Articles

Synthesis and Evaluation of Sphingosine Analogues as Inhibitors of Sphingosine Kinases

Lingkai Wong,[†] Sheryl S. L. Tan,[‡] Yulin Lam,*[,†] and Alirio J. Melendez*[,‡,§]

Department of Chemistry, Faculty of Science, National University of Singapore, 3 Science Drive 3, 117543, Singapore, Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, 2 Medical Drive, 117597, Singapore, and Immunology, Infection and Inflammation, Faculty of Medicine, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow G12 8TA, Scotland, U.K.

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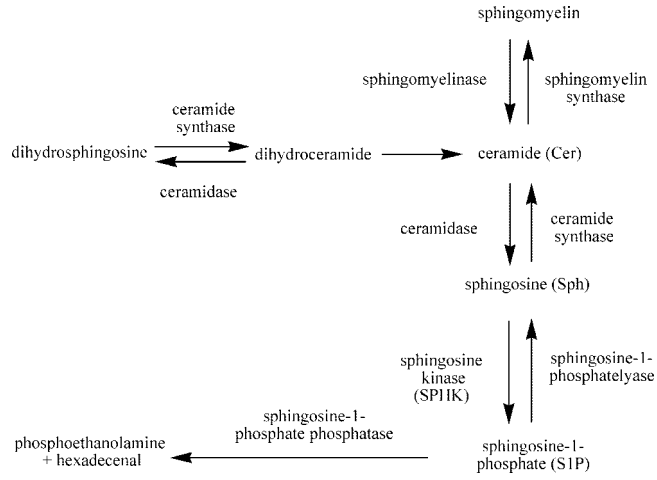
Sphingolipid-metabolizing enzymes control the critical balance of the cellular levels of sphingolipids, including the apoptotic inducing ceramide (Cer) and the proliferative inducing sphingosine 1-phosphate (S1P). The production of S1P, catalyzed by the action of sphingosine kinases (SPHKs), is known to be critical for many cellular processes. However, it is suggested that SPHK, and/or its catalytic product S1P, plays critical roles in various diseases including autoimmune diseases, cancer, and allergies. However, there is a great limitation of specific pharmacological inhibitors for SPHKs. In this paper, we describe a novel and stereoselective method of synthesizing SPHKs inhibitors. We generated a number of novel compounds and identified a number of specific inhibitors of human SPHKs. These compounds demonstrated inhibition of SPHKs at micromolar concentrations, making them more potent than dimethylsphingosine (DMS), a well-known inhibitor of SPHKs. In particular, one of the inhibitors was found to be selective toward a particular isoform of SPHK.

Introduction

Sphingolipids, which are major components of membrane lipids, have emerged as important contributors in the regulation of the physiological and pathophysiological functions.[1] Sphingolipid metabolites (Scheme 1), such as ceramide (Cer), sphingosine (Sph[a]), and its phosphorylated product, sphingosine 1-phosphate (S1P), belong to a new class of potent bioactive molecules, which have been shown to be involved in several cellular processes, including cell proliferation, differentiation, migration, and apoptosis.[2,3] Ceramide is formed when the major membrane sphingolipid, sphingomyelin, is hydrolyzed by sphingomyelinase. Ceramide has been shown to induce apoptosis in proliferating cells,[4,5] and it can also be further metabolized by ceramidase to produce sphingosine. Sphingosine, in turn, can rapidly be phosphorylated by sphingosine kinases (SPHKs) to produce S1P. In contrast to ceramide, SIP has been shown to promote cell proliferation and has been established to be a central player in the development and onset of several diseases including in autoimmune diseases, allergy, cardiovascular diseases, and cancer.[6] The balance between the different sphingolipid metabolites in mammalian cells is controlled by the enzymes in the signaling pathway shown in Scheme 1. S1P is made by the action of SPHKs; therefore, these enzymes represent interesting targets for drug discovery and for the development of new therapeutics to treat the wide range of diseases where S1P has been shown to play a key role.[6]

Scheme 1. Metabolism of S1P in Mammalian Cells



SPHK is a key enzyme in modulating the levels of cellular sphingolipids. To date, two isoforms of SPHK, namely, sphingosine kinase 1 (SPHK1)[7−9] and sphingosine kinase 2 (SPHK2),[10] have been cloned, synthesized, and characterized from mammalian sources. Earlier studies have shown that SPHKs can be inhibited by a number of organic compounds,[11,12] but many of these inhibitors demonstrate only moderate inhibition and are not specific or selective. The best inhibitors known to date are the analogues of sphingosine, such as *N*,*N*-dimethylsphingosine (DMS) and dihydrosphingosine (DHS) (Figure 1).[1,11−13] However, these two compounds inhibit not only both the isoforms of human SPHK but other enzymes such as protein kinase C (PKC)[14] and sphingosine-dependent protein kinase.[15] To better understand the roles of the different isoforms of SPHK, it would be highly desirable to obtain inhibitors that are specific to either of the SPHKs able to inhibit only one of the two different SPHK

* To whom correspondence should be addressed. For Y.L.: phone, (65) 65162688; fax, (65) 67791691; e-mail, chmlamyl@nus.edu.sg. For A.J.M.: phone, +44 141 330 8415; fax, +44 141 330 4297; e-mail, a.melendez-romero@clinmed.gla.ac.uk.
[†] Faculty of Science, National University of Singapore.
[‡] Yong Loo Lin School of Medicine, National University of Singapore.
[§] University of Glasgow.
[a] Abbreviations: SPHK, sphingosine kinase; Sph, sphingosine; S1P, sphingosine 1-phosphate; TNFα, tumor necrosis factor α.

