Isolation of Mycobactins from Various Mycobacteria

THE PROPERTIES OF MYCOBACTINS S AND H

By A. J. WHITE AND G. A. SNOW

Imperial Chemical Industries Ltd., Pharmaceuticals Division,
Alderley Park, Macclesfield, Cheshire

(Received 28 October 1968)

Mycobactin S has been isolated from *Mycobacterium smegmatis* and from *Mycobacterium* sp. Olitzky & Gershon, strain 2, and mycobactin H from *M. thermoresistible*; all three organisms were grown on synthetic media of low iron content. These two mycobactins are mixtures of compounds having the same nucleus but differing in their fatty side chains. The nucleus of mycobactin S has a chemical structure identical with that of mycobactin T but differs in the optical configuration at the β -carbon atom of the hydroxy acid fragment; the configuration in mycobactin S is *S* whereas that in mycobactin T is *R* (the previous assignment of this configuration was incorrect). The cobactin fragment of mycobactin H is identical with that of mycobactin S, but the mycobactinic acid moiety differs in having methyl groups at position 6 in the benzene ring and at position 5 in the oxazoline ring. The configurations of all the asymmetric centres have been established for both mycobactins. Improved and simplified methods for the extraction and purification of mycobactins are described.

Most species of mycobacteria grown in media deficient in iron produce mycobactins capable of promoting the growth of *Mycobacterium johnei*. The mycobactins from *M. phlei* and *M. tuberculosis* have already been characterized, and thin-layer chromatography of ferric complexes has shown that other species of mycobacteria produce mycobactins differing among themselves and differing from those already known (White & Snow, 1968). Mycobactins S and H have now been isolated from *M. smegmatis* and *M. thermoresistible* respectively, and their structures have been determined. A *Mycobacterium* sp. designated strain 2 was reported by Olitzky & Gershon (1965) to yield extracts by ultrasonic disintegration that permitted the multiplication of *M. leprae* in Eagle's medium. This strain produces a mycobactin identical with mycobactin S.

MATERIALS AND METHODS

For details of solvents, methods of mass spectrometry and vapour-phase chromatography see White & Snow (1968). The Varian HA 100 spectrometer was used for n.m.r. measurements. Recent supplies of the grade of alumina previously used for thin-layer chromatography (White & Snow, 1968) have given unsatisfactory results. For the present work basic alumina type T (catalogue no. 1105) from E. Merck A.-G., Darmstadt, Germany, has been used instead; it gives

separations comparable with those previously obtained, though the R_F values are different. Since R_F values vary from one run to another it was found preferable to express results as R_R values, i.e. R_F of the compound/ R_F of ferric mycobactin R (White & Snow, 1968). Mycobactins and their metal complexes were conveniently detected on thin-layer plates by spraying with a mixture of ammonium vanadate soln. (50 mm) and conc. HCl (1:1, v/v); this gave intensely blue spots of the acid vanadyl complexes.

Growth and extraction of organisms

The organisms used were *M. smegmatis* (N.C.I.B. 8548), *M. tuberculosis* H 37 Rv, *M. thermoresistible* (N.C.T.C. 10409) and *Mycobacterium* sp. strain 2 (Olitzky & Gershon, 1965). White & Snow (1968) described conditions for the growth of the first two organisms on defined media of low iron content. *Mycobacterium* sp. strain 2 was grown as for *M. smegmatis* but time of growth was 3 weeks; *M. thermoresistible* was grown on the same medium for 3 weeks at 42° and was subcultured from the liquid medium.

The organisms were filtered off through muslin in a glove box and the filtrates were sterilized and discarded. The quantities given in the description refer to organisms grown on 10 l. of medium. For organisms other than *M. thermoresistible* the moist cells were suspended in aq. 70% (v/v) ethanol (1 l.) for 2 days at room temperature to kill them and to extract the mycobactin; they were then filtered off. [With *Mycobacterium* sp. strain 2 the cells were further extracted with 500 ml. of boiling ethanol; the extract was decanted, cooled and kept at 2° for 24 hr. A white waxy precipitate (850 mg.) was filtered off. The filtrate was

Table 1. *Extraction and concentration of mycobactins*

Results show yields (g.) at different stages of typical extractions.

