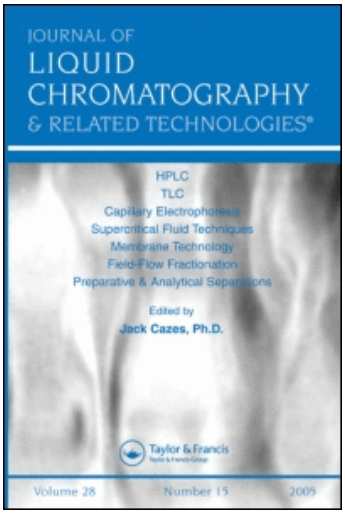

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Separation of a Homologous Series of Long-Chain Fatty Alcohols (C_{49}-C_{58}) From *Mycobacterium Tuberculosis* H37Ra by High Performance Liquid Chromatography

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SEPARATION OF A HOMOLOGOUS SERIES OF LONG-CHAIN FATTY ALCOHOLS (C_{49}-C_{58}) FROM *MYCOBACTERIUM TUBERCULOSIS* H37Ra BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Fatty alcohols were obtained from the saponified chloroform-methanol extract of Mycobacterium tuberculosis H37Ra by extraction with diethyl ether and partially purified by silicic acid column chromatography. The long-chain fatty alcohols (C_{49}-C_{58}) were separated from the shorter-chain alcohols by Sephadex LH-20 column chromatography and further fractionated into saturated and unsaturated alcohols by argentation thin-layer chromatography. These two fractions were analyzed by proton nuclear magnetic resonance spectroscopy, derivatized to the 3,5-dinitrobenzoyl esters, and fractionated on a C_{18}-bonded silica column by high performance liquid chromatography (HPLC). Complete separation of esters differing by two carbon units and partial separation

of esters differing by a single carbon unit were achieved. Each of these HPLC fractions was analyzed by mass spectrometry. The major saturated components were identified to be the dicyclopropyl C_{51} and C_{53} fatty alcohols. These fatty alcohols were structurally related to the α-mycolic acids.

INTRODUCTION

We have previously separated the homologous series of α-methoxy-, and keto-mycolic acids from Mycobacterium tuberculosis H37Ra as their p-bromophenacyl esters using high performance liquid chromatography (HPLC) (1,2). Mycolic acids are α-alkyl, β-hydroxy fatty acids containing cyclopropane rings. The present physiological significance of mycolic acids is that isoniazid, a drug widely used for the treatment of tuberculosis, inhibits their synthesis (3-6). We have purified the saturated and unsaturated C_{16}-C_{56} fatty acids from M. tuberculosis H37Ra, which are potential precursors of mycolic acids. These acids included the C_{34}-C_{56} fatty acids containing cis-cyclopropane rings (7-11).

In the present communication, we report on the isolation and purification of homologous series of long-chain fatty alcohols from M. tuberculosis H37Ra which are structurally related to mycolic acids. These new fatty alcohols were fractionated by HPLC as their 3,5-dinitrobenzoyl derivatives. Complete separation was achieved with components differing by only two carbon units.

MATERIALS

Acetonitrile, p-dioxane, chloroform, and methanol were obtained from the Burdick and Jackson Laboratory, Muskegon, MI. The 3,5-dinitrobenzoyl chloride reagent was obtained from Regis Chemical Company, Morton Grove, IL.

Growth of Bacteria

Cells of M. tuberculosis H37Ra were grown at 37°C in glycerol-alanine-salts medium in a New Brunswick 28-liter fermentor (6) and harvested at late-log growth (17-21 days).

Isolation and Purification of Long-Chain Fatty Alcohols

The scheme for the isolation and purification of long-chain fatty alcohols from M. tuberculosis H37Ra is shown in Figure 1. Harvested cells (1.0 Kg, drained weight) were extracted twice overnight with 8 liters each of chloroform-methanol (2:1, v/v). The pooled extract was evaporated to dryness and saponified by refluxing for 4h in 600 ml of 5% KOH (w/v) in ethanol-water (1:1, v/v). The sample was acidified with 6N HCl (pH 1-2) and extracted three times with equal volumes of diethyl ether. The pooled ether extract was washed with an equal volume of water and evaporated to dryness. The ether extract (6 g) was applied to a silicic acid column (450 g of Bio Sil HA, minus 325 mesh, Bio Rad Laboratories, Richmond, CA). The column was eluted in a stepwise gradient of 5 and 8% diethyl ether in petroleum ether to remove the fatty acids. The alcohols were eluted with 25% diethyl ether in petroleum ether into three pooled fractions totaling 320 mg. This preparation contained 650 mg of mycolic acids, 300 mg of C_{30}-C_{40} fatty acids, and 2.85 g of C_{16}-C_{26} fatty acids. The first pooled fraction (120 mg) was enriched with long-chain fatty alcohols whereas the other two fractions contained the shorter-chain fatty alcohols. The first pooled fraction was further fractionated by thin-layer chromatography (TLC) on silica gel G plates (Analtech Inc., Newark, DE) using the petroleum ether-diethyl ether (3:2, v/v) system to remove the remaining traces of fatty

