STRUCTURAL ANALYSIS OF MOLECULAR SPECIES OF NOCARDOMYCOLIC ACIDS FROM *NOCARDIA ERYTHROPOLIS* BY THE COMBINED SYSTEM OF GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

Ikuya YANO* and Kunihiko SAITO
Department of Medical Chemistry, Kansai Medical School, Fumizono-cho, Moriguchi, Osaka 570

and

Yoshiya FURUKAWA and Masamichi KUSUNOSE
Toneyama Hospital, National Sanatorium and Toneyama Institute for Tuberculosis Research,
Osaka City University Medical School, Toyonaka, Osaka 560, Japan

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1. Introduction

Mycolic acids are high molecular β-hydroxy fatty acids with long-chain branches in the α-position, which occur abundantly in certain groups of bacteria such as Mycobacteria [1, 2, 3], Corynebacteria [4, 5] and Nocardia [6, 7, 8]. The common structures of these acids have been well investigated. However, owing to the instability of mycolic acids at high temperature and the complexity of their homologues, gas chromatographic analysis of the individual acids has not yet been established. Recently, we have succeeded in separating the individual molecular species of the mycolic acids from Nocardia by the combination of gas chromatography and mass spectrometry as their trimethylsilyl derivatives. The present paper describes that the homologues of nocardomycolic acids from *Nocardia erythropolis* grown on a high glucose medium [9, 10] can be separated into 15 major acids according to their carbon numbers, which comprise saturated acids ranging from C_{32} to C_{46} with a smaller amounts of monoenes. In all cases, the branches at the α-position are C_8 to C_{14}.

2. Materials and methods

Nocardia erythropolis was kindly supplied by Dr. M. Mayama, Shionogi Research Laboratories, Osaka. The cells were incubated at 30° for 48 hr on a rotary shaker in a medium containing 1% glucose, 1% peptone and 0.5% yeast extract (Difco) with pH adjusted to 7.0. After the cells were harvested by centrifugation, lipids were extracted with chloroform–methanol (2:1, v/v) and washed by the method of Folch et al. [11], then hydrolysed with 10% methanolic KOH for 2 hr. After unsaponifiable materials were removed with diethyl ether, the mixture was acidified with 1 N HCl. Fatty acids were then extracted and transmethylated with 5% HCl–methanol for 2 hr under reflux.

The resultant fatty acid esters were separated into polar and non-polar esters on a thin-layer plate of Silica gel H (Merck) with a solvent of light petroleum (b.p. 35°–60°)–diethyl ether (85:15, v/v). The polar esters were recovered from the thin-layer plate with diethyl ether. The solution was then evaporated to dryness, and the residue was dissolved in pyridine, after which trimethylchlorosilane and hexamethyldisilazane were added. After the mixture was left for 20 min at room temp, the resultant trimethylsilyl ethers were extracted with hexane, and injected into a gas chromatograph-mass spectrometer (Shimadzu

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* To whom correspondence should be addressed.

and LKB, model 9000 apparatus), in which gas chromatographic effluents were directly conducted into analytical tubes of a mass spectrometer through a Ryhage-type molecular separater. The column (1.5 m X 4 mm) coated with 1% OV-1 on Chromosorb W as stationary phase was operated at 265°, and the molecular separater and the ion source were kept at 300°. The ionization current was 60 μA, the electron energy 70 eV and the accelerating voltage 3 KV.

3. Results and discussion

Thin-layer chromatography (TLC) of the methyl-esters of the fatty acids from *N. erythropolis* gave 2 major spots, one corresponding to non-polar acid esters, and the other migrating close to the mycolic acid esters from Mycobacteria. The latter accounted for approx. 40% of the total fatty acids from *N. erythropolis* and argentation TLC showed almost a single spot corresponding to saturated hydroxy fatty acid esters. Mass spectrometric analysis revealed the presence of 3 groups of prominent ion peaks, the first being at m/e 520, 548, 576 and 604, due to M-18, the second, at m/e 186, 214, 242 and 270, due to α–β cleavage, and the third, at m/e 215, 243, 271 and 299, due to β–γ cleavage.

The pyrolysis products of these polar esters were identified gas chromatographically as methyl decanoate, laurate, myristate and palmitate with long chain aldehydes ranging from C_{20} to C_{28}. These results suggested that these compounds were the mixtures of α-branched chain β-hydroxy fatty acid esters ranging from C_{31} to C_{47} [9].

For further characterization, the trimethylsilyl derivatives of these fatty acid esters (nocardomycolic acids) were submitted to gas chromatography. Fig. 1 shows that trimethylsilyl derivatives of the nocardomycolic acids consist of 15 major components designated as P_1 to P_{15}. Mass spectra of each component were taken and 3 of them (peaks 7, 11 and 15) are reproduced as bar graphs in fig. 2. Mass spectra of peak 7 showed the presence of the parent ion peak at m/e 666 (M), a more intense peak at m/e 651 (M-15) due to elimination of a methyl group and a metastable peak at m/e 576 (M-90) due to loss of trimethylsilanol.

