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Extractives of Fungi. VI*
Gas Chromatographic–Mass Spectrometric Investigations of the Lipids of *Trametes lilacino-gilva* (Berk.) Lloyd

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Abstract

The lipid constituents of *T. lilacino-gilva* were examined by gas chromatography and gas chromatography–mass spectrometry.

The free fatty acids of the organism were found to include a number of saturated and monounsaturated acids with odd carbon-chain lengths, such compounds being rarely reported before from fungi.

The neutral lipid fraction contained fatty alcohols and wax esters together with ethyl esters of fatty acids. In addition, this fraction contained a number of triterpenoids.

The triterpenoid acid fraction was shown to be considerably more complex than was indicated by previous work. In addition to the two triterpenoid acids (tumulosic acid and its 3-*O*-acetyl derivative), and their dehydro-analogues previously reported from this organism, two additional triterpenoid acids (plus their dehydro-analogues) were identified as eburicoic acid and its 3-*O*-acetyl derivative.

Introduction

Earlier papers in this series have described the isolation of novel compounds from various species of fungi, and the chemical modification (Batey *et al.* 1972; Kazlauskas *et al.* 1972) of tumulosic acid to increase its antibiotic activity. The earlier investigation of the lipids of *Trametes lilacino-gilva* (Berk.) Lloyd was directed principally toward the triterpenoid acid fraction since this was the most abundant and thus most accessible via classical chemical techniques.

The relatively recent availability of a modern gas chromatographic–mass spectrometric (GCMS) facility enabled a collaborative investigation into the lipid constituents of that organism in order to characterize the free fatty acids and neutral constituents in addition to the minor triterpenoid acids.

Materials and Methods

Organisms

Cultures of the following organisms were obtained from the Mycology Collection, School of Botany, University of New South Wales: *Trametes lilacino-gilva* (strain No. 626), *Daedalea trabea* (strain No. 574), *Polyporus sulphureus*, *Trametes feei* (strain No. 8766).

Fungal Culture

Organisms were usually grown in previously autoclaved Roux flasks to which had been added 500 ml of filtration-sterilized medium. The cultures were grown for various periods of time, usually at 25°C; between 10 and 100 flasks constituted a single 'batch'. Additionally, *T. lilacino-gilva* was

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grown for various periods of time in either shaken Erlenmeyer flasks (1 litre capacity; 200 ml of medium) or a 10-litre working capacity 'Porton'-type fermenter. The growth media compositions are detailed separately in Table 1. Fungal cultures were harvested either by filtration or by centrifugation.

Extracts

Freeze-dried fungal mycelium (50 g) was extracted in a Soxhlet apparatus with diethyl ether (2 litres) for 24 h. After removal of the solvent in a rotary evaporator, the dry extract (10 g) was twice washed with light petroleum (100 ml); the residue, after drying, was designated the triterpenoid acid fraction. The light petroleum solution was washed with dilute acid (2M HCl), distilled water, and aqueous alkali ($NaHCO_3$); the light petroleum fraction was again washed with distilled water, dried (Na_2SO_4), and the solvent evaporated to yield the fraction designated as neutral lipids. The aqueous alkaline washings were acidified (HCl) and the free cellular fatty acids back-extracted into ethyl acetate which was washed with distilled water, dried (Na_2SO_4), and evaporated to dryness.

Table 1. Composition of growth media

Weights of constituents are for 1 litre of medium

Constituent	Medium			
	WD_5	C	D	E
Glucose	50 g	50 g	100 g	100 g
$(NH_4)_2SO_4$	3·0 g	2·22 g	2·22 g	3·33 g
$(NH_4)H_2PO_4$	—	1·35 g	1·35 g	2·03 g
KH_2PO_4	2·0 g	0·4 g	0·4 g	0·6 g
L-Glutamic acid	2·0 g	—	—	—
$CaCl_2.6H_2O$	0·6 g	—	—	—
$MgSO_4.7H_2O$	0·25 g	0·25 g	0·25 g	0·25 g
TlCl	1·0 mg	—	—	—
$ZnSO_4.7H_2O$	1·0 mg	1·0 mg	1·0 mg	1·0 mg
$MnCl_2.4H_2O$	1·0 mg	—	—	—
$MnSO_4.H_2O$	—	0·5 mg	0·5 mg	0·5 mg
H_3BO_3	1·0 mg	—	—	—
$FeCl_3.6H_2O$	0·5 mg	—	—	—
$FeSO_4.7H_2O$	—	0·5 mg	0·5 mg	0·5 mg
$CuSO_4.5H_2O$	0·1 mg	0·1 mg	0·1 mg	0·1 mg
KI	0·1 mg	—	—	—
Vegemite	1·0 g	—	—	—
Thiamine hydrochloride	—	0·1 g	0·1 g	0·1 g