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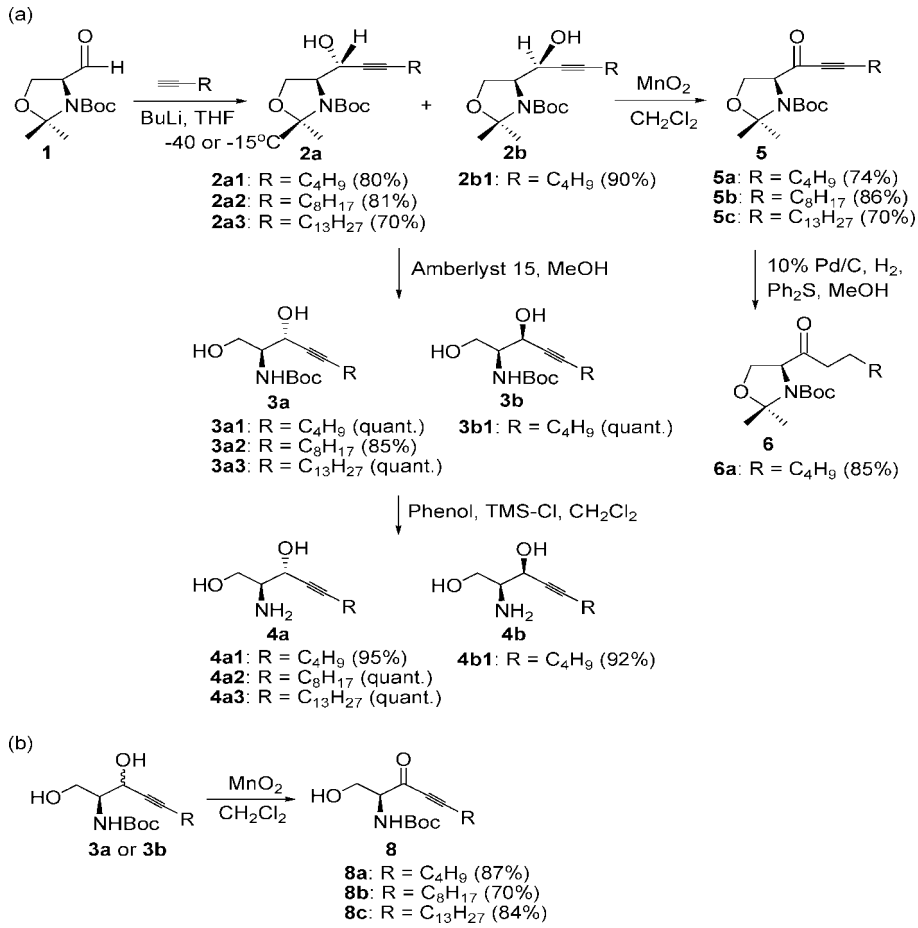
Scheme 2. (a) Synthesis of Sphingosine Analogues and (b) Oxidation of Compound **3**



isoforms. Thus, as part of our efforts to develop and evaluate the role of the different SPHKs, we set out to develop sphingosine analogues as novel and specific SPHK inhibitors, which can be used as laboratory reagents and potentially as novel drug candidates. We have synthesized a new series of sphingosine analogues **4a** and **4b** (Scheme 2) and evaluated them for their inhibitory activities. In our inhibitor design, we have included the *N*-alkyloxycarbonyl pharmacophore, as earlier studies of some of these compounds have shown favorable inhibition of either of the SPHK isoforms.[16−18] Analogues of DMS were not explored because we perceive such analogues would most probably exhibit similar physiological effects as DMS.[14] We herein present the synthesis of sphingosine analogues **4a** and **4b** and the investigation of these compounds and their synthetic intermediates for their (i) role in the inhibition of either isoform of the SPHKs, (ii) specific functionality to the inhibitory function, and (iii) cytotoxicity and PKC specificity.

Chemistry

Compounds **4a** and **4b** were prepared from *S*-(−)-1,1-dimethylethyl-4-formyl-2,2-dimethyloxazolidine-3-carboxylate **1** using the synthetic strategy shown in Scheme 2. Acetylides of various chain lengths, obtained by treating alkynes with *n*-butyllithium (BuLi, 1.6 M in hexanes), were coupled with **1**. According to an earlier reported procedure,[19] this coupling reaction does not occur stereoselectively, thus giving both the erythro isomer **2a** and the threo isomer **2b** in nearly equal quantities. To obtain specifically the naturally occurring erytho isomer, Williams[20] and Bielawska[21] have reported the use of hexamethylphosphinetriamide (HMPT) as an additive. Since HMPT is a harmful reagent, we sought to develop a more benign procedure for this reaction. We found that temperature is a contributing factor in determining the stereoselectivity of the reaction (Table 1). Lowering the reaction temperature to −15 °C and using 1.16 equiv of BuLi gave solely the threo-isomer **2b** (Table 1, entry 1), while decreasing the reaction temperature further provided predominantly the erythro-isomer **2a** (Table 1, entries 2−5). These observations could be explained using the Felkin−Anh model[22] (Figure 2a), which has been commonly used to demonstrate the stereoselective reaction of a carbonyl group with an adjacent stereogenic center. According to the Felkin−Anh transition state, the most reactive conformation has the electronegative nitrogen atom lying perpendicular to the carbonyl group (i.e., G1 and G2 in Figure 2a). At low temperatures, the gauche conformer G1 is preferred, as it would enable the nucleophile to approach the carbonyl group at the Bürgi−Dunitz angle via the least hindered trajectory. Hence, **2a**, the kinetic control Felkin−Anh product, is predominant. However, at a higher reaction temperature, the reverse in stereoselectivity (Table 1, entry 1) could be attributed to the gauche conformer G2, which undergoes nucleophilic addition

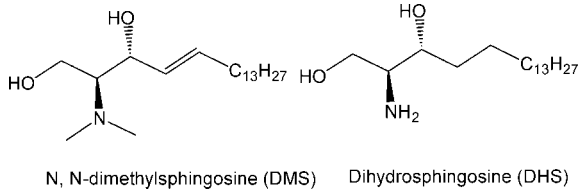
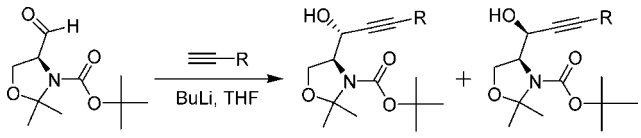


Figure 1. Structures of SPHK inhibitors DMS and DHS.

Table 1. Synthesis of **2a** or **2b**



1 **2a** **2b**

entry	reagents: alkyne (equiv)	reagents: BuLi[a] (equiv)	temp (°C)	ratio of diastereoisomers **2a**/**2b**
1	1.36	1.16	−15	**2b** only
2	1.36	1.16	−23	3:1
3	1.36	1.16	−40	**2a** only
4	1.36	1.16	−50	9:1
5	1.36	1.16	−78	7:1
6	1.36	1.56	−15	**2b** only
7	1.36	1.56	−23	1:7
8	1.36	1.56	−40	1:7

[a] 1.6 M BuLi in hexanes.

via a thermodynamically more stable transition state. This transition state is also most populated when a larger excess of BuLi is used (Table 1, entries 6−8), implying that it is stabilized by chelation (Figure 2b), and is also the most reactive because the Lewis acidic metal atom increases the reactivity of the carbonyl group.

Subsequent ring-opening of **2a** and **2b** using strongly acidic, macroreticular resin with sulfonic acid functionality yielded **3a** and **3b**, respectively, in high yields.[23] Attempts to remove the Boc protecting group by treating **3a** and **3b** with 4 M HCl resulted in a complex mixture that was difficult to purify. Hence, a mixture of 1 M trimethylsilane chloride and 1 M phenol in dichloromethane[24] was used, and this gave the deprotected product **4a** and **4b** in near quantitative yields.

Compound **2a** and/or compound **2b** was also oxidized with manganese dioxide to give compound **5** in good yields[23] (Scheme 2). Treatment of **5** with the strongly acidic, macroreticular resin with sulfonic acid functionality, however, did not provide the ring opened product **8**. To circumvent this problem, we proceeded to oxidize **3a** and/or **3b**, which provided compound **8** in good yields. To further increase the diversity of the analogues, the C≡C bond in **5a** was selectively reduced to C−C bond,[25] giving **6a** in over 83% yield. By use of this synthetic strategy, 19 analogues of sphingosine were synthesized (Figure 3) and evaluated for their inhibitory activity against SPHK1 and SPHK2.

