Structural Analysis of Mycolic Acids from the Cell Wall Skeleton of *Rhodococcus lentifragmentus* AN-115

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The structures of the mycolic acids from the cell wall skeleton of *Rhodococcus lentifragmentus* AN-115 were established. Field desorption mass spectrometry of underivatized mycolic acids showed that the molecular species of the mycolic acids were distributed from C₃₈ to C₅₀ and the major components were C₄₄, C₄₆ and C₄₈ mono- and dienoic β -hydroxy fatty acids. Pyrolysis gas chromatography-mass spectrometry confirmed that the mycolic acids consisted of a saturated straight C₁₂ and C₁₄ α -chain and an unsaturated β -chain, having mainly C₂₉ and C₃₁ carbons with one or two double bonds. The double bonds in the β -chain were established as the *cis* isomers by nuclear magnetic resonance. The absolute configurations of the α - and β -asymmetric carbons were both established as R using molecular rotation data.

INTRODUCTION

The cell walls of Gram-positive bacteria of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* have a common construction consisting of mycolic acids, arabinogalactan and peptidoglycan (Lederer, 1971; Michel & Bordet, 1976; Barksdale & Kim, 1977). Mycolic acids are defined as long chain α -branched β -hydroxy fatty acids whose structures have been extensively studied (Michel & Bordet, 1976; Minnikin & Goodfellow, 1980; Tomiyasu & Yano, 1984). Mycolic acids from *Nocardia* have a range from C₃₆ to C₆₆ usually with a saturated straight chain at the α -position (α -chain) and an unsaturated straight chain at the β -position (β -chain) containing from one to three double bonds. Similar mycolic acids have been characterized from members of the genus *Rhodococcus* (Minnikin & Goodfellow, 1980).

The present report deals with the structures of the mycolic acids from the cell wall skeleton of *Rhodococcus lentifragmentus* AN-115, including determinations of the molecular species distribution, carbon numbers of α - and β -chains, geometrical isomerism of the double bonds and the absolute configuration.

METHODS

Organism. The cell wall skeleton was prepared, according to Fujioka *et al.* (1985), from '*Nocardia rubra*' AN-115 preserved in the Research Laboratories of Fujisawa Pharmaceutical Co. (Osaka, Japan). Organisms labelled '*N. rubra*' should now properly be considered as members of the species *Rhodococcus lentifragmentus* (Tsukamura, 1985).

Mycolic acids. About 200 mg of the cell walls of *R. lentifragmentus* was hydrolysed with 10 ml 5% (w/v) KOH in methanol at 100 °C for 3 h in a sealed tube. After acidification with 10 ml 1 M-HCl, the sample was extracted three times with 25 ml diethyl ether. The combined ethereal solution was rinsed twice with water and then evaporated to dryness. The mycolic acids (*R_f* 0.49) were purified by thin-layer chromatography (TLC) using Merck silica gel 60 plates and chloroform/methanol (9:1, v/v).

Methyl mycolates. The mycolic acids were esterified in HCl saturated methanol under reflux for 1 h. The sample was evaporated to dryness and the methyl mycolates (*R_f* 0.3) were purified by TLC using hexane/diethyl ether

Fig. 1. Field desorption mass spectrum of the mycolic acids from the cell wall skeleton of *R. lentifragmentus* AN-115.

(2:1, v/v). The diastereoisomeric methyl mycolates were prepared by the alkaline isomerization method of Etémadi (1967*a*).

Preparation of trimethylsilyl derivatives of the methyl mycolates. The mycolic acids in 2 ml chloroform were esterified with 0.4 ml *N,N*-dimethylformamide dimethyl acetal for 10 min at room temperature and then silylated with 0.4 ml acetonitrile/bis(trimethylsilyl)acetamide/trimethylchlorosilane (75:25:4, by vol.) for 10 min at room temperature.