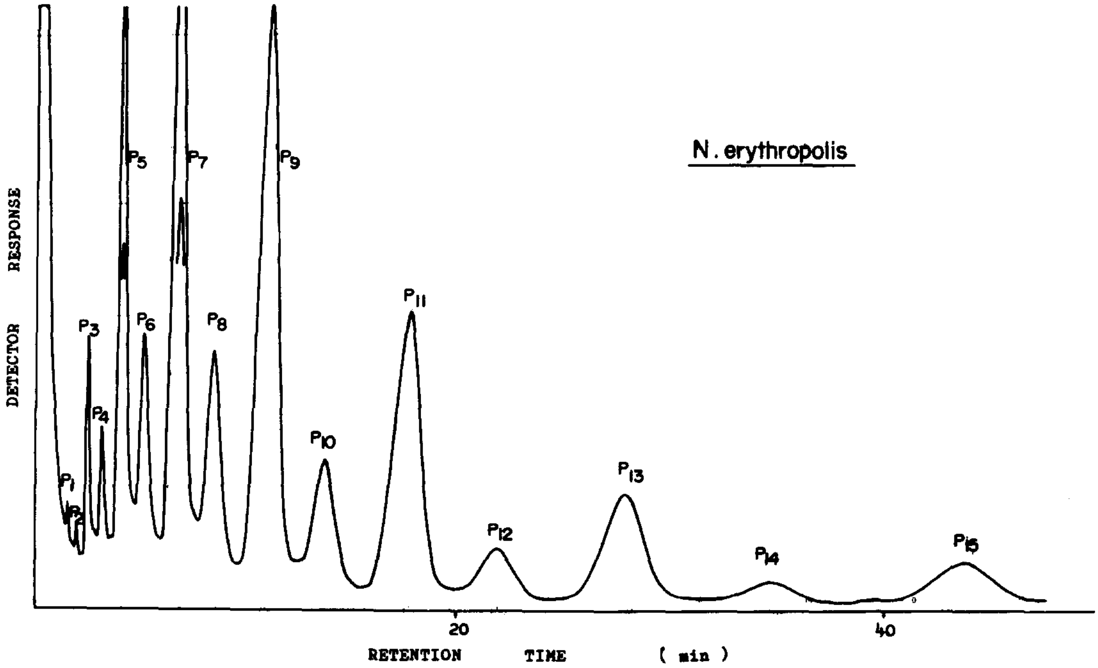


Fig. 1. Gas-liquid chromatogram of the trimethylsilyl derivatives of the methyl esters of α-branched chain β-hydroxy fatty acids from *N. erythropolis*. Conditions for GLC are described in the text.

