Biochem. J. (1968) **108**, 593
Printed in Great Britain

Methods for the Separation and Identification of Mycobactins from Various Species of Mycobacteria

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(*Received* 12 *February* 1968)

A single pure component was isolated from mycobactin P by countercurrent distribution; its side chain is *n-cis*-octadec-2-enoyl; its purity and molecular structure were confirmed by mass spectrometry of its aluminium complex. The separation of ferric and of aluminium complexes of mycobactins by thin-layer chromatography is described. *Mycobacterium terrae, M. marinum* and *M. smegmatis* produce mycobactins that differ among themselves and from mycobactins P and T. A nomenclature for the mycobactins and their derivatives is suggested.

The mycobactins from *Mycobacterium phlei* have been shown (Snow, 1965*a*) to consist of a family of compounds having the same nucleus but differing in their side chains; the mycobactins from *Mycobacterium tuberculosis* (Snow, 1965*b*) are also a family based on a slightly different nucleus. The separation of one member of the mycobactin P group is reported here. The production of mycobactins by several other species of mycobacteria was demonstrated, and methods are described for distinguishing and identifying distinct groups of mycobactins.

NOMENCLATURE

The terms mycobactin P and mycobactin T have been used to describe families of compounds from *M. phlei* and *M. tuberculosis* respectively. We propose to retain this nomenclature and to distinguish between individual members of each family by a suffix indicating the number of carbon atoms and the presence of unsaturation in the side chain; this can be extended if necessary to describe branching in the side chain. For other mycobactins we propose to use a separate letter for each family in which there is chemical evidence for a difference in the nucleus, or where the behaviour in separation methods of the kind described in this paper suggests that such a difference probably exists. Mild alkaline degradation of a mycobactin splits the molecule into two fragments, the mycobactic acid and the cobactin. These will be identified by the same letters as the mycobactin from which they are derived; the individual members of the mycobactic acid families will be defined by suffixes where necessary. Some redundancy of lettering may be expected in naming these fragments since different mycobactins may be identical in one or other fragment.

As an example of the proposed nomenclature the main component of mycobactin P (Snow, 1965*a*), which has now been separated in a pure form, is described as mycobactin P 18-*cis*-Δ_2.

MATERIALS AND METHODS

Solvents. Cyclohexane was 'special for spectroscopy' grade from British Drug Houses Ltd., Poole, Dorset; the use of this grade (free from aromatic compounds) was found to be essential both for countercurrent distribution and for thin-layer chromatography. Propane-1,2-diol was reagent grade from Hopkin and Williams Ltd., Chadwell Heath, Essex. 2-Methylpropan-2-ol was reagent grade from Hopkin and Williams Ltd.; it was redistilled and the fraction with b.p. 82–83° was used.

Chemicals for media. L-Asparagine was reagent grade from Hopkin and Williams Ltd.; it was found to contain less than 5μg. of Fe/g. Other chemicals were of A.R. grade.

Media for growth of bacteria. For both medium I and medium II two separate solutions were prepared as shown in Table 1. The first solution was adjusted to pH 6·8. Both solutions were then autoclaved and mixed aseptically. The water was distilled in glass and then deionized by passage through a mixed-bed resin. All vessels for preparation of medium and growth of bacteria were rigorously cleaned with HNO_3, washed and rinsed with deionized distilled water. Both media contained less than 150μg. of Fe/l.

Growth of organisms. All the organisms were grown first on slopes of Lowenstein–Jensen egg medium (Mackie & McCartney, 1949), usually for 7–10 days; with *M. tuberculosis* H 37R_V 14–21 days' growth was necessary. The organisms were washed off aseptically from two or three slopes with the appropriate liquid growth medium and diluted to 100ml. with medium. Portions (1ml.) of this suspension were used to inoculate medium (100ml. quantities) contained in round screw-cap bottles of 560ml. capacity. The bottles were stacked on their sides and incubated; growth occurred mainly on the surface of the liquid. Strains of organism, medium and time of incubation are given in Table 4. *M. phlei* and *M. smegmatis* cultures grown in liquid medium for 1 week could be used to inoculate fresh medium (1ml. of inoculum/100ml. of medium). With the other organisms inoculation required fresh isolates from slopes.