Results and Discussions

SPHK Assay. Tables 2 and 3 show the results of the inhibitory function tests of the compounds on SPHK1 and SPHK2. The compounds are tested, in an initial SPHK assay, at a standard 10 μM concentration to filter out potential inhibitors. Potential inhibitors are determined by their ability to inhibit SPHKs by at least 50% at 10 μM.

From Tables 2 and 3, it can be seen that only **5c** (Table 2, entry 9) and **8c** (Tables 2 and 3, entry 13) met the initial criterion of 50% at 10 μM. **5c** shows potential specificity on the inhibition of SPHK1 only, whereas **8c** shows inhibition for both isoforms of SPHK. The rest of the compounds show either little or no inhibition. This initial screen, using a fixed concentration of 10 μM, only provides a rapid identification of compounds that show potential ability to inhibit SPHK; further additional work was necessary to verify their potencies. Thus, we decided to investigate the substrate specificity and kinetics of inhibition by DMS and our compounds. The expressed huSPHK1 and

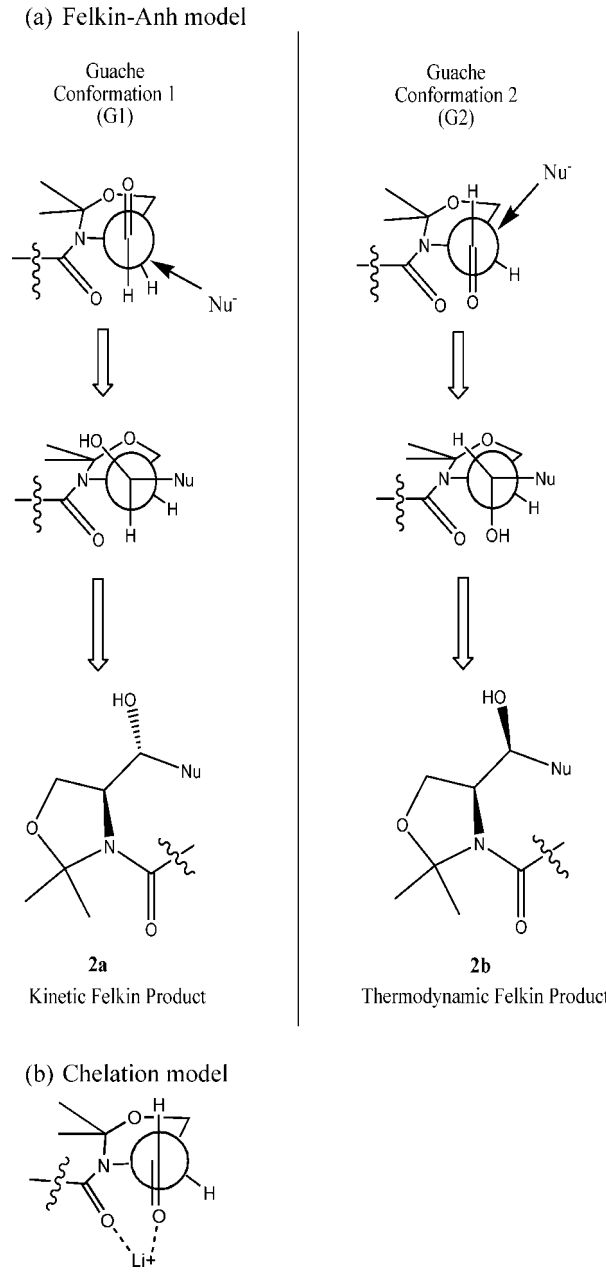


Figure 2. (a) Felkin−Anh model. (b) Chelated transition state.

huSPHK2 show typical Michaelis−Menten kinetics, very similar to that of the original cloning publications.[8,10] The K_m value for D-*erythro*-sphingosine for SPHK1 was 3.5 μM, and for SPHK2 it was 4 μM. The K_i value for DMS for SPHK1 was 6 μM, and for SPHK2 was 12.5 μM. In agreement with the IC_{50} values shown in Table 4, we show here that SPHK1 is inhibited by compound **5c** (K_i = 3 μM) and by compound **8c** (K_i = 1 μM).

Therefore, the effects of compounds **5c** and **8c** were determined at multiple concentrations, and IC_{50} values for each compound were calculated using Graphpad Prism software. As shown in Table 4, compounds **5c** and **8c** demonstrated IC_{50} values at low micromolar range for the inhibition of SPHK1. In fact, their IC_{50} values are lower than that of DMS, making them more potent SPHK inhibitors than DMS.

From Table 2 (entry 4) and Table 3 (entry 3), it can be seen that some of the data from the SPHK assay showed an increase in the sphingosine kinase's activity. This phenomenon is especially noticeable in the SPHK2 assay result for **4a3** (Table

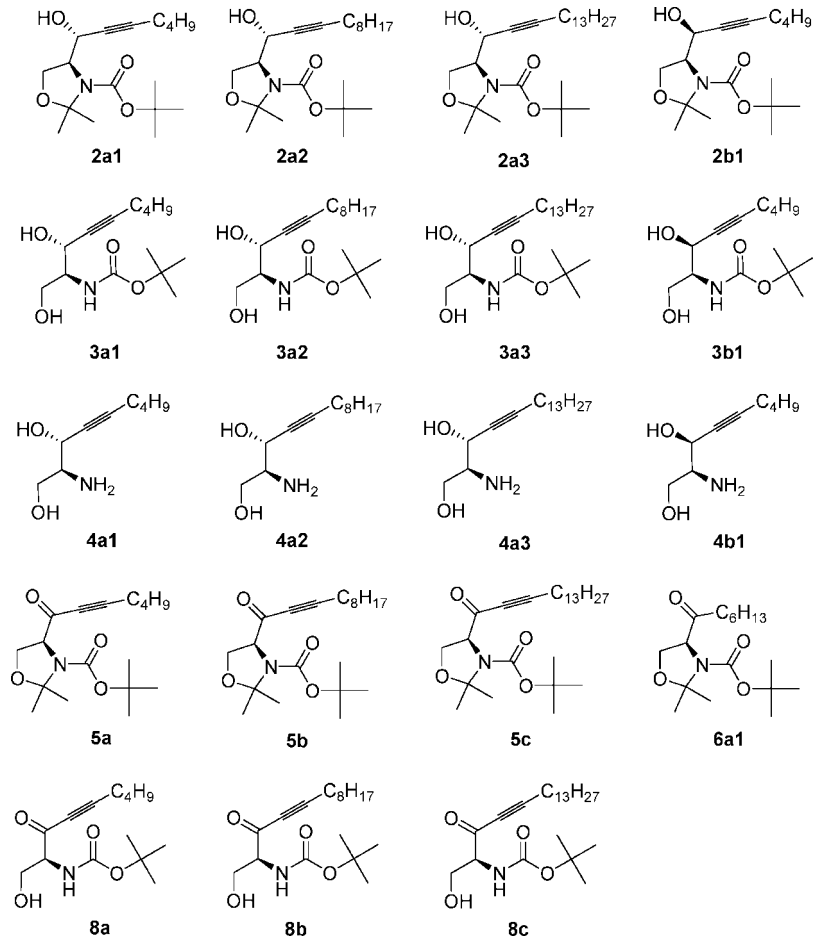


Figure 3. Structures of analogues synthesized.

