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Novel fluorescent ceramide derivatives for probing ceramidase substrate specificity

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ABSTRACT

Ceramidases are key regulators of cell fate. The biochemistry of different ceramidases and of their substrate ceramide appears to be complex, mainly due to specific biophysical characteristics at the water-membrane interface. In the present study, we describe the design and synthesis of a set of fluorescently labeled ceramides as substrates for acid and neutral ceramidases. For the first time we have replaced the commonly used polar NBD-dye with the lipophilic Nile Red (NR) dye. Analysis of kinetic data reveal that although both the dyes do not have any noticeable preference for the substitution at acyl or sphingosine (Sph) part in ceramide towards hydrolysis by acid ceramidase, the ceramides with acyl-substituted NBD and Sph-substituted NR dyes have been found to be a better substrate for neutral ceramidase.

1. Introduction

Ceramide takes a central position in sphingolipid biosynthesis and metabolism. It is the common lipid anchor for the membrane constituents like sphingomyelin, gangliosides and other glycosphingolipids (GSLs) (Fig. 1).[1] Furthermore, it is the precursor of the lower sphingolipids involved in signaling events. The biochemistry of ceramide appears to be relatively complex, since ceramide can appear in a variety of structural modifications not only in the sphingosyl-, but also in its *N*-acyl part[2,3] and not all forms of ceramide are equally recognized by the respective metabolic enzymes.

Ceramidases are lipolytic enzymes that cleave the *N*-acyl moiety of ceramide to form the lysolipid sphingosine.[4] Ceramide, sphingosine and its derivative sphingosine-1-phosphate (S1P) are signaling lipids triggering contradictory cellular effects. While ceramide is involved in inflammation and initiation of apoptosis, sphingosine and mainly S1P induce cell proliferation and differentiation. Due to this phenomenon, also referred to as the 'sphingolipid rheostat',[5] the different cellular ceramidases are regarded as key enzymes for the determination of cell fate. For ceramide's mode of action there are two alternative models. According to the first model, ceramide can interact with some intracellular proteins like ceramide-activated protein phosphatases (CAPP) or cathepsin D.[6] In an alternative model, ceramide acts as an important mediator of plasma membrane microenvironment enabling receptor clustering within liquid ordered membrane domains,[7] also referred to as 'lipid rafts'.[8]

In total, five ceramidases have been described so far.[9] The acid ceramidase with a pH optimum of ~4.0 was first described by Shimon Gatt[10] and purified from urine,[11] cloned[12] and biochemically characterized[13] by the groups of Sandhoff and Schuchman. This enzyme is mainly localized to the lysosomes, but a portion is secreted extracellularly. A neutral ceramidase was first described in 1969[14] and further described by Stoffel et al.[15]The latter was cloned from human (Hannun)[16] and mouse (Ito).[17] It mainly localizes to the plasma membrane and mitochondriae and has an optimal pH between pH 7 and pH 9. For this enzyme, investigation of substrate specificity revealed that, among the four possible diastereomers only the natural D-*erythro* ceramide is cleaved by this enzyme.[18] In contrast, dihydroceramide, phytoceramide or analogues with shortened alkyl chains were not efficient substrates. Beside these two ceramidases, alkaline ceramidase activity with an pH optimum at ~9 has been described.[19] Alkaline ceramidases are encoded by three different genes, ACER1,[20] ACER2[21] and ACER3.[22]

Mutations in the gene for acid ceramidase can lead to a deficiency in enzyme activity and a lysosomal storage disease, termed Farber's disease.[23] Lang and co-workers have described that an important hallmark of cystic fibrosis is an imbalance between aSMase and aCDase activities caused by an elevated lysosomal pH.[24] While the acid sphingomyelinase, having an activity optimum around pH 5, is still active, the aCDase activity at this pH is significantly reduced. This imbalance leads to an accumulation of ceramide and an increased cell death in lung epithelial cells. Elevated activity of acid ceramidase in contrast, has been observed in a number cancer types and has been related to suppression of apoptotic pathways.[25] Accordingly, pharmacological inhibition of ceramidases should also elevate cellular ceramide levels and lead to a predomination of ceramide-associated effects over those mediated

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by sphingosine or S1P.[26] Indeed, some moderately active inhibitors of the acid ceramidase have been reported with pro-apoptotic and anti-proliferative effects in several cancer models.[9] Among the most prominent inhibitors are B-13[27] and *N*-oleoylethanolamine (NOE)[19] for acid ceramidase and D-*erythro*-*N*-myristoyl-2-amino-1-phenyl-1-propanol (D-*e*-MAPP) for alkaline ceramidases.[27,28] All these compounds have given rise to the development of various derivatives.[9,29]

In order to gain a deeper insight into ceramidase biochemistry, our goal is to develop fluorescent ceramide analogues as in situ probes for intracellular ceramidase activities. Such a goal can be achieved by the development of FRET probes with suitably substituted fluorescent moieties such as Nile Red (NR) and NBD in a single ceramide molecule. This may enable it for real time ratio imaging and thus in situ quantification of enzymatic activities. In contrast to quenched fluorescent probes, real FRET probes provide their own internal standard that allow for determining the ratio of cleaved versus uncleaved probes at any time point, reflected by the ratio of donor fluorescence to acceptor fluorescence.

In the special case of ceramidases, the specific enzyme activity is relatively low. In addition, in the case of the acid ceramidase, high substrate specificity has been reported,[18] but objective data on structure–activity-relationship (SAR) of different ceramides as the substrates of ceramidases is limited. Thus, a more detailed SAR study on ceramides is necessary for a better understanding of substrate specificity toward ceramidases. It should be mentioned that the determination of ceramidase activity was performed previously by using natural ceramide as substrate and the hydrolysis was quantified by the fluorescent emission intensity of produced sphingosine upon its derivatization with *o*-phthalaldehyde (OPA).[11] The ceramidase assays today are more conveniently conducted either by using fatty acid ^{14}C or ^{3}H-labeled ceramides as substrates followed by extraction or TLC-separation or alternatively by using the commercially available fatty acid substituted ceramides such as ω-hexanoyl or dodecanoyl-labeled NBD-ceramides,[30] or the somewhat less common ω-dodecanoyl-labeled BODIPY-ceramides.[31] Tani et al have reported the detailed kinetic studies for the hydrolysis of acyl-NBD-C_{12}-Cer using different cellular lysates of alkaline, neutral as well as acid ceramidases to understand the selectivity of the substrate towards a particular enzyme sub-type.[32] They observed that acyl-NBD-C_{12}-Cer was much more selective towards alkaline and neutral ceramidases but was found to be relatively poor substrate for aCDase. However, ceramide analogues labeled in the sphingosine part are relatively rare. Long before, Sandhoff and co-workers have developed fluorescent sphingosine analogues bearing diphenylhexatrienyl (DPH) fluorophore and subsequently synthesized C6-DPH-Cer. The ceramide analogue was studied for their incorporation behavior towards cultured human fibroblasts, which was similar to that of C6-NBD-Cer.[33] Nieuwenhuizen et al have described a quenched fluorescent probe for bacterial ceramidase, in which the fatty acid was labeled by a fluorescent pyrene and the sphingosine part was substituted with a quenching 3,5-dinitrobenzoyl group.[34] Bedia et al have reported ceramidase probes in which a coumarin dye is attached to the sphinganine part of a dihydroceramide derivative.[35,36] This probe can be used for homogenous high-throughput assays,[37] but a treatment of post-reaction periodate is necessary for the liberation of the fluorescent hydroxycoumarin, making it incompatible with real-time or in situ assays. A detailed structure-activity studies with the variation of fatty acid chain length revealed that the cleavage rate strongly depends on fatty acid length with an optimum at C_{12}.[36]

