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Synthesis of a Novel Ceramide Analogue via Tebbe Methylenation and Evaluation of Its Antiproliferative Activity

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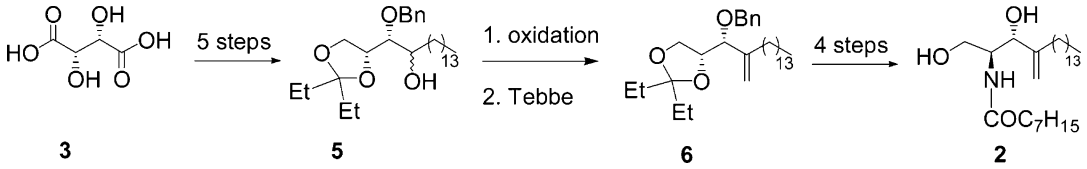
ABSTRACT



A new analogue of (2*S*,3*R*)-ceramide (2) with a methylene group at C4 has been synthesized from D-tartaric acid (3) by using Tebbe methylenation as the key step. Compound 2 exhibited markedly higher antiproliferative activity on mouse embryonic fibroblast (MEF) cells than natural (2*S*,3*R*,4*E*)-ceramide (1).

Ceramide (*N*-acylsphingosine, **1**) is a long-chain aliphatic 2-amido-1,3-diol with a C(4),C(5)-trans double bond (Figure 1).[1] As a key intermediate in the biosynthesis of many sphingolipid mediators, ceramide has been implicated in many physiological events, including the regulation of cell growth and differentiation, inflammation, and in cellular responses to stress stimuli (such as exposure to heat, radiation, oxidative conditions, and chemotherapeutic agents).[2] Ceramide is a messenger for induction of apoptosis, the cell's intrinsic death program.[3]

The well-known biological importance of ceramide has inspired the chemical synthesis of analogues to examine the structural features responsible for some of its physiological functions. In a recent monolayer study of 14 synthetic analogues of **1**, we demonstrated that the (*E*)-C(4)−C(5) double bond of **1** regulates its dipole potential, elastic properties, and packing behavior[4] and appears to be crucial for ceramide's capacity to modulate various fundamental biological functions. Analogues that lack this double bond have reduced apoptotic activity.[5] The ability of **1** to induce apoptosis has been postulated to also require the allylic alcohol group at C3.[6,7]

A deficiency in induction of apoptosis may result in aberrant cell proliferation, and may culminate in tumor formation.[8] An experimental approach in anticancer chemo-

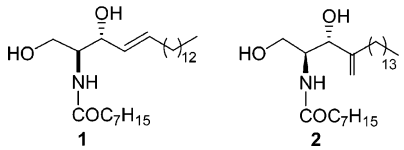


Figure 1. Structures of D-*erythro*-*N*-octanoylceramide (**1**) and Its C4-methylene analogue (**2**).

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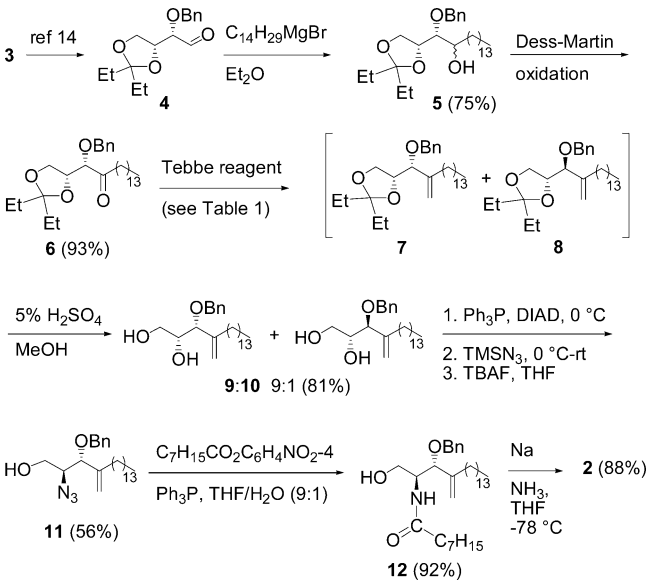
Table 1. Methylenation of Ketone **6**

entry		conditions	ratio of **7**:**8**	overall yield (%)
1	Wittig reaction	CH_2PPh_3, THF, −78 °C to reflux	1.0:0	29
2	Tebbe reagent	2 equiv of Tebbe reagent added to ketone **6** at −78 °C, then warmed to room temperature, 3 h	1.0:1.0	84
3	Tebbe reagent	2 equiv of Tebbe reagent added to ketone **6** at room temperature, 3 h	3.0:1.0	81
4	Tebbe reagent	ketone **6** added to 2 equiv of Tebbe reagent at room temperature, 3 h	9.0:1.0	81

therapy is to employ unnatural analogues of **1** that escape recognition by endogenous ceramide-metabolizing enzymes, and thus are not converted to sphingolipids that stimulate cell proliferation,[9] or inhibit enzymes involved in ceramide turnover.[10] The resulting high intracellular levels of ceramide or its analogues are expected to amplify apoptosis.[11] Our interest in cell-permeable (*N*-octanoyl) analogues of ceramide that can be added exogenously to cells to stimulate apoptosis[5b,12] prompted us to prepare the novel C4-exo-methylene ceramide analogue **2**, in which both the unsaturation at C4 and allylic nature of the C3-hydroxy group are preserved. We also examined the properties of **2** as an antiproliferative agent in cells having normal or dysfunctional apoptosis.[13]