Organism ...	<i>Mycobacterium</i> sp. strain 2	<i>M.</i> <i>smegmatis</i>	<i>M.</i> <i>tuberculosis</i>	<i>M.</i> <i>thermoresistible</i>
Vol. of medium (l.)	120	77	168	108
Cell residue (dried)	87	58	80	77
Fraction A	6.7	3.1	2.8	5.1
Fraction B	3.23	0	1.1	0.9*
Fraction C	3.47	3.1	1.7	4.2

* Sum of fractions B1 and B2 (see the text).

Scheme 1. Purification of mycobactin S from *Mycobacterium* sp. strain 2 by chromatography. Values in brackets show $R_{f\%}$ at 450m μ . All chromatography was on alumina; successive fractions from the column are shown in order from left to right. Fractions marked * were shown by thin-layer chromatography to contain the minor component. Fractions marked † were obtained by washing the column through with CHCl₃; they contained red-brown material that stayed at the origin on thin-layer chromatography.

diluted with water (200ml.) and combined with the original 70% ethanol extract.] Ethanolic FeCl₃ was added slowly to the extract until the maximum red-brown colour had been

formed; CHCl₃ (1-2l.) was added to form two layers. The lower layer was removed, washed with water (4×1l.), dried over Na₂SO₄ and evaporated. The red-brown gummy

residue (A) was extracted with cold methanol until no more colour was removed, leaving an insoluble residue (B). Evaporation of the methanolic extract gave fraction (C).

For the extraction of *M. thermoresistibile* the cells were suspended in ethanol (11.) for 1 day at room temperature and filtered off. Addition of ethanolic FeCl_3 to the filtrate gave a brown-purple solution; CHCl_3 (500 ml.) and water (500 ml.) were added to give two layers. The lower, coloured, layer was washed with water, dried over Na_2SO_4 and evaporated. The residue was extracted with methanol (4×20 ml.), leaving brown-violet insoluble material (B1). The methanol extracts were combined, evaporated to 10 ml. and kept for 18 hr. at room temperature; a purple-black gum (B2) was precipitated. The supernatant solution was evaporated to give a red-brown gum (C).

Table 1 gives the yields from four mycobacterial species at the various stages of the extractions.

Fractions B1 and B2 from extraction of *M. thermoresistibile* were soluble in CHCl_3 , in ether and in light petroleum. Fraction B1 gave a plum-red solution and fraction B2 a violet solution, λ_{max} 515 and 530 $\text{m}\mu$, $E_{1\text{cm}}^{1\%}$ values 20 and 34.7 respectively in CHCl_3 . Yields from 108 l. of medium were: B1, 820 mg.; B2, 70 mg.

Ferric mycobactins

Purification. Similar chromatographic procedures were used for the purification of ferric mycobactins from all these organisms. Scheme 1, used for the products from *Mycobacterium* sp. strain 2, is given as an example.

(i) Stages 1 and 2. Fraction (C) from *Mycobacterium* sp. strain 2 (3.47 g.) was dissolved in cyclohexane-2-methylpropan-2-ol (17:3, v/v) (8 ml.) and applied to a column (40 cm. $\times$ 2 cm.) of neutral alumina suspended in the same

solvent. Development with this solvent continued until the main coloured band had advanced 30 cm., and was then continued with the same solvent but with addition of propan-1,2-diol (1%, v/v). Fractions were collected, mixed with an equal volume of CHCl_3 , washed with water (4×1 vol.), dried over Na_2SO_4 and evaporated. They were examined by thin-layer chromatography; $E_{1\text{cm}}^{1\%}$ was measured for each, and the fraction with the highest value was taken to the second stage of purification, which was an exact repetition of the first.

(ii) Stage 3. A 40 cm. $\times$ 1 cm. column of neutral alumina was used with the same initial solvent mixture; development was continued throughout with this solvent without addition of propane-1,2-diol. In this particular purification the third stage was split as shown in Scheme 1. At stage 3(a) the purest fraction (P) still showed less than the maximum attainable $E_{1\text{cm}}^{1\%}$. To improve the yield of pure material the middle fractions were combined and re-run in stage 3(b) with the same conditions as 3(a). In separations of other ferric mycobactins stage 3 was not split; Table 2 shows the yields and extinctions of fractions obtained during these purifications. Each purification was continued until no further increase in $E_{1\text{cm}}^{1\%}$ at 450 $\text{m}\mu$ of the ferric complex could be achieved. In some purifications less than three chromatographic stages were needed. Most species yielded one mycobactin (comprising a family of compounds with differing side chains) almost exclusively, but there were usually traces of at least one other mycobactin, distinguished by the R_F of its ferric complex on thin-layer chromatography. These are indicated in Table 3. The minor components were eliminated in the course of the purifications.

