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Improved synthesis of a fluorogenic ceramidase substrate

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ABSTRACT

Substantial interest has focused on the roles of sphingolipid metabolizing enzymes in a variety of hyperproliferative and inflammatory diseases. A key family of enzymes involved in these pathologies is the ceramidases. Ceramidases cleave the pro-apoptotic lipid ceramide into a long-chain fatty acid and sphingosine, which can then be further metabolized to the mitogenic and inflammatory lipid sphingosine 1-phosphate. Consequently, development of ceramidase inhibitors would provide useful pharmacologic probes for further studies of sphingolipid metabolism, as well as lead compounds for drug development. This effort has been hampered by the lack of in vitro and cellular ceramidase assays that are amenable to high-throughput screening. Recently, a fluorogenic ceramide analog has been described as a substrate for use in ceramidase assays. The synthesis of this compound has now been substantially improved in terms of both the required effort and the overall yield of the process. Key improvements include: reduction in number of required steps, use of a hydroboration reaction; incorporation of a Mitsunobu reaction; improved acylation by the addition of triethylamine; together providing a fourfold increase in the overall yield. In addition, it has been demonstrated that the ceramide analog can be used in high-throughput assays to identify ceramidase inhibitors. Overall, the improved efficiency in the preparation of this ceramidase substrate should accelerate discovery efforts relating to sphingolipid metabolism.

1. Introduction

Sphingolipids function as structural components of plasma membranes, as well as signaling molecules that trigger profound physiological and pathological responses,[1,2] and their dynamic interconversion offer sites of potential therapeutic manipulation by small molecules (Fig. 1). It is hypothesized that the balance between ceramide and sphingosine-1-phosphate (S1P), plays a pivotal role in cellular survival, differentiation and proliferation.[3–5] For example, ceramide and sphingosine have been shown to induce tumor growth arrest and apoptosis; whereas, S1P promotes tumor cell proliferation and survival. Importantly, stress from radiation therapy or chemotherapy typically elevates ceramide production resulting in tumor cell apoptosis.[6] Deregulation of the ceramide: S1P balance has emerged as an important characteristic in cancer pathology and resistance to therapy.

One mechanism by which cancer cells escape ceramide-induced death is by over-expressing ceramidase enzymes, which cleave the amide bond of ceramide to form sphingosine and a long-chain fatty acid.[7] This allows the cells to more rapidly remove the pro-apoptotic ceramide, facilitating cellular survival, and it has been demonstrated that ceramidases play a crucial role in regulating the ceramide: S1P balance.[8] Therefore, targeting ceramidase and other sphingolipid metabolizing enzymes is an emerging approach toward cancer therapy.[5] In this regard, it has been reported that acid ceramidase[9] is over-expressed in prostate cancer, head and neck cancer, and melanoma.[10–13] It has also been shown that ceramidase inhibitors induce apoptosis in vitro and that ceramide analogs cause apoptosis and inhibit tumor growth in vivo.[9,14,15] Therefore, manipulation of the ceramide/S1P ratio by inhibition of ceramidases may offer an effective strategy in cancer therapy. In addition, our group has identified sphingosine kinase inhibitors that reduce the production of S1P and have significant anticancer activity in animal models.[16,17] It can be postulated that combined inhibition of ceramidase and sphingosine kinase in unison may increase antitumor efficacy beyond that attained by inhibiting either enzyme alone.

Although some inhibitors of ceramidase have been described in the literature,[3] the field suffers from the lack of 'drug-like' ceramidase inhibitors with acceptable pharmacologic properties. Therefore, new ceramidase inhibitors are needed, and the need for the development of assays amenable to high-throughput screening has been recognized.[18] Recently, a significant advance was made in this area by Bedia et al. who identified a ceramide analog, compound (**1**) (Fig. 2), that generates a fluorescent signal indicative of ceramidase activity.[18] They further demonstrated that this ceramidase substrate can be used in drug discovery assays by testing a small library of known dihydroceramide desaturase inhibitors. In an effort to implement this approach into a high-throughput

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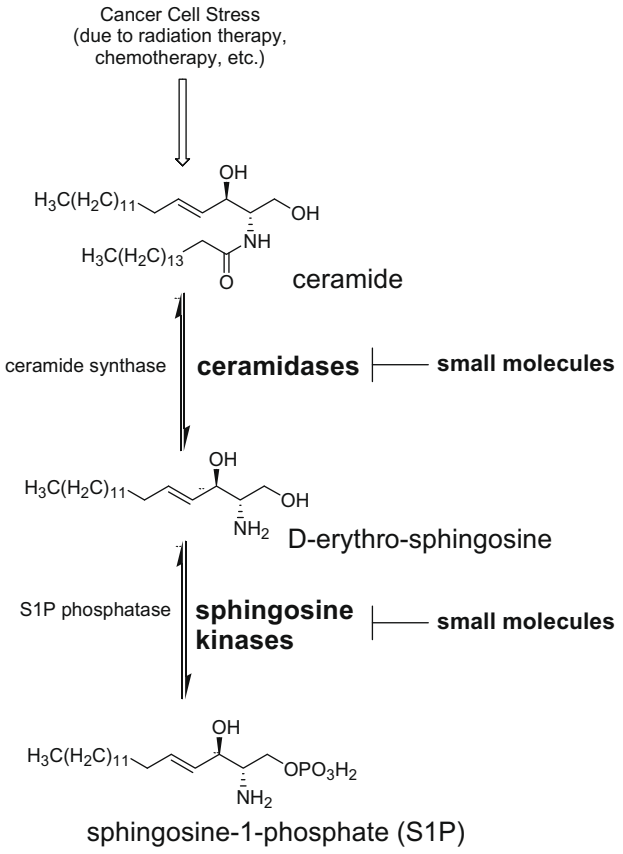


Figure 1. Simplified pathway of sphingolipid metabolism. The interconversion of ceramide, sphingosine and sphingosine-1-phosphate (S1P) is shown.

