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Ceramides with a pentadecasphingosine chain and short acyls have strong permeabilization effects on skin and model lipid membranes

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ABSTRACT

The composition and organization of stratum corneum lipids play an essential role in skin barrier function. Ceramides represent essential components of this lipid matrix; however, the importance of the individual structural features in ceramides is not fully understood. To probe the structure–permeability relationships in ceramides, we prepared analogs of *N*-lignoceroylsphingosine with shortened sphingosine (15 and 12 carbons) and acyl chains (2, 4 and 6 carbons) and studied their behavior in skin and in model lipid membranes. Ceramide analogs with pentadecasphingosine (15C) chains were more barrier-perturbing than 12C- and 18C-sphingosine ceramides; the greatest effects were found with 4 to 6C acyls (up to 15 times higher skin permeability compared to an untreated control and up to 79 times higher permeability of model stratum corneum lipid membranes compared to native very long-chain ceramides). Infrared spectroscopy using deuterated lipids and X-ray powder diffraction showed surprisingly similar behavior of the short ceramide membranes in terms of lipid chain order and packing, phase transitions and domain formation. The high- and low-permeability membranes differed in their amide I band shape and lamellar organization. These skin and membrane permeabilization properties of some short ceramides may be explored, for example, for the rational design of permeation enhancers for transdermal drug delivery.

1. Introduction

The stratum corneum (SC), the uppermost skin layer, provides a barrier against water loss and the entrance of environmental agents. This layer is formed by keratin-enriched corneocytes embedded in a lamellar lipid matrix, which contains an approximately equimolar mixture of ceramides (Cer), free fatty acids (FFA) and cholesterol (Chol). This unique lipid composition with high Cer content is essential for the barrier function of skin [1–3]. For example, lower Cer levels and altered Cer profiles are associated with skin diseases, such as atopic dermatitis and psoriasis [1,4]. In addition to their role in the skin, Cer are also known for their importance in cell signaling processes, such as apoptosis, growth, senescence and differentiation [5,6].

Our knowledge of the structural requirements of Cer for preventing water loss and excessive skin permeability is still rather limited, partly because Cer form a large and diverse class of sphingolipids: in human skin, 15 Cer subclasses have been described [7–9]. The common features of Cer are a relatively small polar head, the ability to create an extensive hydrogen bonding network and strong hydrophobic interactions between their very long saturated hydrocarbon chains, which are probably responsible for the tight arrangement of SC lipids. Previously, we showed that the very long acyl chain in sphingosine Cer (Cer NS) is crucial for a competent skin barrier: its shortening led to an increase in skin permeability with maxima for the Cer with a 4–6C acyl chain length [10,11]. These results were also confirmed using model lipid membranes [12]. In a subsequent study, increased permeability was also found in membranes where Cer with very long 24C chains were replaced by Cer with long 16C chains [13]. A combination of biophysical techniques suggested that compared to native long Cer, Cer with shortened acyls led to a decrease in the proportion of tight orthorhombic packing and a separation of their continuous short Cer-enriched domains with shorter lamellar periodicity. Surprisingly, no such marked increase in permeability upon acyl chain shortening was observed in a series of short dihydroCer [14].

In this work, we focus on the other hydrophobic chain in Cer molecules, the sphingoid base. In our initial study, we observed increased skin permeability upon the treatment of Cer with 12C-sphingosine and 2–6C acyl compared to untreated controls [11]. We were interested in whether further modulation of the sphingosine chain would further increase the barrier-disruptive properties of Cer with short acyl chains (or whether we already reached a limit beyond which no further increase in the permeability of either skin or a model membrane will

Abbreviations: ATR, attenuated total reflectance; Cer, ceramide(s); Chol, cholesterol; CholS, cholesterol sulfate; DFFA, perdeuterated fatty acid(s); FFA, free fatty acid(s); FTIR, Fourier-transform infrared spectroscopy; IND, indomethacin; SAXD, small-angle X-ray diffraction; SC, stratum corneum; TH, theophylline; WAXD, wide-angle X-ray diffraction.

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occur). We believe that defining the Cer structure–permeability relationships is important for the future design of skin barrier modulators, including barrier repair agents [15] and transdermal permeation enhancers [16].

