Chemical and Biological Properties of Mycobactins Isolated from Various Mycobacteria

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Nine different strains of mycobacteria grown on media deficient in iron all produced mycobactins. Most strains produced one mycobactin in great preponderance. Mycobacteria from clearly distinct taxonomic groups gave mycobactins differing in the structure of their nuclei. One group of taxonomically related mycobacteria produced mycobactins having the same nucleus but with different distributions of side chains within the homologous mixtures. Simple methods are described for identifying mycobactins on a small scale; these may be of value in classifying mycobacteria. Structures are proposed for mycobactin A from *Mycobacterium aurum*, mycobactin R from *M. terrae*, mycobactin F, produced together with mycobactin H by *M. fortuitum*, and mycobactins M and N from *M. marinum*. The first three of these differ from known mycobactins in details of substitution and configuration of asymmetric centres in the nucleus. Mycobactins M and N are substantially different, having only small acyl groups (acetyl and propionyl respectively) at the hydroxamic acid centre of the mycobactic acid moiety. Both are homologous mixtures having long-chain saturated 3-hydroxy-2-methyl acid fragments in the cobactin moiety. All mycobactins so far isolated promote almost maximal growth of *M. johnei* at 30 ng./ml. in liquid medium. The activity of some mycobactins extends to much lower concentrations, mycobactin S showing significant growth promotion at 0.3 ng./ml. Mycobactin M or N in combination with mycobactins having a long side chain in the mycobactic acid moiety exerts a mutually antagonistic effect on the growth of *M. johnei*, the mixture giving less growth than either mycobactin separately. Mycobactin M also decreases the growth of *M. kansasii* and *M. tuberculosis* on liquid media. These antagonistic effects are probably caused by a lengthening of the lag phase.

Previous work (Snow, 1965a,b; White & Snow, 1969) on the characterization of mycobactins from *Mycobacterium phlei*, *M. tuberculosis*, *M. smegmatis* and *M. thermoresistibile* has been extended to nine other species of mycobacteria. All these organisms when grown on media deficient in iron produce mycobactins that include six new chemical types. Of these, we were most interested in mycobactins M and N from *M. marinum*, which differ from other mycobactins in the location of their fatty side chains and also show unusual biological properties.

RESULTS AND DISCUSSION

Isolation and purification of ferric mycobactins

Nine strains of mycobacteria were grown on media of low iron content and all gave products showing the properties of mycobactins. The preliminary extraction and concentration followed the

methods previously established, the crude mycobactins being concentrated as their ferric complexes. Yields are given in Table 1. *M. fortuitum* gave the highest yield of mycobactin among the mycobacteria so far examined, whereas *M. aurum*, *M. thamnophaeos* and *M. kansasii* gave quite small amounts.

Isolation of the pure mycobactins was undertaken with extracts from three strains. The crude product from *M. fortuitum* was purified by adsorption chromatography. Impure ferric mycobactin from *M. terrae* was concentrated by adsorption on alumina with subsequent elution; this was followed by a countercurrent separation and a final purification after conversion into the metal-free form. The crude ferric complex from *M. marinum* contained relatively large amounts of a colourless oily impurity that was mostly removed by adsorption on alumina followed by elution. Unlike the products from other mycobacterial species, the ferric mycobactin from *M. marinum* showed the presence of two clearly

Table 1. *Extraction and concentration of mycobactins*

Organism ...	<i>M. terrae</i>	<i>M. fortuitum</i>	<i>M. aurum</i>	<i>M. kansasii</i>	<i>M. marinum</i>	<i>M. balnei</i>	<i>M. piscium</i>	<i>M. thamnophaeos</i>	<i>Mycobacterium</i> sp. A.T.C.C. 9823
Vol. of growth medium (l.)	30	20	10	6	156	10	10	10	10
Dried cell residue (g.)	45.6	22.9	10.1	5.9	136	8.0	9.4	7.4	10.1
Fraction A (g.)	0.62	2.00	0.42	0.10	20.5	0.84	1.40	0.29	0.39
Fraction B (g.) (methanol-insoluble)	0.35	0.65	0.23	(Trace)	2.3	0.57	0.94	0.18	0.26
Fraction C (g.) (methanol-soluble)	0.27	1.32	0.18	0.098	18.2	0.27	0.46	0.10	0.13
Fraction C ($E_{1\text{cm}}^{1\%}$ at 450 nm.)	15.8	13.9	1.7	1.14	1.04	6.6	6.1	2.84	9.5
Calc. yield of ferric mycobactin (mg./101. of medium*)	35	225	8	5	28	43	69	7	30

* Based on an average value of $E_{1\text{cm}}^{1\%} = 41$ at 450 nm. for the pure ferric mycobactin.

separable components, both in substantial amount (White & Snow, 1968). The proportion of the minor component varied from 5 to 25% of the total in different preparations. The second stage of the purification by two successive countercurrent separations gave the major component, ferric mycobactin M, in a nearly pure form. The minor component (ferric mycobactin N) was not completely freed from the major component by this treatment; its purification was completed by repeated preparative t.l.c.

Ferric mycobactin A from *M. aurum* and the ferric mycobactins from *M. piscium* and *Mycobacterium* sp. A.T.C.C. 9823 were purified on a small scale by preparative t.l.c.

Structures and chemical properties of new mycobactins

The structures of the mycobactins discussed in this section are shown in Fig. 1.

Mycobactin R from *M. terrae*. The usual alkali scission applied to this mycobactin gave a mycobactinic acid that yielded salicylic acid, serine, 2-amino-6-hydroxyaminohexanoic acid and long-chain fatty acids; the optical rotation of the mixed amino acids showed both to have the L-form (White & Snow, 1969). Mass spectrometry of the cobactin showed a parent peak corresponding in molecular weight to that from cobactin P (Snow, 1954b), and the n.m.r. spectrum indicated that the two cobactins

were chemically identical. However, they differed in melting point and in optical rotation. Acid hydrolysis of cobactin R gave (+)-2-amino-6-hydroxyaminohexanoic acid, indicating the usual L-configuration at this asymmetric centre. The other hydrolytic product was (+)-3-hydroxy-2-methylpentanoic acid, giving an n.m.r. spectrum that showed it to have the *erythro* configuration (Snow, 1965a). It must therefore be the enantiomer of the hydroxy acid obtained from cobactin P. Its specific rotation, measured on the small sample available, was somewhat lower than the value for the *laevo* acid from cobactin P. The fatty acid component from hydrolysis of mycobactinic acid R was converted into the methyl ester and was examined by vapour-phase chromatography. It showed the presence of the $C_{20}\Delta^2$ acid together with a trace of $C_{18}\Delta^2$ acid (Table 2). The mycobactin produced by *M. terrae* is thus largely a single chemical entity. This was confirmed by mass spectrometry of its aluminium complex, which showed a main parent peak at m/e 907 with a very small peak at m/e 879. The first daughter ion at m/e 668 agreed with the molecular weight of the nucleus deduced from chemical evidence. The structure assigned to mycobactin R is shown in Fig. 1.

Mycobactin A from *M. aurum*. The small amount of this compound available limited its examination to physical methods. The u.v. spectra of the ferric complex and the metal-free product were characteristic of a mycobactin having a methyl group in the

Fig. 1. Structures of mycobactins. Side-chains R_1 are acyl groups having the total number of C atoms shown below; double bonds are indicated where they are known. Numbers 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
R	20 Δ^2	H	H	C_2H_5	CH_3	()	<u>L</u>	L	R	S	L
F*	18, 16, 14, 12, 10 Δ	H	CH_3	CH_3	H	<i>threo</i>			S	()	L
A†	14 Δ^2	CH_3	H	CH_3	H						
M	2	H	CH_3	18, 17, 16, 15 †	CH_3				R§	S§	
N†	3	H	CH_3	18, 17, 16, 15 †	CH_3						

* Structure inferred from mixture with mycobactin H; peculiarities of side-chain distribution are discussed in the text.

