CHROM. 3359

BIOCHEMISTRY OF SPHINGOLIPIDS

XIV. IDENTIFICATION OF SPHINGOSINE BASE DEGRADATION PRODUCTS ORIGINATING FROM DIFFERENT ACID HYDROLYTIC CONDITIONS

Č. MICHALEC AND Z. KOLMAN
Laboratory of Protein Metabolism, Faculty of General Medicine, Charles University, Prague (Czechoslovakia)
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SUMMARY

A systematic paper chromatographic technique for the separation and identification of sphingosine bases and their degradation products formed under different acid hydrolytic conditions is described.

INTRODUCTION

Observations that various degradation products of sphingosine bases were formed during acid hydrolysis of the intact sphingolipids indicated the lability of the parent compounds to these reagents.

During the last few years several authors have studied the nature of these by-products[1–4]. CARTER *et al.*[1] identified 3-O-methyl ethers and WEISS[2], 5-O-methyl ethers of sphingosines. KARLSSON[5–8] observed several by-products, which appeared to be due to allylic rearrangements or to dehydration of sphingosine and related isomers.

In our previous publications[9,10], the effect of different hydrolytic conditions on the sphingosine base content was discussed.

In the present communication the identification of the degradation products formed in these reactions is explored in more detail.

EXPERIMENTAL

Materials

Sphingolipids were isolated from human brain tissue by mild alkaline hydrolysis and chromatography on Florisil and DEAE-cellulose columns. Gangliosides were removed from the initial lipid mixture according to SUZUKI[20]. The purity of all fractions was tested by paper chromatography[11].

Hydrolytic conditions

Aqueous methanolic HCl (AMH), modified aqueous methanolic HCl (MAMH),

10% aqueous HCl with 20% of methanol (AH) and methanolic sulphuric acid (MSA) reagents were used in the same manner as described previously[9,10]. The reaction mixtures were diluted with water and after alkalization the sphingosine bases and their degradation products were extracted with diethyl ether. The ethereal phase was washed with water and dried over sodium sulphate.

The substances isolated from all hydrolytic procedures were converted to their DNP-derivatives[12].

Chromatography

Separation into classes. The DNP-derivatives were separated by preparative chromatography on Whatman No. 3 paper impregnated with silica gel in the following systems:

(1) Petroleum ether (b.p. 60–90°)–diethyl ether (65:35)[10]
(2) Petroleum ether (b.p. 60–90°)–diethyl ether (50:50)
(3) Chloroform.

Separation of individual compounds. The individual classes of DNP-derivatives were further subfractionated by a two-dimensional combination technique on silica gel paper impregnated with sodium tetraborate[10] or on a reversed-phase partition system[10].

Detection. The spots of all DNP-derivatives were detected in ultraviolet light (254 nm).

Semiquantitative determination. After detection, the spots were outlined accurately in pencil, cut out, and eluted with methanol. The eluates were measured against a blank sample at 350 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of crude DNP-derivatives of sphingosine bases and their degradation products obtained from the hydrolytic procedures described above. As can be seen from this figure, MSA-hydrolysis gives the highest yield of O-methyl ethers of sphingosines.

Further characterization of 3-O-methyl ethers and 5-O-methyl ethers is illustrated in Fig. 2. The schematic representation of the two-dimensional techniques is shown in Figs. 3 and 4.

According to KARLSSON[5–8] it is possible to distinguish about eight by-products originating from sphingosines as a result of acid hydrolysis (Table I). It is evident that each type of such hydrolysis gives a somewhat different qualitative and also quantitative profile of these products. This spectrum depends closely on the reaction conditions.

As can be pointed out from the results of GAVER AND SWEELEY[13], hydrolysis of sphingolipids under anhydrous acidic conditions leads to the formation of O-methyl ethers in yields of 50% or more. Although no reagent was found that completely eliminated the formation of these derivatives, the presence of water effectively lowered the production of the ethers to a considerable extent with a concomitant increase in the yield of sphingosine bases[13,14]. Our present findings confirmed these results (Figs. 1 and 5).