Thus, the aim of this study was to synthesize Cer analogs with shortened sphingosine (with 15 carbons) and acyl (2–6 carbons) chains and evaluate their effects on the skin permeability in vitro for comparison to Cer containing 18C- [12] and 12C-sphingosines [11] (Fig. 1). Fig. 1B shows the Cer abbreviations that we use throughout this manuscript: the first number indicates the sphingosine length, and the second number refers to the acyl chain (e.g., Cer18/24 indicates a Cer with 18C-sphingosine chains and 24C acyls). To obtain a more detailed insight into how these short Cer act on a molecular level, we also incorporated the Cer into model SC lipid membranes and studied their permeability. We observed strong skin permeabilization effects of 15C-sphingosine Cer; these effects were even more pronounced in model lipid membranes. To capture any possible trends between membrane biophysics and permeability, we probed the biophysical parameters of the model membranes by Fourier-transform infrared spectroscopy (FTIR) using unlabeled and deuterated lipids and by powder X-ray diffraction in the small-angle (SAXD) and wide-angle (WAXD) ranges.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). TLC plates (Silica gel 60 F_{254}, aluminum back), Silica gel 60 (230–400 mesh) for column chromatography and HPLC columns were obtained from Merck (Darmstadt, Germany). Cer12/2, Cer12/4 and Cer12/6 were prepared in our previous study [11]. Cer18/2, Cer18/4, Cer18/6 and Cer18/24 were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used as received.

2.2. Synthesis of 15C-sphingosine ceramides (Cer15/2, Cer15/4 and Cer15/6; Fig. 1)

The 15C-sphingosine Cer were synthesized by alkynylation of Garner's aldehyde [11,17]. The synthetic scheme (panel A) and list of the studied compounds (panel B) are shown in Fig. 1. The structure and purity of the synthesized compounds were confirmed by FTIR (Nicolet Impact 400 spectrophotometer, Thermo Scientific, Waltham, MA, USA) and ^{1}H and ^{13}C NMR spectra (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C, Palo Alto, CA, USA). The melting points were measured using a Kofler apparatus and are uncorrected.

(*S*)-3-*tert*-butyl-4-methyl-2,2-dimethyloxazolidin-3,4-dicarboxylate (**2**). A solution of *N*-(*tert*-butoxycarbonyl)-L-serine methyl ester **1** (20.0 g, 91.2 mmol), 2,2-dimethoxypropane (DMP, 21.4 g, 205.0 mmol) and p-toluenesulfonic acid monohydrate ($TsOH \cdot H_2O$, 0.1 g, 0.5 mmol) in toluene was heated under reflux for 60 min. Additional DMP (6.8 g, 65.3 mmol) was added, and the mixture refluxed for another 30 min. A part of the solvent was distilled off, and the cooled solution was partitioned between a saturated $NaHCO_3$ solution (100 ml) and diethyl ether (3 × 200 ml). The combined organic layers were washed with saturated $NaHCO_3$ solution (200 ml) followed by brine (120 ml), dried with Na_2SO_4, filtered and concentrated in vacuum. The crude product was purified on silica using 1:1 ethyl acetate/hexane to give a colorless oily product. Yield = 60%, R_f = 0.72 (1:1 hexane/ethyl acetate). The spectra were in accordance with Ref. [17].

(*S*)-*tert*-butyl-4-formyl-2,2-dimethyloxazolidin-3-carboxylate (**3**). The oxazolidine ester **2** (12.0 g, 46.3 mmol) was dissolved in dry toluene (90 ml) and cooled to −78 °C under nitrogen. A 1.0 M solution of diisobutylaluminium hydride (DIBAL-H) in toluene (80 ml) was slowly added to keep the temperature under −65 °C. The reaction mixture was stirred for 2 h at −78 °C under nitrogen until the TLC analysis (4:1 hexane/ethyl acetate, R_f = 0.4) showed that the reaction was complete. The reaction was quenched by the slow addition of methanol (20 ml) to keep the reaction temperature under −65 °C. The resulting white emulsion was slowly poured into ice-cold 1 M HCl (280 ml) and extracted with ethyl acetate (3 × 250 ml). The combined organic layers were washed with saturated NaCl (150 ml), dried with Na_2SO_4, filtered and concentrated in vacuum. The product was separated on a silica column (4:1 hexane/ethyl acetate) as an oily liquid. Yield = 96%. The spectra were in accordance with Ref. [17].