Derivatization

Both the triterpenoid and fatty acid fractions were methylated by addition of ethereal diazomethane, prepared in the normal fashion from 'Diazald' (Aldrich Chemical Company Inc., Milwaukee, Wisconsin) and distilled. For some triterpenoid acid analyses the previously methylated extracts (2 mg) were heated with pyridine (30 μl) and BSTFA (*N,O*-bis-(trimethylsilyl)trifluoroacetamide; 30 μl; Pierce Chemical Co., Rockford, Illinois) in sealed vials at 80°C for 1 h, to prepare the trimethylsilyl ether methyl ester derivatives.

Analyses

GCMS analyses were performed using Finnigan 3200 gas chromatographic–electron ionization mass spectrometric (GC–EIMS) and gas chromatographic–chemical ionization mass spectrometric (GC–CIMS) instruments, with data collection and manipulation being effected with either 6115 or 2300 data systems by the same manufacturer. Typically, electron energies of 70 and 85–150 eV were used together with emission currents of 0·6 and 0·8 mA, for EIMS and CIMS respectively. The ion source temperature was not monitored for the electron ionization source, but was maintained at a nominal 150°C for the chemical ionization source.

For GC–EIMS helium at 30 ml min^{-1} was used as the carrier gas, most of the helium being removed with a glass jet separator before admission of the gas chromatographic effluent to the ion source; the analyser pressure was $1 \cdot 3 \times 10^{-3}$ Pa.

For GC–CIMS the total gas chromatographic effluent was admitted to the ion source, so that the carrier gas acted as (one of) the chemical ionization reactant gas(es). Typically methane at 20 ml min^{-1} was used as the carrier gas and resulted in a source pressure of 119·7 Pa. Other reactant gases such as ammonia and vinyl methyl ether were introduced directly into the ion source so that the total pressure was 133·3 Pa; under these conditions the methane spectrum was supplanted by that of the protonated second reactant gas.

Conventional gas chromatography was performed using a Finnigan 9500 instrument fitted with a flame ionization detector. Nitrogen at 30 ml min^{-1} was used as the carrier gas and injector and detector temperatures were 310 and 320°C respectively.

Protocol

Methylated fatty acids

The methylated fatty acid fractions were examined using both GC–EIMS and GC–methane-CIMS; the data from these two modes of operation were complementary and were usually sufficient to allow identification of the constituents, although positional and geometric isomers could not be differentiated. Vinyl methyl ether-CIMS was used to establish that monoenoic constituents were indeed so, and not cyclopropanoid in nature (Christopher and Duffield 1980). Quantitative estimates of the proportion of each constituent were derived from gas chromatographic analyses (using a flame ionization detector) in the usual fashion. Glass columns for gas chromatography were 1·5 m long (of 2 mm i.d.) and were packed with either 3% OV-1 or 3% OV-17 on 100/120 mesh Gas Chrom Q; usually the initial temperature was 80°C and was held for 1 min after sample injection and thereupon programmed at 6°C min^{-1} to a maximum temperature of 280°C. The interface oven, transfer line and injector were usually held at 280°C. When required, the fatty acid extracts were hydrogenated by dissolving the previously methylated material (5–50 mg) in dry ethyl acetate (5 ml) to which palladium catalyst [50 mg; 10% (w/w) on carbon] was added. The mixture was stirred under hydrogen (atmospheric pressure) for 16 h at 20°C. On completion of the reaction, the catalyst was separated by filtration and the solvent evaporated.

