# 6-Hydroxy-4-sphingenine in human epidermal ceramides

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**Abstract** The solvent-extractable lipids of human epidermal stratum corneum consist predominantly of ceramides. In addition, two non-extractable ceramides are chemically bound to the stratum corneum protein. One of the bound ceramides, constituting 50% of the bound lipids, was previously shown to consist of very long chain ω-hydroxyacids in amide linkage with sphingosine. The second bound ceramide, which forms 25% of the bound lipids, was shown to contain the same hydroxyacids, but the sphingoid base was neither sphingosine nor phytosphingosine. In the present study, the undefined bound ceramide was shown by NMR and chemical procedures to be the ω-hydroxyacid derivative of a new base, 6-hydroxy-4-sphingenine. In addition, a ceramide previously known to constitute 25% of the extractable human stratum corneum ceramides has been found to contain the same novel sphingoid base, amide-linked to long-chain α-hydroxyacids. Finally, a new acylceramide has been isolated and identified that consists of very long chain ω-hydroxyacids in amide linkage with the novel sphingolipid, with fatty acids esterified with the terminal hydroxyl group of the hydroxyacid.—**Robson, K. J., M. E. Stewart, S. Michelsen, N. D. Lazo, and D. T. Downing.** 6-Hydroxy-4-sphingenine in human epidermal ceramides. *J. Lipid Res.* 1994. **35:** 2060–2068.

**Supplementary key words** corneocyte envelope • stratum corneum • acylceramides • phytosphingosine • glucosylceramides • NMR

The barrier to water permeation in mammalian epidermis consists of multiple lipid lamellae between the cells of the stratum corneum (1, 2), together with a covalently bound lipid envelope covering the entire surface of each cornified cell (3, 4). In porcine epidermis, the bound ceramides of the corneocyte envelope consist of a single type: very long chain ($C_{30}$–$C_{34}$) ω-hydroxyacids in amide linkage with $C_{16}$–$C_{22}$ sphingosines (5). The same hydroxyceramide structure was reported to form 50% of the bound stratum corneum lipids of human epidermis, but a further 25% was contributed by a second ceramide having similar ω-hydroxyacids in amide linkage with a base that was neither sphingosine nor phytosphingosine (6). The present study aimed to determine the structure of the unknown sphingoid base. In addition, a search was made for nonbound ceramides that might contain the same unknown base, in analogy with the occurrence in stratum corneum of both bound and nonbound ceramides formed from ω-hydroxyacids and sphingosine. As a result, three ceramides containing the novel sphingoid base 6-hydroxy-4-sphingenine were discovered among the lipids of human epidermal stratum corneum.

## METHODS

### Materials

Chloroform and methanol were HPLC grade and were redistilled immediately prior to use. Deuterochloroform, deuteromethanol, and deuterium oxide were of the highest isotopic purity available from Aldrich Chemical Co (Milwaukee, WI). TLC plates (20 × 20 cm and 5 × 20 cm) coated with silica gel were obtained from Alltech Associates (Deerfield, IL), catalog #16330 and 16331, and were cleaned of lipid impurities before use by development in chloroform–methanol–acetic acid 200:100:3. Ceramides previously isolated and characterized in this laboratory were used as chromatographic standards. A specimen of authentic ω-hydroxyacids having 30 to 34 carbon atoms was prepared by hydrolysis of the giant ring lactones from donkey sebum (7). Authentic D-sphingosine was purchased from Sigma Chemical Company (St. Louis, MO).

### Human stratum corneum lipids

To minimize the possibility of contamination, stratum corneum lipids were obtained from several sources: *a)* from a man who had not used soaps, detergents, or lotions on his

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

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limbs or torso for more than a year; *b)* from desquamated corneocytes collected from material (vernix caseosa) scraped from the skin of newborn babies, avoiding blood and meconium; and *c)* from adult human subjects who had not used lotions or topical medications during the preceeding several weeks.

Extractable stratum corneum lipids were obtained from the adults by pouring ethanol over their arms and legs and collecting the run-off in a stainless-steel basin. To obtain bound stratum corneum lipids, glass microscope slides coated with cyanoacrylate glue were applied to sites on the arms and legs of subject (*a*)) and then peeled off after the glue had polymerized. The glue was then dissolved in dimethylformamide, which was discarded. After several washes with dimethylformamide and then with chloroform–methanol 2:1, the sheets of statum corneum were subjected to mild alkaline hydrolysis with 1 N NaOH in methanol–water 19:1 at 45°C for 30 min. Two volumes of chloroform was then added and stirred. After 15 min, the undissolved tissue was discarded and the remaining solution was shaken with a one-fifth volume of water. The chloroform layer was then separated, washed with water, and evaporated.

Corneocytes were obtained from the vernix caseosa specimens by suspension of the material in chloroform–methanol 2:1 and filtration through delipidized cotton. The collected cells were then washed several times with chloroform–methanol before being subjected to mild alkaline hydrolysis and isolation of the liberated lipids as described above.

