A new 6-hydroxy-4-sphingenine-containing ceramide in human skin

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Abstract A new ceramide consisting of 6-hydroxysphingosine linked to a non-hydroxyacid was found in human epidermal lipid. This ceramide was sought because its fatty acid and sphingoid moieties are present in other combinations in human epidermal ceramides. To isolate the new ceramide, the mixture of ceramides in human epidermal lipid was first separated into fractions by thin-layer chromatography (TLC), and then each fraction was further purified by TLC after acetylation of all hydroxyl groups. TLC after acetylation revealed that one of the fractions isolated in the first TLC step contained two components, namely, the ceramide consisting of sphingosine linked to an α-hydroxyacid and an unknown ceramide. The new ceramide constituted about 9% of the total ceramides, and was shown by NMR spectroscopy to be N-acyl-6-hydroxysphingosine.—Stewart, M. E., and D. T. Downing. **A new 6-hydroxy-4-sphingenine-containing ceramide in human skin. *J. Lipid Res.* 1999.** 40: **1434–1439.**

Supplementary key words NMR • α-hydroxyacids • epidermal lipids

A ceramide is a combination of a fatty acid and a sphingoid base, joined by an amide bond between the carboxyl group of the fatty acid and the amino group of the base. In classic textbook ceramides, the fatty acyl moiety is either non-hydroxylated or α-hydroxylated, while the sphingoid moiety is sphingosine (including both sphingenine and sphinganine) or phytosphingosine (4-hydroxysphinganine). Mammalian stratum corneum, however, contains more classes of ceramides than the four made possible by various combinations of the classical building blocks. Pig stratum corneum, for example, contains two ceramides that have unusually long-chain ω-hydroxyacids. One of these ω-hydroxyceramides is solvent-extractable and has an additional fatty acid esterified to the ω-hydroxyl group (1), while the other ω-hydroxyceramide is protein-bound (2).

Human stratum corneum ceramides were initially thought to be similar to those in pig (3), except for the existence of a second protein-bound ceramide having an ω-hydroxyacid and an additional hydroxyl group on the sphingoid base (4). When we found that this second protein-bound ceramide contains a previously unknown sphingoid moiety, 6-hydroxy-4-sphingenine (6-hydroxysphingosine), it led to the discovery of two additional extractable ceramides in human epidermis (5). One of these previously reported extractable ceramides is analogous to the bound ceramide in consisting of 6-hydroxysphingosine bound to an ω-hydroxyacid, except that the ω-hydroxyl group is esterified to a fatty acid rather than to protein. The other extractable ceramide that we described consists of 6-hydroxysphingosine bound to an α-hydroxyacid. We now report the existence in human epidermis of a third extractable 6-hydroxysphingosine-based ceramide in which the fatty acid is not hydroxylated. This ceramide was overlooked in previous studies because it has chromatographic behavior very similar to the ceramide that contains sphingosine and an α-hydroxyacid.

MATERIAL AND METHODS

Solvents and thin-layer chromatography plates

Chloroform and methanol were "Optima" grade (Fisher Scientific, Pittsburgh, PA). USP ethanol was 190 proof (Pharmco, Brookfield, CT). Deuterated water was purchased from Aldrich Chemical Co. (Milwaukee, WI), and deuterated chloroform from Aldrich and Cambridge Isotope Laboratories, Inc. (Andover, MA). Thin-layer chromatography (TLC) plates, 20 × 20 cm and 20 × 5 cm, precoated with a 0.25 mm layer of silica gel with a $CaSO_4$ binder (Adsorbosil Plus 1), were purchased from Alltech Associates (Deerfield, IL). They were prepared for use by developing them to the top with chloroform–methanol 2:1 and allowing them to dry thoroughly. For analytical TLC, the plates were scored into 6-mm-wide lanes (6).

Collection of lipid and isolation of ceramides

In order to avoid skin surface contamination from the environment, ceramides were isolated from lipids extracted from the discarded contents of epidermal cysts that had been excised for clinical reasons in the Department of Dermatology, University of Iowa. Permission to collect the cyst material was granted by the University of Iowa Institutional Review Board for human subjects.

Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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The contents of each cyst were covered with 5 ml of chloroform–methanol 2:1 and allowed to extract overnight at room temperature in a culture tube having a Teflon-lined cap. After extraction, 1 ml of water was added, the contents of the tube were mixed thoroughly, and the phases were separated by centrifugation. The chloroform layer was removed with a Pasteur pipette, filtered through a plug of lipid-free cotton into a tared culture tube, evaporated to dryness, weighed, and redissolved at a concentration of 20 mg/ml. For comparison purposes, epidermal lipid was also obtained by pouring ethanol over the legs of an informed volunteer. This procedure was approved by the Institutional Review Board. The ethanol was evaporated using a rotary evaporator and the residue was redissolved in chloroform–methanol 2:1. The solution was filtered and the concentration was adjusted as for the cyst lipid.

Isolation of ceramides

Two ceramides were isolated: the new ceramide and the most polar ceramide, which is known to consist of 6-hydroxysphingosine combined with an α-hydroxy fatty acid (5). The latter was needed as a reference ceramide for NMR analysis. The three-step procedure involved TLC of the cyst lipid, acetylation of the recovered ceramides, and TLC of the ceramide acetates. For the first TLC step, 10 mg of lipid was applied to 20 × 20 cm plates in a band 3 cm from the bottom, then the chromatogram was developed twice to the top with chloroform–methanol–acetic acid 190:9:1. To visualize the lipid bands, the chromatogram was sprayed with an ethanolic solution of 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt, 100 mg/l (Eastman Kodak Co., Rochester, NY), allowed to dry, and examined under UV light. Sometimes a third development was necessary to completely resolve the two most polar ceramides. The silica gel in the areas corresponding to the most polar and third most polar bands was scraped from the plate, placed in small glass columns, and eluted with chloroform–methanol 2:1 to recover the lipid. Lipid fractions from several plates were combined to obtain about 1 mg of each fraction. To acetylate the ceramides, the samples were dissolved in a 1:1 mixture of pyridine and acetic anhydride and allowed to react for 2 h at room temperature. The reagents were then removed by evaporation under a stream of nitrogen and the acetylated ceramides were subjected to a second preparative TLC step, using one development with chloroform–ethanol 100:2. This procedure separated the third most polar fraction into two well-resolved bands, the lower of which contained the new ceramide in acetylated form. Acetylation of the ceramides was also useful for the NMR analysis because it eliminated irrelevant exchangeable hydroxyl hydrogens that otherwise might complicate the spectra, and because the acetylated ceramides were soluble in pure deuterated chloroform.

NMR

The purified ceramide acetates were dissolved in 0.5 ml of deuterated chloroform and mixed with 1 ml of deuterated water. The mixture was allowed to stand at room temperature overnight to exchange the amide hydrogens (which otherwise tend to appear at variable and inconvenient positions in the NMR spectra). The mixtures were centrifuged and the chloroform layers were transferred to NMR tubes. One-dimensional proton NMR spectra were obtained at 600 MHz using a Bruker AMX-600 spectrometer.

Estimation of the amount of the new ceramide

Estimating the percentage of the new ceramide in the total ceramides entailed determining the size of the fraction that contained both the new ceramide and the ceramide composed of sphingosine and an α-hydroxyacid (the third most polar band) as a percentage of total ceramides, and then assessing the relative amounts of the two ceramides in that fraction. The percentage of the third most polar band was measured by quantitative TLC analysis of the lipid extracted from six different cysts. Aliquots of each sample, containing 60 μg of lipid, were applied to separate lanes on scored TLC plates. The chromatogram was developed with chloroform–methanol–acetic acid 190:9:1 once to the top. It was then charred by spraying it with 50% aqueous sulfuric acid and heating it slowly to 220°C on a hotplate. The area of the chromatogram that contained the ceramides was scanned with a Shimadzu CS9000U photodensitometer (Shimadzu Corp., Kyoto, Japan) run in reflectance mode and using linearizer setting 2, which incorporates the correction factor suggested by Downing and Stranieri (6). To estimate the percentage of the new ceramide in the mixture, fractions containing the mixture were prepared and acetylated as explained in the paragraph above that describes the isolation and derivatization of ceramides. The percentages of the two ceramide acetates in their mixture were then estimated by quantitative TLC as for the total lipid, except that the developing solvent was chloroform–ethanol 100:2.

RESULTS

Overlap of the new ceramide with an α-hydroxyacid-containing ceramide on TLC

As can be seen in **Fig. 1**, the TLC mobility of the new ceramide (6-hydroxysphingosine combined with a non-hydroxy fatty acid) was very similar to that of a previously known ceramide consisting of sphingosine combined with an α-hydroxy fatty acid. Even multiple developments did not separate the two ceramides in their native form. Comparison with the complete mixture of stratum corneum lipids shows that the mixture of the two inseparable ceramides constitutes the third most polar of the ceramide bands.

