GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF MOLECULAR SPECIES OF CORYNOMYCOLIC ACIDS FROM *CORYNEBACTERIUM ULCERANS*

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1. Introduction

Mycolic acids are very long chain β-hydroxy fatty acids with carbon branch at the α-position and are known to be specific constituents of Mycobacteria [1–3], Corynebacteria [4–6] and Nocardia [7–9]. The structural analysis of these types of fatty acids have been investigated extensively. However, because of their high boiling points and the instability at high temperatures, gas chromatographic analysis of the individual acids has not yet been fully established.

Recently, we have succeeded in separating 15 molecular species of nocardomycolic acids and clarified the branched-chain structures of each component by the combined system of gas chromatography and mass spectrometry [10]. The present paper describes that the homologues of corynomycolic acids obtained from *Corynebacterium ulcerans* can be also separated clearly into 12 major acids according to their total carbon numbers and the degree of unsaturation. The structural analysis of their trimethylsilyl derivatives revealed that the major fatty acids are C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, C_{30} and C_{32} saturated acids with smaller amounts of mono- and di-enoic acids. The branches at α-position are C_{12} or C_{14} in all cases.

2. Materials and methods

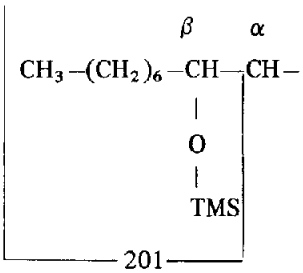
C. ulcerans was kindly supplied by Prof. M. Hori, Institute for Microbiological Disease, Osaka University, Osaka. The cells were grown at 30° for 48 to 72 hr on a shaker in a medium containing 1% glucose, 1% peptone and 0.5% yeast extract (Difco) with pH adjusted to 7.0. After the cells were harvested by centrifugation, lipids were extracted with chloroform–methanol (2:1, v/v), washed by the methods of Folch et al. [11], and then hydrolysed with 10% methanolic KOH for 2 hr. After nonsaponifiable materials were removed with diethyl ether, the mixture was acidified with 1 N HCl. Fatty acids were then extracted and transmethylated with 5% HCl dry methanol for 2 hr under reflux. The resultant fatty acid methyl esters were separated into polar and non-polar esters on a thin-layer plate of Silica gel G (Merck) with a solvent system of light petroleum–diethyl ether (80:20, v/v). The polar esters were recovered from the thin-layer plate with diethyl ether. After the solution was evaporated to dryness, the residues were trimethylsilylated by the method described previously [10].

The trimethylsilyl derivatives of the polar esters were injected into a combined gas chromatograph-mass spectrometer (Shimadzu & LKB, model 9000 apparatus), in which gas chromatographic effluents were directly conducted into high vacuum (10^{-6} torr) analytical tubes of mass spectrometer through a Ryhage-type molecular separater. The column (2.0 m × 4 mm i.d.) coated with 1% OV-1 on Chromosorb W as stationary phase was operated at 250°, and the molecular separater and the ion source were kept at 300°. The ionization current was 60 μA, the electron energy was 70 eV and the accelerating voltage, 3 KV.

3. Results and discussion

Thin-layer chromatography of the methyl esters of the fatty acids from *C. ulcerans* gave two major spots, one corresponding to non-polar acid esters, and the other migrating exactly the same to the corynomycolic acid esters from *Corynebacterium diphtheriae*. The polar esters accounted for approx. 60% of the total fatty acids from *C. ulcerans* and the argentation thin-layer chromatogram showed three major spots corresponding to saturated, monoenoic and dienoic hydroxy fatty acid esters. The pyrolysis of these polar esters yielded homologues of aldehydes ranging from C_6 to C_{16} and fatty acid methyl esters of C_{14} and C_{16}. The mass spectrometric analysis of these polar esters showed similar ion peaks to corynomycolic acid esters reported earlier [5,6]. From these results, it was suggested that these esters were the mixture of α-branched-chain β-hydroxy fatty acid esters ranging from C_{21} to C_{33}.