In the present study we describe the straightforward synthesis for the development of two novel Nile Red-substituted fluorescent ceramide analogues. Together with the commercially available acyl-NBD-Cer and the literature-known Sph-NBD-Cer, these ceramides were tested as substrates for the recombinant acid and neutral ceramidases in micellar assays. In addition, detailed kinetic studies are performed with all the substrates for the determination of different kinetic parameters such as Michaelis constant (K_M), maximum velocity (V_{max}), catalytic constant (k_{cat}) and the ratio of k_{cat} and K_M, specificity constant (η) to understand their suitable positional selectivity. (See Fig. 2)

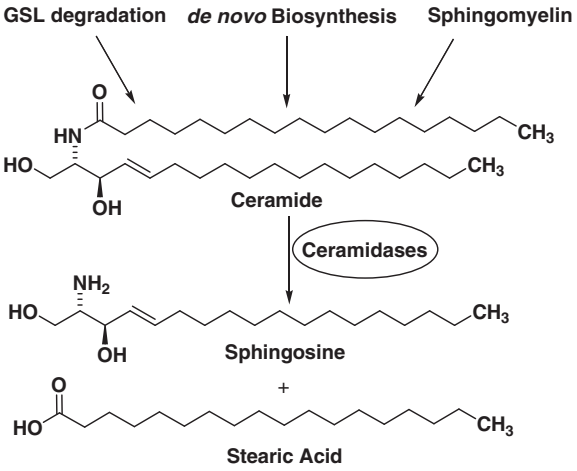


Figure 1. Schematic representation for the catalytic hydrolysis of ceramide by ceramidases.

2. Results and discussion

In order to make use of the positive results obtained by Schultz and Co-workers with the NR-modified phosphoglycerolipids as substrates of phospholipase A_2 (PLA_2),[38,39] we planned to synthesize NR-labeled ceramides and to evaluate their properties as ceramidase substrates in comparison to the commercially available NBD-labeled ceramides. Although NR is a hydrophobic dye with a selectivity to lipids and membranes,[40] to the best of our knowledge no NR-labeled sphingolipids have been reported so far. In contrast to NR, NBD is a relatively polar dye, which only reluctantly integrates into membranes.[41,42] Unlike pyrene dyes, which are very hydrophobic but under most conditions are only poorly fluorescing molecules, NR has its highest quantum yield in nonpolar solvents or lipid environments. These features suggest that NR-labeling may be favored even for singly labeled ceramidase substrates. In order to verify this hypothesis, we synthesized a set of three ceramide analogues, with the incorporation of either NBD- or NR-labeling in the fatty acid or in the sphingosyl part of the ceramide molecule (Fig. 2). The set was completed for the SAR studies with the inclusion of acyl-NBD-Cer, commercially obtained from Avanti Polar Lipids Inc.

In order to guarantee a modular synthesis of sphingosyl-modified ceramides with a minimum number of synthetic steps, we followed a strategy developed by Nussbaumer et al employing olefin metathesis as a key step.[43,44] Starting from Garner's aldehyde **4** (Scheme 1), the allyl alcohol **5** was synthesized using vinylmagnesium bromide.[45] Subsequently, the building block **5** was subjected to olefin metathesis reaction using the 2nd generation Hoveyda-Grubbs catalyst to form either protected sphingosine **6** or the NR- or NBD-modified compounds **7a** or **7b**, respectively. Acidic deprotection yielded natural sphingosine **8** or the NR- or NBD-modified sphingosines **9a** and **9b**. Finally, acylation of the sphingosine derivatives led to the formation of fluorescent ceramides **1**, **3a** and **3b** (Scheme 1). Probe **1** was synthesized by the reaction of sphingosine **8** with NR-acyl-*N*-oxysuccinimide **11** (Scheme 2).

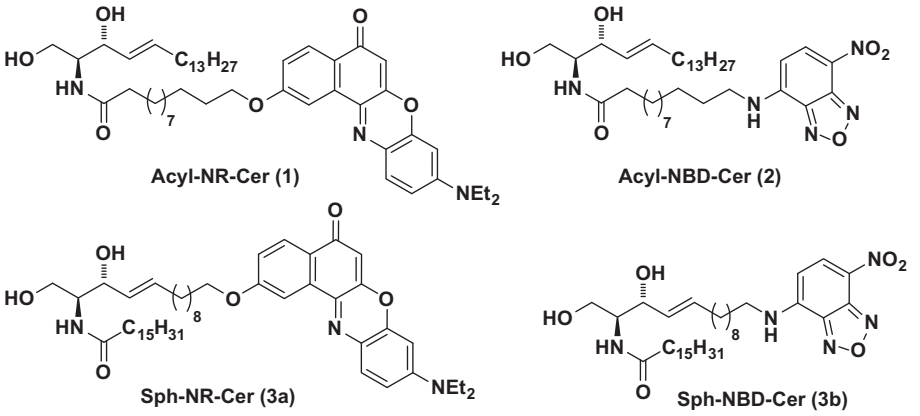
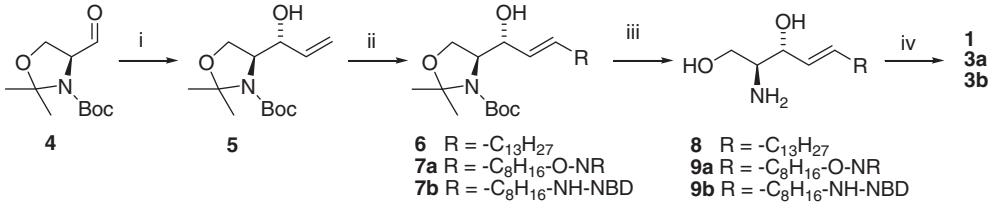
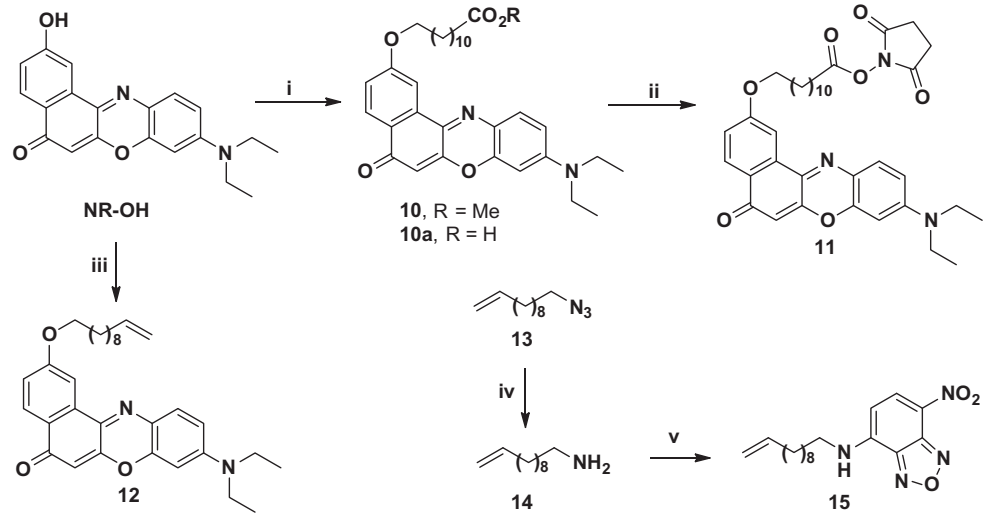


Figure 2. Chemical structures of the fluorescent ceramide derivatives evaluated in this study: The sphingosine-labeled derivatives **3a** and **3b** and the acyl-NR-Cer analogue **1** as well as the commercially available acyl-NBD-Cer (**2**).



