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CHARACTERIZATION OF THE *iso*-BRANCHED SPHINGANINES FROM THE CERAMIDE PHOSPHOLIPIDS OF *BACTEROIDES MELANINOGENICUS**

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SUMMARY

The anaerobic bacterium *Bacteroides melaninogenicus* contains ceramide phospholipids.

The major sphinganines from the ceramides are 17-methyl octadecasphinganine (63%), normal octadecapshinganine (21%), 15-methyl hexadecasphinganine (12%) and possibly 16-methyl heptadecasphinganine (4%), as characterized by gas chromatography and mass spectrometry.

INTRODUCTION

The phospholipids of the obligate anaerobe *Bacteroides melaninogenicus* contain 50 μmoles lipid phosphate per g dry wt. Between 50–60% of the phospholipid is not hydrolyzable in mild alkali which is quite unusual for phospholipids derived from bacteria. The mild alkali-stable fraction contains ceramide phosphorylethanolamine, ceramide phosphorylglycerol[1] and a trace of ceramide phosphorylglycerol phosphate[2]. Preliminary characterization of the sphinganines derived from the ceramides indicated the presence of saturated bases containing 17, 18 and 19 carbon atoms[1]. The 17- and 19-carbon sphinganines appeared to be branched. In this study the major sphinganines have been characterized as *iso*-branched sphinganines of 17, 19 and possibly 18 carbon atoms and normal octadecasphinganine by a combination of gas chromatography and mass spectrometry.

METHODS

Bacteria

The CR2A strain of *B. melaninogenicus* was supplied by R. W. Gibbons. The medium, the procedures for growth, harvesting, storage and monitering against con-

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tamination have been described[3]. Victor Rizza kindly provided the bacteria used in this study.

Isolation of the sphinganines

The lipids were extracted from the bacteria by the procedure of BLIGH AND DYER[4]. This procedure was modified by adding 1 M KCl containing 7 mM acetic acid to the final mixture[2]. Saponification of the residue after extraction indicated that 95% of the fatty acids in the bacteria had been extracted. The lipids (12.0 μmoles of phosphate) were then hydrolyzed at 100° for 3 h in 2 M methanolic·HCl containing 9.5% water[5]. After cooling, an equal volume of water was added and the mixture was extracted 2 times with 2 vol. of light petroleum (b.p. 30–60). The aqueous portion was then adjusted to pH 11 with KOH and extracted 2 times with 2 vol. of light petroleum. The light petroleum fractions from both acidic and alkaline extractions were combined, reduced in volume with a stream of N_2 and loaded onto a 1 g (11 × 50 mm) silicic acid column (Unisil, 100–200 mesh). Fatty acid methyl esters were eluted with chloroform. The sphinganines (5.5 μmoles) were eluted with chloroform–methanol (1:1, v/v) and methanol as described[1]. The sphinganine fraction was then applied as a band to a silica gel G thin-layer plate and chromatographed in one dimension in a solvent of chloroform–methanol–acetic acid–water (42:12:3.15:1, by vol.). The central portion of the thin-layer plate was covered and the plate sprayed with ninhydrin[6]. Ninhydrin-reacting lipid at R_F 0.52 was detected on the exposed ends of the thin-layer plate. Authentic *n*-octadecylsphinganine has an R_F of 0.52 in this system[1]. The portion of the band containing the sphinganines at R_F 0.52 that had been protected was recovered from the silica gel G. *N*-Acetyl-*O*-trimethylsilyl ethers of the sphinganines were prepared from a part of the sphinganine fraction[7]. Another part of the sphinganine fraction was cleaved with periodate[8], the aldehydes recovered and oxidized with silver oxide[9] and the resulting fatty acids methylated[10].

Gas–liquid chromatography

N-Acetyl-*O*-trimethylsilyl ethers of the sphinganines were applied to 6 ft × 0.125 inch glass columns containing 2% (w/v)OV-1 on 100/200 mesh Gas Chrom P and were chromatographed isothermally at 210° in the LKB-9000 mass spectrometer. Fatty acid methyl esters derived from the sphinganines were chromatographed isothermally at 180° on 3% OV-1 on 100/200 mesh Gas Chrom P. OV-1 is a methyl silicone polymer.