It follows quite clearly from our experiments that in all the acid hydrolyses

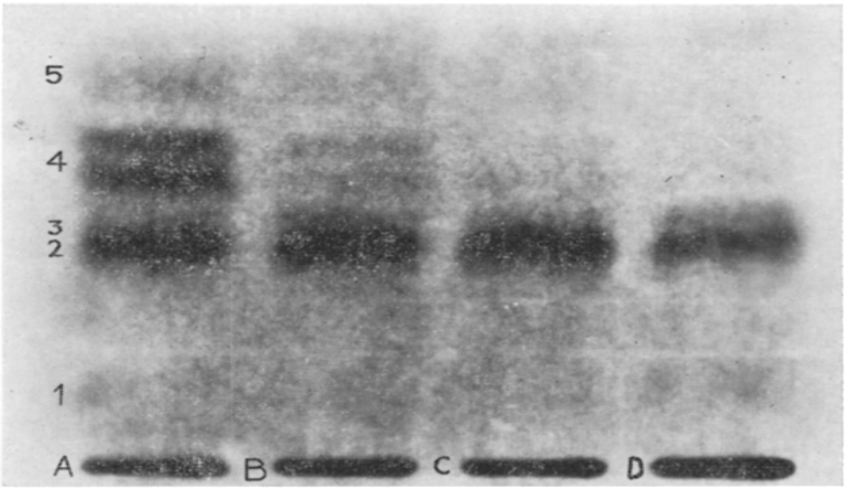

Fig. 1. Separation of DNP-derivatives of sphingosine bases and their degradation products isolated after different acid hydrolytic procedures on human brain sphingolipids. Paper: Whatman No. 3 impregnated with silica gel; solvent system: petroleum ether (b.p. 60–90°)–diethyl ether (50:50); detection: U.V. light (254 nm). A = MSA; B = AMH; C = MAMH; D = AH (for abbreviations see the text). 1 = 1,5-Dihydroxy monoenoic derivatives; 2 + 3 = sphingosines (*erythro* and *threo* isomers) + dihydrosphingosines; 4 = 5-O-methyl ethers; 5 = 3-O-methyl ethers + dienoic derivatives.

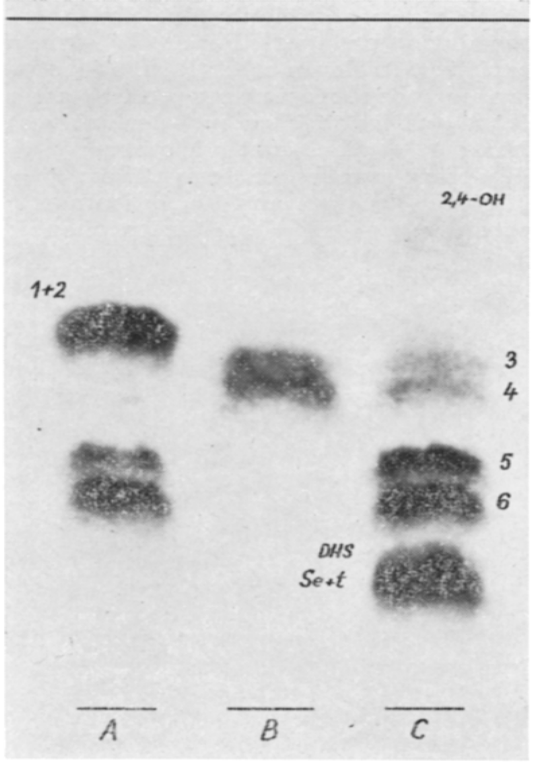

Fig. 2. Separation of DNP-derivatives of sphingosine bases and their degradation products isolated after MSA-hydrolysis of human brain sphingolipids. Paper: Whatman No. 3 impregnated with silica gel; solvent system: petroleum ether (b.p. 60–90°)–diethyl ether (65:35); detection: U.V. light (254 nm). A = Standard mixture of dienoic derivatives (1+2), 5D-O-methyl ethers (5) and 5L-O-methyl ethers (6); B = 3D-O-methyl ethers (3) and 3L-O-methyl ethers (4); C = MSA hydrolysate (DHS-dihydrosphingosines, Se + t = *erythro* and *threo* sphingosines); 2,4-OH = 2,4-dinitrophenol.

studied, the concentration of 5-O-methyl ethers formed by allylic rearrangements is much higher than of 3-O-methyl ethers. This is in agreement with the findings of WEISS[2] (Fig. 6). 5-L-Methoxy isomers predominate in all cases (Fig. 6).

