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Note

Separation of homologues of methyl ester and 3-O-acetyl methyl ester derivatives of the corynomycolic acid fraction from *Corynebacterium pseudotuberculosis*

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Mycolic acids are high-molecular-weight α-branched, β-hydroxylated fatty acids that characteristically undergo pyrolytic cleavage between the C-α–C-β linkage. The resulting fatty acid unit (merofatty acid) bears the α-branch and the first two carbon atoms of the whole molecule (R_1–CH_2–COOH). On the other hand, the fatty aldehyde unit (meraldehyde) corresponds to the main chain of mycolic acid less the first two carbon atoms, and therefore displays a carbon chain from methyl terminal to C-β(R_2–CHO) (Fig. 1). The number of carbon atoms of the α-branch centre either around C-16, as in representatives of the genera *Artrobacter*[1], *Brevibacterium*[2], *Corynebacterium*[3], *Nocardia*[4] and *Rhodococcus*[5,6], or C-24, in *Mycobacterium tuberculosis*[7], and C-22 in the majority of representatives of the genus *Mycobacterium*[8].

In general, the α-branch is represented by an alkyl group, and an alkenyl group may occur in small proportions. For example, the corynomycoldienic acid fraction and a small proportion of the corynomycolenic acid fraction from *C. diphtheriae* and *C. pseudotuberculosis* under pyrolysis liberate the monounsaturated fatty acid unit in which the double bond is located at C-9.

The β-unit displays progressive structural complexity with increasing chain length. Thus, the approximately C_{32} mycolic acids (corynomycolic acids) have either a saturated or a monounsaturated hydrocarbon chain with a double bond at C-9 from the carbonyl group of the meraldehyde unit[2,9]. The approximately C_{40} mycolic acids (short-chain nocardomycolic acids) contain in the β-unit a carbon chain of about C_{24}–C_{32}, and in *R. rhodochrous* only saturated and monounsaturated hydrocarbon chains have been reported[5,10]. However, approximately C_{50} mycolic acids (nocardic acids[4] or nocardomycolic acids) show an approximate chain length in the range C_{34}–C_{40} in which up to four double bonds may be found[11]. Therefore, the structure of the β-chain unit and also the amount of the corresponding homologue respond to the

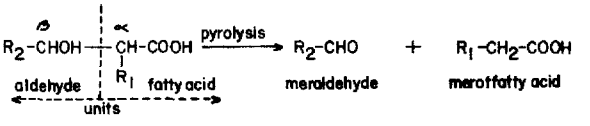

Fig. 1. Scheme of pyrolytic fragmentation of mycolic acids.

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complexity of composition of the nocardic acid fraction from *N. astéroides*. Similar reasoning can be applied to approximately C_{60} mycolic acids (long-chain nocardomycolic acids)[6] found in *R. bronchialis*[6], *R. aurantiacus* and *R. sputi*[12].

For approximately C_{80} mycolic acids, the structure of the β-unit becomes more complex, and in addition to the occurrence of double bonds in the hydrocarbon chain, there may be an additional side-chain from the introduction of a methyl or cyclopropyl group[13]. Moreover, other functional groups may be present, such as carbonyl and methoxyl[14].

Although a great deal of work has been done on mycolic acids, the investigation of procedures for the isolation of individual mycolic acids on the basis of the number of carbon atoms is limited. An interesting method is the separation of *p*-bromophenacyl mycolates into their homologues by reversed-phase column liquid chromatography (LC)[15,16]. However, the development of alternative procedures was thought to be of interest in order to provide another means of isolating individual homologues from a complex mixture of mycolic acids as methyl ester or O-acetyl methyl ester derivatives.

In this paper, the separation of C_{30}, C_{31}, C_{32} and C_{34} mycolic acids from *C. pseudotuberculosis* as methyl ester or 3-O-acetyl methyl ester derivatives by reversed-phase LC is described.

EXPERIMENTAL

All reagents were of analytical-reagent grade; commercial and analytical-reagent grade solvents were distilled before use. LiChrosorb-grade solvents were obtained from E. Merck (Darmstadt, F.R.G.).

Crude methyl corynomycolate from *C. pseudotuberculosis* was prepared essentially as described[17], and the methyl corynomycolate fraction was obtained by repeated crystallization using methanol. The corynomycolic acid fraction was obtained from methyl corynomycolate after alkaline hydrolysis[18], followed by preparative thin-layer chromatography (TLC) (solvent C, see below) and subsequent LC on silicic acid (Carlo Erba, Milano, Italy) [eluted with 40% (v/v) diethyl ether in *n*-hexane]. A standard corynomycolic acid fraction was from *C. diphtheriae*[18].