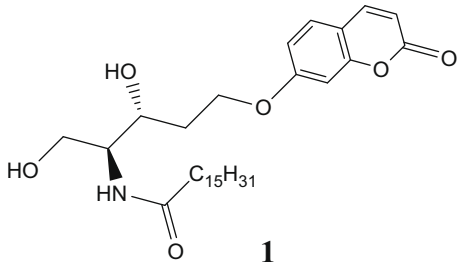


Figure 2. Compound (**1**): *N*-((2*S*,3*R*)-1,3-dihydroxy-5-((2-oxo-2*H*-chromen-7-yl)oxy)pentan-2-yl)palmitamide.

screening effort, we sought to improve their method for the synthesis of compound (**1**). Herein, we provide a new synthetic route toward compound (**1**) that reduces the number of steps by two and increases the overall yield fourfold. Additionally, we demonstrate that this compound can be used as a substrate in a high-throughput assay for ceramidase inhibitors.

2. Results and discussion

2.1. Synthesis of *N*-((2*S*,3*R*)-1,3-dihydroxy-5-((2-oxo-2*H*-chromen-7-yl)oxy)pentan-2-yl)palmitamide (1)

In 2007, Bedia et al. published a seven-step method to synthesize a fluorogenic ceramide analog (**1**) that is potentially useful in the identification of ceramidase inhibitors.[18] Briefly, Garner's aldehyde was converted to (*S*)-*tert*-butyl 2,2-dimethyl-4-((*S*)-oxiran-2-yl)oxazolidine-3-carboxylate (**2**) by a Corey–Chaykovsky epoxidation,[19] and then transformed by treatment with dimethylsulfonium methylide[20,21] to yield the corresponding allylic alcohol (*S*)-*tert*-butyl 4-((*R*)-1-hydroxyallyl)-2,2-dimethyloxazolidine-3-carboxylate (**3**). Hydroboration of (**3**) using borane in THF produced a mixture of the two possible regioisomers, (*S*)-*tert*-butyl 4-((*R*)-1,3-dihydroxypropyl)-2,2-dimethyloxazolidine-3-carboxylate (**4-b**) and (4*S*)-*tert*-butyl 4-((1*S*)-1,2-dihydroxypropyl)-2,2-dimethyloxazolidine-3-carboxylate (**4-a**). After separation of the isomers, (**4-b**) was mesylated and then coupled with umbelliferone cesium salt[22] to form (*S*)-*tert*-butyl 4-((*R*)-1-hydroxy-3-((2-oxo-2*H*-chromen-7-yl)oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (**6**). After removal of the protective groups by trifluoroacetic acid, the ceramide analog (**1**) was obtained by acylation with palmitoyl chloride by the Schotten–Baumann reaction with an overall yield of 2%.

