Identification of a xylose-containing cerebroside in the salt gland of the herring gull

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Abstract

A pentose-containing cerebroside has been identified in the salt gland of the herring gull, using mass spectrometry of acetyl and trimethylsilyl derivatives. A detailed interpretation of the spectra allowed a conclusion concerning the major long-chain base (the Cm homolog of sphingosine) and the major fatty acids (Ct2-C2s 2-hydroxy fatty acids), using reference spectra of synthetic galactosylceramides. A six-membered glucose ring (aldopyranose) was demonstrated by mass spectrometry of the acetyl derivative of periodic acid-oxidized and sodium borodeuteride-reduced pentosylceramide. By gas-liquid chromatography and mass spectrometry of methanolysis products, the pentose was shown to be identical with xylose. The procedures were applied to 25-50 µg of glycolipid.

Supplementary key words sphingosine · 2-hydroxy fatty acids · pentosylceramide · periodic acid oxidation · sodium borodeuteride reduction · mass spectrometry

MATERIALS AND METHODS

Preparation of cerebroside

The preparation of sphingolipids from salt glands of the herring gull has been described elsewhere (3). The unknown cerebroside was isolated by TLC, using silica gel G (Fluka A.G., Buchs, Switzerland, batch 117736 K) and a spreader adjusted to 0.25 mm (C. Desaga, Heidelberg, Germany). The slurry was prepared by shaking 30 g of adsorbent for 90 sec with 55 ml of water containing 1% sodium borate decahydrate (Na2B4O7·10H2O). The plates were dried for 30 min at 120°C immediately before use and were developed in chloroform–methanol–water 65:25:4 (v/v/v). The bands were made visible in iodine vapors. The iodine was then allowed to evaporate at room temperature for several hours until no color remained. The fast-moving band was scraped off and transferred to a small column of silicic acid (more than 325 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.). Elution was with chloroform–methanol 9:1 (v/v).

Preparation of derivatives of intact cerebroside for mass spectrometry

About 25 µg of material was acetylated in 50 µl of acetic anhydride–pyridine 1:1 (v/v) overnight at room temperature. Water was added and the mixture was extracted with diethyl ether. The organic phase was evaporated and the residue was taken for mass spectrometry. About 25 µg of the cerebroside was silylated for at least 10 min in 20 µl of hexamethyldisilazane–trimethylchlorosilane–pyridine 2:1:10 (v/v/v) at room temperature in a test tube with a Teflon-lined screw cap. For mass spectrometry all of the material was transferred to a
carboxyl group at position 1 was obtained by methanolysis of the total cerebroside fraction, by GLC (9) and by GLC-MS (LKB 9000). Standards of arabinose, xyllose, ribose, and xylose were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Fig. 1 shows a thin-layer chromatogram of the total cerebroside fraction of herring gull salt gland. The fast-moving component is the unknown material; the slow-moving and middle compounds correspond to galactosylceramide and glucosylceramide, respectively. Their relative proportions are indicated in the gas-liquid chromatogram of trimethylsilyl ethers of methyl glycosides of the total fraction (Fig. 1). The amount of total cerebroside is about 0.45 amole/g dry weight of salt gland, which corresponds to about 100 μg per bird. Figs. 2 and 3 show the mass spectra of the acetylated and silylated fast-moving compound, respectively. For the detailed interpretation of fragments, information from a series of homogeneous galactopyranosylceramides was used; these compounds contained normal and 2-hydroxy fatty acids and di- and trihydroxy long-chain bases.1 The base peak of the acetylated derivative (Fig. 2) is m/e 43, due to acetyl ions. The second most intense peak of acetylated hexosylceramides (8) is at m/e 331, which corresponds to the complete glycoside residue, absent in the present case. Instead, m/e 259, corresponding to a pentosyl residue, is the second peak. In analogy with hexosyl derivatives, the peaks found below m/e 259 (217, 199, 157, 139, 115, 97) are due to successive losses of molecular ketene (mass 42) and molecular acetic acid (mass 60). The presence of a pentose residue is confirmed by fragments obtained from the silylated derivative (Fig. 3). Concerning hexosylceramides, the fragment at m/e 451, corresponding to the complete glycoside, is a low-intensity ion; however, the ion at m/e 361 (451 — 90), due to a loss of molecular trimethylsilyloxyl, is stronger. Fragments at m/e 349 and 259 are analogously found in the present case. Finally, fragments of the long-chain base and of the fatty acid (e.g., at m/e 339 and 411, respectively; see below) are in agreement with a glucose-binding position at carbon 1 of the long-chain base.

