Structures of the 2-hydroxy unsaturated fatty acids of pig brain sphingolipids

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SUMMARY The structural characterization of the 2-hydroxy monounsaturated fatty acids of pig brain sphingolipids is described. The acids were isolated via the copper chelate and converted to the 2-methoxy methyl esters. The ether esters were separated using column, thin-layer, and gas-liquid chromatography, and the positions of the double bonds were determined by oxidative ozonolysis. Seventeen chemically defined hydroxy acids were found, 15 of which have not been previously reported. These include the odd- and even-numbered hydroxy acids 22 to 26 carbon atoms long. Each acid consisted of three to four positional isomers which appear to have been derived from the corresponding non-hydroxy unsaturated acids. The proportion of isomers in the hydroxy acids structurally related to palmitoleate (ω7) was higher than in the non-hydroxy monoenoic acids. Components tentatively identified as 2-hydroxy dienoic acids, 22 to 26 carbons long, and 27h:1 were found by gas-liquid chromatography, and small amounts of 2-hydroxy trienoic acids were also tentatively identified by thin-layer chromatography of the mercuric acetate adducts.

Recently we have examined the unsaturated non-hydroxy fatty acids of pig brain sphingolipids by a combination of silver nitrate–silica gel chromatography, GLC,[1] and ozonolysis (1). The acids were found to be C_{22} to C_{26} monoenes and $C_{24,25,26}$ dienes, and each acid consisted of two to four positional isomers. From the positions of the double bonds, it appeared that most of the acids were formed from oleic, palmitoleic, linoleic, or other shorter chain fatty acids by chain elongation. Some appeared to be derived by 1-carbon oxidation of higher homologues (2–4).

Brain sphingolipids are distinctive in that they contain a high percentage of 2-hydroxy fatty acids. Although GLC studies in this laboratory (5, 6) have shown the presence of several unsaturated hydroxy acids, only 24h:1 has been isolated and further characterized as a mixture of two isomers, $24h{:}1^{15}$ and $24h{:}1^{17}$ (7). Recently it was shown that the saturated acid, 24:0, can be converted by brain to the analogous hydroxy acid (8, 9). It seemed possible that a comparison of the isomeric structures of the unsaturated non-hydroxy and hydroxy acids would indicate a composition compatible with the existence of a similar hydroxylation system acting on the unsaturated acids.

EXPERIMENTAL METHODS

Most reagents, apparatus, and procedures employed in this study have been described previously (1).

The pig brain sphingolipid preparation used before for the isolation of normal unsaturated acids (1) yielded 1.13 g of a mixture of hydroxy acid methyl esters. The esters were saponified (10), purified by precipitation of the copper chelate (11), and converted to the 2-acetoxy methyl esters which were stored for about 4 months under nitrogen at −20°. The unsaturated esters were freed from the saturated esters by silica gel chromatography (12) of the mercuric acetate adducts (5). Because of a change in experimental plan, the 2-acetoxy compounds were converted to the 2-methoxy derivatives (5). The ether-esters were isolated as a monoene group by silver nitrate–silica gel column chromatography with SkB[1]–benzene 8:2; and as a diene group, with SkB–benzene 6:4 (1).

The individual monoenes were isolated by GLC using a thermal conductivity detector and an 8 ft × 0.5 in.

[1] Abbreviations used: DEGS is a polymeric form of diethyleneglycol succinate; SE-30 is a General Electric silicone gum; SkB is Skellysolve B, a commercial hexane from Skelly Oil Co., Kansas City, Missouri; TLC is thin-layer chromatography; GLC is gas-liquid chromatography.

In fatty acid abbreviations, the first number indicates the chain length, the second number indicates the number of double bonds, the superscripts indicate the positions of the double bonds, h indicates a 2-hydroxy group, and d indicates the acid is an α, ω-dicarboxylic acid. The position of a fatty acid double bond, as measured from the ω-end, is indicated by a prefixed ω, as in ω7.

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o.d. steel column packed with 20% SE-30 on Chromosorb W, 60–80 mesh.[2] A linear temperature program of 240 to 350° was used. Thin-layer chromatography of the isolated esters showed the presence of material moving faster than the ester spots, evidently the result of bleeding from the column, so the esters were further purified using silica gel chromatography as described above. The column loading was 15 mg ester per g silica gel; the impurity was eluted with the first solvent mentioned above and the ester was eluted with the second. The resultant esters were then examined by oxidative ozonolysis and GLC with SE-30 and DEGS columns.

