Secondary fatty alcohols of *Mycobacterium xenopi*

SRINIVAS ALUGUPALLI and LENNART LARSSON*

Department of Medical Microbiology, University of Lund, Sölvegatan 23, 223 62 Lund, Sweden

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Secondary alcohols of *Mycobacterium xenopi* were studied by gas chromatography and gas chromatography–mass spectrometry. Mycobacterial cells were hydrolysed and the liberated alcohols separated by extraction and analysed both underivatized and as trimethylsilyl-, methyl ether- and pentafluorobenzoyl derivatives. Seven straight-chain secondary alcohols containing from 18 to 24 carbon atoms and two branched-chain secondary alcohols with 21 and 23 carbon atoms were present in all of the studied strains.

Introduction

Mycobacteria and related organisms characteristically contain high-molecular-mass 3-hydroxy-2-alkyl branched fatty acids known as mycolic acids (Minnikin, 1982). Wax-ester mycolates have been found in only a limited number of mycobacterial species, among them the *Mycobacterium avium* complex, *M. terrae*, *M. phlei* and *M. xenopi* (Minnikin *et al.*, 1985). These mycolates are readily hydrolysed to yield ω -carboxy mycolates and free secondary alcohols (Jimenez & Larsson, 1986); the latter are useful chemical markers in species identification of mycobacteria by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) (Jantzen *et al.*, 1989; Jimenez & Larsson, 1986).

The most commonly found secondary alcohols in mycobacteria that contain wax-ester mycolates are 2-octadecanol and 2-eicosanol (Luquin *et al.*, 1991). In addition, 2-docosanol has also been demonstrated in *M. xenopi* and *M. phlei* (Larsson *et al.*, 1989; Luquin *et al.*, 1989; Toriyama *et al.*, 1982). Recently, we detected 2-nonadecanol in strains of the *M. avium* complex after extracting alcohols from whole-cell hydrolysates and identification by GC–MS (Alugupalli *et al.*, 1992). In the present study, the same methodology was used for detailed analysis of the secondary alcohols of *M. xenopi*.

Methods

Chemicals. Pentafluorobenzoyl chloride (PFBO-Cl) was purchased from Janssen; *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), silver oxide and methyl iodide from Fluka; pyridine from Merck; triethylamine from Sigma; and the solvents acetonitrile and hexane from Lab Scan. All the chemicals were of analytical grade.

Micro-organisms. Fifteen clinical isolates of *M. xenopi* and one reference strain (NCTC 10042) were examined. The mycobacteria were identified by standard biochemical tests. The bacteria were grown in Middlebrook 7H10 medium at 37 °C for 6 weeks, autoclaved (120 °C for 30 min), washed twice with distilled water, and lyophilized.

Sample preparation for GC. A portion (5 mg dry wt) of each strain was heated in 1 ml 30% (w/v) methanolic KOH at 80 °C for 30 min in glass test-tubes fitted with Teflon-lined screw-caps. After cooling, the alkaline hydrolysates were extracted with 2 ml hexane. The hexane phase was used for both GC and GC–MS analysis of alcohols (see below).

A separate portion (5 mg dry wt) of one of the mycobacterial strains was mixed with 1 ml 1 M-methanolic HCl and heated at 80 °C for 30 min (Jimenez & Larsson, 1986). After cooling, the reaction mixture was shaken with 2 ml hexane. The hexane phase was separated and used for combined GC analysis of fatty acids and fatty alcohols.

Gas chromatography. A Varian model 3500 gas chromatograph equipped with a flame-ionization detector and a split/splitless injector (working in the splitless mode) was used. Analyses were performed on a fused-silica capillary column (25 m/0.25 mm i.d.) coated with cross-linked OV-1. Nitrogen, at a flow-rate of 2.5 ml min⁻¹, was used as carrier gas. Both the injector and the detector were held at 280 °C, and the column temperature was programmed from 140 to 280 °C, at a rate of 8 °C min⁻¹.