† Tentative structures.

‡ Saturated alkyl groups having the number of C atoms shown.

§ Relative configuration at d and e is *erythro*; absolute configuration is uncertain.

benzene ring at position 6 and a Δ^2 side chain. The mass spectrum of its aluminium complex showed a single preponderant parent peak at m/e 809 (other parent peaks were less than 1% of this) and a first daughter ion at m/e 654. If the molecule conforms to the structural pattern of other mycobactins the molecular weight of the daughter ion accommodates only one methyl group in the nucleus besides that on the benzene ring. This is most likely to be at C-3 in the hydroxy acid fragment, giving a cobactin structure of the type found in mycobactins S or T. The mass of the parent ion indicates a C_{14} Δ side chain. The probable structure of mycobactin A is given in Fig. 1.

Mycobactins from M. fortuitum. Although the ferric mycobactin isolated from *M. fortuitum* gave a single spot on t.l.c. in the systems normally used, several observations have shown the product to be a mixture of two ferric mycobactins having different nuclei. These two types have not been separated and degradations have been carried out on the un-separated mixture. The usual alkaline degradation gave a crystalline cobactin identical in melting point, optical rotation, n.m.r. and mass spectrum with cobactin S (White & Snow, 1969). The crude cobactin was also examined, in case preferential crystallization of one cobactin had occurred, but cobactin S was the only compound found. The mycobactin acid hydrolysed with acid gave a fatty acid fraction, an amino acid fraction containing only threonine, 2-amino-6-hydroxyaminohexanoic acid and a trace of lysine, and an aromatic fraction con-

taining both *m*-cresol and salicylic acid. Thus the nuclei of the two mycobactins differ solely in their aromatic components. One probably has the same nucleus as mycobactin H (White & Snow, 1969) (the identity of the asymmetric centres in the mycobactin acid moiety have not been proved), whereas the second component lacks the methyl group in the benzene ring. This previously undescribed type is named mycobactin F. Further confirmation of the nature of the mixture was obtained from the n.m.r. spectrum of the metal-free material. This showed the presence of two very similar compounds, differing only in the aromatic ring. The ratio of the compound with a methyl group in the benzene ring to that without a methyl group was estimated to be 5:2 (Greatbanks & Bedford, 1969). Further, the u.v. spectrum of the mixture could be matched by a summation of spectra of the two types in the appropriate ratio. The mass spectrum of the mixed aluminium complexes showed the usual primary degradation (White & Snow, 1968, 1969) by loss of the side chains beyond the double bond, giving rise to two daughter peaks at m/e 668 and 654; the first of these corresponded to the degradation of aluminium mycobactin H and the second, 14 mass units lower, agreed with the degradation expected for the postulated structure of aluminium mycobactin F. The height ratio of these degradation peaks was 4:5, but this ratio need not reflect the abundance of the parent compounds, since it will be influenced by differences in volatility or ease of degradation between compounds having different nuclei. The fatty

Table 2. *Components present in various mycobactins: evidence from vapour-phase chromatography and mass spectrometry*

Vapour-phase chromatography results show separation of the methyl esters of the long-chain fatty acids obtained by acid hydrolysis of the mycobactic acids; retention times are in min. Mass-spectrometric measurements were on the aluminium complexes of the mycobactins. R, mycobactin R; A, mycobactin A; dihydro-P, dihydro-mycobactin P; H + F, mixture of mycobactins from *M. fortuitum* (the peaks of the mass spectrum are allocated to the mycobactin F or the mycobactin H group assuming that all side chains have even numbers of C atoms as shown by the vapour-phase chromatography results).

C atoms in side chain	Vapour-phase chromatography						Mass spectrometry of aluminium complexes					
	Methyl esters from acid hydrolysis products						R	H + F		A	Dihydro-P	
	Reference compound		R		H + F		m/e	m/e		m/e	m/e	
	Com- pound	Reten- tion time	Reten- tion time	Abund- ance	Reten- tion time	Abund- ance		H	F			Abundance
10								739		2		
12					0.73	5		753		6		
					0.93	23	28	767		23		
14					1.69	Trace		781		45		
					2.1	Trace		795		2	809	
16	Δ^2 cis	3.6			3.7	6		809		3		
	Δ^2 trans	4.5			4.4	14	20	823		8		867 11
18	Δ^2 cis	7.9			7.9	8		837		14		881* 5
	Δ^2 trans	9.7		Trace	9.7	44	52	(879) 851		23		895 82
20	Δ^2 cis	17.5	16.9	8	17.9	Trace	907	865		45		909* 2
	Δ^2 trans	21.0	21.3	92	21.0	Trace						
				First daughter ion ...			668	668 654			654	656

* Peaks corresponding to C_{17} and C_{19} side chains (cf. results with mycobactin P; White & Snow, 1968).

acids obtained by hydrolysis of the mixed mycobactic acids were converted into methyl esters and separated by vapour-phase chromatography. The retention times (Table 2) agreed with those of esters of a series of *trans* Δ^2 acids together with smaller amounts of the corresponding *cis* acids, all the acids having even numbers of carbon atoms. However, the distribution of chain lengths was unusual in showing two maxima at C_{18} and C_{12} with only traces of the C_{14} compounds. The mass spectrum of the aluminium complex derived from the mixed mycobactins also showed a complex pattern of primary peaks with two maxima (Table 2). If all the ten peaks listed were parent ions it seems likely that alternate peaks were derived from the mycobactin H and mycobactin F components, since the molecular weights of these nuclei differed by increments of 14 whereas the acids obtained by degradation differed by increments of 28 mass units. This would suggest that both the mycobactin F and the mycobactin H in the mixture have this unusual distribution of side-chain lengths. Mycobactin H from *M. thermoresistibile* had C_{18} Δ and C_{20} Δ side

chains in equal proportion with no indication of a C_{12} component (White & Snow, 1969). Fig. 1 gives the probable structure of the mycobactin F component from *M. fortuitum*.

The production by the same organism of two mycobactins, one containing a salicylic acid fragment and the other a 6-methylsalicylic acid fragment, poses even more acutely the problem of biosynthesis already discussed in relation to mycobactin T (Snow, 1965b). *M. fortuitum* would be an advantageous organism to use in a study of this biosynthetic puzzle.

Mycobactins from M. marinum. Most work has centred on the major component, mycobactin M. The alkali degradation of this compound gave products differing in physical properties from those given by other mycobactins. The cobactin fraction was unusual in being insoluble in water, but soluble in ether; it also differed in being the major degradation product by weight. The mass spectrum showed it to be a mixture of homologous compounds, the parent ions having peaks at m/e 468, 454, 440 and (426) with peak-height ratios 65:19:16:(trace).

