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Thermospray mass spectral analyses of corynomycolic acids and their derivatives

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Abstract

Thermospray mass spectral (TSP-MS) analyses were carried out on methyl corynomycolates, their 3-O-acetyl and 3-O-benzoyl derivatives, and on corynomycolic acids and their 3-O-acetyl derivatives, using an ion generating solvent system consisting of water/isopropanol (99:1, v/v) containing 0.1 M ammonium acetate. Methyl corynomycolates generated three groups of peaks corresponding to adducts M-18 + H, M + H and M + NH_4, while two groups of peaks representing adducts M − 60 + H and M + H + NH_4 were seen in the spectra of 3-O-acetyl methyl corynomycolates. The 3-O-benzoyl methyl corynomycolates gave a series of peaks representing the adducts M − 122 + H, M + 2H and M + H + NH_4. In the spectra of 3-O-acetyl corynomycolic acids, a series of peaks which represented M − 60 + H and M + NH_4 was observed, and in turn, mass spectra of corynomycolic acids revealed peaks that represented the adducts M − 18 + H and M + NH_4. Therefore, methyl corynomycolates, 3-O-acyl derivatives of methyl corynomycolates, 3-O-acetylated derivatives of corynomycolic acids and the underivatized corynomycolic acids all exhibited the formation of an adduct of the anhydro compounds. These anhydro forms were generated by a generalized process.

Keywords: Corynomycolic acids; Mycolic acids; Thermospray; Mass spectrometry

1. Introduction

Bacteria belonging to the genera *Corynebacterium*, *Rhodococcus*, *Nocardia*, *Gordona* and *Mycobacterium* typically synthesize high molecular weight α-branched-β-hydroxylated fatty acids called mycolic acids [1]. The following groups of mycolic acids may be considered: (1) those with a carbon chain length centered around C_{32}, named corynomycolic acids and isolated from *C. diphtheriae* [2–4]; (2) those with a carbon chain length

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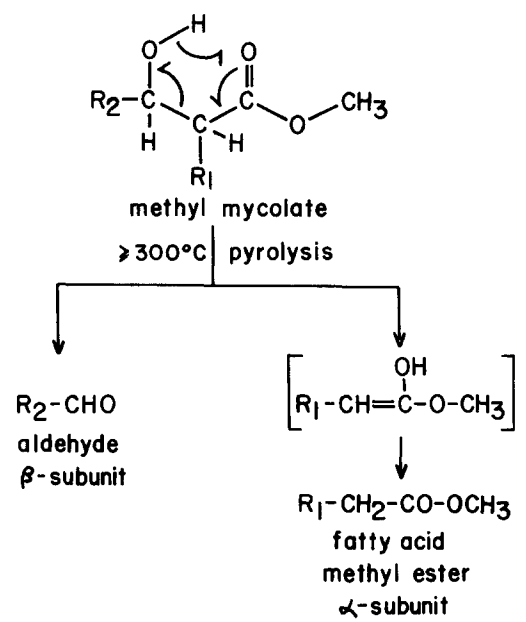
centered around C_{50}, named nocardic or nocardomycolic acids, isolated from *N. asteroides* [5,6]; (3) those with a carbon chain length centered around C_{40}, the short chain nocardomycolic acids, isolated from *R. rhodochrous* (previously classified as *N. rhodochrous*) [6]; (4) those with a carbon chain length centered around C_{60}, the long chain length nocardomycolic acids, isolated from *G.bronchialis* [7,8]; and (5) those with a carbon chain length centered around C_{90}, typified by mycolic acids isolated from *M. tuberculosis* [1].

After the discovery of these various-sized mycolic acids, investigations seeking to use these α-branched-β-hydroxylated long chain fatty acids for taxonomical purposes established that mycolic acids of a specific size range are characteristic of representative organisms within a given genus. There are, however, exceptions in which more than one group of mycolic acids may be found together within a single species. For example, *N. asteroides* 10905 was shown to contain both corynomycolic and nocardomycolic acids [6]. Pommier and Michel [9] reported the occurrence of two groups of mycolic acids, namely corynomycolic acids and nocardomycolic acids, in five (31%) of sixteen strains of *N. otitidiscaviarum* which they examined. Nocardomycolic acids were always found in the bound cell wall lipid preparations from these organisms while corynomycolic acids were absent.