### Analysis and fractionation of the epidermal lipids

For analytical TLC, the ceramides from stratum corneum were resolved by one or more developments of the thin-layer chromatograms in a mixture of chloroform–methanol–acetic acid 190:9:1, followed by one development with ether–acetic acid 100:1. For visualization of analytical chromatograms, the developed plates were sprayed with 50% sulfuric acid and then heated to 220°C on a hot plate. For preparative TLC, the chromatograms were first developed with ether–acetic acid 100:1 to move nonpolar lipids to the top of the plate. The ceramides were then resolved by two developments with chloroform–methanol–acetic acid 190:9:1. The chromatograms were sprayed with 0.1% dichlorofluorescein in ethanol, dried, and viewed under UV. The fluorescent bands were scraped into glass tubes and the lipids were eluted with chloroform–methanol 2:1. After the eluates were evaporated and redissolved in chloroform–methanol, fluorescein was removed by passage of the solution through a 1-in column of magnesium hydroxide contained in a cotton-plugged Pasteur pipette. These eluates were then assayed by analytical TLC.

### Chemical analyses

For identification of the ceramides that contain phytosphingosine, TLC plates containing sodium arsenite were prepared in the laboratory (8). Aliquots of the isolated ceramide fractions were applied to the plates in separate lanes and the chromatograms were developed in chloroform–methanol 95:5.

To identify ester linkages, each ceramide fraction was subjected to mild alkaline hydrolysis. For this, a solution of a ceramide in chloroform–methanol 2:1 was heated with an equal volume of 1 N NaOH in methanol–water 19:1 at 45°C for 1 h. An aliquot of the resulting solution was then analyzed by TLC.

For identification of amide-linked acids, each ceramide was hydrolyzed at 80°C in 1 ml of a mixture prepared from 8.6 ml of concentrated HCl, 9.6 ml of water, diluted to 100 ml with methanol. After 18 h the solution was evaporated to dryness with a stream of $N_2$. The residue was treated with 1 ml of 10% $BCl_3$ in methanol at 50°C for 10 min to ensure that all of the fatty acids were in the form of methyl esters. The solution was again evaporated to dryness and the residue was dissolved in chloroform–methanol. Aliquots of this solution were analyzed by TLC, using the methyl esters of stearic acid, DL-$\alpha$-hydroxypalmitic acid, and the $\omega$-hydroxyacids from donkey skin equolides as reference standards.

Hydrogenation of ethylenic bonds was performed by passing hydrogen through ceramide solutions in ether–methanol 2:1 for 20 min at 20°C, using Pt catalyst.

Acetylation of ceramides and sphingosine was carried out in acetic anhydride–pyridine for 2 h at 45°C. The reagents were then evaporated with a stream of nitrogen and the residual product was purified by TLC using chloroform–methanol 100:2 as developing solvent.

### NMR spectroscopy

Approximately 1 mM solutions of the sphingolipids in deuterochloroform–deuteromethanol 2:1 were used in obtaining proton NMR spectra in a 600-MHz spectrometer (Bruker AMX-600). NMR spectra were also obtained using higher concentrations of the fully acetylated sphingolipids in deuterochloroform, and also after shaking the deuterochloroform solutions with $D_2O$ to exchange the amide proton.

## RESULTS

### TLC resolution of the epidermal ceramides

**Figure 1** demonstrates the resolution of the ceramides liberated from protein binding in stratum corneum, and of the seven groups of ceramides recovered by surface extraction of the skin of four of the adult subjects.



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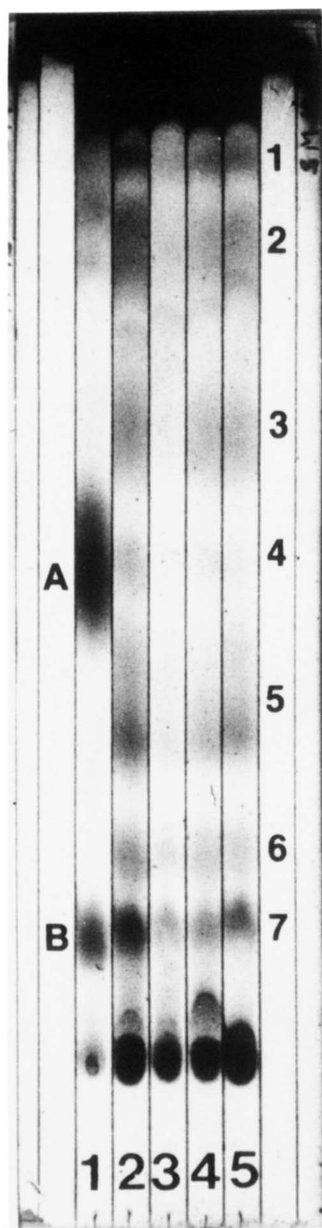


**Fig. 1.** Thin-layer chromatograms of human epidermal lipids, developed twice with chloroform–methanol–acetic acid 190:9:1 (to 20 cm) then with ether–acetic acid 100:1 (to 20 cm) and charred with 50% $H_2SO_4$. Lane 1: Lipids liberated from protein binding to stratum corneum of subject *(a)*: A: ceramide A; B: ceramide B; Lane 2: Extractable ceramides 1 through 7 from subject *(a)*: Lanes 3–5: Extractable ceramides 1 through 7 from three other adult males.

