Nonhydroxylated 1-O-acylceramides in vernix caseosa

Eva Harazim,†*, Vladimír Vrkoslav,*, Miloš Buděšínský,*, Petr Harazim,§ Martin Svoboda,§ Richard Plavka,*, Zuzana Bosáková,† and Josef Cvačka†*

Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences,* CZ-166 10 Praha 6, Czech Republic; Department of Analytical Chemistry,† Faculty of Science, Charles University, CZ-128 43 Praha 2, Czech Republic; Department of Concrete and Masonry Structures,§ Faculty of Civil Engineering, Czech Technical University in Prague, CZ-166 29 Praha 6, Czech Republic; and Department of Obstetrics and Gynecology,*** First Faculty of Medicine, Charles University and General University Hospital in Prague, CZ-128 00 Praha 2, Czech Republic

Abstract Vernix caseosa, the waxy substance that coats the skin of newborn babies, has an extremely complex lipid composition. We have explored these lipids and identified nonhydroxylated 1-O-acylceramides (1-O-ENSs) as a new class of lipids in vernix caseosa. These ceramides mostly contain saturated C11–C38 ester-linked (1-O) acyls, saturated C12–C39 amide-linked acyls, and C16–C24 sphingoid bases. Because their fatty acyl chains are frequently branched, numerous molecular species were separable and detectable by HPLC/MS: we found more than 2,300 molecular species, 972 of which were structurally characterized. The most abundant 1-O-ENSs contained straight-chain and branched fatty acyls with 20, 22, 24, or 26 carbons in the 1-O position, 24 or 26 carbons in the N position, and sphingosine. The 1-O-ENSs were isolated using multistep TLC and HPLC and they accounted for 1% of the total lipid extract. The molecular species of 1-O-ENSs were separated on a C18 HPLC column using an acetonitrile/propan-2-ol gradient and detected by APCIMS, and the structures were elucidated by high-resolution and tandem MS. Medium-polarity 1-O-ENSs likely contribute to the cohesiveness and to the waterproofing and moisturizing properties of vernix caseosa.

Harazim, E., V. Vrkoslav, M. Buděšínský, P. Harazim, M. Svoboda, R. Plavka, Z. Bosáková, and J. Cvačka. Nonhydroxylated 1-O-acylceramides in vernix caseosa. J. Lipid Res. 2018. 59: 2164–2173.

Supplementary key words ceramides • lipids • lipidomics • mass spectrometry • skin

Human skin is the largest complex organ in the human body with mainly protective function. The outermost layer of the skin is the stratum corneum, consisting of corneocytes filled with keratin filaments and surrounded by a continuous lipid matrix. The extracellular lipids form a lamellar structure composed of ceramides, cholesterol, and FAs. The stratum corneum provides a major barrier to water loss and the permeation of exogenous substances (1, 2). The skin begins to form during embryogenesis in the third week of gestation. The initial layer of ectodermal cells undergoes gradual differentiation to create a stratified epithelium. By the end of the fourth week, the skin consists of a basal layer and the periderm, which protects the fetus from amniotic fluid and ensures glucose uptake. An intermediate layer is then formed by the proliferation of keratinocytes from the spinous layer between the basal layer and the periderm. The stratum corneum begins to be shaped in the eighteenth week. The periderm cells are completely replaced by the twenty-third week of gestation. At about the same time, vernix caseosa starts to be produced (3, 4).

Vernix caseosa is a cheese-like substance that coats the fetal skin from the middle of gestation (5). It protects the fetus from the amniotic fluid, prevents the loss of water and electrolytes, and aids in postbirth adaptation of a newborn’s skin. The main components of the vernix caseosa are water, sebum, desquamated epithelial cells, and lanugo hair. Chemically, it is a rich mixture of lipids and proteins. Even though it has been studied for many decades, the chemical constituents of vernix caseosa have not been comprehensively characterized yet. The enormous chemical complexity of vernix caseosa lipids has fascinated scientists, but they are still far from completely understanding the biological roles of individual lipid classes. Earlier studies of lipid composition focused on abundant neutral (nonpolar) species (6–16), with significantly less attention

Abbreviations: CID, collision-induced dissociation; CN, number of carbons; DB, number of double bonds; ECN, equivalent carbon number; 1-OEAS, hydroxylated 1-Oacylceramide; 1-OENS, nonhydroxylated 1-Oacylceramide; FAME, fatty acid methyl ester; NARP, nonaqueous reversed-phase.

To whom correspondence should be addressed.

e-mail: josef.cvacka@uochb.cas.cz

The online version of this article (available at http://www.jlr.org) contains a supplement.

Copyright © 2018 Harazim et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at http://www.jlr.org
being paid to polar lipids, mostly represented by ceramides (12, 17, 18).