Electron impact mass spectrometric analyses of mycolic acids have been done primarily as methyl ester derivatives [7,10,11]. The introduction of gas-liquid chromatography (GLC) coupled to mass spectrometry improved significantly the analytical means for studying the structure and composition of nocardomycolic acid homologues. Development of derivatization procedures increased the volatility of mycolic acids, thus decreasing the pyrolytic fragmentation observed with methyl mycolates [12]. The high temperature required to separate mycolic acids methyl esters by GLC resulted in pyrolytic cleavage forming two subunits (1): the α-subunit is a fatty acid methyl ester, while the β-subunit is an aldehyde [10]. To prevent this fragmentation, trimethylsilylation of mycolic acids resulted in methyl 3-trimethylsilyl nocardomycolates which were suitable for GLC analysis. Using these procedures, the structural composition of nocardomycolic acids from *N.corallina* [12] and *N. rubra* [13] were determined. Even though trimethylsilyl derivatives of methyl nocardomycolates are separated by GLC, their mass spectra have intense peaks representing fragmentation ions corresponding to the methyl ester of the fatty acid α-subunit and to the meroaldehyde β-subunit of the mycolic acid molecule. As a consequence, the ion peak that represents the molecular ion minus trimethylsilylol (M-90) is weak, or often missing, by standard electron impact mass spectrometry [13].

Acetylation reactions of mycolic acid methyl esters result in 3-O-acetyl derivatives that are suitable for gas chromatography combined with mass spectrometric analysis [14]. As with the trimethylsilyl derivatives of mycolic acids, peaks representing the molecular ion minus acetic acid (M-60) or the molecular ion minus acetic acid and the methoxyl group (M-91) are found in the mass spectra in significantly reduced intensities relative to the fragment ions which represent the fatty acid and the meroaldehyde subunits [14]. Addi-



Scheme 1.

tional fragmentation of mycolic acid molecules occurs using electron impact mass spectrometry. Consequently, the determination of the mycolic acid size may be complicated.

To provide an additional tool for studying mycolic acids, the molecular weight of these lipid compounds in the form of methyl ester derivatives were determined by using thermospray mass spectral (TSP-MS) analysis [15]. In these studies, it was found that methyl mycolates formed adducts either with a hydrogen ion or with an ammonium ion. It was thought that, along with methyl ester derivatives, other mycolic acid derivatives could be used for TSP-MS analysis. In this communication, results of studies carried out on C_{32}-mycolic acids and a variety of related derivatization products are presented. In addition, a solvent system for TSP-MS that contains lower amounts of organic solvents than in previous studies [15] is reported.

2. Materials and methods

2.1. Chemicals and solvents

Reagents were of analytical grade and solvents were distilled before use. Palmitic and oleic acids were from Sigma Chemical Co., Saint Louis, MO, USA; methyl palmitate and methyl oleate were from Polyscience Corporation, Evanston, IL, USA. Corynomycolic acid was from *C. pseudotuberculosis*. Methyl corynomycolate obtained by esterification of corynomycolic acid was purified by crystallization in *n*-hexane and in methanol.

2.2. Chromatographic adsorbents

Silicic acid was from Carlo Erba, Milano, Italy, and silica gel H and neutral aluminum oxide were from E. Merck, Darmstadt, FRG. Silica gel 60 F_{254} precoated plastic sheets for thin-layer chromatography (TLC) was from E. Merck.