Analytical methods. Field desorption mass spectra were taken with a Jeol JMS-D300 mass spectrometer equipped with a Jeol JMA-2000 mass data analysis system. The sample was applied to a Jeol silicon emitter, the heating current being controlled from 15 to 20 mA. Proton magnetic resonance spectra were recorded in CDCl_3 using tetramethylsilane as an internal standard on Jeol JNM-PMX 60 and Jeol PFT-100 instruments. Methyl mycolates were pyrolysed at 280 °C for 10 min *in vacuo*. The product was dissolved in chloroform and applied to a Hitachi M-80 mass spectrometer fitted with 1.5% OV-17 on a Chromosorb W HP column (2 mm $\times$ 50 cm). The conditions of gas chromatography-mass spectrometry (GC-MS) were as follows: column temperature, 160–290 °C; injection temperature, 295 °C; ionizing energy, 20 eV; total emission current, 100 μA . Gas chromatograms of the trimethylsilylated methyl mycolates were taken on a Shimadzu GC-7A gas chromatograph fitted with a Diasolid ZS column (3 mm $\times$ 100 cm) under the following conditions: column temperature, 240–340 °C; injection temperature, 350 °C; flame ionization detector temperature, 350 °C. Molecular rotations were measured in chloroform at 20 °C with a Jasco J-20 spectropolarimeter.

RESULTS AND DISCUSSION

Field desorption MS has been used for characterization and determination of the M_r distribution of synthetic polymers, since it provides predominantly molecular ions or quasi-molecular ions without, or with very few weak, fragment ions (Schulten, 1981). The field desorption mass spectrum of the mycolic acids of the *R. lentifragmentus* cell wall is shown in Fig. 1. It is interesting to note that the distribution of mass spectral peaks essentially corresponds to the peaks in the gas chromatogram of the trimethylsilylated methyl mycolates (Fig. 2).

The composition of each molecular species, determined by MS, and their molecular formulae are summarized in Table 1, which shows that the molecular species of the mycolic acids ranged from C_{38} to C_{50} and the major components were C_{44} , C_{46} and C_{48} with one or two double bonds. Most of the minor components also contained one or two double bonds with the exception of some of the short chain components such as $\text{C}_{38:0}$, $\text{C}_{40:0}$ and $\text{C}_{41:0}$. Very small amounts of $\text{C}_{45:3}$ and $\text{C}_{48:3}$ were also observed, so that trienoic components were also present in the mycolic acids. In agreement with Tomiyasu & Yano (1984), it was also apparent that the longer chain components contained greater numbers of double bonds. The present results indicate that field desorption MS is a useful method for determining detailed molecular species distribution as well as M_r values of mycolic acids without derivatizations. It may also be useful for larger, more involatile mycolic acids where GC or GC-MS operations may be difficult.

Fig. 2. Gas chromatogram of the trimethylsilyl derivatives of the methyl mycolates from the cell wall skeleton of *R. lentifragmentus* AN-115.

Table 1. Field desorption mass spectrometric analysis of the mycolic acids from the cell wall skeleton of *R. lentifragmentus* AN-115

M_r^*	Formula	Carbon and double bond no.	Peak no.†
580	$C_{38}H_{76}O_3$	$C_{38:0}$	3
592	$C_{39}H_{76}O_3$	$C_{39:0}$	4
594	$C_{39}H_{78}O_3$	$C_{39:1}$	4
606	$C_{40}H_{78}O_3$	$C_{40:1}$	5
608	$C_{40}H_{80}O_3$	$C_{40:0}$	5
622	$C_{41}H_{82}O_3$	$C_{41:0}$	6
632	$C_{42}H_{80}O_3$	$C_{42:2}$	7
634	$C_{42}H_{82}O_3$	$C_{42:1}$	7
636	$C_{42}H_{84}O_3$	$C_{42:0}$	7
646	$C_{43}H_{82}O_3$	$C_{43:2}$	8
648	$C_{43}H_{84}O_3$	$C_{43:1}$	8
650	$C_{43}H_{86}O_3$	$C_{43:0}$	8
660	$C_{44}H_{84}O_3$	$C_{44:2}$	9
662	$C_{44}H_{86}O_3$	$C_{44:1}$	9
664	$C_{44}H_{88}O_3$	$C_{44:0}$	9
672	$C_{45}H_{84}O_3$	$C_{45:3}$	10
674	$C_{45}H_{86}O_3$	$C_{45:2}$	10
676	$C_{45}H_{88}O_3$	$C_{45:1}$	10
678	$C_{45}H_{90}O_3$	$C_{45:0}$	10
688	$C_{46}H_{88}O_3$	$C_{46:2}$	11
690	$C_{46}H_{90}O_3$	$C_{46:1}$	11
692	$C_{46}H_{92}O_3$	$C_{46:0}$	11
702	$C_{47}H_{90}O_3$	$C_{47:2}$	12
704	$C_{47}H_{92}O_3$	$C_{47:1}$	12
714	$C_{48}H_{90}O_3$	$C_{48:3}$	13
716	$C_{48}H_{92}O_3$	$C_{48:2}$	13
718	$C_{48}H_{94}O_3$	$C_{48:1}$	13
730	$C_{49}H_{94}O_3$	$C_{49:2}$	14
732	$C_{49}H_{96}O_3$	$C_{49:1}$	14
744	$C_{50}H_{96}O_3$	$C_{50:2}$	15
746	$C_{50}H_{98}O_3$	$C_{50:1}$	15