Properties. The products from *Mycobacterium* sp. strain 2 and from *M. smegmatis* were shown to have similar physical

Table 2. Purification of mycobactins by adsorption chromatography

Results show yields and extinction values at 450 $\text{m}\mu$ after successive chromatographic purification stages.

Organism		Crude extract	Stage 1	Stage 2	Stage 3
<i>Mycobacterium</i> sp. strain 2	Wt. (g.)	3.47	0.69	0.43	0.21*
	$E_{1\text{cm}}^{1\%}$	7.4	27.1	38.6	42.8
<i>M. smegmatis</i>	Wt. (g.)	3.1	0.6		0.37
	$E_{1\text{cm}}^{1\%}$	13.2	37.0		42.8
<i>M. tuberculosis</i>	Wt. (g.)	1.69	0.104	0.043	0.021
	$E_{1\text{cm}}^{1\%}$	3.65	17.7	32.0	42.2
<i>M. thermoresistibile</i>	Wt. (g.)	0.65			0.12
	$E_{1\text{cm}}^{1\%}$	8.8			39.7†

* Includes stages 3(a) and 3(b).

† Converted into the metal-free form, which was purified by precipitation; reconversion into the ferric complex gave a product with $E_{1\text{cm}}^{1\%}$ 41.2.

Table 3. Thin-layer chromatography of ferric mycobactins from different mycobacteria

Adsorbent: basic alumina type T. Solvent: cyclohexane-2-methylpropan-2-ol (9:1, v/v). Results show mean R_F values (observed R_F/R_F for ferric mycobactin R). R_F for mycobactin R varied from 0.49 to 0.63,

Source organism ...	<i>M. phlei</i>	<i>M. smegmatis</i>	<i>Mycobacterium</i> sp. strain 2	<i>M. thermoresistibile</i>
Main component	0.82	0.19	0.19	0.38
Minor component (present as trace)		0.69	0.69	0.92

properties and to give the same degradation products. The purified compound, *ferric mycobactin S*, was a deep red-brown glass, $\lambda_{\max}$ in methanol 450 m μ ($E_{1\text{cm}}^{1\%}$ 42.8) with inflexions at 330, 275 and 245 m μ . The product from *M. thermoresistibile*, *ferric mycobactin H*, was a red-brown glass, $\lambda_{\max}$ in methanol 449 and 255 m μ ($E_{1\text{cm}}^{1\%}$ 41.2 and 251 respectively) and inflexions at 330 and 280 m μ .

Mycobactins

Mycobactins S and H. These were prepared from the corresponding ferric complexes by the method used for mycobactin T (Snow, 1965b). *Mycobactin S* was a creamy white powder, m.p. 163°, $[\alpha]_D^{25} +10.1 \pm 0.1^\circ$ (c 1.2 in methanol), $\lambda_{\max}$ in methanol 243, 249 and 304 m μ ($E_{1\text{cm}}^{1\%}$ 193, 193 and 63.5 respectively) and inflexion at 258 m μ . *Mycobactin H* was a creamy white powder, m.p. 158°, $[\alpha]_D^{25} +15.9 \pm 0.1^\circ$ (c 1.42 in methanol), $\lambda_{\max}$ in methanol 249.5 and 310 m μ ($E_{1\text{cm}}^{1\%}$ 152 and 39.6 respectively) and inflexions at 254 and 265 m μ .

Aluminium complexes of mycobactins S and H. The method used with mycobactin T (Snow, 1965b) was applied to prepare the aluminium complexes of mycobactins S and H. *Aluminium mycobactin S* was purified by dissolving it in cyclohexane at room temperature; on cooling the solution an amorphous product was obtained as a pale-cream powder, m.p. 120–123°, $\lambda_{\max}$ in methanol 258 and 337 m μ ($E_{1\text{cm}}^{1\%}$ 190 and 66 respectively) with inflexion at 283 m μ . (*Aluminium mycobactin T* had $\lambda_{\max}$ in methanol 256 and 336 m μ .) *Aluminium mycobactin H* was prepared in small amount for mass spectrometry; its other physical properties were not examined. Results from the mass spectrometry of aluminium mycobactins are given in Table 4.

Alkaline cleavage of mycobactins and subsequent examination of the products. The degradation methods used for mycobactin T (Snow, 1965b) were applied to mycobactins S and H. The crude yields of alkaline scission products from mycobactin S (59 mg.) and mycobactin H (83 mg.) were mycobactinic acids (43 and 59 mg. respectively) and cobactins (18 and 26 mg. respectively).

