

Identification of some Neutral Lipids of *Thiobacillus thioparus* using Gas Chromatography-Chemical Ionization Mass Spectrometry

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Abstract

The neutral lipid fraction of two strains of *T. thioparus* was examined using gas chromatography-chemical ionization mass spectrometry. Both strains were found to produce fatty acid ethyl esters, long-chain alcohols and wax esters. In addition, one strain produced substantial quantities of squalene, although no sterols or triterpenoids could be detected. However, in the second strain, although squalene was present at greatly reduced levels, cholesterol, lanosteryl acetate and 24,25-dihydrolanosteryl acetate were identified.

Introduction

In the past decade, the fatty acid profiles of many species of thiobacilli have attracted interest (see, for example, Levin 1971, 1972), principally because of their possible utility as a taxonomic criterion (Agate and Vishniac 1973) for a genus which is difficult to assess by classical techniques and therefore is sometimes contentious (Hutchinson *et al.* 1965). Since the aim was to generate reference lipid profiles for use as a taxonomic aid for members of the genus *Thiobacillus*, grown under standardized conditions, no attempt was made to quantitate the levels of individual constituents in other than relative terms. However, little or no attention has been given to the neutral lipids of these bacteria, or indeed to bacteria in general. Prior to 1960 (Asselineau and Lederer 1960), squalene, the normal precursor of sterols and triterpenoids, had not been reported in a bacterial system although it was subsequently identified in several bacteria (see, for example, Bird *et al.* 1971) by gas chromatography-mass spectrometry (GCMS).

Triterpenoids (including sterols) are widely distributed throughout the biosphere, and in excess of 500 compounds of established structure are known, the bulk of which are confined to the plant kingdom (Connolly and Overton 1972). Formerly, all procaryotic organisms were thought unable to synthesize sterols (Fiertel and Klein 1959), but sterols have now been identified in several species of bacteria (Buetow and Levedahl 1964; Schubert *et al.* 1968; Bird *et al.* 1971), albeit at much lower levels than are commonly found in other life forms.

Materials and Methods

Organisms

Cultures of *Thiobacillus thioparus*, strains 01 and BJR-451, were obtained from the culture collection of the School of Biological Technology, University of New South Wales.

Culture

The thiobacilli were normally inoculated into 1 litre of thiosulfate medium (Vishniac and Santer 1957) and grown in 3-litre Erlenmeyer flasks shaken at 32°C for 10–14 days. Cultures were harvested by centrifugation for 10 min at 12000 *g*, washed by resuspension in distilled water, and centrifuged again.

Extracts

The wet biomass (0.1–0.3 g) was lyophilised and the dry residue extracted exhaustively with diethyl ether–hexane (1 : 1); the organic extract was washed with acid (2 M HCl), alkali (2 M NaOH) and, finally, distilled water. After drying over sodium sulfate the solvent was removed in a rotary evaporator, the residue being redissolved in 25 μ l of ethyl acetate prior to GCMS analysis.

Analyses

GCMS analyses were performed using a Finnigan 3200 chemical ionization GCMS system, with data collection and manipulation being effected with either 6115 or 2300 data systems (Finnigan Corp., Sunnyvale, Calif.). Typically an electron energy of 80–150 eV was used, together with an emission current of 0.8 mA. The ion source was held at a nominal 150°C.

The total gas chromatographic effluent was admitted to the ion source so that methane, used as the gas chromatographic carrier gas at 20 ml min⁻¹, also acted as (one of) the reactant gas(es); using methane alone the ion-source pressure was 120 Pa. For some analyses, ammonia was admitted separately to the ion source such that the total pressure was 133 Pa. Under these conditions the ammonia spectrum supplanted that of the methane and the system offered data supplementary to that achieved with methane chemical ionization mass spectrometry.

Most analyses were performed using a 1.5 m long glass column of 2 mm i.d. packed with 3% OV 1 on 100/120 mesh Gas Chrom Q; the initial temperature of 100°C was held isothermal for 1 min after injection and thereafter programmed at the rate of 8°C min⁻¹ to a maximum of 310°C. The transfer line, interface oven and injector were all maintained at 300°C.

Authentic Compounds

Squalene, cholesterol, lanosterol and 24,25-dihydrolanosterol were purchased from Sigma Chemical Co., St. Louis, Missouri; lanosterol and its dihydro analogue were acetylated using pyridine–acetic anhydride at room temperature overnight.

Fatty acid standards were purchased from Applied Science Laboratories Inc., State College, Pennsylvania; these were esterified by heating at 80°C for 40 min with anhydrous ethanol–1.25 M HCl.

Long-chain alcohol standards were prepared by lithium aluminium hydride reduction in dry ether of the corresponding fatty acid methyl esters. Both standards and sample extracts were analysed before and after acetylation (pyridine–acetic anhydride at room temperature), and those components designated as alcohols on the basis of their initial mass spectral characteristics all increased in molecular weight (by 42 atomic mass units) as a result of the acetylation, providing confirmation of a single free hydroxyl group.