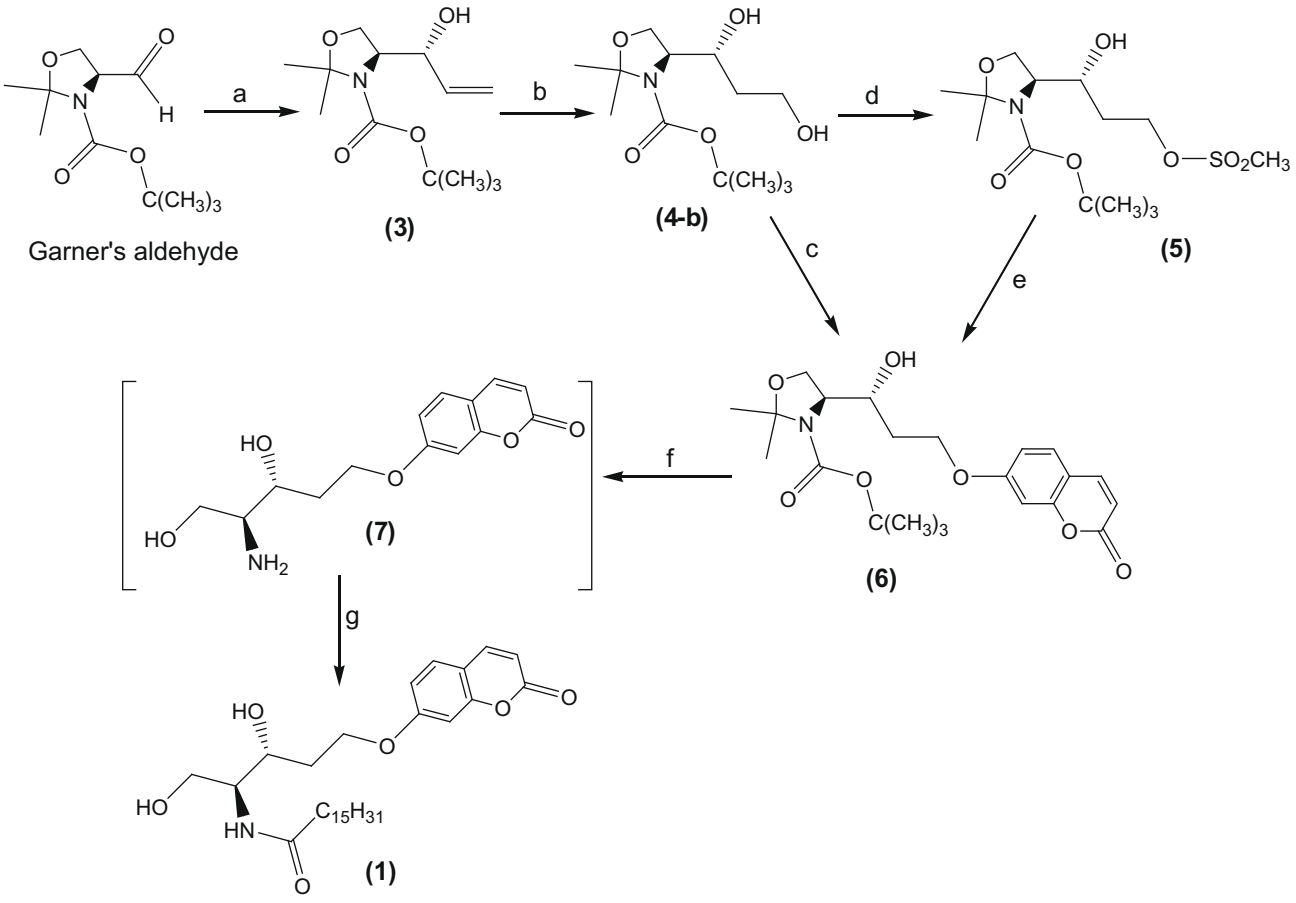
During our effort to synthesize compound (**1**), we identified a more efficient route (Scheme 1). First, we found that the Corey–Chaykovsky reaction did not provide substantial stereoselectivity in the epoxidation of Garner's aldehyde. Therefore, instead of two reactions to obtain (*S*)-*tert*-butyl 4-((*R*)-1-hydroxyallyl)-2,2-dimethyloxazolidine-3-carboxylate (**3**), we prefer the one-step Grignard reaction with vinyl magnesium bromide, even though the purification of compound (**3**) is somewhat difficult.[23–25] Second, the hydroboration–oxidation of compound (**3**) with borane–THF produced a mixture of (**4-a**) and (**4-b**), and this low regioselectivity is likely due to an inductive effect of the 2'-OH. One way to circumvent this limitation is to eliminate the 2'-OH influence by protection of the 2'-OH such that the hydroboration will favor the terminal position of the double bond. However, this would add reaction steps to the synthesis of compound (**1**). Another way to overcome this shortcoming is to use a more selective hydroboration reagent so that the reaction might not be affected by the 2'-OH. Therefore, we utilized 9-borabicyclo[3.3.1]nonane (9-BBN), which resulted in improved selectivity for the hydroboration–oxidation,[26,27] such that only (**4-b**) was produced.

Displacement of primary and secondary alcohols under Mitsunobu conditions is a versatile synthetic procedure to form alkyl aryl ethers.[28] Several reasons motivated us to consider using a Mitsunobu reaction to synthesize compound (**6**). First, it allows the preparation of compound (**6**) with one-step less than using mesylation–substitution. Secondly, the pK_a of the acidic umbelliferone reactant is 7.7, which is much less than 15 as is generally required for the Mitsunobu reaction conditions.[29] Thirdly, formation of the ether bond with the primary alcohol was expected to be sterically favored over reaction at the secondary alcohol. Together, these factors allowed us to obtain compound (**6**) via a Mitsunobu reaction without protection of the secondary hydroxyl. Although the reaction conditions can be further optimized for chemical yield, the Mitsunobu reaction is a preferred alternative to the mesylation–substitution in the synthesis of compound (**6**). To our surprise, we were not able to obtain pure compound (**6**) from (**4-b**) using tosylation–substitution.

Finally, the yield of the fluorogenic ceramide analog (**1**) from intermediate (**6**) was 20% using the original Schotten–Baumann reaction.[7] We found that by using triethylamine as the base and hexadecanoic acid 2,5-dioxopyrrolidin-1-yl ester as the acylating agent,[30] the yield of compound (**1**) could be significantly improved, providing a chemical yield up to 80%. The overall yield of the present scheme is 8%, which is fourfold greater than the original approach described by Bedia et al. In conclusion, we have improved the synthetic method for the fluorogenic ceramide analog (**1**) with fewer reaction steps and a better chemical yield compared to the original methodology.