Scheme 1. Synthetic routes to fluorescent-labeled ceramide analogues. Reagents and conditions: (i) Vinylmagnesium bromide, THF, −78 °C, 1.5 h; (ii) For **6**: 1-Pentadecene, Hoveyda-Grubb's catalyst, CH_2Cl_2, 12 h, 40 °C; for **7a**: **12**, Hoveyda-Grubb's catalyst, CH_2Cl_2, 12 h, 40 °C; for **7b**: **15**, Hoveyda-Grubb's catalyst, CH_2Cl_2, 12 h, 40 °C; (iii) TFA, CH_2Cl_2, 45–180 min; (iv) for **1**: **8**, **11**, DIPEA, $CHCl_3$, 40 h; for **3a**: **9a**, palmitic acid, EDC, HOBT, DIPEA, CH_2Cl_2; for **3b**: **9b**, palmitoyl chloride, pyridine, 1 h.



Scheme 2. Synthetic routes to functionalized fluorescent dyes. Reagents and conditions: (i) (a) Methyl-12-bromo-dodecanoate, K_2CO_3, DMF, 80 °C, 4 h; (b) Me_3SiOK, Et_2O, 48 h; (ii) *N*-Hydroxysuccinimide, D-MAP, DCC, $CHCl_3$, 0 °C-RT, 12 h; (iii) 11-Bromo-1-undecene, NaH, THF, reflux, 72 h; (iv) $LiAlH_4$, THF, 0 °C, 30 min; (v) NBD-Cl, DIPEA, MeOH, 15 h.

The NR-sphingosine **9a** was coupled to palmitic acid using EDC/HOBT yielding **3a** and NBD-sphingosine **9b** was reacted with palmitoyl chloride to form **3b**.

The conjugated dyes were synthesized as shown in Scheme 2. The precursor hydroxyl-NR (NR-OH) was synthesized as described previously[46] and coupled to methyl-12-bromo-dodecanoate in the presence of potassium carbonate to yield **10**. The methyl ester was then hydrolyzed via silyl ester formation. The respective carboxylic acid (**10a**) was isolated and purified and then reacted with *N*-hydroxysuccinimide to yield the activated ester **11**. The NR-alkene **12** for metathesis reaction was formed by Williamson ether synthesis with 11-bromo-1-undecene. The NBD-alkene **15** was synthesized by reaction of the amine **14** with NBD chloride.[47]

Before carrying out the detailed experiments for the determination of kinetic parameters, we optimized the assay conditions with the variation of important parameters such as the incubation time and enzyme concentrations (Supplementary data). Incubation times of 150 min and 60 min for aCDase and nCDase, respectively were considered for all the kinetic studies as linear increase of hydrolysis by ceramidases was observed for all the substrates with

fixed concentrations of substrates and enzymes. Furthermore, the concentration of both aCDase (50 μg/mL) and nCDase (20 ng/mL) was optimized by the variation of enzyme concentrations at fixed concentrations of substrates (20 μM) using the optimized incubation times. The values of these parameters are optimized for the standard assay method to ensure a linear progress of the hydrolysis of substrates in the presence of ceramidases. Due to the known lipophilic properties of NR in contrast to the relatively poor lipophilicity of NBD, we expected that the novel NR-modified ceramides would better mimic the natural ceramide and thus should be better substrates for ceramidases. However, when we tested the modified ceramides as substrates for ceramidases (aCDase and nCDase) under standard assay conditions, we unexpectedly observed a significantly lower cleavage rate for acyl-NR-Cer **1** as compared to the corresponding acyl-NBD-Cer **2** (aCDase: **1**, 6%; **2**, 10%; nCDase: **1**, 3%; **2**, 10%). On the other hand, the enzymatic cleavage of Sph-NR-Cer was found to be almost equal or lower than the corresponding Sph-NBD-Cer (aCDase: **3a**, 6%; **3b**, 6%; nCDase: **3a**, 4%; **3b**, 6%). In the present assay condition, the ceramidase-catalyzed hydrolysis of NBD-substituted ceramides was more or at least equal to the corresponding NR-based analogues and there was no case in which the NR-dye was superior to the NBD-dye with respect to the cleavage rate. However, in order to have more generalized data and to understand the positional selectivity of NBD and NR dyes to ceramide, we decided to evaluate dose-dependency and to determine the Michaelis-Menten kinetic parameters such as K_M, V_{max}, k_{cat} and the ratio of k_{cat} to K_M (η) values for all the substrates with the variation of substrate concentrations at a fixed concentration of enzymes.

The Michaelis constant K_M is the substrate concentration needed for half-maximum catalytic velocity and thus represents the enzyme's affinity to a given substrate. In contrast, the maximum catalytic velocity V_{max} is a measure for the enzyme's ability to cleave a given substrate, assumed that all the existing enzyme is bound or saturated by substrate. Thus, the ideal substrate should have low K_M and high V_{max} values. Evaluation of the kinetic parameters however showed in most cases that low K_M values were associated with low V_{max} values or vice versa. In general, K_M values and also V_{max} values for NR-ceramides were lower as compared to NBD-ceramides (Table 1). The only exception from this trend is provided by the aCDase-mediated cleavage of acyl-modified ceramides. To obtain a more generalized comparison, we have further determined the parameters such as catalytic constant (k_{cat}) and specificity constant (η) as a ratio of k_{cat} and K_M. While k_{cat} value represents the turn over number of an enzyme for a particular substrate, the specificity constant, the ratio of k_{cat} to K_M is useful to understand the comparative specificity of different substrates to a particular enzyme active site. Therefore, specificity constant is the best parameter for determining the relative specificity of NR- and NBD-ceramides toward aCDase and nCDase in the present study.

As shown in Table 1, the specificity constants of all the NR-Cer and NBD-Cer were almost comparable for the hydrolysis by aCDase, although a 1.3 times enhancement of specificity of acyl-NR-Cer has been observed than Sph-NR-Cer, the corresponding NBD-based ceramides did not show any positional selectivity. However, a more pronounced effect is observed for the hydrolysis of ceramide analogues by nCDase. For example, around 1.4 times enhancement in the specificity has been observed upon the incorporation of NR group from acyl part (**1**) to sphingosine part (**3a**) of ceramide. On the other hand, the specificity of acyl-NBD-Cer (**2**) is found to be almost double of Sph-NBD (**3b**) analogue of ceramide. These observations clearly suggest that incorporation of NBD-moiety at the acyl part and NR-moiety at the sphingosine part could be a better choice for the development of a FRET probe as a substrate of nCDase.

