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PAPER

Design and synthesis of pyrrolidine-containing sphingomimetics†

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Based on the structures of natural sphingolipids, we designed heterocyclic sphingoid base mimetics in which the conformational restriction is introduced by incorporation of a pyrrolidine moiety between the 2-amino group and the C-4 carbon atom of the sphingoid base. Our synthesis features a regioselective nucleophilic ring opening of a cyclic sulfate with cyanide and subsequent manipulation of the cyanide group. During the course of synthesis, Staudinger-type reductive cyclization of 1,3-azido carboxylic acid and 1,4-azido alcohol offers a direct route to the five-membered pyrrolidone and pyrrolidine products. The preliminary biological evaluation indicates that the designed pyrrolidine analog is biologically active and its cytotoxic effect is associated with the induction of apoptosis.

Introduction

Sphingoid bases are long-chain aliphatic compounds typically possessing a 2-amino-1,3-diol functionality.[1] These compounds serve a fundamental backbone of more complex metabolic derivatives such as ceramide and sphingosine-1-phosphate. These and all sphingoid base-containing compounds are referred to as sphingolipids. Because sphingolipids play important roles in membrane structure and in cell regulation as second messengers,[2] they have been attractive targets for synthetic and medicinal chemists over the last few decades.[3]

The increased appreciation and understanding of the biological roles of sphingolipids raise the promising possibility that sphingolipid metabolites are biologically validated starting points for the development of novel therapeutics.[2a] However, the physicochemical properties of sphingolipids themselves are not entirely suitable for drug development. Thus, chemical modification of the structure of sphingolipids, especially the sphingoid base moiety, is deemed reasonable and necessary to confer drug-like properties.

The typical sphingoid bases are D-*ribo*-phytosphingosine (**1**, Fig. 1) and D-*erythro*-sphingosine (**2**). In principle, sphingoid bases can adopt multiple conformations depending on the environment, because they are acyclic molecules and their amino and hydroxyl groups can be engaged in several inter-/intramolecular hydrogen bonds.[4] Interestingly, there are several sphingoid base-like natural products that have a cyclic moiety in their polar region.[1b,5] The representatives of this class are pramanicin (**3**),[6] pachastrissamine (**4**),[7] and penaresidines (**5**).[8] These heterocyclic compounds are conformationally more restricted than the prototype sphingoid bases **1** and **2** and exhibit various interesting biological activities.[9]

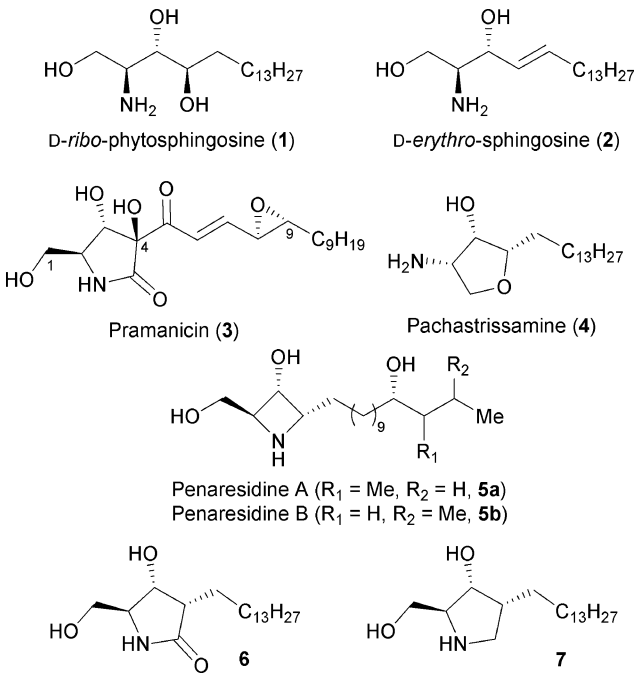

Fig. 1 Chemical structures of compounds **1–7**.

There are also synthetic sphingoid base-like compounds that have been designed and synthesized to provide conformational restriction, especially in the polar region of the sphingoid base.[10]

Because the conformationally restricted analogs of sphingoid bases might be useful in the investigation of the biological functions of sphingolipids and might serve as valuable starting points for the development of novel therapeutics, we have been engaged in the design and synthesis of heterocyclic sphingoid base-like compounds. In this regard, we have previously reported sphingoid base mimetics, wherein the conformational restriction is introduced by incorporation of a piperidine moiety between the

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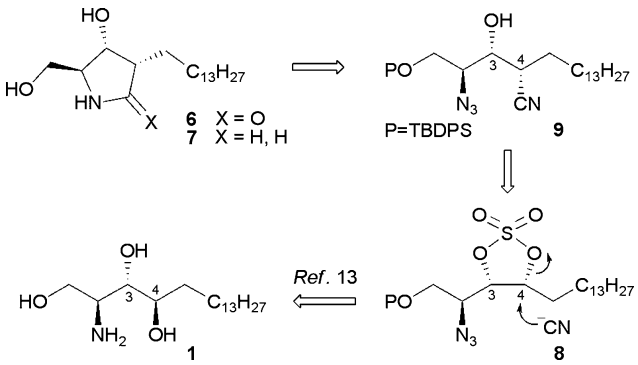
† Electronic supplementary information (ESI) available: Copies of ^{1}H NMR and ^{13}C NMR spectra of compounds **6–7** and **9–15**. See DOI: 10.1039/c1ob05324h

2-amino group and the C-4 carbon atom of the sphingoid base.[11] These mimetics are much more effective than acyclic sphingoid bases **1** and **2** at inhibiting cancer cell growth. This preliminary biological result indicated that the polar region of the sphingoid bases is amenable to conformational restriction by incorporation into a heterocyclic ring, providing a basis for further investigation on the structural modification.

