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Synthesis of constrained ceramide analogs and their potent antileukemic activities

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Abstract—Constrained ceramide analogs were designed and synthesized by binding terminal alcohol and amine of ceramide with additional carbonyl functional group as 3-acetyl (**3**), 3-propionyl (**4**), 3-benzoyl (**5**), and 3-hexadecanoyl-4-(1-hydroxyhexadec-2-enyl)-oxazolidin-2-ones (**6**). Compounds **4** and **5** showed potent antileukemic activities against human leukemia HL-60 cells with good correlation between cell death and DNA fragmentation.

Sphingolipids and glycosphingolipids, including ceramide, sphingomyelin, cerebroside, and ganglioside are unique membrane components of all eukaryotic cells.[1] They play important roles in the regulation of cell proliferation, survival, and cell death.[2] Among them ceramide is one of the most important molecules as a second messenger of sphingolipid signaling.[2] Ceramide is generated from sphingosine by ceramide synthase with an additional fatty acid chain. Sphingomyelin, one of the membrane phospholipids, is hydrolyzed to ceramide[3] as a second messenger by sphingomyelinase[4] in response to extracellular agents and stresses including chemotherapeutic agents,[5] tumor necrosis factor-α (TNF-α),[6] and ionizing radiation.[7]

The main structural components of ceramide are a long alkyl chain with 2-amino-1,3-diols and C4,5-*trans*-olefin as a sphingosine backbone and a *N*-acyl group. In mammalian tissue, the most common sphingoid base is D-*erythro*-C18-sphingosine[(2*S*,3*R*,4*E*)-2-amino-octadec-4-en-1,3-diol, (*E*)-sphing-4-enine] and the typical acyl group attached with amine has the chain length of 16–24. Dihydroceramide (DHCer) with saturated D-*erythro*-C18-sphinganine does not have any biological activity that ceramide shows possibly due to the lack of essential allylic alcohol.[8] In particular, cell-permeable ceramides with short acyl group such as C2-Cer (*N*-acetyl-D-*erythro*-C18-sphingosine) and C8-Cer (*N*-octyl-D-*erythro*-C18-sphingosine) inhibit tumor cell growth and induce apoptosis in leukemia cell lines.[2,9] For this reason many synthetic and natural ceramide analogs were prepared and evaluated as anticancer agents by altering the backbone and by changing two hydroxy and one amine functional groups.[10] Most of them were less active than C2-Cer. Furthermore, acyclic ceramide analogs bearing primary alcohols in sphingoid backbone would have a chance to perturb metabolic pathways by its possible involvement in various biological reactions. Constrained ceramide analogs free from primary alcohol may overcome this drawback without metabolic perturbation. It was shown by uracil ring compound **2** by which the polar portion of ceramide was replaced. Additional long and short alkyl chains were introduced to mimic the sphingoid backbone of ceramide at C-5 and C-6 of the uracil ring positions, respectively.[11] There is no allylic alcohol available and the uracil ring has aromaticity in the compound, which are quite different from the real conformational alignment of acyclic C2-Cer. In this report are described the synthesis and biological activities

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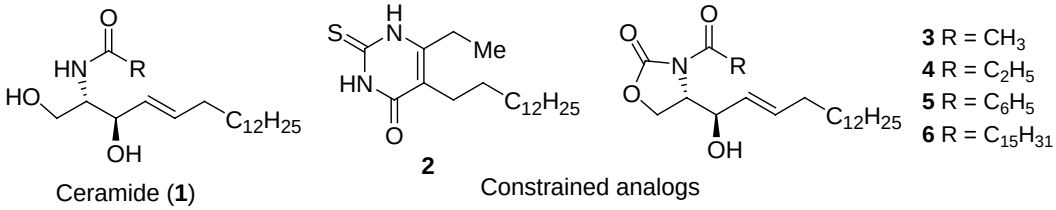


of new constrained ceramide analogs which are closer to the real conformational and electronic properties of ceramide on the basis of oxazolidin-2-one ring (**3**–**6**). These molecules retain allylic alcohol that is one of the essential structural elements to keep the biological activity with the generation of reactive oxygen species.[8]

