Romanian Biotechnological Letters *Vol. 16, No. 5, 2011*

ORIGINAL PAPER

Preparation of Ceramide and Sphingosine by Chemical and Biochemical Methods – An Instrument for the Evaluation of Compatibility between Sphinganine and Fatty Acids

Received for publication, September 20, 2011
Accepted, April 12, 2011

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Key words: ceramide, sphingosine, N-acylation, ^{1}H and ^{13}C NMR spectra.

Abstract

Two complementary methods for the preparation of ceramide and sphingosine have been elaborated. (i) Galacto- or glucocerebrosides were oxidized with periodate and the newly formed aldehyde groups were reduced to alcohols by reaction with $NaBH_4$. By this treatment, susceptibility of sugar cleavage residues to acidic hydrolysis was increased, so they were removed by mild acidic hydrolysis. The product of this ensemble of reactions was ceramide but it could be converted to sphingosine by a stronger acidic hydrolysis. (ii) Sphingolipids (sphingomyelin, glucocerebrosides, galactocerebrosides), isolated from adequate sources, were cleaved to their chemical constituents by an energetic acidic hydrolysis. From this mixture, sphingosine was isolated by partition and column chromatography, and N-acylated with the choice fatty acid. Finally, both ceramides and sphingosine were analysed by chemical and physical methods (IR, NMR) per se or in peracetylated form. The implications of a mathematical equation describing molecular diversity of ceramides, in connection with their biochemical transformations has been also discussed.

Introduction

Sphingosine ((2S,3R,4E)-1,3-dihydroxy-2-amino-4-octadecene) and ceramide (N-fatty acyl sphingosine) were discovered by Thudichum as complete or partial hydrolysis products of cerebroside (β-D-galactopyranosyl-1'-ceramide) or sulfatide (β-D-(3-O-sulfo)galactopyranosyl-1'-ceramide) [1]. However, their structure was elucidated concomitantly with the structure of galactocerebroside and sphingomyelin [2,3,4]. Confirmation of the structure of sphingosine and ceramide was completed by their total chemical synthesis [5,6], and even more by the synthesis of cerebrosides [7] and other glycosphingolipids [8]. Biosynthesis and catabolic paths of ceramide and its enzymatic cleavage to sphingosine can be also considered structural arguments, since metabolical pathways constitute themselves as such as they become more complete [9].

A remarkable diversity of chemical modifications has been undertaken on ceramide and sphingosine *per se* or within glycosphingolipids. In fact, all chemical transformations made on sphingolipids could be applied on ceramides. Removing of fatty acid from glycosphingolipids produces the so-called lyso-derivatives [10,11]. Although initially they were prepared by chemical means, subsequently it was found that they are natural compounds [12,13]. By linking a single type of fatty acid to the amino group, a more homogenous ceramide/glycosphingolipid has been obtained [14]. Hydrogenation of sphingosine with tritium gave rise to radioactively labeled glycosphingolipids [15]. Limitations of this method

were demonstrated, since some biological activities of sphingosine essentially depend on *trans*-double bond [16]. Tritium was introduced in the molecule of sphingosine within sphingolipids by a more subtle method: hydroxy group on C-3 was selectively oxidized to a keto group and then reconstituted by tritiation [17,18]. By a similar method, ^{3}H was introduced to C-1 of sphingosine [19]. The substitution of N-acyl group from ceramides with an N-alkyl one (N-hexyl), disclosed new biochemical properties, i. e. inhibition of glucosyl ceramide β-glucosidase [20].

In this paper two methods for the preparation of ceramide and sphingosine have been elaborated. The products were characterized by chemical and physical (IR, NMR) methods. Biochemical methods for the approach of the two compounds are also comparatively discussed, in connection with a mathematical equation describing molecular diversity of ceramides and gangliosides.

Materials and methods

Materials

Galactocerebroside (**i**) and sulfatide were prepared from bovine brain as indicated [14,21,22], glucocerebroside from soybean [23] and sphingomyelin was from Sigma. All the other reagents, solvents or materials – $CDCl_3$ containing TMS, $NaIO_4$, $NaBH_4$, HCl, NaOH, chloroform, methanol, ethanol, hexane, diethyl ether, Ac_2O, pyridine, ethylene glycol, silica gel for column chromatography, ready-to-use glass plates covered with silica gel 60 for thin layer chromatography (TLC) – were either from Sigma or Merck.

Methods

NMR Spectra Registration

^{1}H and ^{13}C NMR spectra of synthesis intermediates were registered in $CDCl_3$ containing TMS.

