N-Acyl migration in ceramides

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Abstract **Upon exposure of truncated ceramides, such as *N*-acetyl-sphingenine, and long-chain ceramides to moderate acidic conditions, three derivatives are formed. Two of them turned out to be *O*-acylated sphingenine, 1-*O*- and 3-*O*-acyl-sphingenine, and the other was identified as sphingenine. Truncated dihydroceramides (e.g., *N*-acetyl- and *N*-hexanoyl-sphinganine) also show this type of rearrangement, which is therefore not related to the presence of the allylic hydroxy group or to the length of the *N*-acyl chain. Of particular concern is the fact that the *O*-acylated compounds, which can be considered sphingoid base analogs, can be formed in chloroform or chloroform-methanol mixtures upon storage. For long-term storage, methanol or dichloromethane is the preferred solvent. A procedure to document the presence/formation of such *O*-acylated sphingoid bases in ceramide solutions in the picomole range, based on reversed-phase chromatography after derivatization of their amino group with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, is presented.—** Van Overloop, H., G. Van der Hoeven, and P. P. Van Veldhoven. ***N*-Acyl migration in ceramides.** ***J. Lipid Res.*** **2005.** 46: **812–816.**

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When analyzing lipid kinase activity in bacterial lysates using labeled truncated ceramide analogs, the presence of unknown labeled compounds was noticed in the organic extracts. With *N*-[^{14}C]acetyl-sphingenine, prepared as described previously (1), as substrate, two additional radioactive spots, one with a lower (derivative X) and one with a higher relative mobility (R_f; derivative Y) than the substrate, were observed after TLC in an acidic solvent (**Fig. 1A**). These lipids did not comigrate with any known commercially available sphingolipid. As the formation of these derivatives was not dependent on the presence of bacterial proteins (Fig. 1A), the reaction and extraction conditions were investigated. This revealed that the acidification of the assay mixture with HCl, to halt the reaction and to improve the extraction of the ceramide phosphate in the organic phase, was to blame. This was confirmed using unlabeled *N*-acetyl-sphingenine. After treatment of this lipid with 0.5 N HCl, three extra iodine-reactive spots were formed (Fig. 1B). One derivative comigrated with sphingenine; the others resembled derivatives X and Y with regard to R_f values. All three stained with ninhydrin, suggesting that a migration of the acetyl group from the amino to one of the two adjacent hydroxy groups of the sphingoid base gave rise to the unknown ceramide derivatives X and Y (**Fig. 2**). The conversions were concentration dependent and were even noticed when using 0.1 N HCl (Fig. 1B, C; **Table 1**). Other acids, such as H_2SO_4 and $HClO_4$, in equivalent normality as 0.5 N HCl, also affected *N*-acetyl-sphingenine, but 5% acetic acid and 0.5 N H_3PO_4 did not (Fig. 1B, C; data not shown).

Attempts to isolate the unknown lipids by column chromatography or to characterize them by gas chromatography-mass spectrometry were not successful, probably because of (some) reversions during these steps. However, mass spectral analysis (positive mode, LCQ-duo MSn Ion-trap; ThermoFinnigan) of the spots on the silica plate revealed that the parent *N*-acetyl-sphingenine and derivatives X and Y possess the same mass [$(M+H)^+$ 342] and show similar fragmentation (main fragments 324, 282, 264), in support of the acyl migration. The more polar *O*-acyl derivative X likely represents 1-*O*-acyl-sphingenine, as it gave rise, upon in situ periodate treatment of the silica plate, to a fatty aldehyde (data not shown). Why the difference in mobility between the 1-*O*- and 3-*O*-acyl derivatives is so pronounced is not clear. Perhaps when the amino group is situated in the middle of the molecule, as in the 1-*O*-acyl derivative, a more polar compound is formed. This is consistent with our observations that 1-*O*-butanoyl-2-amino-1-hexadecanol, formed by HCl treatment of *N*-butanoyl-2-amino-1-hexadecanol, a ceramide analog lacking the secondary hydroxy group, has a lower R_f value than the *N*-acylated parent molecule in an acidic solvent (data not shown).