Table 1. *Compositions of media for the growth of bacteria*

	Medium I	Medium II
First solution		
KH_2PO_4	1g.	1g.
Na_2HPO_4	2g.	2g.
Glycerol	10ml.	30ml.
L-Asparagine	5g.	5g.
Water	500ml.	500ml.
Second solution		
Glucose	50g.	10g.
$MgSO_4,7H_2O$	0·2g.	0·2g.
Water	500ml.	500ml.

Isolation of crude mycobactins. Cells from each organism were harvested by filtration and were suspended in ethanol for 24hr. to extract the mycobactin; the bacterial residue was filtered off and the extract was mixed with an equal volume of chloroform, and ethanolic $FeCl_3$ solution was added slowly until there was no further increase in the formation of the red-brown colour of ferric mycobactin. Sufficient water was added to form two layers. The lower layer containing ferric mycobactins was washed with water, dried over Na_2SO_4 and evaporated to dryness.

The residue was extracted with methanol at room temperature and the extract, separated from insoluble material, was evaporated to dryness. The crude preparations of ferric mycobactins thus obtained were examined by thin-layer chromatography. In some cases the iron was removed by acid treatment and the metal-free product was converted into the aluminium complex by a method similar to that used for mycobactin T (Snow, 1965*b*).

Mass spectrometry. Measurements were made with the Associated Electrical Industries MS9 double-focusing mass spectrometer, with the solid-sample insertion probe at 70ev.

Vapour-phase chromatography. The Pye 104 model 24 gas chromatograph was used with the flame-ionization detector.

Countercurrent distributions. Cyclohexane–2-methylpropan-2-ol–propane-1,2-diol–water (23:10:10:1, by vol.) mixture was equilibrated at 25° and allowed to settle. An automatic countercurrent machine having 120 tubes (upper and lower phases 20ml. each) was charged with the lower phase of the mixture, the first tube being left empty. Crystalline ferric mycobactin P (Snow, 1965*a*) was dissolved in a mixture of 20ml. of each of upper and of lower phase and the mixed solution was placed in the first tube. Development was carried out with upper phase in the solvent reservoir; 190 transfers were made. The contents of tubes 52–100 containing coloured material were removed and the E_{450} values of upper and lower phases were measured for each tube. From these the partition coefficients were calculated. Solutions from tubes 78–100 were combined; chloroform (1 vol.) was added and the mixture was washed with water (6 × 1vol.), then dried over Na_2SO_4 and evaporated, leaving a red–brown glassy residue. This was crystallized from cyclohexane–2-methylpropan-2-ol (19:1, v/v) to give *ferric mycobactin P* 18-cis-Δ_2, 73·2mg., m.p. 199·5–202°, as thin brown plates (second crop 17·2mg.).

The ferric complex was similarly recovered from tubes 52–77 and was submitted to a second countercurrent distribution similar to the first. Tubes 81–100 in the second distribution gave material (65mg.) identical with fraction I; tubes 64–80 gave fraction II (55mg.), and tubes 45–63 gave fraction III (6·0mg.). Fractions II and III were crystallized similarly to fraction I (melting points in Table 2). Iron was removed from the ferric complexes by the method described by Snow (1965*a*). The metal-free product from fraction I was *mycobactin P* 18-cis-Δ_2 (physical data in Table 2).

Identification of the side-chain component in mycobactin P 18-cis-Δ_2. The metal-free complex from fraction I (42mg.) was converted into the corresponding mycobactic acid P (Snow, 1954); the product (22mg.) was oxidized with periodate and the fatty acid fraction (12mg.) was isolated (Snow, 1965*a*). The fatty acid was converted into the methyl ester by treating its silver salt with methyl iodide (Gehrke & Goerlitz, 1963); the ester was submitted to vapour-phase chromatography; the methyl esters of *cis*- and *trans*-Δ^2-C_{16}–C_{20} acids were used as reference compounds. The conditions were: glass column, 150cm. × 4mm. internal diam.; packing, 2% methyl silicone gum rubber SE30 (F & M Division, Hewlett Packard, Slough, Bucks.) on acid-washed Chromosorb G treated with dimethylchlorosilane, 80–100 mesh (Perkin-Elmer Ltd., Beaconsfield, Bucks.); temperature, 183°; argon, 50ml./min. Retention times are given in Table 3.