The intermediate and upper regions of the mass spectrum of the silylated derivative (Fig. 3) may be used to define the major species of long-chain base and fatty acid. No peaks corresponding to phytosphingosine (m/e 299) or its 20-carbon homolog (m/e 327) are present. The peak at m/e 311 (sphingosine) is of relatively low inten-
FIG. 1. Thin-layer chromatogram (left) of total cerebrosides of the salt gland of the herring gull, and a gas-liquid chromatogram (right) of the trimethylsilyl derivatives of methyl glycosides obtained by methanolysis of total cerebrosides. The numbers indicate cerebrosides of xylose (1), glucose (2), and galactose (3). GLC was performed on a 6-ft glass column (I.D. 3 mm) packed with 3% OV-1 coated on 100-120-mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa.) Column temperature was 160°C and the argon gas flow was 60 ml/min. The instrument was an F & M model 402 gas chromatograph with a flame ionization detector (Hewlett-Packard Co., Avondale Div., Avondale, Pa.).

Xylose-containing Cerebroside

sity. Instead, m/e 339 is strong, corresponding to the 20-carbon homolog of sphingosine. The double bond is probably at position 4, as for other salt gland sphingolipids (3), but this cannot be deduced from the spectrum. The molecular-ion region is around m/e 1170-1185. This corresponds to a silylated pentosylceramide containing the 20-carbon homolog of sphingosine and a fatty acid with a mass of about 440. This is equivalent to a normal fatty acid with about 27 carbon atoms or a hydroxy fatty acid with 24 carbon atoms. Consideration of the peaks in the intermediate region and comparison with the reference material\(^1\) conclusively give the major fatty acid as 2-hydroxytetraacerosanic acid. The most intense ion in the molecular-ion region is m/e 1170, which is the expected molecular ion minus 15, characteristic for silylated derivatives. The peak at m/e 820 corresponds to ceramide, and m/e 411 is a specific hydroxy fatty acid fragment, due to a cleavage between carbon atoms 1 and 2. Furthermore, the peak at m/e 570 is due to a rearrangement ion, containing the complete fatty acid and carbon atom 2 and the amino group of sphingosine, plus 103.\(^1\)

Peaks that support these conclusions are found in the spectrum of the acetyl derivative (Fig. 2), although the interpretation of this spectrum alone is less conclusive concerning the ceramide part than is the spectrum of the silyl derivative. Ions at m/e 776, 760, and 686 originate in the ceramide part, and the ion at m/e 667 is probably due to a loss of carbon atoms 3-24 of the fatty acid and 59 mass units, corresponding to the α-acetoxyceramide group. The peak at m/e 292 is a rearrangement ion of the C\(_{20}\) long-chain base.

Peaks at m/e 760 + 58, 776 + 58, and M + 58 found for the acetyl derivative (Fig. 2) have not been found in the reference material of hexosylceramides; this is consistent with a substitution of one hydrogen of the ceramide part with an acetoxy group. However, no indication of an extra hydroxy group is present in the silylated derivative, and an admixture of this type should have given a double-spot appearance on the chromatogram (Fig. 1). The lack of material has prevented further studies, e.g., to find out if there is a relationship between the intensity of these ions and sample pressure.

The series of peaks differing by 14 mass units around the major fatty acid-containing peaks at m/e 1185, 820, 570, 498, and 411 (Fig. 3) indicates a mixture of 2-hydroxy fatty acids in the compound, mainly with 22, 23, 24, and 25 carbon atoms. Small amounts of normal acids may also be present, according to the spectra, although the absence of a double spot in Fig. 1 is evidence against this.

The following conclusions are allowed on the basis of the interpretation of the mass spectra obtained from acetyl (Fig. 2) and trimethylsilyl (Fig. 3) derivatives of the fast-moving compound in Fig. 1, and by comparison with a reference material of homogeneous hexosylceramides.\(^1\) The compound is a pentose-containing cerebroside with the glycose in position 1 of the base. The major long-chain base is the 20-carbon homolog of

\(^1\) Karlsson, Samuelsson, and Steen

\(\text{Xylose-containing Cerebroside}\)
Fig. 2. Mass spectrum of the acetylated fast-moving cerebroside shown in Fig. 1. The top formula represents the major molecular species identified.
sphingosine, and the major fatty acids are saturated 2-hydroxy fatty acids with 22–25 carbon atoms. Small amounts of sphingosine are also indicated, but phytosphingosine is absent.

The peak m/e 103 (Fig. 3) is of similar intensity to that of silyl derivatives of galactopyranosylceramides1 and was first thought to originate in a primary alcohol group (-CH3OSi(CH3)3), thus indicating that the pentosylceramide was in the furanose form (five-membered ring). It has, however, been shown for trimethylsilyl derivatives of simple carbohydrates (10) that about half of the ions at m/e 103 are rearrangement ions. Mass spectra of

![Diagram](image-url)

**Fig. 3.** Mass spectrum of the trimethylsilyl derivative of the fast-moving cerebroside shown in Fig. 1. The top formula represents the major molecular species identified.
Fig. 4. Mass spectra of acetyl derivatives of periodic acid-oxidized and sodium borodeuteride-reduced cerebroside. The oxidation was performed in 0.002 M (A), 0.02 M (B), and 0.2 M (C) periodic acid. The peak at m/e 292 is a rearrangement fragment of the C₆₀ long-chain base.