A. One-Dimensional NMR Studies

NMR experiments were performed on a Bruker Avance DRX 400 spectrometer using 400 and 100 MHz for ^{1}H and ^{13}C frequencies, respectively.

B. Two-Dimensional NMR Experiments

The ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe.

IR spectra were recorded as KBr pellets or Nujol solutions on a Bruker Equinox 55 FT-IR spectrometer.

TLC was made with solvent systems and visualization agents formerly indicated [21]. Moreover, two solvent systems, particularly suitable for ceramides have been used: $CHCl_3$-MeOH-AcOH (65:2.5:4, v/v) and $CHC1_3$:MeOH:NH_4OH (80:20:2, v/v).

Ceramide (N-fatty acyl sphingosine). Gluco- or galactocerebrosides (**i**) (40-50 mg) were solved in 1 mL of chloroform and 3.5 mL ethanol and oxidized [24,25] for 22 hr at room temperature in the dark with 2 mL solution of $NaIO_4$ (0.5 M). Then enough ethylene glycol in 6 mL water was added to destroy the excess of periodate. Upper phase, containing salts and formic acid, was discarded, and a solution consisting of 6 mL methanol, 3.5 mg $NaBH_4$ and 2 mL 0.1 N NaOH was added to the lower phase and left for 22 hr at room temperature to reduce **ii**. This treatment was destined to reduce the aldehyde residues produced by periodate oxidation of pyranoside. Partial acidic hydrolysis of **iii** was made by adding 0.5 mL 6 M HCl and preserving overnight at room temperature. By mixing the latter solution with 10 mL chloroform and 15 mL water, two layers appeared, the lower one containing the ceramide (**iv**). The ceramide solution was dried, filtered, evaporated to dryness and the residue chromatographed on a column of silica gel in a gradient of methanol in chloroform. Finally, a small portion of prepared ceramide was peracetylated and NMR spectra registered.

Sphingosine. Galacto-, glucocerebrosides or sphingomyelin (**v**) were heated for 18 hr at 70°C [26] in a solution of 1 N HCl in methanol containing 10 M water. Fatty acids formed by hydrolysis as well as their methyl esters were separated by extraction with hexane. Then the solution was brought to alkaline pH by adding 5 N NaOH, and sphingosine was extracted with diethyl ether. The sphingosine solution was dried, filtered and the long chain base was separated by column chromatopgraphy. A small portion was analyzed by NMR in peracetylated form.

Ceramide reconstruction. Sphingosine was reacted with lauroyl chloride in the presence of a base catalyst [11]; the synthetic ceramide (**vi**) was submitted to mild alkaline hydrolysis and purified by column chromatography on silica gel.

Results and discussion

The two ways to arrive at ceramide and sphingosine are presented in Fig. 1.

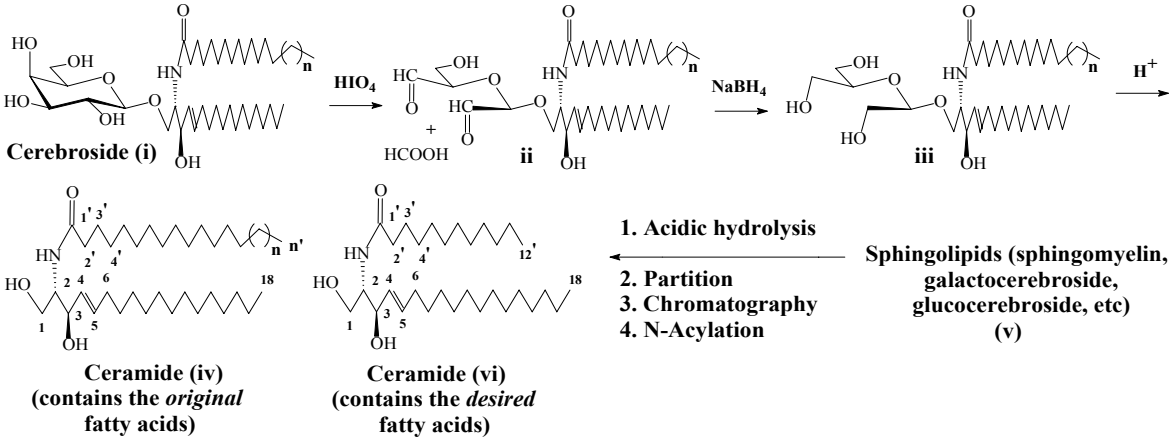

Figure 1. Two independent preparative ways to arrive at ceramide and sphingosine.