Scheme 1. Synthesis of C4-Methylene-ceramide Analogue **2**



Scheme 1 outlines the synthesis of C4-methylene-ceramide analogue **2**. Aldehyde **4**, which was prepared from D-tartaric acid as previously reported,[14] reacted with tetradecylmagnesium bromide to give a diastereomeric mixture of alcohol **5**. After the mixture was oxidized to ketone **6** with the Dess−Martin reagent,[15] various methods were tested to carry out the methylenation reaction (Table 1). Wittig reaction gave a low yield of (2*R*,3*R*)-alkene **7** (without any accompanying (2*R*,3*S*)-diastereomer), and changing the base (KOBu-*t* or NaH) or refluxing the reaction mixture did not improve the yield (entry 1). When 2 equiv of Tebbe reagent[16] was added slowly to a solution of ketone **6** in THF at −78 °C, and the reaction mixture was allowed to warm with stirring for 3 h, the yield was high but epimerization occurred at C3 to give a mixture of alkenes **7** and **8** in a 1:1 ratio (entry 2). Since epimerization may have resulted from the slow addition of the Tebbe reagent to the carbonyl group of compound **6** at low temperature, we added 2 equiv of Tebbe reagent to ketone **7** at room temperature. Entry 3 shows that the ratio of **7** to **8** was increased to 3:1. High diastereoselectivity was attained when ketone **6** was added to 2 equiv of Tebbe reagent slowly at room temperature for 30 min, with stirring at room temperature for an additional 3 h; a 9:1 ratio of alkenes **7** and **8** was obtained (entry 4). The mixture of **7**/**8** was treated with 5% H_2SO_4 to give diols **9** and **10** in 81% yield for the two steps.[17] Then, diol **9** was converted to azido alcohol **11** in a one-pot reaction.[18] This was accomplished by adding the diol to a mixture of diisopropyl azodicarboxylate (DIAD) and Ph_3P at 0 °C. After 3 h, $TMSN_3$, was added to accomplish the azide substitution reaction. Hydrolysis of

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(16) For a review of Tebbe methylenation, see: Hartley, R. C.; McKiernan, G. J. *J. Chem. Soc., Perkin Trans. 1* **2002**, 2763−2793.

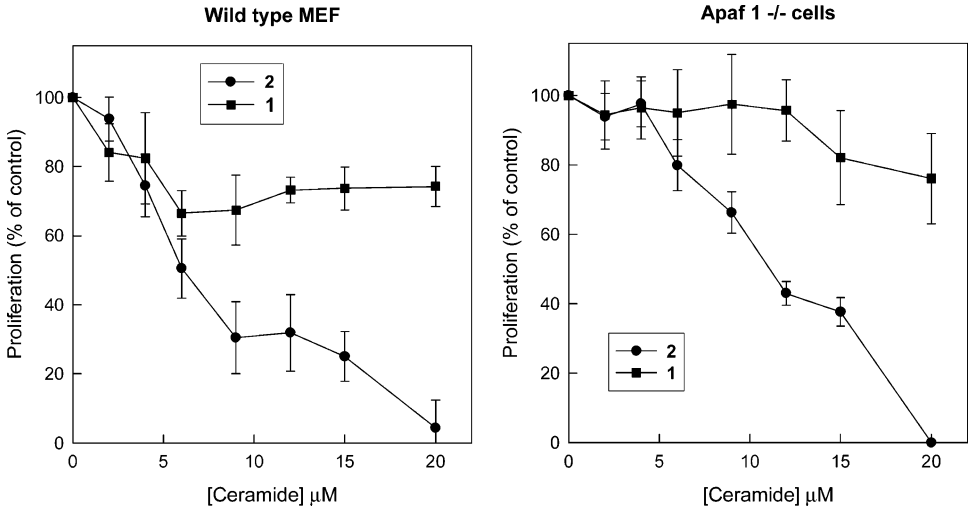

Figure 2. Antiproliferative activities of **1** and **2**.[23]

the concomitant silyl ether of the primary hydroxy group and purification by column chromatography provided azido alcohol **11** in 56% yield. Reduction of the azido group with PPh_3, followed by amide formation in a one-pot reaction, gave amide **12** in 92% yield. The benzyl group of amide **12** was removed by Birch reduction (Na, liquid NH_3, 30 min) to give product **2** in 88% yield.

