Linoleate-rich acylglucosylceramides of pig epidermis: structure determination by proton magnetic resonance

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Abstract The structure of the linoleate-rich acylglycosylceramides isolated from pig epidermis has been reinvestigated. Gas–liquid chromatographic analysis of the alditol acetates produced from the sugar component indicated that 90% of the hexose is glucose while the remaining 10% is galactose. The predominance of the β-D-glucosyl group was confirmed by 360 MHz proton magnetic resonance spectroscopy. The magnetic resonance method was also used to prove that the ester-linked linoleic acid is actually attached to the ω-hydroxyl group of the long chain hydroxyacid, not to the sugar as had been reported previously. A key spectral feature supporting this new structural assignment was a triplet at 3.82 ppm, which indicates methylene protons between another methylene and an ester linkage. After saponification, this signal moved to 3.33 ppm, a chemical shift expected for a methylene bearing a free hydroxyl group. Furthermore, all of the sugar ring protons could be accounted for both before and after acetylation. No evidence was found to suggest that an ester is attached to the sugar ring in the native material. It is concluded that the principal porcine epidermal acylglycosylceramide is 1-β-D-glucosyl-N-(ω-O-linoleoyl)-triacontanoylsphingosine. —**Abraham, W., P. W. Wertz, and D. T. Downing.** Linoleate-rich acylglucosylceramides of pig epidermis: structure determination by proton magnetic resonance. *J. Lipid Res.* 1985. **26:** 761–766.

Supplementary key words long chain hydroxyacid • ω-hydroxyl group • glycosphingolipid

In 1978, Gray, White, and Majer (1) isolated an unusual glycolipid from human and porcine epidermis. This novel lipid was said to be a glucosylceramide with an 18-carbon dienoic acid esterified to the 3'-hydroxyl group of the sugar ring. The long-chain base consisted of a mixture of 16- through 20-carbon sphingosines and dihydrosphingosines, and the amide-linked fatty acid was said to have 35 carbon atoms, two double bonds and two hydroxyl groups. The double bonds and hydroxyl groups were said to be located on the central five carbon atoms of this pentatriacontadienoic acid.

These workers suggested that the ester-linked fatty acid in the acylglucosylceramide was probably linoleic acid (1). Since linoleic acid is known to be essential for the formation of a functional water barrier in the stratum corneum (2), it was proposed that the acylglucosylceramide may be a functionally significant component of the barrier lipids (3). However, it was also demonstrated that the level of acylglucosylceramide is maximal in the granular layer of the epidermis, and is absent from the stratum corneum (4, 5).

In a more recent study (6), the nature of the long-chain base was confirmed, and the ester-linked fatty acid proved to be mainly linoleic acid. The amide-linked fatty acid, however, was shown to consist of a series of long-chain ω-hydroxyacids, the major species of which were a 30-carbon saturated entity and a 32-carbon monoene. The earlier assignments regarding the identity of the sugar and the position of attachment of the ester-linked fatty acid seemed soundly based, and therefore were not reinvestigated at that time.

Since the length of the major amide-linked hydroxyacids is just sufficient to span a typical lipid bilayer, it was proposed that the acylglucosylceramide may serve as a transmembranal element, and as such might possibly function in aggregation of the lamellae which have been observed in epidermal lamellar granules (7). Within the context of this hypothesis, the linoleoyl chain would serve to hold adjacent membranous structures in close apposition. This suggestion accounted for the essentiality of linoleic acid for barrier formation, and is consistent with the distribution of the glycolipid within the epidermis.

In related studies, we showed that an acylceramide fraction from pig epidermis also contains esterified fatty acids containing a high proportion of linoleate, but in this case the acids were found to be esterified with the hydroxyl function of the ω-hydroxyacids (8). It seemed

Abbreviations: GLC, gas–liquid chromatography

possible that the original assignment of the structure of the acylglucosylceramide was incorrect, and that this series also bears the linoleate-rich fatty acids on the hydroxyacid rather than on the glucose. In the present study we have reinvestigated this aspect of the sphingolipid structures, using high-field proton NMR spectroscopy of the acylglucosylceramide and some of its derivatives. Some recent proton NMR studies of glycosphingolipids (9–15) illustrate the use of this technique in the structural investigation of these lipids.

