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Isolation and characterization of carboxymycobactins as the second extracellular siderophores in *Mycobacterium smegmatis*

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The structure of a chloroform-extractable siderophore from the supernatant of a nonpathogenic mycobacteria, *Mycobacterium smegmatis*, has been determined and it closely resembles the structure of mycobactin, the intracellular siderophore found in all mycobacteria. The difference in structures is that the extracellular siderophore has a family of short carboxylic chains attached to the mycobactin nucleus instead of a long alkyl chain and hence the name 'carboxymycobactin' is proposed to distinguish it from mycobactin itself as well as from the major siderophore that is produced – exochelin MS.

Keywords: carboxymycobactin, exochelin, iron, *Mycobacterium smegmatis,* mycobactin

Introduction

In response to iron deficiency, mycobacteria, in common with most other microorganisms, produce extracellular siderophores. In the fast-growing, saprophytic species a water-soluble, peptido-hydroxamate siderophore occurs with evidently different structures between species (Sharman *et al.* 1995a,b). These are termed the exochelins. In the slow-growing, pathogenic species, a chloroform-extractable siderophore occurs (Macham *et al.* 1975) that has been characterized in *Mycobacterium arium* and *Mycobacterium tuberculosis* (Gobin *et al.* 1995, Lane *et al.* 1995) as a variation in the structure of mycobactin, which is an intracellular siderophore found in almost all mycobacteria both saprophytic and pathogenic alike (Snow 1970). In this extracellular siderophore, the long alkyl chain of mycobactin has been replaced by a shorter acyl chain that terminates in a carboxylic acid group and hence the name carboxymycobactin has been given (Lane *et al.* 1995). The term 'exochelin' should now be confined to the water-soluble, non-extractable siderophores as the two types of siderophore are of different types.

Horwitz and colleagues, who have also reported on the structures of the carboxymycobactins from *M. tuberculosis* (Gobin *et al.* 1995) and *M. avium* (Wong *et al.* 1996), have described these as occurring principally as their methyl esters though the highest concentrations of the esterified forms occurred in older (8 week) cultures whereas we (Lane *et al.* 1995) were unable to recognize equivalent materials in *M. avium*, *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) or *M. tuberculosis.* However we only examined the siderophores taken from younger (4–5 week) cultures and at this stage would regard the carboxy forms as the active form and the esters as possibly being artefactual.

Although no exochelin-type siderophore has been recognized in culture filtrates of the pathogenic mycobacteria (M. Ewing and C. Ratledge, unpublished work), a carboxymycobactin has been recovered from *M. smegmatis* where it constitutes no

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more than 10% of the total iron binding capacity (Ratledge & Ewing, 1996). Another carboxymycobactin from another saprophytic mycobacterium has been recognized in culture filtrates of *Mycobacterium neoaurum* (Tan Eng Lee and C. Ratledge, unpublished work) and the occurrence of a second siderophore along with the exochelin might then be common amongst the saprophytes. Our previous and preliminary description of some of the properties of carboxymycobactin MS (i.e. from *M. smegmatis*) (Ratledge & Ewing 1996) indicated, principally on the basis of its UV spectrum, that it possibly had the same relationship to mycobactin S as had carboxymycobactins MAv, MT and MB (i.e. from *M. avium, M. tuberculosis* and *M. bovis* BCG) to their equivalent mycobactins.

We report here the full structure of carboxymycobactin MS which, although having the predicted structure, nevertheless shows some variation in the constitution of the acyl chain to the alkyl chain of mycobactin.

Materials and methods

Organism and growth

Mycobacterium smegmatis NCIMB 8548 was grown with shaking at 37°C on 100 ml lots of glycerol/asparagine medium at pH 8.8 under iron deficient conditions (Ratledge & Hall 1971) in 250 ml conical flasks for six or seven days to optimize production of carboxymycobactin (Ratledge & Ewing 1996).

Isolation of carboxymycobactin

The procedure of Ratledge & Ewing (1996) was followed.