NMR spectrum of the new ceramide compared with the spectrum of a 6-hydroxysphingosine-containing ceramide of known structure

The complete NMR spectrum of the new ceramide (in fully acetylated form) is shown in **Fig. 2a**, and, for comparison, the spectrum of the ceramide consisting of 6-hydroxysphingosine combined with an α-hydroxy fatty acid (also fully acetylated) is shown in Fig. 2b. The similarities in the two spectra indicate that both ceramides contain 6-hydroxysphingosine, while the differences result from the absence of an acetoxy group on carbon-2 of the fatty acid in the new ceramide. In the spectrum of the reference ceramide, there is a peak representing the three hydrogens of the carbon-2 acetoxy group (Fig 2b, peak 4), that is absent in the spectrum of the new ceramide (Fig. 2a). The peak for the single hydrogen on carbon-2 of the α-hydroxy fatty acid of the reference ceramide (the 2′ hydrogen) can be better seen in the partial spectrum shown in **Fig. 3b**. The peak for this hydrogen was moved down-field to 5.1 ppm by the presence of the acetoxy group on the same carbon. The peak for the two 2′ hydrogens of the new ceramide does not appear in this portion of the spectrum.

The structure of the 6-hydroxysphingosine moiety (5) was based primarily on the NMR signals for the hydrogens on sphingoid carbon-5 and carbon-6. As can be seen in

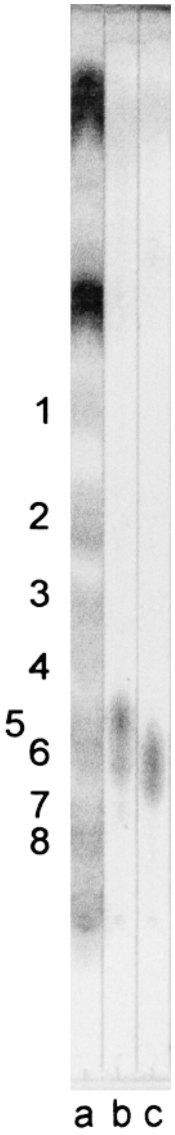

Fig. 1. Similar TLC mobility of human ceramides 5 and 6. A total lipid extract (lane a) was obtained in vivo from leg skin. Ceramide 5 (lane b) and ceramide 6 (lane 3) were isolated as one fraction from epidermal cyst lipid, separated from each other in acetylated form, and then converted back to the native form by mild alkaline hydrolysis (1 N KOH in 95% methanol for 60 min at 50°C). The chromatogram was developed once to the top in chloroform–methanol–acetic acid 190:9:1 and charred by spraying with 50% aqueous sulfuric acid and then heating slowly to 220° on an aluminum slab placed on a hotplate. See Table 1 for the structures of the ceramides.

both of the spectra in Fig. 3, the peak for the hydrogen on carbon-6 is an unresolved doublet of triplets because of triplet splitting by the pair of hydrogens on carbon-7 and doublet splitting by the single hydrogen on carbon-5. The NMR signal for the carbon-5 hydrogen is a doublet of doublets because of splitting by the single hydrogen on carbon-6 and by the single hydrogen on carbon-4. In the absence of the 6-hydroxy group, the carbon-5 hydrogen gives a doublet of triplets because of triplet splitting by two hydrogens on carbon-6, plus doublet splitting by the single hydrogen on carbon-4. It is interesting to note that in the spectrum of the ceramide having an α-hydroxy fatty acid (Fig. 2b and Fig. 3b), the peak for the hydrogen on carbon-6 appears close to the peak for the hydrogen on carbon-2 of the fatty acid moiety because of similar chemical environments, i.e., both share a carbon with an acetoxy group and both are adjacent to a double bond.

Percentage of the new ceramide

The average percentage of the new ceramide in the total ceramides from six samples of epidermal cyst lipid was 9.2% ± 2.1% SD. The ceramide containing sphingosine combined with an α-hydroxyacid, with which the new ceramide co-chromatographs, constituted 1.8% ± 1.3% SD.