(*S*)-*tert*-butyl-4-((*R*)-1-hydroxytridec-2-yn-1-yl)-2,2-dimethyloxazolidin-3-carboxylate (**4**). Hexamethylphosphoramide (HMPA, 5.50 g, 30.7 mmol) and Garner's aldehyde **3** (3.50 g, 15.3 mmol) in THF were added to the mixture of 1.6 M solution of butyl lithium (BuLi) in hexane (13.7 ml, 21.9 mmol) and dodec-1-yne (3.05 g, 18.3 mmol) in dry THF (25 ml) at −50 °C under nitrogen, and the mixture was stirred for 2 h. The reaction was warmed to room

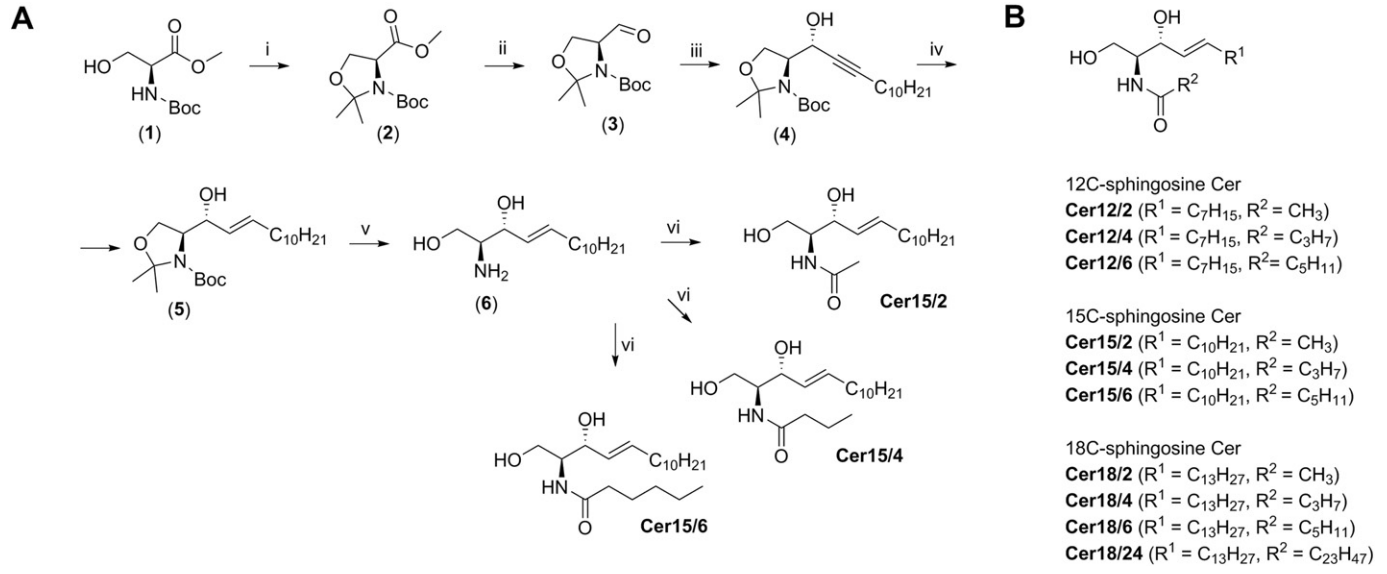

Fig. 1. Preparation of 15C-sphingosine Cer (panel A) and a list of the studied Cer analogs (panel B). Reagents and conditions: i) DMP, TsOH, 100 °C, toluene, 90 min (60%); ii) DIBAL-H, −78 °C, toluene, 120 min (96%); iii) dodecyne, BuLi, HMPA, −50 °C, THF, 60 min (61%); iv) Li, $EtNH_2$, −78 °C, 180 min (70%); v) 1 M HCl/dioxane, 100 °C, 120 min (88%); and vi) acyl chloride or succinimidyl ester, CH_2Cl_2, overnight (51–68%).