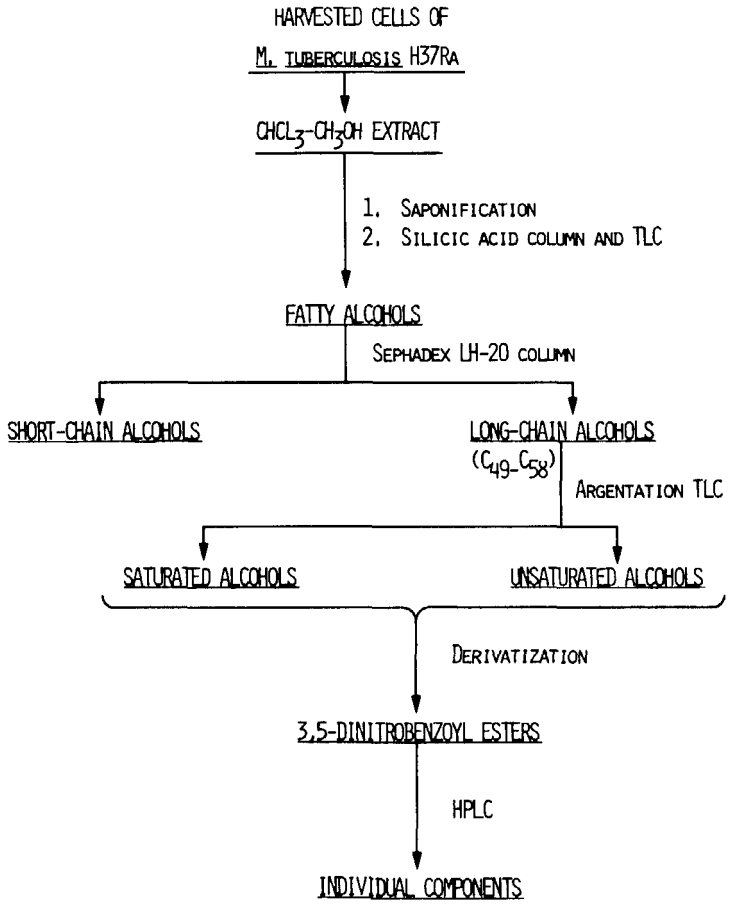

FIGURE 1. Scheme for the isolation and purification of long-chain fatty alcohols from M. tuberculosis H37Ra.

acids and then fractionated on a Sephadex LH-20 column. Two pooled fractions were obtained - the long-chain fatty alcohols (C_{49}-C_{58}) and the short-chain fatty alcohols (dry weights not determined). The long-chain alcohols (major fraction) were separated into the saturated and unsaturated alcohols by using an $AgNO_3$-impregnated silica gel G column (Hi-Flosil-Ag, Applied Science Division, Laboratory Data

Control/ASD, Park Ridge, IL). The saturated alcohols (26 mg) were eluted from the column with 10% diethyl ether in petroleum ether and the unsaturated alcohols (7 mg) were eluted with 20% diethyl ether in petroleum ether.

The saturated (13 mg) and unsaturated fatty alcohols (3.5 mg) were derivatized as the dinitrobenzoyl esters by adding 500 mg and 250 mg respectively of dinitrobenzoyl chloride in 5 ml of tetrahydrofuran along with 2-3 drops of pyridine and heating at 60°C for 4 hrs with stirring.

HPLC Fractionation

HPLC was performed with a system consisting of: two Waters model 6000A solvent delivery systems, a Waters model 660 solvent programmer, a Waters model U6K universal liquid chromatograph injector, a Waters radial compression separation system, and a Perkin-Elmer variable wavelength detector (model LC-55). Waters 0.8 mm x 10 cm, Radial Pak A cartridge C_{18}-bonded silica) was used to fractionate the saturated fatty alcohol esters. A 4 mm x 30 cm Varian Micro Pak MCH octadecylsilane (monomeric) C_{18}-bonded silica column was used specifically to fractionate the unsaturated fatty alcohol esters because it was superior.

Instrumental Analyses

Fourier transform proton nuclear magnetic resonance (NMR) spectra were obtained with a Bruker model HX-90E spectrometer at 90 MHz. Mass spectra were obtained on an AEI model MS 902 mass spectrometer using an ionization potential of 70 ev. and source temperature of 150-170°C. Samples were introduced at the inlet with a probe.