Removing of sugar from galactocerebroside (R_F 0.20) produced a less polar compound, ceramide (R_F 0.75), while cleavage of the latter compound gave rise to sphingosine, and the polarity increased again, still to a different level from galactocerebroside (R_F 0.41). Both compounds contained the envisaged IR absorption bands i. e. ceramide: 3250 cm^{-1} (NH and OH), 2890 and 2830 cm^{-1} (CH_2 and CH_3), 1650 and 1552 cm^{-1} (amide bands I and II), 1080 cm^{-1} and 1050 cm^{-1} (OH), 970 cm^{-1} (*trans* double bond). Sphingosine was devoid of absorption bands 1650 and 1552 cm^{-1}, it presented instead positive reaction to ninhydrin. In NMR spectra, the three constituents –sphingosine, sugar, fatty acid, interfere in a very small degree or at all, so they were clearly seen. NMR data of this paper were correlated with our previous results concerning glycosphingolipids [14,21,22] as well as with data from biochemical literature [4,16].

Ceramide (N-fatty acyl sphingosine)(Fig. 1). ^{1}H-NMR ($CDCl_3$; δ ppm; *J* Hz): 0.86 (t, 6.8, terminal methyls of sphingosine and fatty acid), 1.26 (br s, methylenes groups of sphingosine and fatty acid), 1.98-2.05 (m, H-6), 2.01, 2.03 (s, Me group of Ac), 4.05 (m, H-1b), 4.31 (d, 6.4, H-1a), 4.44 (d, 7.6, H-2), 5.30 (dd, 7.2, 6.8, H-3), 5.38 (dd, 7.6, 4.8, H-4), 5.80 (d, 15.2, H-5), 6.34 (d, 8.8, NH).

^{13}C-NMR ($CDCl_3$; δ ppm): 14.1 (terminal methyls of sphingosine and fatty acid), 20.9 (Me group of Ac), 22.7 (C-17), 29.4-29.7 (intermediate methylenes groups of sphingosine and fatty acid), 31.9 (C-16), 32.5 (C-6), 50.6 (C-2), 61.2 (C-1), 73.2 (C-3), 129.9 (C-5), 137.3 (C-4), 169.7 (O=*C*-CH_3), 170.4 (O=*C*-NH).

Sphingosine ((2S,3R,4E)-1,3-dihydroxy-2-amino-4-octadecene). ^{1}H-NMR ($CDCl_3$; δ ppm; *J* Hz): 0.86 (t, 6.8, terminal Me group), 1.26 (br s, CH_2 intermediate groups), 1.43-1.50 (m, H-7), 1.97-2.09 (m, 2H, H-6), 2.05, 2.07, 2.09 (3s, CH_3 of Ac), 4.06 (m, H-1b), 4.30 (m, H-1a), 4.40 (m, H-2), 5.28 (m, H-3), 5.39 (d, 6.8, H-4), 5.76 (dt, 9.2, 3.6, H-5), 6.34 (d, 8.8, N*H*).

^{13}C-NMR ($CDCl_3$; δ ppm): 14.1 (terminal Me group), 20.6, 20.7, 20.8 (Me groups of Ac), 22.7 (C-17), 28.9-29.7 (intermediate CH_2 groups), 31.9 (C-16), 32.3 (C-6), 50.7 (C-2), 62.6 (C-1), 73.8 (C-3), 130.0 (C-5), 137.4 (C-4), 169.8 and 170.0 (O=*C*-CH_3), 171 (O=*C*-NH).

Ceramides constitute a vast and extremely diverse group of natural compounds. The high diversity of ceramides is conferred by the two constituents, sphingosine (or long chain base), a generic term, and fatty acid. Sphingosine is a chiral polyhydroxy hydrophobic base having 2-4 asymmetric carbon atoms per molecule. The most widespread and studied sphingosine is 4-sphingenine – trans 1,3-dihydroxy 2-amino 4-octadecene ((2S,3R,4E)-1,3-dihydroxy 2-amino 4-octadecene). Addition of a molecule of water to 4-sphingenine produces the so-called phytosphingosine, the prefix phyto being artificial, because the compound was found also in animal tissues. Chain length of sphingosine is also variable, 14-20 carbon atoms [27]; the degree of unsaturation oscillates from complete saturation (phytosphingosine) to two or even three double bonds, usually situated on C-4, C-8 and C-10. The hydrocarbon chain of sphingosine is alternatively linear, iso, anteiso or 9-methyl; anteiso hypostasis is associated with a supplementary chiral center [28] while 9-methyl group is found on an unsaturated carbon [29]. One aspect should be mentioned concerning linear, iso, anteiso forms: while ^{1}H NMR spectra cannot distinguish between the three types, this can be done by measuring δ value of terminal carbons in ^{13}C NMR spectra: linear, 14.2; iso, 22.8, both Me groups; anteiso, 11.4 (terminal Me) and 19.3 (anteiso Me) [28]. Even a cyclopropanated hydrocarbon chain has been found in sphingosine [30]. In reality, these factors – the degree of unsaturation, hydroxylation, chain form – change simultaneously and no wonder that in such circumstances at least 70 molecular types of sphingosine have been discovered till now [27].