Details of the degradation of the cobactins and mycobactinic acids and of the identification of the resultant products are given only where they differ from methods described in earlier papers.

Mycobactinic acid S. An ether solution of the crude product (240 mg.) concentrated to 3 ml. gave creamy-white crystals (71 mg.) of *mycobactinic acid S*, m.p. 91.5°, $[\alpha]_D^{20} +29.6 \pm 0.1^\circ$ (c 0.47 in methanol), $\lambda_{\max}$ in methanol 243.5, 250 and 305 m μ ($E_{1\text{cm}}^{1\%}$ 245, 242 and 83 respectively) and inflexion at 259 m μ . The crude product (52 mg.) hydrolysed with acid gave an ether-soluble fraction from which salicylic acid (10 mg.), identified by i.r. spectrum, was obtained by sublimation under reduced pressure; the residue was distilled under high vacuum, giving a crystalline product (18 mg.) consisting of fatty acids (fraction I). The water-soluble fraction from the hydrolysis was evaporated and the residue was redissolved in water and evaporated several times to remove free HCl. The final dried residue (16 mg.) had $[\alpha]_D^{20} +14.5^\circ$ (c 0.75 in m-HCl). Paper electrophoresis and paper chromatography showed the presence of serine and 2-amino-6-hydroxyaminohexanoic acid; previous experiments have shown that similar treatment of other mycobactinic acids gives these amino acids as their mono- and di-hydrochlorides respectively. An equimolecular mixture of these salts of the L-amino acids would have $[\alpha]_D +14.3^\circ$ in m-HCl. Crude mycobactinic acid S (47.4 mg.) was oxidized with periodate, and the fatty acids (11 mg.) were separated (fraction 2). Fractions 1 and 2 were converted into methyl esters by treating their silver salts with CH₃I (Gehrke & Goerlitz, 1963); the products were examined by vapour-phase chromatography (Table 5).

Mycobactinic acid H. The product (59 mg.) was a pale-yellow glass. After acid hydrolysis the ether-soluble material was removed and the aqueous solution was evaporated. The residue was redissolved in water and evaporated several times to remove free HCl. The dried glassy residue (38 mg.) had $[\alpha]_D^{25} +5.2 \pm 0.1^\circ$ (c 3.76 in m-HCl); it was examined by paper chromatography and paper electrophoresis alongside reference compounds (Table 6), and shown to contain threonine, 2-amino-6-

Table 4. Mass spectrometry of aluminium mycobactins

m/e, Mass number of parent peaks; *R*, relative peak heights (for aluminium mycobactin S: *a*=product from *M. smegmatis*, *b*=product from *Mycobacterium* sp. strain 2); *N*, number of carbon atoms in mycobactin side chain.

Aluminium mycobactin									
T			S			H			
<i>m/e</i>	<i>R</i>	<i>N</i>	<i>m/e</i>	<i>R</i>		<i>N</i>	<i>m/e</i>	<i>R</i>	<i>N</i>
				<i>a</i>	<i>b</i>				
895	12	21	879	9	6	20	907	50	20
893	10		851	36	38	18	879	50	18
881	24		823	46	50				
879	25		795	9	6	14			
867	7	19							
865	8								
853	7	18							
851	7								
First daughter ions									
640			640				668		

Table 5. *Vapour-phase chromatography of methyl esters of fatty acids from the degradation of mycobactin acid S*

Reference compounds were methyl esters of Δ^2 -normal fatty acids. For column conditions see White & Snow (1968). Fraction 1: product from acid hydrolysis; fraction 2: product from periodate oxidation.

		Retention time (min.)							
		C ₁₆		C ₁₈		C ₂₀			
		<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>		
Fraction 1	1.7	2.2	3.8	4.6	8.2	9.9	17.9	22.1	
Fraction 2	1.7	2.2	3.7	4.7	8.0	10.1	17.4	22.7	
Approx. % of total in peak for fraction 2	3	Trace	3.6	4.7	7.9	10.3	17.5	23.0	
			46	Trace	42	Trace	9	Trace	

Table 6. *Paper chromatography and paper electrophoresis of water-soluble products from acid hydrolysis of mycobactin acid H*

Paper chromatography: solvent butan-1-ol–butan-2-one–water–cyclohexylamine (10:10:5:2, by vol.). Results are R_F values. Paper electrophoresis: *m*-acetic acid–0.75 *M*-formic acid buffer (pH 2), 3 kv for 90 min. Spots were revealed with ninhydrin. Values in *italics* indicate spots that also react with triphenyltetrazolium chloride and NaOH (Snow, 1954). Results show distance run (cm.).