The formation of 1,5-dihydroxy monoenoic derivatives is relatively small except in AH-hydrolysis where about 18 % was found.

Only traces of dienoic products were identified in AMH, MAMH and MSA

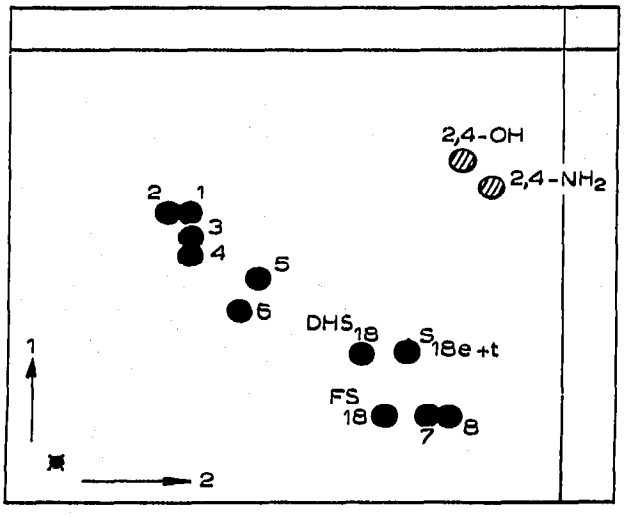

Fig. 3. Two-dimensional combination of adsorption and reversed-phase partition paper chromatography of DNP-derivatives of sphingosine bases and their degradation products. Paper: Whatman No. 3 impregnated with silica gel; solvent systems: 1st dimension: petroleum ether (b.p. 60–90°)–diethyl ether(65:35); 2nd dimension: methanol–tetralin–water (90:10:10; upper phase) after impregnation with tetralin; detection: U.V. light (254 nm). 1 = Dienoic(*cis,trans*)-derivatives; 2 = dienoic(*trans,trans*)-derivatives; 3 = 3D-O-methyl ethers; 4 = 3L-O-methyl ethers; 5 = 5D-O-methyl ethers; 6 = 5L-O-methyl ethers; 7 = 1,5D-dihydroxy monoenoic derivatives; 8 = 1,5L-dihydroxy monoenoic derivatives; S_{18e+t} = C_{18}-sphingosine (*erythro* and *threo* isomers); DHS_{18} = C_{18}-dihydrosphingosine; FS_{18} = C_{18}-phytosphingosine; 2,4-OH = 2,4-dinitrophenol; 2,4-NH_2 = 2,4-dinitroaniline.

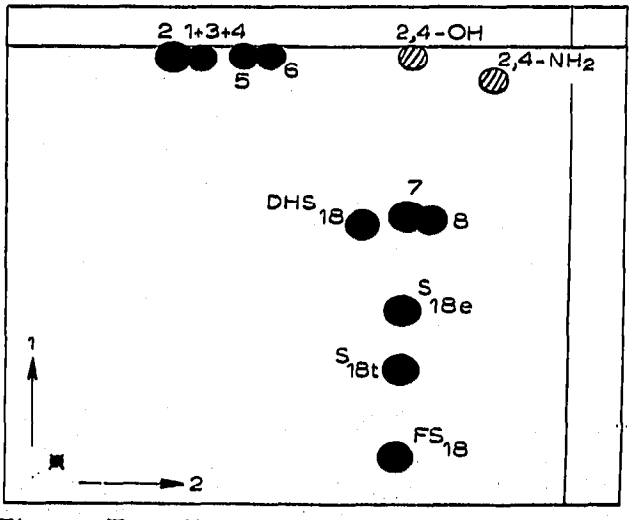

Fig. 4. Two-dimensional adsorption chromatography of DNP-derivatives of sphingosine bases and their degradation products. Paper: Whatman No. 3 impregnated with silica gel and 0.05 *M* $Na_2B_4O_7$; solvent systems: 1st dimension: chloroform–methanol (99:1); 2nd dimension: methanol–tetralin–water (90:10:10; upper phase) after impregnation with tetralin; detection: U.V. light (254 nm). Designation of spots is as in Fig. 3.