Ceramides are bioactive lipids found in low amounts in animal tissues, where they are important for cell growth, division, and programmed cell death (19). In the skin, ceramides play a key role in maintaining the barrier function, and changes in their content are associated with a number of diseases, such as atopic dermatitis and psoriasis (20–23). Chemically, they are sphingolipids consisting of fatty acyls linked to sphingoid bases by amide bonds. Mammalian tissues mostly contain sphingoid bases with 18 carbons, although chains with 12–28 carbons have also been identified (24–28). The large structural diversity of ceramides stems from the high number of FAs typically found in the skin and from several possibilities of ester bond formation from the hydroxy groups, both on sphingoid bases and on hydroxy FA chains (28). To date, 15 subclasses of free ceramides have been described in the human stratum corneum (23, 29–32) and 10 of them have also been found in vernix caseosa (17). Ceramides can be analyzed after their hydrolysis as FA esters and sphingoid bases (33, 34), or in the form of intact molecules using chromatography and/or MS. Chromatography is performed both in normal-phase (27, 30, 32, 35–37) and reversed-phase (31, 38, 39) modes. Concerning MS, ceramides are usually probed by ESI (25, 27, 38–47) or APCI (25, 32, 35–38, 48, 49).

In this work, we report on nonhydroxylated 1-O-acylceramides (1-O-ENSs) discovered during a search for unknown lipids in vernix caseosa. The 1-O-ENSs were isolated using adsorption chromatography on silica gel in several steps and their general structure was established using high-resolution and tandem MS, derivatization, and NMR. The molecular species within this class were comprehensively characterized using nonaqueous reversed-phase (NARP)-HPLC with APCI-MS.

**MATERIALS AND METHODS**

**Materials**

Vernix caseosa (1–2 g) was collected from healthy newborn subjects delivered at full term (gestation weeks 39–42) immediately after the delivery. The samples were stored in amber glass vials at −80°C. The study was approved by the Ethics Committee of the General University Hospital, Prague (910/09 S-IV) and the samples were collected with written informed parental consent.

**Chemicals and reagents**

LC-MS grade methanol, hexane, acetonitrile, and propan-2-ol (Sigma-Aldrich, St. Louis, MO) were used as received. Diethyl ether and chloroform (both from Penta, Czech Republic) were distilled in glass from analytical-grade solvents. Rhodamine 6G and primulin and chloroform (both from Penta, Czech Republic) were distilled in glass from analytical-grade solvents. Rhodamine 6G and primulin were dissolved in methanol (1 mg/ml) and stored at −20°C until needed. The whole HPLC/MS system was controlled by Xcalibur software (Thermo Fisher Scientific). The collected mobile phase with lipids (100 µl/min) was directed via a switching divert valve either into a glass flask to collect the lipids (ts = 35–75 min) or into waste. The column was maintained at 30°C and the gradient was programmed from hexane (A) and hexane/propan-2-ol (96:4 by volume) (B) as follows: 0 min, 20% of B; 30 min, 90% of B; 60 min, 47% of B. The sample (10 µl) was injected with an Accela autosampler (Thermo Fisher Scientific, San Jose, CA). The chromatographic separation was monitored using an LCQ Fleet spectrometer equipped with an APCI probe (Thermo Fisher Scientific). The APCI vaporizer and heated capillary temperatures were set to 270°C and 170°C, respectively. Nitrogen served as both the sheath and auxiliary gas at a flow rate of 15 and 15 arbitrary units, respectively. The MS spectra of the positively charged ions were recorded in the range of m/z 250–2,000. The whole HPLC/MS system was controlled by Xcalibur software (Thermo Fisher Scientific). The collected mobile phase with lipids was evaporated using a rotary evaporator (30°C, 170 mbar). The HPLC separation was repeated 22 times, providing 1.1 mg of

![1-O-acylceramides in vernix caseosa](https://example.com/image.png)
were extracted with hexane. The reaction products were inserted into the EI source of GCT Premier (Waters). The 70 eV EI mass spectra were recorded in the range of \( m/z \) 25–600.

**GC/EI-MS and direct-probe EI-MS**

FA methyl esters (FAMEs) were analyzed using a 6890N GC system (Agilent, Santa Clara, CA) coupled to a 5975B quadrupole mass spectrometer and equipped with an HP-5ms column (30 m × 250 \( \mu \)m, 0.25 \( \mu \)m; Agilent). The carrier gas was helium at 1 ml/min. The injector was held at 280°C and operated in the splitless mode (2 \( \mu \)l of sample injected). The temperature program was as follows: 140°C (0 min), then 4°C/min to 320°C (10 min). The 70 eV EI mass spectra were recorded in the range of \( m/z \) 25–600; a solvent delay of 3.5 min was used. The temperatures of the transfer line, ion source, and quadrupole were 280°C, 230°C, and 150°C, respectively. For the EI-MS analysis without chromatographic separation, the sample was loaded on a direct insertion probe and inserted into the EI source of GCT Premier (Waters). The 70 eV EI mass spectra were recorded in the range of \( m/z \) 25–600.

**Direct infusion MS and HPLC/API-MS**

The structure of intact lipids in F-3 was also elucidated by APCI-MS performed on an LTQ Orbitrap XL hybrid FT mass spectrometer having either an APCI or ESI probe installed (Thermo Fisher Scientific). The spectrometer was coupled to an HPLC system consisting of a Rheos 2200 quaternary gradient pump (Flux Instruments) and a PAL HTS autosampler (CTC Analytics, Zwingen, Switzerland). The system was controlled by Xcalibur software (Thermo Fisher Scientific).