Constrained ceramide analogs with oxazolidin-2-one ring were designed to put the primary alcohol at C-1 and amide at C-2 of ceramide together by additional carbonyl group. This conformation was deduced by early observation that there are strong hydrogen-bonds between alcoholic hydrogen and the amide nitrogen.[12] An analog, *N*-hexadecanoyl compound **6**, mimicked naturally occurring acyclic C16-Cer whose conformational similarity was evaluated by the comparison of minimum energy conformers calculated by the density functional model.[13] The overlay structure of those two compounds, C16-Cer and **6**, shows similarity in Figure 1.

All of the designed compounds were synthesized from commercially available (2*S*)-aziridine-2-carboxylate **7** shown in Scheme 1. The ester was reacted with *N*,*O*-dimethylhydroxylamine in the presence of *i*-PrMgBr at 0 °C to form the Weinreb's amide **8** in 92% yield followed by addition of 1-pentadecynyl lithium to yield **9** in quantitative yield. Acyl aziridine **9** was selectively reduced to *threo*-amino alcohol **10** to have the right stereochemistry of ceramide with $NaBH_4$ and $ZnCl_2$ via the chelation controlled transition state in 98% yield.[14] Then alkyne **10** was reduced by LAH to yield *N*-[(*R*)-α-methylbenzyl]aziridin-2(*S*)-yl-hydroxy-1-pentadecene in 86% yield whose hydroxy group was protected by TBDMS in 93% yield. The aziridine ring was regioselectively opened by the reaction with acetic acid in CH_2Cl_2 to give acyclic compound **11** in 73%. The *O*-acetyl group in **11** was hydrolyzed by KOH in ethanol to yield the free alcohol at which stage oxazolidin-2-one ring was introduced by the reaction with 1,1′-carbodiimidazole in 70% yield for two steps. The α-methylbenzyl group attached on the nitrogen was removed by Na in liq NH_3 at −78 °C to give the backbone ring **12** in 77% yield. The parent ring (**12**) was ready for acylation on the nitrogen using acid chloride in the presence of a suitable base. Many different bases including NaH, alkoxides, and alkyllithium were tried in vain until we found sodium hexamethyldisilazane as an effective base being applicable to various acyl chlorides. The target molecules **3**–**6**

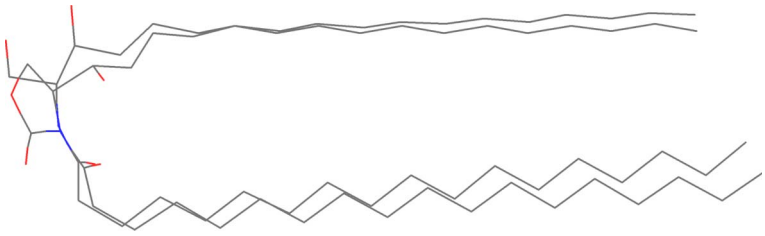
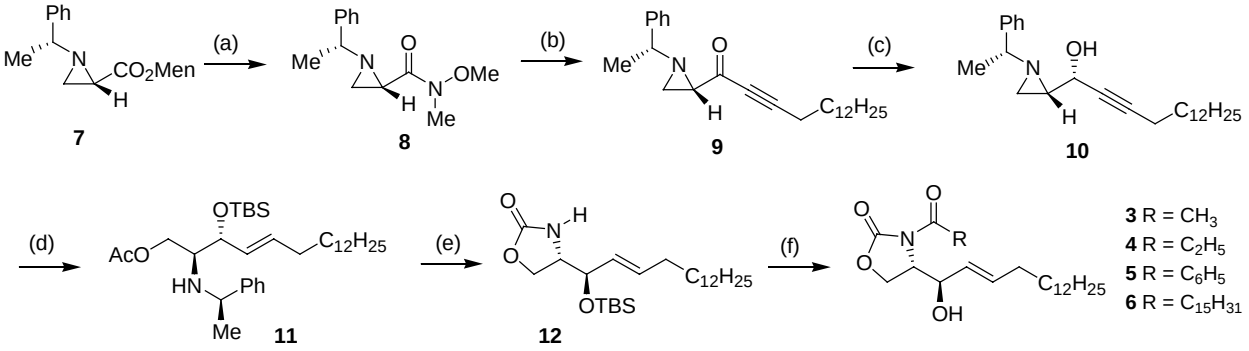


Figure 1. Overlay of naturally occurring C16-ceramide and its oxazolidin-2-one analog **6**.



