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IDENTIFICATION OF THE LEPROSY BACILLUS AND RELATED MYCOBACTERIA BY ANALYSIS OF MYCOCEROSATE PROFILES

by

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Summary - Members of the phthiocerol dimycocerosate family of waxes were extracted from *Mycobacterium bovis* BCG, *Mycobacterium tuberculosis*, *Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium ulcerans* and a skin biopsy from a leprosy patient. The waxes were degraded by alkaline hydrolysis and the mycocerosic acids converted to pentafluorobenzyl ester. Profiles of the esters, recorded using electron-capture gas-chromatography, gave characteristic profilesfor the mycocerosates from *M. leprae* but those from *M. bovis, M. tuberculosis* and *M. kansasii* were superficially similar. The mycocerosate profiles from *M. marinum* and *M. ulcerans* were similar, but distinct from the others. Selected ion monitoring negative ion-chemical ionisation gas chromatography-mass spectrometry of of the pentafluorobenzyl esters allowed the analysis of mycocerosate isomers not revealed on gas chromatography alone. *M. bovis* and *M. tuberculosis* had similar profiles of C_{29}, C_{30} and C_{32} mycocerosates; an additional C_{33} component was also present in *M. kansasii.* The mycocerosates from *M. marinum* and *M. ulcerans* were C_{27}, C_{29} and C_{30} and those from *M. leprae* were distinct in having C_{29}, C_{30}, C_{32}, C_{33} and C_{34} components. These methods have excellent potential for use in the detection of mycobacterial disease by direct analysis of infected tissue without prior cultivation of the causative agent.

Introduction

One approach to the identification and classification of incultivable bacteria, such as the leprosy bacillus, is to use direct chemical analysis of characteristic structural components. The identification of lipids characteristic of mycobacteria in armadillo-grown organisms was decisive in assigning the causative agent of leprosy to the genus *Mycobacterium* (12). In particular, the determination of a characteristic pattern of mycobacterial mycolic acids (12,14), a unique phenolic glycolipid (9) and waxes of the phthiocerol dimycocerosate type (6,10) established beyond doubt the mycobacterial nature of the leprosy bacillus.

The rapid detection of mycobacteria can be achieved by direct chemical analysis of specific lipids in infected tissue, as reviewed elsewhere in this journal (20). Methods are currently available for the sensitive detection of tuberculostearic acid (10-methyloctadecanoic acid) in sputum, serum and cerebrospinal fluid (4, 7, 11, 20). Tuberculostearic acid is, however, widely distributed among mycobacteria and actinomycetes so it is not possible to accurately identify a particular pathogen in the absence of supporting data. A more specific class of fatty acids are the multimethyl-branched mycocerosic (mycoceranic) acids (Table 1) which are the acyl components of the phenolic glycolipids and phthiocerol-based waxes (8,13,18).

Table 1
Structures of mycobacterial mycocerosic acids

$$CH_3.(CH_2)m.(CH_2.\underset{\displaystyle CH_3}{\underset{|}{CH}})p.COOH$$

Overall carbons

C_{27};	m = 16,	p = 3
C_{29};	m = 18,	p = 3
C_{30};	m = 16,	p = 4
C_{32};	m = 18,	p = 4
C_{33};	m = 16,	p = 5
C_{34};	m = 20,	p = 4

Profiles of mycocerosic acids can be determined by simple gas chromatography but there is a problem in that certain types of acids have essentially the same retention times (13,18). For example a C_{29} mycocerosate with three methyl branches overlaps with a C_{30} acid with four methyl branches (Table 1). Pentafluorobenzyl esters of mycocerosic acids can be separated into their individual components by reverse phase high performance liquid chromatography (HPLC) (18) but this is not a sensitive procedure. Sensitive detection of mycocerosates can be made by use of pentafluorobenzyl esters and an electron-capture gas chromatograph (EC-GC) (16), but the individual components are not separated. The precise composition of a mycocerosate mixture can be determined in an ultra-sensitive manner by negative-ion chemical ionisation (NI-CI) gas chromatography-mass spectrometry (GC-MS) (15) and this procedure has been applied to mycocerosates from *M. leprae* (2,19) and *M. tuberculosis* (15). In this communication, profiles of mycocerosates from *M. leprae* have been compared by EC-GC and NI-CI GC-MS with those from related mycobacteria.