Preparation of 2-Methoxy Dicarboxylic Acid Esters. These esters were needed as standards for identifying the products of ozonolysis. A mixture of the even-numbered commercially available dicarboxylic acids, 8d:0 to 14d:0, was brominated in thionyl chloride (13) with an equimolar amount of bromine, then hydrolyzed to the hydroxy acids (14). The crude mixture was purified through the copper chelate and converted to the dimethyl esters. (Incidentally, the chelate was rather bluish, showing the presence of the non-hydroxylated ω-carboxyl group.) The hydroxy esters were next purified with a Florisil column, using SkB–ether 9:1, which left the dihydroxy esters on the column.

Examination with TLC (chloroform–acetic acid 24:1) showed the hydroxy dimethyl esters at R_F 0.61, with a faint spot just below. The R_F value for the non-hydroxy dimethyl esters was 0.73; for the dihydroxy esters, 0.30. The faint lower spot in the monohydroxy esters might represent a monohydroxy ester with a double bond next to the ω-carboxyl group, arising from dehydrohalogenation of dibromo acid during hydrolysis.

The 2-hydroxy esters were methylated with methyl iodide and silver oxide and purified again by Florisil chromatography, using SkB–ether 97:3. Thin-layer chromatography of the methoxy diesters (SkB–ether 85:15) gave a single spot with an R_F of 0.11; the R_f of the unsubstituted diesters was 0.22. The retention times of the individual ether diesters on GLC showed a parallel relation to those of the normal diesters. With the DEGS column, the C_{10} ether diester came out after the C_{12} diester; with the SE-30 column, the C_{10} ether diester came out just after the C_{11} diester. The retention times relative to 10d:0 of the methoxy diesters of 10d:0, 12d:0, and 14d:0 were 1.22, 1.39, and 1.55 on DEGS (R_t of 10d:0 was 18.7 min); and 1.21, 1.56, and 1.86 on SE-30 (R_t of 10d:0 was 12.8 min).

RESULTS

Validation of the Methodology. Analysis of the individual ether esters by TLC showed only one spot using Silica Gel G[3] and SkB–ether 85:15. The R_F values were 0.35 for the ether esters and 0.60 for the non-hydroxy ester standard. It is interesting to note that the unsaturated esters yield a distinctively different color when the plate is sprayed with bromothymol blue containing NaOH (15). Indeed, this spray is unexpectedly specific although the colors change on storage of the plate and are difficult to describe. It is possible to distinguish qualitatively the various degrees of unsaturation in fatty esters: saturated, monoenes, dienes, and trienes. Dicarboxylic acid methyl esters yield white spots on a blue background, sulfatides (developed with an ammoniacal solvent) yield bright yellow spots, sphingosine and psychosine yield blue spots against a pale background, and cerebrosides a greenish yellow.

Analysis of the individual ether esters by GLC on DEGS showed that the 23h:1 was contaminated with 2.9% of 24h:1, while the others contained less than 1% of homologous esters.

The infrared spectra of the isolated ether esters were very similar to each other and to those of the non-hydroxy unsaturated esters (1). The major differences, with respect to the non-hydroxy esters, were (*a*) a split carbonyl absorption band (5.72 and 5.77 μ), (*b*) the expected, strong C—O—C absorption at 8.8 μ arising from the methoxyl group, and (*c*) inversion of the relative intensities of the bands between 8.3 and 8.6 μ. No evidence for a *trans* double bond was obtained.

The retention times of the ozonolysis products were compared with those of standard monocarboxylic and 2-methoxy-dicarboxylic acid esters. Thin-layer chromatography of the products showed two spots corresponding to the standard ether-diesters and monoesters, together with paler spots of by-products (with lower mobilities).

Occurrence of Positional Isomers. As shown in Table 1, each ether-ester proved to consist of three or four positional isomers. The last column in the table shows that $24h{:}1^{15}$ is the most common acid, as in the case of the non-hydroxy monoenes. However, it is much less predominant and not much greater in amount than $24h{:}1^{17}$.

Table 1 does not show 27h:1, which was seen in the gas chromatogram but not isolated. A fraction from the silver nitrate–silica gel column (SkB–benzene 6:4) appeared to consist of 2-hydroxy dienes, C_{22} to C_{26}, including odd-numbered acids. The esters were tentatively identified as dienes on the basis of their later elution from the silver nitrate–silica gel columns and from their relative retention times on the DEGS column. Although the hydroxy esters had been stored in dilute solution under supposedly protective conditions, it was evident from TLC of the mercuric acetate adducts

[2] Model 720, F & M Scientific Corporation, Avondale, Pennsylvania.