RESULTS AND DISCUSSION

Fig. 2 shows the HPLC separation of the saturated dinitrobenzoyl derivatives into two major and six minor frac-

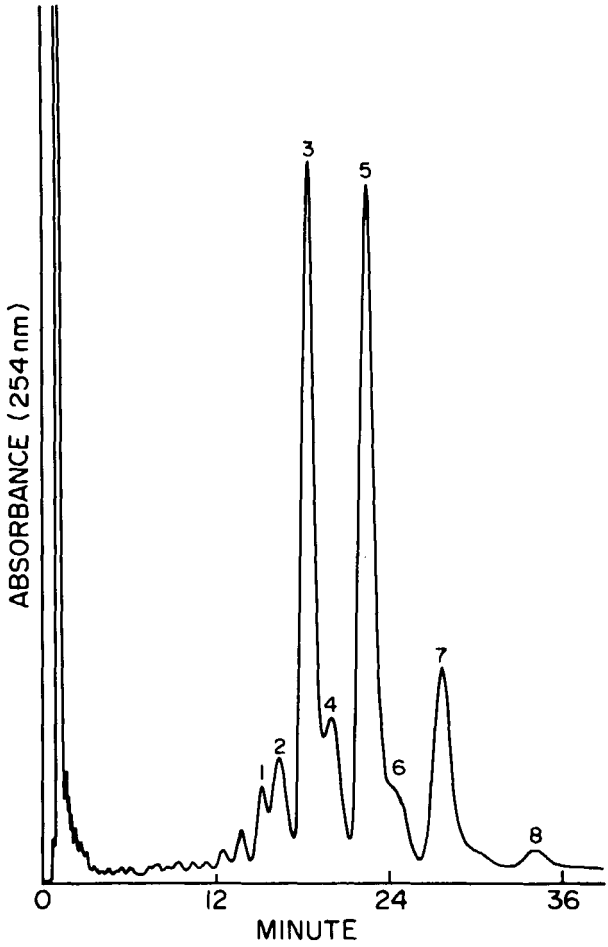

FIGURE 2. HPLC fractionation of the dinitrobenzoyl derivatives of saturated C_{50}-C_{58} fatty alcohols. Waters Radial Pak A cartridge was used. Mobile phase was p-dioxane-acetonitrile (3:2, v/v) at a flow rate of 3 ml/min.

tions. The mass spectra of these individual fractions showed molecular ion peaks (M) at m/e 894, 922, 950, and 978 for components 1, 3, 5 and 7 respectively (Table 1). Intense peaks due to M-60 (M-2xNO) fragments appeared at m/e 834,

TABLE 1
Mass Spectral Identification of the Peaks Obtained from HPLC Fractionation of the 3,5-Dinitrobenzoyl Ester of Saturated Fatty Alcohols. Refer to Fig. 2.

HPLC peak	No. of carbon[1]	m/e		
		M	M-60	M-195+1
1	C_{49}	894	834	700
2	C_{50}, C_{52}			714, 742
3	C_{51}	922	862	728
4	C_{52}, C_{54}			742, 770
5	C_{53}	950	890	756
6	C_{52}, C_{56}			770, 798
7	C_{55}	978	918	784
8	C_{57}		946	812

[1]All components are thought to contain two cis-cyclopropane rings.

862, 890, and 918 respectively for this same component series. This series also gave intense peaks due to the M-195+1 (the free alcohol) fragments appearing at m/e 700, 728, 756, and 784 respectively. M-212 fragments were also observed. The mass spectral fragmentations of the 3,5-dinitrobenzoyl ester is shown in Fig. 3.

Each of these HPLC fractions differed by two carbons (28 atomic mass units [amu]). Smaller, incompletely resolved shoulder fractions (peaks 2, 4, and 6) appeared which differed by a single carbon unit (14 amu) from their neighboring major peaks. Each of these peaks contained two separate components of which one appeared to be in the more polar series.

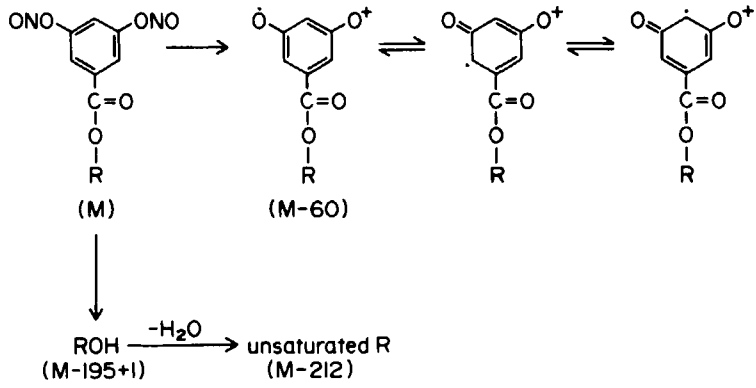

FIGURE 3. Mass spectral fragmentations of a 3,5-dinitrobenzoyl ester of a fatty alcohol.