Materials and Methods

Growth of organisms. *Mycobacterium bovis* BCG (Danish sub-strain), *M. kansasii* ATCC 12478, *M. marinum* ATCC 927 and *M.tuberculosis* H37Rv were cultivated as described previously (13,17). *M. ulcerans* HB7748 was available

from a previous study (3). A large skin biopsy from a leprosy patient was collected at the JALMA Institute, Agra, India.

Lipid extraction. Non-polar lipids were extracted from freeze-dried biomass (50 mg) and the skin biopsy with 2 ml methanolic saline (10 ml 0.3% aqueous NaCl added to 100 ml methanol) and 2 ml petroleum ether (b.p. 60-800C) for 15 min, the upper layer was removed and the residue was re-extracted with half the volume of extractants (13). The petroleum ether extracts were evaporated to dryness under a stream of nitrogen. The extracts were examined by two-dimensional TLC on Merck 5554 aluminium-backed silica-gel sheets (6.6 cm x 6.6 cm) using petroleum ether (b.p. 60-800C)/ ethyl acetate (98:2) with three developments in the first direction followed by petroleum ether (b.p. 60800C)/ acetone (98:2) in the second direction (13). Separated components were revealed by spraying with 5% ethanolic molybdophosphoric acid and heating at 1500C for 15 min. The total dimycocerosates of the phthiocerol family (PDIMs) were isolated by preparative TLC by the use of a 10 cm x 10 cm Merck 5735 plastic backed silica gel sheet using the above solvent systems. Separated components were detected with 0.01% ethanolic rhodamine 6G, cut from the sheets and extracted with diethyl ether (3 x 1 ml). The diethyl ether was removed from the extracts with a stream of nitrogen at 370C.

Pentafluorobenzyl esters of mycocerosic acids. Mycocerosic acids were liberated from PDIMs esters by heating overnight at 1000C with 30% methanolic potassium hydroxide (1 ml) and toluene (1 ml) in an 8.5 ml tube with a PTFE-lined cap. After cooling, the solution was acidified with 10% aqueous hydrochloric acid (3 ml) and, after centrifugation, the toluene layer was transferred to another tube. The aqueous layer was washed with toluene (1 ml) and this extract added to the previous toluene layer. The combined organic layer was washed with water (3 ml) and evaporated to dryness under nitrogen. The fatty acid extract was heated at 1000C for 3 h with 5% aqueous tetrabutylammonium hydroxide (2 ml) followed by mixing at room temperature with pentafluorobenzyl bromide (0.025 ml) in dichloromethane (1 ml) for 1 hour. The upper aqueous layer was discarded and the lower organic layer washed with dilute hydrochloric acid (1 ml) followed by water (1 ml). The organic layer was dried over anhydrous magnesium sulphate, filtered and evaporated at 370C under nitrogen. The title compounds were purified using a C-18 reverse-phase Bond Elut column. The column was initially conditioned with solvent A [acetonitrile/toluene (80:20)] (2 ml). The reaction mixture was then applied to the column in 0.5 ml of solvent A and the column washed with a further 2ml of the same solvent. The title compound was then washed off the column using solvent B [acetonitrile/toluene (20:80)] (2ml). The purity of the mycocerosate PFB esters was checked by thin layer chromatography using the solvent system petroleum ether (b.p. 60-800C)/acetone (98:2), (R_f 0.7).

Electron-capture gas-chromatography (EC-GC). A Packard 427 instrument was used with an electron-capture detector and a 10 m Altech RSL-300 polyphenylmethylsiloxane wide-bore bonded capillary column, internal diameter 0.53 mm. Nitrogen was used as a carrier gas. The initial temperature was 1700C for 5 mins followed by an increase of 40C/ min until a final temperature of 2900C was reached. The relative intensities of the signals were normalized.