2.3. Chromatographic techniques

TLC was carried out using the following solvent systems: *n*-hexane/diethyl ether/acetone/acetic acid (70:30:11:1, v/v/v/v), solvent A; *n*-hexane/diethyl ether/acetone/acetic acid (70/20/5/1, v/v/v/v), solvent B; chloroform/methanol (100:0.4, v/v), solvent C; petroleum ether/acetone (90:10, v/v), solvent E; petroleum ether/acetone (95:5, v/v), solvent F. Lipids on silica gel plates were detected using iodine or by spraying with 10% (v/v) aqueous sulphuric acid, then heating the plates for 10–15 min at 110°C. Lipids on silver nitrate-impregnated plates were observed by spraying with 0.05% (w/v) rhodamine B in ethanol/water (1:1, v/v) followed by 20% (v/v) glacial acetic acid in water.

2.4. Preparation of methyl corynomycolate, methyl corynomycolenate and methyl corynomycoldienate from C. diphtheriae

Methyl corynomycolate and methyl corynomycolenate were prepared as previously described [16]. Isolation of methyl corynomycoldienate was carried out as follows: the weakly stained band found at (R_f 0.15) on silver nitrate impregnated-silica gel plates in the preparation of methyl corynomycolate (R_f 0.7), and methyl corynomycolenate (R_f 0.3), using solvent C, was scraped from several preparative plates. The lipid material was purified on silica gel plates using the same solvent system; the band was scraped and elution was done as usual. The recovered material was filtered on a column of silicic acid/silica gel (1:1, w/w) equilibrated in chloroform, and elution conducted with chloroform. Solvent was evaporated under a stream of nitrogen, and the remaining material was a transparent and viscous liquid.

2.5. Preparation of corynomycolic, corynomycolenic and corynomycoldienic acids from C.diphtheriae.

To about 1–2 mg of methyl ester, 1 ml chloroform/methanol (1:2, v/v) and 0.1 ml 5 N KOH in methanol/water (3:1, v/v) were added. The mixture was incubated for 5 h at 60°C. After cooling, 3 ml chloroform and 1 ml water were added. The mixture was acidified with 6 N HCl; chloroform was withdrawn with a Pasteur pipette. The remaining aqueous phase was re-extracted with 1 ml

chloroform thrice. The pooled chloroform phase was washed with distilled water until neutral. The chloroform extract was filtered through chloroform-washed cotton wool in a Pasteur pipette; then the solvent was evaporated under a stream of nitrogen. The lipid material was filtered on a column of silicic acid/silica gel (1:1, w/w) equilibrated with chloroform. The column was eluted with chloroform, and solvent was evaporated with a stream of nitrogen. The material corresponding to corynomycolic acid was a white solid, while those corresponding to corynomycolenic and corynomycoldienic acids were transparent, viscous liquids.

2.6. Preparation of acetylated compounds

About 1–10 mg of lipid material was acetylated using 0.5–1.0 ml pyridine and 0.25–0.5 ml acetic acid anhydride [17]. The mixture was left overnight at room temperature, then 0.5–1.0 ml methanol was added. Solvents and reagents were evaporated under a stream of nitrogen, and the reaction product was dissolved in a small volume of chloroform and loaded onto a small silicic acid/silica gel column (2 cm × 0.6 cm) conditioned in chloroform. The column was eluted with chloroform which was evaporated under a stream of nitrogen to leave a colourless material that was further analyzed by TLC using either solvent B or E.

2.7. Mass spectral analyses

Mass spectral analyses were carried out using a Hewlett Packard HP-1090 liquid chromatograph coupled by way of a thermospray probe with a Hewlett Packard 5988A mass spectrometer. The sample, in chloroform solution, was introduced through the injection port equipped with a 20 μl loop. The carrier and ion generating solvent system consisted of distilled water/isopropanol (99:1, v/v) containing 0.1 M ammonium acetate. The flow rate was 1.0 ml/min and the mixture was directly directed into the ion source through the thermospray system. The ion source was set at 210°C; the probe temperature was kept around 195–205°C. The chemstation unit was used both as a control and data acquisition unit for the mass spectrometer. The mass spectra were printed on a Hewlett Packard plotter.