Formation of desferricarboxymycobactin

As ferricarboxymycobactin is soluble in chloroform but desferricarboxymycobactin is soluble in water, it was not possible to use the previous procedures used to desferriate either mycobactin or exochelin. The following procedure, however, was found to be satisfactory. Concentrated H_2SO_4 was added dropwise to approximately 10 mg ferricarboxymycobactin in 10 ml chloroform until the colour had disappeared (~pH 0). The solution was then immediately and repeatedly shaken with 50–75 ml lots of a saturated solution of $(NH_4)_2SO_4$ until the final washings were above pH 5. This procedure effectively removed all the iron complexed to the carboxymycobactin without extraction of the desferricarboxymycobactin which, though soluble in water, was not partitioned appreciably into the saturated $(NH_4)_2SO_4$ solution. The chloroform solution was dried by adding a few mg of anhydrous $MgSO_4$ and then carefully decanted into iron-free glassware and the solvent evaporated under vacuum. Some ferricarboxymycobactin did re-form during the latter stages but this was less than 5% of the total material (see Results).

A portion of the desferricarboxymycobactin was re-ferriated by adding a drop of 1% $FeCl_3$ in ethanol and the resulting ferricomplex compared to the original material by HPTLC (Ratledge & Ewing 1996). No difference in Rf values of the various components of the carboxymycobactin was found.

NMR spectroscopy

A variety of classical NMR experiments were run on a 1.2 mg desferri sample of carboxymycobactins dissolved in 0.7 ml of $DMSO_{d6}$ at 30°C and performed using a Varian INOVA 750 NMR spectrometer. Selected experiments were also run at 80°C under similar conditions. All spectra are reference to the solvent signal at 2.49 p.p.m. and 39.5 p.p.m. in the proton and carbon dimensions respectively. One dimensional proton spectra were recorded with 64K data points. The two dimensional COSY, ROESY, HMQC and HMBC experiments were typically recorded with 2 K data points in F2 and 512 increments in F1. The two dimensional data were zero-filled and weighted by shifted sine bell or shifted sine bell squared functions in both dimensions prior to Fourier transformation. Phase sensitive two dimensional data sets were collected using the States method to achieve quadrature detection in F1. ROESY spectra were recorded with a mixing time of 250 ms and a spin locking field of 3 kHz. The HMBC spectra were run optimized for long range proton–carbon couplings of 10 Hz.

Nanospray mass spectrometry

All the Nanospray ESI experiments were performed on a Finnigan MAT TSQ7000 Mass Spectrometer. The voltage applied was 0.6 kV and the Capillary temperature was 100°C. The MS/MS experiments were performed at –40eV and 1.7 mTorr argon.

High resolution ESI mass spectrometry

All the accurate mass determinations were performed in positive mode electrospray ionization on a Micromass AutospecQ Magnetic Sector instrument. The data were acquired in the voltage scan mode, range 735–790 amu at 5 s decade^{-1} scan speed under continuum data mode. The internal standard reference calibration used ammoniated PEG solution with reference peaks at 740.46438 and 784.49059.

HPLC conditions

HPLC of the carboxymycobactins was carried out on a Spherisorb C18, 5 μm, 15 cm × 4.6 mm i.d. column, with a gradient of water + 0.1% formic acid/90% acetonitrile + 0.09% formic acid (100:0 v/v) for 2 min then to water + 0.1% formic acid/90% acetonitrile + 0.09% formic

acid (0:100 v/v) over 15 min then holding at this concentration for a further 10 min. The elution rate was 1.0 ml min^{-1} and the eluate was monitored continuously at 450 and 304 nm.

Results

NMR

The NMR spectra run on a desferri sample of carboxymycobactin dissolved in $DMSO_{d6}$, included one dimensional proton and two dimensional COSY, ROESY, HMQC and HMBC experiments. Despite the light brown colour of the solution suggesting the presence of some ferri-material, good NMR spectra were obtained (Figure 1).

The NMR data collated indicated the presence of carboxymycobactin related structures (Figure 2) similar to the mycobactin structure characterized for *M. smegmatis* (Snow 1970), but containing the carboxymycobactin's characteristically shortened R_1 sidechain terminating in a carboxylic acid (Lane *et al.* 1995). There was no evidence in the NMR spectra for methyl ester signals, which would give rise to intense singlet resonances in the region of 3 to 4 p.p.m. Proton spectra at 80°C were also recorded to move the residual water signal (3.30 p.p.m. at 30°C) which has a temperature dependent chemical shift. No methyl ester signals were uncovered at higher temperature. Comprehensive NMR assignments were made using the data collected (Table 1).

