Ornithine lipid of *Mycobacterium tuberculosis*: its distribution in some slow- and fast-growing mycobacteria

MARIE-ANTOINETTE LANÉELLE,* DANIELE PROMÉ, GILBERT LANÉELLE and JEAN-CLAUDE PROMÉ

Centre de Biochimie et Génétique Cellulaires du CNRS et Université P. Sabatier, 118 route de Narbonne, 31062 Toulouse Cédex, France

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An ornithine-amide lipid is present in *Mycobacterium tuberculosis*. Its structure was established by a combination of chemical analysis and mass spectrometry. 3-Hydroxyoctadecanoic and 3-hydroxyeicosanoic acids (and homologues) were found to be linked through an amide bond to the α -amino group of L-ornithine, the hydroxyl group of the fatty acid being esterified mainly by tuberculostearic acid (10-methyloctadecanoic acid). This ornithine-amide lipid was detected in several other slow-growing pathogenic mycobacteria by thin layer chromatography, but not in an avirulent strain (H37 Ra) of *M. tuberculosis*. In each case mass spectrometry showed that all the structures were identical, thus revising an earlier reported structure for the lipid from *M. bovis*.

Introduction

Ornithine- or lysine-amide lipids are the most frequently detected amino acid lipids that are devoid of phosphorus. Their most common basic structure is an ornithine residue acylated on its α -amino group by a long-chain hydroxy-fatty acid, this hydroxy group being itself acylated by a non-hydroxylated fatty acid (Fig. 1).

Isolation of these lipids by the usual chromatographic procedures is hampered by their polarity which is close to that of phospholipids, particularly phosphatidylethanolamine which is relatively abundant in many bacterial genera. Purification difficulties could explain several discrepancies in published work between the proposed structures, particularly concerning the presence (Promé *et al.*, 1969; Thiele & Schwinn, 1973) or the absence (Kawai & Yano, 1983) of a diol residue esterifying the carboxyl group of ornithine. Moreover, diol lipids have been detected in some bacteria (Demartean-Ginsburg & Miquel, 1962; Batrakov & Bergelson, 1978).

Ornithine- and lysine-amide lipids have some similarities with phosphatidylethanolamine, and Wilkinson (1972) postulated that they could replace this commonly found phospholipid in the membrane: when *Pseudomonas fluorescens* was grown in phosphate-limited medium, an ornithine-amide lipid was the major lipid component (Minnikin & Abdolrahimzadeh, 1974).

Abbreviations: FAB, fast-atom bombardment; CID-MIKE spectrum, collision induced dissociation-mass analysed ion kinetic energy spectrum.

			Mol. mass (Da)
Main	$x=14, y=3$	R-COOH = Tuberculostearic acid	694
components	$x=16, y=3$	R-COOH = Tuberculostearic acid	722
Minor	$x=14, y=4$	R-COOH = Tuberculostearic acid	708
components	$x=14, y=4$	R-COOH = Stearic acid	694
	$x=16, y=3$	R-COOH = Palmitic acid	680

Fig. 1. Structure of the amino acid amide-lipid from *M. tuberculosis* strain Canetti. Ornithine, $y=3$; lysine, $y=4$.

Ornithine- and lysine-amide lipids have been found in many Gram-negative bacteria (for a review see Wilkinson, 1988) including several pathogens, e.g. *Brucella* (Promé *et al.*, 1969; Thiele & Schwinn, 1973), *Bordetella* (Thiele & Schwinn, 1973), *Flavobacterium* (Asselineau *et al.*, 1988; Kawai *et al.*, 1988a) and *Pseudomonas* (Kawai *et al.*, 1988b). They have not been detected in Gram-positive bacteria (for a review see O'Leary & Wilkinson, 1988), except in the order Actinomycetales, e.g. in *Mycobacterium bovis* (BCG) (Promé *et al.*, 1969) and in some *Streptomyces* species (Batrakov & Bergelson, 1978).

There is a growing interest in mycobacterial lipids. Some of them are toxic for host cells or are effectors for the immune system (Lederer, 1979; Mehra *et al.*, 1984) and their biological activities can partly explain the

pathogenic effects of mycobacteria. Biologically active mycobacterial lipids are mainly glycolipids, but ornithine- and lysine-amide lipids, have been shown to be haemagglutinins (Kawai & Yano, 1983), and their *O*-deacylated derivatives are haemolytic (Kawai *et al.*, 1988b). Moreover, they may play a role in bacterial development in the host by their ability to substitute for phosphatidylethanolamine.