The sample for direct-infusion APCI-MS was dissolved in chloroform (250 \( \mu \)g/ml) and delivered by a syringe pump (3 \( \mu \)l/min) to a low-dead-volume T-piece, where it was mixed with acetonitrile, flowing at 200 \( \mu \)l/min into the APCI source. The vaporizer and capillary temperatures were set to 320°C and 180°C, respectively, and nitrogen was used as the sheath and auxiliary gas at a flow rate of 65 and 10 arbitrary units, respectively. The spectra of positively charged ions were recorded. As regards direct ESI-MS in the negative ion mode, the transesterification products were infused into the ion source at the flow rate of 3 \( \mu \)l/min. The ion source, capillary, and tube lens voltages were set to 4 kV, −16 V, and −120 V, respectively, and the capillary temperature was 275°C. The sheath gas and auxiliary gas were operated at 50 and 10 arbitrary units, respectively.

The molecular species of 1-O-ENSs in F-3 were separated using a Nova-Pak C18 stainless-steel column (300 × 3.9 mm, particle size 4 \( \mu \)m; Waters) at 30°C. The autosampler injected 10 \( \mu \)l of the sample and the injection system was washed with chloroform:acetonitrile (1:1, v/v). The gradient was mixed from acetonitrile (A) and propan-2-ol (B) as follows: 0 min: 30% of B, 0.70 ml/min; 60 min: 69% of B, 0.35 ml/min; 80 min: 82% of B, 0.25 ml/min; 106 min: 99% of B, 0.25 ml/min; 130 min: 99% B, 0.25 ml/min. The APCI vaporizer and heated capillary temperatures were set to 315°C and 180°C, respectively; the corona discharge current was 5 \( \mu \)A. The sheath gas and auxiliary gas (nitrogen) were set to 70 and 10 arbitrary units, respectively. The MS method encompassed eight scan events: 1) the full scan in the range of \( m/z \) 400–2,000 for Orbitrap; 2) the collision-induced dissociation (CID) MS/MS of the first most abundant ion from the precursor mass list with a normalized collision energy of 26–29% and isolation with 2 Da for a linear ion trap; 3–8) the CID MS3 of the first to sixth most abundant fragments recorded in the previous event using a normalized collision energy of 28% and isolation with 2 Da. The same sample was analyzed four times, each time with a different inclusion list corresponding to 1-O-ENSs with 0, 1, 2, and 3 double bonds. The precursor mass list was calculated for \([M + H – H_2O]^{-}\) and the total number of carbons (CN) in the range of 50–80. The mass errors in the Orbitrap spectra were within the range of 0.1–3.0 ppm. All recorded chromatograms were interpreted manually using an in-house developed Excel macro. The relative proportions of the 1-O-ENS molecular species were calculated in two steps. In the first step, a full-scan trace from Orbitrap was used to create reconstructed ion chromatograms for individual \( m/z \) values (\([M + H – H_2O]^{-}\)). The peak areas were integrated and expressed in relative values. In the second step, the relative peak areas were divided using the relative proportions of FA loss fragments in the MS2 step and the relative proportions of sphingoid base loss fragments (i.e., the loss of alkadiene) in the MS3 step.

**NMR**

The NMR spectra of samples in CDCl3 were measured on a Bruker AVANCE-III-HD 600 instrument (\(^1\)H at 600.13 MHz and \(^1\)C at 150.9 MHz) equipped with a cryoprobe at 25°C. The spectra were referenced to the TMS (\(^1\)H) or solvent peak (\(^1\)C, using \( \delta_{\text{ppm}}(\text{CDCl}_3) = 77.0 \) ppm). The partial structural assignment of proton and carbon signals was achieved through a combination of 1D-\(^1\)H and 1D-APT-\(^1\)C-spectra with homonuclear 2D-\(^1\)H,H-COSY, heteronuclear 2D-\(^1\)H,C-HSQC, and 2D-\(^1\)H,C-HMBC spectra.
The notation of ceramides

This work uses the ceramide nomenclature introduced by Motta et al. (51) and extended by Rabionet, Gorgas, and Sandhoff (28). The 1-O-ENs are abbreviated as follows: fatty acyl (ester-linked); sphingoid base; fatty acyl (amide-linked). Fatty chains are given as the CN: number of double bonds (DB). Sphingoid chains are labeled with the prefix “d” to designate dihydroxy bases. Therefore, for instance, 22:0;18:1;24:0 corresponds to 1-O-ENs composed of sphingosine with ester-linked FA22:0 and amide-linked FA24:0 (2-aminoocattoc-4-ene-1,3-diol).