TABLE I

MAIN DEGRADATION PRODUCTS OF C_{18}-SPHINGOSINE (1,3-DIHYDROXY-2D-AMINO-4-*trans*-OCTADECENE) FORMED AS A RESULT OF VARIOUS ACID HYDROLYSES[7]

Designation	*Degradation product*
1	1-Hydroxy-2D-amino-3,5(*cis,trans*)-octadecadiene
2	1-Hydroxy-2D-amino-3,5 (*trans,trans*)-octadecadiene
3	1-Hydroxy-2D-amino-3D-methoxy-4-*trans*-octadecene
4	1-Hydroxy-2D-amino-3L-methoxy-4-*trans*-octadecene
5	1-Hydroxy-2D-amino-5D-methoxy-3-*trans*-octadecene
6	1-Hydroxy-2D-amino-5L-methoxy-3-*trans*-octadecene
7	1,5D-Dihydroxy-2D-amino-3-*trans*-octadecene
8	1,5L-Dihydroxy-2D-amino-3-*trans*-octadecene

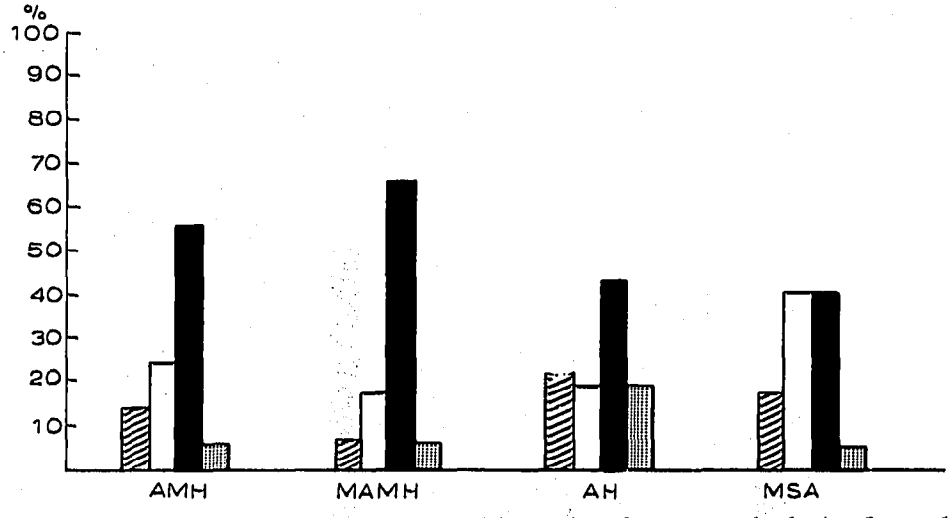

Fig. 5. Average percentage of sphingosine bases and their degradation products after various hydrolytic procedures of human brain sphingolipids. AMH, MAMH, AH and MSA, see text. (▨) Dienoic derivatives + 3-O-methyl ethers; (□) 5-O-methyl ethers; (■) sphingosines (*erythro* and *threo* isomers) + dihydrosphingosines; (▩) 1,5-dihydroxy monoenoic derivatives.