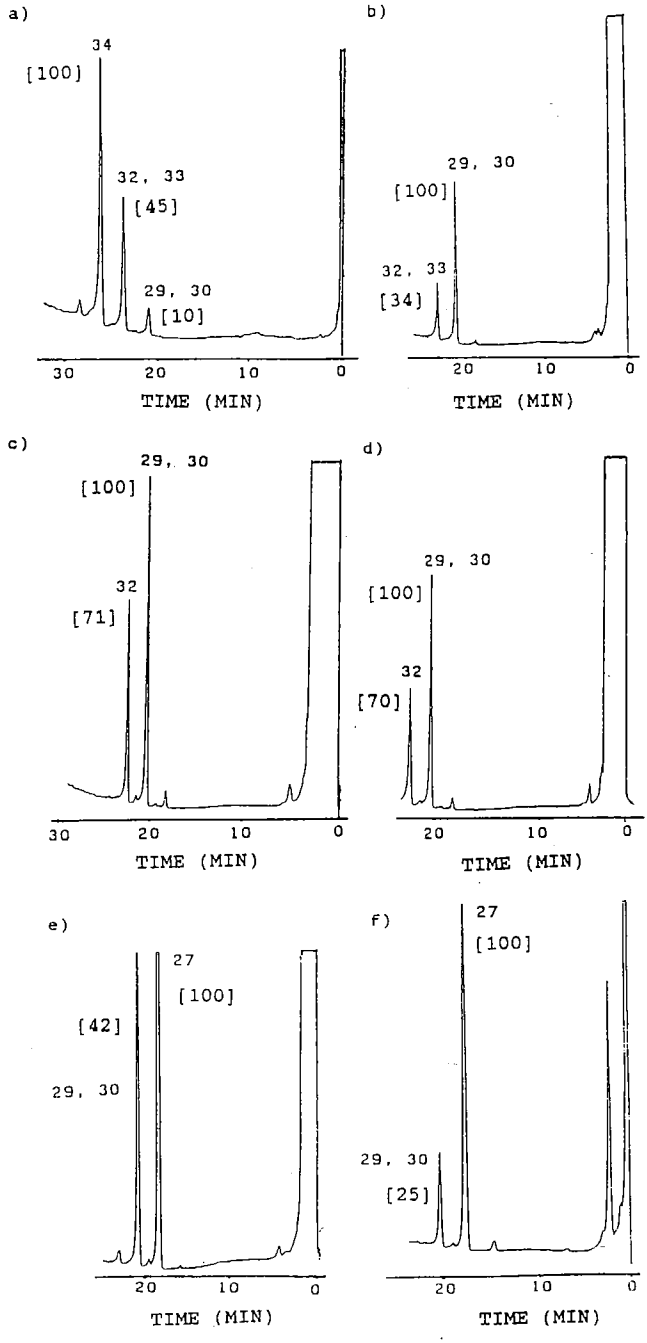

Figure 1

Electron-capture gas-chromatography of mycocerosate pentafluorobenzyl esters from a) M.leprae, b) M. kansasii, c) M. tuberculosis, d) M. bovis, e) M. marinum and f) M. ulcerans. Abbreviations: Numbers above peaks correspond to number of carbonsin underivatised compound. Values in square brackets are the relative proportions normalised to the highest peak.

Gas-chromatography mass-spectrometry (GC-MS). Negative-ion chemical ionization (NI-CI, methane), selected-ion monitoring (SIM) GC-MS experiments were carried out on a 12 m bonded SE 30 fused silica capillary column (Hewlett Packard) linked to a VG 305 mass spectrometer with a source temperature of 1800C. The relative intensities of the signals were normalized.

Results and Discussion

Profiles of the mycocerosate PFB esters recorded by EC-GC are shown in Fig. 1 and the corresponding NI-CI GC-MS SIM traces are given in Figs. 2-7. As noted above, mycocerosates overlapped depending on the chain length and number of methyl branches. Derivatives of mycocerosic acids had the following retention times on EC-GC: C_{27} (17.85 min), C_{29} and C_{30} (20.80 min), C_{32} and C_{33} (23.50 min), C_{34} (25.9 min). The EC-GC traces of all the samples (Fig. 1a-f) demonstrated that superficial inspection allowed some mycobacteria to be distinguished from each other. For example, *M. leprae* is clearly separated by the presence of the C_{34} component (Fig. 1a) and *M.marinum* (Fig. 1e) and *M. ulcerans* (Fig. 1f) have relatively shorter mycocerosates than the others.

