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Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts

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Summary

Monounsaturated fatty acid double-bond position and geometry have been determined for microbial monocultures and complex microbial consortia by capillary GC-MS of their dimethyl disulphide (DMDS) adducts. The technique has permitted (*i*) chromatographic separation and positive identification of adducts derived from *cis/trans* isomers, (*ii*) characterization of long-chain monounsaturated components (up to 26:1), and (*iii*) the identification of a wide range of monounsaturated components derived from methanotrophic soil material. The methanotrophic soil sample contained a high relative proportion of the novel phospholipid ester-linked fatty acid 18:1 Δ 10c. The DMDS procedure offers a simple and rapid approach that can be routinely applied to microbial fatty acids derived from environmental samples and monocultures.

Key words: *Biological markers – Community structure – Gas chromatography-mass spectrometry – Monounsaturated fatty acids – Structural verification*

Introduction

Precise determination of monounsaturated fatty acid double-bond position and geometry is essential for the correct interpretation of complex data sets, in order that membrane fatty acids can be used as biomarkers in the fields of taxonomy, ecology,

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organic geochemistry and clinical microbiology. A number of derivatization procedures have been applied to sedimentary and microbially derived fatty acids to achieve structural identification [1–5]. These methods are not, however, applied routinely to environmental and microbial samples for a variety of reasons, such as lengthy sample workup, the method's failure to provide data for long-chain (greater than 24:1) components, and the degree of difficulty in interpreting relative proportions of *cis* and *trans* isomers.

A single-step derivatization procedure followed by gas chromatography-mass spectrometry (GC-MS) involving the simple and rapid iodine-catalyzed addition of dimethyl disulphide (DMDS) to linear alkenes has recently been reported [6]. Similarly, the DMDS addition has also been used in the identification of standard monounsaturated acetates and fatty acid methyl esters derived from pheromone extracts of moth species [7]. The latter application achieved chromatographic separation of adducts derived from *cis* and *trans* isomers.

In this report we present data obtained by means of the DMDS procedure for monounsaturated fatty acids from microbial monocultures and complex soil consortia. Structural confirmation has been performed by capillary GC-MS of the adducts. The main criteria for the selection and use of this method were (*i*) confirmation of double-bond configuration for relatively novel monounsaturated fatty acids isolated from soil samples, (*ii*) distinction between *cis* and *trans* isomers, (*iii*) identification of long-chain monounsaturated, 24:1 and 26:1, fatty acids from a marine alga, (*iv*) the absence of contaminating byproducts, and (*v*) low cost and convenience of the reaction. The method has permitted the achievement of these goals.

Materials and Methods

Sample preparation

Sample extraction, fractionation of total lipid, and methylation of the phospholipid ester-linked fatty acids were as previously reported [8]. No further separation of the resulting methyl esters was required for this analysis.

Gas chromatography

Fatty acid methyl esters (FAME) were taken up in hexane with methylnonadecanoate (19:0) as the internal injection standard. Separation of FAME was performed by high-resolution gas chromatography with a Hewlett Packard 5880A gas chromatograph (GC) equipped with a flame ionization detector. Samples were injected at 50 °C in the splitless mode with a Hewlett Packard 7672 automatic sampler onto a non-polar cross-linked methyl silicone capillary column (50 m × 0.2 mm, i.d., Hewlett Packard). The oven temperature was programmed from 50 to 160 °C at 10 °C per min, then at 4 °C per min to 300 °C. Hydrogen was used as the carrier gas (1 ml/min). The injector and detector were maintained at 300 °C.

Tentative peak identification, prior to GC-MS analysis, was based on comparison of retention times with those obtained for standards from Supelco Inc. (Bellefonte, PA) and Applied Science Laboratories Inc. (State College, PA) and previously

identified laboratory standards. Peak areas were quantified with a Hewlett Packard 3350 series programmable laboratory data system operated with an internal standard program.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses were performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet. The same column type described above was used for analysis. Samples were injected in the splitless mode at 100 °C with a 0.5 min venting time, after which the oven temperature was programmed to 300 °C at either 3 or 4 °C per min. Helium was used as the carrier gas. MS operating parameters were: electron multiplier between 1300 and 1400 V, transfer line 300 °C, source and analyzer 250 °C, autotune file DFTPP normalized, optics tuned at m/z 502, electron impact energy = 70 eV. Mass spectral data were acquired and processed with a Hewlett Packard RTE-6/VM data system.