2.2. In vitro analyses of the fluorogenic ceramide analog

Although Bedia et al. expertly described the kinetics of hydrolysis of compound (**1**) by ceramidase and tested a small series of



Scheme 1. Reagents and conditions: (a) Vinylmagnesium bromide/THF/−75 °C /2 h; (b) (1) 9-BBN/THF/rt/2 h; (2) H_2O_2/50 °C/1 h; (c) umbelliferone/TPP/DEAD/THF/rt/24 h; (d) MsCl/Et_3N/CH_2Cl_2/MsCl/0 °C/30 min; (e) Cs_2CO_3/umbelliferone/acetone/65 °C/5 h; (f) TFA/CH_2Cl_2/rt/2 h; (g) palmitic acid succinimide/Et_3N/THF/80 °C/12 h.

known dihydroceramide desaturase inhibitors,[18] it remained necessary to demonstrate the use of the assay in a high-throughput format. Therefore, the potential for use of compound (**1**) to measure ceramidase activity in a screening assay was assessed. First, to demonstrate that compound (**1**) could be catabolized to the sphingosine analog (**7**), the compound was incubated in tissue culture medium either alone or in the presence of SKOV3 human ovarian carcinoma cells for 24 h at 37 °C and analyzed by HPLC. As shown in Panel A of Figure 3, exposure of compound (**1**) to cells leads to an increase in the area under the curve (AUC) of peak A. Mass spectrometric analyses confirmed that peak A is consistent with the mass of the sphingosine analog (**7**), and that peak B is consistent with the mass of the ceramide analog (**1**). These results demonstrate that ceramidases in the SKOV3 cells are able to catalyze the conversion of compound (**1**) to the sphingosine analog (**7**), and that these compounds are easily resolved by HPLC.

Time course experiments were conducted to assess the rate of the conversion of compound (**1**) to the sphingosine analog (**7**), and to demonstrate that the assay can be performed in a 96-well format, which is more conducive to high-throughput screening. In these experiments, compound (**1**) was incubated in tissue culture medium with or without SKOV3 cells for varying times, and the samples were then analyzed either by HPLC or by fluorescence intensity on a platereader after cleavage by $NaIO_4$ to release umbelliferone as described by Bedia et al.[18] For those samples analyzed by HPLC, the amount of compound (**7**) produced was calculated at each time point and plotted in Panel B of Figure 3. As the data illustrates, the amount of compound (**1**) hydrolyzed to the sphingosine analog (**7**) by the SKOV3 cells increased in a nearly linear fashion from 1 to 24 h. This linear kinetic profile provides excellent flexibility for the evaluation of ceramidase activity in a variety of growth conditions. To demonstrate that compound (**1**) could be used in a screening platform, it was incubated with SKOV3 cells as above; however, the resultant sphingosine analog (**7**) was converted to free umbelliferone by reaction with $NaIO_4$, which has greater fluorescence intensity than the bound fluorophore precursor in compounds (**1**) and (**7**).[18] As shown in Panel C of Figure 3, the fluorescence signal increased in a linear fashion for at least 24 h, virtually in parallel with the increase in compound (**7**) quantified by HPLC. This demonstrates that the free umbelliferone created by reaction of compound (**7**) with $NaIO_4$ does produce greater fluorescence than the precursor ceramide analog (**1**), and that this method can be used to measure ceramidase activity in a microtiter plate format.

To further demonstrate the usefulness of the analog in identifying ceramidase inhibitors, we evaluated the effects of a known ceramidase inhibitor, *N*-oleoylethanolamine,[31] using both assay formats. Compound (**1**) was incubated with or without cells in the presence of *N*-oleoylethanolamine at final varying concentrations for 5 h at 37 °C, and subsequently analyzed by the HPLC and platereader methods as discussed above. As demonstrated in Figure 4, *N*-oleoylethanolamine caused dose-dependent decreases in ceramidase activity as quantified by HPLC (Panel A) and fluorescence intensity analyses (Panel B). The IC_{50} values determined by the two methods were very similar, specifically 24 and 28 μM for the HPLC and platereader methods, respectively. These values are lower than values reported by others, 71 and 76 μM, using rat and pig liver tissue,[32] which may reflect greater sensitivity of the human enzymes.