## Bound hydroxyceramides of human stratum corneum

The two ceramides isolated from the protein-bound lipids of adult human stratum corneum provided NMR spectra that were very similar except in the region characteristic of protons attached to ethylenic carbons (**Fig. 2**). Both spectra showed ethylenic proton resonances equivalent to a total of two protons per molecule. In the case of ceramide A, the position and pattern of the resonances was the same as for the 4,5-unsaturation of sphingosine, with a doublet of doublets for the proton on carbon-4, and an overlapping doublet of triplets for the carbon-5 proton. The four main C-4 proton resonances each showed additional fine triplet splitting due to allylic coupling with the pair of protons on C-6. Likewise, allylic coupling of the C-5 protons with the lone C-3 proton split each of the five main resonances into doublets.

For ceramide B, the carbon-5 proton resonances formed a doublet of doublets, each with doublet fine splitting. The doublet of doublets assigned to the C-4 proton also showed doublet fine splitting. These aspects of the ethylenic proton spectra, in Fig. 2, were interpreted as resulting from an additional hydroxyl group on carbon-6, so that the new sphingoid base is 6-hydroxy-4-sphingenine. The corresponding protein-bound ceramides from the vernix corneocytes showed spectra that were virtually identical with those from the adult stratum corneum. In each case, catalytic hydrogenation eliminated the signals in the ethylenic proton region of the spectra.

Both of the protein-bound ceramides from human stratum corneum were previously shown to be converted by strong acid hydrolysis to a mixture containing ω-hydroxyacids having 28 to 34 carbon atoms (6). The differences in the chromatographic and spectroscopic properties of the two ceramides, therefore, result from the additional hydroxyl group in the 6-position of the sphingoid base in the case of ceramide B. The lack of vicinal hydroxyl groups in the new hydroxysphingosine was also demonstrated by its unchanged mobility on TLC plates containing sodium arsenite.

## Free ceramides of human stratum corneum

As noted previously (9), only two of the nonbound ceramides of human stratum corneum showed the increased migration on sodium arsenite that is characteristic of vicinal hydroxyl groups, as in phytosphingosine. These were the ceramide that is the third-most mobile on standard silica gel TLC (ceramide 3), and the less abundant of the two components of the least-mobile ceramide (ceramide 6). It was previously postulated that the more abundant of the ceramide 6 components (hereinafter designated as ceramide 7) was not affected by arsenite, in spite of supposedly containing phytosphingosine, because of esterification of one of the vicinal hydroxyls by an α-hydroxyacid. However, it was shown recently that neither ceramide 6 nor ceramide 7 contains an ester linkage (10), an observation that was confirmed in the present study. In the present study, the NMR spectrum of ceramide 7 showed that its sphingoid base is the same as that in bound ceramide B, i.e., 6-hydroxy-4-sphingenine. Unlike bound ceramide B, ceramide 7 contains an α-hydroxyacid amide-linked to the sphingoid base. This was further confirmed by TLC of the fatty acid methyl esters obtained after acid hydrolysis of the ceramides. The NMR spec-