The proton spectrum recorded at 80°C led to a sharpening of the otherwise broad double bond signals at 6.28 and 5.97 p.p.m. respectively. This enabled the fine structure of the double bond signals to be observed and accurate coupling constants measured. A two dimensional HMBC experiment was also run at 80°C and a correlation from the double bond proton at 5.97 p.p.m. to the carbonyl at 166.0 p.p.m. could then be seen. The broadness of the double bond signals at 30°C followed by sharpening at 80°C, may be indicative of rate processes occurring in the vicinity which are intermediate on the NMR timescale at the lower temperature and rapid at higher temperature.

Subsequent HPLC/MS studies indicated that the sample was primarily a mixture containing an homologous series of carboxymycobactins with variations in the length of the R_1 sidechain. However, little apparent 'doubling' of the NMR signals was observed. This is because of the minimal effect on the characteristic chemical shifts within each homologue due to variations in the R_1 chain length, particularly on signals remote from this site of change.

HPLC/UV/MS

Gradient positive ion electrospray (ESI) HPLC/UV/MS of the desferricarboxymycobactin sample in DMSO (Figure 3) afforded a major series of eight components that exhibited a λ max at 304 nm with molecular weights 14 daltons apart. These data were

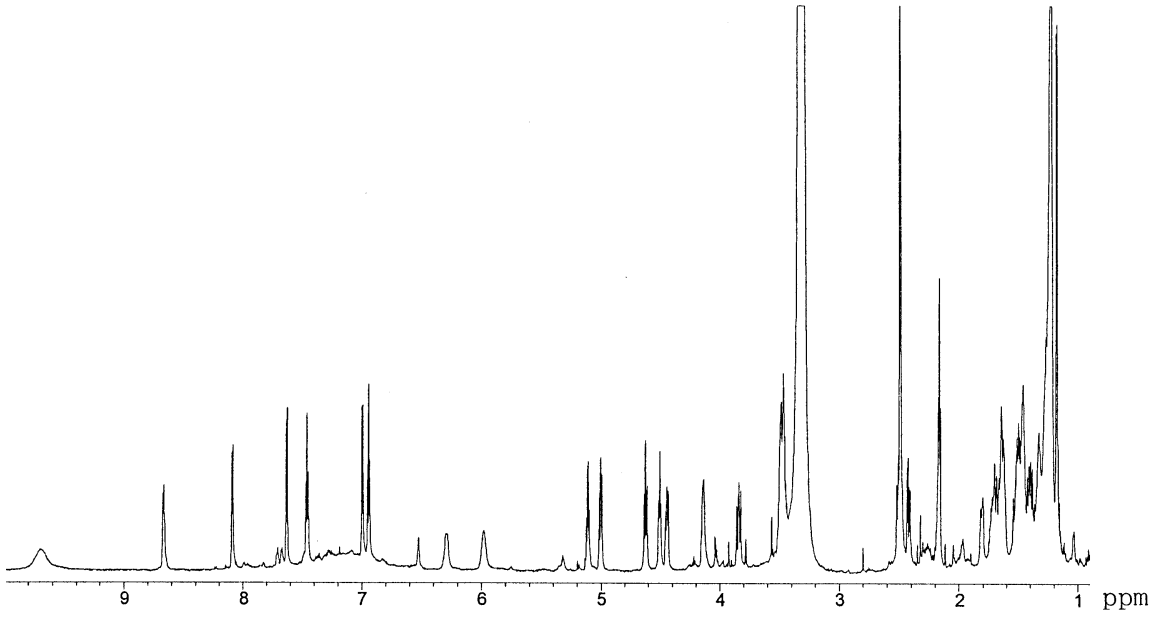

Figure 1. ^1H spectrum of carboxymycobactin from *M. smegmatis* in $DMSO_{d6}$ at 750 MHz.