Acetyl derivatives of periodic acid-oxidized and sodium borodeuteride-reduced pentosylceramide strongly indicate an aldopyranose derivative (Fig. 4). We have found this structural modification to be very helpful in the characterization of glycolipids by mass spectrometry on a microgram scale.² The preparation needs no transfers, and the amount of material is usually below 50 µg. The deuterium labeling is used to differentiate between, for example, hexapyranosyl- and hexafuranosylceramides, which otherwise produce the same chemical structures and mass spectra.² Mass spectra of oxidized glycolipid (omitting the reduction step) are not suitable for detailed information concerning the carbohydrate residue.³ Three different concentrations of periodic acid were used. 0.002 M acid quantitatively oxidizes glucopyranosyl- and galactopyranosylceramides and also the terminal galactose of lactosylceramide, but leaves the glucose moiety intact. 0.2 M acid in addition oxidizes the glucose ring of lactosylceramide quantitatively. As shown in Fig. 4A, 0.002 M periodic acid leaves considerable amounts of unoxidized pentosylceramide. Peaks at m/e 259, 217,
Fig. 5. Partial formulas of possible isomers of pentosylceramide. From top: aldopentapyranosylceramide, aldopentafuranosylceramide, and ketopentosylceramide. The stereochemistry given is arbitrary. Oxidation sites are indicated by dashed lines. Products of oxidation, reduction, and acetylation are shown with expected fragmentation under electron impact.

199, 157, and 139 are the same as those in Fig. 2. 0.02 M (Fig. 4B) and 0.2 M (Fig. 4C) oxidant give a complete oxidation of the glyose ring. In all three spectra (Fig. 4A–C), strong peaks are found at m/e 88 and 191, and these are the only major peaks in Fig. 4B, in addition to the base peak at m/e 43, due to acetyl ions. Fig. 5 presents the isomeric alternatives for a pentose in glycosidic linkage to ceramide. In analogy with spectra obtained from oxidized hexosylceramides, high intensity fragments are expected from cleavage at the ring oxygen and at the glycosidic oxygen. The peaks appearing after oxidation and reduction of pentosylceramide correspond to an aldopentapyranosylceramide (top formulas in Fig. 5). In Fig. 4B, no evidence for the presence of an aldopentafuranose (expected fragments at m/e 263 and 160) or a ketopentose (expected fragments at m/e 263 and 88) was obtained. Peaks at m/e 118 and 177 in Fig. 4C may be due to an overoxidation in 0.2 M periodic acid. A possible overoxidation to carboxylic acid at carbon atom 2 of the pentose may be followed by a decarboxylation, which is favored by the electron-withdrawing oxygens at the α-carbon, producing the compound shown in Fig. 6.

The peak at m/e 177, which is 118 plus 59 mass units (acetoxyl), remains unexplained.

At present it is not possible to interpret the stereochemistry of glycolipids from mass spectra. Thus, we do not know if the configuration of the glycosidic linkage is β, if the hydroxyl group of the fatty acid is α, or if the configuration of the base is β-erythro as for galactosylceramide of brain (11). By GLC–MS of methanalysis products the pentose, however, was shown to be identical with xylose but to behave differently from arabinose, lyxose, and ribose on GLC. The α and β aldopyranose
forms of xylose (12) were eluted later and were completely separated from the other pentoses. The references and the pentosylceramide were treated identically, and the proportion of α and β anomers (about 2 to 1, see Fig. 1) was the same for xylose and the unknown.

As far as we know this is the first conclusive evidence for the natural existence of a pentose-containing cerebroside. A recent study on marine invertebrates (13) has, however, demonstrated a complex glucose pattern of total lipid hydrolysates, including arabinose and xylose. TLC of the lipids revealed mixtures of glycolipids, including rapidly moving cerebrosides. The pentosylceramide reported here has not been detected in the salt gland of eider duck or domestic duck. Whether the salt gland lipid is synthesized de novo or is of dietary origin (marine invertebrates) is an open question. Although the furanose form of glucose seems to exist in glycolipids of invertebrates (14), xylose is present as a pyranose in mammalian glycoproteins (15) in β linkage to serine.

Mass spectrometry alone is thus capable of giving on a microgram scale detailed and conclusive information concerning fatty acid and long-chain base composition of an unknown cerebroside, as well as information concerning the glucose type (hexose or pentose) and glucose ring size. The combination of preparative TLC and MS may prove to be a powerful tool in structural microanalysis of glycolipids, as in the identification of biosynthetic products or of lipid components of enzymes and membranes.

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