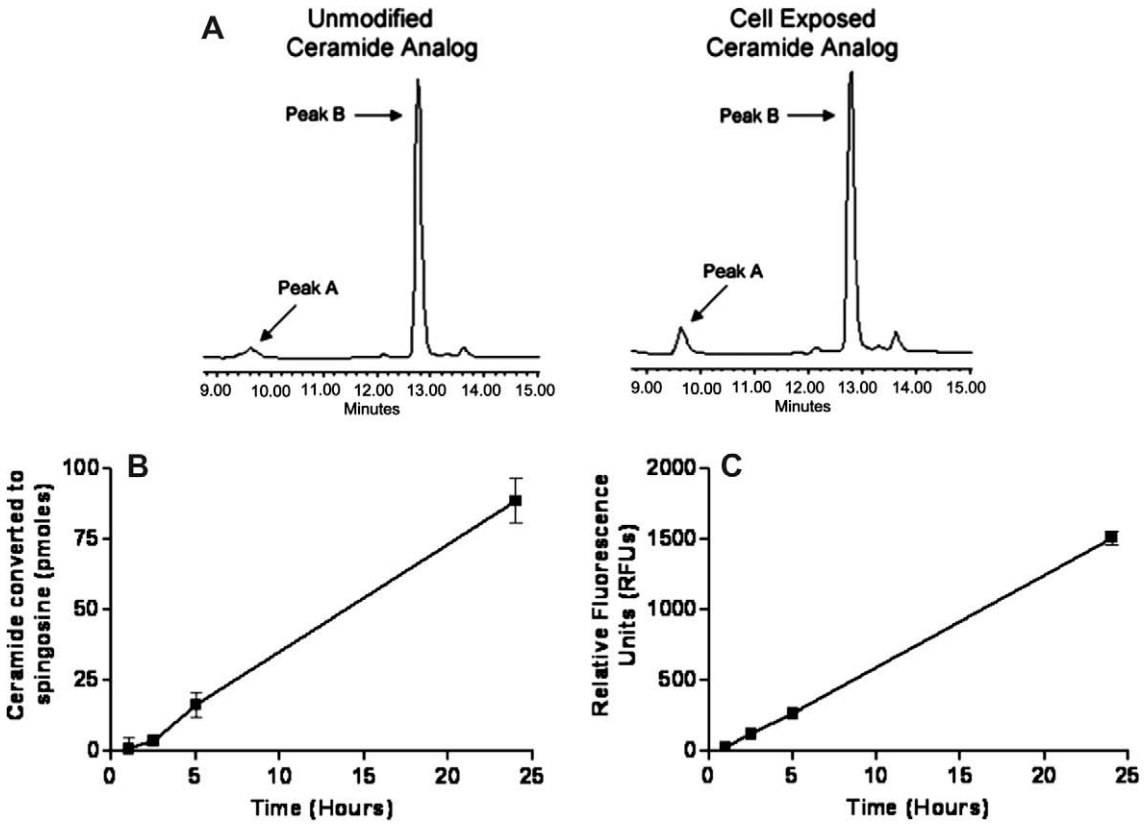


Figure 3. Cellular conversion of compound (**1**) to compound (**7**). Panel A. The ceramide analog (**1**) was incubated in media alone or in the presence of SKOV3 cells for 24 h at 37 °C and subjected to HPLC fractionation as described in the Experimental Section. Panel B. The ceramide analog (**1**) was incubated with SKOV3 cells for the indicated times at 37 °C, and the amount of (**1**) converted to sphingosine analog (**7**) was calculated from HPLC chromatograms. Values represent mean ± SD for triplicate samples). Panel C. Incubations identical to those described for Panel B were conducted and the amount of (**1**) converted to sphingosine analog (**7**) was determined using the platereader assay detailed in the Section 3. Values represent mean ± SD of six samples.

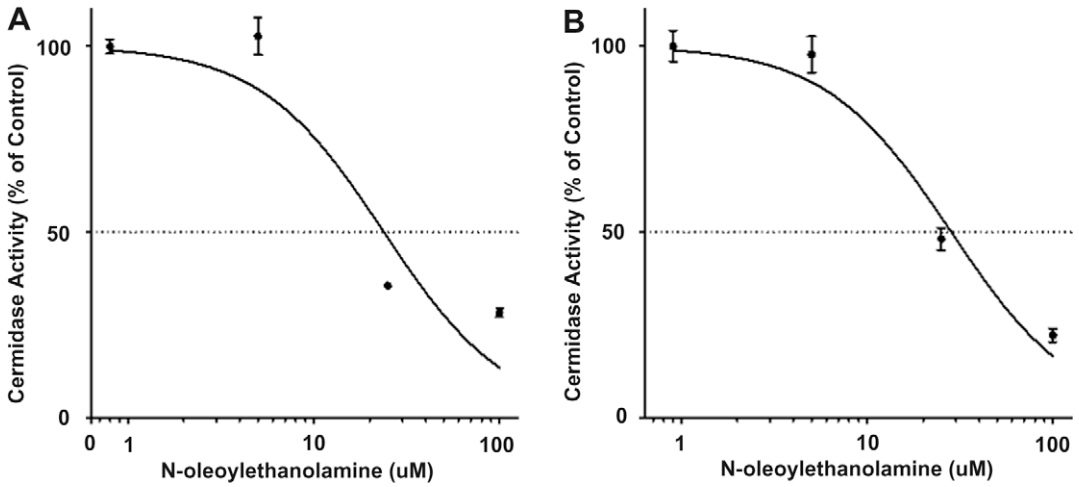


Figure 4. Inhibition of ceramidase activity by *N*-oleoylethanolamine. The ceramide analog (**1**) was incubated in media without cells or media with cells containing 0, 5, 25, or 100 μM *N*-oleoylethanolamine for 5 h at 37 °C as described in the Section 3. The samples were analyzed by either HPLC assay (Panel A, values represent the mean ± SD of triplicate samples) or the platereader assay (Panel B, values represent the mean ± SD of six samples).

To confirm that the fluorescence intensity assay can be conducted in a high-throughput mode, we completed a pilot screen of 7690 compounds from the ChemBridge Corporation DIVERSet collection in the Drug Discovery Core at the Medical University of South Carolina. For this screen, the pools of 10 compounds per sample were assayed at a final concentration of 30 μM for each compound, and samples were tested in triplicate. A compound pool was considered to be a hit if it reduced ceramidase activity by at least 75% when compared to DMSO-treated controls, and approximately 4% of the samples were classified as active by this criterion. While confirmatory assays using individual ceramidase isozymes are needed, these data confirm that the ceramide analog

(**1**) can be used in assays to evaluate ceramidase inhibitors, including high-throughput screening in microtiter plates.

2.3. Conclusions

In conclusion, we have developed a synthetic approach that decreases the overall effort required to synthesize the fluorogenic ceramide analog (**1**), while simultaneously increasing yield. In addition, we have shown that compound (**1**) can be used in cell-based assays to evaluate ceramidase inhibitors, and that these assays can be applied to high-throughput screens in a 96-well, and likely a 384-well, format. The improved efficiency in the preparation of this ceramidase substrate, along with the amenability of the analog to high-throughput screening, should accelerate drug discovery efforts relating to targets in the sphingolipid metabolism pathway.