Separation method	Hydrolysis product	Lysine	2-Amino-6-hydroxy-amino-hexanoic acid	Serine	Allothreonine	Threonine
Chromatography	0.16 (trace)	0.16				
	0.23		0.23	0.31	0.45	
	0.60					0.60
Electrophoresis	17.9			19.9		17.9
	31.2		31.5			
	32.2	32.0				
	(trace)					

hydroxyaminohexanoic acid and a trace of lysine. The residue would contain mainly threonine hydrochloride and 2-amino-6-hydroxyaminohexanoic acid dihydrochloride. L-Threonine and L-2-amino-6-hydroxyamino hexanoic acid have $[\alpha]_D^{25} -14.9^\circ$ and $+23.9^\circ$ respectively in *m*-HCl and thus an equimolecular mixture of the hydrochlorides would have $[\alpha]_D^{25} +5.5^\circ$ in *m*-HCl. The ether-soluble material was steam-distilled. Extracts of the distillate gave u.v. and i.r. spectra characteristic of *m*-cresol.

Cobactin S. The crude product crystallized from acetone gave *cobactin S*, m.p. 123° , $[\alpha]_D^{25} -54.7 \pm 0.1^\circ$ (c 0.62 in water) (Found: C, 52.2; H, 7.9; N, 12.2; mol.wt. from mass spectrum 230. $C_{16}H_{18}N_2O_4$ requires C, 52.1; H, 7.9; N, 12.2%; mol.wt. 230). A preliminary acid hydrolysis gave an ether-extractable fraction that was shown by n.m.r. spectroscopy to be an approximately equimolar mixture of 3-hydroxybutyric acid and crotonic acid. On repetition, *cobactin S* (46 mg.) was refluxed for 6 hr. with 5 *M*-HCl; the solution was cooled and extracted with benzene (10 $\times$ 1 ml.) to remove the crotonic acid and was then continuously extracted with ether (4 ml.). The residue from the ether extract was a colourless oil, $[\alpha]_D^{25} +22.5 \pm 0.1^\circ$ (c 1.22 in water), identified as *S*-(+)-3-hydroxybutyric acid (wt. of acid by titration: 11.6 mg.); n.m.r. showed the correct spectrum and confirmed the substantial absence of crotonic acid. *R*-(−)-3-Hydroxybutyric acid has $[\alpha]_D^{20} -24.8^\circ$ (c 3.26 in

water) (McKenzie, 1902). The aqueous solution after ether extraction was evaporated; the residue (32 mg.) was shown by paper electrophoresis to contain 2-amino-6-hydroxyaminohexanoic acid as the sole amino acid; by assuming that the residue was the dihydrochloride, $[\alpha]_D^{24}$ calculated for the free amino acid was $+27.0^\circ$ (c 1.17 in *m*-HCl) ($[\alpha]_D^{25}$ for the L-isomer in *m*-HCl $+23.9^\circ$; Snow, 1954).

Cobactin T. The product from a new preparation of mycobactin T had m.p. 139.5° , $[\alpha]_D^{21} -88.9 \pm 0.5^\circ$ (c 0.51 in water); mol.wt. by mass spectrometry 230. A portion (16 mg.) was refluxed for 6 hr. with 5 *M*-HCl (1 ml.). The solution was cooled and was continuously extracted with ether (4 ml.). Evaporation of the aqueous solution remaining gave a residue of the dihydrochloride of L-2-amino-6-hydroxyaminohexanoic acid, having $[\alpha]_D^{25} +23^\circ$ in *m*-HCl (calculated on the content of the free amino acid, determined by its reducing value; Snow, 1954). The ether extract, carefully evaporated, gave an oil, which was shown by n.m.r. to be a mixture of 3-hydroxybutyric acid and crotonic acid in molar ratio 2.4:1, and by titration to contain 45.5 μ moles of acid. The sodium salt had $[\alpha]_D^{24} -11 \pm 1^\circ$ (c 0.4 in water) based on the estimated content of sodium 3-hydroxybutyrate. [*R*-(−)-Sodium 3-hydroxybutyrate has $[\alpha]_D^{17} -13.8^\circ$, c 1.36 in water (McKenzie, 1902).]