To understand the suitability of these singly fluorescent-labeled ceramides as substrates for the inhibition studies, we employed two literature known inhibitors such as B-13[27,48] and DP-24a[49] for their effects towards aCDase-catalyzed hydrolysis of the substrates in the present study (Fig. 3). As NBD and NR-based ceramides have different V_{max} and K_M values, the substrate concentrations for the inhibition studies were chosen similar to their K_M values. A noticeable inhibition of aCDase activity was observed for all the substrates in the presence of 50 μM concentration of inhibitors. As shown previously for the aCDase-mediated hydrolysis of natural ceramide,[49] DP-24a has been found to be a better inhibitor than B-13 for the aCDase-catalyzed hydrolysis of all the NR- and NBD-substituted ceramide analogues.

The limits of NBD-labeled lipids have been extensively discussed in the report by van Meer and Liskamp. The NBD is a relatively polar dye and thus does not behave like many sphingolipids, which are thought to mainly concentrate in liquid ordered domains of membranes.[42] In the course of searching for an alternative dye for labeling sphingolipids we concentrated on NR, which has been shown to readily integrating into lipid membranes.[50] In our study, labeling with NR in most cases generally leads to lower K_M but nonetheless the NBD labeled ceramides are cleaved more efficiently. It may be speculated that this behavior is at least in part an inherent result of the substrates' biophysical properties. However, the behavior of the NR-Cer analogues cannot be predicted as their entry and distribution in cells has not been studied yet. As shown in a detailed study by Sandhoff and Co-workers that glucosylceramides with short lipid chains are most efficiently cleaved by the lysosomal enzyme glucocerebrosidase.[51] For long natural chain lengths, the enzymatic activity drops down to zero, unless a

Table 1
Determination of percentage hydrolysis and kinetic parameters of fluorescent ceramides as substrates for aCDase or nCDase

		Acyl-NR-Cer **1**	Acyl-NBD-Cer **2**	Sph-NR-Cer **3a**	Sph-NBD-Cer **3b**
aCDase	Hydrolysis (%)*	6.1 ± 1.1	9.9 ± 0.8	6.3 ± 1.1	5.6 ± 0.4
	V_{max} (pmol min^{-1})	7.2	4.4	3.9	12.2
	K_M (μM)	110.3	86.9	81.8	203.6
	k_{cat} (pmol/min/μg of protein) × 10^{-2}	14.3	8.82	7.8	24.4
	k_{cat}/K_M (η, lit/min/μg of protein) × 10^{-9}	1.3	1.0	1.0	1.2
nCDase	Hydrolysis (%)*	3.3 ± 0.2	9.9 ± 0.7	3.9 ± 1.0	6.2 ± 0.2
	V_{max} (pmol min^{-1})	10.0	62.4	5.3	31.8
	K_M (μM)	85.3	189.9	36.4	192.9
	k_{cat} (pmol/min/ng of protein)	0.50	3.1	0.26	1.6
	k_{cat}/K_M (η, lit/min/ng of protein) × 10^{-9}	5.9	16.4	7.3	8.2

*Enzymatic hydrolysis of substrates (20 μM) was studied after 150 min for aCDase (60 μg/mL) and 60 min for nCDase (20 ng/mL) at 37 °C in NaOAc buffer of pH 4.5 and 7.0, respectively. For the determination of kinetic parameters, the substrate concentration was varied from 5 to 150 μM or 5 to 180 μM at fixed concentration of enzymes under standard assay condition. All the data shown in the Table represent Mean ± SD from three independent measurements. All the kinetic parameters are determined from the Lineweaver-Burk plots and each data point in the plot represents Mean ± SD from three independent experiments (Supplementary data).

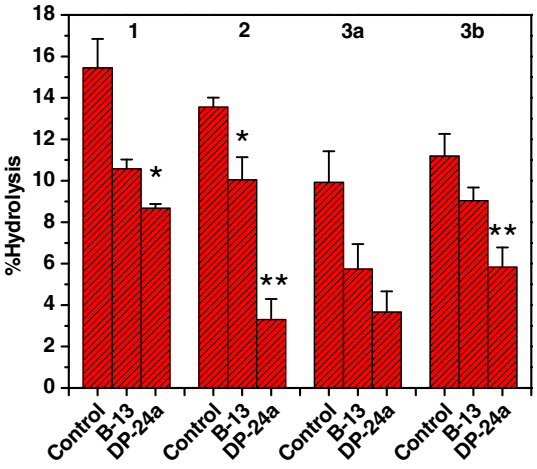


Figure 3. Hydrolysis of different substrates **1** (80 μM), **2** (80 μM), **3a** (55 μM) and **3b** (160 μM) by aCDase (30 μg/mL) in the presence of 50 μM of inhibitors such as B-13 or DP-24a under standard assay condition. Control value represents the hydrolysis of substrates in the absence of any inhibitors. Each data point in the plot represents Mean ± SD from three independent experiments. Statistical significance of inhibition: *, $0.01 < p < 0.05$; **, $0.001 \leqslant p \leqslant 0.01$.

sphingolipid activator protein (SAP) is present that probably acts as a 'liftase' making the substrate available to the soluble enzyme.[52] Also the measurement of membrane lateral forces support the idea that membrane perturbation is an important hallmark of enzymatic reactions at the water lipid interface.[53] In micellar assays, the lateral forces are minimized but nonetheless, high lipophilicity may still reduce the enzyme's ability to detach the substrate from the lipid phase, while NBD is additionally perturbing the membrane surface making NBD-labeled substrates better accessible to the enzymes. The high V_{max} but also high K_M values of the NBD substrates seem to support such a theory. Another important factor may be the difference in size of NR and NBD moieties and the effective chain length of NR and NBD-based ceramide analogues. Due to the bigger size of the NR moiety with four fused ring systems, the effective chain length is much higher than that of the corresponding NBD analogues. Ettmayer and co-workers have reported the effect of linker chain length as well as the effect of different fluorescent dyes in sphingosine derivatives towards the phosphorylation by sphingosine kinases and their cellular distribution.[54] In the present study, instead of having the same aliphatic linker chain lengths for both of NR- and NBD-based substrates, the overall chain length are eventually different and therefore it would be worth to study the effect of chain lengths on their kinetic as well as cellular behavior.

3. Conclusion

In the present study, for the first time, we report the design and synthesis of fluorescent ceramide derivatives labeled with the lipid-selective dye Nile Red (NR). A detailed comparative SAR study has been performed with a combined set of singly labeled NR- and NBD-ceramide analogues for their specificity as substrates toward aCDase and nCDase. In contrast to most other studies, exclusively purified recombinant enzymes were used. For the known and commercially available acyl-NBD-Ceramide **2**, the obtained enzymatic data (new) for aCDase matches quite well with a previous report (old) by Tani et al.:[32] K_M (new/old) = 86.9/59.0 μM; V_{max} (new/old) = 4.4/0.8 pmol/min. taking into account that V_{max} very much depends on the specific activity of the used enzyme. For nCDase, however the values significantly differ: K_M (new/old) = 189.9/22.3 μM; V_{max} (new/old) = 62.4/4.7 pmol/min. The reason for this discrepancy in the Michaelis constant is unclear, but it is very likely not just due to the different specific activity of the used enzymes.

In the present study, NR-ceramides exhibited lower rates of cleavage than that of NBD-ceramides. Determination and detailed analysis of kinetic parameters reveal the pronounced specificity of ceramide analogues with NR moiety at the sphingosine part or NBD group at the acyl part of singly labeled ceramide probes especially for the hydrolysis by nCDase, while the aCDase does not show any significant specificity.