TLC was carried out either on plates coated in the laboratory with silica gel H (Merck) or on plastic sheets precoated with silica gel 60 F_{254}, layer thickness 0.2 mm (Art. No. 5735, Merck). The developing solvents were (A) *n*-hexane–diethyl ether–acetone–acetic acid (70:10:10:1), (B) *n*-hexane–diethyl ether–acetone–acetic acid (70:20:5:1), (C) *n*-hexane–diethyl ether–acetone–acetic acid (70:30:11:1) and (D) chloroform–acetone–methanol–water (50:60:2.5:3) (all proportions by volume). The precoated sheets were developed overnight in solvent D, then air dried in a hood; the solvent-free plates were kept in the original box and used without further heat activation.

The TLC plates were developed at room temperature (23–26°C) without previous chamber saturation.

Detection of spots on the plates was carried out under iodine vapour or by spraying with 20% (w/v) potassium dichromate in 10% (v/v) sulphuric acid and heating the plates at 110°C for approximately 10 min.

LC was carried out using a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 2/2 pump with a Rheodyne 7125 injection port and a syringe-loading sample injector (20 μl

loop). The mobile phase was ethanol–distilled water–acetonitrile (90:10:5, v/v/v) containing 5% (v/v) of *n*-hexane (for methyl corynomycolate) or ethanol–distilled water (90:10, v/v) containing 20% (v/v) of *n*-hexane (for 3-O-acetyl methyl corynomycolate), at a flow-rate of 1 ml/min.

A stainless-steel column (250 mm × 4.6 mm I.D.) containing Spherisorb octadecylsilane–silica gel (ODS, 5 μm) was obtained from Spectra-Physics (Santa Clara, CA, U.S.A.). The detector was an SP-8400 variable-wavelength ultraviolet–visible spectrophotometer from Spectra-Physics, set at 215 nm. An SP-4100 integrator (Spectra-Physics) was used, the chart speed was set at 0.5 cm/min and attenuation 4 was used. For preparative purposes (with methyl corynomycolate), several runs were carried out by collecting 15 drops/min using an LKB (Bromma, Sweden) 7004 drop counter connected to an LKB Ultrorac 7000 fraction collector; each cycle was started from the same tube. Fractions corresponding to the most abundant homologue were concentrated to dryness with a stream of nitrogen in a hood. The white powdery residue was dissolved in a small volume of benzene and submitted to analytical chromatography for purity determination.

RESULTS AND DISCUSSION

The TLC behaviour of the methyl corynomycolate derivative from corynomycolic acid fraction of *C. pseudotuberculosis* is shown in Table I. It migrates with an R_F value that is intermediate between those of the unesterified form and the O-acetylated derivative. The melting point, refractive index and specific optical rotatory power are given in Table I, and compared with those of the acid and the 3-O-acetyl derivative.

Separation of the methyl corynomycolates fraction by LC on octadecylsilane–silica gel into homologues (methyl esters of C_{30}, C_{31}, C_{32} and C_{34} corynomycolic acid) with increasing retention times was achieved (Fig. 2, trace A). The component attributed to the methyl ester of C_{31} corynomycolic acid was inferred by plotting the logarithm of retention time against the number of carbon atoms in the mycolic acids. On the basis of the detector response and of the percentage peak area, the components accounted for approximately 13.1, 2.3, 80.6 and 4.1%, corresponding to methyl C_{30}, C_{31}, C_{32} and C_{34} corynomycolate, respectively. Previous studies on the composition of the saturated corynomycolic acid fraction from *C. pseudotuberculosis* as O-acetyl

TABLE I

TLC BEHAVIOUR AND PHYSICO-CHEMICAL CHARACTERISTICS OF CORYNOMYCOLIC ACID FRACTION FROM *C. PSEUDOTUBERCULOSIS* AND OF METHYL- AND 3-O-ACETYL METHYL ESTER DERIVATIVES

For the composition of mobile phases A–C, see Experimental. n_D of immersion oil (E. Merck) = 1.514.