Mass spectrometry

Material was eluted directly from the gas chromatograph of the LKB-9000 combined gas chromatograph mass spectrometer and was examined with the ion source at 290° and an accelerating voltage of 3500 V. Spectra were recorded at 70 eV with a trap current of 60 μA in about 10 sec (m/e 15–900)[11].

RESULTS

Gas chromatography

The retention times of the *N*-acetyl-*O*-trimethylsilyl ethers of the sphinganines and of the fatty acid methyl esters derived from the sphinganines were compared

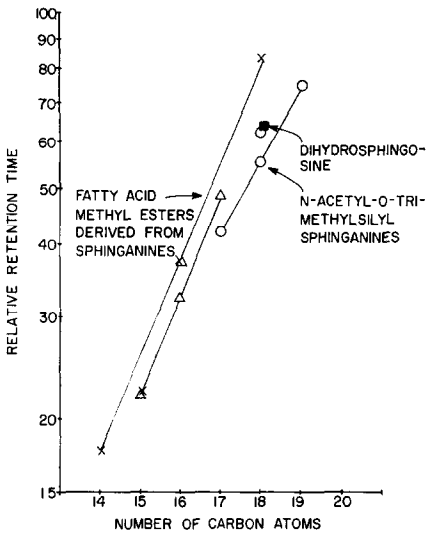

Fig. 1. Logarithm of the retention times in gas chromatography *vs.* the number of carbon atoms. The left-hand graphs indicate the relative retention times of the methyl esters of the fatty acids derived from the sphinganines (△) by periodate cleavage, oxidation and methylation. Authentic methyl esters are indicated (×). The right-hand graph shows the relative retention times of *N*-acetyltrimethylsilyl derivatives of the sphinganines (○). The authentic dihydrosphingosine derivative is indicated as (■). The fatty acid methyl esters were chromatographed isothermally at 180° and the sphinganine derivatives at 210° on 1% OV-1.

to standards and the results suggested that branched 17:0, 18:0, 19:0 and normal 18:0 sphinganines were present. This is illustrated in Fig. 1.

Mass spectrometry

The molecular ions of *N*-acetyl-*O*-trimethylsilyl ethers of sphingolipid bases are usually not detected[11]. The 17-, 18- and 19-carbon sphinganine derivatives showed prominent M−15 ions at *m/e* 458, 472 and 486, representing the loss of methyl groups from the trimethylsilyl ethers[11]. Each component also showed an ion at an M−103 (loss of the terminal trimethylsilyloxymethylene moiety)[11]. This is Fragment b of the 15-methyl hexadecasphinganine derivative illustrated in Fig. 2. Each component had a peak at M−174 representing the fragmentation and cleavage with charge retention on the terminal two carbons with their substituent groups as illustrated by ion d in Fig. 2. An additional ion in the mass spectrum of each compound was observed at M−276, representing the cleavage between C-3 and C-4, illustrated by ion f in Fig. 2. Ions at M−15−90 (loss of methyl and a trimethylsilanol group) and M−103−90 (loss of the terminal trimethylsilyloxymethylene residue and a trimethylsilanol group) were also detected. An ion at *m/e* 247, represented cleavage at the C-2–C-3 bond with transfer of the trimethylsilyl group from O on C-3 to N on C-2, and a secondary ion was found at *m/e* 157 represented the loss of a trimethylsilanol group from the *m/e* 247 (ref. 11). These two ions were prominent in the mass spectra of all the derivatives. The mass spectrum of the 18-carbon *N*-acetyl-*O*-trimethylsilyl sphinganine was identical to that of authentic *N*-acetyl-*O*-trimethylsilyl-*n*-octadecasphinganine.