3. Experimental section

3.1. General methods

All commercial chemicals were of reagent grade, and anhydrous solvents were purchased from Aldrich or freshly distilled in our own lab. (*S*)-*tert*-Butyl 4-formyl-2,2-dimethyloxazolidine-3-carboxylate (Garner aldehyde) was from Astatech, Inc., and *N*-oleoylethanolamine was from Sigma–Aldrich. Screening compounds consisted of the DIVERSet library from ChemBridge Corporation, which were dissolved in DMSO at a concentration of 10 mM. Proton magnetic resonance and carbon magnetic resonance spectra were recorded on a Varian INOVA 400 MHz Spectrometer. Radial chromatography was performed on a Harrison Research Chromatotron using EMD 60 PF_{254}-containing gypsum as the solid phase. Thin layer chromatography was performed with Sigma–Aldrich silica gel aluminum-backed plates with UV visualization. Optical rotation was measured with a Rudolph Autopol IV polarimeter.

3.2. Synthesis of (*S*)-*tert*-butyl 4-((*R*)-1-hydroxyallyl)-2,2-dimethyloxazolidine-3-carboxylate (3)

(*S*)-*tert*-Butyl 4-formyl-2,2-dimethyloxazolidine-3-carboxylate (Garner aldehyde) (680 mg, 2.97 mmol) was dissolved in anhydrous THF (10 mL) under argon. The solution was chilled to less than −75 °C, followed by the addition of 1 M vinyl magnesium bromide in THF (9 mL). The addition rate was controlled to keep the temperature below −70 °C. After the addition, the reaction was continued at this low temperature for 2 h, and then the temperature was allowed to gradually rise to −40 °C. Saturated NH_4Cl (5 mL) was added to quench the reaction, and the mixture was then warmed to the room temperature and extracted with ether (3 × 50 mL). The ether solutions were combined and dried over Na_2SO_4. After primary purification by a small silica column sequentially eluted with 0%, 5%, 10% and 15% ethyl acetate in hexane, the collected fractions were analyzed by TLC (25% ethyl acetate in hexane, visualized by 0.3% ninhydrin in butanol/acetic acid (97:3)). Although the reaction resulted in a mixture of diastereomers, the *S*-configuration, that is, the target compound, was the preferred product by a ratio of approximately 6:1,[24] and the diastereomers were resolved by chromatography. The fractions containing the target compound were concentrated and purified by radial chromatography (silica) eluted with the same sequence of ethyl acetate in hexane to afford compound (**3**): a colorless oil, 374 mg, 1.46 mmol, Yield = 49% (R_f = 0.25 in 25% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.87 (m, 1H), 5.36 (m, 1H), 5.20 (m, 1H), 3.80–4.40 (m, 5H), 1.55 (s, 3H), 1.48 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.2, 137.0, 116.2, 94.5, 81.2, 74.1, 64.8, 62.0, 28.4 (3C), 26.4, 24.6.

3.3. Synthesis of (*S*)-*tert*-butyl 4-((*R*)-1,3-dihydroxypropyl)-2,2-dimethyloxazolidine-3-carboxylate (4-b)

(*S*)-*tert*-Butyl 4-((*R*)-1-hydroxyallyl)-2,2-dimethyloxazolidine-3-carboxylate (**3**) (328 mg, 1.29 mmol) was placed in a three-mouth flask (100 mL), and dissolved in anhydrous THF (3 mL) under the protection of argon. The solution was chilled in an ice-water bath for a few minutes, followed by addition of 9-BBN in THF (0.5 M, 4 mL). The temperature of the reaction was allowed to gradually rise to room temperature, and the reaction was continued for 1 h at which time another portion of 9-BBN (0.5 M, 3 mL) was added. After incubation for another hour, the mixture was chilled in the ice-water bath, and ethanol (2 mL) was added to quench the reaction, followed by 3 N NaOH (2 mL) and 30% hydrogen peroxide (2 mL). The solution was then warmed to 50 °C for 1 h, at which time brine (20 mL) was added and the mixture was extracted with ether (3 × 50 mL). The collected organic phases were dried over Na_2SO_4 and then passed through a pad of silica eluted with ethyl acetate. The concentrated residue was purified by radial chromatography on silica eluted with 50–75% ethyl acetate in hexane. Only one major component, the target compound (**4-b**), was observed by TLC (silica, ethyl acetate) with an R_f of 0.24 when visualized by tolidine-chlorine. However, an impurity with an R_f of 0.21 was detected by staining with PMA, necessitating careful purification of the target compound. Compound (**4-b**) is an off-white semisolid: 189 mg, 0.69 mmol, Yield = 54%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (m, 1H), 3.74–4.18 (m, 6H), 3.38 (br s, 1H), 1.66 (m, 2H), 1.58 (s, 3H) 1.50 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.6, 94.5, 81.5, 73.6, 65.0, 62.3, 61.4, 33.9, 28.4 (3C), 26.4, 24.1.