Scheme 1. Reagents and conditions: (a) $NH(OMe)CH_3$, *i*-PrMgBr, THF, 0 °C (92%); (b) $HCCHCH_2C_{12}H_{25}$, *n*-BuLi, −78 °C then 0 °C, 2 h (99%); (c) $ZnCl_2$, $NaBH_4$, MeOH, −78 °C, then −15 °C, 1 h (98%); (d) i—LAH, 0 °C, 60 °C, 8 h (86%); ii—DMAP, *t*-$BuMe_2SiCl$, rt (93%); iii—AcOH, CH_2Cl_2, rt, (73%); (e) i—KOH, EtOH, rt, 1 h (81%); ii—CDI, DBU, rt, 24 h (87%); iii—Na, NH_3, −78 °C (77%); (f) i—RCOCl, NaHMDS, 0 °C; ii—Bu_4NF, THF, 0 °C.

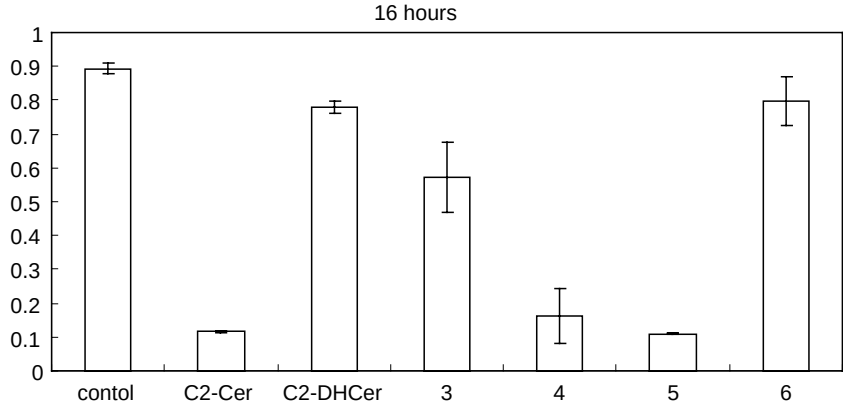


Figure 2. Percent cell survival of HL-60 cells after treatment with 20 μM of C2-Cer, C2-DHCer, and oxazolidin-2-ones **3**–**6** for 16 h.

were prepared from the parent ring compound **12** by the same reaction sequence with the corresponding acid chlorides followed by desilylations in 62%, 71%, 55%, and 58% yield, respectively.[15]

All of these compounds were evaluated as potential antileukemic agents against human leukemia HL-60 cells with C2-Cer and C2-DHCer as positive and negative references, respectively, by the MTT assay whose results are shown in Figure 2.[16] Human leukemia HL-60 cells were treated with 20 μM of each constrained ceramide analog with references for 16 h. Four tested compounds (**3**–**6**) showed activities with drastic difference by the *N*-acyl substituents of the oxazolidin-2-one ring. *N*-Acetyl compound **3** mimicking C2-Cer has much lower activity than C2-Cer itself while a great deal of improvement was observed in the compound **4** with additional one carbon as *N*-propanoyl group. Compound **5** possessing benzoyl rather than alkanoyl group showed a little better activity than C2-Cer. Compound **6** with long alkyl chain does not show any activity due to low cell permeability to produce its antileukemic activity which was also observed in natural C16-Cer.[9]