One drawback of known fluorescently labeled ceramides like NBD-Cer or BODIPY-Cer[55] is the fact that they do not integrate into liquid ordered domains or 'lipid rafts' of plasma membranes.[41,42] In this regard, the polyene ceramide developed by Thiele and coworkers is most promising.[56] However, the latter requires two-photon-excitation, a technique unavailable in many laboratrories.

Given the higher lipophilicity of our novel ceramides, it will be interesting to test their behavior in living cells, which might be different from commercially available substrates commonly used for such studies.

4. Experimental

4.1. Acid ceramidase (aCDase) assays

The recombinant human aCDase was expressed from insect cells as described previously.[57] For details, see supplementary data. The acid ceramidase assay was performed in sodium acetate buffer (200 mM, pH 4.5) with 200 mM sodium chloride and 0.1% Triton-X100. The stock solutions of the fluorescent substrates and all the inhibitors were prepared in DMSO. Human recombinant acid ceramidase (aCDase) was used as the enzyme source for the cleavage of the fluorescent substrates. The total volume of the assay mixture was 100 μL. A final concentration of 20 μM of all the substrates was used to study the effect of incubation times and enzyme concentrations (50 μg/mL). Different substrate concentrations (**1** and **2**: 80 μM; **3a**: 55 μM; **3b**: 160 μM) were used for the inhibition studies with 50 μM of inhibitors and 30 μg/mL of aCDase. The substrate concentration was varied at fixed concentration of enzyme for the determination of kinetic parameters. In all the assay mixtures the volume of DMSO was maintained less than 5% (v/v). The assay mixture was incubated for 150 min at 37 °C. The enzymatic reaction was quenched by adding 200 μL of chloroform/methanol (2:1) mixture. The assay mixture was vortexed well and centrifuged to separate the organic and aqueous layers. 30 μL of the organic layer was transferred into new sets of vials and the total amount was spotted on non-fluorescent silica gel TLC plates pre-coated on aluminium sheets (Mobile phase: For **1** and **2**: Cyclohexane/Ethyl acetate/Acetic acid = 40:60:2; **3a**: Ethyl acetate/Acetic acid = 50:1; **3b**: Dichloromethane/Methanol/Acetic acid = 20:1:1). The fluorescent spots of the cleaved and uncleaved substrates on the TLC plates were detected by Fluorescent Imaging System (Kodak Image Station 4000 MM PRO) using 550 nm and 600 nm as excitation and emission wave lengths, respectively for the Nile-red linked substrates and 430 nm and 550 nm as excitation and emission wave lengths, respectively for the NBD-linked substrates. The percentage ceramidase activity and the inhibition were determined by the quantification of the relative fluorescent intensity of the cleaved and uncleaved substrates, which both together were set as 100%. The reaction rate was calculated from the percentage conversion with the known concentration of substrates and the reaction time using the quantification software in fluorescent imaging system. Within the dynamic range of the detector less than 0.5% substrate cleavage were detectable. No further calibration was applied.

4.2. Neutral ceramidase (nCDase) assays

Human recombinant neutral ceramidase (nCDase) (R&D Systems GmbH) was used as the enzyme source for the cleavage of the fluorescent substrates. The neutral ceramidase assay was performed in sodium acetate buffer (200 mM, pH 7.0) with 200 mM sodium chloride and 0.1% Triton-X100. The stock solutions of the fluorescent substrates were prepared in DMSO. The total volume of the assay mixture was 100 μL. The final concentration of the substrates and enzyme were 20 μM and 20 ng/mL, respectively. The substrate concentration was varied at fixed concentration of enzyme for the determination of kinetic parameters. In all the assay mixtures the volume of DMSO was maintained less than 5% (v/v). The mixture was incubated for 60 min at 37 °C and the enzymatic reaction was quenched by adding 200 μL of chloroform/methanol (2:1) mixture. The mixture was vortexed thoroughly and centrifuged at 10,000 rpm for 2 min to separate the organic layer. 30 μL of the organic layer was transferred into new sets of vials and the percentage hydrolysis was determined as mentioned for the aCDase activities.

4.3. General synthesis

Most of the commercially available chemicals were purchased from Sigma–Aldrich. The acyl-NBD-Cer **2** was purchased from Avanti Polar Lipids, Inc. Solvents were freshly distilled whenever required for the reaction. All the moisture sensitive reactions were carried out under dry argon atmosphere. Thin layer chromatographic (TLC) studies are performed on pre-coated silica gel 60 F_{254} on aluminum sheets (Merck KGaA). ^{1}H (500 MHz and 300 MHz) and ^{13}C (125 MHz and 75 MHz) NMR spectra were obtained on a Bruker Avance III 500 MHz and Bruker Avance DPX 300 MHz NMR spectrometers. Chemical shifts are cited with respect to Me_4Si as internal (^{1}H and ^{13}C) standard. Mass spectral studies were carried out on a Hewlett-Packard GCMS 5995-A mass spectrometer with ESI-MS mode analysis.

4.4. Synthesis of 10

To a stirred solution of NR-OH (0.12 g, 0.35 mmol) and methyl-12-bromo-dodecanoate (0.12 g, 0.39 mmol) in dry DMF (7 mL) was added K_2CO_3 (0.15 g, 1.07 mmol) and the mixture was heated at 80 °C for 4 h. Thin layer chromatography indicated almost complete disappearance of NR-OH and the appearance of a relatively non-polar spot. The solvent was evaporated under reduced pressure and the residue was diluted with ethyl acetate and washed with water followed by brine solution. The combined organic extract was dried over sodium sulfate and the solvent was evaporated to obtain the crude product as deep red solid. The product was purified by silica gel column chromatography using ethyl acetate and cyclohexane as eluents. Solvent was evaporated to obtain the product as deep red solid. Yield: 0.17 g (88.1%). R_f: 0.55 (1:1 Ethyl acetate & Cyclohexane). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 1.22 (t, J = 7.0 Hz, 6H), 1.29 (br, 12H), 1.47–1.52 (m, 2H), 1.58–1.63 (m, 2H), 1.81–1.86 (m, 2H), 2.29 (t, J = 7.5 Hz, 2H), 3.40 (q, J = 7.0 Hz, 4H), 3.65 (s, 3H), 4.12 (t, J = 6.4 Hz, 2H), 6.23 (s, 1H), 6.36 (d, J = 2.7 Hz, 1H), 6.57 (dd, J_1 = 2.7 Hz, J_2 = 9.1 Hz, 1H), 7.11 (dd, J_1 = 2.6 Hz, J_2 = 8.7 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 7.96 (d, J = 2.5 Hz, 1H), 8.16 (d, J = 8.7 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 12.6, 24.9, 26.1, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 34.1, 45.0, 51.4, 68.3, 96.2, 105.2, 106.5, 109.4, 118.2, 124.6, 125.4, 127.6, 131.0, 134.0, 139.8, 146.7, 150.6, 151.9, 161.8, 174.3, 183.2. ESI-MS: m/z calcd for $C_{33}H_{42}N_2O_5$: 547.3166 $[M+H]^+$; observed: 547.3165.