As part of our ongoing sphingolipid research focused on the design and preparation of conformationally constrained sphingomimetics, we designed the heterocyclic compounds **6** and **7** (Fig. 1). These compounds can be considered cyclized mimetics of sphingoid bases that bind the amine at C-2 and the carbon at C-4 with the carbonyl or methylene linkages. The structure of natural pramanicin (**3**) and penaresidines (**5**) led us to the above design. The basic skeleton, stereochemistry, and chain length of pramanicin (**3**) were retained, but its oxidized functionalities on C-4 to C-9 were removed, including a quaternary hydroxyl group and an epoxide group. This manoeuvre yielded the structure of substituted pyrrolidone.[6] Further removal of the carbonyl oxygen of **6** resulted in the pyrrolidine compound **7**.[12] Pyrrolidine **7** can be also considered a five-membered ring homologue of penaresidines (**5**) with a simplified side chain. Herein, we report new sphingoid base mimetics, their efficient synthetic route, and preliminary activity studies.

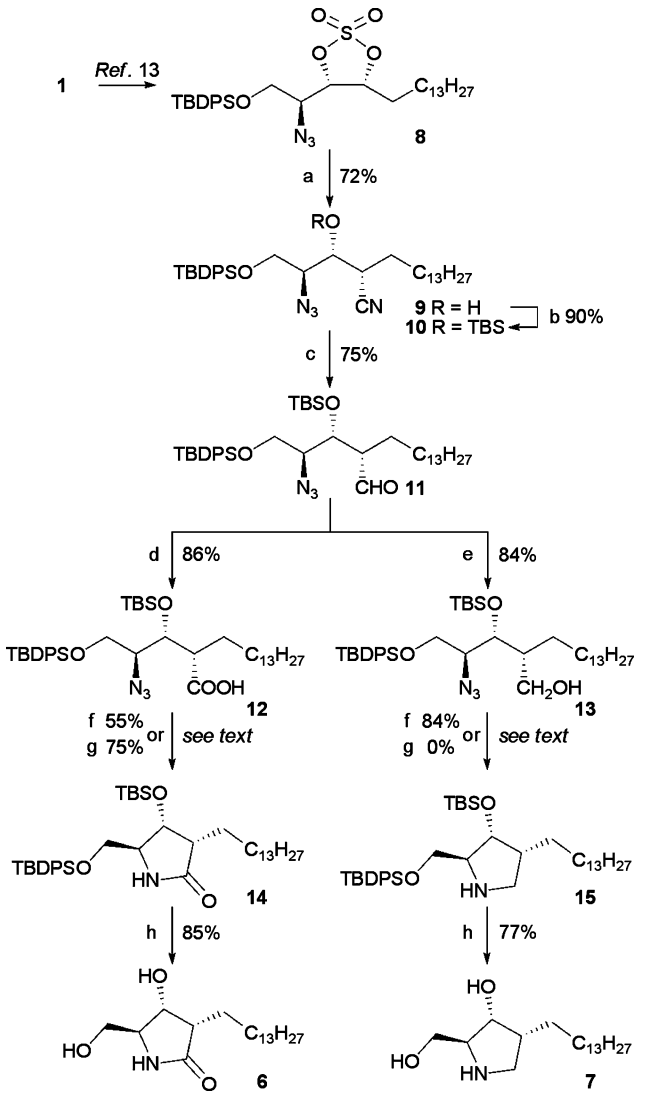
Results and discussion

We envisioned that the designed heterocyclic compounds **6** and **7** could be accessible from the acyclic compound **9** through the manipulation of its azido and nitrile groups (Scheme 1). The requisite azido nitrile **9** might, in turn, arise from the regioselective nucleophilic ring opening of cyclic sulfate **8** with cyanide. This prediction was based on our previous observation that the ring opening of cyclic sulfate **8** with iodide occurred exclusively at C-4 with clean inversion.[13]



Scheme 1 Retrosynthetic plan for heterocyclic compounds **6** and **7**.

Cyclic sulfate **8** can be easily obtained from phytosphingosine (**1**) in high overall yield, as previously reported by our group.[13] The reaction of cyclic sulfate **8** with NaCN in DMF at room temperature, after acidic hydrolysis of the intermediate *O*-sulfate, afforded the C-4 cyanide compound **9** in 72% yield (Scheme 2). As in previous ring opening reaction of **8** with iodide,[13] the C-4 substituted product **9** was the only regioisomer detected in ^{1}H NMR spectra of the crude product. The excellent regioselectivity might be attributed to the differences in the steric and electronic



Scheme 2 (a) i) NaCN, DMF, 1 h, ii) $H_2SO_4/H_2O/THF$ (1 : 1.5 : 50), 1 h; (b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C to rt, 1 h; (c) DIBAL-H, toluene, −78 °C, 1 h; (d) $NaClO_2$, $NaH_2PO_4{\cdot}2H_2O$, 2-methyl-2-butene, *t*-BuOH/H_2O (1 : 1), 1 h; (e) $NaBH_4$, MeOH/CH_2Cl_2 (2 : 1), 0 °C, 30 min; (f) PPh_3, toluene, reflux, 3 h; (g) PPh_3, THF/H_2O (100 : 1), reflux, 48 h; (h) Bu_4NF, THF, 0 °C to rt, 2 h; TBSOTf = *tert*-butyldimethylsilyl trifluoromethanesulfonate; DIBAL-H = diisobutylaluminium hydride.