The n.m.r. spectrum showed the pattern expected for a cobactin type of structure with methyl on the α -carbon and a long alkyl chain on the β -carbon of the hydroxy acid part of the molecule. This was confirmed by acid hydrolysis, which gave the expected hydroxy acid mixture, together with a mixture of the corresponding unsaturated acids derived from them. Both were identified by the n.m.r. spectrum; n.m.r. evidence also showed that the hydroxy acids had the *erythro* configuration (Snow, 1965a). The other acid hydrolysis product was 2-amino-6-hydroxyaminohexanoic acid. Cobactin M was thus a mixture of compounds represented by formula (I) (the absolute configuration of the asymmetric centres has not been established). The other primary product of alkali degradation of mycobactin M was a mycobactic acid. However, it differed markedly from the usual type of mycobactic acid, being soluble in water as well as in ether. The u.v. spectrum indicated the presence of a 2-(2-hydroxyphenyl)oxazoline group. The n.m.r. spectrum showed many of the characteristics of a mycobactic acid but lacked evidence of a long fatty side chain. A methyl group was assigned to the oxazoline ring. The physical evidence was confirmed by acid hydrolysis, which gave salicylic acid, threonine

and 2-amino-6-hydroxyaminohexanoic acid but no long-chain fatty acid fraction. The mycobactic acid gave a weak reaction with triphenyltetrazolium chloride and alkali (Snow, 1954a), suggesting that it contained some material having a free hydroxy-amino group, but the presence of a hydroxamic acid group was confirmed by the reddish colour given by ferric chloride in acid solution; the phenolic group gives little colour under these conditions (Snow, 1954b). The mycobactin was therefore thought to contain a short-chain acyl hydroxamic acid that was partly degraded to the hydroxyamino compound during the alkali treatment used to prepare the primary degradation fragments. The acid corresponding to this acyl group was not definitely identified in the products of acid hydrolysis, which was done with rather small quantities, though there were indications of the presence of acetic acid on examination by vapour-phase chromatography.

The mass spectrum of aluminium mycobactin M (Table 3) showed three principle parent peaks spaced at intervals of 14 mass units. The relative heights of these peaks was similar to the relative heights of the three main peaks in the mass spectrum of cobactin M. The mass of each aluminium mycobactin M parent peak exceeded that of the corresponding cobactin M peak by 413 mass units. (The mass spectrum of the aluminium complex showed traces of two higher homologues, and the cobactin spectrum a trace of a lower homologue). The mycobactins previously investigated have comprised families of compounds differing only in the acyl group of the hydroxamic acid in the mycobactic acid moiety. In mycobactin M the mycobactic acid moiety is evidently constant and variation occurs

Table 3. Mass spectrometry of aluminium complexes of mycobactins M and N from *M. marinum* and of mycobactins from related mycobacteria

m/e, mass number of parent peaks; *R*, relative peak heights.

C atoms in cobactin side chain (R_4 in Fig. 1)	Source organism							
	<i>M. marinum</i>							
	<i>M. piscium</i>		Mycobacterium sp. A.T.C.C. 9823		Aluminium mycobactin M		Aluminium mycobactin N	
	<i>m/e</i>	<i>R</i>	<i>m/e</i>	<i>R</i>	<i>m/e</i>	<i>R</i>	<i>m/e</i>	<i>R</i>
14			839	3			853	Trace
15	853	34	853	14	853	18	867	14
16	867	12	867	8	867	25	881	34
17	881	54	881	70	881	55	895	47
18			895	3	895	2	909	5
19			909	1	909	Trace	923	Trace
First prominent degradation peak	432		432		432		446

Fig. 2. Absorption spectra: —, (a) mycobactin M, (b) ferric mycobactin M; - · - · -, (a) mycobactin S, (b) ferric mycobactin S; - - - -, dihydromycobactin P; · · · · ·, mycobactin P. All spectra were measured on solutions in methanol. Molecular weights were calculated from the relative abundances of the different side-chain components of each mycobactin determined by mass spectrometry of the aluminium complexes. The values were: mycobactin M, 850; mycobactin S, 813; mycobactin P, 867; dihydromycobactin P, 869.

in the chain length of the substituent on the β -carbon atom in the hydroxy acid of the cobactin moiety. Taking account of the hydrolytic products of the mycobactinic acid fragment the molecular-weight difference between cobactin M and aluminium mycobactin M can be explained by assuming that in mycobactin M the hydroxamic acid of the mycobactinic acid moiety carries an acetyl group. The n.m.r. spectrum of mycobactin M gave a methyl peak, which was clearly in agreement with the presence of an acetohydroxamic acid group. Other mycobactins have long-chain Δ^2 -alkenohydroxamic acid groups, which make a substantial contribution to their absorption spectra at wavelengths below 300 nm. (Snow, 1965b). In mycobactin M the lack of an $\alpha\beta$ -double bond in the acyl group considerably decreased this absorption. This was apparent in the much decreased E_{249}/E_{304} ratio (2.35) compared with that found for mycobactin S (3.0) and in the greater prominence of the peaks at lower wavelength (Fig. 2). The effect of saturation in the side chain was also seen in the absorption spectrum of a product obtained from mycobactin P by catalytic reduction, which showed similar differences compared with the parent compound. Saturation of the side chain similarly affected the absorption spectra of the ferric mycobactins (Fig. 2).

Besides the differences already discussed, mycobactin M also differed from other mycobactins in the type of degradation undergone during mass spectrometry of the aluminium complex. With other aluminium mycobactins the primary degradation

occurred with loss of the mycobactinic acid side chain immediately beyond the $\alpha\beta$ -double bond (White & Snow, 1969). Thus all the components of a homologous mixture of mycobactins gave the same first daughter ion, which was characteristic of the common nucleus. With aluminium dihydromycobactin P, where the side chain was saturated, breakdown occurred immediately beyond the carbonyl group giving a daughter ion at m/e 656. The first prominent fragment ion in the mass spectrum of aluminium mycobactin M was at m/e 432.1326. This degradation apparently involves a break at the ester link with loss of the whole cobactin fragment, retention of the aluminium atom and proton transfer. The calculated mass of the fragment $C_{19}H_{23}AlN_3O_7$ is 432.1351, in good agreement with the observed value. Since the variable side chain is lost, all components of the aluminium mycobactin M mixture give the same fragment ion. The combined evidence shows mycobactin M to have the structure shown in Fig. 1.

The mycobactin (mycobactin N) that is present in smaller amount in *M. marinum* has been examined in less detail. Its n.m.r. spectrum was closely similar to that of mycobactin M, but the methyl group adjacent to the oxo group was apparently replaced by an ethyl group (Greatbanks & Bedford, 1969). Mycobactin N was therefore thought to resemble mycobactin M but with a propionohydroxamic acid in place of an acetohydroxamic acid in the mycobactinic acid moiety. The u.v. spectrum of mycobactin N and its ferric complex were closely similar to

those of mycobactin M, indicating identity of the aromatic ring structures and saturation in the mycobactinic acid side chain. The mass spectrum of the aluminium complex of mycobactin N (Table 3) supported the deductions already made from other physical measurements. As with aluminium mycobactin M there was no daughter ion in the m/e 640–680 region, but the first prominent degradation peak was at 446, 14 mass units above the corresponding peak in the aluminium mycobactin M spectrum. The three main parent peaks were also 14 mass units higher in the spectrum for aluminium mycobactin N, and showed a distribution of abundance similar to the parent peaks in the aluminium mycobactin M spectrum. These results agree with the presence in mycobactin N of an extra methylene group in the mycobactinic acid side chain in a structure otherwise identical with that of mycobactin M. The tentative structure is given in Fig. 1.

Mycobactins from mycobacteria resembling M. marinum. In the classification of mycobacteria a number of species isolated from reptiles and fish, or from superficial human infections arising from injuries during swimming, have been regarded as closely similar to *M. marinum*. Strains described as *M. balnei*, *M. piscium*, *M. thamnophaeos* and *Mycobacterium* sp. A.T.C.C. 9823 (isolated from a long-necked terrapin, *Chelodina longicollis*) were examined for mycobactin production. All grew well but showed considerable variation in the amount of mycobactin formed (Table 1). The mycobactins were not all fully purified, but t.l.c. of the ferric complexes showed that mycobactin M was the principal product in every case. The mycobactins from *M. piscium* and *Mycobacterium* sp. A.T.C.C. 9823 were converted into their aluminium complexes, which were examined by mass spectrometry. Both resembled mycobactin M in showing the strongest parent peak at m/e 881 and the first prominent degradation peak at m/e 432. However, there were differences in the ratios of the relative heights of the parent peaks, corresponding to different distributions in the lengths of the side chains (Table 3).