4.5. Synthesis of 10a

To a stirred suspension of **10** (0.20 g, 0.36 mmol) in dry Et_2O (10 mL) was added an excess amount of potassium trimethylsilanolate (0.70 g, 5.49 mmol) under argon atmosphere. The mixture was stirred at room temperature for 2 days. The solvent was evaporated and the crude mixture was purified by silica gel column chromatography. The non-polar impurities and unreacted reactants were eluted with ethyl acetate and cyclohexane mixture and the product was eluted with 15% methanol in ethyl acetate. The solvent was evaporated to afford the product as deep red solid. Yield: 0.11 g (54.8%). R_f: 0.34 (100% Ethyl acetate). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 1.24–1.30 (m, 18H), 1.46–1.54 (m, 2H), 1.59–1.70 (m, 2H), 1.81–1.88 (m, 2H), 2.34 (t, J = 7.4 Hz, 2H), 3.47 (q, J_1 = 6.3 Hz, J_2 = 13.4 Hz, 4H), 4.16 (t, J = 6.6 Hz, 2H), 6.31 (s, 1H), 6.45 (d, J = 2.5 Hz, 1H), 6.65 (dd, J_1 = 2.6 Hz, J_2 = 9.0 Hz, 1H), 7.16 (dd, J_1 = 2.4 Hz, J_2 = 8.8 Hz, 1H), 7.60 (d, J = 9.1 Hz, 1H), 8.04 (d, J = 2.4 Hz, 1H), 8.21 (d, J = 8.8 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 12.6, 24.8, 26.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.7, 34.0, 45.1, 68.4, 96.3, 105.3, 106.6, 109.5, 118.3, 124.7, 125.5, 127.7, 131.0, 134.0, 140.0, 146.8, 150.7, 152.0, 161.9, 183.4. ESI-MS: m/z calcd for $C_{32}H_{40}N_2O_5$: 533.3015 $[M+H]^+$; observed: 533.3010.

4.6. Synthesis of 11

The acid obtained in the first step (**10a**) was further transformed to **11** in analogy to literature methods:[47]To a solution of **10a** (45.0 mg, 0.084 mmol), *N*-hydroxysuccinimide (10.7 mg, 0.093 mmol) and a catalytic amount of D-MAP (2.0 mg) was added DCC (20.9 mg, 0.10 mmol) in chloroform (5 mL) at 0 °C. After 1 h, the mixture was allowed to warm to room temperature and the reaction continued overnight. Thin layer chromatography indicated almost complete disappearance of the starting acid **10a**. The solvent was evaporated and the crude mixture was used directly for the synthesis of **1** without any further purification.

4.7. Synthesis of 1

Compound **1** was synthesized analogously to methods described previously.[47] To a solution of **11** (45.0 mg, 0.071 mmol) in chloroform (5 mL) was added a solution of sphingosine **8** (64.1 mg, 0.21 mmol) in chloroform (2 mL) with diisopropylethylamine (DIPEA, 27.7 mg, 0.21 mmol). The resulting solution was stirred at room temperature for 40 h. The crude product was purified by silica gel column chromatography. The unreacted compounds and non-polar impurities were eluted with ethyl acetate and cyclohexane mixture and the product was eluted with 2% methanol in ethyl acetate. The solvent was evaporated to afford the product as deep red solid. Yield: 26.8 mg (46.2%). R_f: 0.17 (100% Ethyl acetate). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, J = 6.2 Hz, 6H), 1.25–1.29 (br, 40H), 1.45–1.54 (m, 2H), 1.60–1.64 (m, 2H), 1.81–1.90 (m, 2H), 2.05 (q, J_1 = 6.9 Hz, J_2 = 13.5 Hz, 2H), 2.18–2.23 (m, 2H), 3.48 (q, J = 7.1 Hz, 4H), 3.66–3.76 (m, 2H), 3.81–3.94 (m, 2H), 4.11–4.20 (m, 2H), 5.54–5.58 (m, 1H), 5.74–5.83 (m, 1H), 6.30 (s, 1H), 6.40 (d, J = 7.2 Hz, 1H), 6.47 (d, J = 2.6 Hz, 1H), 6.67 (dd, J_1 = 2.7 Hz, J_2 = 9.1 Hz, 1H), 7.17 (dd, J_1 = 2.6 Hz, J_2 = 8.7 Hz, 1H), 7.61 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 2.5 Hz, 1H), 8.20 (d, J = 8.7 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 12.6, 14.1, 22.7, 25.8, 25.9, 29.1, 29.2, 29.3, 29.4, 29.5, 29.7, 31.9, 32.3, 36.8, 45.1, 54.6, 60.8, 62.5, 68.3, 74.6, 77.2, 96.3, 105.1, 106.7, 109.6, 118.3, 124.8, 125.4, 127.7, 128.8, 128.9, 131.1, 134.0, 134.1, 139.9, 146.9, 150.8, 152.2, 161.9, 174.0, 183.5. ESI-MS: m/z calcd for $C_{50}H_{75}N_3O_6$: 814.5734 $[M+H]^+$; observed: 814.5733.

4.8. Synthesis of 7a

To a solution of (*S*)-*tert*-Butyl-4-((*R*)-1-hydroxyallyl)-2,2-dimethyloxazolidine-3-carboxylate **5** (0.15 g, 0.58 mmol)[45,44,32] and 9-(Diethylamino)-2-(undec-10-enyloxy)-5*H*-benzo[*a*]phenoxazin-5-one **12** (0.10 g, 0.21 mmol) in dichloromethane (5 mL) was added a catalytic amount of Hoveyda-Grubbs 2nd Generation catalyst (5.0 mg). The reaction mixture was stirred for 24 h at rt. The solvent was evaporated and the crude product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluents to afford the product as red solid. Yield: 54 mg (33.3%). R_f: 0.23 (1:1 Ethyl acetate & Cyclohexane) ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 1.21 (t, J = 7.1 Hz, 6H), 1.39 (m, 4H), 1.50 (s, 19H), 1.59 (m, 4H), 1.87 (m, 2H), 2.07 (dd, J_1 = 6.9, J_2 = 13.5 Hz, 2H), 3.48 (q, J = 7.1 Hz, 4H), 3.89 (br, 1H), 4.03 (br, 2H), 4.18 (t, J = 6.5 Hz, 2H), 4.32 (br, 1H), 5.47 (dd, J_1 = 4.4, J_2 = 15.7 Hz,1H), 5.76 (m, 1H), 6.31 (s, 1H), 6.46 (d, J = 2.7 Hz, 1H), 6.66 (dd, J_1 = 2.7, J_2 = 9.1 Hz, 1H), 7.17 (dd, J_1 = 2.6, J_2 = 8.7 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 2.6 Hz, 1H), 8.22 (d, J = 8.7 Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): δ = 12.6, 26.0, 26.3, 28.3, 29.1, 29.2, 29.4, 29.4, 29.5, 32.2, 32.4, 45.0, 62.1, 64.6, 68.3, 94.4, 94.5, 96.3, 105.3, 106.6, 109.4, 118.2, 124.6, 125.5, 127.7, 130.9, 131.0, 134.0, 140.1, 146.8, 150.7, 152.0, 161.9, 183.3. ESI-MS: m/z calcd for $C_{42}H_{57}N_3O_7$ $(M+H)^+$: 716.4269; observed 716.4269.

