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Scalable Synthesis of Human Ultralong Chain Ceramides

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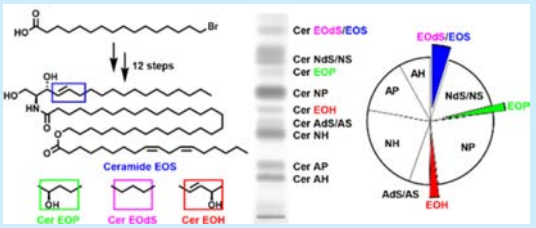
Supporting Information

ABSTRACT: Ceramides with ultralong chains (≥30 carbons), also known as acylceramides, play a critical role in the survival of mammals on dry land. An efficient and scalable synthesis of four major classes of ultralong human skin ceramides is reported. The key approach involves the use of a succinimidyl ester that acts as a protective group, helps overcome the extremely low solubility, and simultaneously activates the fatty acid for its clean and high-yielding attachment to a sphingoid base.

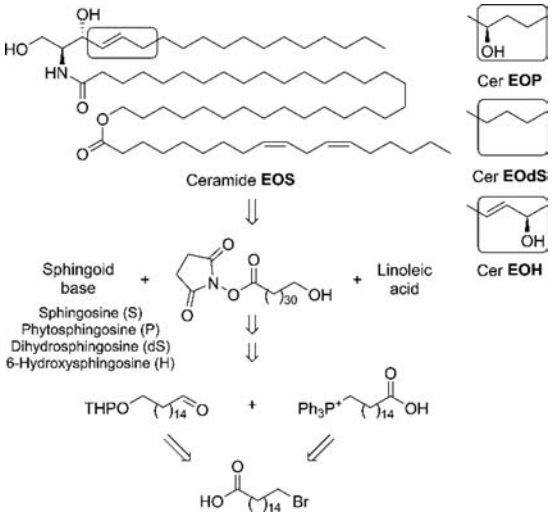


Ceramides (Cer) are central molecules in the sphingolipid metabolism. In mammals, Cer are important regulators in various cellular (e.g., cell growth, metabolism, differentiation, and death)[1] and extracellular (e.g., epidermal water barrier) processes.[2] Cer consist of a sphingoid base and a fatty acid attached to the amino group. The structural diversity of this group of lipids is highly intriguing; for example, in human skin, 15 different subclasses of free Cer have been identified,[3] resulting in over 400 molecular species of Cer when their chain length is also considered. The acyl chain length in Cer ranges from 14 to 24 carbons in most mammalian tissues; however, epidermal keratinocytes and male germ cells largely produce sphingolipids with ultralong chains (≥30 carbons).[4]

The biosynthesis of ultralong sphingolipids requires specific enzymes, such as elongase ELOVL4[5] and Cer synthase CERS3,[6] and is critical for the survival of terrestrial mammalian species. This is because ultralong Cer, which are minor but essential components of the extracellular lipid matrix in the uppermost skin layer, the *stratum corneum*,[7] protect the body against water loss and against the penetration of toxins, allergens, or bacteria.[2,3,7,8] Major defects in the biosynthesis of epidermal ultralong Cer lead to neonatal death.[6,9] Minor alterations in the levels of these lipids are found in several skin diseases, such as atopic dermatitis or lamellar ichthyosis.[3,10] The structures of the major free epidermal ultralong Cer classes are given in Scheme 1. They consist of a sphingoid base (sphingosine in **Cer EOS**, phytosphingosine in **Cer EOP**, dihydrosphingosine in **Cer EOdS**, and 6-hydroxysphingosine in **Cer EOH**),[11] an ultralong ω-hydroxylated fatty acid (with 28–34 carbons), and an ester-linked linoleic acid, which is also essential for their function.[12] For the shorthand nomenclature of epidermal Cer, see the Supporting Information.

A deeper understanding of the role of these unusually long sphingolipids in human (patho)physiology or the potential therapeutic use of such lipids is hampered by their limited availability. A few complete syntheses of acylCer have been reported,[13] but their synthesis remains a challenge because of their poor solubility and low reactivity. Isolation of these Cer compounds from the skin is also difficult. In this study, we developed a practical, high-yielding, and scalable synthesis of all the major subclasses of free ultralong epidermal Cer, namely **Cer EOS**, **Cer EOP**, **Cer EOdS**, and **Cer EOH**. We also show that the use of these authentic acylCer standards greatly improved quantitative analysis of human skin barrier Cer.