RESULTS

General structure elucidation

The mass spectra of unknown lipids infused into the APCI source showed protonated molecules accompanied by more abundant water-loss fragments in the range of m/z 750–1,200 (Fig. 2). The exact masses of the protonated molecules were consistent with the elemental compositions CnH2n-xO4N+ (x = 0, 2, 4, or 6). The presence of nitrogen pointed to structures related to ceramides. In the next step, the lipids were transesterified using conditions strong enough to cleave both ester- and amide-linked FAs. Infusion experiments using an ESI source operated in the negative ion mode revealed signals consistent with sphinganine (chloride adduct at m/z 336.3), sphingosine (chloride adduct at m/z 334.3), and other sphingoid bases (Fig. 3A). High-temperature GC/MS and direct-probe EI-MS showed a number of mostly saturated FAMEs with 8–32 carbons having straight and methyl-branched chains, but no hydroxyl group (supplemental Fig. S1a; supplemental Table S1). The masses of the intact lipids were too high to be explained by a sphingoid base linked to one FA. Therefore, two fatty acyls in the structures were assumed. The absence of hydroxy FAs led us to a structure with the two fatty acyls linked directly to the sphingoid base by means of one amide and one ester bond. To confirm the hypothesis, transesterification was performed once again, but this time using milder conditions preserving amide bonds. Products having a sphingoid base linked to a fatty acyl by an amide bond were sought in the reaction mixture. Indeed, APCI-MS showed ions corresponding to CnH2n-xO3N+ (x = 2, 4, 6, or 8) and their water-loss products consistent with Cers (NS) and Cers (NdS) (Fig. 3B). These Cers have free hydroxyl groups on C1 and C3 of the sphingoid chain. Although both of them can be esterified, the C1 hydroxy group was considered more likely to be involved in the ester bond formation. Many ceramides, e.g., sphingomyelins and glycosphingolipids, are functionalized on C1, whereas the C3 hydroxy group remains unmodified. Therefore, we hypothesized that the unknown lipids were, in fact, 1-O-ENs (Fig. 4). To test the hypothesis, we compared the MS and NMR data of the lipids isolated from vernix caseosa with a commercial standard (17:0 ceramide-1-O-18:1). The mass spectra of the standard (Fig. 5) were consistent with the spectra recorded for the unknown lipids. NMR spectra were taken for the isolated lipid class, i.e., for a rich mixture of molecular species. Although no conclusions could be made regarding the side chains, the chemical shifts for the central part of the molecules observed in 1H and 13C NMR spectra were almost identical to those obtained from the standard (supplemental Fig. S2). Hence, the hypothesis was confirmed and the lipids isolated from vernix caseosa were identified as 1-O-ENs. Later, it was confirmed...
that the retention time of the commercial standard in the optimized NARP-HPLC system (tR = 40.7 min) is in agreement with the retention of 1-O-ENSs isolated from vernix caseosa.

The NARP-HPLC/MS method for 1-O-ENSs

To comprehensively characterize 1-O-ENSs in vernix caseosa, a HPLC/MS method for the separation and identification of the molecular species was developed. The chromatographic conditions were optimized with the mixture of 1-O-ENSs isolated from vernix caseosa. Good separation was achieved on a 30 cm Nova-Pak C18 column, with a gradient elution of propan-2-ol in acetonitrile. The data-dependent MS detection method consisted of three scanning events. In the full-scan APCI spectra, 1-O-ENSs showed abundant dehydrated protonated molecules [M + H – H2O]+ accompanied by protonated molecules at much lower intensities. The full-scan spectra were used for deducing the total CN and DB. Dehydrated protonated molecules were selected for CID in the ion trap. The MS2 spectra provided ions consistent with the neutral loss of ester-linked FA ([M + H – H2O – FA]+), the combined neutral loss of ester-linked FA and alkadiene from the sphingoid base chain ([M + H – H2O – FA – CnH2n-2]+), and protonated amides corresponding to the amide-linked FA. In the case of the most common sphingoid base, sphingosine, the eliminated alkadiene corresponded to hexadecadiene. In addition, protonated doubly dehydrated sphingoid base ions (m/z 264.3 for sphingosine) were formed. These low-mass ions were not always detected because of the ion trap cut-off effect. The MS3 spectra were utilized to identify ester-linked FAs (Fig. 5A). Further fragmentation of the ions formed by the elimination of FA (MS3 spectra) led to the neutral loss of alkadiene ([M + H – H2O – FA – CnH2n-2]+) and protonated doubly dehydrated sphingoid base ions. Fragments of protonated amides corresponding to amide-linked FAs were detected as well. The MS3 spectra made it possible to identify amide-linked FAs and sphingoid bases (Fig. 5B). The APCI mass spectra closely resembled the ESI spectra of 1-O-ENSs published previously (52).

The 1-O-ENSs in vernix caseosa

The NARP-HPLC data revealed an enormous complexity of 1-O-ENSs in vernix caseosa. The chromatogram contained many coeluting peaks (Fig. 6A), which could be partially resolved using reconstructed chromatograms. For instance, reconstructed chromatograms for selected monounsaturated 1-O-ENSs provided several broad peaks. Interestingly, the pattern was very similar for monounsaturated species differing in the total CN (Fig. 6B–D). The relatively large peak widths could be explained by the coelution of species with the same total CN, but various lengths of...

Fig. 4. The structure of 1-O-ENSs.

Fig. 5. The ion-trap APCI mass spectra of the 1-O-ENS standard 17:0 ceramide-1-O18:1; MS2 of m/z 798.8 ([M + H – H2O]+) (A) and MS3 of m/z 516.5 ([M + H – H2O – FA18:1]+) (B).