Table 2. SPHK1 Assay Results of Compounds at 10 μM with DMS as Comparison[a]

entry	compd[b]	SPHK activity level (%)	percentage inhibition (%)
1	**2a3**	111.93 ± 9.80	NA
2	**3b1**	117.21 ± 3.92	NA
3	**4a1**	131.64 ± 6.20	NA
4	**4a2**	86.34 ± 4.53	13.66
5	**4a3**	75.63 ± 7.26	24.37
6	**4b1**	95.15 ± 8.82	4.85
7	**5a**	90.92 ± 9.55	9.08
8	**5b**	84.85 ± 5.13	15.15
9	**5c**	45.34 ± 2.00	54.66
10	**6a1**	95.29 ± 1.50	4.71
11	**8a**	106.02 ± 10.55	NA
12	**8b**	95.80 ± 5.80	4.20
13	**8c**	43.06 ± 9.17	56.94
14	DMS	58.04 ± 0.73	41.96

[a] Values are reported as percentages (%) and over the basis level of 100% (maximum SPHK activity from the SPHK1 overexpression cells). NA = not applicable because no inhibition observed. [b] Compounds used are of ≥95% purity.

Table 3. SPHK2 Assay Results of Compounds at 10 μM with DMS as Comparison[a]

entry	compd[b]	SPHK activity level (%)	percentage inhibition (%)
1	**2a3**	123.36 ± 15.10	NA
2	**3b1**	111.03 ± 5.89	NA
3	**4a1**	108.86 ± 7.62	NA
4	**4a2**	116.46 ± 12.45	NA
5	**4a3**	152.49 ± 8.16	NA
6	**4b1**	95.71 ± 15.33	4.29
7	**5a**	114.70 ± 12.29	NA
8	**5b**	62.81 ± 14.85	37.19
9	**5c**	117.06 ± 3.86	NA
10	**6a1**	126.29 ± 13.21	NA
11	**8a**	117.27 ± 4.41	NA
12	**8b**	89.88 ± 7.31	NA
13	**8c**	45.88 ± 9.14	54.12
14	DMS	66.97 ± 12.63	33.03

[a] Values are noted in percentage (%) and over the basis level of 100% (maximum SPHK activity from the SPHK2 overexpression cells). NA = not applicable because no inhibition was observed. [b] Compounds used are of ≥95% purity.

Table 4. Determination of IC_{50} Value of Compounds with DMS as Comparison[a]

entry	compd	IC_{50} (μM)
1	**5c**	3.3 ± 0.2
2	**8c**	1.2 ± 0.1
3	DMS	5.7 ± 1.5

[a] Data represent the concentrations required to inhibit SPHK1's activity by 50% and are the mean ± standard deviation of triplicate experiments.

2, entry 5) and has been attributed to the formation of sphingosine 1-phosphate (S1P) during the SPHK assay. Earlier studies have shown that S1P is capable of activating the kinase through specific G-protein-coupled receptors (GPCRs).[26] Though not a part of this current research project, it is worth mentioning that there is a growing list of agonists that have been reported to increase SPHK activity.[26] This phenomenon, however, was not observed in compounds that could inhibit the enzyme because there is a possibility that these potential inhibitors could block or bypass the agonist-induced effects. Another plausible reason for the activation of the sphingosine kinase could be due to the compound added being a better substrate for SPHK than sphingosine itself. If the compounds are substrate for SPHK, then they could be phosphorylated by the γ[^{32}P]ATP and thus

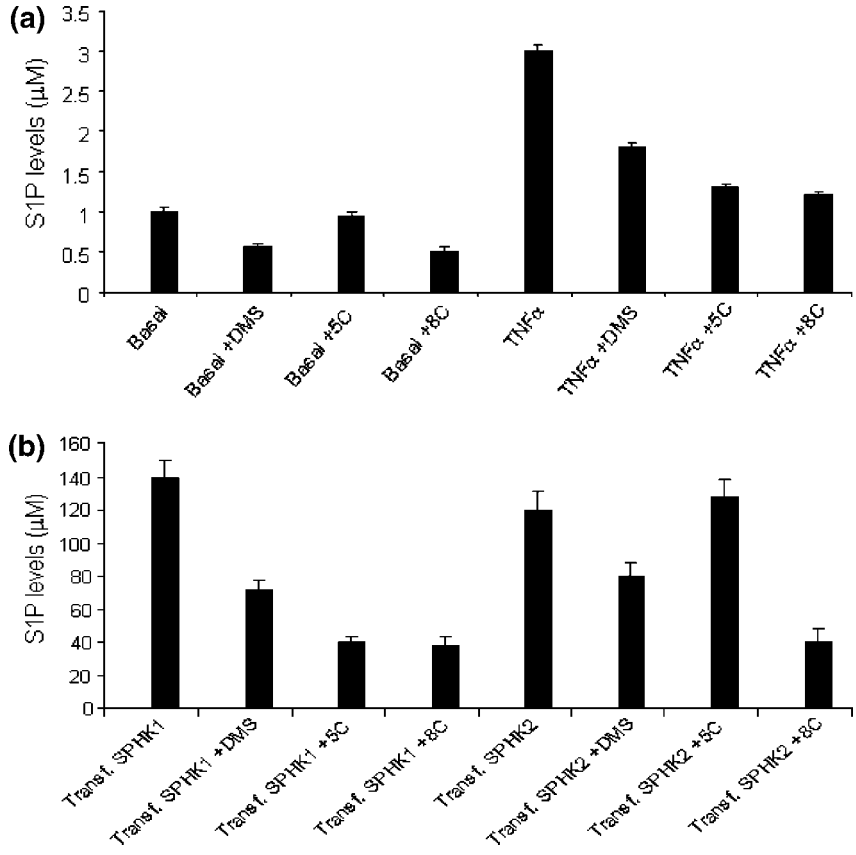


Figure 4. (a) S1P assay results at 2 h following the addition of inhibitors, with or without the addition of TNFα, in the U937 cell line. Compounds were added at 5 μM concentration. (b) S1P assay results in U937 cells transfected with either huSPHK1 or huSPHK2 plasmids. S1P levels were measured at 2 h following the addition of inhibitors. Compounds were added at 5 μM concentration.

increase the radioactive count during the SPHK assay. This in turn would translate to an increase in the SPHK activity.

To understand the full potentials of the compounds' abilities to function as sphingosine kinases inhibitors, intact cell assays, cytoxicity test, and PKC specificity test were performed.