Scheme 1. Structures and Retrosynthesis of Ultralong Human Skin Ceramides



Scheme 1 shows the structures and retrosynthesis of target ultralong Cer. Linoleic acid, sphingosine, phytosphingosine, and dihydrosphingosine were commercially available, while the fourth sphingoid base, 6-hydroxysphingosine,[14] was prepared according to published procedures.[15] The key fragment is 32-

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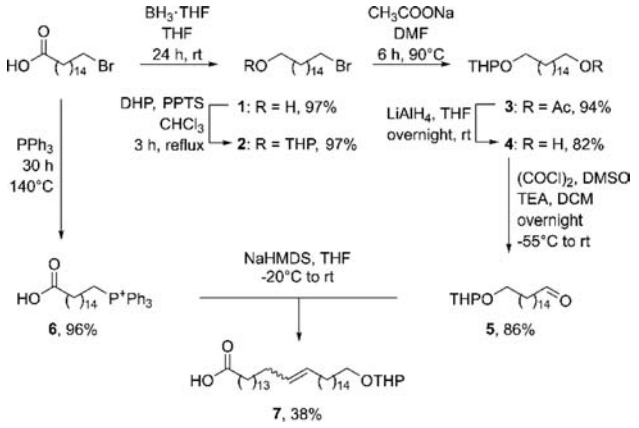
hydroxydotriacontanoic acid, which is the most abundant member of ω-hydroxy fatty acids in human acylceramides.[16]

Our synthesis started from 16-bromohexadecanoic acid, which is commercially available and inexpensive. First, we attempted to prepare free 32-hydroxydotriacontanoic acid using a coupling reaction of a Grignard compound and an alkyl halide in the presence of Li_2CuCl_4, as previously described.[13b,17] We did not obtain any product, although a similar reaction was previously successful for preparing chains containing up to 24 carbons.[17,18] Our second choice was the Wittig reaction because it was previously used for the construction of ultralong chains.[13a,b] 16-Bromohexadecanoic acid was reduced to alcohol **1** using a BH_3·THF complex, and the alcohol was protected with THP group (compound **2**, Scheme 2). The direct

Scheme 2. Synthesis of the Ultralong ω-Hydroxy Acid Backbone

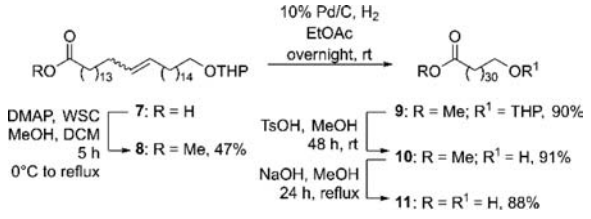


substitution of bromine for a hydroxyl group did not proceed well, and the yields were low, even after prolonged reflux in 2 M NaOH (virtually no product was detected). Therefore, we first converted bromide **2** to ester **3** and then reduced it to alcohol **4** in very good yields under mild conditions. Swern oxidation[19] of alcohol **4** provided aldehyde **5** in 86% yield. Dess–Martin oxidation[20] or oxidation using pyridinium chlorochromate led to lower yields (55% and 50%, respectively).

The second partner for the Wittig reaction, the phosphonium salt **6**, was directly obtained from the same starting material, 16-bromohexadecanoic acid, by its reaction with triphenylphosphine. The reaction proceeded best without a solvent (as a melt).[13b] The Wittig reaction was difficult due to the low solubility and reactivity of the 16-carbon fragments **5** and **6**. Despite numerous efforts, our best yield of acid **7** was only 38% using over 2 equiv of NaHMDS in THF. Other strong bases, such as NaH, LiHMDS, or LDA, were used with the same or lower yields. Attempts to protect the carboxyl yielded a difficult to purify waxy solid, causing even lower yields of the Wittig reaction. In 2012, Tashiro and Mori used the Grubbs metathesis for the formation of similar 30-carbon chains, but their yields were similarly low (45%).[13c] The starting compounds **5** and **6** can be easily prepared on a large scale; thus, we considered that this yield early in the reaction sequence was satisfactory.