Fig. 6. NARP-HPLC/ACPI-MS chromatograms of 1-O-ENSs from vernix caseosa: the base peak chromatogram (A) and reconstructed chromatograms for the 1-O-ENS molecular species 68:1, m/z 1011.0223 (B); 67:1, m/z 997.0064 (C); and 66:1, m/z 982.9908 (D).
individual chains or positions of the double bonds. The chromatographic separation of 1-O-ENSs with the same total CN was most likely caused by methyl branching of the aliphatic chains. We had already shown that the methyl branching of lipids significantly decreased their retention in RP-HPLC (53). The MS² spectra taken in the separated peaks looked similarly, indicating almost the same distribution of the fatty acyl chain length (supplemental Fig. S3). Hence, various degrees of methyl branching seemed to be a reasonable explanation for the peak separations. The peak patterns looked differently in the reconstructed chromatograms for 1-O-ENSs with a different DB; nevertheless, similarly to monounsaturated 1-O-ENSs, the profiles were alike within each group (supplemental Fig. S4). The complexity of 1-O-ENSs in vernix caseosa can be further illustrated using chromatograms reconstructed for the neutral loss of a specific ester-linked FA observed in MS². As for the monounsaturated 1-O-ENSs, the profiles consisted of dozens of peaks and looked almost the same regardless of the FA (Fig. 7A–C). The reconstructed chromatograms, however, changed dramatically when the neutral loss of the same FA was plotted for 1-O-ENSs with a different total DB (Fig. 7C–E).

Representative mass spectra of 1-O-ENSs from vernix caseosa are given in Fig. 8. They show 1-O-ENSs eluting in the peak tₚ= 70.5 min. The precursor selected for fragmentation was a dehydrated protonated molecule of monounsaturated 1-O-ENSs with 62 carbons (m/z 926.9) (Fig. 8A). The ion trap APCI MS² of m/z 926.9 ([M + H – H₂O]⁺) (B) and MS³ of m/z 614.7 ([M + H – H₂O – FA₂₀:₀]⁺) (C).
reversed-phase chromatography, the retention of lipids depends on the number of carbon atoms and double bonds in acyl chains. Therefore, the retention times of lipids are usually in good agreement with their ECN values calculated as the total CN minus twice the DB, i.e., ECN = CN − 2DB (14, 16, 54, 55). This is also the case of 1-O-ENSs, which makes the validation possible (Fig. 9).

The most abundant 1-O-ENSs identified in vernix caseosa are summarized in Table 1. The list of 30 molecular species represents almost one-third of the total intensity of lipids within this class. For a complete list of 1-O-ENSs see supplemental Table S2. Ester-linked fatty acyls were mostly saturated; they consisted of 11–38 carbons, where even-number chains predominated (Fig. 10A). Although amide-linked fatty acyls spanned a similar range of chain lengths (12–39 carbons), their profile was significantly different (Fig. 10B). The fatty acyl profiles calculated from LC/MS data were in good agreement with the GC/MS data recorded for transesterified samples (supplemental Fig. S1). Some additional peaks in GC/MS traces (particularly FAME 16:0 and FAME 18:0 in supplemental Fig. S1a) indicated minor impurities of other lipids in the sample (fraction F-3). Concerning sphingoid bases, they appeared to contain 16–24 carbons and up to two double bonds. Sphingosine (d18:1) was the most abundant, but sphinganine (d18:0) and d19:1 were also detected in significant amounts (Fig. 10C). The most abundant 1-O-ENS in vernix caseosa proved to be 24:0;18:1;26:0, which formed 2.3% of these lipids.

**DISCUSSION**

Ceramides are essential lipids for the skin barrier function, protecting the body against desiccation and microbial infections. Together with FFAs and cholesterol, they form lamellar membranous structures surrounding corneocytes in the stratum corneum. They are also important in vernix caseosa, where barrier lipids typically do not form lamellar structures. The level of barrier lipids is significantly lower than in the stratum corneum, and the dominant components of vernix caseosa are nonpolar lipids. Such composition ensures the protection of the fetal skin from direct contact with the amniotic fluid and facilitates the maturation of epidermis in utero (12, 56).

Barrier lipids in vernix caseosa are products of the developing skin, which is reflected in a similar composition of ceramides in the stratum corneum and vernix caseosa (12, 18, 57). Most of the ceramide subclasses existing in the skin have also been found in vernix caseosa. To date, EOS (Cer 1), NS (Cer 2), NP (Cer 3), EOH (Cer 4), AS (Cer 5), AP (Cer 6), AH (Cer 7), NH (Cer 8), and EOP (Cer 9) have been detected in vernix caseosa (12, 17, 18), together with EAS possibly corresponding to hydroxylated 1-O-acylceramides (1-O-EASs) (17). Therefore, the discovery of 1-O-ENSs in vernix caseosa is not very surprising.

The very first mention of 1-O-ENSs comes from 1977, when they were tentatively identified in rat brain after the injection of precursor ceramides (58). As regards endogenously expressed lipids, 1-O-ENSs together with 1-O-EASs were identified in humans and mice in 2013 as new classes of epidermal ceramides (31). Their masses were in the range of 750–1,000 Da, and they contained sphingosine and long to very long acyl chains with an even CN. They were found to comprise 5% of all esterified ceramides in the epidermis of mice. In humans, their total amount accounted for approximately 30% of the corresponding unmodified parent ceramides (NS). The most abundant 1-O-ENSs contained 24:0 or 16:0 chains in ester-linked FAs, whereas amide-linked acyls mostly consisted of hydroxy-16:0 or 16:0 chains. The biosynthesis of these ceramides was suggested to take place at endoplasmic reticulum-related sites and involve acylation by DGAT2 (31). Mouse epidermal 1-O-ENSs have recently been investigated using direct infusion MS (52). Approximately 710 molecular species composed mainly of saturated long-chain acyls and sphingosine were found. The high number of isobaric species illustrated the complexity of this lipid class. The double bond in unsaturated 1-O-acyl and Nacyl chains appeared to be located at the position n-9.
The 1-O-ENs analyzed in this work were entirely nonhydroxylated species (1-O-ENs). They were isolated using adsorption chromatography on silica gel, which ensured efficient separation from other lipids of different polarity, including 1-OEASs, if they were present. In total, 1.1 mg of 1-OENs were isolated, which corresponds to approximately 1% of lipids in vernix caseosa. As ceramides comprise about 50% of stratum corneum lipids and approximately 1% of lipids in vernix caseosa. As ceramides comprising 1-O-ENs in the lipids of vernix caseosa and stratum corneum is approximately the same.