* Both M^+ and $M + H^+$ ions were observed.

† Corresponds to the peak number in Fig. 2.

Fig. 3. Structure of the $C_{46:2}$ mycolic acid from the cell wall skeleton of *R. lentifragmentus* AN-115.

Methyl mycolates can be easily pyrolysed into methyl esters of fatty acids and meroaldehydes (Etémadi, 1967*b*). Pyrolysis followed by GC-MS of the methyl mycolates showed that the α -chain was saturated C_{10} , C_{11} , C_{12} , C_{13} , C_{14} , C_{15} and C_{16} , and the β -chain was unsaturated C_{23} , C_{24} , C_{25} , C_{26} , C_{27} , C_{28} , C_{29} , C_{30} , C_{31} , C_{32} and C_{33} mainly with one or two double bonds, the major components being shown in *italic type*.

The chemical shifts in the proton magnetic resonance spectrum (100 MHz) of the mycolic acids are as follows: methyl protons, 0.89 p.p.m. (triplet); methylene protons, 1.25 p.p.m. (singlet); methylene protons vicinal to olefin groups, 2.01 p.p.m. (broad singlet); α -methine protons, 2.34 p.p.m. (broad singlet); β -methine protons, 3.66 p.p.m. (broad singlet); olefinic protons, 5.32 p.p.m. (multiplet). Analysis of the olefinic signal pattern at about 5.3 p.p.m. on proton magnetic resonance spectra can provide information to distinguish between *cis* and *trans* configurations of non-conjugated alkenoic acids (Purcell *et al.*, 1966; Frost & Gunstone, 1975). Oleic and elaidic acids were chosen as suitable materials for comparing the splitting patterns of the mycolic acid olefinic protons. The splitting pattern of the signal for the mycolic acids was very similar to that of oleic acid but not to that of elaidic acid, showing that the mycolic acids had exclusively *cis* configuration.

Four optical isomers are theoretically possible for mycolic acids because they have two asymmetric carbon atoms at the α - and β -positions. A method for determining the absolute configuration of mycolic acids has been developed (Asselineau & Asselineau, 1966; Tocanne & Asselineau, 1968). The molecular rotation values of methyl mycolates, having R configurations at both α - and β -asymmetric carbon atoms, were about $+40^\circ$ and those of diastereoisomers, having the opposite configuration at the α -carbon, were about -10° in several kinds of mycolic acids (Asselineau *et al.*, 1970), including mycolic acids having double bonds only in the β -chain. Our molecular rotation data of the methyl mycolates from *R. lentifragmentus* and their diastereoisomers about the α -carbon, obtained by the method of Etémadi (1967*a*), were $+39^\circ$ and -7° respectively. These values show that both asymmetric carbon atoms of these mycolic acids adopt the R configuration.

Previous GC-MS studies of trimethylsilylated methyl mycolates have demonstrated the precise distribution of M_r , double bond numbers and α - and β -chain structures of the mycolic acids from *R. lentifragmentus* ('*N. rubra*') (Yano *et al.*, 1978; Tomiyasu *et al.*, 1981; Tomiyasu & Yano, 1984). Our field desorption MS of the underivatized mycolic acids and pyrolysis followed by GC-MS of the methyl mycolates provides the same result, namely that the mycolic acids from *R. lentifragmentus* AN-115 ranged from C_{38} to C_{50} , C_{44} , C_{46} and C_{48} being the major components, and their double bonds of 0 to 3 were located in the β -chains, which were mainly C_{29} and C_{31} . A tendency for the longer chain components to contain greater numbers of double bonds was also observed.

The chemical structure of a mycolic acid with carbon number 46 and two double bonds is illustrated in Fig. 3 as a representative mycolic acid from the cell wall skeleton of *R. lentifragmentus* AN-115.

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