Thin-layer chromatography. To obtain reliable results a very thin (0·13mm.) adsorbent layer was necessary. After coating, the plates were dried at 110° for 2hr., cooled in a desiccator over silica gel and used at once. The adsorbent was alumina GF (E. Merck A.-G., Darmstadt, Germany). The metal complexes were applied as the smallest possible spot (no more than 2mm. diam.) and the complexes then ran as small discrete spots without tailing. Ferric complexes were detected as visible red–brown spots or as dark spots in u.v. light. Aluminium complexes were detected by blue fluorescence in u.v. light or by spraying with dilute ethanolic $FeCl_3$ to give red–brown spots.

RESULTS AND DISCUSSION

Separation of a single component from mycobactin P by countercurrent distribution. In trials with various solvent mixtures the most suitable was a quaternary system of cyclohexane, 2-methylpropan-2-ol, propane-1,2-diol and water, forming two phases of almost equal volume at 25°. This gave partition coefficients (upper/lower phase) for ferric mycobactins P and T (both with mixed side chains) 0·66 and 0·39 respectively. Ferric mycobactin P (with mixed side chains) was submitted to a 190-transfer distribution in this system. The distribution (Fig. 1) showed a single peak at tubes 78–79. A theoretical distribution curve calculated for K0·695 closely fitted the experimental points from tube 78 onwards, but in earlier tubes the experimental points were considerably higher than the theoretical curve. The partial separation implied by this result was confirmed by measurement of the E_{450} values of both layers in each tube and calculation of partition coefficients; these rose from 0·56 at tube 56 to 0·69 at tube 78 and then remained constant within the experimental error of measurement. Solutions from

tube 78 onwards were therefore combined and the ferric mycobactin P was recovered and crystallized (fraction I). The ferric mycobactin from the earlier tubes was submitted to a second distribution similar to the first, giving further material identical with fraction I and two other fractions (II and III) with lower partition coefficients; these fractions were also crystallized. Iron was removed from each of the fractions and the melting points and optical rotation of the metal-free products were determined (Table 2). The metal-free product from fraction I was also degraded and the methyl ester of the fatty acid side chain was examined by vapour-phase chromatography. Results are presented in Table 3. Both the constancy of its partition coefficient and the purity of the fatty acid degradation product show that fraction I is substantially a single chemical entity in which the side chain is *n-cis*-octadec-2-enoyl. Fractions II and III are mixtures that must contain mycobactins of other chain lengths. However, the large negative rotation of the metal-free product from fraction III demonstrates the presence of a small amount of material that must differ in some other way, probably at an asymmetric centre in the nucleus.

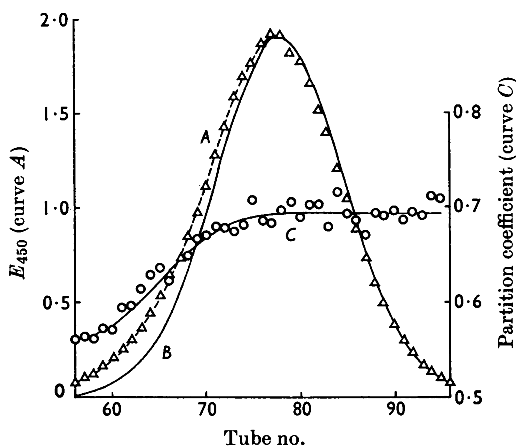

Fig. 1. Countercurrent distribution of ferric mycobactin P. The solvent and other details are given in the text; 190 transfers were made. Curve A (△-- △), E_{450} of lower phase; curve B (——), theoretical distribution for K 0·695; curve C (○—○), partition coefficient.