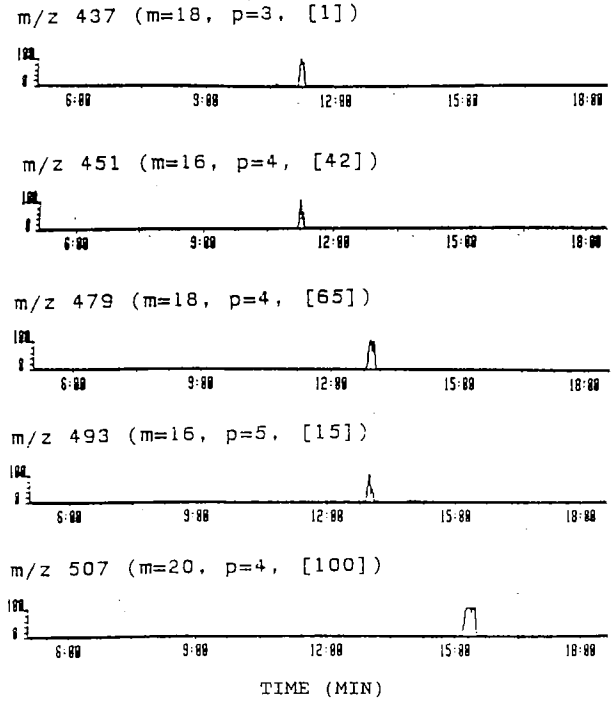

Figure 2
Selected ion monitoring (SIM) negative ion-chemical ionisation (NI-CI) gas chromatography-mass spectrometry (GC-MS) of mycocerosate pentafluorobenzyl (PFB) esters from *M. leprae*. Values in square brackets are normalized intensities and the terms m and p refer to structure in Table 1.

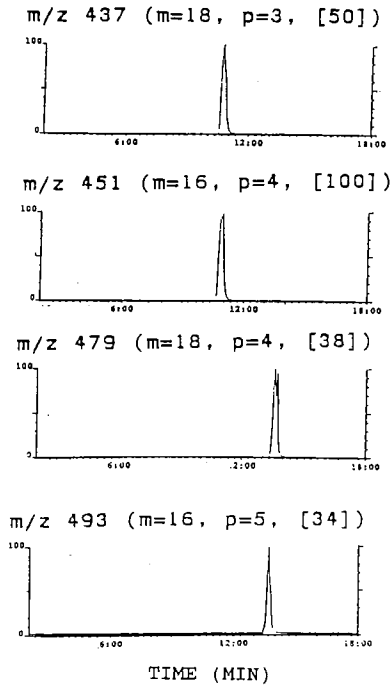

Figure 3
SIM NI-CI GC-MS of mycocerosate PFB esters from *M. kansasii*.
See Fig. 2 for explanation of values.

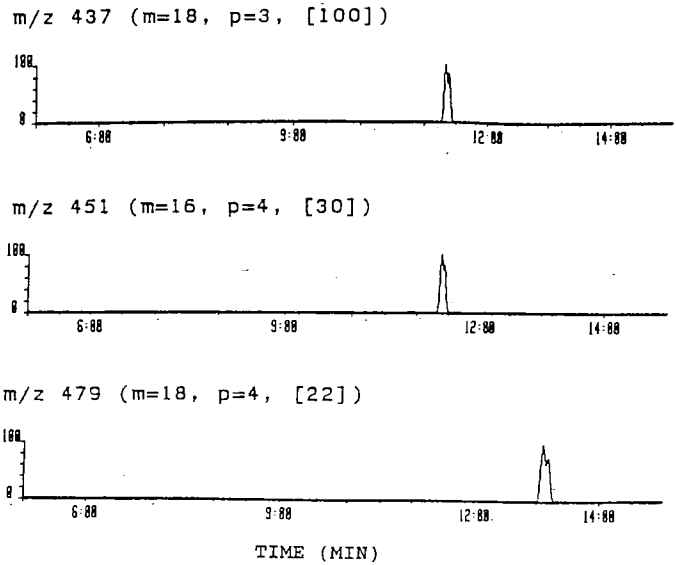

Figure 4
SIM NI-CI GC-MS of mycocerosate PFB esters from *M. tuberculosis*.
See Fig. 2 for explanation of values.

