BRANCHED-CHAIN SPHINGOSINES FROM TETRAHYMENA PYRIFORMIS

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In a previous paper (Carter et al., 1966) we reported the discovery of a branched-chain sphingolipid base (19-methyl-C_{20}-phytosphingosine) in Crithidia fasciculata, a flagellated Protozoan. In continuing this examination of the sphingolipids of Protozoa, we have now looked at the ciliated Protozoan Tetrahymena pyriformis. The only sphingolipid previously reported in this organism was sphingomyelin (Taketomi, 1961), but the fatty acid and long-chain base components were not characterized.

In this paper we report the characterization of two previously undescribed branched-chain sphingosines,[2] isolated from Tetrahymena pyriformis. These new long-chain bases contain a total of 17 and 19 carbon atoms and have the double bond between carbons 4 and 5. They occur as constituents of ceramides and ceramide aminoethylphosphonates, neither of which has been previously reported in this organism.

MATERIALS AND METHODS

Tetrahymena pyriformis cells, harvested in the stationary phase, were kindly supplied by Dr. George Kidder of Amherst. The cells were lyophilized, and the total lipids were extracted by the procedure of Folch et al. (1957). The lipids were separated into the so-called neutral and polar lipid fractions by

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sphingosine	1,3 dihydroxy-2-amino-4-octadecene
dihydrosphingosine	1,3 dihydroxy-2-amino-octadecane

elution from a silicic acid column (Unisil, 100-200 mesh, Clarkson Chemical Co., Williamsport, Pa.) with $CHCl_3$ and MeOH respectively. The polar lipids were separated by a combination of DEAE cellulose (Rouser et al., 1963) and silicic acid column chromatography.

Mild-alkaline hydrolysis was carried out by dissolving up to 20 mg of lipid in 0.5 ml of $CHCl_3$-MeOH 2:1 in a 100 x 13 mm screwcapped tube with Teflon lined cap. After adding 0.5 ml freshly prepared 1 N NaOH in MeOH the tube was left at room temperature, with occasional mixing, for 30 min. The alkali was then neutralized by the addition of 1 N HCl, and the appropriate volumes of $CHCl_3$, MeOH, and H_2O were added to give a Folch partition of $CHCl_3$:MeOH:H_2O of 8:4:3. After shaking the mixtures were centrifuged, and the clear upper MeOH-H_2O layer was carefully removed. The lower $CHCl_3$ phase was taken to dryness under a stream of nitrogen, and the fatty acid methyl esters and mild-alkali stable (MAS) lipids were separated by elution from a silicic acid column with $CHCl_3$ and MeOH respectively.

Methanolysis of sphingolipids was carried out using 1 N MeOH-HCl made 10 M with respect to water, in 100 x 13 mm screw capped vials with Teflon lined caps, in a hot air oven at 75-80°C for 16 hr. The fatty acid methyl esters and sphingolipid bases were separated by the extraction procedure described by Kates (1964).

Silica Gel G-HR (Brinkmann Instruments, Westbury, N. Y.) was used for all thin-layer chromatography (TLC). Polar lipids were separated in lined tanks with $CHCl_3$-MeOH-H_2O 100:42:6, and long-chain bases (LCB) were separated using $CHCl_3$-MeOH-NH_4OH 100:25:2.5. Spots were detected both by spraying with ninhydrin and by charring on a hot aluminum block after spraying with 5 N H_2SO_4 in MeOH.

Gas-liquid chromatography (GLC) of fatty acid methyl esters was carried out as previously described (Carter et al., 1966). GLC of the trimethylsilyl ethers of the LCB (TMSi-LCB) has been described (Carter and Gaver, 1967).

Colorimetric LCB analyses and periodate oxidations were carried out

as described previously (Carter et al., 1966).

A modification of the procedure of Tinoco and Miljanich (1965) was used for the permanganate oxidation of the LCB.

Hydrogenations were performed at about 15 psi, using 5% palladium on charcoal (Baker Co., Inc., Newark, N. J.) as catalyst (Sweeley and Moscatelli, 1959).

RESULTS

Total lipids, extracted by the Folch procedure, accounted for 8% of the dry weight of Tetrahymena pyriformis. The polar lipid fraction, eluted from silicic acid with MeOH, represented 27% by weight of the total lipid and contained about 12.6% long-chain base (LCB) based on colorimetric analysis of methanolysis products.

About 50% by weight of the polar lipid fraction was stable to a mild-alkaline hydrolysis. The mild-alkali-stable (MAS) contained essentially all of the LCB; and, based on TLC, contained at least four major components, two of which were ninhydrin positive.

The MAS material was methanolyzed and the LCB fraction was isolated. On TLC one major ninhydrin-positive spot with the same R_f as C_{18}-sphingosine was found. GLC of the trimethylsilyl ether derivatives of the LCB (TMSi-LCB) gave two major peaks which did not correspond to any of the known LCB. The unknowns had retention times of 0.54 and 1.09 relative to C_{18}-dihydrosphingosine and equivalent chain lengths (ECL) of 16.25 and 18.25 based on C_{18}-dihydrosphingosine as 18.00. C_{18}-sphingosine under these conditions has an ECL of 17.65.