For further characterization, the gas chromatographic analysis of trimethylsilyl derivatives of these polar esters were carried out and the gas chromatograms are shown in fig. 1. The data indicates that the corynomycolic acids from *C. ulcerans* consisted of at least 12 major components designated as P_1 to $P_{7''}$. Mass spectra of each components were taken and 6 of them (P_2, P_4, P_6, P_7, $P_{7'}$ and $P_{7''}$) are partially reproduced (from m/e 101) as bar graphs in fig. 2. Mass spectra of all these components possessed a base peak at m/e 73, showing trimethylsilylether derivatives [12]. In peak 2, the molecular weight is indicated by mass ion peaks at m/e 427 (M-15) and m/e 352 (M-90) formed by elimination of a methyl group and trimethylsilanol, though the parent ion peak (M) at m/e 442 is very weak. On the other hand, the most intense peak at m/e 201 is very likely formed by the cleavage of the C–C bond between α and β position and indicative of the structure of A).



A)

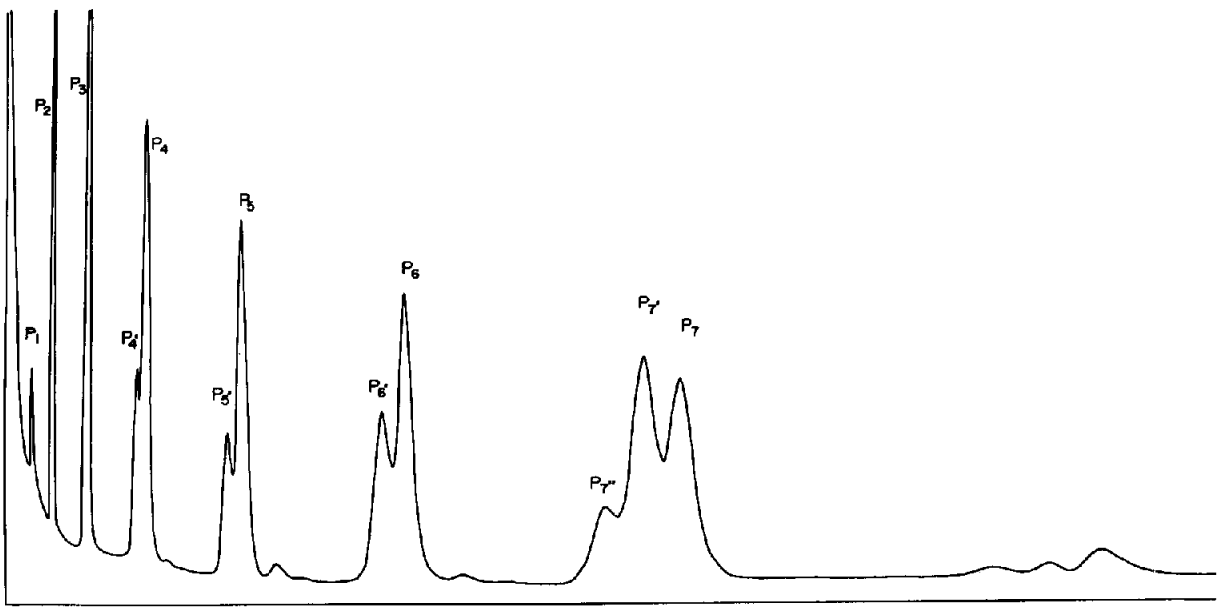


Fig. 1. Gas-liquid chromatogram of the trimethylsilyl derivatives of the methyl esters of corynomycolic acids from *C. ulcerans*. Conditions for gas-liquid chromatography are described in the text.