4.9. Synthesis of 9a

To a solution of protected NR-sphingosine **7a** (120 mg, 0.17 mmol) in dichloromethane (10 mL) was dropwise added 2 ml of Trifluoroacetic acid at room temperature. The reaction mixture was stirred for 45 min. The reaction mixture was poured into 50 ml ice cooled saturated $NaHCO_3$solution and extracted with ethyl acetate (3 × 40 mL). The combined organic layer was washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using dichloromethane and methanol mixtures as eluents. The pure product was obtained as wine red colored solid. Yield: 54 mg (55.3%). R_f: 0.05 (100% Ethyl acetate). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 1.21 (t, J = 7.2 Hz, 6H), 1.32 (t, J = 7.3 Hz, 10H), 1.45 (m, 2H), 1.78 (m, 2H), 1.97 (dd, J_1 = 6.9, J_2 = 13.9 Hz, 2H), 3.42 (dd, J_1 = 7.1, J_2 = 14.3 Hz, 4H), 3.52 (dd, J_1 = 7.3, J_2 = 14.6 Hz, 2H), 3.79 (d, J = 5.6 Hz, 1H), 4.10 (t, J = 6.4 Hz, 2H), 4.39 (m, 1H), 5.38 (dd, J_1 = 6.4, J_2 = 15.5 Hz,1H), 5.76 (m, 1H), 6.24 (s, 1 H), 6.42 (d, J = 2.5 Hz, 1H), 6.64 (dd, J_1 = 2.5, J_2 = 9.1 Hz, 1H), 7.10 (dd, J_1 = 2.4, J_2 = 8.7 Hz, 1H), 7.56 (d, J = 9.1 Hz, 1H), 7.99 (d, J = 2.4 Hz, 1H), 8.11 (d, J = 8.7 Hz, 1H). ^{13}C NMR ($CDCl_3$; 125 MHz, ppm): δ = 12.5, 25.9, 28.8, 29.1, 29.1, 29.2, 29.3, 29.4, 32.2, 42.0, 45.1, 52.8, 68.4, 96.2, 104.7, 106.6, 110.0, 115.3, 118.3, 126.3, 127.6, 131.2, 135.4, 139.2, 146.9, 151.1, 152.4, 162.0, 183.8. ESI-MS: m/z calcd for $C_{34}H_{45}N_3O_5$: $(M+H)^+$ 576.3432; observed: 576.3430.

4.10. Synthesis of 3a

To a solution of NR-sphingosine **9a** (34 mg, 0.059 mmol) in dichloromethane (5 mL) was added palmitic acid (15 mg, 0.059 mmol), EDC (11.3 mg, 0.059 mmol) and HOBT (8.0 mg, 0.059 mmol) with 0.2 mL of DIPEA. The reaction mixture was stirred for 16 h at rt. The solution was poured into 20 mL ice-water and extracted with ethyl acetate (3 × 30 mL). The combined organic layer was washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluents to afford the product as wine red solid. Yield: 16 mg (34.0%). R_f: 0.95 (100% Ethyl acetate). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.87 (t, J = 7.0 Hz, 3H), 1.24–1.38 (m,40H), 1.52 (m, 2H), 1.63 (m, 2H), 1.85 (m, 2H), 2.03 (m, 2H), 2.23 (m, 2H), 3.48 (q, J = 7.1 Hz, 4H), 3.71 (dd, J_1 = 3.3, J_2 = 11.2 Hz, 1H), 3.91 (dt, J_1 = 3.6, J_2 = 7.3 Hz, J_3 = 7.4 Hz, 1H), 3.96 (dd, J_1 = 3.8, J_2 = 11.3 Hz, 1H), 4.18 (t, J = 6.4 Hz, 2H), 4.32 (m, 1H), 5.53 (tdd, J_1, J_2 = 1.3 Hz, J_3 = 6.4, J_4 = 15.5 Hz,1H), 5.77 (m, 1H), 6.33 (d, J = 7.6 Hz, 1H), 6.35 (s, 1H), 6.50 (d, J = 2.5 Hz, 1H), 6.70 (dd, J_1 = 2.4, J_2 = 9.1 Hz, 1H), 7.17 (dd, J_1 = 2.4, J_2 = 8.7 Hz, 1H), 7.63 (d, J = 9.1 Hz, 1H), 8.06 (d, J = 2.6 Hz, 1H), 8.21 (d, J = 8.7 Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): δ = 12.6, 14.1, 22.6, 24.7, 24.9, 26.0, 28.2, 28.3, 28.9, 29.0, 29.1, 29.1, 29.2, 29.2, 29.3,29.4, 29.4, 29.6, 29.6, 29.6, 31.9, 32.3, 33.8, 33.9, 34.2, 45.0,63.0, 68.3, 96.3, 105.2, 106.6, 109.5, 118.2, 124.7, 125.4, 127.7, 128.3, 131.0, 134.0, 139.2, 140.0, 146.8, 150.7, 152.0, 161.9, 173.9, 178.2, 183.3. ESI-MS: m/z calcd for $C_{50}H_{75}N_3O_6$: $(M+H)^+$ 814.5729; observed 814.5729.