Identification of the wax esters relied totally upon mass spectral evidence. The protonated molecular ions and alkyl (or NH₄⁺) adduct ions gave positive evidence for the molecular weights of the compounds.

Results and Discussion

During GCMS investigations of the total lipid extracts of several species of thiobacilli, aimed at confirming the identity of several minor fatty acids, a major constituent with a relatively long retention time was present consistently in the extracts of *T. thioparus* 01. It was identified, by its retention time and mass spectrum, as squalene. Repeated extraction, fractionation and characterization of the lipids of this organism failed to reveal the presence of any sterols or triterpenoids, although squalene, their logical precursor, was invariably present in large (16% of the neutral lipid fraction; estimated using gas chromatography with a flame ionization detector)

quantities irrespective of the growth conditions used. This suggested that this particular strain may have had a metabolic deficiency which prevented squalene cyclization, forcing its accumulation and precluding the formation of sterols.

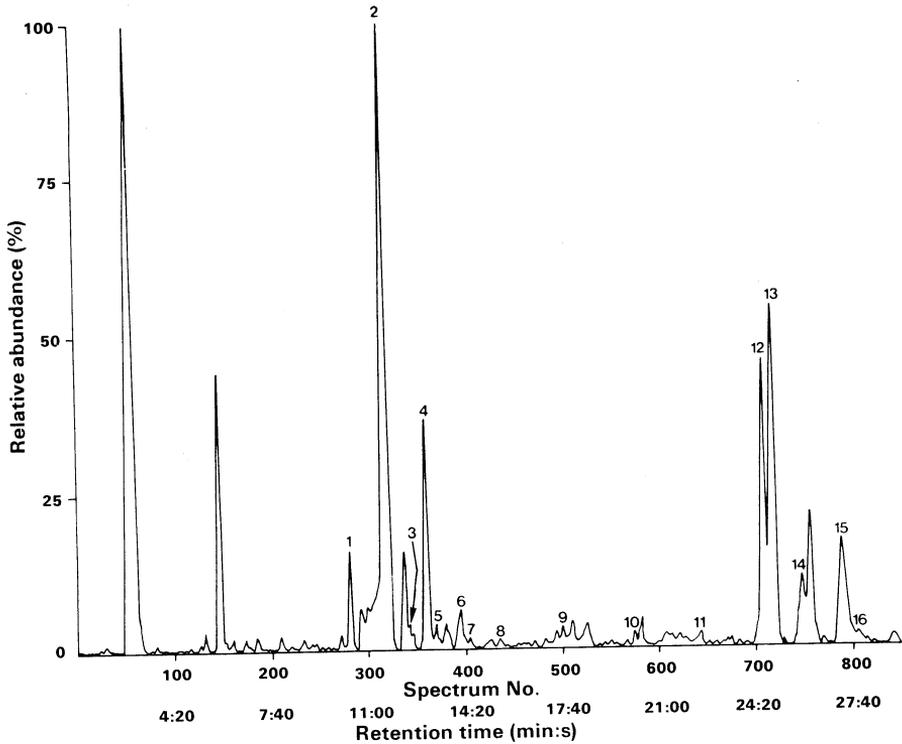


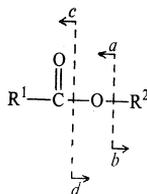
Fig. 1. Total ion chromatogram trace by gas chromatography–methane chemical ionization mass spectrometry of the neutral lipid fraction of *Thiobacillus thioparus* BJR-451 grown in shaken flasks. Key to constituents: 1, M = 278, dibutyl phthalate; 2, M = 278, dibutyl phthalate; 3, M = 284, ethyl hexadecanoate; 4, M = 268, octadecenol; 5, M = 270, octadecanol; 6, M = 310, ethyl octadecanoate; 7, M = 312, ethyl octadecanoate; 8, ?, phthalate ester; 9, M = 390, dioctyl phthalate; 10, M = 410, squalene; 11, M = 386, cholesterol; 12, M = 470, dihydrolanosteryl acetate; 13, M = 468, lanosteryl acetate; 14, M = 506, 16 : 0/18 : 1 wax ester; 15, M = 534/532, 18 : 0/18 : 1 and 18 : 1/18 : 1 wax esters; 16, M = 530, 18 : 2/18 : 1 wax ester. Constituents 1, 2, 8 and 9 almost certainly result from solvent contamination by plasticizers and are commonly detected in gas chromatography–mass spectrometry studies of biological extracts. Constituents 1 and 2 yielded identical mass spectra and are therefore presumed to be isomeric butyl phthalates.