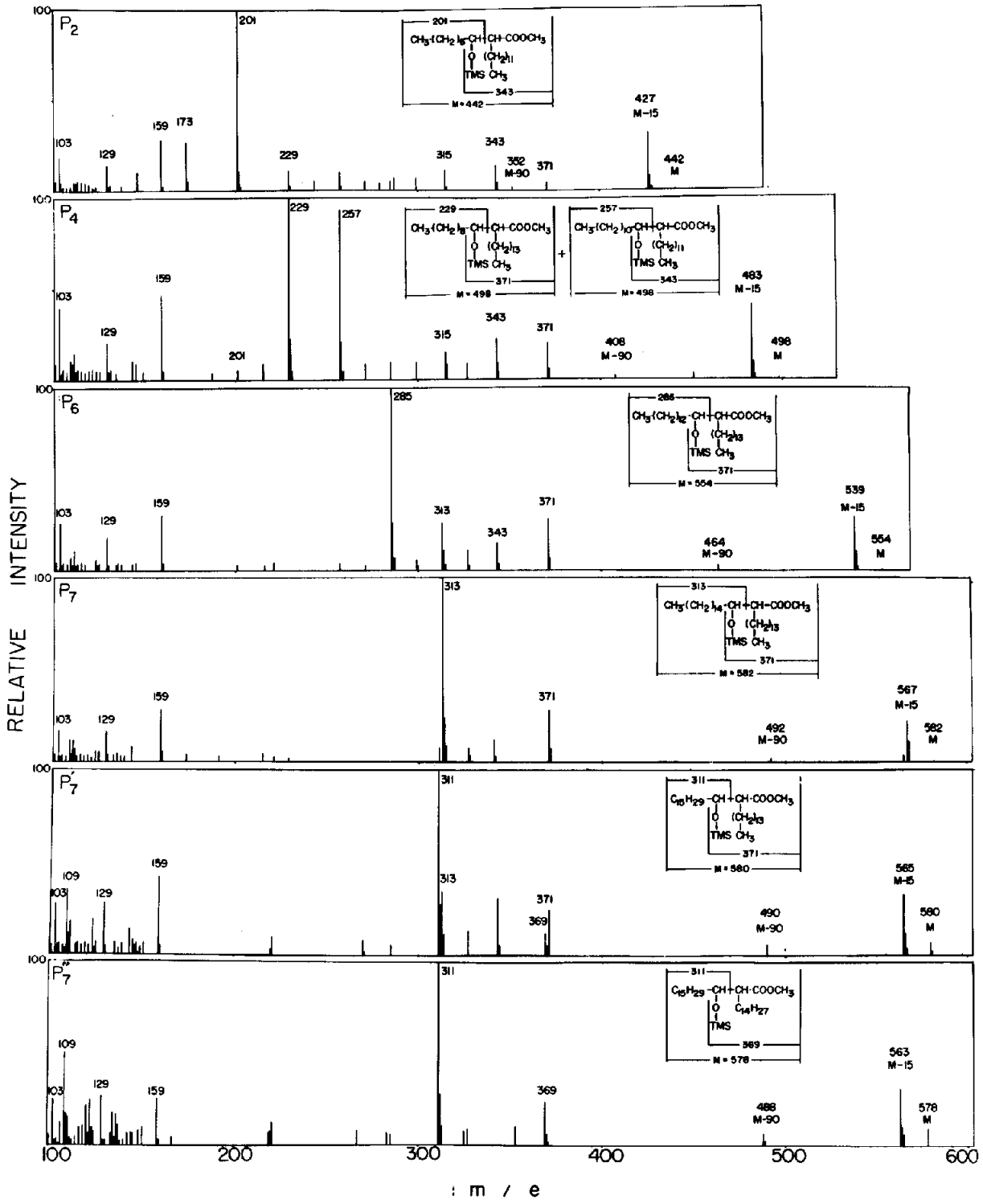
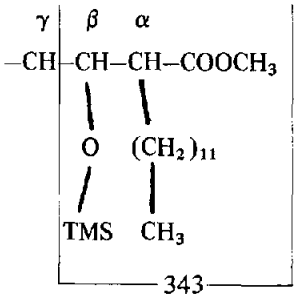


Fig. 2. Mass spectra of peaks P_2, P_4, P_6, P_7, $P_{7'}$ and $P_{7''}$ of fig. 1. Conditions for mass spectrometry are described in the text.

Furthermore, from the total carbon numbers of original fatty acid corresponding to peak 2, the ion peaks derived from $\beta-\gamma$ cleavage are also expected [10]. This peak can be observed at m/e 343 in fig. 2, indicating the structure of B).



B)

From the above results, peak 2 was identified as $C_{26}H_{54}O_3Si$ derived from β-hydroxy C_{22} fatty acids with a carbon branch of C_{12} at α-position.