Neutral lipids

The neutral lipid fraction was examined using EIMS, methane-CIMS and ammonia-CIMS; gas chromatographic conditions were as detailed above for the fatty acid analyses. Ethyl esters were confirmed by comparison of their mass spectra and retention times of authentic fatty acids which had been esterified using ethanol/HCl. The fatty alcohols were confirmed by comparison with authentic material both before and after acetylation at room temperature with pyridine/acetic anhydride.

Triterpenoid acid fraction

The triterpenoid acid fraction was chromatographed using either a 1·8 m long glass column (2 mm i.d.) packed with 1·5% OV-1 on 100/120 Chromosorb W (AW/DMCS) or the columns detailed above for the fatty acid analyses. Typically the initial temperature was 200°C and was held constant for 1 min, then programmed at 6°C min^{-1} to a maximum of 310°C. The interface oven, transfer line and injector temperatures were also increased to 310°C. EIMS, methane-CIMS and ammonia-CIMS were all employed for identification of constituents, and gas chromatography using a flame ionization detector for quantitative analyses.

Authentic Compounds

Most fatty acid standards were obtained from Applied Science Laboratories Inc., State College, Pennsylvania. Methyl heptadec-10-enoate and methyl nonadec-10-enoate were purchased from NuChek Prep Inc., Elysian, Minnesota. Lanosterol, cholesterol, ergosterol and squalene were purchased from Sigma Chemical Co., St Louis, Missouri. Triterpenoid acids, previously isolated and characterized, were received as gifts. Fatty alcohols were synthesized by lithium aluminium hydride reduction in diethyl ether of the corresponding methyl esters.

Results and Discussion

Free Fatty Acids of T. lilacino-gilva

The results of analyses of the methylated free fatty acid fraction of the organism by gas chromatography and GCMS are presented below; both EIMS and methane-CIMS were used:

Free fatty acid	Quantity	Free fatty acid	Quantity	Free fatty acid	Quantity
Octatrienoic	Trace	Hexadecanoic	9%	Methyloctadecenoic	Trace
Methylnonanoic	Trace	Hexadecenoic	Trace	Nonadecenoic	Trace
Decanoic	Trace	Heptadecanoic	2%	Nonadecadienoic	Trace
Decatrienoic	Trace	Heptadecenoic	1%	Eicosanoic	Trace
Methyldecenoic	Trace	Octadecanoic	4%	Docosanoic	Trace
Undecanoic	Trace	Octadecenoic	40%	Tricosanoic	Trace
Dodecanoic	Trace	Octadecadienoic	42%	Tetracosanoic	Trace
Tridecanoic	Trace	Octadecatrienoic	1%	Pentacosanoic	Trace
Tetradecanoic	Trace	Methyloctadecanoic		Hexacosanoic	Trace
Pentadecanoic	Trace	acids	1%		

There is a paucity of data concerning fungal fatty acid profiles, but the results are generally in accord with analyses (Mitsuhashi *et al.* 1973; Endo *et al.* 1975) of other species of Polyporaceae where hexadecanoic, octadecenoic and octadecadienoic acids are reported to be the major constituents. Whilst fatty acids with an even number of carbon atoms are held to be universal in occurrence, those with an odd number of carbon atoms or branched chains are reported more rarely. *T. lilacino-gilva* was found to contain odd-carbon fatty acids, including heptadecanoic and heptadecenoic acids; the former compound has been reported (Sumner 1973) in five other species of Polyporaceae although the mono-unsaturated analogue appears never to have been reported previously in fungi. In the light of the occurrence in some bacteria (O'Leary 1962) of C_{17} cyclopropane fatty acids, it was desirable to establish positively the nature of the C_{17}, apparently monoenoic, constituent. A technique (Christopher and Duffield 1980) was developed by which, using a vinyl methyl ether–methane mixture as the reactant gas for CIMS, the mass spectra for cyclopropane and mono-ene fatty acid methyl esters could be differentiated. Using this technique, the C_{17} constituent, and indeed all of the other monoenes present, were positively confirmed as such.

Neutral Lipids of T. lilacino-gilva

The neutral lipid fraction of the organism generally showed quantitative, but not qualitative, variations from sample to sample. The major constituents identified in this fraction were found to be naturally occurring fatty acid esters, together with smaller amounts of fatty alcohols, wax esters, hydrocarbons and steroids.