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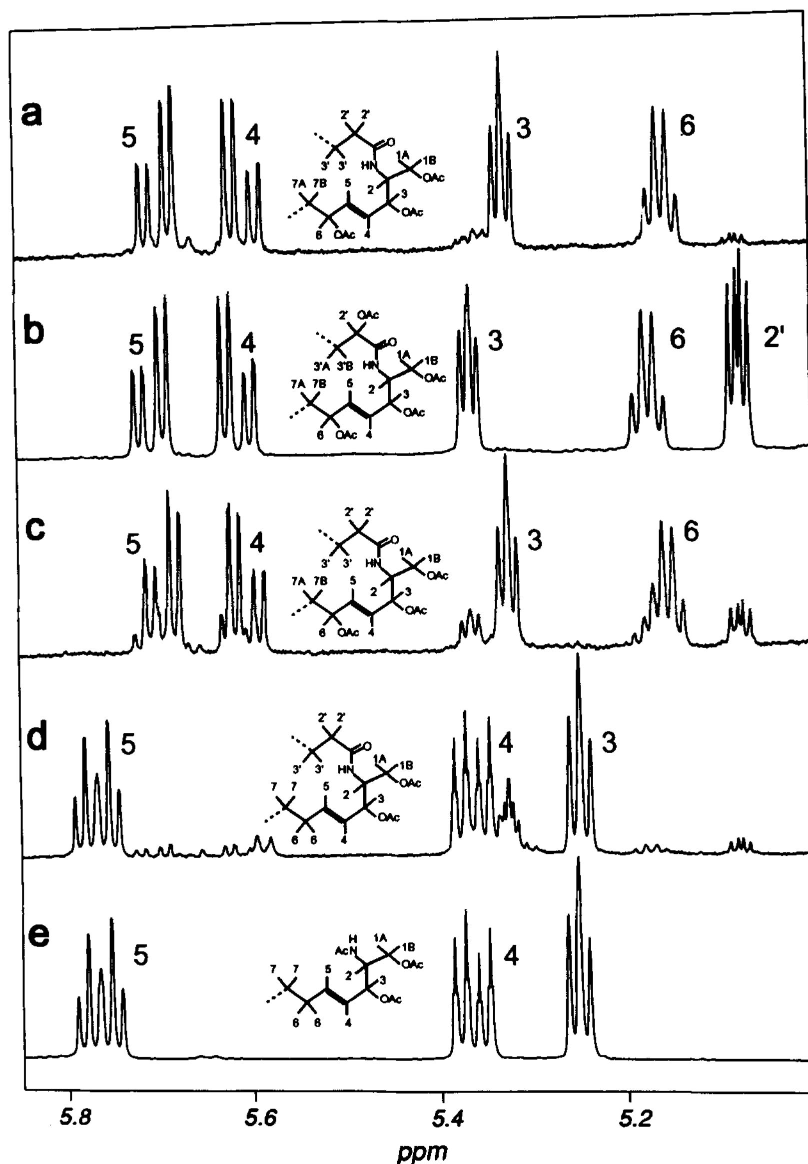


**Fig. 2.** ^{1}H NMR spectra of various sphingolipids after hydrolysis of ester groups (where necessary), peracetylation, and $D_2O$ exchange. (a) ceramide 4; (b) ceramide 7; (c) bound ceramide B; (d) bound ceramide A; (e) sphingosine.

trum, arsenite migration, and acidic hydrolysis of ceramide 6 confirmed the presence of α-hydroxyacids in amide linkage with phytosphingosine. The structures and complete NMR spectra of ceramide 7 and bound ceramide B are compared in **Figure 3.**

Among the series of ceramides from the extractable stratum corneum lipids was a previously overlooked component that migrated on TLC below ceramide 3, and is now designated as ceramide 4. Mild alkaline hydrolysis of ceramide 4 produced fatty acids and a less-mobile ceramide that had an NMR spectrum identical with that of bound ceramide B. TLC comparison with reference fatty acids showed that the acids liberated from ceramide 4 were in the 18-carbon range rather than longer-chain materials. The new acylceramide is therefore an analog of ceramide 1, differing only in the replacement of sphingosine by 6-hydroxysphingosine.

### Interpretations of the NMR spectra

Interpretations included correlation of the coupling constants for each of the protons, in addition to chemical shifts and numbers of protons assessed from integration. These data are summarized in **Tables 1 through 5,** in which peak areas obtained by electronic integration are



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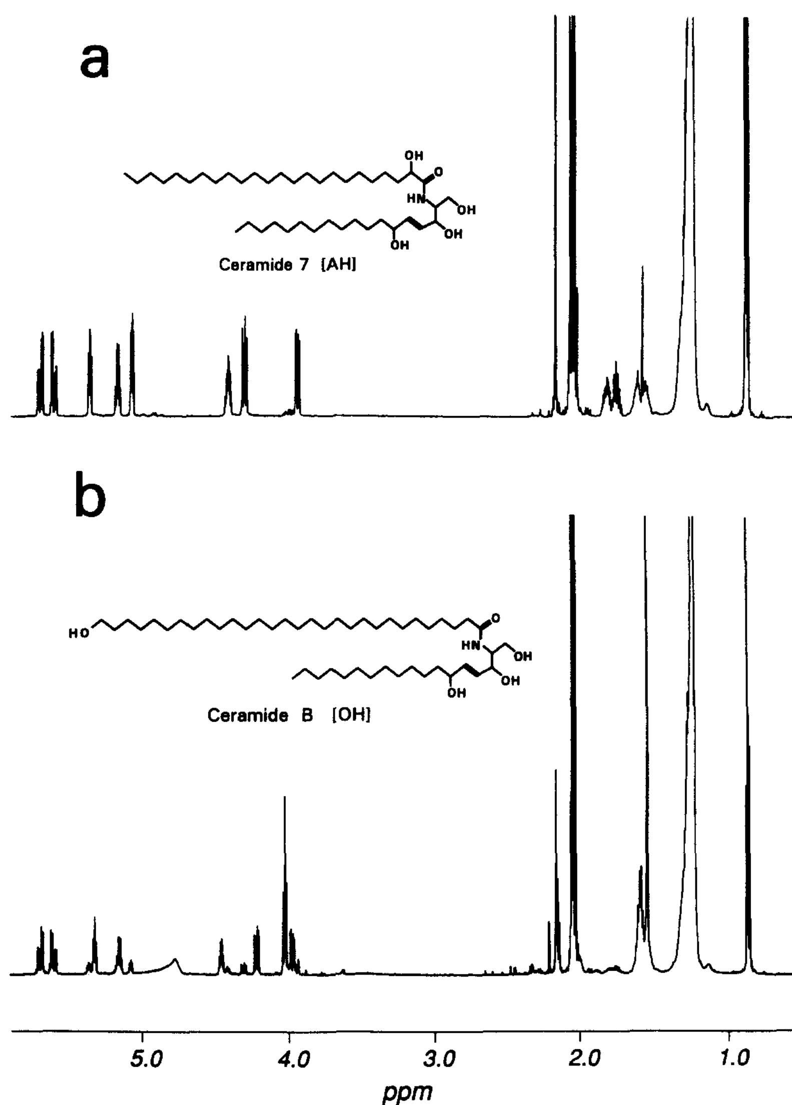