Mycobactin K from M. kansasii. The low yield of the mycobactin from *M. kansasii* has restricted investigation to a few simple tests. In the usual system of t.l.c. the ferric complex showed an R_F value different from those of other mycobactins, though rather close to the value for ferric mycobactin M. The difference between the two compounds was confirmed by two-dimensional chromatography on silica gel, which gave a clear separation of the two applied as a mixture; the R_F values of ferric mycobactins K and M in this system were respectively 0.28 and 0.32 with ethyl acetate and 0.56 and 0.49 with propan-2-ol as developing solvent.

The structure of mycobactin K has not been determined.

Dissociation of ferric mycobactins in acid conditions

The ferric complexes of the mycobactins, like other complexes of sideramines with trivalent metals, dissociate in strongly acid conditions (Snow, 1969). The dissociation behaviour of ferric mycobactin S is illustrated in Fig. 3. The ferric complex dissolved in chloroform was shaken with acids of various concentrations and the dissociation was determined from the decrease in E_{450} in the chloroform layer. Similar sigmoid curves were obtained with other ferric mycobactins. When fixed concentrations of ferric mycobactin and acid were used, the degree of dissociation differed from one type of ferric mycobactin to another (Table 6) and could be a means of distinguishing them. The marked difference in ease of dissociation in acid between the different ferric mycobactins is surprising in view of their close structural similarities.

Promotion of growth of M. johnei by various mycobactins

The assay of mycobactins by measurement of the growth of *M. johnei* (Wheater & Snow, 1966) has been modified. In order to obtain a consistent response the inoculum needed to be carefully controlled. The organism was normally maintained on media containing a large excess of mycobactin P. For assay purposes it was subcultured in a liquid medium containing sufficient mycobactin P to give

Fig. 3. Dissociation of ferric mycobactin S by mineral acid. Portions of a CHCl_3 solution of the ferric complex were shaken with equal volumes of HCl at graded concentrations. The dissociation was calculated from the diminution in E_{450} in the CHCl_3 layer.

Fig. 4. Growth curves for *M. johnei* in the presence of mycobactin S (a) and mycobactin H (b). Mycobactin concentrations (ng./ml.): ●, 0.3; ▲, 1; ■, 3; ▼, 5; ○, 10; △, 16; □, 30; ▽, 100. Each point is the mean of three replicates.

slightly less than optimum growth. This culture was grown for not more than 10 days, and was then used to prepare the inoculum for immediate use. The cells were removed from the medium containing mycobactin, resuspended and standardized by turbidity measurement. For inoculation a large volume of diluted inoculum was used, so that dispensing errors were negligible. With these modifications replicates gave very good agreement. However, the assay method still showed some difference in response from one experiment to another; comparisons are therefore quoted for single experiments and values should be regarded as relative rather than absolute.

All the mycobactins so far isolated stimulated the growth of *M. johnei* to at least 95% of its maximum rate when they were added to the medium at a concentration of 30 ng./ml. However, there was a spread of activity at lower concentrations. In the comparison between mycobactins P and T, Wheeler & Snow (1966) observed that the course of growth of *M. johnei* differed, and consequently an exact comparison of activities could not be made. Now that a much larger number of mycobactins has been examined, the growth curves appear to fall into two distinct patterns, as shown in Fig. 4. In both types the onset of growth was delayed for several days, and in the experiment illustrated measurement of

turbidity was begun on the eighth day after inoculation. In the growth pattern represented by mycobactin H the curves for the lower concentrations showed a marked extension of the initial lag, and the rate of growth gradually increased over the period of measurement. The growth response to increasing concentrations was very steep; little growth was detectable with mycobactin H below 3 ng./ml. and almost maximum growth was observed at 16 ng./ml. In the pattern represented by mycobactin S there was no noticeable prolongation of the lag even at the lowest concentrations, the curves showing maximum growth rate from the time that measurements began. The growth response was spread over a wider range of concentrations. With mycobactin S significant growth occurred at a concentration of 0.3 ng./ml., an observation confirmed repeatedly in other experiments. A comparison of activity could be made between mycobactins giving the same growth pattern, but between the two patterns the comparison could only be approximate. The grouping of the mycobactins and their relative activities are shown in Table 4. The pattern of growth with mycobactins M and N was somewhat different from the other members of the group, but was close enough for a reasonable comparison to be made. The mixed mycobactins from *M. fortuitum*, containing about 70% of mycobactin H and 30% of mycobactin F,

Table 4. *Relative activities of different mycobactins in promoting the growth of M. johnei*

Mycobactins are divided into two groups according to the pattern of growth. Values show activities relative to mycobactins P and T.

Group P							
Mycobactin	P	Dihydro-P	A	H	R	M	N
Relative activity	(1.0)	2.0	1.5	1.2	1.0	1.6	2.0
Group T							
Mycobactin	T	S	F + H*				
Relative activity	(1.0)	1.6	1.1				

* Mixture containing F and H in the ratio 2:5.

showed a much higher activity than mycobactin H; most of this activity was presumably due to the mycobactin F component. Thus mycobactin F itself may have an activity higher than that of mycobactin S. Variations of substitution within the general mycobactin structure cause only limited changes in growth-promoting activity towards *M. johnei*. The high activity of dihydromycobactin P shows that the double bond adjacent to the carbonyl group in the side chain of this and most other mycobactins is not necessary for growth-promoting action. The features of the mycobactin structure that are essential to activity were not determined. Cobactin P and mycobactin acid P, the products of alkaline cleavage of mycobactin P, show no growth-promoting action (Snow, 1954b). It is probably significant that these relatively large fragments do not contain the three iron-binding centres that are characteristic of bacterial growth factors and consequently do not form iron complexes of such high stability constants as the ferric mycobactins. On the other hand, the water-soluble sideramines, which have iron bound as a hexadentate complex, cannot replace the mycobactins as growth promoters for *M. johnei*. The specificity of the mycobactins may lie in some distinct structural requirement or may depend on the physical properties that distinguish the mycobactins so clearly from other microbial iron-chelating growth factors.

Mutual antagonism of growth-promoting activity in mixtures of mycobactins M and N with other mycobactins

Among the mycobactins the biggest variation from the parent structure is seen in mycobactins M and N. These mycobactins stimulated the growth of *M. johnei* at the usual concentrations. With most mycobactins concentrations above that required for maximum growth have no further effect, but with mycobactins M and N growth was noticeably depressed by higher concentrations. Investigation of this effect led to the unexpected observation that mixtures of mycobactin M or N with mycobactin P

Table 5. *Growth promotion of M. johnei by mycobactins P and M or N, and their mutually antagonistic action*

Values show increases in nephelometer readings after 14 days growth; each value is the mean of three replicates.