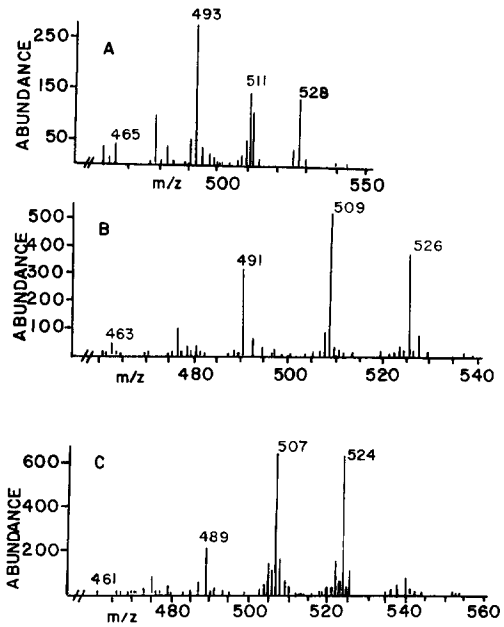


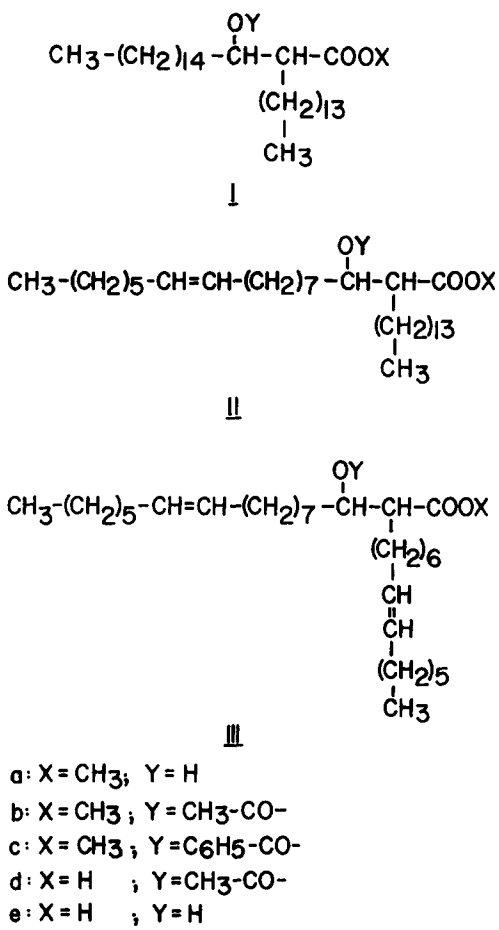
Fig. 1. Mass spectra of methyl corynomycolate (A), methyl corynomycolenate (B) and methyl corynomycoldienate (C).

3. Results and discussion

Thermospray mass spectral analysis of the major $C_{32:0}$, $C_{32:1}$ and $C_{32:2}$ homologues of corynomycolic acids and their derivatives was carried out using 0.1 M ammonium acetate in distilled water/isopropanol (99:1, v/v). The mass spectra (Fig. 1A) of methyl corynomycolate (Ia; 2) from *C. diphtheriae* revealed peaks at m/z 493 that corresponded to the adduct of the dehydration product of methyl corynomycolate, anhydro methyl corynomycolate, i.e., M-18 + H. In addition, peaks observed at m/z 511 and 528 represented adducts M + H and M + NH_4, respectively. The mass spectrum of methyl corynomycolenate (Ib) showed peaks at m/z 491, 509 and 526 that corresponded to M-18 + H, M + H and M + NH_4, respectively (Fig. 1B). Similarly, the spectrum of methyl corynomycoldienate (Ic) exhibited peaks

at m/z 489, 507 and 524 that corresponded to adducts M-18 + H, M + H and M + NH_4, respectively (Fig. 1C). Peaks seen at m/z 465, 463 and 461 in Fig. 1A, Fig. 1B and Fig. 1C represent adducts M-18 + H of lower homologous components, corresponding to methyl ester derivatives of corynomycolic acid in $C_{30:0}$, corynomycolenic acid in $C_{30:1}$ and corynomycoldienic acid in $C_{30:2}$, respectively.