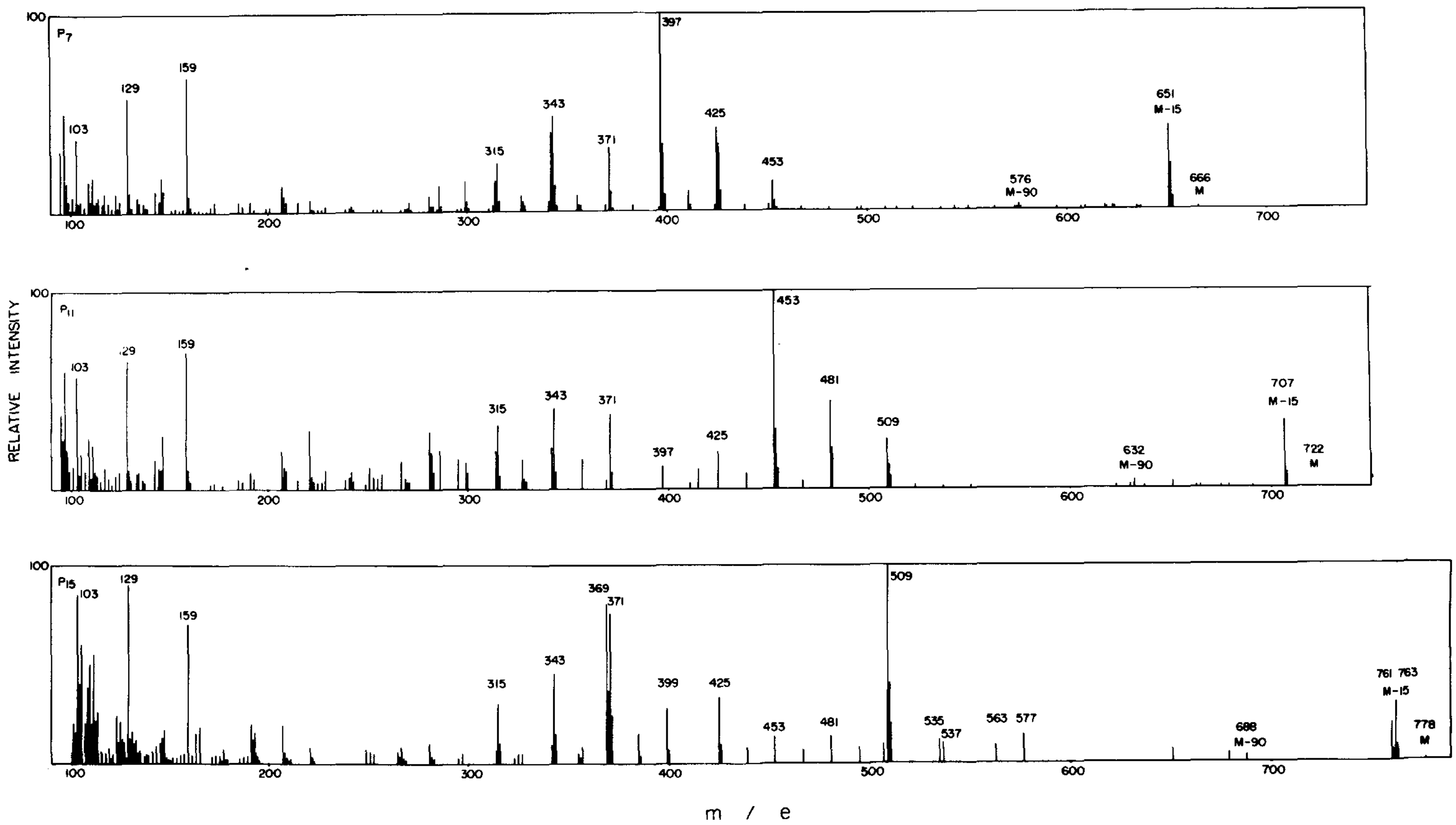


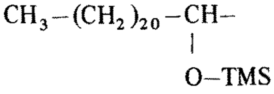
Fig. 2. Mass spectra of peak P_7 (upper), P_{11} (middle) and P_{15} (lower) of fig. 1. Conditions for mass spectrometry are described in the text.

Table 1
Gas-liquid chromatographic and mass spectrometric identification for trimethylsilylether derivatives of α-branched chain β-hydroxy fatty acid from *N. erythropolis.*

Peak no.	M	M-15	M-90	Component	Carbon number of original fatty acid	Branched-chain structure
P_1	582	567	492	$C_{36}H_{74}O_3Si$	C_{32}	m=18, n= 9 or m=20, n= 7
P_2	596	581	506	$C_{37}H_{76}O_3Si$	C_{33}	m=19, n= 9 or m=20, n= 8
P_3	610	595	520	$C_{38}H_{78}O_3Si$	C_{34}	m=18, n=11 or m=20, n= 9
P_4	624	609	534	$C_{39}H_{80}O_3Si$	C_{35}	m=19, n=11 m=20, n=10 or m=21, n= 9
P_5	638	623	548	$C_{40}H_{82}O_3Si$	C_{36}	m=20, n=11 or m=22, n= 9
P_6	652	637	562	$C_{41}H_{84}O_3Si$	C_{37}	m=21, n=11 or m=20, n=12
P_7	666	651	576	$C_{42}H_{86}O_3Si$	C_{38}	m=20, n=13 or m=22, n=11
P_8	680	665	590	$C_{43}H_{88}O_3Si$	C_{39}	m=22, n=12 or m=23, n=11
P_9	694	679	604	$C_{44}H_{90}O_3Si$	C_{40}	m=22, n=13 or m=24, n=11
P_{10}	708	693	618	$C_{45}H_{92}O_3Si$	C_{41}	m=24, n=12 or m=25, n=11
P_{11}	722	707	632	$C_{46}H_{94}O_3Si$	C_{42}	m=24, n=13 or m=26, n=11
P_{12}	736	721	646	$C_{47}H_{96}O_3Si$	C_{43}	m=25, n=13 etc.
P_{13}	750	735	660	$C_{48}H_{98}O_3Si$	C_{44}	m=26, n=13 or m=28, n=11
P_{14}	764	749	674	$C_{49}H_{100}O_3Si$	C_{45}	m=27, n=13 m=29, n=11 or m=28, n=12
P_{15}	778	763	688	$C_{50}H_{102}O_3Si$	C_{46}	m=28, n=13 etc.