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Supplementary data

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References and notes

1. Kolter, T.; Sandhoff, K. *Angew. Chem., Int. Ed.* **1999**, *38*, 1532.
2. Hannun, Y. A.; Obeid, L. M. *J. Biol. Chem.* **2011**, *286*, 27855.
3. Merrill, A. H., Jr. *Chem. Rev.* **2011**, *111*, 6387.
4. Park, J. H.; Schuchman, E. H. *Biochim. Biophys. Acta.* **2006**, *1758*, 2133.
5. Spiegel, S.; Milstien, S. *Nat. Rev. Mol. Cell. Biol.* **2003**, *4*, 397.
6. Hannun, Y. A.; Obeid, L. M. *Nat. Rev. Mol. Cell. Biol.* **2008**, *9*, 139.
7. London, E. *Curr. Opin. Struct. Biol.* **2002**, *12*, 480.
8. Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569.
9. Canals, D.; Perry, D. M.; Jenkins, R. W.; Hannun, Y. A. *Brit. J. Pharmacol.* **2011**, *163*, 694.
10. Gatt, S. *J. Biol. Chem.* **1963**, *238*, 3131.
11. Bernardo, K.; Hurwitz, R.; Zenk, T.; Desnick, R. J.; Ferlinz, K.; Schuchman, E. H.; Sandhoff, K. *J. Biol. Chem.* **1995**, *270*, 11098.
12. Li, C. M.; Hong, S. B.; Kopal, G.; He, X.; Linke, T.; Hou, W. S.; Koch, J.; Gatt, S.; Sandhoff, K.; Schuchman, E. H. *Genomics* **1998**, *50*, 267.
13. Koch, J.; Gartner, S.; Li, C. M.; Quintern, L. E.; Bernardo, K.; Levran, O.; Schnabel, D.; Desnick, R. J.; Schuchman, E. H.; Sandhoff, K. *J. Biol. Chem.* **1996**, *271*, 33110.
14. Nilsson, A. *Biochim. Biophys. Acta.* **1969**, *176*, 339.
15. Stoffel, W.; Melzner, I. *Hoppe Seylers Z. Physiol. Chem.* **1980**, *361*, 755.
16. El Bawab, S.; Roddy, P.; Qian, T.; Bielawska, A.; Lemasters, J. J.; Hannun, Y. A. *J. Biol. Chem.* **2000**, *275*, 21508.
17. Tani, M.; Okino, N.; Mitsutake, S.; Tanigawa, T.; Izu, H.; Ito, M. *J. Biol. Chem.* **2000**, *275*, 3462.
18. El Bawab, S.; Usta, J.; Roddy, P.; Szulc, Z. M.; Bielawska, A.; Hannun, Y. A. *J. Lipid. Res.* **2002**, *43*, 141.
19. Sugita, M.; Willians, M.; Dulaney, J. T.; Moser, H. W. *Biochim. Biophys. Acta.* **1975**, *398*, 125.
20. Mao, C.; Xu, R.; Szulc, Z. M.; Bielawska, A.; Galadari, S. H.; Obeid, L. M. *J. Biol. Chem.* **2001**, *276*, 26577.
21. Xu, R.; Jin, J.; Hu, W.; Sun, W.; Bielawski, J.; Szulc, Z.; Taha, T.; Obeid, L. M.; Mao, C. *FASEB J.* **2006**, *20*, 1813.
22. Sun, W.; Xu, R.; Hu, W.; Jin, J.; Crellin, H. A.; Bielawski, J.; Szulc, Z. M.; Thiers, B. H.; Obeid, L. M.; Mao, C. *J. Invest. Dermatol.* **2008**, *128*, 389.
23. Ehlert, K.; Frosch, M.; Fehse, N.; Zander, A.; Roth, J.; Vormoor, J. *Pediatr. Rheumatol. Online. J.* **2007**, *5*, 15.
24. Teichgraber, V.; Ulrich, M.; Endlich, N.; Riethmuller, J.; Wilker, B.; De Oliveira-Munding, C. C.; van Heeckeren, A. M.; Barr, M. L.; von Kurthy, G.; Schmid, K. W.; Weller, M.; Tummler, B.; Lang, F.; Grassme, H.; Doring, G.; Gulbins, E. *Nat. Med.* **2008**, *14*, 382.
25. Liu, X.; Elojeimy, S.; Turner, L. S.; Mahdy, A. E.; Zeidan, Y. H.; Bielawska, A.; Bielawski, J.; Dong, J. Y.; El-Zawahry, A. M.; Guo, G. W.; Hannun, Y. A.; Holman, D. H.; Rubinchik, S.; Szulc, Z.; Keane, T. E.; Tavassoli, M.; Norris, J. S. *Front. Biosci.* **2008**, *13*, 2293.
26. Radin, N. S. *Eur. J. Biochem.* **2001**, *268*, 193.
27. Bielawska, A.; Linardic, C. M.; Hannun, Y. A. *J. Biol. Chem.* **1992**, *267*, 18493.
28. Bielawska, A.; Greenberg, M. S.; Perry, D.; Jayadev, S.; Shayman, J. A.; McKay, C.; Hannun, Y. A. *J. Biol. Chem.* **1996**, *271*, 12646.

29. Delgado, A.; Casas, J.; Llebaria, A.; Abad, J. L.; Fabrias, G. *Biochim. Biophys. Acta.* **2006**, *1758*, 1957.
30. Nikolova-Karakashian, M.; Morgan, E. T.; Alexander, C.; Liotta, D. C.; Merrill, A. H., Jr. *J. Biol. Chem.* **1997**, *272*, 18718.
31. Dagan, A.; Agmon, V.; Gatt, S.; Dinur, T. In *Methods Enzymol*; Merill, A. H., Hannun, Y. A., Eds.; Academic Press: San Diego, 1999.
32. Tani, M.; Okino, N.; Mitsutake, S.; Ito, M. *J. Biochem.* **1999**, *125*, 746.
33. Antes, P.; Schwarzmann, G.; Sandhoff, K. *Eur. J. Cell. Biol.* **1992**, *59*, 27.
34. Nieuwenhuizen, W. F.; van Leeuwen, S.; Gotz, F.; Egmond, M. R. *Chem. Phys. Lipids.* **2002**, *114*, 181.
35. Bedia, C.; Casas, J.; Garcia, V.; Levade, T.; Fabrias, G. *ChemBioChem* **2007**, *8*, 642.
36. Bedia, C.; Camacho, L.; Abad, J. L.; Fabrias, G.; Levade, T. *J. Lipid. Res.* **2010**, *51*, 3542.
37. Xia, Z.; Draper, J. M.; Smith, C. D. *Bioorg. Med. Chem.* **2010**, *18*, 1003.
38. Wichmann, O.; Schultz, C. *Chem. Commun.* **2001**, 2500.
39. Wichmann, O.; Wittbrodt, J.; Schultz, C. *Angew. Chem., Int. Ed.* **2006**, *45*, 508.
40. Greenspan, P.; Mayer, E. P.; Fowler, S. D. *J. Cell. Biol.* **1985**, *100*, 965.
41. Wang, T. Y.; Silvius, J. R. *Biophys. J.* **2000**, *79*, 1478.
42. van Meer, G.; Liskamp, R. M. J. *Nat. Meth.* **2005**, *2*, 14.
43. Nussbaumer, P.; Ettmayer, P.; Peters, C.; Rosenbeiger, D.; Hogenauer, K. *Chem. Commun.* **2005**, 5086.
44. Peters, C.; Billich, A.; Ghobrial, M.; Hogenauer, K.; Ullrich, T.; Nussbaumer, P. *J. Org. Chem.* **2007**, *72*, 1842.
45. Ojima, I.; Vidal, E. S. *J. Org. Chem.* **1998**, *63*, 7999.
46. Briggs, M. S. J.; Bruce, I.; Miller, J. N.; Moody, C. J.; Simmonds, A. C.; Swann, E. *J. Chem. Soc., Perkin. Trans.* **1997**, *1*, 1051.
47. Novotny, J.; Pospechova, K.; Hrabalek, A.; Cap, R.; Vavrova, K. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6975.
48. Raisova, M.; Goltz, G.; Bektas, M.; Bielawska, A.; Riebeling, C.; Hossini, A. M.; Eberle, J.; Hannun, Y. A.; Orfanos, C. E.; Geilen, C. C. *FEBS Lett.* **2002**, *516*, 47.
49. Proksch, D.; Klein, J. J.; Arenz, C. *J. Lipids* **2011**, *2011*, 971618.
50. Mukherjee, S.; Raghuraman, H.; Chattopadhyay, A. *Biochim. Biophys. Acta.* **2007**, *1768*, 59.
51. Wilkening, G.; Linke, T.; Sandhoff, K. *J. Biol. Chem.* **1998**, *273*, 30271.
52. Furst, W.; Sandhoff, K. *Biochim. Biophys. Acta.* **1992**, *1126*, 1.
53. Giehl, A.; Lemm, T.; Bartelsen, O.; Sandhoff, K.; Blume, A. *Eur. J. Biochem.* **1999**, *261*, 650.
54. Ettmayer, P.; Billich, A.; Baumruker, T.; Mechtcheriakova, D.; Schmid, H.; Nussbaumer, P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1555.
55. He, X.; Li, C. M.; Park, J. H.; Dagan, A.; Gatt, S.; Schuchman, E. H. *Anal. Biochem.* **1999**, *274*, 264.
56. Kuerschner, L.; Ejsing, C. S.; Ekroos, K.; Shevchenko, A.; Anderson, K. I.; Thiele, C. *Nat. Methods* **2005**, *2*, 39.
57. Schulze, H.; Schepers, U.; Sandhoff, K. *Biol. Chem.* **2007**, *388*, 1333.