $[\alpha]_D^{20} + 30 \pm 10^\circ$ in ethanol. The aqueous solution from the original hydrolysis was evaporated to a pale-brownish gum (fraction V) (8.6 mg.).

Examination of water-soluble hydrolysis products. About 50 μ g. of each product, I and V, was submitted to paper chromatography with ethyl methyl ketone-propionic acid-water (15:5:6, by vol.) and to paper electrophoresis at pH 2 with *m*-acetic acid–0.75*M*-formic acid and at pH 5 with 0.04*M*-acetate buffer. In each case comparison was made with lysine, serine and 2-amino-6-hydroxyamino-hexanoic acid dihydrochloride, run separately and as a mixture. The separated components were detected by ninhydrin and by triphenyltetrazolium chloride and alkali (Snow, 1954a). Results are shown in Table 3.

Oxidation of mycobactic acid T with periodate. Mycobactic acid T (7.9 mg.) was dissolved in ether (0.2 ml.) and 0.1*N*-NaOH (0.11 ml.) added; the ether was removed with a stream of air, leaving a turbid solution. Then 0.25 ml. of 0.1*M*- $NaIO_4$ was added. After 1 hr. at 20° the solution was acidified with 0.15 ml. of 0.1*N*-HCl. The flocculent precipitate was extracted out with $CHCl_3$ (2 × 2 ml.); the extract was washed, dried with $MgSO_4$ and evaporated almost to dryness. Light petroleum (b.p. 40–60°) (2 ml.) was added and the brown flocculent precipitate (4.3 mg.) was spun down. The supernatant was evaporated to give an almost-

white partly-crystalline residue (fraction VI) (3.4 mg.). This product was examined by infrared spectroscopy and by vapour-phase chromatography.

Vapour-phase chromatography of long-chain fatty acid products. Each product, IIIA, IIIB and VI, was dissolved in ether (0.1 ml.) and allowed to react with a slight excess of ethereal diazomethane. The methyl esters (0.5–1 mg.) dissolved in ether (2–4 μ l.) were applied to column P (stationary phase, succinic ester of diethylene glycol) on a Perkin–Elmer Fractometer; conditions were: temp. 227°; carrier gas H₂ at 60 ml./min.; thermal-conductivity detector. The results are given in Table 2.

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