**Fig. 3.** Structures and complete $^{1}H$ NMR spectra of (a) ceramide 7; (b) bound ceramide B, both after acetylation and $D_2O$ exchange.

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TABLE 1. 600 MHz proton NMR data for authentic triacetyl-4-sphingenine

| Assigned | PPM | Integral | Multiplicity | Coupling Constants (Hz) | | |
|---|---|---|---|---|---|---|
| 1A | 4.015 | 1.00 | 2 × 2 | 1A,1B = 11.6 | 1A,2 = 3.9 | |
| 1B | 4.277 | 0.99 | 2 × 2 | 1B,1A = 11.6 | 1B,2 = 6.2 | |
| 2 | 4.400 | 1.00 | 2 × 2 × 2 | 2,1A = 4.0 | 2,1B = 6.1 | 2,3 = 5.9 |
| 3 | 5.250 | 1.01 | 2 × 2 | 3,2 = [6.0] | 3,4 = [7.4] | |
| 4 | 5.365 | 1.01 | 2 × 2 × 3 | 4,3 = 7.4 | 4,5 = 15.4 | 4,6 = 1.3 |
| 5 | 5.765 | 1.00 | 2 × 3 | 5,4 = 15.3 | 5,6 = 6.4 | |
| 6 | 2.006 | [2] | 2 × 2 × 3 | 6,5 = 6.9 | 6,7 = 7.1 | 6,4 = 1.6 |
| 7 | 1.321 | [2] | [3 × 3] | | | |
| $CH_2$ | 1.227 | [20] | m | | | |
| $CH_3$ | 0.854 | 3.03 | 3 | n,n-1 = 7.0 | | |
| $C-COCH_3$ | 2.046 | [3] | 1 | | | |
| $C-COCH_3$ | 2.039 | [3] | 1 | | | |
| $N-COCH_3$ | 1.956 | [3] | 1 | | | |

The assignments of chemical shift and coupling constants for triacetylsphingosine are consistent with those published previously for 220-MHz spectra (20).

TABLE 2. 600 MHz proton NMR data for human stratum corneum bound ceramide A triacetate

| Assigned | PPM | Integral | Multiplicity | Coupling Constants (Hz) | | |
|---|---|---|---|---|---|---|
| Base | | | | | | |
| 1A | 4.010 | [1] | 2 × 2 | 1A,1B = 11.6 | 1A,2 = 3.9 | |
| 1B | 4.277 | 0.99 | 2 × 2 | 1B,1A = 11.6 | 1B,2 = 6.1 | |
| 2 | 4.423 | 1.00 | 2 × 2 × 2 | 2,1A = 4.0 | 2,1B = 6.1 | 2,3 = 6.0 |
| 3 | 5.248 | 1.00 | 2 × 2 | 3,2 = [6.0] | 3,4 = [7.4] | |
| 4 | 5.365 | 1.01 | 2 × 2 × 3 | 4,3 = 7.4 | 4,5 = 15.4 | 4,6 = 1.3 |
| 5 | 5.767 | 1.01 | 2 × 3 | 5,4 = 15.3 | 5,6 = 6.6 | |
| 6 | 2.000 | [2] | [2 × 2 × 3] | 6,4 = [1.6] | 6,5 = [6.9] | 6,7 = [7.1] |
| 7 | 1.321 | [2] | [3 × 3] | | | |
| $CH_2$ | 1.227 | 87.5 | m | | | |
| $CH_3$ | 0.854 | 4.16 | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.043 | [3] | 1 | | | |
| C-$COCH_3$ | 2.036 | [3] | 1 | | | |
| Acid | | | | | | |
| 2 | 2.134 | 2.72 | 3 | 2,3 = 6.3 | | |
| n | 4.028 | [3] | 3 | n,n-1 = 6.8 | | |
| n-1 | 1.581 | [2] | m | | | |
| C-$COCH_3$ | 2.024 | [3] | 1 | | | |

shown to 3 significant figures, those obtained by graphical means are shown to 2 significant figures, and those inferred by inspection, but not measureable directly because of overlap with other protons, are shown in brackets. Likewise, multiplicities and coupling constants that were inferrable but not directly measurable also are shown in brackets.