The major neutral compounds identified in the organism were:

Ethyl decanoate
Ethyl hexadecanoate
Ethyl hexadecenoate
Ethyl heptadecanoate
Ethyl heptadecenoate
Ethyl octadecanoate
Ethyl octadecenoate
Ethyl octadecadienoate
Ethyl docosanoate
Ethyl tricosanoate
Hexadecenyl octadecenoate
Octadecanyl octadecenoate
(and/or octadecenyl octadecanoate)
Octadecenyl octadecenoate
(and/or octadecanyl octadecadienoate
octadecadienyl octadecanoate)
Octadecadienyl octadecenoate
(and/or octadecenyl octadecadienoate)
Tridecane
Tetradecane

Ethyl tetracosanoate	Pentadecane
Ethyl pentacosanoate	Hexadecane
Ethyl hexacosanoate	Heptadecane
Methyl octadecenoate	Octadecane
Octadecenol	Docosane
Hexadecanyl octadecenoate (and/or hexadecenyl octadecanoate)	

Fatty acids were usually present as natural ethyl esters although methyl octadecenoate was present in some of the extracts examined. GCMS data on the wax esters did not allow positive separate identification of the alcohol and acid moieties involved, but several esters containing C_{16} and/or C_{18} acids and alcohols were present and were only partially resolved under the chromatographic conditions employed. The principal alcohol identified was octadecenol, although some extracts contained what appeared to be a nonadecadienol. Additionally saturated hydrocarbons in the range C_{12}–C_{23} were present in most extracts. As is almost inevitable in GCMS examinations of biological extracts, common plasticizers, such as dibutyl and dioctyl phthalates and tri(*n*-butyl)phosphate, were detected.

The minor neutral triterpenoids identified in the organism were squalene, lanosterol, 24,25-dihydrolanosterol, ergosterol, lanosteryl acetate and 24,25-dihydrolanosterol acetate. Several of these compounds correspond to anticipated intermediates in the synthesis of lanosterol, ergosterol and eburicoic acid. The identification of what appeared to be agnosterol, the conjugated diene analogue of lanosterol, raises the possibility of origin of the triterpenoid acid dehydro-analogues from this compound rather than from lanosterol *per se*.

A single batch of mycelium, grown in a fermenter using a synthetic medium (Table 1, medium D; glucose and thiamine hydrochloride as the only organic materials) at a dissolved oxygen tension approximately 50% that of saturation, yielded a comparatively large quantity (4·2 g) of petroleum-soluble neutrals. Although most of the compounds discussed above were detected, their relative composition was at variance with the other extracts examined. The major constituents and their approximate proportions as determined by gas chromatography and a flame ionization detector are given in the following tabulation:

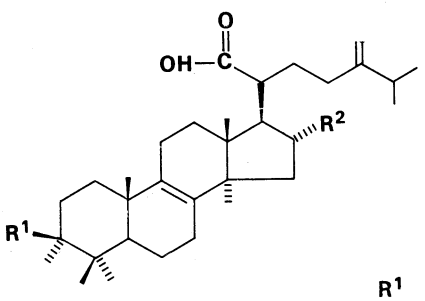
Fatty acid	Quantity	Fatty acid	Quantity
Octadecenol	35%	Hexadecenol	2%
Dibutyl phthalate	14%	Ethyl octadecenoate / Ethyl octadecadienoate	2%
Octadecadienol	12%	Nonadecenol	1%
Octadecenyl octadecanoate / Octadecanyl octadecenoate	10%	Hexadecanol	Trace
Unidentified: aromatic?	9%	Ethyl hexadecenoate	Trace
Hexadecenyl octadecanoate / Octadecenyl hexadecanoate	7%	Squalene	Trace
		Ergosterol	Trace
Unidentified: triterpenoid?	4%	Dihydroergosterol	Trace
Dioctyl phthalate	4%		

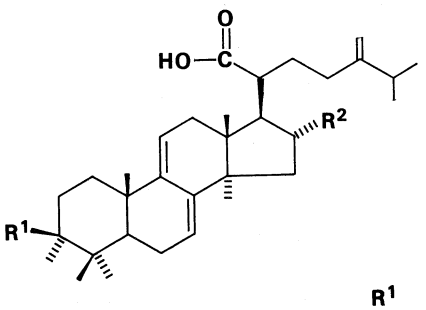
Additionally several unidentified compounds of relatively low molecular weight (120–160) were detected. These were possibly hydrocarbon or monoterpenoid in nature and included several which were apparently isomeric.

