Free Nocardomycolic Acids in the Classification of Nocardias and Strains of the 'Rhodochrous' Complex

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INTRODUCTION

Good characters are at a premium in taxonomy, especially where classification is confused and complex. Chemotaxonomic characters are being increasingly used and have been partly responsible for improvements in the classification of actinomycetes (Lechevalier, Lechevalier & Gerber, 1971). The analyses of lipid fractions have been found to be particularly useful in separating strains of *Nocardia* and *Mycobacterium*. These taxa contain β -hydroxy-acids of high molecular weight which have a long chain in the α -position and are collectively called mycolic acids. The mycobacteria produce a wide range of mycolic acids which may contain methyl branches and oxygen functions (oxo, methoxy and carboxy) other than the β -hydroxy-acid system. The mycolic acids of nocardias, termed nocardomycolic acids, are less complicated and consist mainly of long chains containing a varying number of *cis*-unsaturated linkages (Michel, Bordet & Lederer, 1960). The structures of the nocardomycolic acids of *Nocardia asteroides* ATCC9969 as determined by Bordet, Etémadi, Michel & Lederer (1965) are given:

Acids of this general type occur invariably as mixtures of homologues; more detailed analyses of the nocardomycolates of *Nocardia asteroides* (Bordet & Michel, 1969) and other species of *Nocardia* (Maurice, Vacheron & Michel, 1971) have been made.

Methyl esters of mycolic acids on pyrolysis produce a meromycolic aldehyde and a straight-chain ester.

The straight-chain esters derived from nocardomycolic esters can be distinguished from those of the true mycolates by their shorter chain length. The detection of these differences in structure using either mass spectrometry (Etémadi, 1967) or pyrolysis gas chromatography (Lechevalier, Horan & Lechevalier, 1971) provides a means of distinguishing between mycobacteria and nocardias.

Mordarska (1968) and Mordarska & Mordarski (1969) analysed lipids by means of thin-layer chromatography (t.l.c.) and found a component which appeared to be specific for nocardias. This lipid was subsequently called lipid characteristic of *Nocardia* or LCN-A (Mordarska & Réthy, 1970) and its detection provided a reliable way of distinguishing nocardias and 'rhodochrous' strains from other nocardioform bacteria (Mordarska, Mordarski & Goodfellow, 1972). This report presents evidence to show that the lipid LCN-A's are free nocardomycolic acids.

RESULTS

Nocardia caviae NCTC 1934 and a representative of the 'rhodochrous' complex (*Nocardia calcaria* NCIB 8863) were grown in modified Sauton's medium, tested for purity, harvested, dried and the lipids extracted with ethanol:diethyl ether (1:1), as described previously by Mordarska *et al.* (1972). The partially extracted cells of *N. caviae* and the 'rhodochrous' strain were exhaustively extracted with chloroform and the residue hydrolysed by heating under reflux with 2.5 N-methanolic KOH in benzene for 6 h. The hydrolysate was neutralized with dilute HCl and extracted with diethyl ether; evaporation of the ethereal extract gave a wax which consisted mainly of nocardomycolic acid (Ionedá, Lederer & Rozanis, 1970).

Thin-layer chromatograms (Merck silica gel PF 254 + 366) were, in all cases, developed with hexane:diethyl ether:acetic acid (80:20:1, by vol.). The ethanol-ether extracts of both strains contained a component having the main characteristic of LCN-A lipids in that no migration was observed when the chromatogram was redeveloped in methanol (Mordarska *et al.* 1972). The nocardomycolic acids from each strain showed identical chromatographic behaviour to the LCN-A lipid of the corresponding strain. As previously noted (Mordarska *et al.* 1972), the LCN-A lipid and nocardomycolic acid from *Nocardia caviae* had a slightly greater chromatographic mobility (R_F 0.18) than the corresponding components from the 'rhodochrous' strain (R_F 0.14).

Nocardomycolic acids and LCN-A lipids were isolated by preparative t.l.c. and esterified with ethereal diazomethane. The methyl esters of the nocardomycolic acid and LCN-A of *Nocardia caviae* had R_F 0.55 on t.l.c. and those from the 'rhodochrous' strain had R_F 0.48; the difference in mobility was thus maintained after esterification.

Pyrolysis gas chromatography (Perkin-Elmer F 11, 2 m $\times$ 3 mm column of 2½% OV-1 on Chromsorb G at 200 °C, inlet 270 °C, N₂ as carrier gas) of the methyl esters of LCN-A and nocardomycolic acid from *Nocardia caviae* released methyl esters of straight-chain acids having 14 and 16 carbon atoms; esters of acids having 12, 14 and 16 carbons were released from the corresponding esters of the 'rhodochrous' strain. Mass spectrometry (A.E.I., MS 9) of these esters confirmed the release of straight-chain esters (peaks at m/e 214, 242 and 270) as determined by gas chromatography.

DISCUSSION

The chromatographic and spectral behaviour of the methyl esters of the lipid LCN-A and nocardomycolic acid from *Nocardia caviae* are practically identical, as are those from the 'rhodochrous' strain. These results show that the so-called LCN-A lipids characteristic for *Nocardia* have properties consistent with those of free nocardomycolic acids. The results obtained lead to the partial structure

for the nocardomycolates of *N. caviae* ($m = 11, 13$), and for the 'rhodochrous' strain ($m = 9, 11, 13$). An explanation of why the nocardomycolates of the latter have a lower mobility than those of *N. caviae* must await findings from additional studies on the chemical structure of these molecules. The results explain the characteristic immobility of lipid LCN-A on t.l.c. when using methanol as eluant, since nocardomycolic acids are insoluble in methanol.

The results also explain the basis of the technique used to detect lipid LCN-A (Mordarska *et al.* 1972) and why this method can be confidently used to distinguish nocardias and strains of the 'rhodochrous' complex from allied taxa. Thus the nocardomycolates have a comparatively low molecular weight (40 to 60 carbon atoms) and are soluble in ethanol-ether (1:1), whereas the mycolates from mycobacteria have a higher molecular range (80 to 90 carbon atoms) and can be extracted with chloroform but are insoluble in such ethanol-ether mixtures.

The relative insolubility of the mycolic acids of mycobacteria in ethanol-ether mixtures forms the basis of a simple chemical method used to distinguish *Mycobacterium* from *Nocardia* (Kanetsuna & Bartoli, 1972). In this method bacteria are saponified and the ethereal extract treated with an equal volume of ethanol. Mycobacteria give a heavy precipitate of mycolic acids (melting point 45 to 70 °C) while nocardias form either no precipitate or only tiny amounts which have a much higher melting point. This method is therefore a reliable way of distinguishing mycobacteria from strains of allied taxa.

The procedure reported here requires no sophisticated equipment and does not involve the chemical transformations which are necessary in the mycolic ester pyrolysis method of Lechevalier *et al.* (1971) and in the mycolic acid precipitation technique of Kanetsuna & Bartoli (1972). The latter technique is only capable of distinguishing mycobacteria from other organisms; the present procedure gives a positive reaction only with nocardias and 'rhodochrous' strains and is, therefore, complementary. As a confirmatory procedure the LCN-A or mycolic acid precipitate of Kanetsuna & Bartoli (1972) might be esterified and its identity checked by the gas chromatographic technique of Lechevalier *et al.* (1971) or by other physical and chemical procedures.

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