In each of the spectra, the two protons on C-1 of sphingosine were nonequivalent and their NMR signals occurred at widely separated chemical shifts. This added to the complexity of the signals for the C-2 proton, which was further split by coupling with the amide proton. Proton exchange with $D_2O$ simplified the spectra by eliminating the coupling with the amide proton in most cases. However, for ceramide 7 tetraacetate, $D_2O$ exchange of the amide proton was largely unsuccessful, so that its coupling with the proton on C-2 was not eliminated (Table 5).

In the new sphingosine moiety, the two C-7 protons are also nonequivalent, although this was not immediately apparent because of coincidence of the signals with those from the n-1 protons of the ω-hydroxyacid, as in bound ceramide B (Table 3) and in ceramide 4 (Table 4). In ceramide 7, the nonequivalency of the C-7 protons was revealed because the α-hydroxyacid resonances did not obscure the region of interest. However, the C-3 protons of the α-hydroxyacid were also nonequivalent, providing additional complexity (Table 5). In each case of nonequiva-

TABLE 3. 600 MHz proton NMR data for human stratum corneum bound ceramide B tetraacetate

| Assigned | PPM | Integral | Multiplicity | Coupling Constants (Hz) | | |
|---|---|---|---|---|---|---|
| Base | | | | | | |
| 1A | 3.977 | 1.1 | 2 × 2 | 1A,1B = 11.6 | 1A,2 = 4.6 | |
| 1B | 4.217 | 1.0 | 2 × 2 | 1B,1A = 11.6 | 1B,2 = 6.4 | |
| 2 | 4.457 | 1.0 | 2 × 2 × 2 | 2,1A = 4.6 | 2,1B = 6.4 | 2,3 = 4.8 |
| 3 | 5.322 | 1.0 | 2 × 2[ × 2] | 3,2 = [4.8] | 3,4 = [6.6] | [3,5 = 0.7] |
| 4 | 5.603 | 1.0 | 2 × 2 × 2 | 4,3 = 6.6 | 4,5 = 15.6 | 4,6 = 0.9 |
| 5 | 5.693 | 1.0 | 2 × 2 × 2 | 5,4 = 15.6 | 5,6 = 6.6 | 5,3 = 0.7 |
| 6 | 5.153 | 1.0 | 2 × 3[ × 2] | 6,5 = 6.5 | 6,7 = 6.3 | [6,4 = 0.9] |
| 7 | 1.586 | [2] | [2 × 3] | | | |
| $CH_2$ | 1.227 | 74.0 | m | | | |
| $CH_3$ | 0.857 | 4.18 | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.037 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.042 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.050 | 3.0 | 1 | | | |
| Acid | | | | | | |
| 2 | 2.134 | 2.0 | 3 | 2,3 = 7.4 | | |
| n | 4.027 | 2.0 | 3 | n,n-1 = 6.8 | | |
| n-1 | 1.581 | [2] | m | | | |
| C-$COCH_3$ | 2.023 | 3.0 | 1 | | | |



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TABLE 4. 600 MHz proton NMR data for human ceramide 4 after deacylation and acetylation

| Assigned | PPM | Integral | Multiplicity | Coupling Constants (Hz) | | |
|---|---|---|---|---|---|---|
| Base | | | | | | |
| 1A | 3.979 | 1.1 | 2 × 2 | 1A,1B = 11.6 | 1A,2 = 4.5 | |
| 1B | 4.221 | 1.0 | 2 × 2 | 1B,1A = 11.6 | 1B,2 = 6.4 | |
| 2 | 4.457 | 1.01 | 2 × 2 × 2 | 2,1A = [4.5] | 2,1B = [6.3] | 2,3 = [5.3] |
| 3 | 5.325 | 1.0 | 2 × 2[ × 2] | 3,2 = [5.3] | 3,4 = [6.5] | [3,5 = 0.8] |
| 4 | 5.603 | 1.0 | 2 × 2 × 2 | 4,3 = 6.5 | 4,5 = 15.6 | 4,6 = 0.8 |
| 5 | 5.696 | 1.1 | 2 × 2 × 2 | 5,4 = 15.7 | 5,6 = 6.4 | 5,3 = 0.8 |
| 6 | 5.161 | 1.0 | 2 × 3[ × 2] | 6,5 = 6.4 | 6,7 = 6.5 | [6,7 = 0.8] |
| 7A | 1.550 | | [2 × 2 × 3] | | | |
| 7B | 1.600 | | [2 × 2 × 3] | | | |
| $CH_2$ | 1.230 | [84.5] | m | | | |
| $CH_3$ | 0.858 | [6.7] | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.051 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.042 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.037 | 3.0 | 1 | | | |
| Acid | | | | | | |
| 2 | 2.149 | 2.17 | m | | | |
| 3 | 1.587 | | m | | | |
| n | 4.028 | 2.0 | 3 | n,n-1 = 6.8 | | |
| n-1 | 1.581 | [2] | m | | | |
| $CH_2$ | 1.229 | [84.5] | m | | | |
| $CH_3$ | 0.857 | [6.7] | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.024 | 3.0 | 1 | | | |

lency of geminal methylene protons, the feature was consistent with the respective carbons being adjacent to chiral centers.