Hydrogenation of the LCB fraction and re-examination of the products by TLC and GLC demonstrated the presence of at least one double bond in each of the major unknown LCB. TLC of the hydrogenated LCB fraction revealed one major ninhydrin-positive component with the same R_f as C_{18}-dihydrosphingosine. GLC of the same fraction gave two major peaks with ECL of 16.65 and 18.65 (retention times of 0.62 and 1.25 relative to C_{18}-dihydrosphingosine). The change in ECL, + 0.40, suggested the presence of an allylic double bond.

Furthermore, the ECL of the hydrogenated LCB, indicated a branched-chain structure. Under the conditions used it is known that the addition of a methyl group to give an isopropyl structure, increases the ECL by 0.65. For example, C_{20}-phytosphingosine has an ECL of 21.45 while 19-methyl C_{20}-phytosphingosine has an ECL of 22.10.

Periodate oxidation of the hydrogenated LCB fraction provided added evidence for the branched-chain structure and for the 1,3 dihydroxy-2-amino structure. The aldehydes obtained were treated with $NaBH_4$, and the alcohols were analyzed by GLC of the TMSi-derivatives. Two major products were found which corresponded to branched-chain alcohols containing a total of 15 and 17 carbon atoms with ECL of 14.65 and 16.65. Thus periodate oxidation of the hydrogenated LCB resulted in the loss of two carbon atoms. The N-acylated LCB were resistant to periodate.

The position of the double bond was demonstrated by oxidation of the LCB fraction with $KMnO_4$ and analysis of the products by GLC. The two major products were branched chain fatty acids containing a total of 13 and 15 carbon atoms, with ECL, on SE-30, of 12.65 and 14.65 respectively (based on saturated straight chain fatty acid methyl ester standards).

Based on the above data the two major LCB from Tetrahymena are branched-chain sphingosines containing a total of 17 and 19 carbon atoms respectively, each with the double bond between carbons 4 and 5. Based on the ECL of the fatty acid methyl esters and alcohols, the compounds probably have an isopropyl structure. Therefore, the new bases are identified tentatively as 15-methyl C_{16}-sphingosine and 17-methyl C_{18}-sphingosine. Evidence was also found for minor amounts of the corresponding saturated bases as well as other branched-chain sphingosines (methyl C_{17} and methyl C_{19}-sphingosine).

Isolation of two sphingolipids from Tetrahymena pyriformis was accomplished by a combination of DEAE cellulose and silicic acid column chromatography. Chromatography of the total polar lipid fraction on DEAE cellulose gave two LCB containing fractions which together accounted for over 90% of the total LCB.

One major sphingolipid, a ceramide, was eluted from DEAE cellulose with $CHCl_3$-MeOH 9:1 and represented about 12% by weight of the polar lipid fraction. It was eluted from a silicic acid column with 5% MeOH in $CHCl_3$, contained 48% LCB, had the same R_f on TLC as a beef lung ceramide, was resistant to mild-alkaline hydrolysis and periodate oxidation, and gave an infrared spectrum typical of ceramides. GLC analysis of the LCB fraction from the ceramide mixture showed the presence of both of the new branched-chain LCB. The predominate fatty acid was a branched-chain acid containing a total of 17 carbon atoms (66%) along with stearic (19%) and smaller amounts of palmitic and a 19 carbon branched-chain acid.

The other major sphingolipid was eluted from DEAE cellulose with $CHCl_3$-MeOH 7:3 and represented about 15% by weight of the polar lipid fraction. It was eluted from a silicic acid column with 25% MeOH in $CHCl_3$ but not with 20% MeOH in $CHCl_3$, contained about 47% LCB, and was stable to mild-alkaline hydrolysis. This sphingolipid also contained both of the new LCB. The fatty acid composition was very similar to the fatty acid composition of the ceramide, with a 17 carbon branched-chain fatty acid being the predominate acid (74%) along with stearic (22%) and smaller amounts of palmitic and a 19 carbon branched-chain fatty acid. Hydrolysis with 2 N HCl for 12 hr at 100°C followed by paper chromatography (BuOH-HAc-H_2O 60:15:25) of the products indicated the presence of LCB and a compound with the same R_f as 2-aminoethylphosphonate. No ethanolamine was detected. Based on these results it appears that this sphingolipid is ceramide aminoethylphosphonate. The infrared spectrum of the purified sphingolipid is identical to published spectra of ceramide aminoethylphosphonate (Hori *et al.*, 1964; Rouser *et al.*, 1963).

A lipid having the same chromatographic properties as sphingomyelin and containing LCB was detected, but it accounted for less than 3% of the polar lipid fraction.

As recently reported, Thompson (1967) failed to detect sphingosine in *Tetrahymena pyriformis*, however, the fraction which he examined (lipids eluted

from silicic acid with 14% MeOH) would not have contained ceramide aminoethylphosphonate.

Based on the results with Crithidia fasciculata (class Flagellata) and Tetrahymena pyriformis (class Ciliata) it would appear that branched-chain LCB are characteristic of the Phylum Protozoa. However, we now have evidence (unpublished results) that C_{20}-sphingosine is the major LCB in both light and dark grown Euglena gracilis (class Flagellata). The Protozoa, therefore, contain an unusual diversity of sphingolipid bases.

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