Triterpenoid Acids of T. lilacino-gilva

Gas chromatographic analyses of the methylated ether extract of *T. lilacino-gilva* grown in shake flasks with medium WD5 showed that three major constituents were

present: the previously reported tumulosic (2) and 3-*O*-acetyltumulosic (3) acids were identified from gas chromatographic and GCMS data while the third constituent remained unidentified. The proportion of tumulosic acid tended to increase only marginally with time, and then only in the latter stages of growth. In experiments in which the synthetic medium, C, was substituted for WD5, similar results were obtained except that during the earlier stages of growth, low levels of 3-*O*-acetyleburicoic acid (4) identified by comparison of GCMS data of authentic material, previously unreported in this organism, were also detected; although low in absolute terms, this constituent reached the significant level of 16% of the total ether extract after 4 days incubation of the cultures.





		R^1	R^2
(1)	Eburicoic acid	OH	H
(2)	Tumulosic acid	OH	OH
(3)	3-*O*-Acetyltumulosic acid	OAc	OH
(4)	3-*O*-Acetyleburicoic acid	OAc	H

		R^1	R^2
(5)	Dehydroeburicoic acid	OH	H
(6)	Dehydrotumulosic acid	OH	OH
(7)	3-*O*-Acetylhydrotumulosic acid	OAc	OH
(8)	3-*O*-Acetylhydroeburicoic acid	OAc	H

Table 2 reports the results of gas chromatographic analyses of the ether extracts of *T. lilacino-gilva* grown under a variety of conditions. Tumulosic acid (2) and its 3-*O*-acetyl derivative (3) were invariably present, but the presence of the unidentified compound and 3-*O*-acetyleburicoic acid (4) proved variable. The unidentified compound was present in still cultures and flask cultures shaken at low speed (where aeration was limited) but was not detected under conditions where higher aeration rates prevailed. Although 3-*O*-acetyleburicoic acid (4) was detected in all still cultures, it was usually seen only in some of the shaken cultures and then only in the latter stages of growth. Free eburicoic acid (1) confirmed by GCMS data, previously unreported in the organism, was detected at a significant, but low, level in one still culture, although its presence in most cultures at levels $< 0{\cdot}1\%$ of the total ether extract could be demonstrated with GCMS.

Previously these triterpenoids have been reported generally as mixtures containing variable amounts of the dehydro-analogues, and no separation of these mixtures has yet been reported; none could be achieved in the current study employing either non-polar (OV-1) or moderately polar (OV-17) silicone phases for gas chromatography. Consequently prominent ions in the spectra of both authentic compounds and the constituents of the fungal extract were invariably accompanied by a second ion two mass units lower which was assumed to be due to the dehydro-analogue.

The triterpenoid acid methyl esters were of low volatility for GCMS and thus a second derivatization step was used to produce the methyl esters/trimethylsilyl (TMS) ethers (where applicable). Methane and ammonia were employed as a chemical

Table 2. Results of gas chromatographic analysis of methylated triterpenoid acid fraction of *Trametes lilacino-gilva* 626 as influenced by the conditions of culture

Growth conditions	Growth time (days)	Extract ether weight[A]	Triterpenoid acids present[A]					
			Ergosterol	Eburicoic acid	3-*O*-Acetyl-eburicoic acid	Tumulosic acid	Unidentified triterpene	3-*O*-Acetyl-tumulosic acid
Still culture (Roux flask) with medium WD_5 at 25°C								
Batch No. 184	105	22	—	—	6	17	10	67
190	95	22	—	—	12	39	22	27
194	140	52	—	—	17	48	17	18
202	148	44	—	—	13	52	18	17
203	120	47	—	2	18	50	14	16
Flasks with medium E at various temperatures, shaken at different speeds								
Moderate speed, 25°C	22	18	—	—	—	78	—	22
	35	32	3	—	4	77	—	16
Slow speed, 30°C	22	18	—	—	—	20	50	30
	35	32	—	—	—	18	52	30
Moderate speed, 30°C	22	32	—	—	—	88	—	12
	35	42	—	—	—	89	—	11
Fast speed, 30°C	22	40	2	—	—	90	—	8
	35	38	1	—	10	86	—	3
Moderate speed, 37°C	22	9	—	—	14	73	—	13
Fermenter with medium D and supplementary glucose at 25°C	54	—	—	1	28	52	—	19

[A] As percentage of total dry weight.