Levels of S1P in Intact, Live Cells. In order to maximize the potential of our inhibitors in penetrating cells and thus in inhibiting SPHKs in live cells, we directly measured the levels of endogenously generated S1P. Figure 4a shows the basal S1P levels in human U937 cells (a human monocytic cell line), pretreated or not with the inhibitors for 2 h. DMS and compound **8c** inhibit basal S1P levels in these cells, suggesting that the basal levels of S1P are generated primarily through the action of SPHK2. However, following stimulation with the cytokine TNFα (known to stimulate SPHK1 in these cells),[27] DMS and compound **5c** indeed inhibit the levels of S1P, whereas compound **8c** does not inhibit the TNFα-induced levels of S1P. In order to further prove the independent role of each of the inhibitors, we performed experiments in cells transfected with either huSPHK1 or huSPHK2 and incubated with the inhibitors for 2 h. Figure 4b indeed shows that compound **5c** is specific for SPKH1 whereas both DMS and compound **8c** inhibit the levels of endogenously generated S1P in both SPHK1 and SPHK2 transfected cell.

MTT Assay. Figure 5 shows the results of MTT assay using U937 cells, a human monocytic cell line, for DMS, **5c**, **8c** at three different incubation timings. Vehicular control, dimethyl sulfoxide (DMSO), was also included to serve as control as the testing compounds were dissolved in DMSO. The amount of DMSO used in the assay was controlled at 0.5% of the total volume. This is to ensure that DMSO does not result in cytotoxicity of cells. Compound **5c** is shown to be the least toxic compound among the two compounds. Its cytoxicity is comparable with, if not less toxic than, that of DMS. However it is observed that compound **8c** is very cytotoxic to cells (Figure 5). To confirm our observation that **5c** is a better inhibitor than DMS, we subjected **5c** in another MTT assay testing, using the HL60 cell line, a human promyelocytic leukemia cell line. From Figure 6, it was observed that **5c** is less toxic toward HL 60 cells compared to DMS. Both MTT assay results, using two different cell lines, allowed us to determine that **5c** is not toxic toward cells and remains as a potential candidate as SPHK1's inhibitor. In the MTT assay testing, it was observed that the proliferation rate of both cell lines, when subjected to 10 μM **5c**, was moderately down-regulated. In comparison with control, it was observed that the proliferation rate of cells was slowed by at least 10% during the incubation period from 24 to 72 h. We hypothesized that **5c** could be antiproliferative. This could be an important finding because SPHK1 is often found to be overexpressed in cancer cells; thus, **5c** may be an effective therapeutic agent against resistant cancer tumors. Though not part of our research focus, more work will be needed to prove our hypothesis. With elimination of **8c** as a potential inhibitor of SPHK through the MTT assay, **5c** is then subjected to a testing in a protein kinase C (PKC) assay to determine its specificity toward SPHK1.

PKC Assay. Detection of protein kinase C using a nonradioactive assay was chosen to evaluate compounds. Pure human PKC α protein was used to incubate with PKC specific peptide with or without the presence of **5c** or DMS. **5c** and DMS as a control were tested under five concentrations: 10, 25, 50, 75, 100 μM in this assay. Procedures are provided in the Experi-

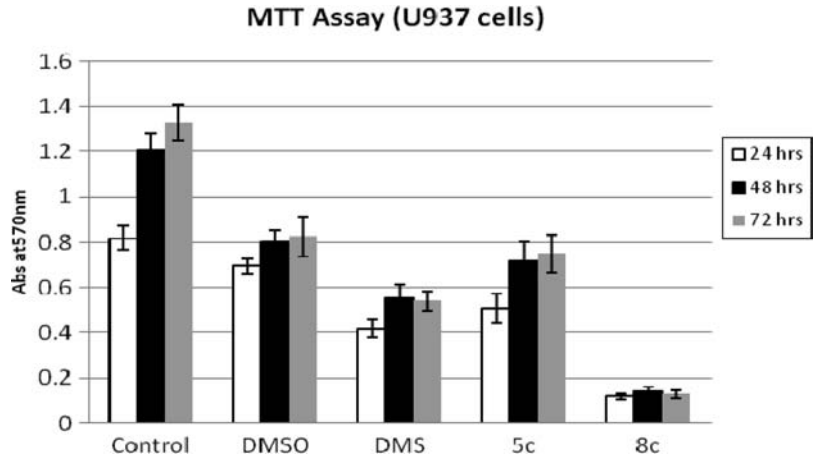


Figure 5. MTT assay results at various incubation times using U937 cell line. Compounds were added at 10 μM concentration.

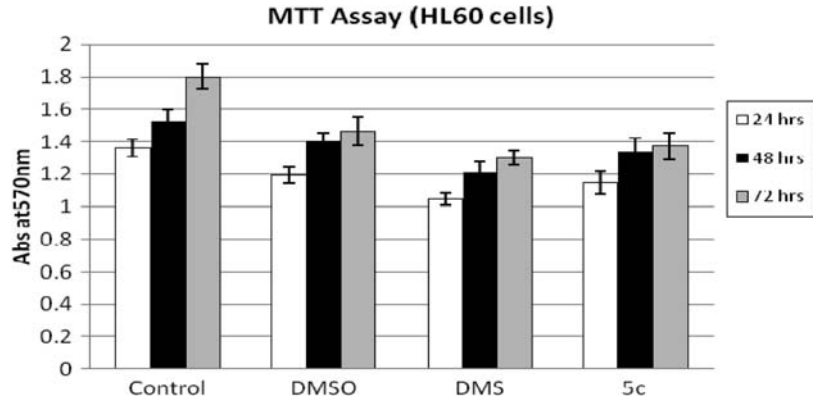


Figure 6. MTT assay results at various incubation times using HL60 cell line. Compounds were added at 10 μM concentration.

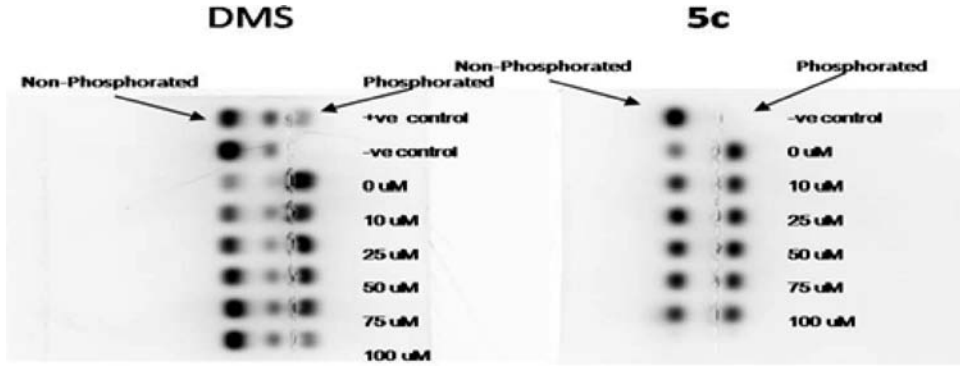


Figure 7. Gel photograph of the PKC assay for DMS and compound **5c**. Inhibition of PKC activity results in decreased intensity of the phosphorylated substrate/band and an increased intensity of the nonphosphorylated band and vice versa.

mental Section. Electrophoresis was used to separate incubation product. The phosphorylation process of compounds by protein kinase C protein changes the phosphorylated peptide's net charge from +1 to −1. This change in the net charge of the substrate allows the phosphorylated and nonphosphorylated substrate to migrate toward the negative electrode (Figure 7).