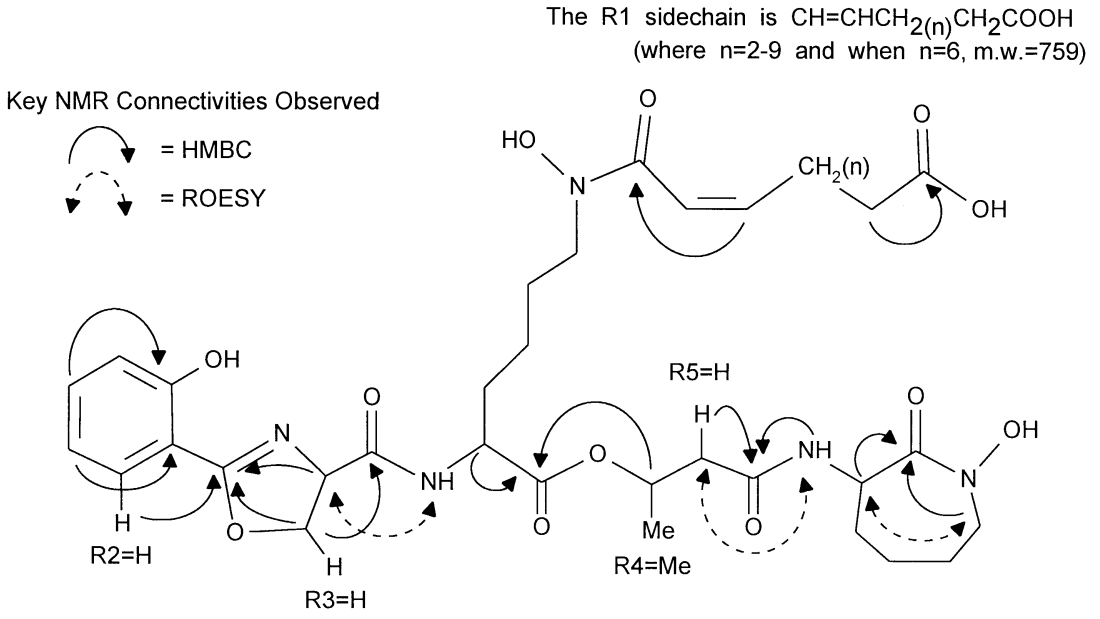

Figure 2. Carboxymycobactin structures isolated from *M. smegmatis* with key NMR connectivities observed. R_1 to R_5 indicate the sidechain variations commonly seen in mycobactin/carboxymycobactin related structures.

Table 1. NMR Assignments

	^{1}H (couplings Hz)	^{13}C		^{1}H (couplings Hz)	^{13}C
Cyclo lysine			*3-hydroxy butanoic acid*		
α	4.44 (ddm 11, 7.5, m)	50.5	1	–	168.0
ββ′	1.69, 1.39	30.0	2	2.50 (dd 14, 7.5)	41.0
γ′s	not determined		2′	2.42 (dd 14, 6.5)	41.0
δ, δ′	1.66, 1.42	25.0	3	5.10 (m)	68.5
ϵ, ϵ′	3.83 (m 16, 11.5), 3.47	52.0	4	1.17 (d 6.5)	19.0
NH	8.08 (d 7.5)	–			
C=O	–	168.5			
Lysine			*R1*	C(=O)CH=CH(CH$_2$)$_n$CH$_2$COOH	
α	4.13 (ddd 8.5, 7.5, 4.5)	52.5	1	–	166.0
β, β′	1.71, 1.62	29.5	2	6.28 (dm 11.5 broad)	119.5
γ′s	not determined		3	5.97 (dt 11.5, 6.5 broad)	144.5
δ′s	1.52	25.5	4	2.49	28.0
ϵ′s	3.48 (broad m)	46.5	5	1.32	28.0
NH	8.66 (d 7.5)	–	CH$_2$′s	1.50–1.20	~24.0–30.0
C=O	–	171.0	CH$_2$	2.16 (t 7.5)	33.5
			COOH	11.77 (broad)	174.5
Serine			*2-hydroxy benzoic acid*		
α	5.00 (dd 10, 8)	67.0	1	–	110.0
β	4.62 (dd 10, 8.5)	69.0	2	–	159.5
β′	4.50 (dd 8.5, 8)	69.0	3	6.99 (d 8.5)	116.5
C=O	–	169.5	4	7.45 (ddd 8.5, 7.5, 2)	134.0
			5	6.93 (dd 8.0, 7.5)	119.0
			6	7.63 (dd 8.0, 2)	128.5
			C=N	–	166.0
			OH	9.69 (broad)	–

(NOH signals – broad 7.6 to 6.6 p.p.m.)