Sample preparation for GC–MS. The preparations used for GC analysis of alcohols (see above) were also studied by GC–MS. The alcohols were analysed both underivatized and as trimethylsilyl (TMS), methyl ether (METH) or pentafluorobenzoyl (PFBO) derivatives prepared from the hexane extracts (after evaporation to dryness) according to the following procedures.

* Author for correspondence. Tel 46-46173289; fax 46-46189117.

Abbreviations: EI, electron impact; NICI, negative ion-chemical ionization; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; METH, methyl ether; PFBO, pentafluorobenzoyl; TMS, trimethylsilyl.

Fig. 1. Mass spectra of one of the two branched-chain secondary alcohols detected (peak no. 7, see Fig. 2). The alcohol was analysed in the EI mode underivatized and as TMS- and METH derivatives and in the NICI mode as a PFBO derivative. %FS, percentage full-scale deflection.

TMS derivatives were formed by adding BSTFA (50 μ l) and pyridine (10 μ l) to the sample and then heating at 80 $^{\circ}$ C for 15 min. After cooling to room temperature, pyridine was removed from the reaction mixture with a stream of nitrogen, and hexane was added as final solvent.

METH derivatives were formed by mixing the sample with 60 mg silver oxide and refluxing in 2 ml methyl iodide for 5 h. The reaction mixture was then filtered and the filtrate was collected, dried under a stream of nitrogen, and dissolved in hexane.

PFBO derivatives were prepared by mixing the sample with 35% (v/v) PFBO-Cl (40 μ l) and 2% (v/v) triethylamine (20 μ l) (both in acetonitrile) and heating at 80 $^{\circ}$ C for 30 min. After cooling to room temperature, hexane (1 ml) and water (1 ml) were added and the mixture was shaken. The organic phase was collected, evaporated and dissolved in hexane.

Gas chromatography-mass spectrometry. A VG Trio-1 S GC-MS system connected to a Hewlett-Packard model 5890 gas chromatograph was used. The gas chromatograph was equipped with a fused-silica capillary column (25 m/0.25 mm i.d.) containing cross-linked OV-1 as the stationary phase. Splitless injections were made using a Hewlett-Packard model 7673 autosampler. Helium, at an inlet pressure of 7 p.s.i., was used as the carrier gas. The temperature of the column was programmed to rise at 20 $^{\circ}$ C min $^{-1}$ from 120 to 260 $^{\circ}$ C; both the injector and the interface (between GC and MS) were kept at 260 $^{\circ}$ C. The ionization was performed at 70 eV. The ion-source temperature was 200 $^{\circ}$ C in the electron impact mode and 150 $^{\circ}$ C in the negative ion-chemical ionization (NICI) mode (utilizing methane as reagent gas).

Results

Mass spectra

Altogether nine secondary alcohols were detected, and seven of these were identified as 2-octadecanol, 2-

nonadecanol, 2-eicosanol, 2-heneicosanol, 2-docosanol, 2-tricosanol and 2-tetracosanol. The electron impact (EI) mass spectra of the underivatized alcohols exhibited fragmentations at m/z 45, m/z ($M - 1$), m/z ($M - 15$), m/z ($M - 18$) and m/z ($M - 46$). The mass spectra of the TMS-derivatized alcohols revealed m/z 117 [$\text{CH}_3\text{CHO-Si}(\text{CH}_3)_3$] as the most abundant ion produced, and prominent ions of m/z 73 [$(\text{CH}_3)_3\text{Si}$], m/z 75 [$(\text{CH}_3)_2\text{Si=OH}$] and the molecular-specific ion of m/z ($M - 15$) were also observed. The spectra of the METH derivatives showed m/z ($M - 15$), m/z ($M - 32$) and m/z ($M - 60$) as the specific fragments, whereas the molecular ion (M) was the major fragment in the NICI mass spectra of the PFBO-derivatized alcohols.

The remaining two alcohols eluted just after 2-eicosanol and 2-docosanol. These alcohols exhibited, respectively, the same mass spectra as 2-heneicosanol and 2-tricosanol, both when analysed underivatized and when analysed as TMS-, PFBO- and METH derivatives (Fig. 1).