characteristics between the C-3 and C-4 carbons. Our computational studies on the truncated system of **8**, shown in Fig. 2, revealed that the electron densities on the cyclic sulfate carbon atoms are different. The C-4 carbon displays a lower electron density and less steric hindrance than the C-3 carbon. Thus, the

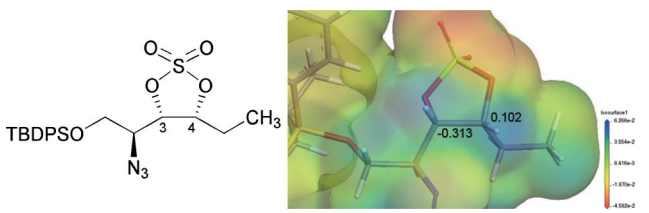


Fig. 2 The electrostatic potential map of the truncated system of **8** calculated at the PBE/DNP level.

C-4 carbon would be more susceptible to nucleophilic attack than the C-3 carbon.

For the efficient transformation of the cyano group, it was desirable to protect the free hydroxyl group of **9**. Thus, the hydroxyl group was protected as its silyl ether using TBSOTf to give **10** in 90% yield (Scheme 2). The cyano group of **10** was reduced to the corresponding aldehyde **11** (75%) using DIBAL-H, and then the aldehyde group of **11** was oxidized with $NaClO_2$ to give carboxylic acid **12** (86%). In a parallel reaction, aldehyde **11** was reduced with $NaBH_4$ to give azido alcohol **13** (84%).

For the Staudinger reduction of the azide functionality,[14] the 1,3-azido carboxylic acid **12** was treated with PPh_3 in refluxing anhydrous toluene. This treatment resulted in concomitant *in situ* cyclization to afford γ-lactam **14** in 55% yield.[15,16] Similarly, the azido alcohol **13** also gave the cyclized product **15** in 84% yield under the same reaction conditions. Removal of the silyl protecting groups in compounds **14** and **15** with Bu_4NF in THF afforded the desired target compounds pyrrolidone **6** and pyrrolidine **7** in 85% and 77% yields, respectively. The structures of **6** and **7** were supported by spectral and analytical data.

In our synthetic process, Staudinger-type transformations offer a direct route to the five-membered pyrrolidone and pyrrolidine products. Despite its synthetic potential, the Staudinger-type reductive cyclization of 1,3-azido carboxylic acids and 1,4-azido alcohols has not been widely reported. To our knowledge, the *in situ* conversion of a 1,3-azido carboxylic acid to a γ-lactam *via* the Staudinger reaction has not been described previously, although ring closure through the activation of the carboxy group has been reported extensively.[17,18] Only one case of the direct reductive cyclization of a 1,4-azido alcohol to a pyrrolidine ring has been previously reported,[19] although the formation of aziridine from 1,2-azido alcohol has been widely explored.[20,21]

One plausible mechanism leading to **14** and **15** from **12** and **13** might be that the initially formed iminophosphoranes (**16a** and **16b**, Fig. 3) react with an intramolecular acid or hydroxyl group to form the seven-membered [1,3,2]oxazaphosphepanone or [1,3,2]oxazaphosphepane. However, this mechanism is questionable because there have been no reports on the existence of seven-membered [1,3,2]oxazaphosphepane ring systems. To obtain clues on the mechanism of this unusual Staudinger cyclization, the reactions of **12** and **13** with PPh_3 were carried out in the presence of H_2O in refluxing THF. Under these reaction conditions, 1,3-azido carboxylic acid **12** was cleanly converted to the cyclized product **14** in 75% yield. However, 1,4-azido alcohol **13** gave none of the cyclized product **15**, but instead gave a 78% yield of the corresponding primary amine **17**. From these results, the reaction mechanisms might be assumed to be as follows. The formation of **14** might include the initial hydrolysis of iminophosphorane **16a** followed by subsequent condensation of the resulting amine with the acid group, although the prior nucleophilic attack of the

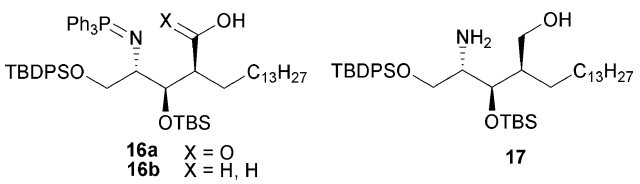


Fig. 3 Chemical structures of iminophosphoranes **16** and amine **17**.

Table 1 Anti-proliferative effects of sphingolipids on cancer cells

Cell line	IC_{50}[e] (μM)			
	1	**2**	**6**	**7**
B16 Melanoma[a]	30.4	29.6	59.6	6.4
A549[b]	29.8	38.6	>75	6.0
SK-HEP1[c]	13.7	27.9	>75	8.1
HCT116[d]	17.4	21.9	>75	4.0

[a] Mouse B16 melanoma cells. [b] Human lung carcinoma cells. [c] Human hepatocarcinoma cells. [d] Human colorectal carcinoma cells. [e] 50% inhibition concentration.

iminophosphorane nitrogen atom on the carbonyl carbon could not be excluded. For the formation of **15** from iminophosphorane **16b**, the direct nucleophilic attack of the imino nitrogen atom at the carbon atom bearing a hydroxyl group might be one possible pathway. Nevertheless, more systematic experimental and theoretical studies are needed to clarify the mechanisms.