The original mixed product designated mycobactin P and the purified component fraction I were also examined by mass spectrometry after conversion into their aluminium complexes (Snow, 1965*b*). In both cases the main parent peak was at m/e 893, corresponding to the accepted formula with a C_{18} side chain containing one double bond. In the purified compound no peaks other than those due to natural isotopes could be seen around this mass number; the first degradation peak was at m/e 682, corresponding to breakage of the side chain immediately beyond the double bond, leaving the radical R·CO·CH:CH· (where R represents the undegraded nucleus). The mass spectrum of the aluminium complex of the mixed product showed a

Table 3. *Vapour-phase chromatography of methyl ester of fatty acid degradation product from fraction I of ferric mycobactin P separation*

Reference compounds were methyl esters of Δ^2-normal fatty acids. For column conditions see the text. Approximate proportions in the fatty acid mixture from unseparated mycobactin P were: C_{14}, 3; C_{16}, 5; C_{18}, 85; C_{20}, 5, all Δ^2-*cis* (see Snow, 1965*b*).

	Retention time (min.)					
	C_{16}		C_{18}		C_{20}	
	cis	*trans*	*cis*	*trans*	*cis*	*trans*
Reference compounds	3·8	4·6	8·2	9·9	17·9	22·1
Degradation product	3·8		8·0	9·9	17·5	
% of total in peak	Trace		>99·5	Trace	Trace	

Table 2. *Separation of ferric mycobactin P mixture by countercurrent distribution*

Two successive 190-transfer runs were made with the two phases of the mixture cyclohexane–2-methylpropan-2-ol–propane-1,2-diol–water (23:10:10:1, by vol.).

Iron complex	Mixture before separation	Fraction I	Fraction II	Fraction III
Amount in fraction as % of original mixture		51 (as crystals)	19	2
K (upper/lower phase)	0·66	0·69		
m.p. of crystallized product	199–201°	199·5–202°	193°	181–182·5°
Metal-free material				
m.p.	165–166·5°	168·5°	164·5°	
$[\alpha]_D^{20}$ in methanol	−0·54 ± 0·1° (*c* 0·73)	+1·43 ± 0·1° (*c* 1·09)	−1·68 ± 0·1° (*c* 0·77)	−58·5 ± 0·5° (*c* 0·1)

peak at m/e 865 (about 7% of the height of the main peak), small peaks at m/e 907 and 879 and very small peaks at m/e 921 and 837. The peaks at m/e 921, 865 and 837 correspond to the compounds with C_{20}, C_{16} and C_{14} side chains, each with one double bond, that were expected from the results of vapour-phase chromatography of degradation products (Snow, 1965*a*). The minor components giving peaks at m/e 907 and 879 differ in molecular structure from the main component by CH_2. The two probably have the same nucleus with side chains differing by C_2H_4. It seems unlikely that the side chains are C_{19} and C_{17}, since these would probably have shown up in the vapour-phase chromatography of the degradation products, and thus they more probably differ from mycobactin P by CH_2 in the nucleus, and have side chains with even numbers of carbon atoms. The severe conditions required for mass spectrometry of the aluminium complexes include a relatively high probe temperature and a high amplification of the signal; it is therefore thought unjustifiable to attempt a quantitative assessment of the relative amounts of the different components by measurement of the peak heights in the spectrum.

Mycobactins from various mycobacteria: identification by thin-layer chromatography. Mycobactins P and T were originally isolated from *M. phlei* and *M. tuberculosis* grown on beef infusion broth (Francis, Macturk, Madinaveitia & Snow, 1953; Snow, 1965*b*). Similar yields of these mycobactins have now been obtained from organisms grown on defined media of low iron content. These media have also been used to grow several other species of mycobacteria; extracts from three species contained products having the properties of mycobactins. No satisfactory growth of *M. rhodocrous* (N.C.I.B.8574) could be obtained on these media. This species grew well on nutrient–agar, but the cells gave no detectable mycobactin. However, the assignment of this organism to the genus *Mycobacterium* is in considerable doubt (Gordon, 1966). The total yield of mycobactin in each extract was determined by measurement of E_{450} of the iron complex, corrected for residual absorption after dissociation of the complex with acid (Snow, 1965*b*). The conditions of growth and yields of mycobactin for the different strains are shown in Table 4.