Chromatography

All of the *M. xenopi* strains contained the nine alcohols described. As shown in Fig. 2, the detection of alcohols was facilitated by the use of the selective extraction procedure. In comparison, combined fatty acid and alcohol analysis revealed only 2-eicosanol, 2-docosanol and branched-2-tricosanol (see Discussion) as clearly distinguished peaks.

Fig. 2. Gas chromatograms of a strain of *M. xenopi*, obtained after selective extraction of alcohols (a) and by combined fatty acid and alcohol analysis (b). The fatty acids were derivatized to methyl esters whereas the alcohols were underivatized. (a) 1, 2-octadecanol; 2, 2-nonadecanol; 3, 2-eicosanol; 4, branched-2-heneicosanol; 5, 2-heneicosanol; 6, 2-docosanol; 7, branched-2-tricosanol; 8, 2-tricosanol; 9, 2-tetracosanol. (b) TBP, tuberculopalmitate; TBS, tuberculostearate; numbers of carbon atoms and double bonds are indicated using standard nomenclature.

Discussion

We recently reported that hexane extraction of alkaline hydrolysates followed by GC-MS analysis can be successfully applied to the detection of mycobacterial secondary alcohols: using this procedure, 2-nonadecanol, as well as the previously reported 2-octadecanol and 2-eicosanol, was detected in strains of the *M. avium* complex (Alugupalli & Larsson, 1992). In the present study on *M. xenopi*, we detected not only 2-eicosanol and 2-docosanol, which have previously been found in *M. xenopi* (Larsson *et al.*, 1989; Luquin *et al.*, 1989), but also 2-octadecanol, 2-nonadecanol, 2-heneicosanol, 2-tricosanol and 2-tetracosanol; the identities of the alcohols were ascertained by GC-MS. Thus, the EI mass spectra of the TMS-derivatized alcohols exhibited abundant ions of m/z 117 (breakdown of C_2-C_3) and m/z ($M - 15$), whereas the NICI spectra of the PFBO-derivatized alcohols exhibited the molecular ion as the major fragment (Alugupalli & Larsson, 1992). The EI spectra of the free alcohols revealed fragments at m/z 45, m/z ($M - 15$) and m/z ($M - 18$), all characteristic of secondary alcohols (Luquin *et al.*, 1989).

Also detected in all strains were two compounds that eluted just after 2-eicosanol and 2-docosanol (Fig. 2, peaks 4 and 7). When analysed underivatized, both of the compounds showed an abundant peak at m/z 45, indicative of a secondary free alcohol. The EI spectra of their TMS derivatives as well as the NICI spectra of their PFBO derivatives were identical with those of 2-heneicosanol and 2-tricosanol, respectively. To investigate whether the compounds were iso- or anteiso alcohols, METH derivatives were prepared. This was done since it is known that METH derivatives of straight-chain, iso and anteiso fatty alcohols produce different EI mass spectra: the characteristic fragments of straight-chain alcohols are m/z ($M - 60$), m/z ($M - 32$), and m/z ($M - 15$); those of iso alcohols are m/z ($M - 88$), m/z ($M - 60$), m/z ($M - 32$) and m/z ($M - 15$); and those of anteiso alcohols are m/z ($M - 89$), m/z ($M - 61$), m/z ($M - 32$) and m/z ($M - 15$) (Karlsson *et al.*, 1973). However, the mass spectra of the two METH-derivatized alcohols were found not to differ from those of METH-derivatized 2-heneicosanol and 2-tricosanol: all exhibited major ions at m/z ($M - 15$), m/z ($M - 32$) and m/z ($M - 60$) (Fig. 1). Considering these data, as well as the retention time characteristics of the two compounds, we have drawn the conclusion that they are branched-chain (but not iso or anteiso) secondary alcohols, one with 21 carbon atoms and the other with 23.

The method described is useful for the determination of microbial fatty alcohols. It remains to be investigated whether a detailed study on the distribution of secondary alcohols in *Mycobacterium* could provide further information applicable in species identification or chemotaxonomy.

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