The resulting E/Z isomers of unsaturated acid **7** were not separated because the acid was hydrogenated in the next step. The direct hydrogenation of acid **7** did not proceed (due to its low solubility). Protection using *tert*-butyldiphenylsilyl ester gave better results,[21] but the yields were low (approximately 20%) due to the low stability of these esters. Thus, we prepared methyl ester **8** of acid **7** using water-soluble carbodiimide (*N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide; WSC) in DCM/MeOH (Scheme 3). The yield of this reaction was

Scheme 3. Synthesis of 32-Hydroxydotriacontanoic Acid



47%; however, prolonging the reaction time led to decomposition of ester **8**. Other attempts to prepare ester **8** gave even lower yields or did not proceed at all. Esterification of **7** under acidic conditions led to the deprotection of THP or decomposition. Methylation of **7** using methyl iodide or dimethyl sulfate (in the presence of a base) led to very low yields (5% and 15%, respectively), even after the temperature was increased or the reaction time was prolonged. Reaction with diazomethane provided **8** in 50% yield, but the capacity of the diazomethane generator limited its use in a larger scale preparation of **8**. The methylation was followed by catalytic hydrogenation of **8** using Pd/C with a high yield of ester **9** without requiring further purification. The THP group was removed, and gratifyingly, hydroxy ester **10** was sufficiently pure after simple crystallization from $CHCl_3$/MeOH. The methyl ester was removed using methanolic NaOH to yield 32-hydroxydotriacontanoic acid **11**. This acid is a natural constituent of the skin barrier. It is attached to the glutamate residues of involucrin via its ω-hydroxyl and constitutes a part of the corneocyte lipid envelope.[16b]

Our original plan was to continue the synthesis of the target Cer from acid **11**, but the conversion of this acid to a suitable derivative was nearly impossible due to its low solubility and low reactivity. Thus, we searched for a suitable protective group that could be introduced earlier in the synthetic procedure and could increase the solubility of the ultralong acid, possibly overcoming the low yield of methylester **8**. We speculated that using succinimidyl ester, which is commonly used for carboxyl activation in *N*-acylation reactions,[22] could serve this purpose and could be sufficiently stable during the subsequent reactions. Then, in the final step, succinimidyl ester will be directly used for the formation of the target Cer. An optimal change to the reaction sequence appeared to be a conversion of the product of the Wittig reaction, unsaturated acid **7**, to its succinimidyl ester **12** (Scheme 4) (we also attempted to introduce this group earlier or later in the reaction sequence but without improvement). The best method of forming succinimidyl ester **12** was using disuccinimidyl carbonate with DIPEA. This reaction leads to a 64% yield on a large scale (on a scale of approximately 100 mg, the yield can be improved up to 80%). Using *N*-hydroxysuccinimide with WSC gave lower yields of ester **12**. Hydrogenation of **12** gave ester **13** without cleavage of the succinimidyl group. The reaction was completed within 48 h with 81% yield on a gram scale. On a scale of up to 100 mg, the reaction yield was over 90%. The ratio of the starting compound and product was followed by ^{1}H NMR because the

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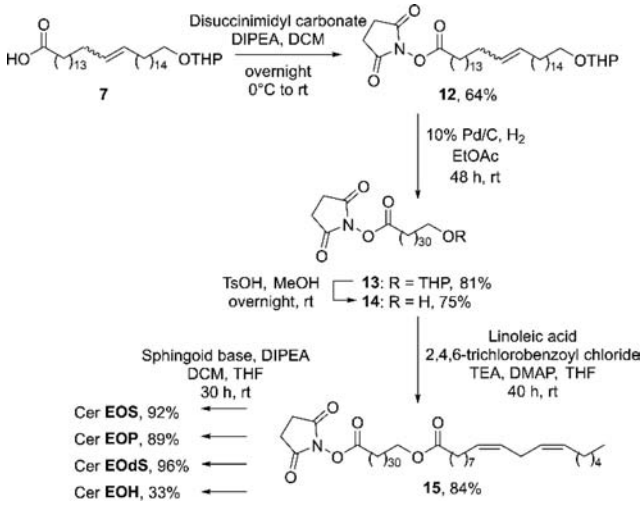
Scheme 4. Synthesis of the Target Ultralong Cer EOS, Cer EOP, Cer EOdS, and Cer EOH



R_f values of **12** and **13** were the same. After acidic deprotection of THP, succinimidyl ester **14** was obtained.

In the next step, we planned to attach linoleic acid to the activated ω-hydroxy acid **14** prior to its reaction with the sphingoid base to overcome the requirement to protect/deprotect the sphingoid bases[13c,23] because some of the compounds for this step are the most expensive and unstable components in the synthesis. This step leading to diester **15** appeared to be surprisingly difficult. Numerous reagents and conditions, as follows, were tested without success: linoleoyl chloride, linoleic acid with WSC, Mitsunobu reaction,[24] reaction in ionic crystals,[25] or activation using 2-chloro-1-methylpyridinium iodide.[13b] Finally, the Yamaguchi esterification was shown to be an excellent reaction to form the linoleoyl ester **15** in very high yield (over 80%).[26] Compound **15** served as a precursor for all four natural ultralong Cer. **Cer EOS**, **Cer EOP**, **Cer EOdS**, and **Cer EOH** were obtained by reacting the precursor **15** with the respective sphingoid base in the presence of DIPEA with very high yields, mostly above 90%, which is better than the yield in previous reports using *p*-nitrophenyl esters[27] or EEDQ.[13b] The only exception was **Cer EOH** because of the instability of the starting 6-hydroxysphingosine base.