METHODS

Isolation of acylglycosylceramide and characterization of the sugar

Acylceramides and acylglycosylceramides were isolated from pig epidermis as previously described (6–8). A portion of the glycolipid was treated for 18 hr at 60°C with 1 N HCl in methanol containing 10 M water (16). The reaction mixture was dried under nitrogen, and the residue was partitioned between chloroform and water. The aqueous phase, which contained the liberated sugar, was taken to dryness and redissolved in 0.01 M aqueous potassium carbonate. A small amount of sodium borohydride was added and the mixture was kept at room temperature for 4 hr. Then, the reaction mixture was acidified with acetic acid and passed through a column containing 1 g of Dowex 50 × 4 (H^+) in a Pasteur pipet. This column was washed with several 1-ml portions of water, and the combined eluant and washings were concentrated to dryness. Methanol was added to the residue and removed under a stream of nitrogen. This step was repeated several times. The final residue was treated for 4 hr at 100°C with pyridine–acetic anhydride 1:1. The reagent was removed under a stream of nitrogen, and the alditol acetate was purified by preparative thin-layer chromatography. Standard alditol hexaacetates were prepared from glucose, galactose, and mannose by borohydride reduction followed by acetylation as described above.

The alditol hexaacetates derived from the acylglycosylceramide were identified by GLC on a 50-meter BP-10 vitreous silica capillary column (Scientific Glass Engineering, Inc., Austin, TX). The sample yielded two peaks. The major component comprised 90% of the total and cochromatographed with authentic sorbitol hexaacetate. The minor component cochromatographed with dulcitol hexaacetate. Therefore, the sugar from the epidermal acylglycosylceramide consists of 90% glucose and 10% galactose.

Chemical modifications

Acylceramide and acylglycosylceramide were saponified by treatment with excess chloroform–methanol–10 N NaOH 2:7:1 (by volume) for 1 hr at 37°C. The reaction mixture was then neutralized with 2 N HCl and the products were extracted into chloroform.

Acetylation of the free hydroxyl groups was carried out with pyridine–acetic anhydride 1:1 (by volume). The reaction mixture was warmed to 40°C for 2 hr and evaporated to dryness with a stream of nitrogen. The product redissolved in chloroform.

NMR spectra

Proton NMR spectra were recorded on a Bruker WM-360 spectrometer operating in the Fourier transform mode. The operating frequency was 360 MHz and the spectral width was 3.6 KHz. Three mg of the sample was dissolved in 0.5 ml of solvent. Different solvents ($CDCl_3$, DMSO, and 2:1 mixture of $CDCl_3$–CD_3OD) were tried. The $CDCl_3$–CD_3OD mixture was found to give the best-resolved spectrum. The residual proton signal from $CDCl_3$ (7.25 ppm) was used as internal reference to determine the chemical shifts.

RESULTS

The ^{1}H-NMR spectrum of acylglycosylceramide is shown in **Fig. 1.** The hydroxyl and the amide protons are exchanged in the $CDCl_3$–CD_3OD solvent mixture. The spectrum was not well resolved in $CDCl_3$ or in DMSO, possibly due to self-aggregation of the sphingolipid. The upfield protons arise from the methyls (0.65 ppm) and the methylenes (1.1 ppm) of the aliphatic chains. The broad peak around 1.37 ppm is from the methylenes β to the electronegative groups. The multiplet at 1.8 ppm is from the methylenes adjacent to the double bonds in the sphingosine and in the linoleate fragment, while the methylene protons between the two double bonds in the linoleate appear as a triplet at 2.52 ppm. Two other triplets at 2.05 and 1.90 ppm are due to methylene protons adjacent to the carbonyl group of the ester and the amide, respectively. The signals betwen 2.95 ppm and 4.05 ppm are from the sphingosine and the sugar moiety. Some of these peaks are readily interpretable by inspection. Thus, the anomeric proton from the sugar appears as a clean doublet at 4.02 ppm. The triplet at 3.82 ppm is from the methylene bearing the ester group. Other peaks were assigned by consecutive decoupling experiments and also by comparison with the spectra of model compounds, including glucosylceramides (A) and galactosylceramides (B).

