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Pachastrissamine (Jaspine B) and its Stereoisomers Inhibit Sphingosine Kinases and Atypical Protein Kinase C

Yuji Yoshimitsu, Shinya Oishi*, Jun Miyagaki, Shinsuke Inuki, Hiroaki Ohno, and Nobutaka Fujii*

Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Corresponding Authors:

Shinya Oishi, Ph.D. and Nobutaka Fujii, Ph.D.

Graduate School of Pharmaceutical Sciences

Kyoto University

Sakyo-ku, Kyoto, 606-8501, Japan

Tel: +81-75-753-4551; Fax: +81-75-753-4570,

E-mail (S.O.): soishi@pharm.kyoto-u.ac.jp; E-mail (N.F.): nfujii@pharm.kyoto-u.ac.jp
Abstract: Sphingosine kinases (SphKs) are oncogenic enzymes that regulate the critical balance between ceramide and sphingosine-1-phosphate. Much effort has been dedicated to develop inhibitors against these enzymes. Naturally occurring pachastrissamine (jaspine B) and all its stereoisomers were prepared and evaluated for their inhibitory effects against SphKs. All eight stereoisomers exhibited moderate to potent inhibitory activity against SphK1 and SphK2. Inhibitory effects were profiled against protein kinase C (PKC) isoforms by in vitro experiments. Atypical PKCs (PKCζ and PKCι) were inhibited by several pachastrissamine stereoisomers. The improved activity over N,N-dimethylsphingosine suggests that the cyclic scaffold in pachastrissamines facilitates potential favorable interactions with SphKs and PKCs.

Keywords: jaspine B, pachastrissamine, protein kinase C, sphingosine kinase, sphingolipid

1. Introduction

Sphingolipid metabolites such as ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) play important parts in diverse biological processes.\(^1\) It has been reported that Cer and Sph promote apoptosis and inhibit proliferation, whereas S1P mediates cell proliferation and angiogenesis.\(^2\),\(^3\) The binding of S1P to a family of five specific G protein-coupled receptors termed S1PR\(_{1-5}\) controls various biological functions. S1P can also regulate intracellular processes, but molecular targets are not fully defined.\(^4\) Sphingosine kinases (SphKs), which catalyze the phosphorylation of Sph to form S1P are important lipid kinases.\(^5\) S1P levels are mainly regulated by SphKs, S1P lyase, and S1P phosphatases (Figure 1). Hitherto, two distinct isoforms of SphKs, SphK1, and SphK2, have been reported.\(^6\),\(^7\) SphK1 is overexpressed in various human tumors,\(^8\) thereby impairing the efficacy of chemotherapy.\(^9\) SphK1 inhibition by siRNA-mediated knockdown or pharmacologic inhibition induces apoptosis with elevation of the Cer/S1P ratio,\(^9\) and increases the effectiveness of docetaxel in prostate cancer cell lines.\(^10\)
SphK2 is different in its amino terminus and central region from SphK1. These two enzymes have different kinetic properties and tissue expression, implying that they may have distinct physiological roles. Indeed, in contrast to pro-survival SphK1, the SphK2 induces apoptosis by cytochrome c release. Recent investigations revealed that a selective inhibitor of SphK2 attenuates experimental osteoarthritis. Accordingly, SphKs could be promising drug targets for cancer chemotherapy and inflammatory diseases. So far, a number of sphingosine kinase inhibitors have been reported.

Pachastrissamine, a naturally occurring anhydrophytosphingosine derivative, was isolated from the Okinawan marine sponge, *Pachastrissa* sp. (Figure 2). Pachastrissamine exhibits marked sub-micromolar cytotoxicity against several cancer cell lines. Delgado and co-workers reported the cytotoxicity of pachastrissamine diastereomers with a series of C-2 and C-3 stereochemistries on the tetrahydrofuran core, which were prepared via Sharpless asymmetric dihydroxylation. Rao and co-workers also revealed that pachastrissamine enantiomer is less cytotoxic than pachastrissamine. There has also been significant interest in the molecular mechanisms of cell death induced by pachastrissamine. Salma et al. revealed that pachastrissamine inhibits sphingomyelin synthase to increase the intracellular level of Cer, inducing apoptotic cell death by a caspase-dependent pathway. However, its activity against isolated sphingolipid metabolizing enzymes has yet to be investigated. In the present study, we investigated the inhibitory activities of eight pachastrissamine stereoisomers 1–8 against SphKs in vitro (Figure 2).

2. Results and discussion

2.1. Synthesis of pachastrissamine stereoisomers

Previously, we developed a stereoselective synthesis of four pachastrissamine diastereomers 1–4 with the 4S-configuration. This synthetic route divergently provides four pachastrissamine diastereomers from S-Garner’s aldehyde as a sole chiral pool. We prepared the other enantiomeric stereoisomers 5–8 using this successful approach from R-Garner’s aldehyde (Scheme 1). Briefly, *R-*
Garner’s aldehyde was converted into 11 by treatment with a phosphonium ylide followed by dihydroxylation with OsO₄.¹⁹ The diol 11 was converted into the corresponding bis-tosylate 12 with TsCl, Et₃N and Me₃N·HCl. Treatment of 12 with TsOH successfully constructed the expected tetrahydrofuran ring. Cleavage of the tosyl group with Mg in MeOH gave the (2R,3R,4R)-isomer 5.