DISCUSSION

The present study has demonstrated the presence in human stratum corneum lipid of a previously unrecognized ceramide consisting of 6-hydroxysphingosine in amide linkage with a non-hydroxy fatty acid. The new ceramide necessitates proposing a new numbering system for the human ceramides, as shown in **Table 1**. In the original report identifying the extractable ceramides of pig skin, the ceramides were numbered in order of increasing polarity from 1 to 6a and 6b (1). However, ceramides 4 and 5 are chemically identical except for the chain lengths of the fatty acid moieties, and ceramide 6a apparently was an artifact (7), so there actually are five classes of extractable ceramides in pig stratum corneum, four textbook ceramides plus ceramide 1. With the original discovery of 6-hydroxysphingosine-based ceramides in human skin, the human extractable ceramides were renumbered 1 through 7, with the new ones being ceramide 4, which is analogous to ceramide 1 in having an acylated ω-hydroxyacid, and ceramide 7, which has an α-hydroxyacid (5). The ceramide referred to as ceramide 4/5 in pig was renumbered ceramide 5. The two bound ceramides in human skin were distinguished as ceramide A and ceramide B. Otherwise the corresponding human and pig ceramides had the same designations. Unfortunately, the polarity of the new ceramide requires that it be named human ceramide 6, if the TLC order is to be maintained. Therefore the human ceramides formerly referred as 6 and 7 were renamed ceramide 7 and ceramide 8.

The new ceramide 6 could not be chromatographically purified in its native state because of its very similar polarity to the ceramide consisting of sphingosine linked with an α-hydroxy fatty acid (ceramide 5), but the two ceramides were easily separated by TLC after acetylation of all hydroxyl groups. The separation occurs because acetylation appears to nullify the effect of an α-hydroxyl group on polarity. That is, ceramides that differ only in the presence or absence of an α-hydroxyl group have similar chromatographic mobility when converted to their fully acetylated forms. The reason for this is not clear, but possibly involves hydrogen bonding of the amide hydrogen with the carbonyl of the acetoxy group on the fatty acid α-carbon. This would reduce the ability of the amide group