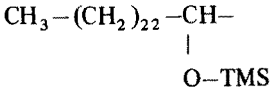
Furthermore, the mass ion peaks due to M-31, M-43, M-47, M-57, M-59, M-71 and M-103 were significant. All these peaks are characteristic of the trimethylsilyl derivatives of long chain hydroxy fatty acid esters or alcohols [12, 13].

On the other hand, the most intense peaks at m/e 397 (base peak), 425 and 453 are indicative of the structure of I, II and III, respectively.



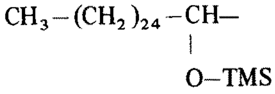
$$\mathrm{CH_3-(CH_2)_{20}-\underset{\substack{|\\ O-TMS}}{CH}-}$$

(I),



$$\mathrm{CH_3-(CH_2)_{22}-\underset{\substack{|\\ O-TMS}}{CH}-}$$

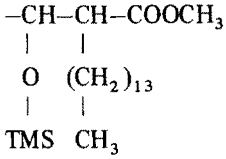
(II) and



$$\mathrm{CH_3-(CH_2)_{24}-\underset{\substack{|\\ O-TMS}}{CH}-}$$

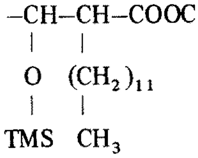
(III)

Since the carbon number of the original fatty acids corresponding to peak 7 was determined to be 38 on the basis of M or M-15, the pairs of carbon numbers derived from the branch at the α-position are also expected. These are the group of peaks at m/e 371, 343 and 315 in fig. 2, indicating the structure of I′, II′ and III′, respectively.



$$-\underset{\substack{|\\ \mathrm{O}\\ |\\ \mathrm{TMS}}}{\mathrm{CH}}-\underset{\substack{|\\ \mathrm{(CH_2)_{13}}\\ |\\ \mathrm{CH_3}}}{\mathrm{CH}}-\mathrm{COOCH_3}$$

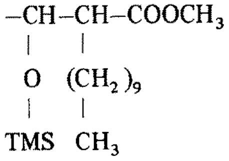
(I′),



$$-\underset{\substack{|\\ \mathrm{O}\\ |\\ \mathrm{TMS}}}{\mathrm{CH}}-\underset{\substack{|\\ \mathrm{(CH_2)_{11}}\\ |\\ \mathrm{CH_3}}}{\mathrm{CH}}-\mathrm{COOCH_3}$$

(II′)

and

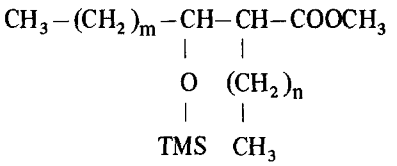


$$-\underset{\substack{|\\ \mathrm{O}\\ |\\ \mathrm{TMS}}}{\mathrm{CH}}-\underset{\substack{|\\ \mathrm{(CH_2)_{9}}\\ |\\ \mathrm{CH_3}}}{\mathrm{CH}}-\mathrm{COOCH_3}$$

(III′)

From the above results, peak 7 was identified as the mixture of C_{38} fatty acid homologues with different carbon skeletons in the straight chain and α-branch. Similarly, peaks 11 and 15 were identified as $C_{46}H_{94}O_3Si$ and $C_{50}H_{102}O_3Si$, derived from C_{42} and C_{46} fatty acids, respectively. Furthermore, it was noted that in the case of peak 15, 2 mass peaks due to M-15 were prominent at m/e 763 and 761 and the latter was 2 mass numbers less, showing the monoenoic acid structure. The occurrence of double peaks at m/e 369 and 371 again indicates that the α-branch possesses one double bond. All of other components (peak 1 to 15) were identified similarly, and the results are summarized in table 1.

The branch structures of the individual components are shown as "m" or "n" numbers in general formula:



$$\begin{array}{llll} CH_3-(CH_2)_m- & CH- & CH- & COOCH_3 \\ & \;| & \;| & \\ & O & (CH_2)_n & \\ & \;| & \;| & \\ & TMS & CH_3 & \end{array}$$

m=18 to 29
n= 7 to 13

Therefore, the total carbon numbers of each of the acids and those derived from α-branch coincided with those obtained by the analysis of pyrolysis products before gas chromatographic separation. These results indicate that the nocardomycolic acids from *N. erythropolis* are highly complex mixtures. Even the acids of the same carbon numbers, which were detected as a single peak on gas chromatograms, were shown to consist of several isomers having different branch structures.

Most recently, Batt et al. [14] reported on the analysis of β-hydroxy fatty acids from *N. corallina*. In our case, however, it was emphasized that a highly complex mixture of odd and even numbered mycolic acids were clearly separated, and the individual molecular species were identified satisfactorily using the direct combination of gas chromatography and mass spectrometry.

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