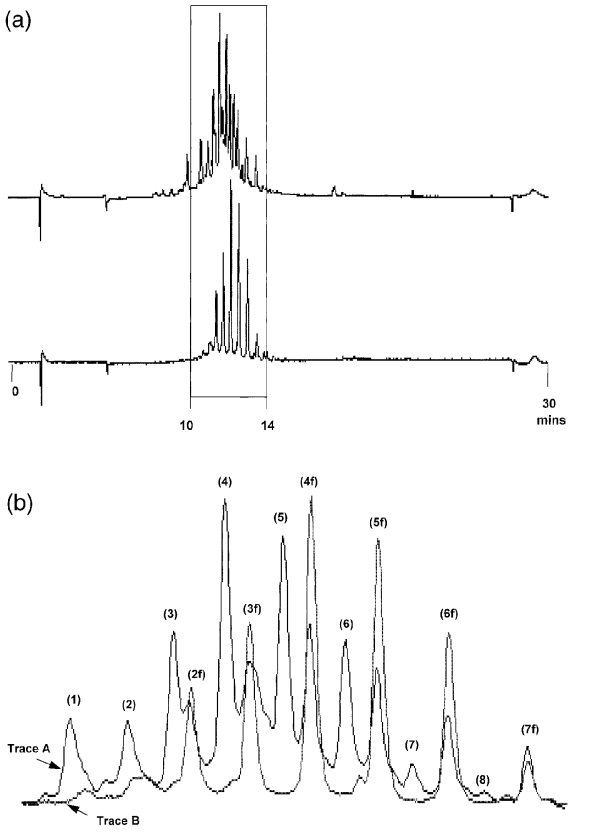

Figure 3. The HPLC-UV chromatograms of desferri- and ferri-carboxymycobactins where f signifies iron complexed.

consistent with a desferried carboxymycobactin homologous series where $n = 2–9$ (Table 2). A second, very minor series of six components exhibited a λ max at 450 nm with molecular weights 14 daltons apart and isotope patterns (Lane *et al.* 1995) characteristic for residual ferried carboxymycobactin homologues where $n = 3–8$ (Table 2). Strong protonated molecular ions allowed unambiguous mol. wt determination for each chromatographic peak/compound (Figure 4).

Mass spectrometry

Molecular formulae for carboxymycobactins nos 4 and 5 were determined using high resolution ESI mass spectrometry (Table 3).

Nanospray ESI

Desferricarboxymycobactin was subjected to MS/MS analysis by nanospray ESI. The major homologous series of eight desferri-carboxymycobactins of *M. smegmatis* and the second minor homologous series of the ferri-forms of the carboxymycobactins could all be observed simultaneously (Figure 5). For the major series, the desferri-carboxymycobactins, the following molecular ions were observed: $M+H^+ = 704, 718, 732, 746, 760, 774, 788$ and 802 (Figure 6). The second series for the ferri-forms of the above series had molecular ions at $M+H^+ = 770, 785, 799, 813, 827$ and 841 (Figure 5). The most abundant carboxymycobactins were nos 4 and 5.

Nanospray MS/MS afforded common fragment ions at *m/z* 100, 171, 207, 213, 334 and 564 (Figure 6) for nos 5, 6 and 7, with *m/z* 334 for the EDC moiety (Figure 7) being the most prominent fragment ion.

The boxes designated A through to F (Figure 7) correspond to the predicted fragment ions resulting from cleavages about the amide or ester bonds.

Discussion

NMR and mass spectrometry data confirm that eight major components exist with R_1 side chains terminating in a free acid. As reported for the carboxymycobactins from *M. avium* (Lane *et al.* 1995), there is no evidence for the presence of the R_1 side chain terminating in a methyl ester as reported by Horwitz and his colleagues (Gobin *et al.* 1995, Wong *et al.* 1996).

The major siderophore in *Mycobacterium smegmatis* is an exochelin which is a trihydroxamate,

Table 2. Carboxymycobactins detected in *Mycobacterium smegmatis*

Compound	M.wt (desferri)	M.wt (ferri)	*n*
(1)	703	–	2
(2)	717	770	3
(3)	731	784	4
(4)	745	798	5
(5)	759	812	6
(6)	773	826	7
(7)	787	840	8
(8)	801	–	9

Table 3. Accurate mass determinations for carboxymycobactins nos 4 and 5 (see Figure 3)

Compound	Formula	Calculated mass	Measured mass	Difference (p.p.m.)
(4)	$C_{36}H_{52}N_5O_{12}$	746.3613	746.3260	1.0
(5)	$C_{37}H_{54}N_5O_{12}$	760.3769	760.3772	0.4