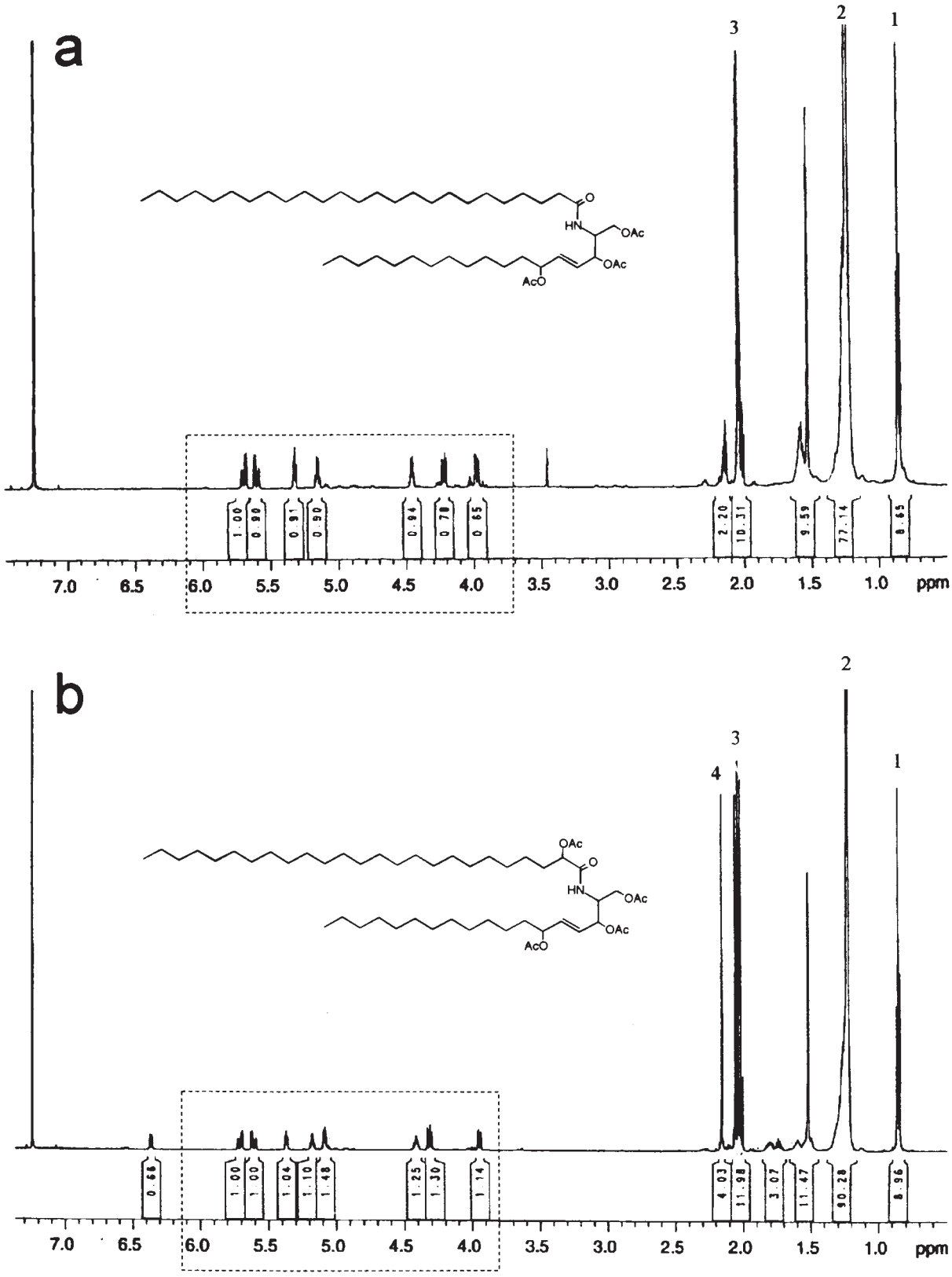

Fig. 2. The complete NMR spectra of fully acetylated ceramides 6 and 8, showing the similarities resulting from the presence of 6-hydroxysphingosine in both. The spectrum (a) of ceramide 6 is similar to that (b) of ceramide 8 except for the absence of peaks attributable to the functional group on the α-carbon of the fatty acid of ceramide 8. The hydrogens of the acetoxy group on the fatty acid of ceramide 8 produce a peak (4) slightly separated from the peak (3) for the acetoxy groups on the sphingosine. Other prominent peaks are attributable to methyl groups (1) and methylene groups (2). The intense peaks at 7.2 ppm are due to chloroform, which was used as an internal chemical shift standard. The peaks at 1.5 and 3.4 ppm are unknown impurities. The numbers that are oriented vertically below the peaks are the number of hydrogens per molecule, as estimated by integration of the peak areas. The portions of spectra enclosed in the boxes are shown enlarged in Fig. 3.