3.4. Synthesis of (*S*)-*tert*-butyl 4-((*R*)-1-hydroxy-3-((methylsulfonyl)oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (5)

(*S*)-*tert*-Butyl 4-((*R*)-1,3-dihydroxypropyl)-2,2-dimethyloxazolidine-3-carboxylate (**4-b**) (38 mg, 0.14 mmol) was dissolved in dichloromethane (4 mL) under argon. The solution was chilled in an ice-bath for 15 min, followed by the addition of triethylamine (23 μl, 0.17 mmol) and methanesulfonyl chloride (12.5 μl, 0.16 mmol) for 30 min before it was quenched by the addition of water (10 mL). The reaction mixture was transferred to a separatory funnel, and an additional 20 mL of dichloromethane was added. The organic phase was sequentially washed with 1 N HCl (10 mL), 5% $NaHCO_3$ (10 mL) and 10% NaCl (10 mL), and then dried over Na_2SO_4. After passing through a pad of silica, the concentrated residue was purified by radial chromatography using a stepped gradient of 25–50–100% ethyl acetate in hexane to afford (**5**) as a colorless oil: 30 mg, 0.085 mmol, Yield = 61%. TLC, R_f = 0.48 (silica, ethyl acetate, visualized by tolidine-chlorine). ^{1}H NMR ($CDCl_3$) δ 4.65 (bd, *J* = 7.6 Hz, 1H), 4.47 (m, 2H), 3.76–4.20 (m, 3H), 3.03 (s, 3H), 1.96 (m, 1H), 1.70 (m, 2H), 1.57 (s, 3H), 1.50 (s, 9H), 1.49 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 155.1, 94.9, 82.0, 70.0, 68.0, 65.4, 62.3, 37.3, 32.2, 28.5 (3C), 26.5, 24.1.

3.5. Synthesis of (*S*)-*tert*-butyl 4-((*R*)-1-hydroxy-3-((2-oxo-2*H*-chromen-7-yl)oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (6)

Method A: Cesium carbonate (131 mg, 0.40 mmol) and umbelliferone (29 mg, 0.18 mmol) were suspended in acetone (4 mL) under argon and stirred at room temperature, during the addition of (*S*)-*tert*-butyl 4-((*R*)-1-hydroxy-3-((methylsulfonyl)oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (**5**) (43 mg, 0.12 mmol) in acetone (2 mL). The mixture was heated to 65 °C under a condenser for 5 h, and the reaction was monitored by TLC (silica, 50% ethyl

acetate in hexane, R_f = 0.21, visualized by UV). Upon completion, the solvent was removed in vacuo, and the residue was transferred to a separatory funnel and extracted with 10% NaCl (20 mL) and ethyl acetate (20 mL). The aqueous phase was extracted with ethyl acetate (3 × 20 mL), and the combined organic solution was dried over Na_2SO_4. After passing through a pad of silica, the concentrated residue was purified by radial chromatography (silica) eluted with 25–50–100% ethyl acetate in hexane, to afford (**6**) as a colorless oil: 21.5 mg, 0.05 mmol, Yield = 43%.

Method B (Mitsunobu reaction): Triphenylphosphine (TPP) (53 mg, 0.20 mmol), umbelliferone (32 mg, 0.20 mmol) and (*S*)-*tert*-butyl 4-((*R*)-1,3-dihydroxypropyl)-2,2-dimethyloxazolidine-3-carboxylate (**4-b**) (51 mg, 0.19 mmol) were dissolved in anhydrous THF (4 mL). The mixture was protected under argon and stirred at room temperature, followed by drop-wise addition of diethylazodicarboxylate (DEAD) (32 μl, 0.20 mmol) in anhydrous THF (1 mL). After incubation at rt for 24 h, ethyl acetate (20 mL) was added and the solution was washed twice with 10% NaCl (10 mL). The collected organic phase was dried over Na_2SO_4, passed through a pad of silica eluted with ethyl acetate, and the concentrated residue was purified by radial chromatography (silica) eluted with 25–50–100% ethyl acetate in hexane to afford the (**6**) as a colorless oil: 32 mg, 0.076 mmol, Yield = 40%, (TLC, ethyl acetate, UV, R_f = 0.51). ^{1}H NMR ($CDCl_3$) δ 7.64 (d, J = 9.6 Hz, 1H), 7.36 (d, J = 8.8 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 6.83 (s, 1H), 6.25 (d, J = 9.6 Hz, 1H), 3.70–4.50 (m, 6H), 2.02 (m, 1H), 1.77 (m, 1H), 1.65 (s, 3H), 1.61 (s, 3H), 1.51 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 162.4, 161.5, 156.0, 143.6, 128.9, 113.1, 112.9, 112.8, 112.6, 101.7, 94.8, 81.8, 70.6, 65.8, 65.4, 62.5, 32.2, 28.5 (3C), 26.5, 24.2.

3.6. Synthesis of 7-(((3*R*,4*S*)-4-amino-3,5-dihydroxypentyl)oxy)-2*H*-chromen-2-one (7)

(*S*)-*tert*-Butyl 4-((*R*)-1-hydroxy-3-((2-oxo-2*H*-chromen-7-yl)-oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (**6**) (17 mg, 0.04 mmol) was dissolved in dichloromethane (4 mL), followed by addition of TFA (0.2 mL) at room temperature. The mixture was stirred for 2 h before the addition of dichloromethane (10 mL), and extraction with water (2 × 15 mL). The combined aqueous phase was lyophilized to afford (**7**) as a white solid residue which after dissolution in methanol and drying under nitrogen became a colorless oil: 10 mg, 0.025 mmol, Yield = 64%. ^{1}H NMR (methanol-d_4) δ 7.89 (d, J = 8.4 Hz, 1H), 7.55 (d, J = 8.4 Hz, 1H), 6.95 (d, J = 9.6 Hz, 1H), 6.94 (s, 1H), 6.25 (d, J = 9.6 Hz, 1H), 4.23–4.28 (m, 2H), 4.11–4.15 (m, 1H), 3.70–3.93 (m, 3H), 2.08 (m, 1H), 1.93 (m, 1H); ^{13}C NMR (methanol-d_4) δ 163.7, 163.3, 157.1, 145.8, 130.5, 114.2, 114.1, 113.5, 102.3, 67.0, 66.1, 58.9, 58.4, 33.6.