Acyl migration between adjacent amino and hydroxy groups is a well-known chemical reaction but has been somewhat overlooked in the research on ceramide-mediated biology. *N*→*O*-Acyl migration has been reported for

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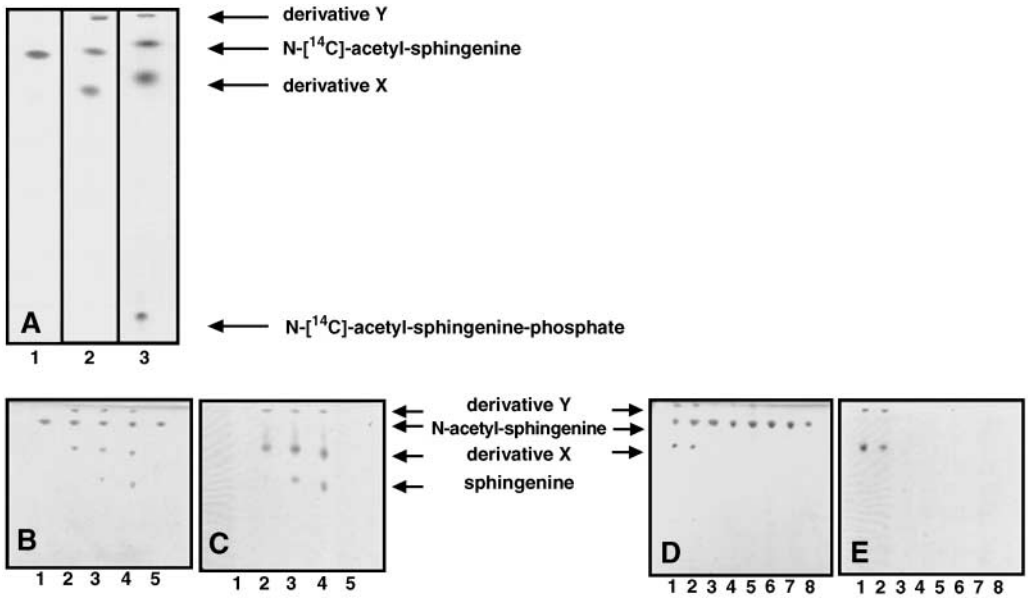

Fig. 1. TLC analysis of truncated ceramides. A: Autoradiograph of extracts from a ceramide kinase assay with *N*-[^{14}C]acetyl-sphingenine. The reaction mixture (200 μl, 100 μM substrate, 7,500 dpm/nmol) was stopped with 1.5 ml of chloroform-methanol (1:2, v/v), followed by phase separation by the addition of 0.7 ml of 0.5 N HCl, 2 M NaCl, and 1 ml of chloroform. The organic phase was analyzed by TLC (silica G plates); the solvent was chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1, v/v), and the plate was exposed to Hyperfilm MP (Amersham Bioscience). Lane 1, standard *N*-[^{14}C]acetyl-sphingenine; lane 2, extract from blank; lane 3, extract from reaction in the presence of bacterial lysate. B, C: *N*-Acetyl-sphingenine was dissolved at 2 mM in chloroform-methanol (1:1, v/v) containing the indicated amount of acid, kept at room temperature for 3 h, and spotted (40 μl) on silica G plates. After development in the system described in A, lipids were transiently visualized with iodine (B), followed by ninhydrin spray (C). Lane 1, 5% acetic acid; lane 2, 0.1 N HCl; lane 3, 0.5 N HCl; lane 4, 1 N HCl; lane 5, standard *N*-acetyl-sphingenine. D, E. *N*-Acetyl-sphingenine was dissolved at 2 mM in the indicated solvents (HPLC grade unless otherwise indicated), kept for 3 h at 60°C, and analyzed as described above (D, iodine; E, ninhydrin). Lane 1, chloroform (Biosolve); lane 2, chloroform-methanol (1:1, v/v); lane 3, methanol (Biosolve); lane 4, dichloromethane (Acros); lane 5, acetone (Fisher Chemicals); lane 6, acetonitrile (Biosolve); lane 7, toluene (Biosolve); lane 8, standard *N*-acetyl-sphingenine. All panels display TLC plates or corresponding autoradiographs scanned from start to front and converted to black-and-white images.