Cobactin from mycobactin H. The *cobactin* fraction

crystallized from acetone had m.p. 122.5° , $[\alpha]_D^{21} -53.0 \pm 0.3^\circ$ (c 1.91 in water), mol.wt. by mass spectrometry 230. The n.m.r. spectrum showed it to be identical with cobactin S.

RESULTS

Extraction and purification. The use of synthetic media of low iron content has proved advantageous for growing mycobacteria for the extraction of mycobactins. The earlier extraction methods have been changed and simplified. A primary extraction with cold aqueous ethanol removes the mycobactins but leaves much of the lipid in the cells. The mycobactins at this stage are converted into their ferric complexes and simple solvent treatments yield crude products containing up to 30% of mycobactin with some mycobacterial strains. Adsorption chromatography with mixtures of cyclohexane, 2-methylpropan-2-ol and propane-1,2-diol is more convenient than with the solvents previously used and loss by irreversible adsorption on the alumina is lessened. Three stages of chromatography were usually sufficient to purify the ferric mycobactins; with the product from *M. smegmatis* the purity after solvent extraction was so high that only two chromatographic stages were needed. In purifying the product from *M. thermoresistibile* a pure product was obtained by one chromatographic treatment of the ferric complex followed by a simple purification of the free mycobactin.

All the organisms considered in this paper produced a single main mycobactin, consisting of a family of compounds with the same nucleus but with side chains of different lengths. The main component was usually accompanied by traces of other mycobactins separable by thin-layer chromatography of their ferric complexes. The R_F values of principal and subsidiary products from the various mycobacteria are shown in Table 3. The amounts of the minor constituents were too small to permit chemical identification, but since they were easily separable they probably differed from the corresponding main component in the nuclei of their structures. The minor components were eliminated in the chromatographic purifications.

The R_F values of the main ferric mycobactins from *M. smegmatis* and *Mycobacterium* sp. strain 2 were identical. The physical properties of the mycobactins and the identity of the degradation products from each also corresponded. There was possibly a slight difference in the ratio of the side-chain components, but this had a negligible effect on the overall properties.

M. thermoresistibile differed from the other mycobacteria in producing, besides mycobactins, a product that gave a purple pigment with ferric iron. The amount of this purple material varied considerably with the conditions of growth of the

organism. It is not known whether it is associated with the biosynthesis of mycobactin H.

Mycobactin S compared with mycobactin T. The absorption spectra of mycobactin S, and of its ferric and aluminium complexes, were identical in form with the corresponding spectra of mycobactin T and its complexes. This showed that both probably contained the same hydroxyphenyl-oxazoline structure. A complete acid hydrolysis of mycobactin S yielded products chemically identical with those from mycobactin T, apart from the compositions of fatty acid mixtures derived from the side chains. However, the two mycobactins differed markedly in the R_F values of their ferric complexes on thin-layer chromatography, and in their optical rotations. It thus seemed probable that there must be a difference in the optical configuration of one or more of the asymmetric centres in the nucleus of the molecule. Alkaline scission of mycobactin S gave the corresponding mycobactinic acid and cobactin. Mass spectrometry of the crystalline cobactin S gave a parent peak having the same m/e value as that from cobactin T. The two differed, however, in melting point and in optical rotation. Acid hydrolysis of both cobactins gave L-amino-6-hydroxyaminohexanoic acid, so the difference between them evidently lay in the hydroxy acid component. In both cases the conditions of hydrolysis caused some degradation of the 3-hydroxybutyric acid to crotonic acid, more of the unsaturated acid being formed from cobactin S than from cobactin T. Crotonic acid was removed from the mixed acids obtained in the cobactin S degradation and the remaining acid was shown to be *S*-(+)-3-hydroxybutyric acid. With the smaller quantity of cobactin T that was available separation was not attempted; the amounts of the two acids were calculated from n.m.r. measurements and titration, and the optical rotation of the sodium salt of the mixture was determined. The rotation was reasonably close to the value expected if the 3-hydroxybutyric acid was *laevo* (*R*). The n.m.r. spectra of the two cobactins were measured in hexadeuteroacetone. The results were interpreted in terms of the structure (I):

Both spectra showed a four-line pattern at τ 5.52 (a), a multiplet at 5.8–6.8 τ (b, c and d), a multiplet at 7.75 τ (e), a multiplet at 8.0–8.8 τ for the remaining ring protons and a doublet at 8.9 τ (f). The hydroxamic acid OH was at 1.4 τ and the amide NH at 2.6 τ . The multiplet at 7.75 τ was more complex

in the cobactin T spectrum than in the cobactin S spectrum. This difference was due to the different influence of the proton (c) in the two diastereoisomers. Integration of the peaks in the n.m.r. spectrum agreed with the interpretation of the contributions from the various protons in the molecule. The optical rotation found for 3-hydroxybutyric acid from mycobactin T differed from that previously reported (Snow, 1965b). The earlier result from the degradation of a very small sample was erroneous.