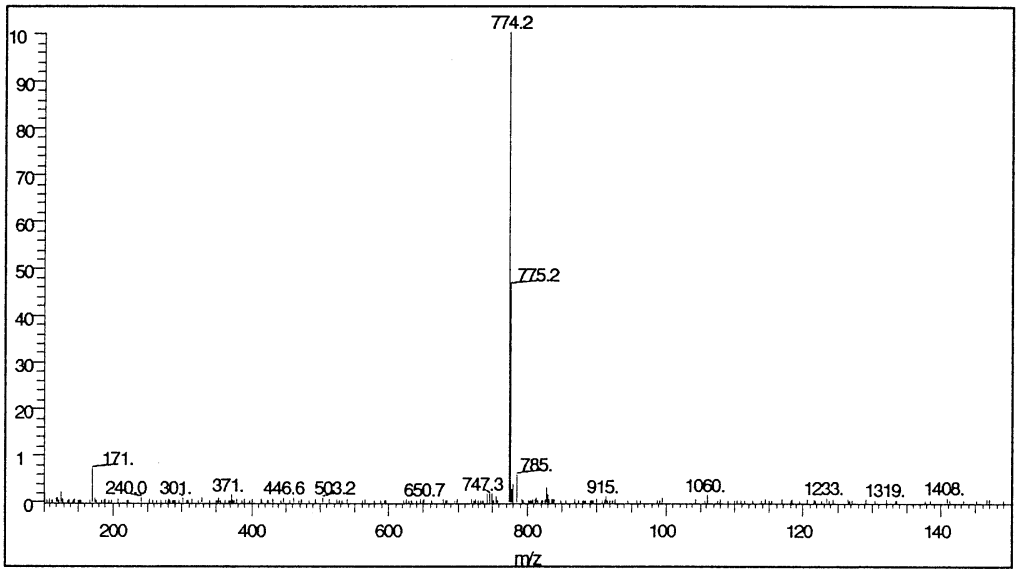

Figure 4. ESI mass spectrum of desferricarboxymycobactin (6).

pentapeptide derivative (Sharman *et al.* 1995a) and the carboxymycobactins are present as secondary siderophores. Thus, to avoid confusion, the term 'exochelin' should now be confined to the water-soluble, non-extractable siderophores and the name 'carboxymycobactin' be used for these mycobactin-like siderophores.

The main differences between the carboxymycobactins of *M. smegmatis* and those previously isolated by our group (Lane *et al.* 1995) from *M. avium* are in the alkyl substituents present in the oxazoline (D) and β-hydroxy acid (B) moieties. The acyl sidechains (F) are the same length in *M. avium* and *M. smegmatis* (C_7 to C_{12}).

The carboxymycobactins of *M. avium* contain threonine in the oxazoline ring ($R_3 = CH_3$) (Lane *et al.* 1995), whereas in *M. smegmatis* the carboxymycobactins contain serine ($R_3 = H$). The β-hydroxy acid substituents in the carboxymycobactins of *M. avium* were R_4 = ethyl and R_5 = methyl, in *M. smegmatis* the substituents are R_4 = methyl and $R_5 = H$. These findings show that the relationship between