Comparison of the spectra of compounds A and B with that of the epidermal acylglycosylceramide indicates that the sugar is mainly glucose. This was confirmed by GLC analysis of the alditol acetates produced by reduction of the sugar as described above. A minor component representing 10% of the total sugar proved to be galactose.

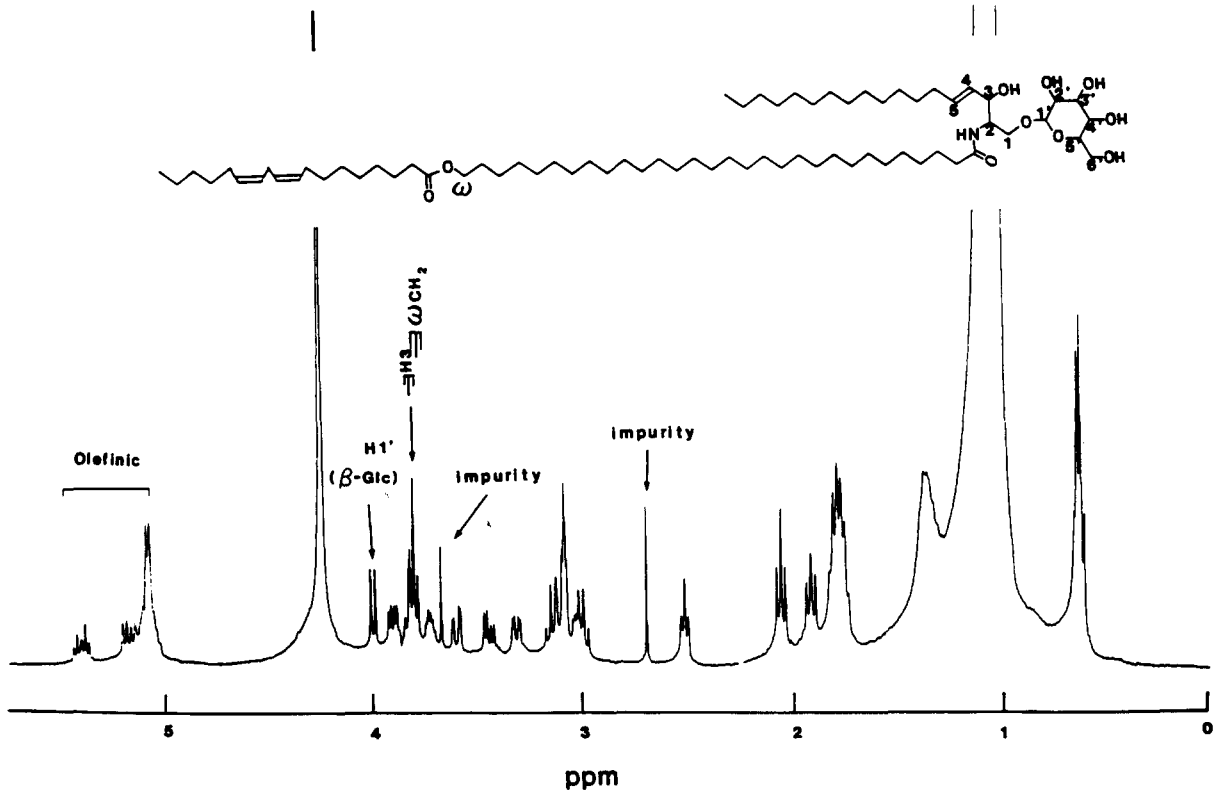

Fig. 1. 360 MHz proton magnetic resonance spectrum of the acylglycosylceramide from pig epidermis.