We report here that ornithine-amide lipids are present in the tubercle bacilli (*M. tuberculosis*-*bovis* complex) and in some other slow-growing mycobacteria. The structure of the compound isolated from *M. tuberculosis* (strain Canetti) was elucidated; this allowed us to revise the structure formerly proposed for the ornithine-amide lipid from *M. bovis* (BCG).

Methods

Mycobacterial strains and cultures. Strains used are listed in Table 1. They were kindly supplied by Dr H. David (Service des Mycobactéries et de la Tuberculose, Institut Pasteur, Paris, France). They were grown on Sauton medium at 37 °C, until a thick surface layer had been produced (3 to 5 weeks). Cells were harvested by decantation, then killed and extracted by addition of CHCl₃/CH₃OH (1:2, v/v).

Lipid extraction and purification. Cells were successively extracted twice with CHCl₃/CH₃OH (2:1, v/v). For preparative-scale purification, the crude lipid fraction was first separated on a Florisil column (Daffé *et al.*, 1987). Ornithine-amide lipids were eluted together with phospholipids using solvent A [CHCl₃/CH₃OH/H₂O (65:25:4, by vol.)]. Pure ornithine-amide lipid from *M. tuberculosis* (strain Canetti) was then obtained by chromatography on a DEAE-Trisacryl column (acetate form; 2 cm diam. × 5 cm long per 100 mg crude lipid loaded). Crude lipid (at least 200 mg) dissolved in CHCl₃ was loaded onto the column and eluted twice with 50 ml portions of the following solvents in succession: CHCl₃; CHCl₃/CH₃OH (9:1, then 7:3 then 1:1). All the ornithine lipid was eluted by 9:1 CHCl₃/CH₃OH.

For rapid screening of the various mycobacteria for the presence of lysine- and ornithine-amide lipids, a sample of the total lipid extract was subjected to mild alkaline deacylation [0.5% KOH in CH₃OH/benzene (2:1, v/v) for 1 h at 37 °C]; the ether-soluble fraction obtained after acidification was spotted onto a thin-layer chromatography (TLC) plate. The *O*-deacylated ornithine- (or lysine-) amide lipid (formula 1 in Results and Discussion) gave a ninhydrin-positive spot on thin-layer chromatograms at *R_F* 0.09 (solvent A), while the intact compound had an *R_F* of 0.44 (just below phosphatidylethanolamine; *R_F* 0.50). Each time amide-linked lipids were detected in small amounts of crude lipid extracts by using the above rapid procedure, fast checking of the structure of the native amide-lipid was determined by mass spectrometry (MS) of material purified on laboratory-made analytical thin-layer plates (Kieselgel G, Merck; solvent A) which were suitable for small-scale preparative work (up to 10 mg per plate). The amide-lipid was scraped off and eluted from the zone just below phosphatidylethanolamine.

Amino acid analysis. Amide bonds were cleaved by acidic hydrolysis of the intact lipid (6 M-HCl overnight at 110 °C). Amino acids were identified either by paper chromatography using solvent B (butanol/acetic acid/water; 4:1:1, by vol.), or by gas-liquid chromatography (GC) of their di-*N*-trifluoroacetyl *R*-(–)-but-2-yl esters, which allowed the stereochemistry of the amino acids to be determined. These

Table 1. Presence of ornithine- or lysine-lipids in slow- and fast-growing mycobacteria

The presence (+) or absence (–) of the lipids was determined by TLC.

Strain	Phosphorus-free lipoamino acids	
	With amide bond	Without amide bond
<i>(a) Slow-growing</i>		
<i>M. tuberculosis</i>		
Canetti 60	+	–
H37 Rv	+	–
H37 Ra	–	–
IP 88628*	+	–
<i>M. bovis</i>		
BCG Pasteur	+	–
IP 14 002 0005	+	–
IP 14 002 0011	+	–
Vallée	+	–
<i>M. marinum</i>		
IP 14 012 0001	+	–
IP 14 012 0003	+	–
ATCC 927	+	–
<i>(b) Fast-growing</i>		
<i>M. smegmatis</i>		
ATCC 607	–	–
<i>M. aurum</i>		
ATCC 23366	–	+
A+†	–	–
<i>M. phlei</i>		
(IST)‡	–	+
ATCC 19249	–	–

* Recent (1988) clinical isolate.