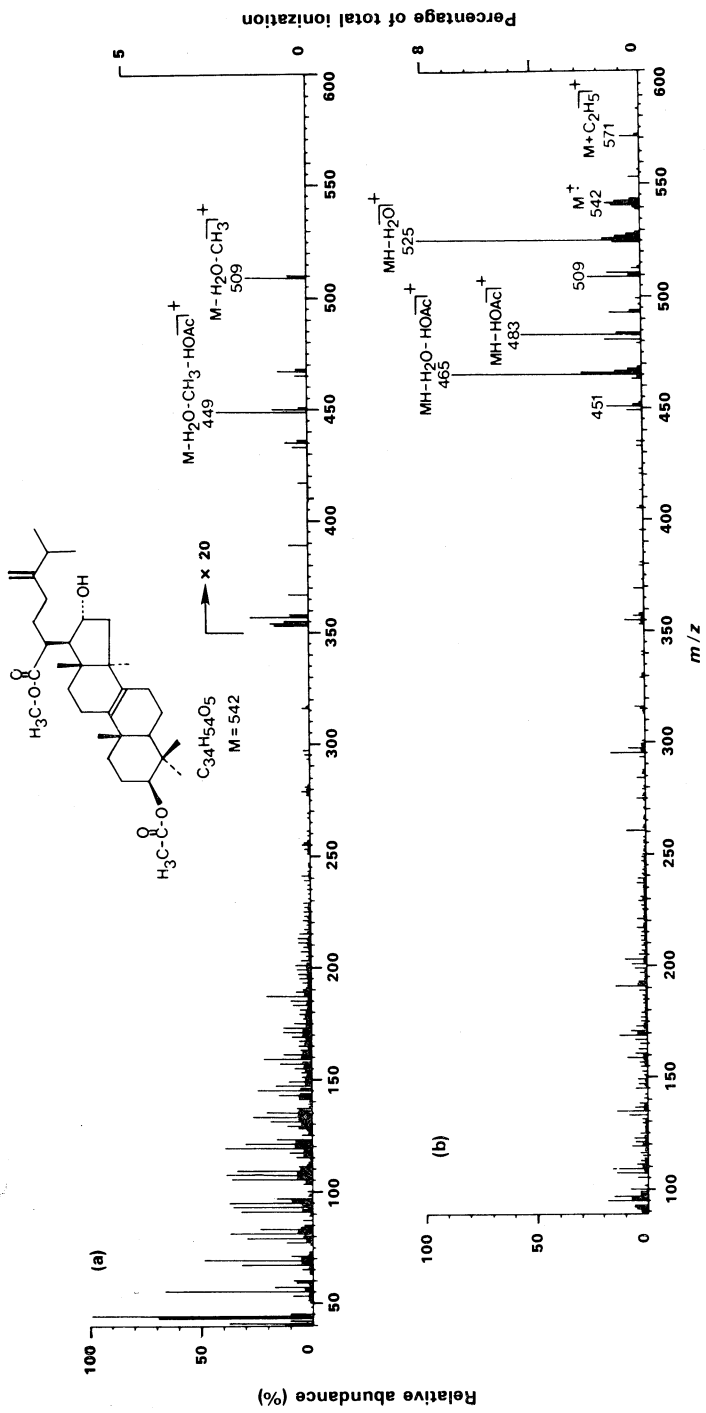


Fig. 1. Comparison of electron ionization (*a*) and methane chemical ionization (*b*) mass spectra of 3-*O*-acetyltumulosic acid methyl ester. In (*a*), the vertical height of each peak from $m/z = 350$ onwards has been magnified 20 times.

ionization reactant gas, and in some instances use of the latter led to the production of a prominent MH^+ ion [and also $(M + NH_4)^+$] whereas methane-CIMS did not, thus allowing confirmation of the molecular weight.

