A NOVEL BRANCHED-CHAIN SPHINGOLIPID BASE FROM CRITHIDIA FASCICULATA

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Since sphingosine[1], but not dihydrosphingosine[1], spared the biopterin requirement of the trypanosomid flagellate, Crithidia fasciculata (Dewey and Kidder, 1964; Kidder, 1964); Kidder concluded that biopterin was required for the biosynthesis of sphingosine and proposed phytosphingosine[1] as an intermediate in the conversion of dihydrosphingosine to sphingosine. Based on the discovery of phytosphingosine in animal tissues, Karlsson (1964) has also suggested this route for the biosynthesis of sphingosine.

In view of the above proposal, studies were undertaken to determine if sphingolipids were indeed present in Crithidia fasciculata, particularly since they had not, with the exception of Tetrahymena pyriformis (Taketomi, 1961), been previously reported in Protozoa.

In this paper we report the characterization of a novel branched-chain sphingolipid base (1),19-methyl C_{20}-phytosphingosine, isolated from Crithidia fasciculata. C_{20}-Phytosphingosine is also present. Both long-chain bases occur as ceramides, with the new base representing 70-80% of the total long-chain base content.

(1) $CH_3-\underset{\displaystyle CH_3}{\underset{|}{CH}}-(CH_2)_{14}-\underset{\displaystyle OH}{\underset{|}{CH}}-\underset{\displaystyle OH}{\underset{|}{CH}}-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CH_2-OH$

[1] sphingosine — 1,3-dihydroxy-2-amino-4-octadecene
dihydrosphingosine — 1,3-dihydroxy-2-amino-octadecane
phytosphingosine — 1,3,4-trihydroxy-2-amino-octadecane

MATERIAL AND METHODS

Crithidia fasciculata cells, grown on a chemically defined medium, were kindly supplied through the generosity of Dr. George Kidder. The lipids were extracted by the method of Folch et al. (1951), and were separated into neutral and polar fractions by silicic acid chromatography (Unisil, Clarkson Chemical Co.). Mild alkali-stable lipids were obtained from the polar lipid fraction by a modification of the method of Dawson, 1960). Fatty acid methyl esters and the mild alkali-stable fraction (MAS) were separated by silicic acid chromatography.

Thin-layer chromatography (TLC) was carried out on MN-Kieselgel G-HR (Brinkmann Instruments). The spots were detected by spraying with 5N H_2SO_4 in MeOH and charring on a hot aluminum block.

The MAS fraction was hydrolyzed by heating for 16 hr., at 76° C, with 1N MeOH-HCl made 20 M with respect to H_2O. A hot-air oven and screw-capped tubes with Teflon-lined caps were used. Fatty acid methyl esters and long-chain bases (LCB) were separated by the method of Kates (1964).

The free LCB were analyzed as their trimethylsilyl ethers (TMS-LCB) by the procedure of Gaver and Sweeley (1965) using an F & M Model 400 Gas Chromatogram with hydrogen flame detector, and a 6' U-shaped, 4 mm I.D., glass column filled with 80-100 mesh Diatoport S coated with 3.8% SE-30 (F & M Scientific Corp.). The temperature of the column was varied from 180° - 220° C.

The method of Lauter and Trams (1962) was used for the quantitative analysis of the LCB. C_{18}-Phytosphingosine was used as standard.

Periodate oxidations were carried out by the method of Sweeley and Moscatelli (1959). The aldehydes were identified by GLC using both the 3.8% SE-30 and an identical column containing 15% diethylene glycol succinate on 100-200 mesh Chromosorb W (F & M Scientific Corp.). The aldehydes were reduced to the corresponding alcohols by treatment with $NaBH_4$ in methanol, and the products were identified by GLC. Pure known

saturated aldehydes and alcohols were used to obtain log retention time vs carbon number plots.

Mass spectra were obtained using an Atlas Model CH-4 with a TO-4 ion source and total electron energy of 70 eV. The samples were evaporated in the ion source.