N-acylated amino alcohols (e.g., *N*-acetyl-ephedrine) (2) and for peptides containing β-hydroxy amino acids (serine or threonine) upon exposure to strong acids (3, 4). Linear peptides are converted into *O*-acyl peptides, and cyclic peptides are isomerized (e.g., cyclosporin A to isocyclosporin A). With regard to lipids, *N*→*O*-acyl migration was documented long ago in the chemical literature for ethanolamides (5). These fatty amides, now referred to as *N*-acylethanolamines, are produced from *N*-acylated phosphatidylethanolamine. Given their endocannabinoid action [e.g., anandamide (*N*-arachidonoyl-ethanolamine)] (6, 7), *N*-acylethanolamines are currently actively investigated, and recently, during their analysis in biological systems, acyl migration was (re)noticed (8). Conditions to induce *N*→*O* migration in anandamide, however, appear more drastic (anhydrous acid, significant heat, sonication) than those for ceramide rearrangements.

Another example of an *N*→*O*-acyl migration in lipids, explaining the instability of *N*-acetylated fumonisin B1, was reported by Norred et al. (9). Fumonisin B1 is a mycotoxin that is well known as a potent inhibitor of sphinganine *N*-acyltransferase (ceramide synthase) (10). Its backbone (2-amino-12,16-dimethyl-3,5,10,14,15-eicosanepentadiol) structurally resembles a sphingoid base, and a free amino group is required to be effective as an inhibitor. Upon storage of *N*-acetylated fumonisin B1, in solid form but especially in acidic solutions, *O*-acetylated fumonisin B1 is formed. As both 3-*O*- and 5-*O*-acetylated toxins are generated, migration is not limited to vicinal amino/hydroxy groups (9).

With regard to acyl migration in sphingolipids, some notes can be found in older papers dealing with their chemical analysis, normally done by acidic hydrolysis (1–2 N HCl, 75°C, 2–18 h). These drastic procedures result in various by-products of sphingoid bases (11). The isomerization at carbon 3 (*threo*-sphingoid bases) and the generation of *O*-methyl-sphingenine have been proposed to rely on the formation of a cyclic intermediate. Upon hydrolysis of lysosphingolipids, lacking the *N*-acyl chain and hence unable to form an oxazolidine ring, the by-products are not seen (12, 13). As far as we are aware, acyl migration under less drastic conditions has not been documented.

Given the abundant use of *N*-acetyl-sphingenine as a water-soluble ceramide analog, and of *N*-acetyl-sphinganine as an inactive analog, as well as various other ceramide analogs (14–18), it seemed of interest to document possible differences in migration between the various ceramides. Moreover, because it cannot be excluded that the *O*-acetyl

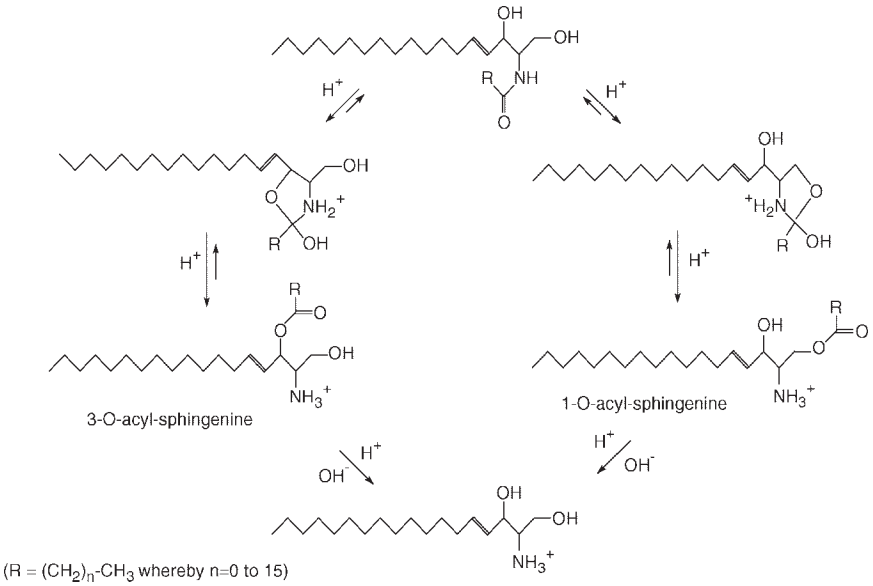

Fig. 2. Rearrangements of ceramide by acyl migration.

derivatives, which can be considered as sphingoid base analogs, possess bioactivity, it appears imperative to verify the stability of these ceramides during storage. To quantify low amounts of derivatives, TLC was not adequate. Therefore, the amino group of the *O*-acyl sphingoid bases was derivatized by means of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (19), followed by reversed-phase HPLC and postcolumn fluorescence detection, similar to a procedure described for the analysis of sphingoid bases (20).