Thin-layer chromatography of the iron or aluminium complexes was found to be a convenient method for characterizing the mycobactins in extracts from mycobacteria. The adsorbent used throughout was alumina GF and many solvent systems were tested. The upper layer from the solvent mixture used in countercurrent separation had a limited use. With ferric mycobactin P (mixture as isolated) separation into a major component (R_F 0·38) and a minor component (R_F 0·31) was observed; this probably parallels behaviour observed on countercurrent separation. This solvent system, however, produced a secondary front on the thin-layer plate and was useless for mycobactins of low R_F value since they were overtaken by the secondary front and then ran with it. More generally useful systems were cyclohexane–2-methylpropan-2-ol–propane-1,2-diol (40:10:1, by vol) and cyclohexane–2-methylpropan-2-ol (9:1, v/v). The former gave higher R_F values. These systems were used to examine the metal complexes of mycobactin-like compounds present in extracts of various species of mycobacteria. Purified ferric and aluminium complexes of mycobactins P and T were also examined. Table 5 shows the R_F values of the major spot for each extract. Most organisms produced only one major type of mycobactin, though extracts from *M. marinum* gave metal complexes showing two main spots, one considerably stronger than the other. All the other mycobacteria produced very small quantities of other mycobactin-like products with iron complexes differing considerably in R_F from the main product. The R_F values of the main spots show that all the organisms mentioned in Table 5 produce distinct mycobactins that probably differ in their nuclei, since side-chain differences appear to affect the R_F only slightly. Distinguishing letters have been given to mycobactins where the metal complexes show such

Table 4. *Conditions of growth of mycobacterial species, and yields of mycobactin from extracts*

For composition of media see the Materials and Methods section. Mycobactin was determined spectrophotometrically as the iron complex in extracts and is expressed as mg. of mycobactin P.

			Incubation			
Species	Reference	Medium used	Temp.	Time (weeks)	Cell yield (mg. dry wt./l. of medium)	Approx. mycobactin yield (mg./l. of medium)
M. phlei	N.C.I.B. 8573	I	37°	3	700–800	11
M. tuberculosis	H 37R_V	II	37	3	100–200	0·1
M. smegmatis	N.C.I.B. 8548	I	37	2	750–900	16
M. marinum	N.C.T.C. 2275	I	28	3	600–700	12
M. terrae	N.C.T.C. 10424	II	37	3	400–600	5

Table 5. *Thin-layer chromatography of metal complexes of various mycobactins*

The adsorbent was alumina GF. The solvent systems were: 1, cyclohexane–2-methylpropan-2-ol–propane-1,2-diol (40:10:1, by vol.); 2, cyclohexane–2-methylpropan-2-ol (9:1, v/v).

		R_F values			
		Ferric complex		Aluminium complex	
Source of mycobactin	Reference letter of mycobactin	Solvent 1	Solvent 2	Solvent 1	Solvent 2
M. terrae	R		0·62		
M. phlei	P	0·76	0·54	0·61	0·43, 0·46
M. marinum	M*		**0·37**, 0·58		**0·31**, 0·49
M. tuberculosis	T	0·46	0·18, 0·22	0·36	0·15
M. smegmatis	S	0·39	0·08	0·28	0·06

* Refers to major component, indicated by **bold** type.

marked differences in R_F that they are almost certain to have different nuclei. Only the more abundant component from *M. marinum* has been lettered, since R_F values for the other component are too close to those of mycobactin P or R to be certainly distinguishable. Further discrimination will call for isolation and chemical degradation. Wherever a comparison has been made, the ratio of the R_F of a ferric mycobactin complex to that of the corresponding aluminium complex is in the approximate range 1·2–1·3:1·0. The reasons for the contiguous double spots with ferric mycobactin T and aluminium mycobactin P in solvent 2 are not known. Thin-layer chromatography provides a quick and convenient method of identifying mycobactins and has already shown the existence of at least five different types. Different species of mycobacteria show considerable specificity in the type of mycobactin that they produce, and this may prove useful for taxonomic purposes.

We thank Mr D. Lowe for selecting suitable conditions for the culture of the micro-organisms and for growing and harvesting them.

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