Like mouse and human epidermal 1-Oacylceramides (31, 52), 1-OENs from vernix caseosa were found to be a highly complex mixture of molecular species. The complexity stems from the high variability of fatty acyls differing in their chain length, methyl branching, and DB. Although 1-OENs exhibit a preference for certain acyls (Fig. 10A, B), the overall incorporation of fatty chains from these “pools” is highly random. Consequently, there are at least 2,300 molecular species of 1-OENs in vernix caseosa, but the real number is very likely even higher. When compared with previous works (31, 52), the higher number of the molecular species identified in this work can be attributed to the analytical method, i.e., MS coupled to thoroughly optimized chromatography. HPLC can efficiently distinguish isobaric 1-OENs differing in chain branching, which is its principal advantage over direct-infusion MS-based approaches. Methylation of acyl chains is known to occur in vernix caseosa ceramides, particularly in acylceramides (17). Methyl-branched 1-OENs have been found to exist in vernix caseosa, which is obvious both from HPLC and GC data. The GC/MS of the transesterified 1-OENs sample revealed three series of FAMEs. Based on their retention behavior (59), we speculate that they corresponded to iso-, anteiso-, and straight-chain esters (supplemental Table S1). Odd-carbon FAMEs were of all three types (iso-, anteiso-, and straight-chain), whereas even-carbon FAMEs lacked anteiso-isomers, likely because anteiso-chains are biosynthesized from the odd-carbon precursor, isoleucine (60). Iso-FAMEs predominated in odd-carbon series, where the most abundant even-carbon FAMEs were of the anteiso type. A very similar profile of branched fatty acyl chains was observed previously in acylated ceramides (17). Besides the methyl branching, fatty acyls in vernix caseosa 1-OENs are characterized by considerable chain lengths. Saturated fatty acyls contained up to almost 40 carbons (38 in ester-linked, 39 in amide-linked), and the most typical chains consisted of 24 carbon atoms. Unsaturated fatty acyls were not common; they were virtually absent from amide-linked chains (about 0.1% of unsaturated chains) and formed approximately 10% of Oacyls. The low content of unsaturated chains is favorable for the resistance to oxidative damage on exposure to air after delivery. Saturated and monounsaturated very-long-chain fatty acyls are commonly found in epidermal ceramides, where they contribute to the barrier function of the skin (23, 30). Odd-carbon chains formed 20% of N-linked acyls and 24% of O-linked acyls in vernix. Sphingosine (d18:1) was by far the most abundant sphingoid base in the 1-OENs of vernix caseosa, like in human and mouse epidermis (31, 52). In addition, the research revealed sphinganine (d18:0), five sphingosine homologs, seven sphinganine homologs, and...
without the need for instruments with MS\textsuperscript{4} capabilities. As far as lipid quantification is concerned, it is important to note that the response factors of individual molecular species depend to some extent on the nature of aliphatic chains. The response is mainly affected by the overall DB: the larger their number, the higher the detection sensitivity (53). Consequently, the quantitative data reported in this work might be slightly biased.

The biological function of 1-O-ENs in vernix caseosa has yet to be clarified. The moderate polarity and the free hydroxyl group of these ceramides might contribute to the cohesiveness of vernix caseosa composed of nonpolar sebaceous lipids and polar barrier lipids. In this way, 1-O-ENs could contribute to the waterproofing and moisturizing properties of vernix caseosa.\textsuperscript{11}

REFERENCES

1. Haake, A., G. A. Scott, and K. A. Holbrook. 2001. Structure and function of the skin: overview of the epidermis and dermis. In The Biology of the Skin. R. K. Freinkel and D. T. Woodley, editors. Parthenon Publishing Group, New York, NY. 19–45.

2. Menon, G. K. 2015. Skin basics; structure and function. In Lipids and Skin Health. A. Pappas, editor. Springer International Publishing AG, Cham, Switzerland. 9–23.

3. Vischer, M. O., and V. Narendran. 2014. Neonatal infant skin: development, structure and function. Newborn Infant Nurs. Rev. 14: 135–141.

4. Vischer, M., and V. Narendran. 2014. The ontogeny of skin. Adv. Wound Care (New Rochelle). 3: 291–303.

5. Hoath, S. B., W. L. Pickens, and M. O. Visscher. 2006. The biology of vernix caseosa. Int. J. Cosmet. Sci. 28: 319–333.