Careful decoupling experiments enabled us to assign the following peaks. The doublet of doublets at 3.90 ppm is due to H1A while H1B appears as a doublet of doublets at 3.32 ppm. It is interesting to note such a large chemical shift difference between these methylene protons. The coupling constants of these protons to the neighboring methine (H2) proton are 3.2 and 4.8 Hz. These differences are indicative of restricted rotation about the C1–C2 bond in the sphingosine. Such restricted rotation was also observed in our model compound A and in galactosyl ceramide (10) earlier. Proton H2 appears as a multiplet around 3.74 ppm. H3 appears as a triplet at 3.83 ppm, partially overlapping with the triplet from the methylene α to the ester group. The doublet of doublets at 3.62 and 3.45 ppm is due to the methylene protons 6'A and 6'B of the sugar ring. H3' and H4' partially overlap to give a complex pattern around 3.15 ppm. The peak at 3.09 ppm showing fine structure is the residual methyl-proton peak from CD_2HOD. The residual hydroxyl-proton peak from CD_3OH appears at 4.25 ppm. H2' and H5' overlap to give the complex pattern around 3.02 ppm. As the sugar ring protons H3' and H4' are coupled to each other as well as to H2' and H5', respectively, and their signals overlap, we were not able to extract the coupling constants. Simultaneous decoupling of protons H2′ and H5′, due to the fortunate overlap of these signals, enabled us to evaluate $J_{3',4'}$. The assignments and the coupling constants are summarized in **Table 1.** The assignment of protons H3' and H4' could be reversed. Typical value of $J_{1',2'} = 7.5$ Hz indicates β-glucosidic linkage.

The olefinic protons are from two different features. The olefinic protons from the sphingosine consist of *1*) a multiplet centered at 5.43 ppm arising from proton H5, and *2*) a multiplet centered at 5.18 ppm from proton H4. A coupling constant between these two protons of 15.9 Hz indicates a *trans* configuration for the double bond, as

TABLE 1. Chemical shifts and coupling constants (in Hz)

Proton	δ (ppm)	J (Hz)	
1A	3.90	$J_{1A,1B} = 11.5$	$J_{1A,2} = 4.8$
1B	3.32	$J_{1B,2} = 3.2$	
2	3.74	$J_{23} = 8.4$	
3	3.83	$J_{34} = 8$	
4	5.18	$J_{45} = 15.9$	
5	5.43		
1'	4.02	$J_{1'2'} = 7.5$	
2'[a]	2.99	$J_{2'3'} = 8$	
3'	3.15	$J_{3'4'} = 8.7$	
4'	3.12	$J_{4'5'} = 8.7$	
5'[a]	3.02	$J_{5'6'A} = 5.6$	$J_{5'6'B} = 3.2$
6'A	3.62	$J_{6'A,6'B} = 12.3$	
6'B	3.45		

[a]Although 2' and 5' partially overlap, we could still assign the upfield triplet to 2' from decoupling experiments.

expected. The tall peak at 5.08 ppm which appears as an unresolved doublet corresponds to the four olefinic protons from the linoleate whose chemical shifts are approximately equal.

The chemical shift value of 3.82 ppm is characteristic of a methylene bearing an esterified hydroxyl group, indicating esterification at the ω-OH position rather than at the 3'-OH of the sugar moiety (6). This is further confirmed by the upfield shift of this triplet from 3.82 ppm to 3.33 ppm in the saponified glycolipid (**Fig. 2**). A similar upfield shift of the ω-methylene signal was also observed upon saponification of the acylceramide.

Additional evidence for the position of the ester linkage can be derived from a close analysis of the spectrum of acetylated acylglucosylceramide (**Fig. 3**). Upon acetylation, we find a downfield shift of the sugar ring protons—H2', H3', and H4' appear as well-resolved triplets from 5.05 to 5.15 ppm. Presence of any ester group at any of the sugar hydroxyls in the original glycolipid would produce a similar effect on the chemical shift of the ring proton on that carbon α to the ester. The fact that protons H2', H3', and H4' are far upfield—3.25 to 3.05 ppm in the original lipid molecule—rules out the possibility of esterification on any of these sugar hydroxyl groups. A similar argument holds true for the hydroxyl on carbon 6' also.

An additional feature of the spectrum of the saponified glycosphingolipid is the presence of a triplet at 5.10 ppm which integrates to 1.5 protons. This can be assigned to two protons of another olefinic bond whose chemical shifts are equal. The fact that this triplet does not integrate to two protons indicates that this double bond is not present in all the molecules, i.e., this additional unsaturation corresponds to 25 mol % for the glycosylceramide.

DISCUSSION

On the basis of these interpretations, the structure of the acylglucosylceramide is revised to that shown in Fig. 1, where linoleate-rich fatty acids are esterified with the ω-hydroxyl group of the hydroxyacid. Conversion to acylceramide, as appears to occur during the final stages of epidermal differentiation, therefore requires only the removal of the sugar moiety. This renders it more likely that the composition of the esterified fatty acids in the acylceramide, which survive desquamation of the horny cells, will reflect the composition of the esterified acids in the acylglucosylceramides, which do not persist beyond the granular layer.