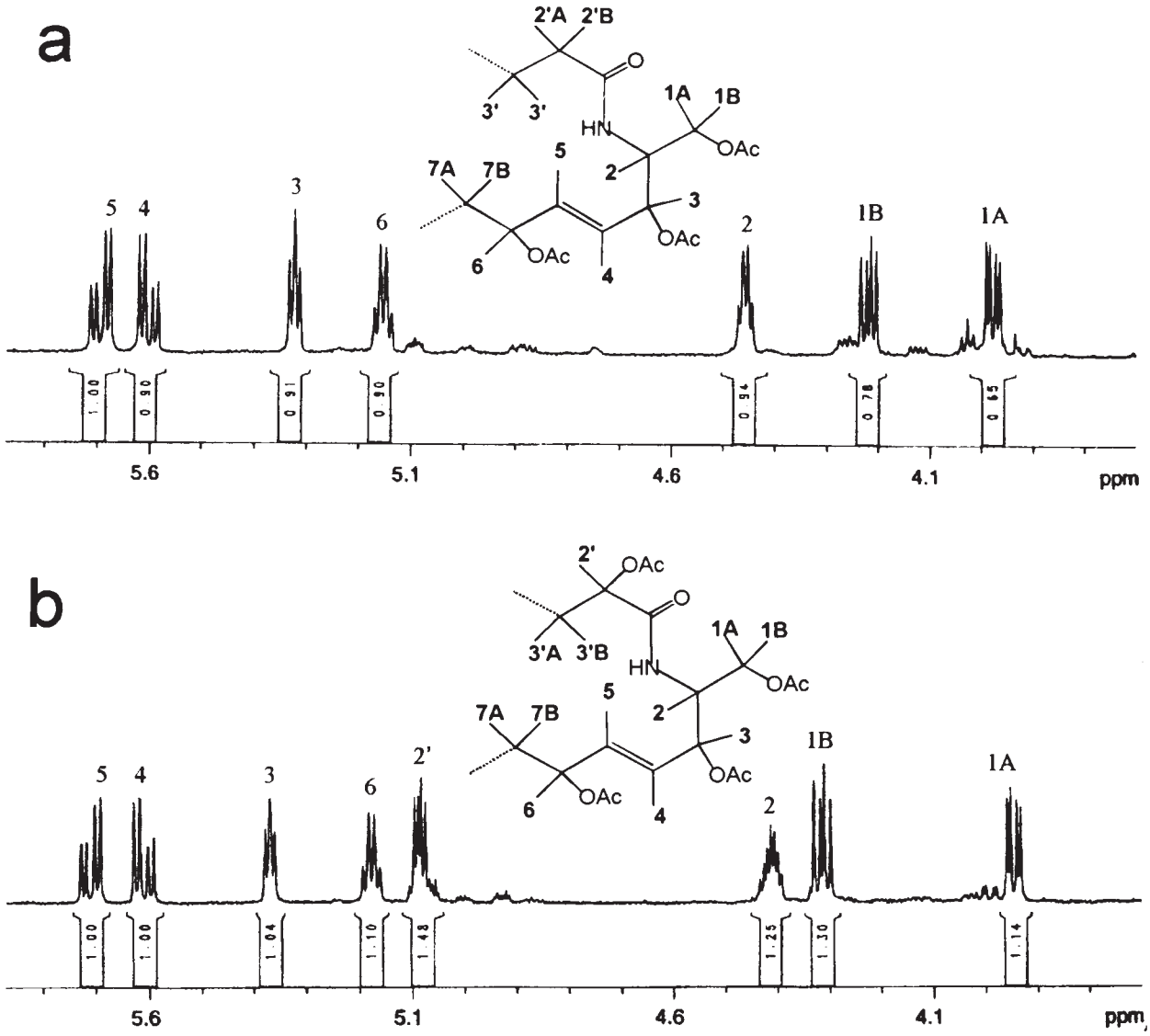

Fig. 3. Partial NMR spectra, showing that ceramide 6 has an non-hydroxy acid and ceramide 8 has an α-hydroxyacid. The peak in spectrum b for the single 2′ hydrogen in ceramide 8 appears in this region of the spectrum (at 5.1 ppm) because of the polar environment provided by the acetoxy group on the same carbon, while the peak in spectrum a for the pair of 2′ hydrogens in ceramide 6 is upfield of this region. See also Results.

to interact with the TLC adsorbent, which may make the acetylated ceramide appear to have fewer polar groups than it actually does have. In any case, acetylation renders ceramide 5, with a hydroxyl group on carbon 2 of the fatty acid, much less polar than the acetylated form of the ceramide 6, with a hydroxyl group on carbon 6 of the sphingoid moiety, even though both have the same number of polar groups.

TABLE 1. Renumbered human stratum corneum ceramides (pig numbering in parentheses)

	Fatty Acid Type			
Sphingosine Type	Non-OH Fatty Acid	α-OH Fatty Acid	Acylated ω-OH Fatty Acid	Protein-Bound ω-OH Fatty Acid
Sphingosine	2 (2)	5 (4,5)	1 (1)	A (A)
Phytosphingosine	3 (3)	7 (6)	not found	not found
6-OH-sphingosine	6	8	4	B

Whether the new ceramide or the previously reported 6-hydroxysphingosine-containing ceramides (5) have specific functions in human stratum corneum has yet to be determined. Ceramides, along with fatty acids and cholesterol (8), form intercellular lamellae between the corneocytes. The bound ceramides, which are attached to the exterior of the corneocytes, also contribute to the lamellae (9). These lamellae constitute the epidermal water barrier. The only ceramide for which a specific biophysical role has been demonstrated is the least polar ceramide (ceramide 1). This unusually long ceramide is necessary to replicate the 13-nm repeat in the X-ray diffraction pattern of native stratum corneum (10, 11), which it may do by binding three bilayers together (9). The similarity of the lamellar ultrastructure in pigs and humans (9) argues

against an important role for 6-hydroxysphingosine-based ceramides as pigs lack these ceramides. On the other hand, Ponec et al. (12) have reported that human epidermal cells in culture are deficient in some of the more polar, hydroxylated ceramides, and cannot form normal lamellae. Both defects are alleviated by the inclusion of vitamin C in the culture medium (12). This result suggests that that 6-hydroxylation could be important insofar as it increases the content of polar ceramides. However, the hydroxylation reaction(s) that are dependent on vitamin C have not been identified, and the 4-hydroxylation of sphingosine (to give phytosphingosine), as well as the α-hydroxylation of fatty acids, also are required for the formation of polar ceramides in skin. If the α-hydroxylation of fatty acids in skin requires vitamin C, skin may differ from brain. Extensive studies by Shimeno, Wali, and Kishimoto (13) and Shigematsu, Hisanari, and Kishimoto (14) on the soluble factors required for the conversion of lignoceroyl Co A to an α-hydroxyacid-containing ceramide in rat brain microsomes have not revealed a requirement for vitamin C.

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