Fatty acid molecules forming ceramides vary also in wide limits: chain length (14-26 or even 34-36 C), hydroxylation (2-hydroxy or ω-hydroxy), unsaturation (saturated or mono-, di-, tri-unsaturated), ramification (liniar, iso-, anteiso) [31-33]. The presence of ω-hydroxy fatty acids in ceramides should be stressed: they were found esterified with a polyunsaturated fatty acid (vitamin F) [32] and it seems extremely plausible that such esters triggered the use of ceramides in skin-care cosmetics [34].

It was stated, about two decades ago, that at least 300 natural glycosphingolipids contain a ceramide in their molecule [35,36]. So their natural variety is of the same order of magnitude, or even higher, as consecrated classes of compounds i. e. aminoacids and nucleotides.

A mathematical equation destined to evaluate molecular diversity of gangliosides has been elaborated [37]. This equation contained a factor, $m \times n \times b$, for the calculation of the number of ceramides that could be formed from *m* types of sphingosines (long chain bases) and *n* types of fatty acids, b being a correction factor, $0 < b \leq 1$. Among a series of example presented in the latter work was this one: in ganglioside GM3 from equine kidney, 17 types of fatty acids and 10 types of long chain bases were found [38]; for b = 1, a number of 170 types of ceramides should be produced. The question was raised if all the theoretical forms of ceramides in this tissue were really biosynthesized. Genetic analysis of ceramide synthases, combined with studies concerning substrate specificity of these enzymes, gave a partial answer to this question, in the last decade [39]. In mammalian tissues, ceramides are synthesized by a family of six ceramide synthases [40], each one using a relatively restricted subset of fatty-acyl-CoAs for N-acylation of the sphingoid long chain base [41,42]. The best studied of these enzymes are ceramide synthases 1 and 5 and they synthesize C18- and C16-ceramide, respectively. On the other hand, ceramide synthases 2 used longer acyl-chain CoAs (C20-C26), showing no activity with C16:0-CoA and very low activity with C18:0-CoA [39]. Demonstration that

sphingosine 1-phosphate was an inhibitor of the latter enzyme [39] showed that ceramide production in a tissue constitutes a complex phenomenon with a complex determinism.

The oxidative-reduction-hydrolysis method [24,25] for ceramides preparation is relatively laborious, still important since all N-fatty acyl linkages from the original glycosphingolipid are preserved. In this way, the picture of relative compatibility between sphingosine and fatty acids can be obtained. Moreover, this method can be indirectly extended to other neutral and acidic glycosphingolipids [43]. Two limits of the oxidative-reduction-hydrolysis method should be mentioned: (i) different steps are not quantitative; (ii) ceramides based on phytosphingosine are destroyed in the periodate oxidation step. This method has been appreciably improved by the incidence of enzymatic processes. An enzyme, endoglycoceramidase (EC 3.2.1.123), cleaving the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids has been found in animals belonging to *Cnidaria*, *Mollusca*, and *Annelida* as well as in microorganims (*Rhodococcus*) [44-47]. At least endoglycoceramidase from *Rhodococcus* [45], jellyfish (*Cyanea nozakii*) [46] and hydra (*Hydra magnipapillata*) [47] were cloned. Is seems extremely plausibly that the exhaustive use of these enzymes as biochemical and analytical tool, concomitantly with other instruments (NMR spectroscopy, MS, chromatography, immunology) will complete the elucidation of this, yet mysterious, phenomenon – compatibility between sphingosine and fatty acids within sphingolipids.

Conclusions

1. Controlled degradation of cerebrosides by oxidation-reduction-hydrolysis, produces ceramides in preparative amounts;
2. Acidic hydrolysis of sphingolipids, followed by partition and chromatography give rise to sphingosine;
3. Ceramide with a single type of fatty acid can be obtained by acylation in alkaline environment;
4. ^{1}H and ^{13}C NMR Spectroscopy of ceramide and sphingosine disclosed all the envisaged chemical functions of prepared compounds.

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