Reformulation of the structure of the acylglucosylceramide does not affect the observation that this materi-

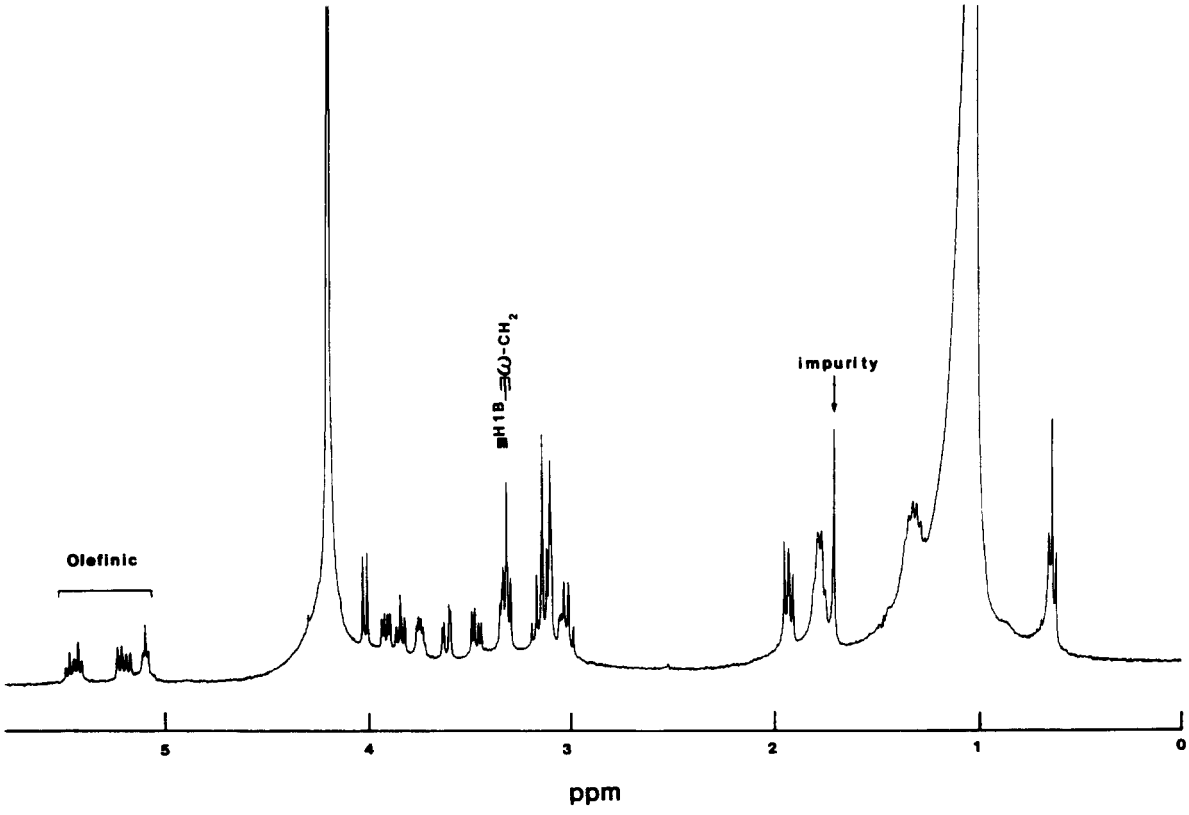

Fig. 2. 360 MHz proton magnetic resonance spectrum of saponified acylglycosylceramide.