Determination of fatty acid double-bond configuration

The DMDS adducts of monounsaturated FAME were formed according to methods similar to those described by Dunkelblum et al. [7] to locate the double-bond positions. A higher temperature was required to achieve complete reaction than reported for the monounsaturated acetates [7]. Samples in hexane (50 μl) were treated with 100 μl DMDS (gold label, Aldrich Chemical Co., Milwaukee, WI) and 1–2 drops of iodine solution (6% w:v in diethyl ether). The reaction took place in a standard 2 ml GC vial (Varian Assoc. Inc., Sunnyvale, CA) fitted with a teflon-lined screw-cap lid. After reaction at 50 °C in a GC oven for 48 h, the mixture was cooled and diluted with hexane (500 μl). Iodine was removed by shaking with 5% (w:v) aqueous sodium thiosulphate (500 μl). The organic layer was removed, and the aqueous layer reextracted with hexane: chloroform (4:1, v:v). The combined organic layers were concentrated under a stream of nitrogen prior to subsequent GC analysis. GC-MS analysis of the DMDS adducts showed major ions attributable to fragmentation between the two CH_3S groups located at the original site of unsaturation (Fig. 1). Discrimination between *cis* and *trans* geometry in the original

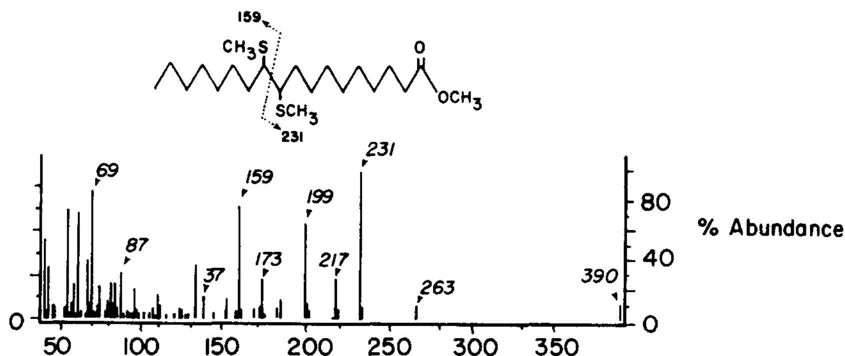

Fig. 1. Mass spectrum of dimethyl disulphide adduct of 18:1Δ10c. Ions at *m/z* 390, 231 and 159 correspond to M+, Δ-fragment and ω-fragment of molecule. A minor contribution from 18:1Δ9c occurs with ions at *m/z* 217 and 173.

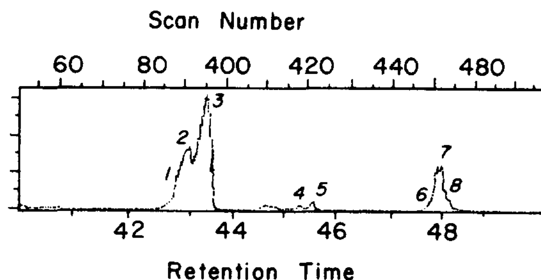

Fig. 2. Reconstructed ion chromatogram of dimethyl disulphide adducts from *Pseudomonas atlantica* monounsaturated fatty acids showing separation of *cis*- and *trans*-derived adduct pairs (2, 3; 4, 5; 7, 8).

monounsaturated FAME was possible. The erythro isomer (originally the *trans* fatty acid) eluted after the threo isomer (originally the *cis* fatty acid) under the GC conditions employed (Fig. 2). The different positional isomers of the same geometry were chromatographically separated under the conditions used in this study.

Fatty acid nomenclature

Fatty acids are designated by total number of carbon atoms:number of double bonds, followed by the position of the double bond from the carboxylic (Δ) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry. The prefix i refers to iso branching.

Results and Discussion

The DMDS adducts of monounsaturated FAME isolated from a methanotrophic soil column [9], *Pseudomonas atlantica* (a slime-producing bacterium) and *Nitzschia cylindrus* (a marine diatom) have been analyzed by capillary GC and GC-MS. Characteristic ion fragments for all monounsaturated FAME are shown in Table 1.

One major criterion for the selection of this technique was achievement of chromatographic separation of *cis* and *trans* acid adducts. A concurrent project in this laboratory has aimed to determine the physiological conditions under which bacteria produce *trans* fatty acids (Guckert and White, unpublished data). A simple and rapid technique that permitted positive identification of double-bond position and geometry and quantification of the two geometrical isomers was required. A reconstructed ion chromatogram showing the DMDS adducts of the monounsaturated FAME from *P. atlantica* is illustrated in Fig. 2. Separation of the adducts of the three *cis/trans* pairs, 16:1Δ9c and t, 17:1Δ9c and t, and 18:1Δ11c and t, occurred under the chromatographic conditions employed for this GC-MS analysis. Better resolution for samples containing overlapping components (where one geometrical isomer dominated) was obtained by GC analysis using hydrogen as carrier gas. We have also noted a further increase in component resolution when a slightly more polar OV1701 column is used.