3.7. Synthesis of *N*-((2*S*,3*R*)-1,3-dihydroxy-5-((2-oxo-2*H*-chromen-7-yl)oxy)pentan-2-yl)palmitamide (1)

(*S*)-*tert*-Butyl 4-((*R*)-1-hydroxy-3-((2-oxo-2*H*-chromen-7-yl)-oxy)propyl)-2,2-dimethyloxazolidine-3-carboxylate (**6**) (21 mg, 0.05 mmol) was dissolved in dichloromethane (5 mL), and the solution was chilled in ice-bath before the addition of TFA (0.25 mL). The mixture was stirred at 4 °C for 10 min, and then stirred at room temperature for 2 h until TLC indicted the completion of the reaction (silica, ethyl acetate, UV). The solvent was removed by rotary evaporation, followed by co-evaporation with toluene twice. The residue was dissolved in THF (3 mL) and triethylamine (2 mL) and palmitic acid succinimide (36 mg, 0.1 mmol) were added, and the mixture was refluxed at 80 °C overnight. After cooling to rt, ethyl acetate (30 mL) was added and the sample was washed with water (15 mL) and 1 N NaOH (15 mL). The organic solution was dried over Na_2SO_4, passed through a pad of silica eluted with 10% methanol in chloroform and concentrated. The residue was purified by radial chromatography (silica) with a stepped gradient of 0–1–3–5–10% methanol in chloroform to afford (**1**) as a white solid: 12 mg, Yield = 81% $[\alpha]_D^{22}$ = 2.33 (c 0.6%, $CHCl_3$), (R_f = 0.37, 10% methanol in chloroform, UV). ^{1}H NMR ($CDCl_3$) δ 7.63 (d, J = 9.6 Hz, 1H), 7.36 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.82 (s, 1H), 6.41 (m, 1H), 6.25 (d, J = 9.6 Hz, 1H), 4.17–4.31 (m, 2H), 3.79–4.08 (m, 4H), 3.57 (br s, 1H), 2.43t, J = 7.6 Hz, 2H), 2.06 (m, 2H), 1.64 (m, 2H), 1.20–1.50 (m, 25H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 174.2, 162.0, 161.4, 156.0, 143.6, 129.0, 113.5, 113.0, 112.8, 101.8, 71.4, 66.2, 62.7, 54.5, 37.1, 33.7, 32.1, 29.4–29.9 (m, 9C), 28.6, 25.9, 22.9, 14.3.

3.8. Ceramidase assays

In a typical experiment, SKOV3 cells (18,000) were plated in each well of 96-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and incubated overnight at 37 °C in a 5% CO_2 environment. To initiate the assay, the media was removed and the cells were washed with sterile phosphate-buffered saline and 100 μl of fresh DMEM containing 16 μM (**1**) was added to the wells. Test compounds (or DMSO as the solvent control) were immediately added, and the samples were incubated for varying times. The hydrolysis of compound (**1**) was measured using the following methods. For inhibitor studies, IC_{50} values were calculated using Graphpad Prism (version 5.0).

HPLC assay. At the end of the incubation period, the reactions were terminated by the addition of 100 μl of MeOH, and the samples were extracted a dichloromethane: 50% MeOH mixture (v/v), and the resulting organic phase was dried under nitrogen. The samples were then resuspended in ethanol and fractionated by HPLC on a Chromalith Performance RP-8e column (VWR) using a 1 ml/min flow rate, and the eluant was monitored for fluorescence at excitation and emission wavelengths of 320 and 390 nm, respectively. Initially, the mobile phase was maintained at 100% water for one min, followed by a linear gradient of 0–100% methanol over 10 min. The mobile phase was then maintained at 100% methanol for 5 min, and then decreased to 0% methanol on a linear gradient over 2 min. Representative chromatograms from non-cell-exposed and cell-exposed compounds are shown in Panel A of Figure 3. Mass spectroscopy demonstrated that peak A is consistent with the mass of the sphingosine analog (**7**) and peak B is consistent with the mass of the ceramide analog (**1**). The amount of the (**1**) catabolized to (**7**) was calculated as: the AUC for peak A/the AUC for peaks A + B times the mass of the substrate in each sample (1600 pmol).

Fluorescence intensity assay. At the end of the incubation period, 100 μl $NaIO_4$ (10 mg/ml in 100 mM phosphate buffer, pH 8.0) and 50 μl bovine serum albumin (2 mg/ml in 100 mM phosphate buffer, pH 8.0) were sequentially added to each well. The samples were incubated overnight at 37 °C to release umbelliferone from (**7**)[18] and analyzed using a SpectraMax M5 platereader (Molecular Devices) at excitation and emission wavelengths of 355 and 460 nm, respectively. The relative fluorescent units (RFUs) for wells that did not contain cells were considered negative controls, and their values were subtracted from the values obtained from each of the wells containing cells.

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