6. Kaerkkaeinen, J., T. Nikkari, S. Ruponen, and E. Haahl. 1965. Ceramides of vernix caseosa. J. Invest. Dermatol. 44: 333–338.

7. Fu, H. C., and N. Nicolaides. 1969. The structure of alkane diols of vernix caseosa. Lipids. 5: 279–282.

8. Ansari, M. N. A., H. C. Fu, and N. Nicolaides. 1970. Fatty acids of the alkane diol diesters of vernix caseosa. Lipids. 6: 170–175.

9. Nicolaides, N., M. N. A. Ansari, H. C. Fu, and G. R. Rice. 1972. The fatty acids of wax esters and sterol esters from vernix caseosa and from human skin surface lipid. Lipids. 7: 506–517.

10. Stewart, M. E., M. A. Quinn, and D. T. Downing. 1982. Variability in the fatty acid composition of wax esters from vernix caseosa and its possible relation to sebaceous gland activity. J. Invest. Dermatol. 78: 291–295.

11. Tollin, M., G. Bergsson, Y. Kai-Larsen, J. Lengqvist, J. Sjövall, W. Griffiths, G. V. Skuladottir, A. Haraldsson, H. Jörnvall, G. H. Gudmundsdottir, et al. 2005. Vernix caseosa as a multi-component defense system based on polypeptides, lipids and their interactions. Cell. Mol. Life Sci. 62: 2390–2399.

12. Rüissmann, R., H. W. W. Groenink, A. M. Weerheim, S. B. Hoath, M. Ponce, and J. A. Bouwstra. 2006. New insights into ultrastructure, lipid composition and organization of vernix caseosa. J. Invest. Dermatol. 126: 1823–1833.

13. Miková, R., V. Vírsková, H. Háková, Z. Hábová, A. Dolečal, R. Plavka, P. Coufal, and J. Čválek. 2014. Newborn boys and girls differ in the lipid composition of vernix caseosa. PLoS One. 9: e99179.

14. Šubíková, L., M. Hoskovec, V. Vírsková, T. Čmelíková, E. Háková, R. Miková, P. Coufal, A. Dolečal, R. Plavka, J. Čválek, et al. 2015. Analysis of 1,2-diol diesters in vernix caseosa by high-performance liquid chromatography - atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A. 1378: 8–18.

15. Háková, E., V. Vírsková, R. Miková, K. Schwarzová-Precková, Z. Bosková, and J. Čválek. 2015. Localization of double bonds in triacylglycerols using high-performance liquid chromatography/atmospheric pressure chemical ionization ion-trap mass spectrometry. Anal. Bioanal. Chem. 407: 5175–5188.