The typical biological activities of sphingoid bases include the modulation of multiple kinases, G protein-coupled receptors, and channels.[22] As a consequence of these actions, sphingoid bases exhibit antiproliferative activity and induce apoptosis in cancer cells.[23] Based on this information, we first examined the cytotoxic activities of sphingomimetics **6** and **7** as part of their preliminary evaluation. As shown in Table 1, pyrrolidine **7** was found to be a more effective initiator of cell death than the prototype sphingoid bases **1** and **2**, whereas pyrrolidone **6** showed less potent cytotoxic activity than **1** and **2**. For example, the IC_{50} values of **6** and **7** against B16 melanoma cells were 59.6 and 6.4 μM, while those of sphingoid bases **1** and **2** were 30.4 and 29.6 μM, respectively. The only structural difference between **6** and **7** is the presence of a carbonyl oxygen group in the heterocyclic ring in **6**. This simple difference influences the biological activity significantly.

To determine whether the cytotoxic effects of **7** involve cellular apoptotic pathways, caspase-3 activation was investigated because induction of caspase-3 proteolytic activity is one of the most important events in apoptosis.[24] As illustrated in Fig. 4, the treatment of B16 melanoma cells with **7** increased the caspase-3-like activity in a concentration-dependent manner. These data

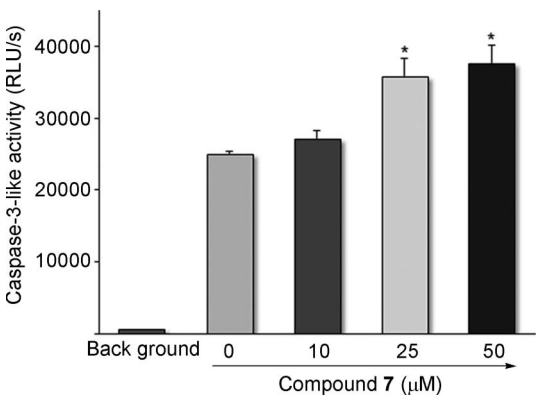


Fig. 4 Effects of **7** on the caspase-3-like activity of mouse melanoma cells. B16 melanoma cells were treated with **7** for 18 h. Caspase-3-like activity was determined by measuring the cleavage of the luminogenic tetrapeptide substrate Ac-DEVD-pNA. Data are expressed as the mean ± S.D. *Statistically significant compared to the control ($*P < 0.05$).

suggest that the cytotoxic effect of compound **7** is associated with the induction of apoptosis, as is the case for sphingoid bases, pramanicin (**3**),[9a,c] and penaresidines (**5**).[8,9g]

Previously, Yves Génisson *et al.* have reported the synthesis of pyrrolidine compound **18** (Fig. 5), which is structurally similar to our pyrrolidine **7** but has a different stereochemistry at C-4 and a different chain length.[12] This C-4 epimeric compound displayed a strong cytotoxicity in B16 melanoma cells, with an IC_{50} of *ca.* 5 μM, which is very similar to that of pyrrolidine **7**.

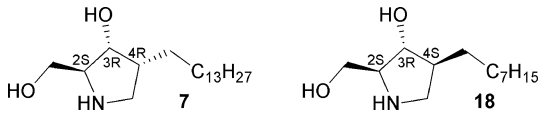


Fig. 5 Structural comparison of compounds **7** and **18**.

Because pyrrolidine **18** was reported to have an inhibitory effect on the activity of glucosylceramide synthase (GCS), we examined whether compound **7** affects GCS gene expression in B16 melanoma cells. As shown in Fig. 6, semi-quantitative PCR (qPCR) analysis of GCS mRNA expression demonstrated that treatment of **7** resulted in the down-regulation of the GCS mRNA expression in a concentration-dependent manner. GCS is an enzyme that catalyzes the transfer of glucose to ceramide in glucosylceramide biosynthesis.[25] In addition to conferring multidrug resistance to tumour cells, GCS also plays an important role in many vital biological functions in cell growth and apoptosis.[26] Accumulating evidence suggests a reduced level of GCS expression resulted in significant effects on the sphingolipid pattern, with proapoptotic ceramide levels being elevated.[27] Thus, although further studies are required to conclusively demonstrate the mechanism of action, the apoptotic cytotoxicity of pyrrolidine **7** might be a result of increased ceramide levels from a down-regulation of GCS expression.

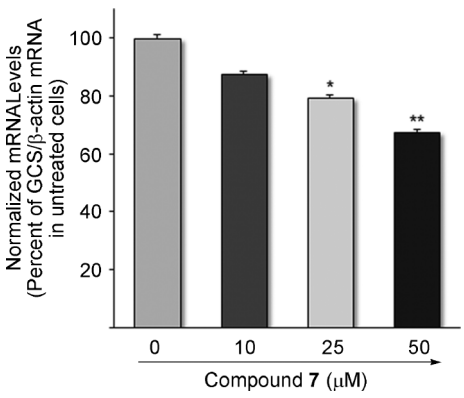


Fig. 6 Down-regulation of GCS mRNA expression in mouse melanoma cells. B16 melanoma cells were treated with the indicated concentrations of **7** (10, 25, and 50 μM) for 24 h. The mRNA levels of GCS were determined by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Using the $2^{-\Delta\Delta CT}$ method as described in the Experimental section, the data are presented as the fold change in gene expression normalized to a housekeeping gene, β-actin, and relative to the untreated control (*first bar*). Data shown are the means ± SEM of four determinations. *Statistically significant compared with the control cultures (*$P < 0.05$, **$P < 0.01$).