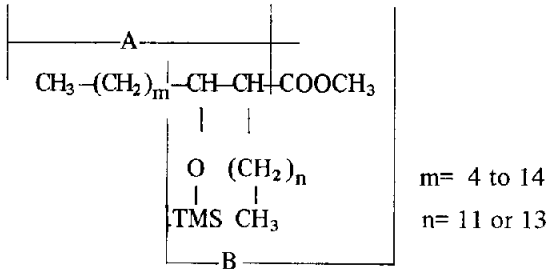
Similarly, peaks 4, 6 and 7 were identified as $C_{30}H_{62}O_3Si$, $C_{34}H_{70}O_3Si$ and $C_{36}H_{74}O_3Si$, derived from C_{26}, C_{30} and C_{32} fatty acids, respectively. All of these components possessed a carbon branch of C_{12} (m/e 343) or C_{14} (m/e 371) at α-position. In the case of peak 4, it is noted that two intense peaks at m/e 229 and m/e 257 due to $\alpha-\beta$ cleavage (and corresponded m/e 371 and m/e 343 due to $\beta-\gamma$ cleavage) occurred apparently, this indicating a mixture of C_{26} fatty acid homologues with different carbon skeletons in straight chain and α-branch. Furthermore, in the case of peak 7′ or peak 7″, mass peaks due to M-15 were present at m/e 565 and 563, two or four mass numbers less than peak 7, showing monoenoic or dienoic structure, respectively. Since the both ion peaks at m/e 311 and 313 or m/e 369 and 371 are prominent in the peak 7′, it is evident that the double bond could be located in both the straight chain and α-branch. All of other components were identified similarly, and the results are summarized in table 1. The retention times of each saturated components on

Table 1

Gas-liquid chromatographic and mass spectrometric identification for trimethylsilyl derivatives of corynomycolic acids from *C. ulcerans*.

Peak no.	M	M-15	M-90	Components	C-number of mycolic acid	Fragment A	Fragment B	Branched chain structure	
P_1	414	399	324	$C_{24}H_{50}O_3Si$	$C_{20:0}$	173	343	m= 4	n=11
P_2	442	427	352	$C_{26}H_{54}O_3Si$	$C_{22:0}$	201	343	m= 6	n=11
P_3	470	455	380	$C_{28}H_{58}O_3Si$	$C_{24:0}$	201	371	m= 6	n=13
P_4	498	483	408	$C_{30}H_{62}O_3Si$	$C_{26:0}$	229 (257)	371 (343)	m= 8 m=10	n=13 or n=11
P_5	526	511	436	$C_{32}H_{66}O_3Si$	$C_{28:0}$	257 (285)	371 (343)	m=10 m=12	n=13 or n=11
P_6	554	539	464	$C_{34}H_{70}O_3Si$	$C_{30:0}$	285	371	m=12	n=13
P_7	582	567	492	$C_{36}H_{74}O_3Si$	$C_{32:0}$	313	371	m=14	n=13
P_4'	496	481	406	$C_{30}H_{60}O_3Si$	$C_{26:1}$	229 (257)	369 (341)	m= 8 m=10	n=13 or n=11
P_5'	524	509	434	$C_{32}H_{64}O_3Si$	$C_{28:1}$	257	369	m=10	n=13
P_6'	552	537	462	$C_{34}H_{68}O_3Si$	$C_{30:1}$	285 (311)	369 (343)	m=12 m=14	n=13 or n=11
P_7'	580	565	490	$C_{36}H_{72}O_3Si$	$C_{32:1}$	313 (311)	369 (371)	m=14	n=13
P_7''	578	563	488	$C_{36}H_{70}O_3Si$	$C_{32:2}$	311	369	m=14	n=13

the gas chromatogram plotted against total carbon numbers possessed a linearity on the semi logarithmic scale. The branch structures of the individual molecular species are shown as "m" or "n" numbers in general formula:



The data obtained by the analysis after gas chromatographic separation agreed well with those of original mixed methyl esters.

The carbon numbers of corynomycolic acids were reported earlier as C_{32} to C_{36} in general [4–6]. However, our results indicated that those of corynomycolic acids from *C. ulcerans* covered a very wide range, and even the acid of same carbon numbers recognized as a single peak on the gas chromatogram were shown to be a mixture of several isomers with different branch structures, as in the case of nocardomycolic acids [10];

In conclusion, from these results and the previous reports [10,12], a highly complex mixture of mycolic acid-type fatty acids ranging from C_{20} to C_{50} can be fully separated according to their total carbon numbers, and the branched-chain structures of individual molecular species can be also determined rapidly using the direct combination system of gas chromatography and mass spectrometry.

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