Mycobactin P (ng./ml.) ...	0	10	30	100	300	1000
Mycobactin M (ng./ml.)						
0	0	59	59	58	59	59
10	56	47	46	46	49	
100	54	42	42	42	43	
1000	43	33	31	31	32	
Mycobactin N (ng./ml.)						
10	51	46	44	46	49	
100	49	41	41	41	41	
1000	48	40	36	37	35	

or mycobactin S gave less growth of *M. johnei* than that given by the individual mycobactins at the same concentration. Results of a typical experiment are shown in Table 5. In this experiment maximum growth was obtained with mycobactin P even at 10 ng./ml. The mutual antagonism is shown with mycobactin P at this concentration and is not significantly different at higher concentrations. In some other experiments the mutual antagonism was lessened at higher concentrations of mycobactin P and the growth measured was similar to that found with mycobactin M or N alone. The nature of this effect is best illustrated by a comparison of the growth curves (Fig. 5). High concentrations of mycobactin M or N prolonged the initial lag. In a mixture of similar concentrations of mycobactin M or N with small amounts of mycobactin P or S this effect became more pronounced. After the prolonged lag the rate of growth rose and approached that given by mycobactin P on its own. The effect was most striking when the concentration of mycobactin M or N was high, but was quite noticeable when mycobactins M and P were both present at 10 ng./ml. The reason for this behaviour is unknown. Mutual

Fig. 5. Growth curves for *M. johnei* in media containing mycobactins P and M separately or together. Concentrations of mycobactins (ng./ml.): ○, 10 P; □, 10 M; △, 10 P + 10 M; ■, 1000 M; ▲, 10 P + 1000 M. Each point is the mean of three replicates.

Fig. 6. Effect of mycobactin M on the growth of *M. tuberculosis*. ○, No addition; □, 3 µg. of mycobactin M added/ml.; △, 3 µg. each of mycobactins M and P added/ml. Filled points show corresponding readings where the medium was supplemented with ferric iron (15 µg./ml.). Each point is the mean of eight replicates.

antagonism of growth has only been found in combinations of mycobactin M or N with other mycobactins and not between combinations of mycobactins having long side chains in the mycobactinic acid

moiety, e.g. mycobactins P and S. Mycobactin M also inhibited the growth of *M. kansasii* and *M. tuberculosis*, which produce their own mycobactins and are not dependent on an exogenous supply. As with *M. johnei* the effect was mainly a prolongation of lag, and once growth started the rate of growth gradually increased with time (Fig. 6). For this experiment the organisms were grown on a medium of low iron content, which is known to increase the endogenous production of mycobactin. The effect was also seen when iron was added to the medium, though growth was somewhat increased both in the presence and absence of mycobactin M. The addition of mycobactin P decreased, but did not abolish the action of mycobactin M. The concentrations of mycobactins M and P used were 3 µg./ml., which approaches the saturation concentration in aqueous media. The growth-inhibitory effect of mycobactin M on *M. tuberculosis* is striking, but is unlikely to have any practical value in inhibiting tuberculous infections.

Significance of mycobactins in the taxonomy of the mycobacteria

Now that a wider range of organisms has been studied, the prospect of using mycobactins as an aid to the classification of mycobacteria seems increasingly attractive. The organisms originally selected for examination were based on Tsukamura's (1967) classification, which has common features with the classifications of Collins (1966) and Wayne (1967). Tsukamura (1967) considered that 36 named strains could be divided into ten clearly distinguished clusters by assessment of 95 varied characteristics. We have chosen a strain from each of nine of these clusters and have grown them on media deficient in iron to promote the production of mycobactins. (Tsukamura's classification included *M. parafortuitum* as the sole representative of one group. The organism received under this name from the culture collection was contaminated, and no satisfactory culture could be isolated; we have therefore been unable to examine it.) Selected organisms from seven of the groups each produced a single chemically distinct type of mycobactin with only the smallest traces of other types. Clear evidence for differences in the structure of the nuclei was obtained for six of these mycobactins. The mycobactin from *M. kansasii* was obtained in quantities too small for critical examination, but by physical tests we distinguished it from the mycobactins produced by other groups of mycobacteria. The organisms representative of the two remaining groups in Tsukamura's classification each produced two different mycobactins in substantial amounts. *M. marinum* gave two closely related products, mycobactins M and N, which differed markedly

from mycobactins produced by strains from other clusters; *M. fortuitum* gave mycobactin H, identical in nucleus with the mycobactin characteristic of *M. thermoresistibile*, together with mycobactin F, which has not been found in any other strain. Thus every one of the nine selected strains produced a chemically distinct mycobactin, and in only one case was the distinction blurred by the additional production of a mycobactin characteristic of another cluster. As we had a special interest in mycobactin M, we examined other strains classified in the same cluster as *M. marinum*. Four named strains from this cluster gave mycobactins that appeared to be identical with mycobactin M. An unclassified mycobacterium, received from Professor A. L. Olitzky, was shown to produce mycobactin S, the mycobactin characteristic of *M. smegmatis* (White & Snow, 1969). This mycobacterium has since been classified as *M. smegmatis* (D. Marks, personal communication). Thus, although the number of strains examined is insufficient to justify a generalization about mycobactin production, the correlation between mycobactin type and Tsukamura's classification is remarkably good. It would be of interest to extend the survey to a much wider range of strains. In particular, examination of the mycobactins may throw further light on the classification of the organisms in group 6 of the scheme proposed by Tsukamura, Mizuno & Tsukamura (1969). The authors point out that the mycobacteria in this

group, which includes *M. avium*, *M. terrae*, *M. gastri*, *M. xenopei* etc., may be too similar to be regarded as separate species, yet as a group they are more heterogeneous in characteristics than are other clusters in the classification scheme. In a later paper Tsukamura (1969) suggests that these strains may all be regarded as belonging to the species *M. avium* with eight defined sub-species. Two more mycobacteria, *M. microti* and *M. novum*, are now considered by Tsukamura *et al.* (1969) as sufficiently distinct to be allocated to separate groups; a study of their mycobactins would provide a further test of the correlations already found.

The practical use of mycobactins for taxonomic purposes clearly depends on reliable and relatively simple methods of obtaining and identifying mycobactins from the organisms. For this purpose the mycobacteria must first be grown in substantial quantity. All the mycobacteria so far investigated grow easily on one of two synthetic media. A sufficiently low iron content can be assured by use of ordinary A.R. chemicals and deionized distilled water. For many organisms surface cultures from 5l. of medium will provide adequate mycobactin for identification. For those that produce only small amounts of mycobactin (e.g. *M. tuberculosis*, *M. aurum* and *M. kansasii*) cultures from 25l. of medium may be needed. The mycobactins are obtained as ferric complexes at a purity varying from 3 to 35% (w/w) by simple solvent extraction. These crude

Table 6. *Properties of mycobactins and their derivatives suitable for their characterization*

T.l.c. method A: adsorbent Al_2O_3 , solvent cyclohexane-2-methylpropan-2-ol (9:1, v/v), R_R refers to the R_F value as a fraction of the R_F of ferric mycobactin R. T.l.c. method B: adsorbent SiO_2 , solvent propan-2-ol. Details of the measurement of the dissociation of ferric complexes are given in the text. Substitution in the benzene ring was determined from the wavelengths of the absorption maxima of the metal-free mycobactin.