Conclusion

We have designed and synthesized conformationally constrained sphingomimetics in which a pyrrolidine moiety was incorporated between the 2-amino group and the C-4 carbon atom of the sphingoid base. For the synthesis of the designed pyrrolidine-containing compounds from phytosphingosine, we employed a regioselective nucleophilic ring opening of a cyclic sulfate with cyanide. The C-4 cyanide group thereby obtained was elaborated further to pyrrolidone and pyrrolidine. In this process, a very unusual Staudinger-type reductive cyclization was utilized. The synthesized pyrrolidine **7** turned out to be much more effective than the prototype sphingoid bases at inhibiting cancer cell growth. Our study suggested that this cytotoxic effect is associated with the induction of apoptosis. In addition, the pyrrolidine **7** down-regulated GCS gene expression. The observed biological results indicate that the polar region of the sphingoid bases is amenable to conformational restriction by incorporation of a pyrrolidine ring. We believe that the presented pyrrolidine analogues could be of value in the investigation of the biological functions of sphingolipids as a chemical tool and in the development of novel therapeutics as a lead scaffold.

Experimental section

A: Preparation of the compounds

General. All chemicals were reagent grade and used as purchased. All reactions were performed under an inert atmosphere of dry argon or nitrogen using dry solvents. Reactions were monitored with TLC analysis using Merck silica gel 60 F-254 thin layer plates. Flash column chromatography was carried out on silica gel (230–400 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded in δ units relative to deuterated solvent as internal reference at 300/400 and 75/100 MHz, respectively. IR spectra were measured on a Fourier Transform Infrared spectrometer. Mass spectra (MS) were recorded using fast atom bombardment (FAB), chemical ionization (CI). High resolution mass spectra (HRMS) were recorded using FAB or CI.

(*R*)-2-((1*R*,2*S*)-2-Azido-3-(*tert*-butyldiphenylsilyloxy-1-hydroxypropyl)hexadecanenitrile (9). To a solution of cyclic sulfate **8** (1.61 g, 2.50 mmol) in DMF (25 mL) was added NaCN (184 mg, 3.75 mmol). This reaction mixture was stirred for 1 h at room temperature. The reaction mixture was concentrated *in vacuo* to remove the DMF, and then THF (10 mL), H_2O (300 μL), and conc. H_2SO_4 (200 μL) were added to the concentrated residue. The mixture was stirred for 1 h at room temperature, diluted with EtOAc and then washed with sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 10 : 1) to give nitrile alcohol **9** (1.06 g, 72%) as a colorless oil. $[\alpha]_D^{20}$ +37.6 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (t, J = 6.6 Hz, 3H), 1.07–1.09 (s, 9H), 1.27 (s, 24 H), 1.48–1.62 (m, 2H), 1.80–1.90 (m, 1H), 2.94 (ddd, J = 2.3, 5.3, 9.8 Hz, 1H), 3.54–3.65 (m, 2H), 3.99 (ddd, J = 4.5, 11.0, 20.9 Hz, 2H), 7.38–7.51 (m, 6H), 7.67–7.73 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 19.1, 22.7, 26.5, 26.7, 27.2, 29.0, 29.2, 29.3, 29.5, 29.57, 29.61, 29.64, 29.65, 31.9, 35.9, 64.0, 64.2, 70.9, 119.3, 127.7, 127.95, 127.99, 129.6, 130.13, 130.17, 132.1, 132.2, 134.8, 135.5; IR

($CHCl_3$) ν_{max} 3444, 2926, 2855, 2101, 1112 (cm^{-1}); HRMS (FAB) calcd for $C_{35}H_{55}N_4O_2Si$ 591.4094 ([M + H]$^+$), found 591.4091.

(*R*) - 2 - ((5*R*,6*S*) - 6 - Azido - 3,3 - diethyl - 10,10 - dimethyl - 9,9-diphenyl-4,8-dioxa-3,9-disilaundecan-5-yl)hexadecanenitrile (10). To a solution of nitrile alcohol **9** (887 mg, 1.50 mmol) in CH_2Cl_2 (7.5 mL) were added 2,6-lutidine (700 μL, 6.00 mmol) and TBSOTf (690 μL, 3.00 mmol) at 0 °C. This reaction mixture was stirred for 1 h at room temperature, quenched with MeOH, poured into sat. $NaHCO_3$, and extracted twice with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 15 : 1) to give nitrile **10** (953 mg, 90%) as a colorless oil. $[\alpha]_D^{25}$ −1.8 (*c* 0.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ −0.16 (s, 3H), 0.03 (s, 3H), 0.81 (s, 9H), 0.87 (t, *J* = 6.7 Hz, 3H), 1.08 (s, 9H), 1.26 (s, 22H), 1.38–1.45 (m, 1H), 1.52–1.54 (m, 1H), 1.66–1.71 (m, 1H), 2.80 (td, *J* = 2.6, 7.7 Hz, 1H), 3.61 (app dd, *J* = 2.2, 5.9 Hz, 1H), 3.65 (app d, *J* = 10.0 Hz, 1H), 3.73 (q, *J* = 6.1 Hz, 1H), 3.81 (dd, *J* = 4.4, 10.1 Hz, 1H), 7.36–7.46 (m, 6H), 7.65 (t, *J* = 6.1 Hz, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ −4.4, −4.2, 14.1, 18.0, 19.1, 22.7, 25.6, 26.7, 27.4, 29.0, 29.3, 29.4, 29.55, 29.62, 29.64, 29.66, 29.8, 31.9, 36.4, 64.7, 66.3, 72.1, 119.4, 127.8, 127.88, 127.92, 129.97, 132.5, 132.6, 135.52, 135.54; IR ($CHCl_3$) ν_{max} 2927, 2856, 2104, 1470, 1428, 1258, 1114, 838, 778, 741, 702 (cm^{-1}); HRMS (FAB) calcd for $C_{41}H_{68}N_4O_2Si_2$ 705.4959 ([M + H]$^+$), found 705.4981.