† Also known as 'strain Rebuffet' in the Institut Pasteur (Paris, France) collection.

‡ Institut de Sérothérapie de Toulouse (France).

derivatives were prepared as follows. The dried amino acids were treated with 0.1 ml trifluoroacetic anhydride for 1 h at room temperature. Excess reagent was allowed to evaporate in a desiccator in the presence of pellets of NaOH. Butyl esters were then prepared by adding 0.1 ml *R*-(–)-2-butanol and 6 µl acetyl chloride, followed by heating for 4 h at 80 °C. The two successive derivatizations were done in the same tightly sealed screw-cap bottle.

The position of the amide linkage in the ornithine-amide lipids was determined by *N*-dansylation. The lipid (1 mg) was dissolved in 0.1 ml CHCl₃/CH₃OH (1:1, v/v). Triethanolamine (1 µl) was added, and a total of 1 mg dansyl chloride dissolved in 0.1 ml CH₃OH was added as two successive 50 µl additions with 1 h allowed after each addition for the reaction to reach completion. After acid hydrolysis (see above) dansylornithine or dansyllysine were identified by TLC (solvent A). Commercial ϵ -dansyllysine (Sigma) and mixtures of synthetic mono-dansyl derivatives of each of the dibasic amino acids were used as references.

Mass spectrometry. The mass spectrometer was a HS-ZAB-2F (VG Analytical) associated with a VG 2035 Data System, using a PDP-8A computer. Fast-atom bombardment (FAB) spectra were generated by a neutral xenon atom beam of 8 keV. Glycerol was used as a matrix. The CID-MIKE (collision-induced dissociation-mass analysed ion kinetic energy) experiments were done using the multichannel recording

system. Helium was introduced into the collision cell located on the second field force area in such a way that a signal arising from the pseudomolecular ion was reduced to approximately 30% of its original value. The scan speed was 800 eV s⁻¹.

Gas-liquid chromatography coupled to mass spectrometry (GC/MS) used the same chromatographic conditions as for GC alone (see below) except that a DANI-3800 chromatograph was used. The mass spectrometer was fitted with an electron-impact ionization source (70 eV). The interface temperature was 200 °C. The scan speed was 1 s per decade.

Miscellaneous techniques. GC was done on a Girdel-30 apparatus equipped with a fused silica capillary column (0.32 mm inside diam. × 25 m long) coated with OV1. Helium was used as carrier. The oven temperature was programmed to rise at 2 °C min⁻¹ from 100 to 280 °C.

Infra-red spectra of solid film lipid samples were obtained on a Perkin-Elmer 1600 FITR.

Trimethylsilyl derivatives were prepared as described previously (Daffé *et al.*, 1983).

Results and Discussion

The infra-red spectrum of the lipoamino acid isolated from *M. tuberculosis* exhibited absorption bands characteristic of an ornithine- or lysine-amide lipid (Kawai *et al.*, 1988b): an N-H stretching band (3320 cm⁻¹), a C=O stretching band of an ester group (1725 cm⁻¹), an ionized carboxyl group at 1580 cm⁻¹ and amide I and II bands (1630 and 1530 cm⁻¹). Infra-red spectroscopy allowed rapid identification of these compounds with about 1 mg of the isolated lipid.

Amino acid analysis after acidic hydrolysis showed that L-ornithine was the main amino acid, with trace amounts of L-lysine, as indicated by GC of the di-*N*-trifluoroacetyl aminoacyl-*R*-(–)-but-2-yl esters.

Dansylation showed that a free δ-amino group of ornithine was present in the lipid; this was confirmed by the absence of a positive ninhydrin reaction after treatment of the lipid with diazomethane, due to its cyclization into a lactam (Thiele *et al.*, 1984), in agreement with the known spontaneous reaction of ornithine esters (Greenstein & Winitz, 1961). Thus the amide bond was on the α-amino group of ornithine.