Mycobactin	Source	Ferric complex			Substitution in benzene ring	Aluminium complex First prominent degradation peak in mass spectrum (m/e)
		T.l.c.		Fraction dissociated by acid under specified conditions		
		Method A R_R	Method B R_F			
R	<i>M. terrae</i>	1.00	0.71		H	668
N	<i>M. marinum</i>	0.93	0.63		H	446
P	<i>M. phlei</i>	0.82	0.64	0.50	Me	682
K	<i>M. kansasii</i>	0.65	0.56			
M	<i>M. marinum</i>	0.60	0.49	0.37	H	432
H	<i>M. thermoresistibile</i>	0.35	0.50	0.45	Me	668
F	<i>M. fortuitum</i>	0.33	0.50		H	654
T	<i>M. tuberculosis</i>	0.31	0.57* 0.63*		H	640
A	<i>M. aurum</i>	0.22	0.39		Me	654
S	<i>M. smegmatis</i>	0.19	0.48	0.28	H	640

* Double spot.

products can be used directly for t.l.c. and for acid dissociation measurements. T.l.c. of the ferric complex provides a good preliminary classification (Table 6) and can distinguish all the mycobactins so far known except for mycobactins H and F. The primary separation is by use of the system of White & Snow (1968, 1969), which distinguishes between most of the ferric mycobactins. A second run on silica gel with propan-2-ol resolves most of the ambiguities of the first separation, and the distinction between ferric mycobactins K and M can be achieved by a two-dimensional method as discussed above. The degree of dissociation of the ferric mycobactin by acid under standard conditions may provide another independent measurement of distinguishing value. The results given in Table 6 show the possibilities of this method, but it is not yet sufficiently explored to assess its value fully. It can only be usefully applied where one type of mycobactin is predominant; a mixture gives ambiguous results.

If further characterization of the mycobactins is required the crude product must be purified. This can be done either by small chromatography columns or by preparative t.l.c. A few milligrams of material of about 90% purity are sufficient for the remaining tests. The ferric complex is easily converted on this scale into the metal-free form and thence into the aluminium complex. The wavelengths of the absorption maxima of the u.v. spectrum of the metal-free product show whether the benzene ring contains a hydrogen atom or a methyl group at position 6 (Snow, 1965*b*). Also, the relative heights of the peaks at high and low wavelengths show whether the side chain on the mycobactinic acid moiety contains the usual $\alpha\beta$ -double bond or whether, as in mycobactins M and N, it is saturated. Finally, the mass spectrum of the aluminium complex provides good evidence distinguishing the nuclei of the mycobactins through the m/e value of the first prominent degradation peak. This should remove any remaining ambiguity. A mixture of two nuclear types, as in the product from *M. fortuitum*, is easily detected unless the two nuclei happen to have the same molecular weight. The mass spectrum also provides information about the number of different side chains in the mycobactin and indicates their relative abundance. Each mycobactin, as isolated by present methods, consists of a number of closely related compounds differing only in the side chain. The chain lengths and relative amounts of the different homologues vary considerably from one mycobactin to another. Mycobactins R and A isolated from *M. terrae* and *M. aurum* respectively were almost entirely homogeneous chemical compounds with one defined side chain, whereas other mycobactins were mixtures of four or more homologues. The lengths and relative abundance of the

side chains in a mycobactin are of less obvious significance than the structure of the nucleus as a guide to the classification of mycobacteria. However, they may help to distinguish closely related strains. Mycobacteria related to *M. marinum* were shown to produce mycobactin M, and among those examined by mass spectrometry (Table 3) the major side chain component was consistently $C_{17}H_{35}$ (R_4 in Fig. 1). The pattern of distribution of other side chains differed markedly between *M. marinum*, *M. piscium* and *Mycobacterium* sp. A.T.C.C. 9823. With *M. marinum* $C_{16}H_{33}$ was the second most abundant side chain whereas with the other two strains $C_{15}H_{31}$ was more abundant than $C_{16}H_{33}$. *Mycobacterium* sp. A.T.C.C. 9823 was distinguished by the high proportion of the $C_{17}H_{35}$ side chain together with smaller amounts of five other chain lengths. It may be significant that the side-chain pattern in mycobactin N resembled that found in mycobactin M derived from the same organism. The diagnostic value of side-chain analysis can be assessed only after many more strains have been examined.

Further research will probably reveal yet more mycobactins. In the past the determination of the chemical structure of a new mycobactin has required a fairly elaborate chemical degradation procedure. However, sufficient experience has now been obtained of the n.m.r. spectra of the mycobactins to enable the variable substituents in the nucleus to be identified (Greatbanks & Bedford, 1969). Thus if 10mg. of a pure metal-free mycobactin can be isolated its chemical structure can probably be characterized by this means alone. The method may not distinguish mycobactins (e.g. S and T) where the nuclei differ only in configuration at an asymmetric centre.

A number of suggested methods of identifying mycobacterial species have been based on compounds produced by the mycobacteria, such as lipids (Marks & Szulga, 1965; Szulga, Jenkins & Marks, 1966; Jenkins, 1969; Reiner, Beam & Kubica, 1967), esterases (Cann & Willox, 1965; Nakayama & Takeya, 1967) and proteins, by antigenic analysis (Tuboly, 1965; Beck & Stanford, 1968; Stanford & Beck, 1968). All these methods involve a separation procedure followed by recognition of different patterns of compounds in the mixtures from different strains of mycobacteria. They are purely empirical since the chemical nature of the components is unknown. Conditions are chosen to show up differences between strains already classified on other grounds, and can be adjusted to detect quite small differences between closely related strains. The use of the mycobactins in taxonomy represents a different approach. Each mycobactin has a chemically defined nucleus and, in most of the mycobacteria so far examined, one predominant mycobactin is produced. A classifica-

tion based on the type of mycobactin present in the cells is thus entirely objective. So far there has been good agreement with other more elaborate classifications. Identification of mycobactins may find a useful place in deciding the taxonomy of the genus.

EXPERIMENTAL

For details of solvents, methods of mass spectrometry and vapour-phase chromatography see White & Snow (1968, 1969). These papers also give details of t.l.c. on alumina; this has been supplemented by t.l.c. of the ferric mycobactins on silica gel G (E. Merck A.-G., Darmstadt, Germany), with propan-2-ol for one-dimensional separations or ethyl acetate followed by propan-2-ol for two dimensional separations. As with the alumina plates, the silica gel was coated as a very thin (0.13 mm.) layer compacted as closely as possible, and the compounds were applied in CHCl_3 solution to give spots no greater than 2 mm. diameter.

Growth of organisms

Conditions of growth and the strains used have already been described for *M. terrae* and *M. marinum* (White & Snow, 1968). *M. balnei* (A.T.C.C. 11564), *M. piscium* (A.T.C.C. 9819), *M. thamnophae* (A.T.C.C. 4445), *M. kansasii* (A.T.C.C. 12478) and *Mycobacterium* sp. (A.T.C.C. 9823) were grown for 3 weeks on medium I at 30°; *M. fortuitum* (N.C.T.C. 8573) and *M. aurum* (N.C.T.C. 10438) were grown similarly at 37°. Other conditions of growth and subculture were as before.

Extraction

Organisms grown at 37° were filtered off through muslin, whereas those grown at 30° were more slimy and were therefore removed by centrifugation. Cells from 10l. of medium were suspended in ethanol (1l.) for 2 days to kill them and to extract the mycobactin; they were then filtered off. Ethanol- FeCl_3 was added slowly to the extract until the maximum red-brown colour had been formed; CHCl_3 (500 ml.) and water (500 ml.) were added to form two layers. The chloroform-soluble fraction (A) was subsequently divided into a methanol-insoluble (B) and a methanol-soluble fraction (C) (White & Snow, 1969). Yields are shown in Table 1. Fractions C containing the ferric mycobactins were used for further purification.

Purification

Values in square brackets show $E_{1\%}^{1\text{cm}}$ at 450 nm. in methanol for the various stages of the purifications.

Ferric mycobactins from *M. fortuitum*. The crude material (1.32 g.) [13.9] was purified by a single treatment using column chromatography (conditions as in stage 3, White & Snow, 1969). This gave a mixture of ferric mycobactins H and F as a red-brown glass (0.32 g.) [42.0].