Mycobactin acid S was hydrolysed with acid to yield serine, 2-amino-6-hydroxyaminohexanoic acid, salicylic acid and fatty acids. The optical rotation of the mixture of amino acids corresponded to both being the L form. The result would not agree with either or both being D. For examination of the side chains the fatty acid mixture from acid hydrolysis and a mixture obtained by oxidation of mycobactin acid S with periodate were separated by vapour-phase chromatography of their methyl esters. The results (Table 5) show that the retention times for the product from periodate oxidation were close to those of the esters of even-numbered *cis*- Δ^2 C₁₄-C₂₀ acids, with trace peaks of the corresponding *trans* acids. The main components were C₁₆ and C₁₈. The retention times for the products from acid hydrolysis were similar, but the peaks corresponding to the *trans* acids were more prominent, though still lower than those due to the *cis* acids. This result contrasts with the findings with mycobactin P, where acid hydrolysis gave only *trans* acids (Snow, 1965a). It suggests that the side-chain structure may be different in mycobactin S, though the ultraviolet-absorption spectrum appears to indicate a contribution from a hydroxamic acid having a Δ^2 -acyl group. Mass spectrometry of the aluminium complex of mycobactin S (Table 4) supported the degradation results. The most abundant parent peaks corresponded to the proposed nucleus with C₁₆ and C₁₈ side chains having one double bond. The first daughter ion at *m/e* 640 corresponded to the loss of the side chain immediately beyond the β -position, a similar breakdown to that found with aluminium mycobactin P (White & Snow, 1968).

Mass spectrometry of the aluminium complex of mycobactin T (Table 4) showed four pairs of peaks at high mass numbers, the members of each pair differing from each other by two mass units and from the next pair by 14 mass units. If these are all parent peaks the results indicate the presence of a family of compounds having side chains C₁₈-C₂₁ both in a saturated series and in a series containing one double bond. It is noticeable that the peak heights for the members of each pair are similar. Investigation of the fatty acids obtained by degradation of mycobactin acid T (Snow, 1965b)

has already shown the complexity of the mixture. The infrared spectrum indicated much less $\alpha\beta$ -unsaturation than was found in mycobactin P and the chain lengths previously assessed by vapour-phase chromatography are confirmed by the present mass-spectrographic results. There may be several series of side chains differing in unsaturation and branching; the vapour-phase separation used would be unable to resolve all the individual esters in a mixture of this complexity. The first daughter ion in the mass spectrum of aluminium mycobactin T was at *m/e* 640, the same as that from aluminium mycobactin S.

Mycobactin H. This mycobactin has been examined in less detail. Its cobactin fragment was identical with cobactin S. The absorption spectra of the mycobactin and of its derivatives were identical with those of mycobactin P and its corresponding derivatives. As with mycobactin P (Snow, 1954), hydrolysis of mycobactin H gave *m*-cresol as the aromatic component. Thus mycobactin H, like mycobactin P, has a methyl group in position 6 of the benzene ring. In the water-soluble fraction from the hydrolysis of mycobactin H, 2-amino-6-hydroxyaminohexanoic acid and threonine were identified by paper chromatography and paper electrophoresis; mycobactins previously examined have given serine rather than threonine. The optical rotation of the mixture agreed with both the amino acids having the L-configuration. The side-chain composition was not determined directly, but mass spectrometry of the aluminium complex indicated an unusually simple mixture of two components present in almost equal amounts. The *m/e* values corresponded to a nucleus having the suggested structure with C₂₀ and C₁₈ side chains containing one double bond. The *m/e* value of the first daughter ion agreed with this interpretation if the same type of primary breakdown as with the other mycobactins is assumed to occur.

The structures of the mycobactins so far examined in detail are summarized in Fig. 1. Variations between different mycobactins have now been shown to involve differences at five points in the molecule. Variations in the side chain affect the physical properties only very slightly, but changes at other points have more marked effects. In particular, mycobactins S and T are easily distinguished although the nuclei probably differ only in the configuration of one asymmetric centre (assuming that the centres b and c in mycobactin T conform to the general pattern of derivation from L-amino acids).