As shown in Figure 4, HPLC separated the unsaturated dinitrobenzoyl derivative of the fatty alcohol into four major (peaks 1, 3, 5, and 7) and three minor (peaks 2, 4, and 6) fractions. Intense peaks due to M-60 fragments appeared at m/e 860, 888, 916, and 944 for peaks 1, 3, 5, and 7 respectively (Table 2). Intense peaks due to M-195+1 appeared at m/e 726, 754, 782, and 810.

Fractionation of components differing by two carbons was achieved. Smaller minor fractions (peaks 2, 4, and 6) appeared which differed by a single carbon unit from their neighboring major peaks. Each of these peaks contained two separate components of which one appeared to be in the more polar series.

Proton Nuclear Magnetic Resonance Studies

The NMR spectrum of saturated long-chain fatty alcohols in $CDCl_3$ showed chemical shifts at 3.70, 3.63, and 3.53 ppm for the presence of the hydroxyl group. A band appeared at

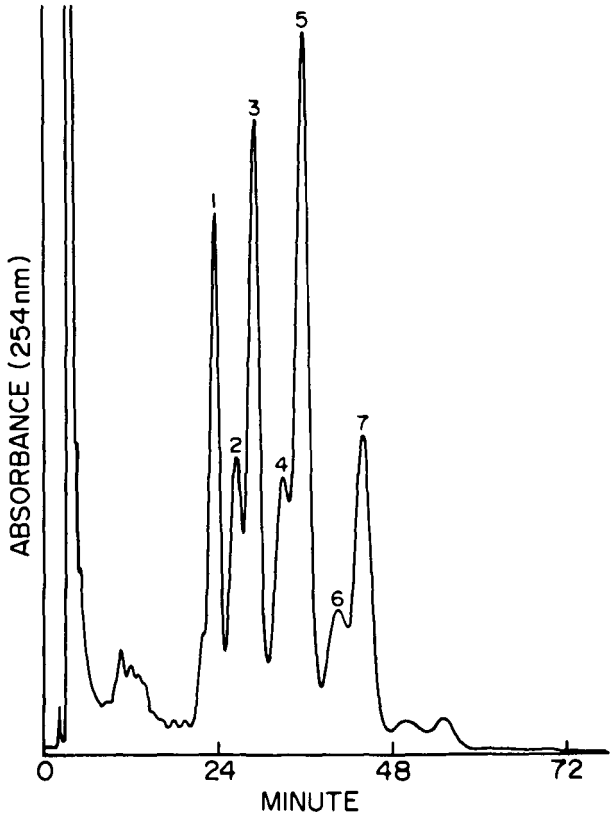

FIGURE 4. HPLC fractionation of the dinitrobenzoyl derivatives of unsaturated C_{50}-C_{58} fatty alcohols. Column used was Varian Micro Pak MCH octadecylsilane. Mobile phase was p-dioxane-acetonitrile (7:13, v/v), at a flow rate of 1 ml/min.

0.87 ppm which was attributed to the protons of the terminal methyl group. Two signals at 0.60 and -0.30 ppm showed the presence of the protons for the cis-cyclopropane ring. A resonance due to hydroxyl proton appeared at 1.49 ppm which disappeared when D_2O was added and a band due to the proton exchange appeared at 4.60 ppm. Similar bands were observed

TABLE 2
Mass Spectral Identification of the Peaks Obtained from HPLC Fractionation of the 3,5-Dinitrobenzoyl Ester of Unsaturated Fatty Alcohols. Refer to Fig. 4.

HPLC peak	No. of carbon[1]	No. of apparent unsaturation	m/e M-60	m/e M-195+1
1	C_{51}	3	860	726
2	C_{52}, C_{54}	3		740,768
3	C_{53}	3	888	754
4	C_{54}, C_{56}	3		768,796
5	C_{55}	3	916	782
6	C_{56}, C_{58}	3		796,824
7	C_{57}	3	944	810

[1]The esters could contain a mixture of double bonds and cis-cyclopropane rings.

for the unsaturated alcohols except that they also showed chemical shifts at 5.41, 5.36, and 5.30 for olefinic protons.

Structure of the Fatty Alcohols

These C_{50}-C_{59} fatty alcohols contained cis-cyclopropane rings in both the saturated and unsaturated series and were isolated as esters of normal fatty acids. They appeared to be related to the α-mycolic acids (2) and the C_{36} dicyclopropyl fatty acid (8). Preliminary evidence suggests that they are products of degradation of mycolic acids.

ACKNOWLEDGMENTS

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