Ferric mycobactin R. Material (0.27 g.) [15.8] from the preliminary extraction of *M. terrae* was dissolved in cyclohexane (10 ml.) and adsorbed on a column (6 cm. $\times$ 1 cm.) of neutral alumina suspended in cyclohexane. The column was washed with cyclohexane (50 ml.) to remove oily im-

purities and the product was then eluted with CHCl_3 (25 ml.), giving a red-brown gum (156 mg.) [27.0]. This product was submitted to countercurrent separation with the solvent system described by White & Snow (1968). Volumes of each upper and each lower phase were 3 ml., and 160 transfers were made. The peak concentration of ferric mycobactin R was at tube 86. Solutions from tubes 61–104 were combined and the ferric mycobactin (78 mg.) [34.2] was extracted. The product was stirred with methanol (2 $\times$ 5 ml.) at -10° and filtered to remove some undissolved impurities. Evaporation of the filtrate gave a red-brown glass (71 mg.) [38.0]. The ferric mycobactin R was not further purified, but was converted into the metal-free product.

Ferric mycobactins M and N. Fraction C from the extraction of *M. marinum* varied considerably in purity from one experiment to another, according to the amount of colourless oily material produced, and varied in the relative amounts of ferric mycobactins M and N present. The purification described was for a product of low purity containing a moderate proportion of ferric mycobactin N. Fraction C (18.2 g.) [1.04] was adsorbed on alumina and eluted as for ferric mycobactin R. The product (2.46 g.) [7.5] was submitted to a countercurrent separation similarly to ferric mycobactin R, but with phase volumes of 20 ml. (160 transfers). The ferric mycobactins were found in tubes 51–100; t.l.c. showed the peak for ferric mycobactin M to be at tube 65 and for ferric mycobactin N at tube 80. No separation of the components was attempted at this stage. The combined product from tubes 51–100 (0.59 g.) [29.7] was submitted to a second countercurrent separation in the same solvents with 325 transfers. Material from tubes 101–150 was nearly pure ferric mycobactin M (302 mg.) [39.7]. Material from tubes 151–220 (275 mg.) [19.1] contained ferric mycobactin N still with some ferric mycobactin M and other impurities. It was purified in small amounts by three successive runs of preparative t.l.c. with the usual solvent system.

Other ferric mycobactins. Ferric mycobactins from *M. aurum*, *M. piscium* and *Mycobacterium* sp. A.T.C.C. 9823 were purified on a small scale by preparative t.l.c. (White & Snow, 1969). Sufficient material was obtained for measurements of absorption spectra and for mass spectrometry. The ferric mycobactins from *M. kansasii*, *M. balnei* and *M. thamnophae* have been studied only in an impure form.

Properties of the ferric mycobactins

None of the present ferric mycobactins was obtained crystalline. All were red-brown glasses; their purity was judged by the $E_{1\%}^{1\text{cm}}$ value at 450 nm. and by constancy of properties on repeated application of purification methods. Their R_R values are given in Table 6.

Ferric dihydromycobactin P was prepared from dihydromycobactin P (see below). It had λ_{max} 253 and 440 nm. ($E_{1\%}^{1\text{cm}}$ in methanol 187 and 42 respectively), inflexions at 283 and 300 nm. The absorption spectra of ferric mycobactins M and S are shown in Fig. 2. **Ferric mycobactin M** had λ_{max} 282 and 450 nm. ($E_{1\%}^{1\text{cm}}$ in methanol 69.1 and 39.7 respectively), inflexions at 295 and 328 nm. The absorption spectra of the ferric mycobactins from *M. thamnophae* and *Mycobacterium* sp. A.T.C.C. 9823 closely resembled that of ferric mycobactin M; the spectrum of ferric mycobactin R resembled that of mycobactin S whereas ferric mycobactin A had a spectrum very similar to that of mycobactin P

(Snow, 1965b). The absorption spectrum of the mixed product from *M. fortuitum* was intermediate between those of ferric mycobactins P and S.

Dissociation of ferric mycobactins in the presence of acid. To demonstrate the dependence of dissociation on acid concentration a solution of the ferric mycobactin of convenient concentration was prepared in CHCl_3 . Measured portions of this solution were shaken mechanically for 15 min. with equal volumes of HCl solutions of graded concentrations from 0.2 M to 4 M. Care was taken to ensure that equilibration was complete. The CHCl_3 layers were separated and dried over Na_2SO_4 , and E_{450} values were measured. The fractional decrease in E_{450} indicated the extent of dissociation of the ferric complex. A typical dissociation curve is shown in Fig. 3. For comparison between different ferric mycobactins equal concentrations (0.25 $\mu\text{mole/ml.}$) of the compounds in CHCl_3 were shaken with a fixed concentration (1.8 M) of HCl exactly as before. The same solutions were also shaken with 4 M-HCl, sufficient to ensure complete dissociation, and the E_{450} values thus corrected for any extraneous absorption. The results were expressed as fraction of the ferric compound dissociated under the chosen conditions (Table 6).

Preparation and properties of the mycobactins

The ferric mycobactins were converted into mycobactins by shaking their chloroform solutions with 5 M-HCl (Snow, 1965b). The mycobactins were dissolved in chloroform and were reprecipitated by addition of ether. Where the ferric mycobactin was not completely pure, this step formed the final stage of the purification. Purity was checked by re-conversion into the ferric complex and measurement of $E_{1\text{cm.}}^{1\%}$ at 450 nm. The mycobactins were all creamy-white powders.

Mycobactin R. This had m.p. 183° , $[\alpha]_D^{25} + 9.1 \pm 0.6^\circ$ (c 0.53 in methanol) and $\lambda_{\text{max.}}$ in methanol 243, 249.5, 304 nm. ($E_{1\text{cm.}}^{1\%}$ 180, 180 and 60 respectively), inflexion at 258 nm. The absorption spectrum resembled that of mycobactin S (Fig. 2).

Mycobactin M. This had m.p. 163° , $[\alpha]_D^{25} + 3.4 \pm 0.1^\circ$ (c 1.82 in methanol) and $\lambda_{\text{max.}}$ in methanol 242, 249 and 305 nm. ($E_{1\text{cm.}}^{1\%}$ 128, 130 and 55 respectively), inflexion at 258 nm. The absorption spectrum is shown in Fig. 2.

Mycobactin N. This had m.p. 166° and $\lambda_{\text{max.}}$ in methanol 242, 249 and 305 nm. ($E_{1\text{cm.}}^{1\%}$ 122, 124 and 54 respectively), inflexion at 257.5 nm. The absorption spectrum resembled that of mycobactin M.

Mixed mycobactins from *M. fortuitum* (probably: 70% mycobactin H and 30% mycobactin F). This had $[\alpha]_D^{25} + 12.4 \pm 0.2^\circ$ (c 1.27 in methanol) and $\lambda_{\text{max.}}$ in methanol 249 and 308 nm. ($E_{1\text{cm.}}^{1\%}$ 159 and 45 respectively). The absorption spectra could be matched by a mixture of 70% mycobactin H and 30% mycobactin S.

Dihydromycobactin P. Mycobactin P (Snow, 1965a) containing mixed side chains (193 mg.) was dissolved in methanol (25 ml.). Platinum on charcoal (5%, w/w) (10 mg.) was added and the solution was shaken for 5 hr. in an atmosphere of H_2 (uptake was about 6 ml. at 20°). The catalyst was filtered off and the solvent evaporated. The residue was dissolved in CHCl_3 and reprecipitated with ether. **Dihydromycobactin P** had m.p. 175° and $\lambda_{\text{max.}}$ 249, 257 and 312 nm. ($E_{1\text{cm.}}^{1\%}$ 112, 111 and 39 respectively), inflexion at 265 nm. The absorption spectrum is shown in Fig. 2.