DISCUSSION

The mycobacteria are a closely-knit group of micro-organisms having biochemical characteristics that distinguish them from other genera; ability to

Fig. 1. Side chains R_1 are acyl groups having the total number of C atoms shown below; double bonds are indicated where they are known; n indicates unbranched chains. Figures show the main types of side chain; those of greatest abundance are shown in bold type. Asymmetric centres are labelled a-f; blanks indicate that the configuration has not been determined; () indicates lack of an asymmetric centre.

Mycobactin	R_1	R_2	R_3	R_4	R_5	a	b	c	d	e	f
P	14, 16, 18, 20 <i>cis</i>-Δ^2 n	CH ₃	H	C ₂ H ₅	CH ₃	()	L	L	<i>S</i>	<i>R</i>	L
T	18, 19, 20, 21 Δ	H	H	CH ₃	H	()			<i>R</i>	()	L
S	14, 16, 18, 20 (<i>cis</i>-Δ^2) *	H	H	CH ₃	H	()	L	L	<i>S</i>	()	L
H	18, 20 Δ	CH ₃	CH ₃	CH ₃	H	<i>R</i>	L	L	<i>S</i>	()	L

* Not certainly established.

synthesize mycobactins is one distinctive feature. The function of the mycobactins is still unsettled, but their specificity and high activity in promoting the growth of *M. johnei* suggest that they play a vital role in the metabolism of these organisms. The mycobactins are probably the specialized counterpart of iron-chelating growth factors that are found in many other bacteria. Although the well-known failure of attempts to culture *M. leprae* *in vitro* has sometimes been ascribed to the lack of a specific growth factor, attempts to promote its growth by supplements of mycobactins P and T have all proved negative. The report by Olitzky & Gershon (1965) that the multiplication of *M. leprae* is stimulated by an extract from an atypical mycobacterium suggested that the mycobactin of this particular strain should be investigated. It proved to be mycobactin S, a mycobactin characteristic of *M. smegmatis*. Since this particular mycobactin has not been examined for the promotion of growth of *M. leprae*, a sample has been sent to Professor A. L. Olitzky for examination. Mycobactin S, like other mycobactins, has only a low solubility in water and the concentration in an ultrasonic extract is likely to be very small; nevertheless the concentrations required for promoting the growth of *M. johnei* are far below the limit of solubility in water. The present results show that the mycobactins from different species of mycobacteria vary in details of structure while retaining an overall molecular similarity. Explanation of this structural variation between species is an interesting biological problem. One possibility is that the optimum suitability of the growth factor for a particular species depends on a precise combination of physical characteristics. In this case mutation and selection

would eventually provide each species with the mycobactin most exactly suited to its needs. However, if only the overall molecular pattern of the growth factor is important, and the various structural variants are biologically interchangeable, the compound produced by a particular species may depend upon the availability of biochemical intermediates; mutation would favour the biosynthetic use of compounds that were most accessible from the general metabolic pool. It may be possible to test this second explanation by a comparative study of the biosynthetic capabilities of different species of mycobacteria and by attempts to direct biosynthesis of the mycobactins by presenting the cells with appropriate precursors. A prerequisite, however, is a fuller understanding of the ways in which products evidently derived from different metabolic pathways are brought together and united in the final complex mycobactin molecule.

We thank Professor A. L. Olitzky for providing *Mycobacterium* sp. strain 2, Mr D. Greatbanks for n.m.r. measurements and their interpretation, Dr B. R. Webster for mass spectrometry, Mr J. T. A. Webster for vapour-phase chromatography and Mr D. Lowe for valuable technical assistance in growing and harvesting the micro-organisms.

REFERENCES

- Gehrke, C. W. & Goerlitz, D. F. (1963). *Analyt. Chem.* **35**, 76.
- McKenzie, A. (1902). *J. chem. Soc.* **81**, 1402.
- Olitzky, A. L. & Gershon, Z. (1965). *Israel. J. med. Sci.* **1**, 1004.
- Snow, G. A. (1954). *J. chem. Soc.* p. 2588.
- Snow, G. A. (1965a). *Biochem. J.* **94**, 160.
- Snow, G. A. (1965b). *Biochem. J.* **97**, 166.
- White, A. J. & Snow, G. A. (1968). *Biochem. J.* **108**, 593.