Preparation	R_F			*Characteristics*		
	A	*B*	*C*	*m.p. (°C)*	n_D	$[\alpha]_D^{25}$
Corynomycolic acid	0.21	0.11	0.31	68–70	–	+ 6.4
Methyl corynomycolate	0.48	0.31	0.54	56–58	–	+11.4
3-O-Acetyl methyl corynomycolate	0.62	0.50	0.64	–	1.453	+16

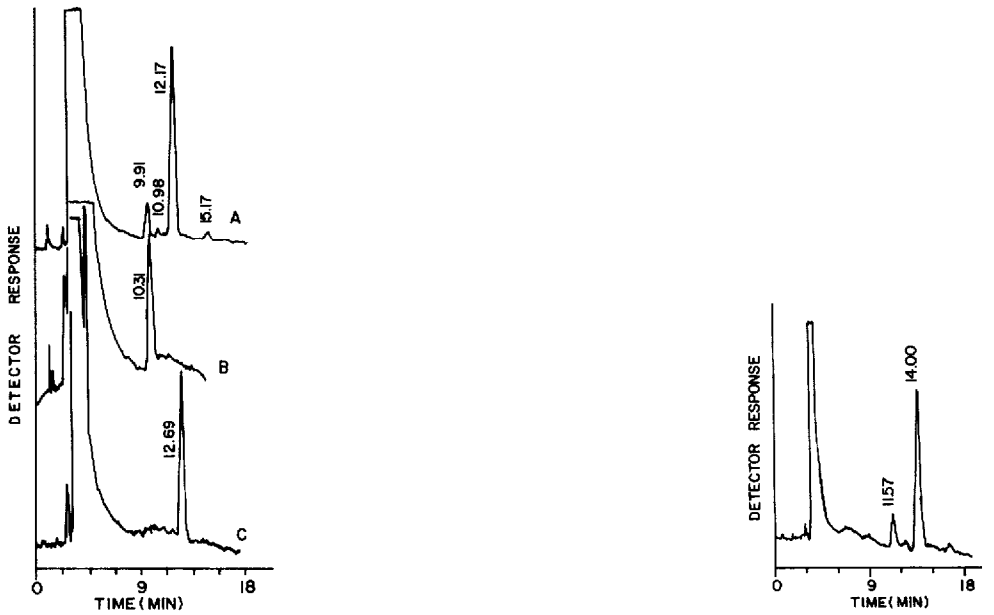

Fig. 2. Separation of methyl ester derivative of corynomycolic acid fraction from *C. pseudotuberculosis* into homologues (trace A) by reversed-phase LC on an octadecylsilane–silica gel column (250 mm × 4.6 mm I.D.). The peaks at 9.91, 10.98, 12.17 and 15.17 min correspond to the methyl ester of C_{30}, C_{31}, C_{32} and C_{34} corynomycolic acid, respectively. Trace B, methyl C_{30} corynomycolate; trace C, metyl C_{32} corynomycolate. Solvent: ethanol–water–acetonitrile (90:10:5, v/v/v) containing 5% (v/v) of *n*-hexane at a flow-rate of 1 ml/min. Detection at 215 nm.

Fig. 3. Separation of 3-O-acetyl methyl ester derivative of the corynomycolic acid fraction from *C. pseudotuberculosis* into homologues by reversed-phase LC on an octadecylsilane–silica gel column (250 mm × 4.6 mm I.D.). Solvent: ethanol–water (90:10, v/v) containing 20% (v/v) of *n*-hexane at a flow-rate of 1 ml/min. Detection at 215 nm.

methyl ester derivatives by gas chromatography combined with mass spectrometry showed mainly the homologues of C_{30}, C_{32} and C_{34} corynomycolic acids[17].

By carrying out several runs, the preparation of a single fraction was possible (Fig. 2, traces B and C; fractions corresponding to methyl C_{30} and C_{32} corynomycolate, respectively). In a similar way, 3-O-acetyl methyl corynomycolate can be separated into homologues by using reversed-phase LC (Fig. 3).

The derivatization procedure used to date for separating homologues of mycolic acids by reversed-phase LC involves the reaction of the carboxyl group with *p*-bromophenacyl bromide, the resulting *p*-bromophenacyl ester derivatives emerging from the column being detected at 254 nm. This technique was used for the separation of homologues of mycolic acids from *M. tuberculosis* $H_{37}Ra$[16] and *M. smegmatis*[15]; recently the usefulness of the same method for identifying the group of mycolic acids from C_{32} to C_{80} was shown, and the utilization of this procedure to classify clinical isolates was proposed[19].

The possibility of detecting methyl mycolate at 215 nm adds an alternative option for the isolation of homologues from a complex mixture of methyl ester derivatives of longer mycolic acids.

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