Aluminium complexes of mycobactins. For mass spectrometry small amounts of the mycobactins were converted into their aluminium complexes by the method used for mycobactin T (Snow, 1965b). Results of mass spectrometry are in Tables 2 and 3.

Degradation of mycobactins

The methods previously applied to mycobactins T (Snow, 1965b), S and H (White & Snow, 1969) have been followed for the mycobactins from *M. terrae* and *M. fortuitum*. The degradation of mycobactin M required different conditions, which are described below.

Mycobactin R. Alkaline scission of mycobactin R (51 mg.) gave crude cobactin R (14 mg.) and mycobactinic acid R (36 mg.). Acid hydrolysis of mycobactinic acid R gave an ether-soluble fraction containing salicylic acid (identified by i.r. spectrum) and a fatty acid fraction that was methylated and examined by vapour-phase chromatography (Table 2); it also gave a water-soluble fraction containing serine, 2-amino-6-hydroxyaminohexanoic acid and a trace of lysine. The dried water-soluble residue had $[\alpha]_D^{25} + 14.6 \pm 0.1^\circ$ (c 2.1 in m-HCl). **Cobactin R** purified by crystallization from acetone had m.p. 171° , $[\alpha]_D^{24} - 46.8 \pm 0.5^\circ$ (c 0.43 in water) and mol.wt. (by mass spectrometry) 258. Acid hydrolysis of cobactin R (10 mg.) and separation of the products by the usual methods gave (+)-erythro-3-hydroxy-2-methylpentanoic acid as a pale-yellow oil, $[\alpha]_D^{25} + 10^\circ$ (c 0.3 in methanol, concentration determined by titration of the acid), identified by n.m.r., which also showed the absence of the corresponding $\alpha\beta$ -unsaturated acid. The other hydrolysis product was identified as 2-amino-6-hydroxyaminohexanoic acid. For comparison, cobactin P had $[\alpha]_D^{24} - 80.2 \pm 0.5^\circ$ (c 0.4 in water).

Mycobactin mixture from *M. fortuitum*. The mixed product (78 mg.) treated with alkali gave a mycobactinic acid (56 mg.) and a cobactin moiety (20 mg.). Acid hydrolysis of the mycobactinic acid moiety gave ether- and water-soluble fractions. Steam-distillation of the ether-soluble material gave a distillate containing *m*-cresol identified by u.v. and i.r. spectra. Separation of the residual material gave salicylic acid and a mixture of fatty acids that were methylated and examined by vapour-phase chromatography (Table 2). The water-soluble fraction contained only threonine, 2-amino-6-hydroxyaminohexanoic acid and a trace of lysine. The crude cobactin fraction was crystallized from acetone with the addition of a little charcoal to give a product that had m.p. 121.5° , $[\alpha]_D^{25} - 57.3 \pm 0.2^\circ$ (c 0.88 in water) and mol.wt. (by mass spectrometry) 230, identified as cobactin S (White & Snow, 1969). A mixed-m.p. determination with cobactin S gave no depression.

Mycobactin M. Mycobactin M (50 mg.) was dissolved in methanol (1 ml.) and m-NaOH (1 ml.) was added. After 45 min. at 20° the solution was neutralized with m-HCl (1 ml.); a gelatinous precipitate formed. The suspension was kept at 1° for 16 hr. and then centrifuged. The precipitate was washed repeatedly by resuspension in water (4 $\times$ 5 ml.) and was finally dried over P_2O_5 at 60° . The product (30 mg.) was dissolved in ether (20 ml.); the solution was washed with water (20 ml.), dried over Na_2SO_4 and concentrated to 2 ml. From this solution, kept at 1° , **cobactin M** was precipitated as a white amorphous powder (19.3 mg.) that had m.p. 129° and $[\alpha]_D^{25} + 3.2 \pm 0.1^\circ$ (c 1.62 in methanol); it was shown by mass spectrometry to be a homologous

mixture [Found: C, 68.0; H, 10.9; N, 5.9. $C_{27}H_{52}N_2O_4$ (the main component) requires C, 69.1; H, 11.1; N, 6.0%]. Cobactin M (48 mg.) was hydrolysed with acid under the usual conditions. The product was extracted with ether. Evaporation of the ether extract (decolorized with a little charcoal) gave a white waxy solid (25 mg.), m.p. 62°, and shown by n.m.r. to be a mixture of long-chain hydroxy acids having the formula $C_nH_{2n+1}\cdot CH(OH)\cdot CH(CH_3)\cdot CO_2H$ (erythro) with 30% of the corresponding $\alpha\beta$ -unsaturated acids (from mass-spectrometric examination of cobactin M, n was mainly 17 with smaller amounts of 16, 15 and 14). The mixed hydroxy acid fraction had $[\alpha]_D^{25} + 12^\circ$ (corrected for the presence of 30% optically inactive unsaturated acid) (c 0.9 in $CHCl_3$). The aqueous solution from the acid hydrolysis contained 2-amino-6-hydroxyamino-hexanoic acid.

The aqueous supernatant, after separation of the crude cobactin from the products of the original alkaline cleavage, was combined with the water washings (total vol. 25 ml.); the solution was fluorescent. It was extracted with ether (6 x 10 ml.), and the extract was washed with water (20 ml.), dried over Na_2SO_4 and evaporated. The product (15.5 mg.) was mycobactin acid M, obtained as a colourless glass having a u.v. spectrum similar in pattern to that of the parent mycobactin. Acid hydrolysis gave an ether-soluble fraction containing salicylic acid, but no long-chain fatty acids; it also gave a water-soluble fraction containing threonine, 2-amino-6-hydroxyamino-hexanoic acid and a trace of lysine. Vapour-phase chromatography indicated the probable presence of acetic acid in the unseparated acid hydrolysate.

Experiments on the promotion and inhibition of growth of M. johnei by mycobactins

M. johnei was grown on slopes as before. The culture was collected by a platinum loop and transferred to Hart's medium containing mycobactin P (10 μ g./ml.). After 10 days this culture was used to inoculate Hart's medium containing mycobactin P (10 ng./ml.); 1 ml. of primary liquid inoculum was added to 200 ml. of medium. After 8–10 days growth on this second medium the cells were centrifuged off and resuspended in Hart's medium containing no added mycobactin. The concentration of this bacterial suspension was adjusted to give a nephelometer reading of 80 on the arbitrary scale used for assay readings (set by reference to a selected solid turbidity standard). This adjusted solution was diluted to 100 times its own volume with blank Hart's medium to give the assay inoculum. For assay purposes *M. johnei* was grown in 6 in. x 1 in. rimless test tubes fitted with stainless-steel caps. Each tube received 10 ml. of blank Hart's medium and 0.2 ml. of propane-1,2-diol containing

any required addition; control tubes received the solvent and medium only. All necessary dilutions were made in propane-1,2-diol before addition to the final medium. Each tube then received 10 ml. of the assay inoculum, which was continuously stirred and was dispensed aseptically with an automatic syringe. Turbidity measurements were made with an EEL nephelometer in which the upper reflector had been modified to accept 1 in.-diameter tubes. The turbidity measurements made immediately after inoculation were used as blanks and were subtracted from all subsequent readings on corresponding tubes. The tubes were incubated and turbidities were read at intervals as in the earlier method. Before each reading the tubes were gently swirled to ensure that all the cells were suspended; otherwise they were left undisturbed. It was found important to apply the same amount of agitation to all tubes to ensure maximum uniformity of readings.

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