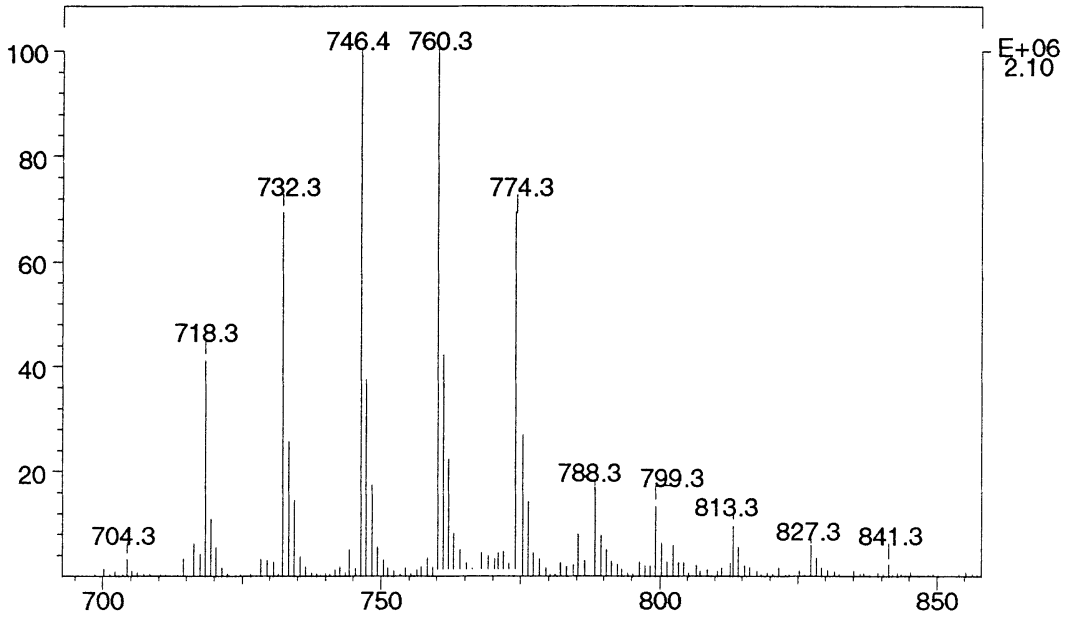

Figure 5. Protonated molecular ions for carboxymycobactin homologues.

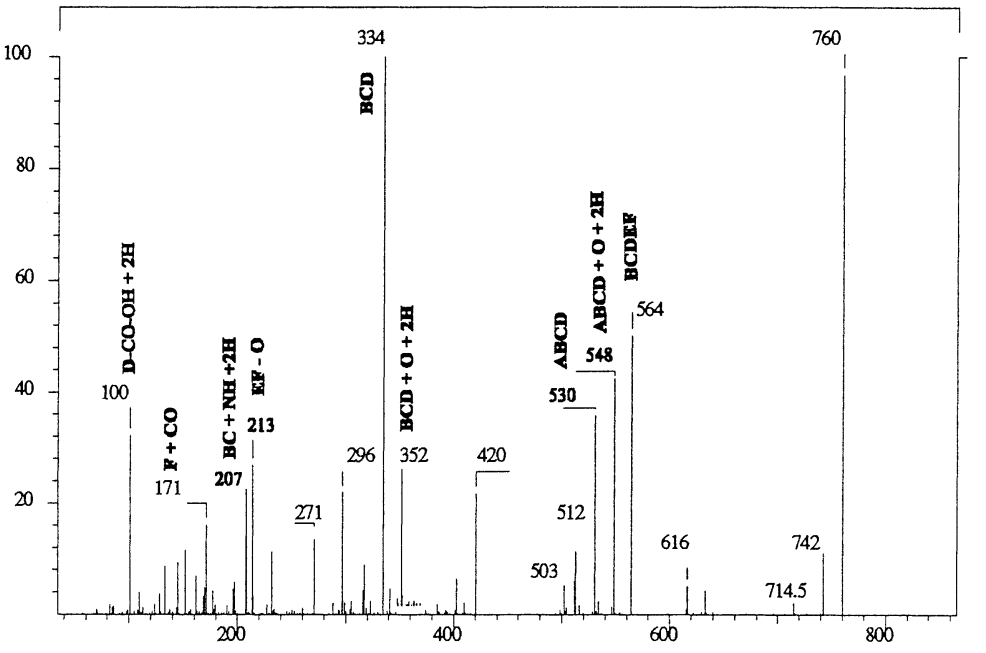

Figure 6. Nanospray MS/MS spectra for carboxymycobactin (5), $M+H^+ = 760$.

carboxymycobactins of *M. smegmatis* and their corresponding mycobactin is identical to that previously discovered for *M. avium*, *M. tuberculosis* and *M. bovis* (BCG; Gobin *et al.* 1995, Lane *et al.* 1995). For this reason the chirality of the three centres can be assumed to be the same as indicated by Snow (1970) and Hough & Rogers (1974).

In a detailed examination of the alkyl chains of mycobactin S, Ratledge & Ewing (1978) reported the following relative % (w/w) distribution as the corresponding fatty acids:

10:1 (*c*2)	3
14:0	21
16:0	17
16:1 (*c*2)	26
17:1*	5
18:0	14
18:1 (*c*2)	14

*probably Δ9

Thus, 95% of the alkyl chains in mycobactin were seemingly derived from fatty acids with an even number of carbon atoms indicating a possible correspondence with the conventional even chain length fatty acids found in lipids. The presence of the unusual *cis*-2 double bond had been noted earlier and is a common feature in mycobactins (Snow 1970).