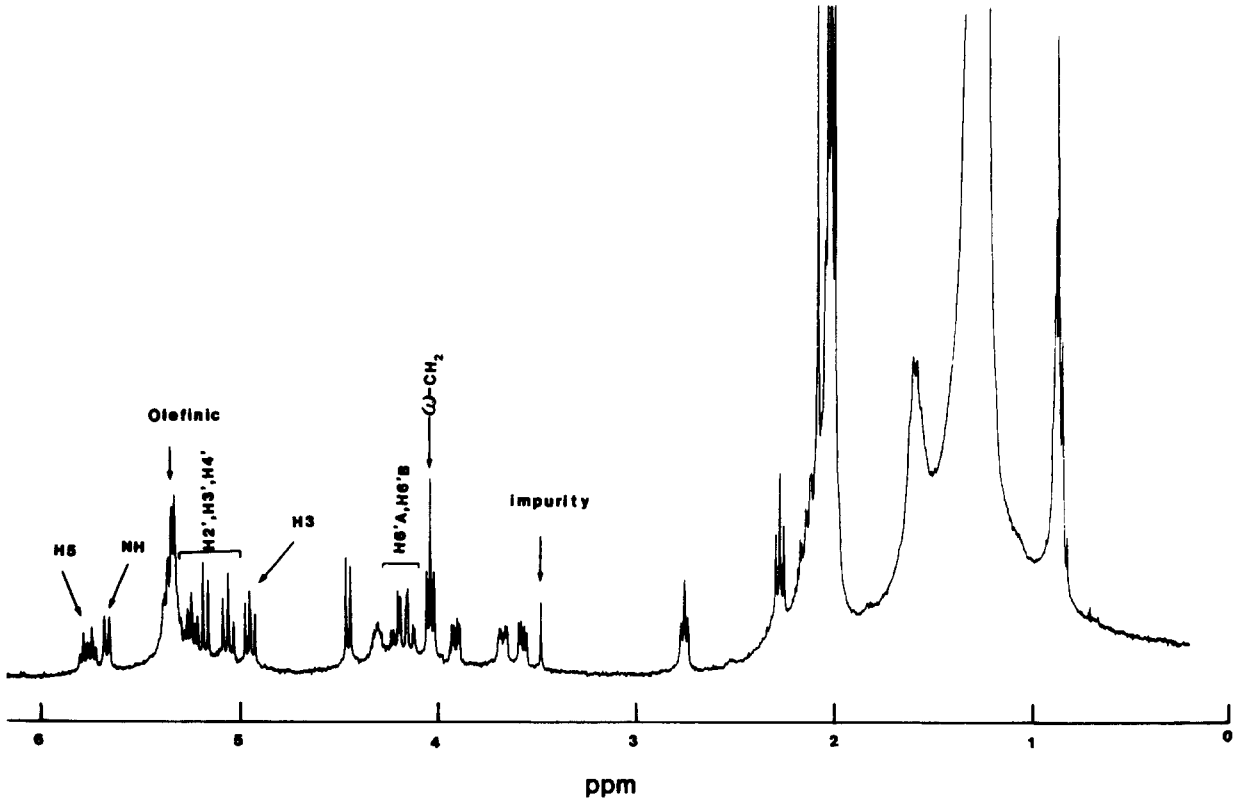

Fig. 3. 360 MHz proton magnetic resonance spectrum of acetylated acylglycosylceramide.

al, when included at low concentration in a liposome-forming mixture of phospholipid and cholesterol, causes the liposomes to become flattened and stacked like coins (17). A similar action was proposed for the observed formation of the stacks of disks that constitute the lamellar granules of epidermal granular cells (7). However, the supposed flattening of vesicles in epidermal cells must now be attributed to attraction and bridging of the internal faces of such vesicles by the linoleate attached to the ω-hydroxyacids of the acylglucosylceramides. The glucose moieties would thereby be arrayed on the external faces of the flattened vesicles, and attraction between vesicles might result both from the surface morphology and from the interaction of glucose molecules. This formulation of vesicle structure would explain the ultrastructure of lamellar granules as seen by electron microscopy, where the exterior surfaces of the disks unite to form dense bands and the apposed internal surfaces are seen as fine lines. The fine lines would result from reactions involving ester and hydroxyl groups, while the dense lines would result from the reactions of aggregated glucose units. A corollary of this structural formulation is that removal of the glucose groups should occur at the same stage of epidermal differentiation as dispersion of the lamellar disks into the broad intercellular lamellae of the stratum corneum, as appears to be the case. The observed adhesion of lipid lamellae in multiple bilayers would continue to be promoted by acylceramide molecules, whose 30-carbon hydroxyacids could span one bilayer and whose linoleate groups would be embedded in adjacent bilayers. A specific role for linoleate in this arrangement would be based on the special relationship with cholesterol, in which the epidermal lipids also abound.

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