Upon examination, the neutral lipid fraction of *T. thioparus* BJR-451 was found to contain sterols and triterpenoids. Squalene, cholesterol, lanosteryl acetate and 24,25-dihydrolanosteryl acetate were all positively identified by comparison of their gas chromatographic retention times and mass spectra with those of authentic compounds recorded under identical conditions. Although found in this second strain, squalene was present only at relatively low levels compared with strain 01, suggesting strain 01 might possess some metabolic irregularity. Several other steroid-like constituents were present in the extracts of strain BJR-451, although definitive spectra of these constituents could not be recorded. Fig. 1 is a typical total ion chromatogram of the neutral lipid fraction of *T. thioparus* BJR-451.

Sterols and triterpenes were observed in the gas chromatography-methane chemical ionization mass spectral examination of *T. thioparus*.^{*} The molecular ions were readily identified in each instance, and for more positive confirmation the extracts were re-examined using ammonia as the chemical ionization reactant gas. This provided MH^+ and $(M+NH_4)^+$ ions to confirm the original assignment made on the basis of the methane chemical ionization mass spectrometric results.

Table 1. Wax esters observed in extracts of *T. thioparus*

Values in parentheses indicate the relative abundance (%) of the ion (m/z) listed



R ¹	R ²	M ⁺	MH ⁺	(M-H) ⁺	a	b	c	d
<i>Diagnostic ions observed in chemical ionization mass spectra of standard docosanyl (C₂₂H₄₅) wax esters</i>								
C ₁₅ H ₃₁ ^A	C ₂₂ H ₄₅	564(16)	565 (11)	563(32)	257(100)	309(2)	239(22)	325(1)
C ₁₃ H ₂₇ ^B	C ₂₂ H ₄₅	536(10)	537(13)	535(23)	229(100)	309(2)	211(30)	325(1)
C ₁₁ H ₃₃ ^C	C ₂₂ H ₄₅	508(6)	509(12)	507(20)	201(100)	309(2)	183(30)	325(1)
<i>Inferred wax esters present and observed ions in extracts of T. thioparus</i>								
C ₁₇ H ₃₃	C ₁₈ H ₃₇			533(48)	283(64)		265(100)	
C ₁₇ H ₃₁	C ₁₈ H ₃₅			531(45)	281(24)		263(60)	
C ₁₇ H ₂₉	C ₁₈ H ₃₃			529(13)	279(13)			
C ₁₅ H ₃₁	C ₁₈ H ₃₅			506(38)	257(100)		239(60)	

^A Palmitate. ^B Myristate. ^C Laurate.

The neutral lipid fraction of both strains of *T. thioparus* showed evidence for the occurrence of ethyl esters of fatty acids. Confirmation of these assignments were obtained by preparation of authentic ethyl esters which showed gas chromatographic retention times, and methane chemical ionization mass spectra, identical to those obtained with the biological extracts.

There was evidence for the presence of long-chain alcohols in extracts of *T. thioparus*. The methane and ammonia chemical ionization mass spectra clearly identified the molecular weights of the unknown alcohols. Positive assignment was achieved by recording identical gas chromatographic retention times and methane chemical ionization mass spectra for authentic alcohols (prepared by lithium aluminium hydride reduction of the respective fatty acid methyl esters). Further evidence was obtained by acetylation of the biological extracts which increased the molecular ions of the components in question by 42 atomic mass units (formation of one acetate group); the spectra and retention times of the acetylated components were identical with those of the standard alcohols after acetylation.

* Tables giving the principal ions observed in the mass spectral examination of sterols, triterpenes, natural fatty acid ethyl esters and fatty alcohols found in extracts of *T. thioparus* have been lodged as an Accessory Publication, copies of which may be obtained from the Editor-in-Chief, Editorial and Publications Service, CSIRO, 314 Albert St, East Melbourne, Vic. 3002.

At comparatively long gas chromatographic retention times (Fig. 1), a series of compounds eluted and these were identified as wax esters (Spencer 1979). Using *n*-docosanyl palmitate, myristate and laurate (see Table 1) as reference standards, one could clearly identify each molecular weight by gas chromatography-methane chemical ionization mass spectrometry. In addition, prominent ions corresponding to the respective protonated acids (m/z 257, 229, 201; see Table 1) and to the acylium ions (m/z 239, 211 and 183, respectively) were observed, and weaker ions (*b* and *d*) helped define the alcohol moiety. The latter could also be deduced since both the total molecular weight and the acid portion of the wax esters were defined by methane chemical ionization mass spectrometry. As shown in Table 1, the wax esters of *T. thioparus* can be defined as esters of $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ and $C_{16:0}$ acids with $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:1}$ alcohols, respectively.

The gas chromatographic conditions and the mass spectral limitations (mass range to 800 atomic mass units) were such that glycolipids, phospholipids and triglycerides would not have been identified nor detected were they present in the extracts.

Acknowledgment

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