The occurrence of a homologous series of acyl chains in the present carboxymycobactin deserves comment. Being an acyl chain, it may be assumed that this is derived from a corresponding dicarboxylic acid: however the virtual absence of saturated chains in carboxymycobactin is in contrast to the approximately equal distribution of both saturated and monounsaturated chains in mycobactin. The greatest difference, though, is that both odd and even chain length acyl chains occur in carboxymycobactins, not just even chain length ones. As fatty acids are usually considered the precursors of long chain dicarboxy acids by ω-hydroxylation and further terminal oxidation, it is not immediately

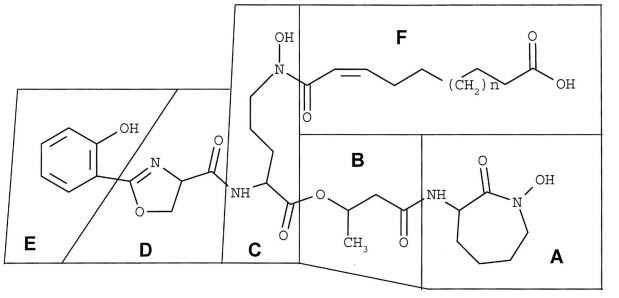

Figure 7. MS/MS fragmentation pattern for carboxymycobactin.

apparent how a homologous series of acyl chains (= diacyl) chains would be synthesized. Usually, fatty acid biosynthesis proceeds by addition of C_2 units (acetyl-CoA) to a growing fatty acyl chain thus resulting in a predominance of even chain fatty acids and, where produced, also of even chain dicarboxylic acids. Thus the synthesis of the acyl chain of carboxymycobactin probably follows a different route from that of conventional fatty acids to account for the odd chain lengths.

A similar homologous series of acyl chains has been noted for the previously described carboxymycobactins both by ourselves (Lane *et al.* 1995) and other workers (Gobin *et al.* 1995, Wong *et al.* 1996). However the novelty of finding a homologous series of acyl chains was not commented upon previously.

The range of the homologous series of acyl chains in carboxymycobactin is from C_7 to C_{13} and is considerably shorter than the corresponding alkyl chain lengths in mycobactins. This could be taken as further indication of a different origin for these chains and, without invoking a complete novel C_1 route of fatty acyl synthesis, the simplest proposal would be to suggest that the dicarboxylic acids arise from cleavage of longer even-chain length fatty acids. Such fatty acids could be precursors of the very long chain mycolic acids found in all mycobacteria and which may contain hydroxyl-, keto- and carboxy groups thus enabling cleavage to occur at selected points. Further speculation though is unwarranted at this stage without additional proof.

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References

Gobin J, Moore CH, Reeve J, *et al.* 1995 Iron acquisition by *Mycobacterium tuberculosis*: isolation and characterization of a family of iron-binding exochelins. *Proc Natl Acad Sci USA* **92**, 5189–5193.

Hough E, Rogers D. 1974 The crystal structure of ferrimycobactin P, a growth factor for the mycobacteria. *Biochem Biophys Res Commun* **57**, 73–77.

Lane SJ, Marshall PS, Upton RJ, Ratledge C, Ewing M. 1995 Novel extracellular mycobactins, the carboxymycobactins from *Mycobacterium avium. Tetrahedron Lett* **36,** 4129–4132.

Macham LP, Ratledge C, Nocton JC. 1975 Extracellular iron acquisition by mycobacteria: role of the exochelins and evidence against the participation of mycobactin. *Infect Immun* **12**, 1242–1251.

Ratledge C, Ewing DE. 1978 The separation of the mycobactins from *Mycobacterium smegmatis* by using high-pressure liquid chromatography. *Biochem J* **175**, 853–857.

Ratledge C, Ewing M. 1996 The occurrence of carboxymycobactin, the siderophore of pathogenic mycobacteria, as a second extracellular siderophore in *Mycobacterium smegmatis. Microbiology* **142**, 2207–2212.

Ratledge C, Hall MJ. 1971 Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. *J Bacteriol* **18**, 314–319.

Sharman GJ, Williams DH, Ewing DF, Ratledge C. 1995a Isolation, purification and structure of exochelin MS, the extracellular siderophore from *Mycobacterium smegmatis. Biochem J* **305**, 187–196.

Sharman GJ, Williams DH, Ewing DF, Ratledge C. 1995b Determination of the structure of exochelin MN, the extracellular siderophore from *Mycobacterium neoaurum. Chemistry & Biology*, **2**, 553–561.

Snow GA. 1970 Mycobactins: iron-chelating growth factors from mycobacteria. *Bacteriol Rev* **34**, 99–125.

Wong DK, Gobin J, Horwitz MA, Gibson BW. 1996 Characterization of exochelins of *Mycobacterium avium*: evidence for saturated and unsaturated and for acid and ester forms. *J Bacteriol* **178**, 6394–6398.