(*S*) - 2 - ((5*R*,6*S*) - 6 - Azido - 3,3 - diethyl - 10,10 - dimethyl - 9,9-diphenyl-4,8-dioxa-3,9-disilaundecan-5-yl)hexadecanal (11). To a solution of nitrile **10** (705 mg, 1.00 mmol) in toluene (10 mL) was added DIBAL-H (2.50 mL, 2.50 mmol, 1.0 M solution in toluene) in a dropwise manner at −78 °C. This reaction mixture was stirred for 1 h at the same temperature and then diluted with Et_2O (10 mL), followed by the addition of H_2O (2.5 mL). The mixture solution was stirred at room temperature for 1 h and then filtered through a pad of Celite. The filtrate was concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 30 : 1) to give aldehyde **11** (526 mg, 75%) as a colorless oil. $[\alpha]_D^{23}$ +17.1 (*c* 0.29, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ −0.10 (s, 3H), 0.02 (s, 3H), 0.77 (s, 9H), 0.86 (t, *J* = 6.6 Hz, 3H), 1.06 (s, 9H), 1.24 (s, 25H), 1.45–1.48 (m, 1H), 1.69–1.71 (m, 1H), 2.21–2.23 (m, 1H), 3.67 (dt, *J* = 2.3, 7.6 Hz, 1H), 3.77 (dt, *J* = 2.3, 7.6 Hz, 1H), 3.91–3.92 (m, 1H), 7.35–7.43 (m, 6H), 7.63 (t, *J* = 6.0 Hz, 4H), 9.67 (d, *J* = 2.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ −4.7, −4.6, 14.1, 17.8, 19.1, 22.7, 25.5, 25.6, 26.7, 27.8, 29.3, 29.4, 29.5, 29.65, 29.67, 31.9, 55.0, 64.8, 66.4, 73.1, 127.83, 129.89, 129.92, 132.7, 132.8, 135.5; IR ($CHCl_3$) ν_{max} 2927, 2855, 2108, 1721, 1469, 1428, 1362, 1318, 1258, 1113, 1007, 837, 778, 740, 702 (cm^{-1}); HRMS (CI) $C_{41}H_{70}N_3O_3Si_2$ calcd for 708.4956 ([M + H]$^+$), found 708.4954.

(*S*)-2-((5*R*,6*S*)-6-Azido-2,2,3,3,10,10-hexamethyl-9,9-diphenyl-4,8-dioxa-3,9-disilaundecan-5-yl)hexadecanoic acid (12). To a solution of aldehyde **11** (140 mg, 0.20 mmol) in *t*-BuOH (1 mL) and 2-methyl-2-butene (130 μL, 1.20 mmol) were added $NaClO_2$ (90 mg, 0.80 mmol) and $NaH_2PO_4{\cdot}2H_2O$ (187 mg, 1.20 mmol) in H_2O (1 mL). This reaction mixture was stirred for 1 h at room temperature, diluted with EtOAc and washed with sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 15 : 1) to give azido carboxylic acid **12** (125 mg, 86%) as a colorless oil. $[\alpha]_D^{25}$ −2.0 (*c* 0.77, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ −0.06 (s, 3H), 0.03 (s, 3H), 0.77 (s, 9H), 0.86 (t, *J* = 6.6 Hz, 3H), 1.06 (s, 9H), 1.24 (s, 24H), 1.36–1.39 (m, 1H), 1.64 (td, *J* = 2.3, 9.6 Hz, 1H), 2.45–2.49 (m, 1H), 3.63–3.72 (m, 2H), 3.80 (dd, *J* = 3.2, 9.3 Hz, 1H), 3.90 (t, *J* = 4.6 Hz, 1H), 7.35–7.43 (m, 6H), 7.65 (t, *J* = 6.4 Hz, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ −4.9, −4.5, 14.1, 17.9, 19.1, 22.7, 25.7, 26.7, 27.7, 28.0, 29.35, 29.43, 29.6, 29.65, 29.69, 31.9, 49.0, 64.4, 66.3, 73.5, 127.8, 129.86, 129.90, 132.8, 132.9, 135.5; IR ($CHCl_3$) ν_{max} 2927, 2856, 2102, 1712, 1464, 1428, 1362, 1258, 1113, 837, 778, 740, 702 (cm^{-1}); HRMS (CI) $C_{41}H_{70}N_3O_4Si_2$ calcd for 724.4905 ([M + H]$^+$), found 724.4905.

(*R*) - 2 - ((5*R*,6*S*) - 6 - Azido - 3,3 - diethyl - 10,10 - dimethyl - 9,9-diphenyl-4,8-dioxa-3,9-disilaundecan-5-yl)hexadecane-1-ol (13). To a solution of aldehyde **11** (140 mg, 0.20 mmol) in CH_2Cl_2 (1 mL) and MeOH (2 mL) was added $NaBH_4$ (11 mg, 0.29 mmol) at 0 °C. This reaction mixture was stirred for 30 min at 0 °C, quenched with sat. NH_4Cl and extracted with twice EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 10 : 1) to give azido alcohol **13** (119 mg, 84%) as a colorless oil. $[\alpha]_D^{25}$ +11.3 (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ −0.08 (s, 3H), 0.02 (s, 3H), 0.78 (s, 9H), 0.86 (t, *J* = 6.7 Hz, 3H), 1.07 (s, 9H), 1.24 (s, 25H), 1.59 (s, 1H), 2.03 (s, 2H), 3.60–3.66 (m, 2H), 3.68–3.73 (m, 3H), 3.80 (dd, *J* = 3.8, 10.5 Hz, 1H), 7.35–7.43 (m, 6H), 7.65 (t, *J* = 6.6 Hz, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ −4.5, −4.3, 14.1, 17.9, 19.1, 22.7, 25.8, 26.7, 27.6, 29.4, 29.55, 29.60, 29.64, 29.7, 29.8, 31.9, 42.8, 62.7, 65.0, 67.6, 75.0, 127.8, 129.86, 129.89, 132.8, 133.0, 135.6; IR ($CHCl_3$) ν_{max} 2927, 2855, 2103, 1471, 1257, 1112, 837, 777, 702 (cm^{-1}); HRMS (FAB) calcd for $C_{41}H_{72}N_3O_3Si_2$ 710.5112 ([M + H]$^+$), found 710.5112.