For *N*-acetyl-sphingenine, after 3 h of exposure to 0.5 N HCl in chloroform-methanol (1:1, v/v) at room temperature, derivative X, derivative Y, and sphingenine were estimated at 12.8, 2.9, and 1.9%, respectively (Table 1; **Fig. 3B**). The conversions of *N*-[^{14}C]acetyl-sphingenine treated with 0.5 N HCl, based on the radioactivity associated with the derivatized products separated via HPLC or based on direct TLC analysis of the acidic ceramide solution, agreed fairly well (8.2% versus 7.9% for X, 3.7% versus 2.8% for Y).

TABLE 1. Influence of acid, unsaturation, and *N*-acyl chain length on *N*-acyl migration in ceramides

Compound	HCl (N)	Derivative X	Derivative Y	Sphingenine	Sphinganine
N-Acetyl-sphingenine[a] (2)[b]	0	0.0	0.0	0.0	N.D.[c]
N-Acetyl-sphingenine[a] (2)	0.1	4.7	0.9	0.3	N.D.
N-Acetyl-sphingenine[a] (2)	0.5	12.8	2.9	1.9	N.D.
N-Acetyl-sphingenine[a] (2)	1.0	13.0	3.3	4.0	N.D.
N-Acetyl-sphinganine[a] (1)	0.5[d]	13.3	8.2	N.D.	7.1
N-Hexanoyl-sphingenine[e] (2)	0.5[d]	12.5	2.6	1.0	N.D.
N-Hexadecanoyl-sphingenine[f] (2)	0.5[d]	10.6	2.0	0.7	N.D.

The indicated (dihydro)ceramides (6 nmol) were dissolved in 100 μl of chloroform-methanol (1:1, v/v) containing the indicated amount of acid and kept at room temperature for 3 h. After the addition of NH_4OH (2-fold molar excess over HCl) and extraction, an internal standard (700 pmol) was added, followed by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, as described in the legend to Fig. 3. The fluorescent derivatives were separated by reversed-phase chromatography on either a Symmetry C_{18} column (150 × 4.6 mm inner diameter, 5 μm; Waters) (*N*-acetyl-sphingenine and *N*-acetyl-sphinganine; 4-hydroxysphinganine as internal standard) or a Nova-Pak C_8 column (150 × 3.9 mm inner diameter, 4 μm; Waters) (*N*-hexanoyl-sphingenine and *N*-hexadecanoyl-sphingenine; sphinganine as internal standard), with fluorescence detection (excitation, 244 nm; emission, 398 nm). The *N*-acetyl-sphingenine products were eluted as described in the legend to Fig. 3. For the analysis of *N*-acetyl-sphinganine, buffer B was replaced by buffer C (acetonitrile-methanol, 90:10, v/v). For *N*-hexanoyl- and *N*-hexadecanoyl-sphingenine, the column was run isocratically for 5 min in buffer A, followed by gradient elution from 100% buffer A to 100% buffer C in 20 min, at a flow rate of 1 ml/min. Results, either single values or means based on two separate experiments, are expressed as percentages of the starting amount of (dihydro)ceramide, assuming that derivatization of the internal standard and the ceramide products, and their fluorescence yield, were identical.

[a] From Avanti Polar Lipids.
[b] Number of experiments.
[c] N.D., Not detected.
[d] In the absence of acid, no derivatives were detectable.
[e] From Acros.
[f] From Larodan.