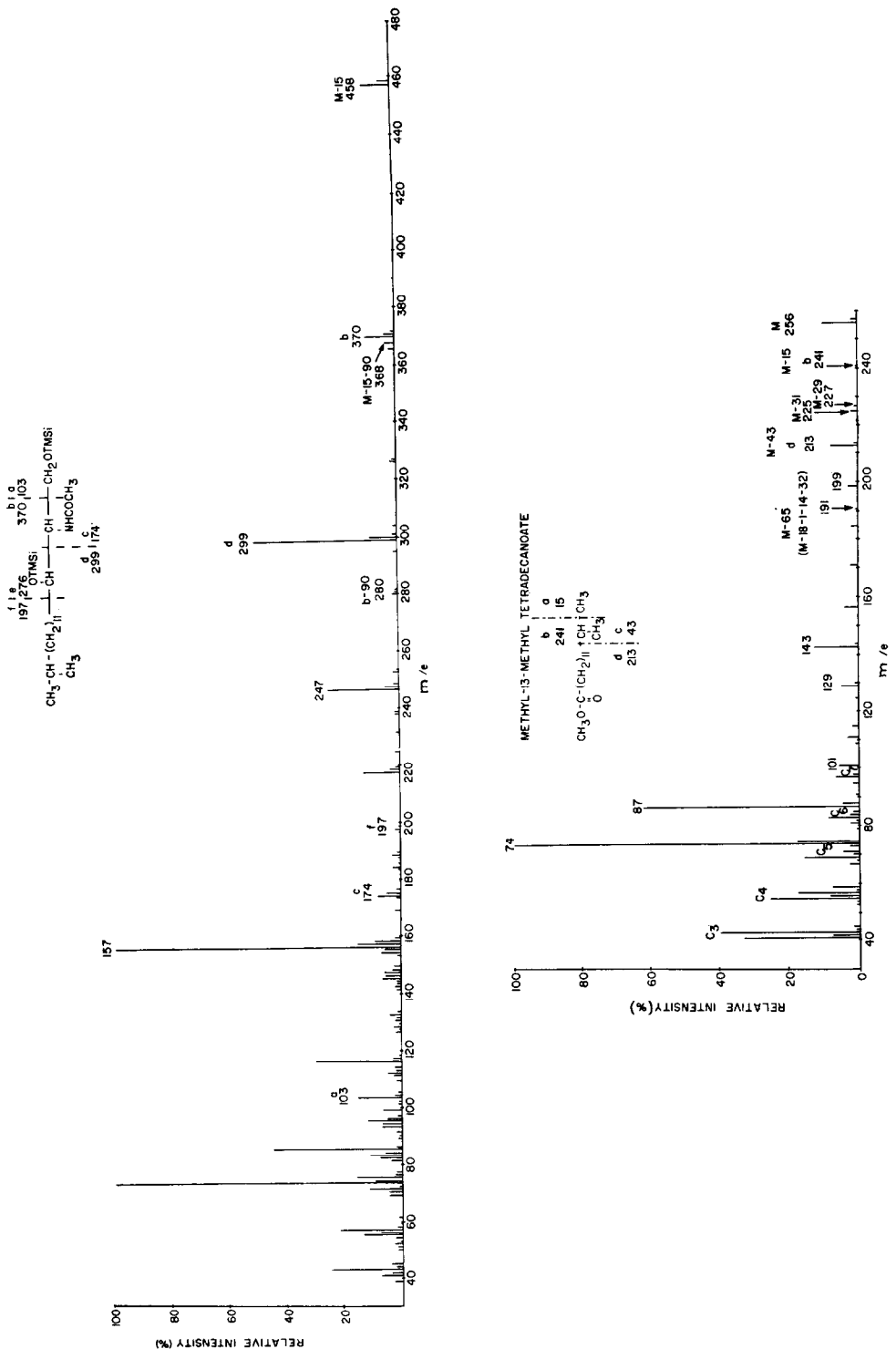

Fig. 2. Mass spectra of the *N*-acetyl-*O*-trimethylsilyl derivative of 15-methyl hexadecasphinganine (mol.wt. 473) (top) and methyl-13-methyl tetradecanoate (mol.wt. 256) derived from the sphinganine (bottom).