Mild alkaline deacylation by transesterification with methanol gave both a mixture of fatty acid methyl esters, in which the major component was identified by GC as tuberculostearic acid (10-methyloctadecanoic acid) and a ninhydrin-positive compound (*R_F* 0.09 with solvent A) corresponding to the *O*-deacylated ornithine-amide lipid (formula 1). After acidic hydrolysis of the latter, a

Formula 1

mixture of 3-hydroxy-acids was obtained, and analysed as methyl esters and *O*-trimethylsilyl derivatives.

Mass spectrometric analysis

The positive FAB mass spectrum of the intact lipid exhibited several signals 14 Da apart, which can be assigned to protonated molecular ions of different homologues (Fig. 2). The two major signals arose at *m/z* 695 and *m/z* 723.

The fragmentation pattern of each ion was determined from the corresponding CID-MIKE spectrum (Fig. 3). From this, *m/z* 695 was found to decompose mainly into *m/z* 397. This indicates that the loss of 298 Da can be attributed to the loss of a C19:0 fatty acid. This fragment is flanked by two others, 18 Da apart, at *m/z* 379 and *m/z* 415 respectively. The former can be attributed to the successive loss of water while the latter corresponds to the loss of a C19:0 ketene from the protonated molecular ion. At low mass range, a characteristic signal at *m/z* 115 corresponds either to the acylium ion from ornithine (formula 2) or to its protonated cyclic form (formula 3).

A similar pattern was observed from *m/z* 723: loss of 298 Da due to formation of the ornithine fragment.

The behaviour of the minor homologues was slightly different. While *m/z* 709 still lost 298 Da (C19:0 fatty acid), it exhibited a peak at *m/z* 129, corresponding to the acylium ion from lysine. *m/z* 691 gave a more complicated pattern, showing the loss of different fatty acids, while forming the ornithine fragment only.

The C19:0 fatty acid which was lost from the protonated molecular ion was clearly identified as tuberculostearic acid by studying the fragmentation of its carboxylate anion. The negative-ion FAB mass spectrum of the intact lipid exhibited a large peak at *m/z* 297, due to a fragmentation corresponding to the deprotonated molecular ion of the C19:0 fatty acid moiety. This fragmentation is the well-known deacylation reaction from ester-containing lipids in negative ion FAB-MS. A CID-MIKE analysis of this ion produced its charge-remote fragmentation spectrum (Tomer *et al.*, 1983). It clearly showed a methyl branch on the ninth carbon atom from the methyl end, as indicated by the weak ninth signal in the regularly spaced fragment series from

Fig. 2. Positive ion FAB mass spectra. A, amino acid amide-lipids from *M. tuberculosis* strain Canetti. B, amino acid amide-lipids from *M. bovis* BCG. This sample is contaminated by phosphatidylethanolamine (see text). Exact mass values are indicated on this Figure; these values are not integer numbers since the mass of H is 1.007 not 1.000. In the corresponding text, mass values are given as the integer just below the exact value (to facilitate calculations).

Fig. 3. CID-MIKE spectrum of the main component (protonated molecular ion) from the ornithine-amide lipid of *M. tuberculosis* strain Canetti – see text for interpretation.

the parent ion. Thus the formation of m/z 297 in the negative-ion FAB mass spectrum indicated the presence of ester-bound tuberculostearic acid.

The characterization of all the lipid components of this compound was finally achieved by GC/MS analysis (electron-impact ionization) of the fatty acids released by acid hydrolysis. These acids were analysed after methy-

lation with diazomethane followed by trimethylsilylation. Such 3-hydroxy-fatty acid derivatives presented a characteristic fragment ion at m/z 175 (formula 4). Spectra having this fragment in the GC/MS analysis were selected by the computer program, and the molecular mass of the corresponding hydroxy-fatty acid derivative was determined from the m/z value of the

intense $M^+ - 15$ ion (i.e. the $M^+ - \text{CH}_3$ ion from trimethylsilyl derivatives, which do not give the molecular ion).

Formula 4

This analysis showed that 3-hydroxyoctadecanoic and 3-hydroxyeicosanoic acids were the main amide-linked components, whereas tuberculostearic acid was the main ester-bound fatty acid, together with small amounts of palmitic and stearic acids. These fatty acids are very characteristic of this type of lipid since the 3-hydroxy-fatty acids were not previously detected in other mycobacterial lipids, and although tuberculostearic acid is specific to mycobacteria and related bacteria, it is not the major fatty acid in these micro-organisms.