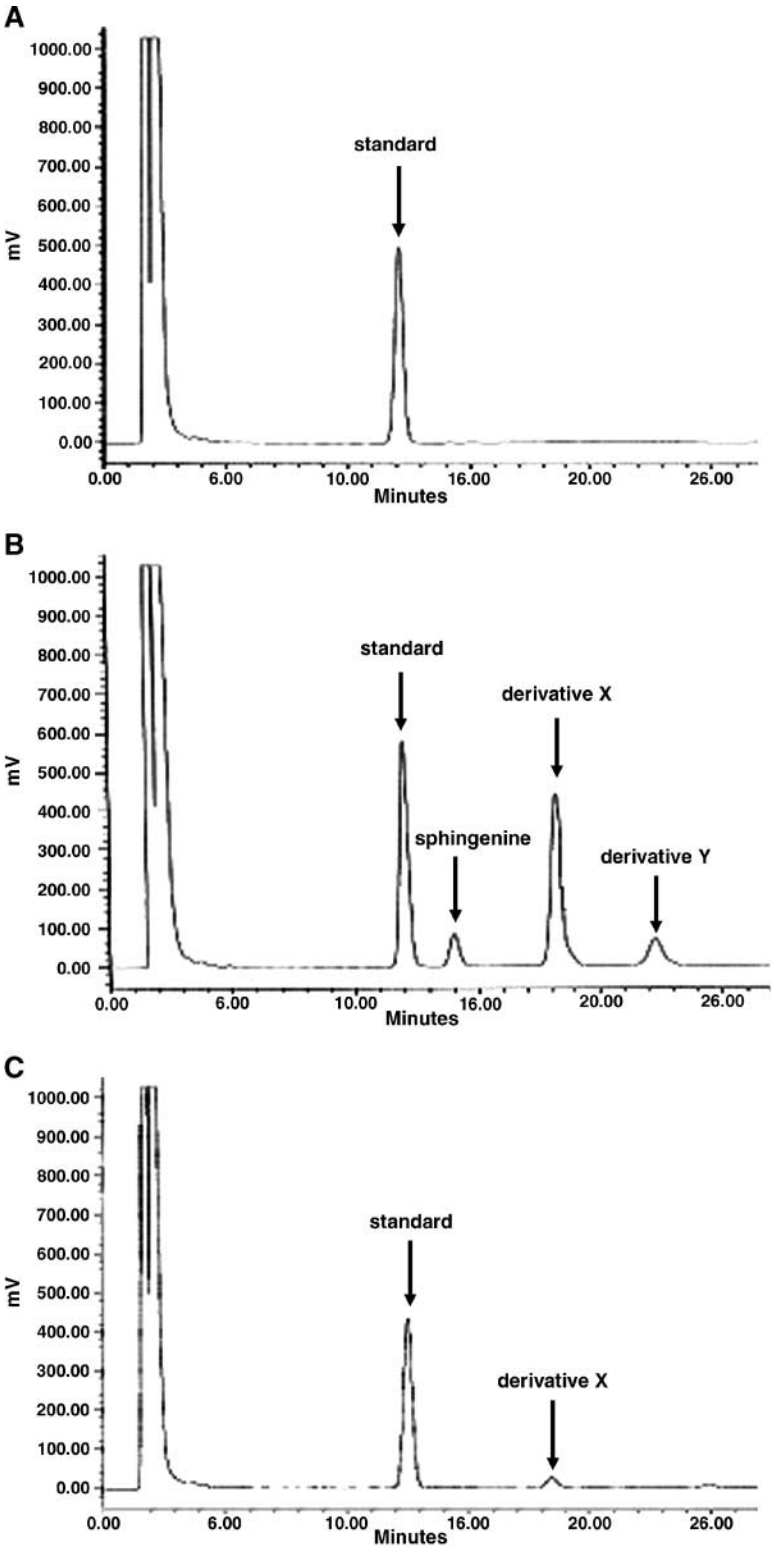

Fig. 3. HPLC analysis of acyl migration in *N*-acetyl-sphingenine upon exposure to acid or storage at room temperature. *N*-Acetyl-sphingenine (Avanti Polar Lipids; 6 nmol) was dissolved in 100 μl of chloroform-methanol (1:1, v/v; 100 μl) (A) or chloroform-methanol containing 0.5 N HCl (B) and kept at room temperature for 3 h. After the addition of 2 N NH_4OH (2-fold molar excess over HCl), methanol and chloroform were added to obtain phase separation (methanol-chloroform-water ratio was 1:1:0.9). An internal standard (700 pmol of 4-hydroxysphinganine; Sigma) was added after the lower phase was dried, and 50 μl of 20 mM triethylamine in methanol, followed by 50 μl of 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, prepared as described previously (19), in tetrahydrofuran, was added. The mixture was kept for 45 min at room temperature, and a volume of 20 μl was injected on a Symmetry C_{18} column (150 × 4.6 mm inner diameter, 5 μm; Waters) via a 717 Plus autosampler (Waters) connected to a 1525 binary HPLC pump (Waters). The column was eluted at a flow rate of 1 ml/min with buffer A (acetonitrile-methanol-water-formic acid-triethylamine, 480:320:188:5:7, v/v) during 25 min, followed by a gradient elution from 100% buffer A to 100% buffer B (acetonitrile-methanol, 60:40, v/v) in 5 min. Elution was monitored by a 2475 multi λ fluorescence detector (Waters; excitation, 244 nm; emission, 398 nm). C: *N*-Acetyl-sphingenine was dissolved at 0.06 mM in $CHCl_3$ and stored at room temperature for 1 week. After the addition of 700 pmol of 4-hydroxysphinganine, the sample was dried, treated with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and analyzed by reversed-phase chromatography as described above.

These values indicate that hydrolysis of the *O*-acyl esters during the extraction/derivatization is negligible and support the validity of the HPLC procedure. The acid-induced rearrangement of ceramides was not limited to *N*-acetylated compounds. *N*-Hexanoyl- and *N*-hexadecanoyl-sphingenine were also converted into ninhydrin-positive products, although the rate of conversion, and certainly further hydrolysis of the *O*-acyl compounds to sphingenine, appears to diminish with a longer acyl chain (Table 1). Based on data obtained with *N*-hexanoyl-[4,5-^{3}H]sphinganine (data not shown), the rearrangements are not dependent on the presence of the 4-*trans* double bond. On the contrary, the double bond suppressed the formation of the 3-*O*-acyl sphingoid base (derivative Y), based on a comparison be-