EXPERIMENTAL AND RESULTS

Extraction of 20 gm (wet weight) of Crithidia cells yielded 553 mg of lipid. The polar lipid fraction represented about 54%, by weight, of the total lipid eluted from silicic acid. Approximately 18% of the polar lipid fraction was stable to mild alkali. The MAS fraction, on TLC, gave only two major spots which corresponded to material which was present before treatment with mild alkali. With $CHCl_3$:MeOH (93:7) as solvent, the two major components of the MAS fraction ran between authentic beef-lung ceramide and pure cerebroside. The separation of the ceramides on TLC is due to differences in their fatty acid composition, as shown later.

Following hydrolysis of the MAS mixture 50% of the weight was recovered in the LCB fraction. Quantitative analysis of this fraction indicated that it was 94% LCB. On TLC, with $CHCl_3$:MeOH:NH_4OH (40:10:1) as solvent, this fraction gave one unresolved major spot, which had the same R_f as pure phytosphingosine and was ninhydrin and periodate positive.

GLC of the TMS-LCB gave two major peaks. The larger peak had an equivalent chain length (ECL) of 20.7 based on phytosphingosine standards, while the second corresponded to C_{20}-phytosphingosine. The area ratio of the major peak to the C_{20}-phytosphingosine peak was about 3:1.

Periodate oxidation of the LCB mixture gave two major aldehydes. The larger component had an ECL of 17.5 on the polar column and 17.7 on the non-polar column and represented about 80% of the total area. The other peak corresponded to n-heptadacanal. Hydrogenation of the mixture of aldehydes, with 5% palladium on charcoal as catalyst, brought about no change in the retention times or in the area ratio of the two peaks.

GLC of the alcohols formed from the aldehydes confirmed the identity of the aldehydes. The larger peak had an ECL of 17.5 on the polar column and 17.7 on the non-polar column, while the smaller peak corresponded to n-heptadecanol. The area ratio of the two peaks was the same as obtained with the aldehydes.

Periodate oxidation of the unhydrolyzed MAS fraction yielded the same aldehydes as were obtained by oxidation of the free LCB, thereby confirming the phytosphingosine structures. Thus, C_{20}-phytosphingosine and a new branched-chain phytosphingosine, containing a total of 21 carbon atoms, are present in Crithidia fasciculata.

The two sphingolipids of the polar lipid fraction were isolated, without prior alkaline treatment, by silicic acid chromatography. The less polar component was eluted with 1% MeOH in $CHCl_3$ and accounted for 10%, by weight, of the polar lipids. The other component was eluted with 3% MeOH in $CHCl_3$ and represented 8% of the total polar lipid. Preparative TLC was employed to remove trace contaminants from each of the two major components.

The infrared spectrum of each of the two purified sphingolipids was very similar and confirmed the ceramide structure.

The fatty acid and LCB composition of each of the purified ceramide fractions were determined by GLC. In the less polar ceramide stearic acid represented over 90% of the total fatty acids, while in the more polar ceramide α-hydroxy stearic acid accounted for over 95% of the total fatty acids. In the less polar ceramide the area ratio of the branched-chain LCB to C_{20}-phytosphingosine was about 2.5:1, whereas in the more polar ceramide the ratio was about 6.0:1.

The nature of the branching in the new LCB was determined by mass spectrometry. The intact ceramides were treated with periodate, and the aldehydes were reduced immediately to the alcohols. The two major alcohols were separated and purified by preparative GLC and were examined by

mass spectrometry. The molecular weight of each alcohol was confirmed: the branched-chain alcohol had a molecular wieght of 270. The identity of the one alcohol as n-heptadecanol was confirmed. The mass spectra of each of the alcohols had major peaks at M-18 and M-(28+18) characteristic of primary alcohols. The mass spectrum of the branched-chain alcohol had in addition a more intense peak at M-(31+43) indicating a terminal isopropyl group. Reduction of authentic methyl 16-methyl heptadecanoate with $LiAlH_4$, purification of the alcohol by preparative GLC, and mass spectrometry of this alcohol confirmed the iso structure. The spectrum of the branched-chain alcohol obtained from the Crithidia ceramides was identical in every respect with the spectrum of authentic 16-methyl heptadecanol.

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