Activation of the cellular apoptosis machinery with exogenous agents may provide a viable therapeutic strategy for the elimination of tumor cells.[19] Since the antiproliferative effects of exogenous short-chain, cell-permeable ceramides have been widely reported,[20] we investigated the effect of **2** on the proliferation of wild-type mouse embryonic fibroblasts (MEFs) and MEFs lacking Apaf-1, a component of the apoptosome (a large complex that activates the caspase cascade in the intrinsic mitochondrial apoptosis pathway).[13c,21] As shown in Figure 2, analogue **2** inhibited the growth of wild-type MEFs with an IC_{50} of 5.9 μM and Apaf-1 −/− cells with an IC_{50} of 11 μM. (The loss of Apaf-1 in the latter cells leads to defects in the execution of cell death by the intrinsic apoptosis pathway.)[13c,21] In contrast, the IC_{50} value of **1** was ≫20 μM for both the wild-type and Apaf-1 null cells. The higher antiproliferative activity of ceramide analogue **2** against these cell lines indicates that variations in the structural features of **1** can lead to significantly enhanced apoptogenic activity.[22] While the sensitivity of the Apaf-1 −/− cells to **2** was less than that

(17) **Representative Procedure for the Preparation of 9.** A solution of 474 mg (1.0 mmol) of ketone **6** in 25 mL of THF was added dropwise over 30 min to a solution of Tebbe reagent (a 0.5 M solution in toluene, 2 mmol) in 25 mL of toluene at room temperature. After the solution had been stirred for 3 h at room temperature, 50 mL of a 1 M aqueous NaOH solution was added; the mixture was extracted with Et_2O (3 × 20 mL), and the extracts were dried (Na_2SO_4). The solvent was evaporated, and the resulting residue was dissolved in 100 mL of MeOH and treated with 5 mL of 5% aqueous H_2SO_4. After the mixture had stirred overnight at room temperature, 5.0 g (36 mmol) of K_2CO_3 was added, and the mixture was filtered; the filtrate was evaporated, and the residue was purified by column chromatography on silica gel (hexane/EtOAc 3:1) to give 328 mg (81% for the two steps) of a 9:1 mixture of **9** and **10** as a colorless oil.

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(22) Results shown in Figure 2 were obtained with a 9:1 mixture of (2*S*,3*R*):(2*S*,3*S*)-**2** vs (2*S*,3*R*)-**1**. To address the possible contribution of the C3 diastereomer to the potency of **2**, we make note of several recent studies that suggest a minimal role of the configuration at C3 in the activation of apoptotic cell death by **1**: (1) One of the best characterized direct targets of ceramide-mediated apoptosis, ceramide-activated protein phosphatase (CAPP), was activated by (2*S*,3*R*)-**1** but not by the (2*S*,3*S*)-diastereomer of **1**.[24] (2) Ceramidase, which regulates the endogenous level of **1** and its subsequent apoptotic responses in cells, catalyzed the hydrolysis of the amide linkage in the natural (2*S*,3*R*)-isomer of **1** but not in the other three stereoisomers; moreover, only a modest stereoselectivity was found for inhibition of ceramidase by the three unnatural isomers.[25] (3) Ceramide-activated protein kinase (CAPK, which has been identified as the kinase suppressor of Ras, KSR, an activator of stress pathways mediated by MAP kinase cascades) was activated by the four stereoisomers of **1** to an equal extent.[5b] In addition, CERT, a cytosolic protein that facilitates intracellular trafficking of (2*S*,3*R*)-**1**, did not recognize the three unnatural stereoisomers of ceramide in a cell-free assay system.[26] These findings indicate that there is no marked selectivity with respect to the configuration at C3 of **1** for interaction with several target proteins and suggest that the presence of the minor amount of the C3 epimer does not account for the improved antiproliferative potency of **2**.

(23) Proliferating cells in 48 well plates were incubated with compounds **1** and **2** at 37 °C for 48 h in a CO_2 incubator. The medium was removed, and the plates were frozen at −80 °C for 3 days. The cell numbers at day 0 and after 48 h of incubation with the ceramides were determined by the CyQuant assay (Molecular Probes). Each point is the average of six independent experiments.

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of the wild type, the ability of **2** to inhibit the growth of these cells suggests that its mechanism of action is not critically dependent on the formation of the apoptosome to induce apoptosis.

In summary, (2*S*,3*R*)-ceramide analogue **2**, which bears an exomethylene group at C4 and an allylic hydroxyl group at C3, was synthesized from D-threitol acetal aldehyde **4**. The key step was Tebbe methylenation of ketone **6** with careful control of the conditions to minimize epimerization at C3. The utility of **2** for inhibition of growth was demonstrated by using mouse embryonic fibroblast cells.

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Supporting Information Available: Experimental procedures for the preparation of all new compounds and their ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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