The intensity of the peak corresponding to M-18 + H decreased as the number of double bonds increased (i.e., the intensity of peak M-18 + H is highest in methyl corynomycolate and lowest in methyl corynomycoldienate). This decreased intensity of peak M-18 + H (493 > 491 > 489) may be attributed to the degree of unsaturation that, in turn, was closely associated with the high volatility of the more unsaturated compounds. Therefore, the intensities of peaks representing M + H, and to a greater extent M + NH_4, increased from methyl corynomycolate to methyl corynomycolenate. Thus, in addition to the formation of adducts with hydrogen and ammonium ions as previously described [15], peaks representing the adducts of the product of dehydration of methyl mycolates were unexpectedly observed in the mass spectra. These spectra were generated using a solvent system containing decreased amounts of organic solvents compared to the previous studies, in which peak M-18 + H was not observed when chloroform/methanol/0.1 M ammonium acetate (40/50/10, v/v/v) were used [15].



Scheme 2.

The use of TSP-MS methodology for analysis of other derivatives of methyl corynomycolate and corynomycolic acid consistently revealed a series of characteristic peaks for each group of derivatives that were analyzed. In the case of methyl 3-O-acetyl corynomycolate (Ib), methyl 3-O-acetyl corynomycolenate (IIb) and methyl 3-O-acetyl corynomycoldienate (IIIb), the mass spectra (Fig. 2A,B,C, respectively) exhibited peaks at m/z 493 and 571, m/z 491 and 569, and m/z 489 and 567, which represented adducts M-60 + H (60 mass units correspond to acetic acid, CH_3COOH) and M + H + NH_4, respectively. Therefore, the ion that corresponds to M-60 + H represents adduct of anhydro methyl corynomycolate. Also, spectra of methyl 3-O-benzoyl corynomycolate (Ic) and methyl 3-O-benzoyl corynomycolenate (IIc) revealed a series of peaks at m/z 493, 616 and 633, and m/z 491, 614 and 631, respectively (Fig. 3A,B). These series of peaks corresponded to the adducts M-122 + H (122 mass units correspond to benzoic acid, C_6H_5COOH), M + 2H and M + H + NH_4. The mass spectra of 3-O-acetyl corynomycolic acid (Id), 3-O-acetyl corynomycolenic acid (IId) and 3-O-acetyl corynomycoldienic acid (IIId) showed peaks at m/z 479 and 556, m/z 477 and 554, and m/z 475 and 552 that corresponded to M-60 + H and M + NH_4, respectively (Fig. 4A,B,C). On the other hand, peaks at m/z 451 and 528, and 448 and 526 corresponded to adducts M-60 + H and M + NH_4 of lower derivatized homologues of corynomycolic acid in $C_{30:0}$

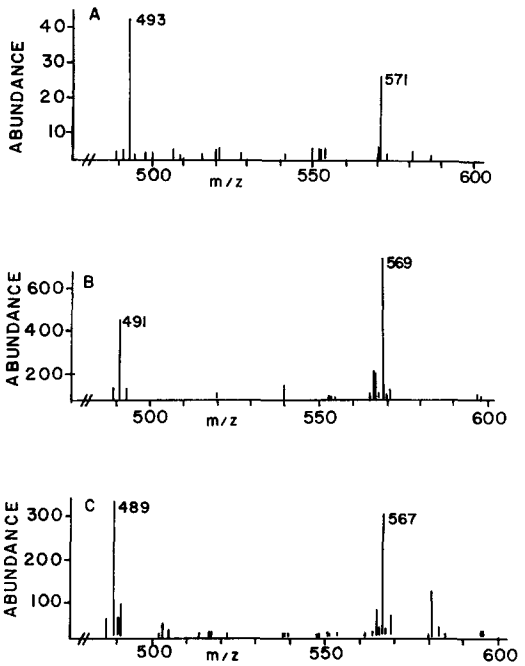


Fig. 2. Mass spectra of 3-O-acetyl methyl corynomycolate (A), 3-O-acetyl methyl corynomycolenate (B) and 3-O-acetyl methyl corynomycoldienate (C).

and $C_{30:1}$, respectively. Occurrence of homologous components was also previously observed [15]. In mass spectrum of 3-O-acetyl corynomycoldienic acid (Fig. 4C), peaks corresponding to the derivatized lower homologue were not clearly noted.