tween *N*-acetyl-sphingenine and *N*-acetyl-sphinganine (Table 1). This is likely attributable to the allylic nature of the 3-hydroxy group.

To quantify conversions during short-term storage, solutions of *N*-acetyl-sphingenine were kept at room temperature, 4°C, or −20°C for 1 or 4 weeks in chloroform, dichloromethane, or methanol before analysis. This short-term storage did not cause any conversions when the solutions were kept at 4°C or −20°C (data not shown). On the contrary, storage at room temperature for 1 week resulted in the formation of derivative X (1.0%) when chloroform was used as a solvent (Fig. 3C). Derivative Y and sphingenine were not found after 1 week. When methanol or dichloromethane was used as a solvent, no migration was observed.

To simulate long-term storage, ceramide solutions were kept at 60°C for 3 h. When this test was performed in methanol, acetone, dichloromethane, toluene, or acetonitrile, no rearrangements were revealed by TLC. In chloroform and chloroform-methanol mixtures, however, the formation of ninhydrin-positive material was observed (Fig. 1D, E). HPLC analysis of *N*-acetyl-sphingenine, stored for 3 years at −20°C and dissolved in chloroform-methanol (1:1, v/v), revealed the presence of 0.8% derivative X (data not shown). Likely, in chloroform or chloroform-containing solvents, some acid-catalyzed migration occurs. Aged chloroform is known to become acidic as a result of water- and/or light-catalyzed generation of radicals and HCl.

Summarizing, our data show that ceramides, contrary to other (fatty) amides, are less stable. As a result of the presence of two hydroxy groups, both vicinal to the amide function, two migration routes are possible, presumably via a five-membered ring intermediate (hydroxyoxazolidine), resulting in the formation of 3-*O*- and 1-*O*-acyl derivatives (Fig. 2). These rearrangements occur readily upon exposure of ceramides to acid. Also upon storage, especially in chloroform, *O*-acylated sphingoid bases can be formed. Hence, our observations call for appropriate storage (in methanol or dichloromethane) of ceramides and ceramide-like compounds. At present, it is unknown whether some of the ceramide-mediated cellular effects could have been caused by *O*-acyl sphingoid bases. The *N*→*O*-acyl ceramide derivatives are likely to be readily converted by cellular esterases to sphingoid bases, which are known to be cytotoxic and to affect multiple cellular functions (13, 14). If it is stable in the cytosol, however, the *O*-acyl derivative will probably be lysomotropic and degraded in the lysosomes.

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