## DISCUSSION

In the living cells of the epidermis, sphingolipids are abundantly present in the form of glucosylceramides, which accumulate in specialized lamellar granules (11). In porcine epidermis, the most abundant of the glucosylceramides consists of ω-hydroxyacids amide-linked to glucosylsphingosine and bearing esterified fatty acids, predominantly linoleic acid, on the ω-hydroxyl group (12, 13). Upon cornification, the glucose is removed from the sphingolipids, producing the mixture of ceramides found in the stratum corneum. During epidermal cornification in nonhuman species, most of the acylceramide that con-

TABLE 5. 600 MHz proton NMR data for human stratum corneum ceramide 7 tetraacetate

| Assigned | PPM | Integral | Multiplicity | Coupling Constants (Hz) | | |
|---|---|---|---|---|---|---|
| Base | | | | | | |
| 1A | 3.943 | 1.00 | 2 × 2 | 1A,1B = 11.6 | 1A,2 = 4.3 | |
| 1B | 4.303 | 1.09 | 2 × 2 | 1B,1A = 11.6 | 1B,2 = 7.8 | |
| 2 | 4.415 | 1.08 | [2 × 2 × 2 × 2] | 2,1A = [4.3] | 2,1B = [7.8] | 2,3 = [4.4] 2,N = [8.9] |
| 3 | 5.362 | 1.02 | 2 × 2[ × 2] | 3,2 = [4.4] | 3,4 = [6.4] | [3,5 = 0.8] |
| 4 | 5.617 | 0.99 | 2 × 2 × 2 | 4,3 = 6.4 | 4,5 = 15.7 | 4,6 = 0.8 |
| 5 | 5.704 | 1.00 | 2 × 2 × 2 | 5,4 = 15.7 | 5,6 = 6.3 | 5,3 = 0.8 |
| 6 | 5.170 | 1.02 | 2 × 3[ × 2] | 6,5 = 6.5 | 6,7 = 6.4 | [6,4 = 0.8] |
| 7A | 1.550 | 1.0 | [2 × 2 × 3] | | | |
| 7B | 1.600 | 1.0 | [2 × 2 × 3] | | | |
| NH | 6.399 | 0.90 | 2 | N,2 = 8.9 | | |
| $CH_2$ | 1.229 | [71.8] | m | | | |
| $CH_3$ | 0.857 | [6.85-n'] | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.022 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.039 | 3.0 | 1 | | | |
| C-$COCH_3$ | 2.056 | 3.0 | 1 | | | |
| Acid | | | | | | |
| 2 | 5.075 | 1.15 | 2 × 2 | 2,3A = 7.6 | 2,3B = 4.5 | |
| 3A | 1.740 | 1.0 | [2 × 2 × 3] | | | |
| 3B | 1.800 | 1.0 | [2 × 2 × 3] | | | |
| $CH_3$ | 1.229 | [71.8] | m | | | |
| $CH_3$ | 0.857 | [6.85-n] | 3 | n,n-1 = 6.9 | | |
| C-$COCH_3$ | 2.156 | 3.0 | 1 | | | |

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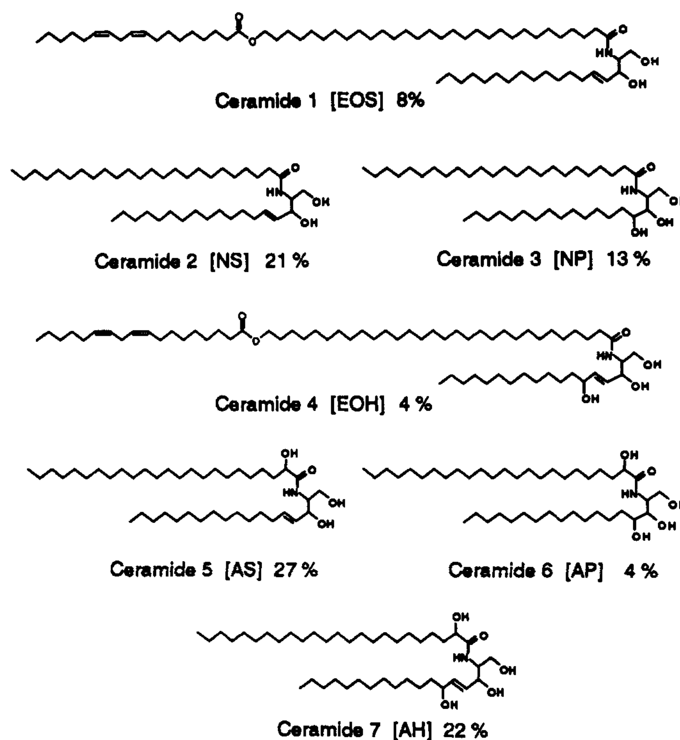
tains the ω-hydroxyacids (ceramide 1) becomes bound to protein, forming the lipid envelope of the corneocyte (4). However, a small proportion of the acylceramide remains free among the solvent-extractable lipids. By analogy with this process, the existence of a second protein-bound ceramide in human stratum corneum suggested that an analogous acylceramide, having an acylated ω-hydroxyacid bound to the novel trihydroxysphingosine, might be found among the unbound ceramides. This analog has now been identified as ceramide 4.