Direct analysis of corynomycolic acid (Ie, 2-tetradecyl-3-hydroxy octadecanoic acid), corynomycolenic acid (IIe, 2-tetradecyl-3-hydroxy octadec-11-enoic acid) and corynomycoldienic acid (IIIe, 2-tetra-7′-enyl-3-hydroxy octadec-11-enoic acid) by TSP-MS (Fig. 5A,B,C) revealed relevant peaks at m/z 479 and 514, m/z 477 and 512, and m/z 475 and 510, respectively. The first member of these three series corresponded to the adduct M-18 + H, while the second represented the adduct $M + NH_4$.

The appearance of peaks at m/z 493, 491 and 489 in mass spectra of methyl, methyl 3-O-acetyl, methyl 3-O-benzoyl derivatives of $C_{32:0}$-corynomycolic acid, $C_{32:1}$-corynomycolenic acid and $C_{32:2}$-corynomycoldienic acid, respectively, suggests occurrence of dehydration, deacetylation

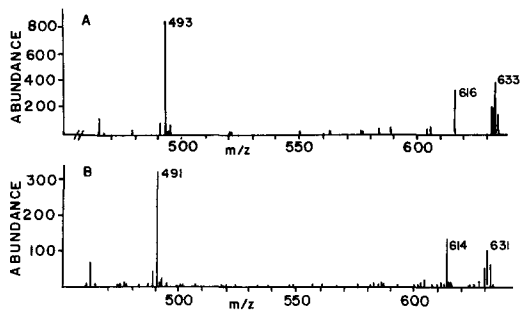


Fig. 3. Mass spectra of 3-O-benzoyl methyl corynomycolate (A) and 3-O-benzoyl methyl corynomycolenate (B).

and debenzoylation process, respectively, and formation of an adduct of the corresponding anhydro form with H. It is worth noting that formation of anhydro form also occurs under electron impact mass spectrometry [7,10,11]. Peaks found at m/z 479, 477 and 475 in TSP-MS of corynomycolic, corynomycolenic and corynomycoldienic acids and their 3-O-acetyl derivatives, respectively, indicate again occurrence of dehydration and deacetylation process that is followed by the formation of adducts of the anhydro forms. Therefore, formation of the anhydro forms may be represented according to 3.

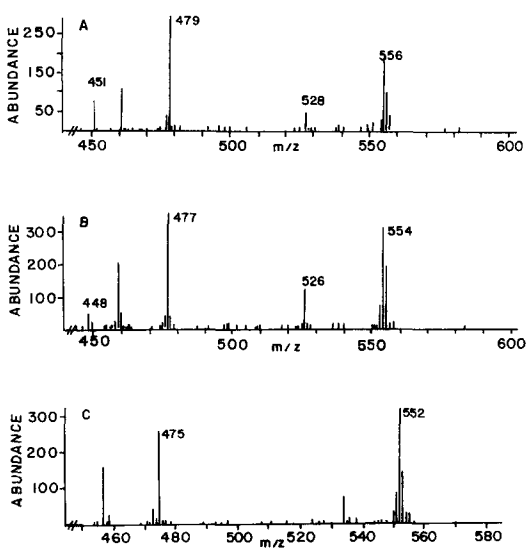


Fig. 4. Mass spectra of 3-O-acetyl corynomycolic acid (A), 3-O-acetyl corynomycolenic acid (B) and 3-O-acetyl corynomycoldienic acid (C).