Application of the DMDS method to the long-chain monounsaturated

TABLE 1

CHARACTERISTIC ION FRAGMENTS OF DERIVATIVES FORMED BY REACTION OF THE PHOSPHOLIPID MONOUNSATURATED FATTY ACID METHYL ESTERS OF MICROBIAL MONOCULTURES AND CONSORTIA WITH DIMETHYL DISULPHIDE (DMDS)

Fatty acid methyl ester	Rt[a]	Peak number[b]	Ion fragments (m/z) of DMDS adducts			
			M+	ω-fragment[c]	Δ-fragment[d]	Source[f]
i15:1Δ9c	17.79		–[e]	–	217	M
i16:1Δ9c	19.74		–	–	217	M
16:1Δ7c	20.34	1	–	173	–	P, M
16:1Δ8c	20.40		–	159	203	M
16:1Δ9c	20.44	2	362	145	217	P, N, M
16:1Δ9t	20.52	3	362	145	217	P, N, M
16:1Δ10c	20.52		–	131	231	M
16:1Δ11c	20.64		362	117	245	M
16:1Δ11t	20.77		362	117	245	M
i17:1Δ9c	21.69		–	159	217	M
17:1Δ9c	22.43	4	376	159	217	P, M
17:1Δ9t	22.55	5	376	159	217	P
18:1Δ9c	24.51	6	–	173	217	P, M
18:1Δ10c'	24.57		390	159	231	M
18:1Δ11c	24.64	7	390	145	245	P, M
18:1Δ11t	24.76	8	390	145	245	P, M
18:1Δ13c	24.86		390	117	273	M
24:1Δ13c	36.07		–	201	273	N
24:1Δ15c	36.16		–	173	301	N
26:1Δ15c	39.69		–	201	301	N
26:1Δ17c	39.79		–	173	329	N

[a] Retention time.
[b] Peak numbers refer to Fig. 2.
[c] ω-Fragment indicates fragment including aliphatic end of the molecule.
[d] Δ-Fragment indicates fragment including carboxylic end of the molecule.
[e] –, Not detected in GC-MS analysis because of insufficient sample material.
[f] Source of lipid: M, methanotrophic soil column; P, *Pseudomonas atlantica;* N, *Nitzschia cylindrus.*

phospholipid FAME of *N. cylindrus* confirmed the presence of 24:1Δ13, 24:1Δ15c, 26:1Δ15c and 26:1Δ17c. The DMDS procedure has previously been applied to the acetates of monounsaturated fatty acids ranging from 12 to 18 carbon atoms long. The data presented here extend the reported working range of this method to monounsaturated fatty acids containing 26 carbon atoms. The use of shorter capillary columns than those utilized in this study will extend this range further. The procedure reported here will permit the identification of long-chain monounsaturated fatty acids from the pathogenic bacterium *Fransicella tularensis.* This bacterium has been previously reported to contain long-chain monounsaturated acids up to 26:1 [10, 11]. Similarly, we plan to apply the procedure routinely to the identification of long-chain components isolated from Antarctic benthic organisms and sediments. Our preliminary studies have indicated the presence of several of these relatively novel long-chain monounsaturated components (unpublished data).

As a further test of the DMDS and subsequent GC-MS procedure, analysis of FAME obtained from the phospholipid fraction of a soil column exposed to natural gas (95% hydrocarbons, 77% methane [9]) was undertaken. Table 1 also includes the sixteen monounsaturated FAME that were positively identified in the soil material. The confirmation of the rarely reported fatty acid 18:1Δ10c by mass spectrometry (Fig. 1), as the major monounsaturated component in the phospholipid fraction, is consistent with the enrichment of a methanotrophic population. This component, to our knowledge, as yet has only been reported as a major phospholipid ester-linked fatty acid in *Methylosinus trichosporium* [12, 13]. Further interpretation of data obtained on fatty acids, including other monounsaturates, present in the soil sample could permit a more complete understanding of the microbial community structure.

Use of the DMDS derivatization procedure followed by GC-MS analysis offers a simple and rapid method for the determination of monounsaturated fatty acid double-bond position and geometry. The method has permitted chromatographic separation and positive identification of *cis/trans* isomers, long-chain components up to 26:1, and several relatively novel components from a complex environmental sample. These separations and identifications are prerequisites to the full exploitation of fatty acid profiles. Although polyunsaturated FAME are similarly derivatized by this procedure, the chromatographic conditions used for this study did not permit elution of these DMDS adducts. The data presented indicate that the DMDS procedure can be easily and routinely applied to monounsaturated fatty acids derived from microbial monocultures and complex consortia.

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