Ornithine-amide lipids from *M. bovis* (BCG strain)

This lipid was present in very small amounts in *M. bovis* and could not be isolated in pure form by TLC, the fraction being contaminated by phosphatidylethanolamine. Nevertheless, the positive FAB mass spectrum of this mixture clearly allowed these two lipids to be distinguished: the ornithine lipids gave odd-numbered m/z values for their protonated molecular ion (due to the presence of two nitrogen atoms) (Fig. 2B) whereas phosphatidylethanolamine gave even-numbered m/z values (due to a single nitrogen atom). The odd-numbered ions possessed the same mass as the lipid from *M. tuberculosis* strain Canetti, namely m/z 723 and m/z 695. Their CID-MIKE spectra resembled those of the Canetti lipids: an abundant ion showing the loss of a C19:0 fatty acid, flanked by two satellites 18 Da apart, and the ornithine fragment at m/z 115. GC/MS analysis of the fatty acids (analysed as methyl ester and trimethylsilyl ether derivatives) revealed that 3-hydroxyeicosanoic and 3-hydroxydocosanoic acids were the main hydroxylated fatty acids linked through an amide bond. The C19:0 fatty acid was identified as tuberculostearic acid.

It is thus clear that ornithine-amide lipid, in contrast to the structure previously postulated (Promé *et al.*, 1969), contains no diol-ester group. The presence of an ethanediol residue, as postulated for ornithine-lipid of *M. bovis* (BCG) (Promé *et al.*, 1969) and *Brucella melitensis* (Promé *et al.*, 1969; Thiele & Schwinn, 1973) probably resulted from contamination of samples with other mycobacterial lipids. Indeed, no attempt was made to quantify this diol and to check if the amount present

corresponded to the postulated structure. Diol-lipid contaminants were likely to occur since it has long been known that ethanediol is present in minor amounts in mycobacteria, linked in various unidentified lipids presenting a large polarity range (Demarteau-Ginsburg & Miquel, 1962).

Ornithine-amide lipids of some other strains

Ornithine-amide lipids were sought in some other strains of the *M. tuberculosis-bovis* complex and in some other mycobacteria. For a rapid survey, lipids were mildly deacylated in order to eliminate nitrogen-containing compounds in which the amino moiety was linked to the lipid moiety by an ester bond (e.g. phosphatidylethanolamine, aminoacyl diglycerides, aminoacyl phosphatidylglycerol). These deacylated products were analysed by TLC and visualized by reaction with ninhydrin. A more precise identification of the native compounds was then achieved by MS.

Ornithine-amide lipids were detected in all the slow-growing pathogenic strains tested, except the avirulent *M. tuberculosis* strain H37 Ra (Table 1). It is worth noting that an ornithine-amide lipid was present in the recent clinical isolate (strain IP 88628) included in our screening. In contrast, these amide-lipids were not detected in the fast-growing strains tested. However, an amino acid ester lipid was present in two out of the six fast-growing strains tested (Table 1): the structure of a lysyl-diglyceride from *M. phlei* was recently elucidated (Lerouge *et al.*, 1988), and the amino acid lipid from *M. aurum* is under investigation.

Conclusions

Ornithine-amide lipids from the slow-growing mycobacteria studied here have the same general structure as those produced by some *Streptomyces* strains and by many Gram-negative bacteria. It is thus likely that most, if not all, ornithine-amide lipids described in the literature have this same general structure (Fig. 1). Mycobacterial ornithine-amide lipids are distinct only in their *O*-acylation by, predominantly, tuberculostearic acid.

It is worth noting that ornithine-amide lipids are not restricted to a few bacterial genera, but are present in a large variety of micro-organisms, including the three pathogenic *Mycobacterium* species examined here. As seen in Table 1, these lipids were not detected in the avirulent *M. tuberculosis* strain H37 Ra. It is known that these lipids exhibit biological effects, e.g. haemolytic

activity and haemagglutination (Kawai *et al.*, 1985), and macrophage stimulation to generate immunoregulatory substances (Kawai & Akagawa, 1989). Thus ornithine-amide lipids could be one of the pathogenicity factors of virulent bacteria.

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