(*R*) - 2 - ((5*R*,6*S*) - 6 - Azido - 3,3 - diethyl - 10,10 - dimethyl - 9,9-diphenyl-4,8-dioxa-3,9-disilaundecan-5-yl)hexadecane-1-ol (14). To a solution of azido carboxylic acid **12** (109 mg, 0.15 mmol) in THF/H_2O (100 : 1, 15 mL) was added PPh_3 (118 mg, 0.45 mmol). This reaction mixture was refluxed for 48 h. The reaction mixture was cooled to room temperature, and then diluted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 5 : 1) to give pyrrolidone **14** (77 mg, 75%) as a colorless oil. $[\alpha]_D^{25}$ −8.2 (*c* 0.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ −0.02 (s, 3H), 0.01 (s, 3H), 0.84 (s, 9H), 0.86 (app t, *J* = 6.4 Hz, 3H), 1.04 (s, 9H), 1.24 (s, 22H), 1.61 (m, 2H), 1.80 (s, 2H), 2.30 (td, *J* = 2.30, 5.67 Hz, 1H), 3.46–3.48 (m, 1H), 3.50–3.54 (m, 1H), 3.57–3.61 (m, 1H), 4.19 (d, *J* = 5.8 Hz, 1H), 5.74 (s, 1H, -NH), 7.35–7.44 (m, 6H), 7.60–7.62 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ −5.1, −4.5, 14.1, 17.9, 19.1, 22.7, 23.7, 25.6, 26.8, 27.7, 29.3, 29.57, 29.64, 29.68, 29.8, 31.9, 46.2, 63.1, 64.4, 71.0, 127.9, 130.0, 132.7, 132.8, 135.48, 135.54, 178.1; IR ($CHCl_3$) ν_{max} 2926, 2855, 1704, 1464, 1428, 1390, 1257, 1113, 837, 775, 740, 702 (cm^{-1}); HRMS (FAB) $C_{41}H_{70}NO_3Si_2$ calcd for 680.4894 ([M + H]$^+$), found 680.4879.

(2*S*,3*R*,4*R*)-2-((*tert*-Butyldiphenylsilyloxy)methyl)-4-tetradecyl-3-(triethylsilyloxy)pyrrolidine (15). To a solution of hydroxymethyl azido alcohol **13** (106 mg, 0.15 mmol) in toluene (7.5 mL)

was added PPh_3 (99 mg, 0.38 mmol). This reaction mixture was refluxed for 3 h. The reaction mixture was cooled to room temperature, and then diluted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (hexane–EtOAc 3 : 1) to give pyrrolidine **15** (84 mg, 84%) as a colorless oil. $[\alpha]_D^{25}$ −8.3 (*c* 0.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ –0.02 (s, 3H), 0.03 (s, 3H), 0.84 (s, 9H), 0.86 (app t, J = 6.0 Hz, 3H), 1.05 (s, 9H), 1.24 (s, 23H), 1.42–1.45 (m, 1H), 1.75–1.82 (m, 2H), 1.89 (br s, 2H), 2.68 (dd, J = 10.2, 10.2 Hz, 1H), 2.96 (dd, J = 7.2, 9.6 Hz, 1H), 3.12 (t, J = 5.2 Hz, 1H), 3.49–3.53 (m, 1H), 3.57–3.60 (m, 1H), 4.04 (d, J = 3.9 Hz, 1H), 7.33–7.42 (m, 6H), 7.61–7.64 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ –4.9, –4.3, 14.1, 18.0, 19.2, 22.7, 25.8, 26.8, 26.9, 28.5, 29.4, 29.66, 29.68, 30.0, 31.9, 44.4, 50.5, 65.1, 68.5, 75.0, 127.7, 129.7, 133.3, 135.6; IR ($CHCl_3$) ν_{max} 2926, 2855, 1739, 1469, 1428, 1365, 1253, 1217, 1113, 836, 775, 740, 702 (cm^{-1}); HRMS (FAB) $C_{41}H_{72}NO_2Si_2$ calcd for 666.5102 ([M + H]$^+$), found 666.5097.

General procedure for removal of the silyl protecting groups on compounds 14 and 15. To a solution of **14** or **15** (68 mg or 67 mg, 0.10 mmol) in THF (2 mL) was added Bu_4NF (0.25 mL, 0.25 mmol, 1.0 M solution in THF) at 0 °C. After stirring for 2 h at room temperature, the solvent was removed *in vacuo*. The crude product was purified using silica gel column chromatography (CH_2Cl_2–MeOH–NH_4OH 100 : 10 : 1) to give **6** or **7**.