We further used the prepared acylCer as authentic standards to improve quantitative Cer analysis. The analysis of barrier Cer in healthy and diseased skin is essential for understanding the (patho)physiological principles regarding how skin homeostasis is maintained/disturbed.[9,28] However, most of the analytical techniques, primarily including HPTLC analysis[9,28a,b] and the new LC–MS techniques,[29] rely on the few commercially available Cer subclasses. We show that using other Cer subclasses for semiquantitation, which is a common practice in HPTLC analysis, leads to errors of up to several hundred percent (Supplementary Figure S2). Within the calibration range (0.1 μg–2 μg), the errors in quantitation are up to 230% (mean 126%) for Cer EOS according to the detector response to Cer NS, up to 250% (mean 64%) for Cer EOP quantitated according to Cer NP, and up to 306% (mean 145%) for Cer EOdS quantitated according to Cer NdS. The worst results were obtained for Cer EOH in which the use of any other Cer subclass led to a marked overestimation of the levels of this lipid class. Thus, the use of appropriate standards is mandatory (see the Supporting Information for details). Figure 1 shows the HPTLC analysis of isolated human skin barrier Cer

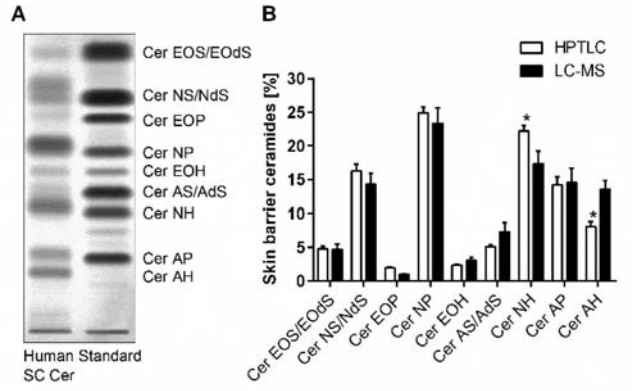


Figure 1. Analysis of Cer subclasses in the human skin barrier. (A) HPTLC plate of a sample of isolated human *stratum corneum* Cer (left) and the corresponding standard Cer (right). (B) Quantification of Cer subclasses based on HPTLC analysis using the prepared Cer standards (mean ± SEM, n = 6) compared with recent data obtained by LC–MS analyses.[29]

compared with Cer standards (panel A), confirming that the prepared acylCer are found in the human skin barrier. Using this widely available and simple technique, nine human Cer subclasses were separated. We did not separate the dihydroCer (Cer EOdS, Cer NdS, and Cer AdS), which coelute with their respective unsaturated Cer (Cer EOS, Cer NS, and Cer AS, respectively). Using these authentic Cer standards, we quantified eight Cer subclasses (Cer AH was determined semiquantitatively using Cer NH). Figure 1B shows a comparison of our isolated and purified human skin barrier Cer fraction with recently published Cer profiles established by LC–MS methods[29] (the only significant differences were found in Cer NH and Cer AH), which are in excellent agreement given the different skin samples analyzed.

In summary, we prepared all the major classes of natural ultralong Cer (**Cer EOS, Cer EOP, Cer EOdS, Cer EOH**). The overall yield of this 12-step synthesis is 11% on a small scale (tens to hundreds of milligrams) and 7% on a gram scale (up to the last precursor **15**; the final Cer were prepared in amounts immediately required for experiments). The key concept is the use of a succinimidyl ester, which acts as a protective group, overcomes the problems of extremely low solubility, and simultaneously activates the fatty acid for its clean and high-yielding attachment to a sphingoid base. This efficient and scalable synthesis and improved analysis introduces a pathway to further explore the biophysical and biochemical properties of ultralong sphingolipids and, consequently, the reasons that the fatty acids are elongated to over 30 carbons in Nature.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02816.

Synthetic and purification procedures and characterization of the compounds; isolation of *stratum corneum* barrier lipids and their analysis (PDF)

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Notes

The authors declare no competing financial interest.

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