The intensity of the bands obtained are calculated, and the percentage inhibition of PKC protein by DMS and **5c** was calculated and plotted (Figure 8) for comparison. It can be seen that **5c** inhibits PKC protein significantly enough only at a high concentration of 100 μM (Figure 8), whereas DMS shows inhibition of PKC protein at 50 μM, as according to the work reported by Igarashi et al.[14] From the PKC assay's results, it could be postulate that **5c** does not inhibit PKC protein and is specific toward SPHK1 protein only.

Conclusion

SPHK1 has been shown to be critically important in the regulation of cancer cell proliferation and apoptosis (reviewed in ref 28). More recently, we have shown a pivotal role for SPHK1 in experimental arthritis[29] and allergic asthma.[30] Thus, SPHK1 represents a potential novel therapeutic target to treat a wide range of disease from cancer to autoimmune diseases, such as rheumatoid arthritis, and allergies such as asthma.[28−31] We have demonstrated a facile and stereoselective synthesis of analogues of sphingosine and have identified several compounds that potently inhibit SPHKs. One of the compounds, **5c**, was

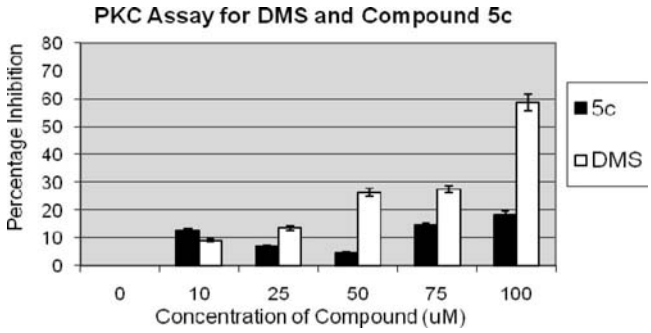


Figure 8. Comparison of inhibitory activity between DMS and **5c**. Relative percentage inhibition is calculated by taking the difference between the absolute inhibition value due to added compound and that for when no compound is added, over the value of absolute inhibition when no compound is added.

shown to be less toxic than DMS. Compound **5c** is a SPHK1 specific inhibitor. Current efforts are ongoing to develop compound **5c** as a lead compound. Understanding the in vivo effects of this new class of targeted compound will be crucial in evaluating their clinical potential.

Experimental Section

Chemistry. General Procedures. All chemical reagents and solvents were obtained from Sigma Aldrich, Merck, Lancaster, or Fluka and were used without further purification. Analytical TLC was carried out on precoated silica plates (Merck silica gel 60, F254) and visualized with UV light or stained with phosphomolybdic acid (PMA) stain. Flash column chromatography was performed with silica (Merck, 70−230 mesh). The purities of the compounds were determined via HPLC using a Shimadzhu LCMS-IT-TOF system with a Phenomenex Luma C18 column (50 mm × 3.0 mm, 5 μm). Detection was conducted at 220 nm, and integration was obtained with a Shimadzhu LCMS solution software. The mobile phase was a gradient with solvent B from 30% to 100% over 9 min and a flow rate of 0.8 mL/min. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. Compounds used in biological assays have purities of at least 95%. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker ACF 300 or AMX 500 Fourier transform spectrometer. Chemical shifts were reported in parts per million (δ) relative to the internal standard of tetramethylsilane (TMS). The signals observed were described as follows: s (singlet), d (doublet), t (triplet), m (multiplet). The number of protons (*n*) for a given resonance was indicated as nH. Mass spectra were performed on a Finnigan/MAT LCQ mass spectrometer under electron spray ionization (ESI). Optical rotations were determined with a JASCO DCP-1000 digital polarimeter and were the average of at least 10 measurements with an RSD value of less than −100%.

Synthesis of (*S*)-*tert*-Butyl-4-((*R*)-1-hydroxyhexadec-2-ynyl)-2,2-dimethyloxazolidine-3-carboxylate (2a3). *S*-(−)-1,1-dimethylethyl-4-formyl-2,2-dimethyloxazolidine-3-carboxylate (also known as Garner's aldehyde) **1** (0.432 mL, 2 mmol) in tetrahydrofuran (12 mL) was added, via a cannula, to a solution of pentadec-1-yne (2.72 mmol) and *n*-butyllithium (1.45 mL, 2.32 mmol, 1.6 M in hexanes) in tetrahydrofuran (25 mL) at −40 °C. The reaction mixture was stirred under nitrogen for 4 h at −40 °C. When TLC monitoring indicated the complete consumption of the starting materials, the reaction mixture was quenched with distilled water (10 mL) and extracted with ethyl acetate. The combined organic layer was washed with ammonium chloride, dried over magnesium sulfate, filtered, concentrated, and purified by column chromatography (ethyl acetate/hexane = 1:5 with 2% acetic acid) to yield **2a3**. $[\alpha]^{25}$ −40.09° (*c* 26.0 × 10^{-3} g/mL, CH_2Cl_2); ^{1}H NMR (300 MHz, C_6D_6) δ 5.11−5.12 (m, 1H, C**H**OH), 4.60 (br s, 1H, CHO**H**), 4.09−4.10 (m, 1H, OCH_2C**H**N), 3.65−3.71 (m, 2H, OC**H**$_2$CH), 2.05−2.08 (t, 2H, *J* = 5.7 Hz, 7.0 Hz, C≡CC**H**$_2$), 1.68 (s, 3H, OCC**H**$_3$), 1.44 (s, 3H, NCC**H**$_3$), 1.37 (s, 9H, OC(C**H**$_3$)$_3$), 1.22−1.30 (m, 22H, CH_2(C**H**$_2$)$_{11}$$CH_3$), 0.87−0.92 (t, 3H, *J* = 6.6 Hz, 7.0 Hz, CH_2C**H**$_3$); ^{13}C NMR (75 MHz, C6D6) δ 154.8, 94.6, 85.6, 80.2, 79.7, 79.2, 65.0, 64.2, 63.8, 63.2, 62.8, 61.9, 32.0, 29.8, 29.6, 29.4, 29.2, 28.9, 28.7, 28.0, 25.8, 25.4, 23.2, 22.7, 18.7, 14.0; HRMS (ESI, M + Na) calcd for $C_{26}H_{47}O_4NNa$, 460.3397; found, 460.3406. Yield: 70%.