(3*S*,4*R*,5*S*)-4-Hydroxy-5-(hydroxymethyl)-3-tetradecylpyrrolidin-2-one (6). As a white solid (28 mg, 85%). $[\alpha]_D^{25}$ +9.7 (*c* 0.26, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.89 (t, J = 6.7 Hz, 3H), 1.28 (s, 23H), 1.39–1.43 (m, 1H), 1.46–1.52 (m, 1H), 1.57–1.65 (m, 2H), 2.47 (td, J = 4.3, 11.0 Hz, 1H), 3.42 (dd, J = 4.9, 4.9 Hz, 1H), 3.55 (d, J = 5.1 Hz, 2H), 4.26 (d, J = 6.0 Hz, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 15.23, 15.24, 24.5, 25.5, 29.7, 31.27, 31.29, 31.5, 31.6, 31.7, 33.9, 48.1, 64.2, 66.1, 72.2, 181.8; IR (MeOH) ν_{max} 3428, 3215, 2912, 2848, 1663, 1471, 1053, 713 (cm^{-1}); HRMS (FAB) calcd for $C_{19}H_{38}NO_3$ 328.2852 ([M + H]$^+$), found 328.2860.

(2*S*,3*R*,4*R*)-2-(Hydroxymethyl)-4-tetradecylpyrrolidin-3-ol (7). As a white solid (24 mg, 77%). $[\alpha]_D^{25}$ −13.5 (*c* 0.15, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.89 (t, J = 6.7 Hz, 3H), 1.28 (s, 26H), 1.57–1.59 (m, 1H), 1.93–1.99 (m, 1H), 2.71 (t, J = 10.7 Hz, 1H), 3.10 (dd, J = 7.2, 10.5 Hz, 1H), 3.15 (td, J = 2.6, 5.9 Hz, 1H), 3.57 (ddd, J = 6.1, 11.5, 17.9 Hz, 2H), 4.00 (dd, J = 2.3, 5.4 Hz, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.4, 23.7, 26.9, 29.0, 30.5, 30.65, 30.74, 30.8, 30.9, 33.1, 44.4, 60.4, 70.9, 73.1; IR (MeOH) ν_{max} 3387, 2918, 2849 (cm^{-1}); HRMS (CI) calcd for $C_{19}H_{40}NO_2$ 314.3059 ([M + H]$^+$), found 314.3059.

B: Bioassay

Cell cultures and drug treatments. Mouse B16 melanoma cells were kindly provided by Skin Research Institute, Amore-Pacific Co., Korea. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% (v/v) fetal bovine serum (FBS). The cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2. Each experiment was carried out at least three times.

Measurements of cell viability. Cell viability was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to formazan.[28] The cells were incubated with the tested compounds for 72 h, following which MTT (0.5 mg mL^{-1} in PBS) solution was added to the media, and then the cells were incubated for 4 h at 37 °C. The media were discarded, and then DMSO was added to dissolve the formazan dye. The absorbance was measured at 570 nm and was then used to calculate the IC_{50} value in comparison to control cells.

Caspase-3 activation assay. Caspase-3 activation assays were performed using a Caspase-GloTM3/7 assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, B16 melanoma cells were seeded in 96-well plates at a density of 1 × 10^4 cells per well. After 24 h, cells were treated with 10, 25, and 50 μM compound **7**. Caspase-Glo 3/7 reagent (100 μL) was then added to each well treated with compound **7** for 18 h. After an incubation of 1 h at room temperature, chemiluminescence was measured with a Centro LB960 luminometer (Berthold Technologies, Vilvoorde, Belgium) and expressed in relative light units per second (RLU s^{-1}).

Real-time RT-PCR. Real-time RT-PCR was employed to determine the expression level of GCS in mouse B16 melanoma cells. Briefly, total RNA was extracted using TRI reagent and was then reverse transcribed at 42 °C for 60 min in 20 μL of Reverse Transcription System (Promega, Madison, WI) with 0.5 μg of oligo(dT)$_{15}$ primer. The levels of GCS mRNA were determined using a MiniOpticon system (Bio-Rad, Hercules, CA) using 5 μL of reverse transcription product, iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA), and primers in a total volume of 20 μL. The standard thermal cycle conditions were employed: 95 °C for 20 s before the first cycle; 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s repeated 40 times; followed by 95 °C for 1 min and 55 °C for 1 min. Specific GCS primers were designed using Roche Applied System (Basel, Swiss) and were provided by Bioneer Corporation (Seoul, Korea). The following sequences were used: GCS forward, 5′-GTTTCAATCCAGAATGATCAGGT-3′; GCS reverse, 5′-AAGCATTCTGAAATTGGCTCA-3′; β-actin (housekeeping gene) forward, 5′-AGCACAATGAAGATCAAGAT-3′; β-actin reverse, 5′-TGTAACGCAACTAAGTCATA-3′. The threshold cycle (CT), indicating the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold from each well, was determined using by MJ Opticon Monitor software. Relative quantification, representing the change in gene expression from real-time quantitative PCR experiments between the treated samples and the untreated controls, was calculated by the comparative CT method as published previously.[29] The data were analyzed using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ = [CT of target gene – CT of housekeeping gene] treated group – [CT of target gene – CT of housekeeping gene] untreated control group. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ represents the fold change in gene expression, normalized to a housekeeping gene (β-actin) and relative to the untreated control.

C: Theorical calculation

Electrostatic potential calculation. Theoretical calculations on the truncated system of **8** was performed using the density functional theory (DFT) method as implemented in the DMol^3 package,[30] which is available as part of the Material Studio 5.5 package. In the DFT calculations, we employed the Perdew, Burke,

and Ernzerhof (PBE) function[31] for the exchange–correlation interaction within a generalized gradient approximation (GGA) and a double numerical basis set including d-polarization functions (DNP) as implemented in the DMol[3].

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