Based upon the previously reported (Pinhey *et al.* 1970) occurrence of tumulosic (2) and 3-*O*-acetyltumulosic (3) acids in this organism, oxygenation in the unidentified compounds was assumed to be at the 3β and 16α positions in preference to alternative locations. Analyses of authentic samples of tumulosic and sulfurenic acids (identical except for 16α- and 15α-hydroxy substituent, respectively) demonstrated that neither gas chromatographic resolution nor mass spectral discrimination between (at least) closely related isomers was possible. Consequently there remains the possibility that what appeared to be a simple mixture of any particular compound and its dehydro-analogue was in reality a more complex mixture which included positional isomers.

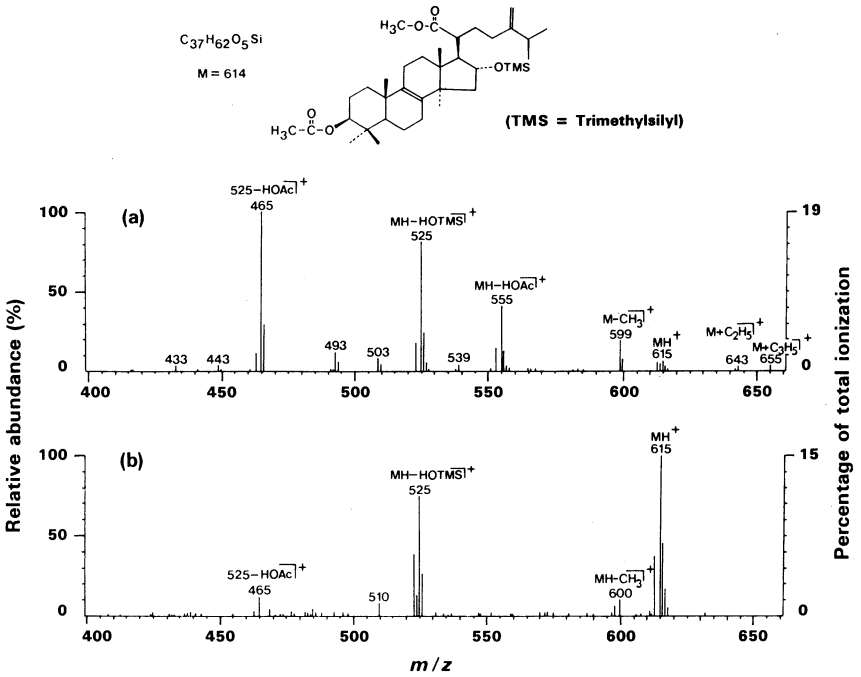


Fig. 2. Comparison of chemical ionization mass spectra of methyl 3-*O*-acetyl-16-*O*-trimethylsilyltumulosate using methane (*a*) and ammonia (*b*) as the reactant gas.

Fig. 1 contrasts the electron ionization and methane chemical ionization mass spectra of methyl 3-*O*-acetyltumulosate and illustrates the absence of the molecular ion under the former conditions, whereas using methane-CIMS both the molecular ion and the confirmatory $(M + C_2H_5)^+$ adduct ion were readily discernible. Fig. 2 records the methane chemical ionization and ammonia chemical ionization mass spectra of methyl 3-*O*-acetyltumulosate, TMS ether, and illustrates how the loss of trimethylsilanol can be used to verify the presence in the parent molecule of a single free hydroxyl substituent.

Two of the hitherto unreported constituents, eburicoic acid (1) and 3-*O*-acetyl-eburicoic acid (4), and their dehydro-analogues (5 and 8) yielded gas chromatographic

retention times and mass spectra identical with authentic samples analysed under identical conditions. Eburicoic acid, first characterized from *Polyporus anthracophilus* by Gascoigne *et al.* (1950, 1951), can be considered the simplest of the known C_{31} lanostane-related triterpenoid acids, and its characterization in *T. lilacino-gilva* together with its 3-*O*-acetyl derivative is not surprising.

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