We decided to find out whether the cytotoxicity induced by these analogs or the reduction of HL60 cells was caused by apoptosis. The characteristics of cell death were verified by the analysis of DNA-fragmentation induced by these molecules. DNA-laddering of fragments was observed after 15 h of incubation of HL-60 cells with 20 μM of C2-Cer, C2-DHCer, and oxazolidin-2-ones (**3**–**6**) shown in Figure 3. This demonstrates cell death was caused by apoptotic process. Similar and a little more amount of DNA fragmentation than C2-Cer was observed by the compounds **4** and **5** bearing propanoyl and benzoyl on the nitrogen, while compounds **3** and **6** with acetyl and hexadecanoyl groups showed less. A good correlation between cell death of HL-60 cells and DNA fragmentation is one solid evidence that the cytotoxicity of the constrained ceramide analogs originated from apoptosis. Additional studies are in progress to find out the mechanistic details of pro-apoptotic process and the structure and activity relationship with diverse oxazolidin-2-ones as constrained ceramide analogs.

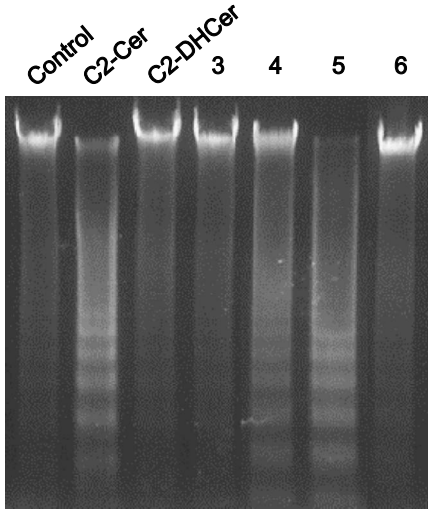


Figure 3. Agarose gel electrophoresis of DNA following treatment of HL-60 cells with 20 μM of C2-Cer, C2-DHCer, and oxazolidin-2-ones **3**–**6**.

Acknowledgments

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15. Compound **3**. Mp 69–70 °C. ^{1}H NMR (300 MHz, $CDCl_3$) 5.89 (dt, J = 11, 5 Hz, 1H), 5.27–5.37 (m, 2H), 4.47 (t, J = 8 Hz, 1H), 4.28 (m, 1 H), 4.02 (m, 1H), 2.11 (s, 3H), 1.37 (m, 25H), 0.89 (t, J = 6 Hz, 3 H). FAB Mass: *m/z* 368 (M^++1). Compound **4**. Mp 91–92 °C. ^{1}H NMR (300 MHz, $CDCl_3$) 5.88 (dt, J = 11, 4 Hz, 1 H), 5.23–5.32 (m, 2H), 4.47 (t, J = 9 Hz, 1H), 4.28 (m, 1H), 4.03 (m, 1 H), 2.35 (q, J = 5 Hz, 2H), 2.08 (q, J = 5 Hz, 2H), 1.37 (m, 23H), 1.14 (t, J = 7 Hz, 3H), 0.89 (t, J = 5 Hz, 3H). FAB Mass: *m/z* 382 (M^++1). Compound **5**. Mp 67–68 °C. ^{1}H NMR (300 MHz, $CDCl_3$) 5.88 (dt, J = 12, 4 Hz, 1H), 5.19–5.33 (m, 3H), 4.44 (t, J = 9 Hz, 1H), 4.26 (m, 1H), 4.01 (m, 1H), 2.34 (t, J = 7 Hz, 2H), 2.07 (q, J = 7 Hz, 2H), 1.68 (m, 3H), 1.24 (m, 48H), 0.89 (t, J = 7 Hz, 3H). FAB Mass: *m/z* 430 (M^++1). Compound **6**. Mp 82–83 °C. ^{1}H NMR (300 MHz, $CDCl_3$) 8.04 (d, J = 8 Hz, 2H), 7.59 (t, J = 8 Hz, 1H), 7.46 (t, J = 8 Hz, 2H), 5.96 (m, 2H), 5.55 (m, 2H), 4.49 (t, J = 8Hz, 1 H), 4.38 (m, 1H), 4.05 (m, 1H), 2.08 (q, J = 7 Hz, 2H), 1.24 (m, 22H), 0.89 (t, J = 7 Hz, 3H). FAB Mass: *m/z* 564 (M^++1).
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