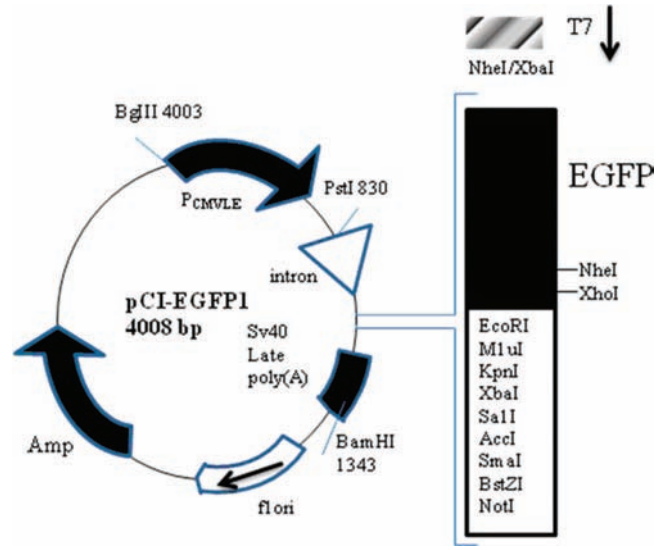


Figure 9. Map of EGFP-SPHK1 plasmid. EGFP-SPHK1 gene is inserted into pcDNA3.1 plasmid.

Synthesis of (*S*)-*tert*-Butyl-4-hexadec-2-ynoyl-2,2-dimethyloxazolidine-3-carboxylate (5c). Compound **2a3** (0.1182 mmol) and anhydrous dichloromethane (4 mL) were placed in a dry round-bottom flask. Manganese dioxide (0.2055 g, 2.369 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then filtered through Celite powder and purified by column chromatography (ethyl acetate/hexane = 1:4) to yield **5c**. $[\alpha]^{25}$ −24.77° (*c* 0.965 × 10^{-3} g/mL, DMSO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.33−4.36 (m, 1H, OCH_2C**H**), 4.04−4.22 (m, 2H, OC**H**$_2$CH), 2.36−2.41 (t, 2H, *J* = 6.9 Hz, 7.1 Hz, C≡CC**H**$_2$), 1.69 (s, 3H, OCC**H**$_3$), 1.54 (s, 3H, NCC**H**$_3$), 1.49−1.50 (m, 4H, C**H**$_2$$(CH_2)_9$C**H**$_2$), 1.42 (s, 9H, OC(C**H**$_3$)$_3$), 1.24 (s, 18H, CH_2(C**H**$_2$)$_9$$CH_2$), 0.85−0.89 (t, 3H, *J* = 5.6 Hz, 7.0 Hz, CH_2C**H**$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 186.2, 127.9, 98.1, 95.3, 80.6, 66.5, 65.7, 65.3, 51.6, 31.8, 29.5, 29.4, 29.3, 28.9, 28.8, 28.3, 28.1, 27.5, 26.1, 25.1, 25.0, 24.2, 22.6, 19.0, 14.0; HRMS (ESI, M + Na) calcd for $C_{26}H_{45}O_4NNa$, 458.3241; found, 458.3220. Yield: 70%.

Pharmacological Studies. Sphingosine Kinase 1 or 2 DNA Plasmid Preparation. Culture Growing. Human SPHK1 plasmid was established in which the SPHK1 gene was fused with an enhanced green fluorescence protein gene (EGFP-SPHK1) (Figure 9). The structure was constructed with restrictive enzymes NheI and *Eco*RI, by inserting PCR A*/B into pcDNA3.1 plasmid (A*, TAT-gCT-AgC-ATg-gAT-CCA-gCg-ggC-ggC; B, AAT-gAA-TTC-TCA-TAA-ggg-CTC-TTC-Tgg).

Human SPHK2 clone, which contained SPHK2 plasmid, was produced by Open Biosystems and purchased from iDNA Technology. SPHK2 gene was inserted into the site constructed by two resistant enzymes NotI and *Sal*I using pCMV-SPORT6 vector. There is no reporter system such as GFP or his-tag within this plasmid. The SPHK1 and SPHK2 bacteria cells were removed from the freezer and warmed to room temperature. Then 200 μL of LB medium was transferred into 50 mL Falcon tubes, and then an amount of 20 μL of the warmed bacteria cells (SPHK1 and 2) was added. The mixtures were shaken gently, and 50 μL of each of the mixture was pipetted into individually prepared culture plates (precoated with AMP) and evenly spread out using a bent glass pipet. The culture plates (containing SPHK1 and SPHK2 cells

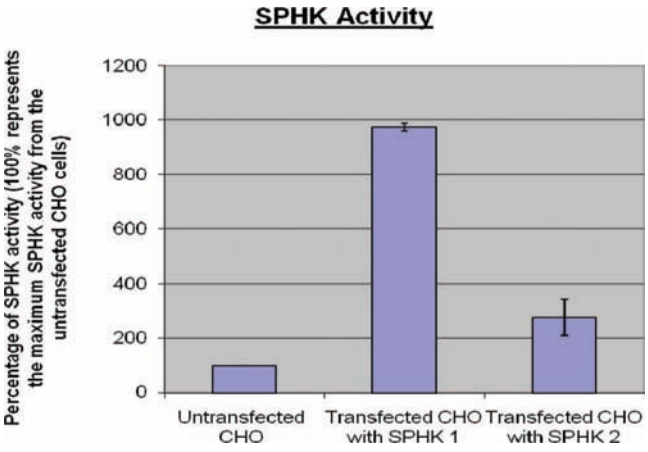


Figure 10. SPHK assay results using cell lysates obtained from the transfected CHO cells.

individually) were then turned upside down and placed overnight in an incubator at 37 °C with 5% CO_2 in a water saturated environment. This environment will allow the cells to grow into single colonies. After the single cell colonies have grown, a single colony was picked using a micropipet tip and dropped into a Falcon tube containing LB medium. The tube was placed in an incubator shaker set at 270 rpm for 7 h or more. The tube was then checked for cloudiness before more medium was added to the tube, and the shaking was continued at 280 rpm for an additional 17 h or more.

Extraction and Purification. The cultured bacteria (containing the DNA-plasmid encoding for SPHK1 and SPHK2 genes) were centrifuged, and the supernatant liquid was poured away. The pellet obtained was redissolved in medium and purified using QIAfilter Plasmid Midi kit, following the protocol listed in the kit, yielding the DNA plasmids for SPHK1 and SPHK2. The concentration of the DNA plasmids is obtained using the Thermo Scientific NanoDrop 1000 Spectrophotometer.