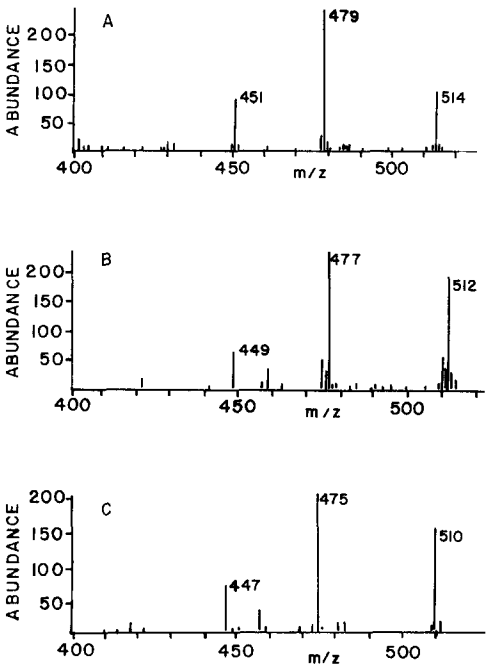


Fig. 5. Mass spectra of corynomycolic acid (A), corynomycolenic acid (B) and corynomycoldienic acid (C).

The possibility of analyzing O-ester derivatives of methyl corynomycolates by TSP-MS suggests the use of a combination of procedures: high performance liquid chromatography (HPLC) for separating the homologous fractions and on line TSP-MS analysis of the emerging compounds.

It is worth noting that the separation of 3-O-acetyl methyl corynomycolates into their homologous components with carbon chain lengths of C_{30}, C_{32} and C_{34} can be achieved by RP-HPLC using an ODS-silica gel column [18]. Similarly, 3-O-benzoyl methyl mycolates in the range from C_{30} to C_{48} may also be separated into individual homologous components by RP-HPLC using an ODS-silica gel column [16]. Therefore, either O-acetylation or O-benzoylation of methyl mycolates combined with RP-HPLC for separating homologous components, followed by TSP-MS analysis of the isolated homologue, represent a potentially important and useful tool for determining the composition and molecular weight of the corresponding underivatized mycolic acids. For simultaneously carrying out RP-HPLC and TSP-MS of methyl 3-O-benzoyl mycolates or methyl 3-O-acetyl mycolates, development of an adequate solvent system is required.

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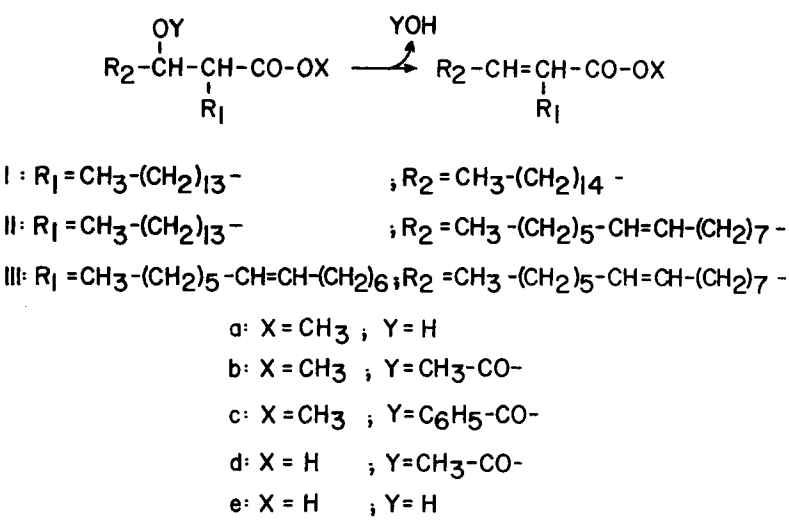

Scheme 3.

from the National Institute of Allergy and Infectious Diseases. Mass spectrometric studies were carried out in the Department of Medical Microbiology and Immunology, UCDavis. These investigations were made possible by a fellowship from FAPESP awarded to T.I. One of the authors (T.I.) wishes to thanks Dr. J. Carneiro da Silva, Director of the Instituto de Ciências Biomédicas, and Dr. M. Krasilchik, Vice-Chancellor of University of São Paulo for encouragements.

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