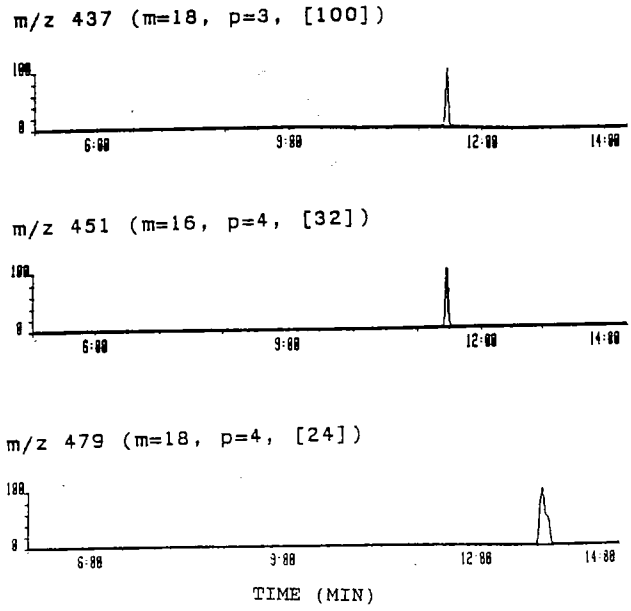

Figure 5
SIM NI-CI GC-MS of mycocerosate PFB esters from *M. bovis*.
See Fig. 2 for explanation of values.

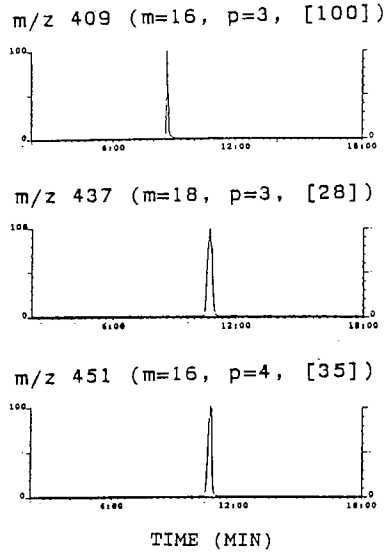

Figure 6
SIM NI-CI GC-MS of mycocerosate PFB esters from *M. marinum*.
See Fig. 2 for explanation of values.

The true composition of the mycocerosates was revealed by SIM NI-CI GC-MS. The PFB esters of the mycocerosates produce predominant peaks corresponding to carboxylate ions (15) and the ions were scanned in the range indicative of mycocerosates using SIM (Figs. 2-7). The homogeneity of the C_{34} component from *M. leprae* was confirmed (Fig 2). As expected from previous studies (5,13,18), the mycocerosates from *M. bovis* and *M. tuberculosis* were closely similar with a characteristic pattern of C_{29}, C_{30} and C_{32} acids (Figs. 4,5). The mycocerosates from *M. kansasii* (Fig. 3), though superficially similar to those from *M. bovis* and *M. tuberculosis* (Fig. 1b-d), were quite distinct in including a substantial proportion of a C_{33} component.

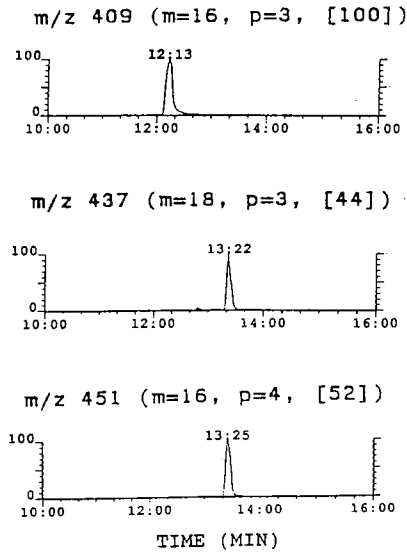

Figure 7
SIM NI-CI GC-MS of mycocerosate PFB esters from *M. ulcerans*.
See Fig. 2 for explanation of values.

Previous studies have indicated a unique relationship between *M. marinum* and *M. ulcerans* (1,3,5), pathogens causing widely different disease. In particular, these two species produce S-mycocerosates with the opposite absolute stereochemistry to the R-mycocerosates from the other species (1,3,5). The relative stereochemistry of the diols from *M. marinum* and *M. ulcerans* is also erythro in contrast to the more common threo arrangement (1,3). The present results (Fig. 1e,f; Figs. 6,7) reinforce the close relationship between these species. The present paper shows the potential value of mycocerosate analysis for the sensitive identification of these mycobacterial species, excepting *M. bovis* and *M. tuberculosis* whose profiles were practically identical as expected. The procedures involved are suitable for analysis of infected material without prior cultivation (2,16,19,20).

Acknowledgements

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