[3] Brinkmann Instruments, Inc., Great Neck, New York.

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(16) before and after storage that considerable loss of dienes and trienes had occurred.

DISCUSSION

All the isomers found in the non-hydroxy acids (1) were found also in the hydroxy acids. From column 3 in Table 1, it can be seen that each acid contains an isomer that could be derived, according to the position of the double bond relative to the ω-end of the molecule, from palmitoleate (ω7), oleate (ω9), and an acid in the ω10 family. In the case of the odd-numbered acids, 23h:1 and 25h:1, the isomers of the ω7, ω9, and ω10 families could have come from the longer even-numbered homologues by 1-carbon degradation, followed by alpha hydroxylation. The odd-numbered acids also contain an isomer presumably derived from an acid in the ω8 family. Since this family is not seen in the even-numbered acids, the parent acid serving for elongation is probably an odd-numbered acid such as $17:1^9$.

Table 1 2-Hydroxy Monoenoic Fatty Acids of Pig Brain Sphingolipids and Their Relative and Absolute Abundances

Ester Isolated	Position of Double Bond		Proportion of Each Isomer	Concentration in Fresh Brain
	From COOH	From CH_3		
			%	*gm/100g*
22h:1	15	7	85	3.2
	13	9	13	0.5
	12	10	2	0.1
23h:1	16	7	58	1.6
	15	8	26	0.7
	14	9	15	0.4
	13	10	1	0.03
24h:1	17	7	41	33
	15	9	55	44
	14	10	4	3.2
25h:1	18	7	51	3.8
	17	8	9	0.7
	16	9	37	2.8
	15	10	3	0.2
26h:1	19	7	68	18
	17	9	28	7.3
	16	10	4	1.0

Because of the qualitative similarity in position of the double bond in both the non-hydroxy and hydroxy acids, it seems likely that the latter are made from the former, in the same way that cerebronate is made from lignocerate (8). There may be a clue to the specificity of the hydroxyl-inserting enzyme system from the observation that the ω7, or palmitoleate, family is seen somewhat more in the hydroxy acids than in the non-hydroxy acids. In every acid except 24h:1 (Column 4, Table 1), the ω7 family is by far the predominant type. The total amounts of hydroxy acids in the major families, ω7 and ω9, are 60 and 55 mg/100 g brain, respectively. In the comparable non-hydroxy acids, the amounts are 86 and 195 mg/100 g.

Table 2 The Relative Abundance of the Hydroxy Acids of Pig Brain Sphingolipids

Acid Type	Ratio of Hydroxy/Non-hydroxy Fatty Acids
22 Monoenes	0.15
23 "	0.31
24 "	0.35
25 "	0.88
26 "	0.97
22 Saturateds	1.6
23 "	2.1
24 "	2.1
25 "	2.3
26 "	1.5

The apparent tendency of the hydroxylating system to attack the ω7 non-hydroxy acids could be construed as indicating a preference for fatty acids having a long saturated chain (counting from the COOH end). This preference is suggested by a comparison of the relative abundances of the hydroxy acids, listed in order of increasing length of the saturated chain (Table 2). The data are derived from the total weights of all the isomeric forms in the hydroxy and non-hydroxy acids, as well as the saturated sphingolipid acids of the same pig brains. The saturated acids are placed below the unsaturated acids because even the longest monoenoic acid, C_{26}, contains only a 19-carbon saturated chain (at most) whereas the shortest saturated acid, C_{22}, contains a 22-carbon saturated chain.

The data in Table 2 reveal that the relative amount of hydroxy acid rises fairly rapidly with increasing chain length within the monoenoic acid group, but only slightly within the other group. The saturated C_{26} acids constitute a minor exception.

Our previous study of the chain-length distribution of 2-hydroxy fatty acids outside the central nervous system showed the presence of two clusters of hydroxy acids centering around C_{16} and C_{24}. It was suggested that the body (outside the brain) contains *two* hydroxylating enzyme systems, with differing chain-length specificities (17).

An alternative explanation for the above abundance data is that the specificity lies in the enzyme system which incorporates the hydroxy and non-hydroxy acids into the sphingolipids.

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