Non-O-acylated N-(ω-hydroxyacyl)sphingosine has not been identified in extractable stratum corneum lipids, nor apparently is the corresponding 6-hydroxysphingosine derivative present unacylated. Conversely, N-(α-hydroxyacyl)-sphingosine has only been found in the unacylated form, and it now seems that N-(α-hydroxyacyl)-6-hydroxysphingosine is also only present nonacylated. The complete array of structures now identifiable among the ceramides of human stratum corneum are set out in **Figure 4.**

In a series of studies by Hamanaka et al., the uncornified cells of human epidermis (14), and cultures of human keratinocytes (15, 16), were found to contain a variety of acylglucosylceramides. One of these was identical with the acylglucosylceramide previously identified in porcine viable epidermal cells (12), while a second was shown to be based on a novel trihydroxy compound, the structure of which was undefined. However, the published

**FREE CERAMIDES OF HUMAN STRATUM CORNEUM**




**PROTEIN BOUND CERAMIDES OF HUMAN STRATUM CORNEUM**

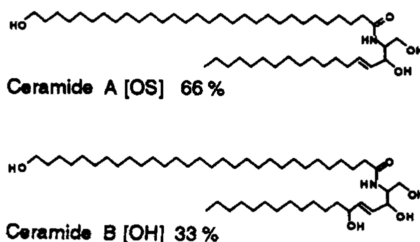



**Fig. 4.** Structures of the complete series of free and protein-bound ceramides of human stratum corneum.



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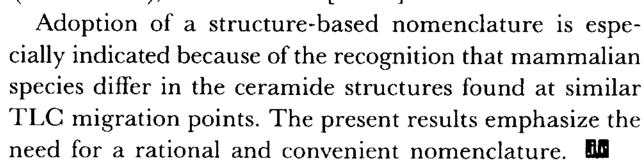
NMR spectra of Hamanaka et al. (14–16) strongly indicate that their new acylglucosylceramide is the metabolic precursor of the new solvent-extractable acylceramide of human stratum corneum, identified in the present study as N-($\omega$-acyloxyacyl)-6-hydroxy-4-sphingenine (ceramide 4) and of the protein-bound N-($\omega$-hydroxyacyl)-6-hydroxy-4-sphingenine (bound ceramide B). Likewise, the glycosylated precursor of the novel N-($\alpha$-hydroxyacyl)-6-hydroxy-4-sphingenine (ceramide 7), identified in the present study, might be found among the complex mixture of glycosphingolipids present in noncornified human keratinocytes (14–16).

In addition to the variety of ceramides and glycosylceramides detected in epidermis, both porcine (17) and human (18) stratum corneum have been shown to contain surprisingly high concentrations of free sphingosine. Additional free sphingoid bases have been detected in human skin (19) that presumably include the novel base identified in the present study.

Progress in elucidation of the epidermal ceramide structures has made possible a nomenclature based on their molecular structure instead of on their mobility on TLC. Previous authors (10) have suggested a system in which a ceramide containing only a nonhydroxylated fatty acid and sphingosine would be designated Cer[NS], an $\alpha$-hydroxyacid/phytosphingosine compound would be Cer[AP], the esterified $\omega$-hydroxyacid/sphingosine derivative would be Cer[EOS], and so on. Under this system, the bound ceramide A would become Cer[OS], and bound ceramide B would be Cer[OH] (for $\omega$-hydroxyacid/hydroxysphingosine). The new nonbound ceramide of human stratum corneum identified in the present study would be designated Cer[AH] for the $\alpha$-hydroxyacid/hydroxysphingosine, while the new acylceramide would become Cer[EOH]. The system could easily be extended to the epidermal glycosylceramides, with the series previously identified in porcine epidermis becoming GlcCer[EOS], GlcCer[NS], GlcCer[NP], GlcCer[OS], GlcCer[AS], and GlcCer[AP] (12). The novel glucosylceramide of Hamanaka et al. would be designated GlcCer[EOH], and its deglycosylated metabolite in the extractable lipids of human stratum corneum, discovered in the present study (ceramide 4), would be Cer[EOH].



Adoption of a structure-based nomenclature is especially indicated because of the recognition that mammalian species differ in the ceramide structures found at similar TLC migration points. The present results emphasize the need for a rational and convenient nomenclature.

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