Mammalian Cell Transfection. Cell line used for the transfection process in this project was the Chinese hamster ovary (CHO) cell line. The cell line was cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of 2 mM L-glutamine, 10 mg/mL streptomycin, and 10 μL/mL penicillin. The cell line was maintained in an incubator at 37 °C, with 5% CO_2 in a water saturated environment. Then 1×10^6 cells were plated in a 10 cm well plate in 15 mL of DMEM a day before actual transfection. On the day of transfection, the medium in the cell culture was removed, the cells were washed with sterile phosphate buffered saline (PBS), and 10 mL of plain DMEM was added as medium. SPHK1 or SPHK2 DNA plasmids obtained were diluted in Opti-MEM I reduced serum medium and mixed gently (24 μg of plasmid in 1.5 mL of Opti-MEM per 10 cm well). Lipofectamine 2000 reagent was diluted in Opti-MEM, mixed gently, and incubated for 5 min (60 μL of Lipofectamine 2000 reagent in 1.5 mL of Opti-MEM per 10 cm well). The diluted DNA and Lipofectamine reagent were combined, shaken gently, and incubated for 30 min. Then 100 μL of the combined DNA−Lipofectamine 2000 complex was added to the wells containing the CHO cells and DMEM medium and the culture plates were placed in the incubator for 48 h at 37 °C with 5% CO_2 in a water saturated environment. After 24 h, the cells were scrapped and transferred to 2.0 mL Eppendorf tubes. Cell lysates are obtained by freeze−thawing the Eppendorf tubes using liquid nitrogen for at least 10 times consecutively. The tubes were then centrifuged at 13 000 rpm for about 5 min, and the supernatant liquid was transferred to a 50 mL centrifuge tube. Concentrations of the cell lysates were measured using the Bradford test template, following the protocol listed for the Bradford test.[32] The success of the transfection process is checked via running SPHK assay using the cell lysates obtained (Figure 10).

^{32}P Based Sphingosine Kinase (SPHK) Assay. Sphingosine−BSA complex (1 mM) was prepared by addition of 20 μL of natural occurring *erythro*-sphingosine (50 mM) dropwise into a glass tube containing 1 mL of BSA solution (4 mg/mL). The glass tube was continually vortexed during the addition. After which, the reaction mixture was sonicated for a minute and stored on ice. The reaction mixture for the sphingosine kinase assay was prepared by first placing the compounds at 0, 10, 25, 50, and 100 μM concentrations into glass tubes containing the cell lysates (40 μg per tube). The total volume was topped up to 180 μL using sphingosine kinase buffer. Then 10 μL of the prepared 1 mM sphingosine−BSA complex was added to the tubes. The reaction mixture in the glass tubes was then vortexed. An amount of 10 μL of the ATP solution (cold and hot ATP combined) was added to each of the samples, and the mixture was then vortexed. The tubes were then placed in a rack in a 37 °C water bath to be incubated for 30 min. After which, the rack was removed from the water bath and placed on ice. Then 20 μL of 1 N HCl and 0.8 mL of chloroform/methanol/HCl (100:200:1, v/v/v) were added and the mixture was vortexed and allowed to stand for 5−10 min. Amounts of 240 μL of chloroform and 240 μL of 2 N KCl were added; the mixture was vortexed and again allowed to stand for 5−10 min. The glass tubes were then centrifuged for 5−10 min. A total of 75 μL of the organic phase (bottom phase, chloroform) was spotted on the TLC plates with three lanes dedicated to each sample tube (i.e., to obtain triplicate readings). Upon drying, the plates were placed in a TLC glass chamber containing the eluent solvent system (1-butanol/methanol/acetic acid/water, 80:20:10:20, v/v/v/v). The plates were exposed individually to a phosphor imager screen for at least 3 h, and the screen was read in a phosphor imager (Typhoon scanner).

Measurement of S1P Levels in Cellular Assays. S1P levels were measured both in untransfected and in huSPHK1 and huSPHK2 transfected U937 cells. The transfection was carried out as described above. Untrasfected cells were stimulated or not with the cytokine tumor necrosis factor-α (TNFα), and cells were incubated or not with the inhibitor for 2 h. Transfected cells were also incubated with or without the inhibitors for 2 h. S1P levels were analyzed using a S1P competitive ELISA kit (Echelon Biosciences) according to the manufacturer's instructions. Sensitivity of the assay was 30 nM.

MTT Assay. U937 cells or HL60 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 1% L-glutamine. The 50 000 cells were placed in wells of three 96-well plates with a cell density of 1×10^6 cells/mL. The compound of interest was added into each well at 10 μM. Dimethylsphingosine was also added at 10 μM to serve as a control. Wells containing plain DMSO served as a vehicular control. The plate was then placed in the incubator for 24, 48, and 72 h of incubation at 37 °C with 5% CO_2 in a water saturated environment. At the end of each individual incubation time point, MTT reagent solution (for 200 samples) was prepared by dissolving 4 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 1 mL of plain RPMI 1640 medium, and 50 μL of the MTT solution was added into each sample well. The plate was then incubated again in the dark for 2 h and centrifuged at 3000 rpm for 5 min. The supernatant was removed using a pipet, and care was taken not to disturb the crystal formation. The crystals were then dissolved in 200 μL (per well) of DMSO/glycine buffer solution (190 μL of DMSO + 10 μL of glycine buffer). The plate was then gently shaken using a vortemp machine for 5 min to ensure equal mixing, and absorbance at 570 nm was taken using a spectrometer.

Protein Kinase C (PKC) Assay. The assay was performed in accordance with Promega's PepTag assay's protocol. Protein kinase C (PKC) α protein and PKC protein were diluted to 2.5 μg/μL. The PKC 5× solution was activated using probe sonicator for 25 s. Master mix containing PKC reaction 5× buffer, C1 peptide, PKC activator 5× solution, and peptide protection solution was prepared. The master mix solution was then aliquoted into individual 0.6 mL Eppendorf tubes (16 μL each) and kept on ice until PKC enzyme was added. Inhibitors of various concentrations were added, and deionized water was used to top up the volume of solution in the Eppendorf tubes to 21 μL (25 μL for negative control assay). The

Eppendorf tubes were incubated in a 30 °C water bath for 2 min. Then 4 μL of PKC α or PKC protein was added into Eppendorf tubes to standard or positive control assay, respectively. The Eppendorf tubes were further incubated in the 30 °C water bath for 30 min. At the end of the incubation time, the Eppendorf tubes were placed in a 95 °C heating block for 10 min to stop the reaction. The Eppendorf tubes were placed on ice and in the dark until loading began. Then 1 μL of 80% glycerol was added to each Eppendorf tube before loading onto gel.

For gel preparation/separation, the horizontal agarose gel apparatus was assembled accordingly. An 0.8% agarose solution (1.6 g in 200 mL 50 mM Tris-HCl, pH 8.0) was prepared and heated in microwave for 2 min and 10 s until all the agarose had been dissolved. The mixture was allowed to cool to 60 °C and subsequently poured into a gel tray, ensuring no bubbles were formed in the process. The gel was allowed to solidify before the comb was removed. The gel was placed in the electrophoresis chamber, and 50 mM Tris-HCl (pH 8.0) solution was poured onto the gel. The PKC assay samples was then loaded into the well (10 μL per well), and the electrophoresis was started at 120 V for about 30 min or until the separation was completed. The gel was then removed, and the photo of the gel was taken using a Kodax imaging machine.

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Supporting Information Available: Spectral data of the analogues and analytical data of **5c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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