Branching probably cannot be detected in the mass spectra of the sphinganine derivatives. Preliminary evidence that the sphinganines were branched was based on gas-chromatographic retention times for three of the four *N*-acetyl-*O*-trimethylsilyl derivatives. Methyl esters (derived from the sphinganines) were analyzed by gas chromatography and mass spectrometry. A mixture of the methyl esters contained four components with retention times characteristic for branched 15:0 (12%), 16:0 (4%), 17:0 (63%) and methyl palmitate (21%). The molecular ions of these components were at *m/e* at 256, 270 (two components), and 284. From the esters presumed to be branched by gas chromatography there was a definite ion at M−65 (located at *m/e* 191, 205 and 219). This ion represents the loss of methylene (14), hydrogen (1), methanol (32) and water (18), and has been found in the mass spectra of every *iso*-branched fatty acid methyl ester examined[12]. The minor ester had the retention time expected for a branched 16:0 ester but the mass spectrum could not be distinguished from that of methyl palmitate. In this instance the characteristic M−65 ion at *m/e* 205 was unfortunately obscured by a similar ion in (bleed) from the silicone liquid phase from the gas-chromatographic column. A slight increase in this ion when the sample was eluted could not be seen above background. Nevertheless, the fact that the mass spectrum is identical to that of methyl palmitate excluded nearly all possibilities but the *iso* 16:0 ester. There was too little of the branched 18:0 sphinganine derivative to recover it and prepare a larger quantity of the 14-methyl pentadecanoic ester for mass spectrometry. The fatty acid derived from the normal 18:0 sphinganine had a mass spectrum identical to that of methyl palmitate as expected. The mass spectrum of methyl 13-methyl tetradecanoate, derived from the 17:0 branched sphinganine, is illustrated in the lower part of Fig. 2.

DISCUSSION

The major sphinganines from the ceramide phospholipids of *B. melaninogenicus* have been characterized as 15-methyl hexadecasphinganine, 17-methyl octadecasphinganine, normal octadecasphinganine and possibly 16-methyl heptadecasphinganine by gas chromatography and mass spectrometry. The configuration at C-2 of the sphinganines has not been determined but the derivatives have the gas-chromatographic retention times predicted for the usual erythro configuration[7,13].

Sphingolipids are exceedingly rare in bacteria and branched-chain sphinganines are rare in nature. Branched-chain sphinganines and 4-hydroxysphinganine were found in the ceramides and ceramide aminoethylphosphonates of protozoa[14,15]. The major fatty acids of *B. melaninogenicus* are branched 15:0 and 17:0, suggesting that the fatty acids and sphinganines may have some common biosynthetic pathways.

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REFERENCES

1 J. P. LABACH AND D. C. WHITE, *J. Lipid Res.*, 10 (1969) 528.
2 D. C. WHITE AND A. N. TUCKER, *Lipids*, 5 (1970) in the press.
3 V. RIZZA, P. R. SINCLAIR, D. C. WHITE AND P. R. COURANT, *J. Bacteriol.*, 96 (1968) 665.
4 E. G. BLIGH AND W. J. DYER, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
5 C. C. SWEELEY AND E. A. MOSCATELLI, *J. Lipid Res.*, 1 (1959) 40.
6 D. C. WHITE AND F. E. FRERMAN, *J. Bacteriol.*, 94 (1967) 1854.
7 H. E. CARTER AND R. C. GAVER, *J. Lipid Res.*, 8 (1967) 391.
8 H. E. CARTER AND H. S. HENDRICKSON, *Biochemistry*, 2 (1963) 389.
9 J. W. FARQUAR, *J. Lipid Res.*, 3 (1962) 21.
10 D. C. WHITE AND R. H. COX, *J. Bacteriol.*, 93 (1967) 1079.
11 A. J. POLITO, J. NAWORAL AND C. C. SWEELEY, *Biochemistry*, 8 (1969) 1811.
12 R. RYHAGE AND E. STENHAGEN, *Arkiv Kemi*, 15 (1960) 291.
13 A. J. POLITO, T. AKITA AND C. C. SWEELEY, *Biochemistry*, 7 (1968) 2609.
14 H. E. CARTER, R. C. GAVER AND R. K. YU, *Biochem. Biophys. Res. Commun.*, 22 (1966) 316.
15 H. E. CARTER AND R. C. GAVER, *Biochem. Biophys. Res. Commun.*, 29 (1967) 886.