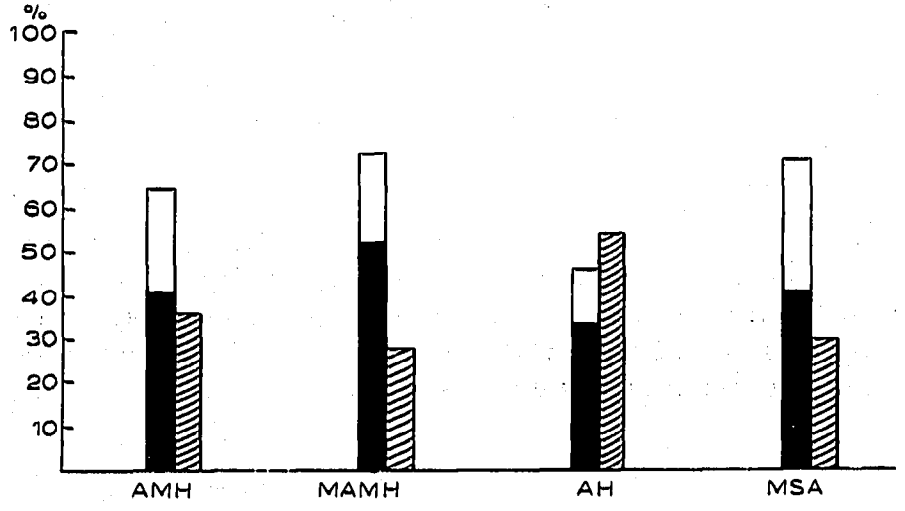

Fig. 6. Distribution of O-methyl ethers after various hydrolytic procedures of human brain sphingolipids. (□) 5D-O-Methyl ethers; (■) 5L-O-methyl ethers; (▨) 3-O-methyl ethers (D + L) + dienoic derivatives.

hydrolytic procedures. In AH-hydrolysis the amounts are higher, but no definite conclusion could be given, since the separation from the 3-O-methyl ether fraction was incomplete.

It has been admitted by numerous authors that during acid hydrolysis of N-acylsphingosines the inversion from *erythro* to *threo* configuration occurs. The *threo* isomer of sphingosine has been isolated, *e.g.* by SEYDEL[15] from aqueous methanolic acid hydrolysates of cerebrosides and by other authors[16,17].

We have found that all acidic hydrolytic conditions lead to considerable formation of *threo* isomer, although its concentration in the intact sphingolipids (*e.g.* sphingomyelins) is negligible (Fig. 7)[18].

In general, when acid hydrolysis has to be used to free the sphingosine bases from sphingolipids, two main problems arise. Firstly, the commonly employed hydrolytic procedures give low yields of bases[9]. Secondly, all these reactions lead to the formation of a lot of undesirable degradation products.

For these reasons the enzymatic hydrolysis of sphingolipids by a specific enzyme system combined with subsequent alkaline hydrolysis is preferable for the precise identification of the sphingosine base profile[7,18].

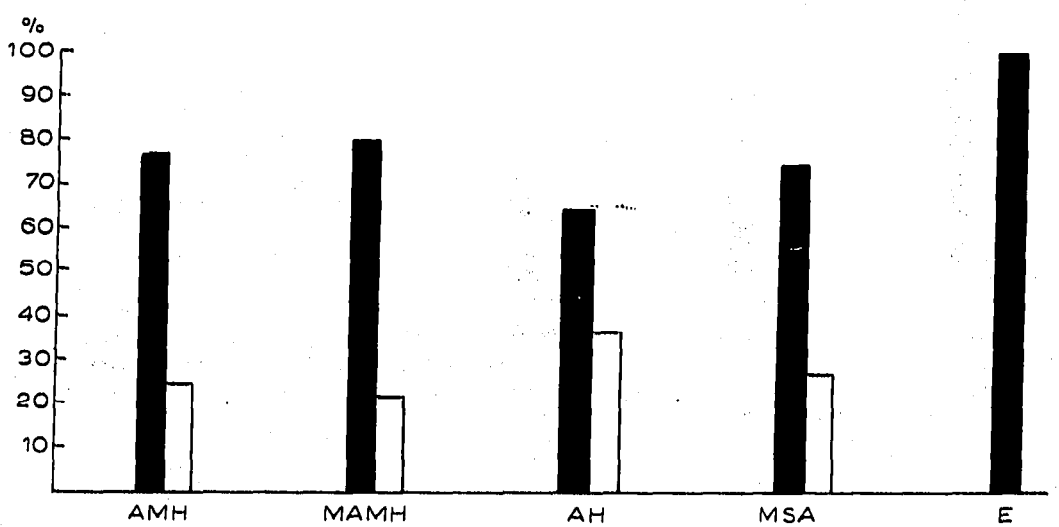

Fig. 7. Formation of *erythro* and *threo* isomers of sphingosines after various hydrolytic procedures of human brain sphingomyelins. E = Enzymatic cleavage with phospholipase C followed by alkaline hydrolysis[9]; (■) *erythro* sphingosines; (□) *threo* sphingosines.

Paper chromatographic techniques were found to be very useful for the separation and characterization of the natural bases as well as their degradation products.

Further techniques for the characterization of the above mentioned substances (*e.g.* chromatographic separation of oxidation products etc.) have been developed in the authors' laboratory, details of which are given in ref. 19.

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