16. Kalužíková, A., V. Vírsková, E. Harazim, M. Hoskovec, R. Plavka, M. Budišinský, Z. Bosková, and J. Čválek. 2017. Cholesterol esters of ω-(O-acetyl)-hydroxy fatty acids in vernix caseosa. J. Lipid Res. 58: 1579–1590.
17. Oku, H., K. Mimura, Y. Tokitsu, K. Onaga, H. Iwasaki, and I. Chinen. 2000. Biased distribution of the branched-chain fatty acids in ceramides of vernix caseosa. Lipids. 35: 373–381.
18. Hoeger, P. H., V. Schreiner, I. A. Klaassen, C. C. Enzmann, K. Friedrichs, and O. Bleck. 2002. Epidermal barrier lipids in human vernix caseosa: corresponding ceramide pattern in vernix and fetal skin. Br. J. Dermatol. 146: 194–201.
19. Futterman, A. H. 2002. Ceramide Signaling (Molecular Biology Intelligence Unit 21). Kluwer Academic/Plenum Publishers, New York, NY.
20. Leray, C. 2012. Simple lipids with two different components. In Introduction to Lipidomics: From Bacteria to Man. CRC Press, Boca Raton, FL. 169–209.
21. Meckfessel, M. H., and S. Brandt. 2014. The structure, function, and importance of ceramides in skin and their use as therapeutic agents in skin-care products. J. Am. Acad. Dermatol. 71: 177–184.
22. Borodzicz, S., L. Rudnicka, D. Mirowska-Guzel, and A. Cudnoch-Jedrzejewska. 2016. The role of epidermal sphingolipids in dermatologic diseases. Lipids Health Dis. 15: 13.
23. Moore, D. J., and A. V. Rawlings. 2017. The chemistry, function and (patho)physiology of stratum corneum barrier ceramides. Int. J. Cosmet. Sci. 39: 366–372.
24. Stewart, M. E., and D. T. Downing. 1995. Free sphingolipids of human skin include 6-hydroxysphingosine and unusually long-chain dihydrosphingosines. J. Invest. Dermatol. 105: 613–618.
25. van Smeden, J., W. A. Boiten, T. Hankemeier, R. Rissmann, J. A. Bouwstra, and R. J. Vreeken. 2011. LC/MS analysis of stratum corneum ceramides. J. Lipid Res. 52: 1469–1476.
26. Pruett, S. T., A. Bushnev, K. Hagedorn, M. Adiga, C. A. Haynes, M. C. Sollars, D. C. Liotta, and A. H. Jr. Merrill. 2008. Biodiversity of sphingoids (“sphingosines”) and related amino alcohols. J. Lipid Res. 49: 1621–1639.
27. Masukawa, Y., H. Narita, H. Sato, A. Naoe, N. Kondo, Y. Sugai, T. Oba, R. Homma, J. Ishikawa, Y. Takagi, et al. 2009. Comprehensive quantification of ceramide species in human stratum corneum. J. Lipid Res. 50: 1708–1719.
28. Rabionet, M., K. Gorgas, and R. Sandhoff. 2014. Ceramide synthesis in the epidermis. Biochim. Biophys. Acta. 1841: 422–434.
29. Ponec, M., A. Weerheim, P. Lankhorst, and P. Wertz. 2003. New acylceramide in native and reconstructed epidermis. J. Invest. Dermatol. 120: 581–588.
30. Masukawa, Y., H. Narita, E. Shimizu, N. Kondo, Y. Sugai, T. Oba, R. Homma, J. Ishikawa, Y. Takagi, et al. 2009. Comprehensive characterization of overall ceramide species in human stratum corneum. J. Lipid Res. 50: 1708–1719.
31. Rabionet, M., K. Gorgas, and R. Sandhoff. 2014. Ceramide synthesis in the epidermis. Biochim. Biophys. Acta. 1841: 422–434.
32. van Smeden, J., W. A. Boiten, T. Hankemeier, R. Rissmann, J. A. Bouwstra, and R. J. Vreeken. 2011. LC/MS analysis of stratum corneum ceramides. J. Lipid Res. 52: 1469–1476.
33. Wu, Z., J. C. Shon, D. Lee, K. T. Park, C. S. Park, T. Lee, H. S. Lee, and K. H. Liu. 2016. Lipidomic platform for structural identification of ceramides with ε-hydroxyacetyl chains. Anal. Bioanal. Chem. 408: 2089–2092.
34. Wu, Z., J. C. Shon, J. Y. Kim, Y. Cho, and K. H. Liu. 2016. Structural identification of skin ceramides containing ε-hydroxyacetyl chains using mass spectrometry. Arch. Pharm. Res. 39: 1426–1432.
35. Luffet, G. P., A. Genette, B. Gamboa, V. Auroy, and J. J. Voegel. 2018. Determination of fatty acid and sphingoid base composition of seven ceramide species in stratum corneum by UHPLC/scheduled-MRM. Metabolomics. 14: 60.
36. Farwana, H., P. Nuhn, R. Neubert, and K. Raith. 2003. Normal-phase liquid chromatographic separation of stratum corneum ceramides with detection by evaporative light scattering and atmospheric pressure chemical ionization mass spectrometry. Anal. Chem. 75: 492–503.
37. Farwana, H., J. Wohlrab, R. H. H. Neubert, and K. Raith. 2005. Profiling of human stratum corneum ceramides by means of normal phase LC/APCI-MS. Anal. Bioanal. Chem. 383: 632–637.
38. Stránský, K., and T. Jursik. 1996. Simple quantitative transsterific-ation of lipids. 1. Introduction. Eur. J. Lipid Sci. Technol. 98: 65–71.
39. Motta, S., M. Monti, S. Sesana, R. Caputo, S. Carelli, and R. Ghidoni. 1995. Ceramide composition of the porcine scale. Biochim. Biophys. Acta. 1182: 147–151.
40. Lin, M. H., J. H. Miner, J. Turk, and F. F. Hsu. 2017. Linear ion-trap MSn with high-resolution MS reveals, structural diversity of 1-O-acylceramides in human stratum corneum. J. Lipid Res. 58: 772–782.
41. Vrkoslav, V., K. Urbanová, and J. Cvaček. 2010. Analysis of wax ester molecular species by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A. 1217: 4184–4194.
42. Christie, W. W. 1987. The separation of molecular species of glycerolipids. In High-Performance Liquid Chromatography and Lipids: A Practical Guide. Pergamon Press, Oxford, 169–210.
43. Zábranská, M., V. Vrkoslav, J. Sobotníková, and J. Cvaček. 2012. Analysis of plant galactolipids by reversed-phase high-performance liquid chromatography/mass spectrometry with accurate mass measurement. Chem. Phys. Lipids. 165: 601–607.
44. Youssef, W., R. R. Wickett, and S. B. Hoath. 2003. Surface free energy characterization of vernix caseosa. Potential role in waterproofing the newborn infant. Skin Res. Technol. 9: 10–17.
45. Robson, K. J., M. E. Stewart, S. Michelsen, N. D. Lazo, and D. T. Downing. 1994. 6-Hydroxy-4-sphingenine in human epidermal ceramides. J. Lipid Res. 35: 2060–2068.
46. Okabe, H., and Y. Kishimoto. 1977. In vivo metabolism of ceramides in rat brain. Fatty acid replacement and esterification of ceramide. J. Biol. Chem. 252: 7068–7073.
47. Stránsky, K., T. Jursik, and A. Viték. 1997. Standard equivalent chain length values of monoenoic and polyenic (methylene interrupted) fatty acids. J. High Resolut. Chromatogr. 20: 143–158.
48. Oku, H., N. Yagi, J. Nagata, and I. Chinen. 1994. Precursor role of branched-chain amino acids in the biosynthesis of iso and anteiso fatty acids in rat skin. Biochim. Biophys. Acta. 1214: 279–287.

1-O-acylceramides in vernix caseosa
2173