Regioselective tosylation of the primary hydroxy group of the d-ribo-phytosphingosine derivative 14 prompted spontaneous cyclization to give the tetrahydrofuran, and removal of the Boc group with TFA provided the desired product, 6.²⁰

The primary hydroxy group of 14 was protected with a TIPS group. Subsequent conversion of the resulting silyl ether to oxazolidinone using MeC(OMe)₃ in the presence of a catalytic amount of BF₃·OEt₂ afforded 17. Protection of the carbamate nitrogen of 17 with Boc₂O and alcoholysis of the oxazolidinone successfully provided 18. Similar to the synthesis of 5, bis-tosylation, desilylation and TBAF-promoted tetrahydrofuran formation afforded the desired core scaffold that was successively deprotected with Mg and TFA to furnish 7.

The silyl ether of 18 was cleaved with TBAF in THF to give the triol 21. Selective monotosylation of the primary hydroxy group followed by base treatment afforded tetrahydrofuran 22. The Boc group was removed with TFA to give 8.

2.2. Sphingosine kinase inhibition by pachastrissamine stereoisomers

To evaluate the SphK inhibitory activity of pachastrissamines, in vitro SphK inhibition assays were undertaken for SphK1 and SphK2 based on the partitioning of Sph and S1P using the LabChip3000 system.²¹ N,N-dimethylsphingosine (DMS) was employed as a reference SphK inhibitor (Table 1).²² Interestingly, all of the pachastrissamine stereoisomers exhibited an inhibitory effect on both SphKs. Among the compounds tested, (2R,3S,4R)-isomer 7 and (2S,3S,4R)-isomer 8 exhibited most potent inhibitory activity upon SphK1 and SphK2, respectively, which are more potent than DMS. These results indicate that a primary hydroxy group in the substrate Sph, which is
phosphorylated by SphKs, may not be essential for enzyme recognition. The stereochemistry of the accessory amino and hydroxyl groups on the tetrahydrofuran core has significant influence on SphK inhibitory potency. This apparently ambiguous recognition of stereochemistry resembles the potent SphK inhibitory effect by D- and L-threeo-sphingosines. Inhibitory activity of 7 and 8 may be due to sphingosine-like conformation of hydroxy and amino groups. The hydrophobic tetradecyl moiety probably significantly contributes to binding to SphK by hydrophobic interactions, corresponding to known inhibitors with sphingosine-based structures. Whereas the cis-olefin-containing hydrocarbon is indispensable for SphK inhibition by B-5354c, the saturated aliphatic chain of pachastrissamines subserves effective SphK suppression.

2.3. Protein kinase C (PKC) inhibition by pachastrissamine stereoisomers

DMS was also reported to work as a protein kinase C (PKC) inhibitor. Hence, the bioactivity of pachastrissamine 1, and potent SphK inhibitors 7 and 8 were profiled against each PKC isoform (Figure 3). Although numerous studies on Sph itself and DMS were revealed, little is known about its isoform selectivity. The individual PKC isoform is involved in different cellular process, so the importance of the selectivity profiles of inhibitors is emphasized. For example, PKCζ has been implicated in epidermal growth factor (EGF)-stimulated chemotaxis of cancer cells, whereas PKCı is an oncogenic protein required for the transformed growth and tumorigenesis of human cancer cells. Pachastrissamines 1, 7 and 8 exerted complete inhibition of PKCζ and PKCı at 10 μM, whereas modest inhibition against the other PKCs was observed (see the Supplementary data). The inhibitory effects of pachastrissamine isomers at 3 μM against PKCζ and PKCı were ~50%, which were more potent than the reference DMS (Figure 3). To our best knowledge, this is the first report on the Sph-based PKC inhibitors with selectivity for atypical PKC isozymes in vitro.

The catalytic domain (motifs required for ATP-substrate binding and catalysis) of PKC is highly conserved among three subfamilies: conventional PKCs (PKCa, β1, β2, γ), novel PKCs (PKCd, ε,
Conventional PKCs and novel PKCs have a C1 domain that binds diacylglycerol (DAG) for kinase activation. In the in vitro assay system for the present study, conventional PKCs/novel PKCs were activated by the addition of DAG. The addition of DAG for conventional PKCs/novel PKCs may diminish the potential inhibitory effects by pachastrissamines. In contrast, atypical PKCs, including distinct nucleotide binding loop and ATP binding pocket, bind phosphatidylinositol 3,4,5-trisphosphate (PIP3) and Cer (not DAG) in the atypical C1 domain, whereas they lack a DAG binding site in the regulatory domain. Considering that sphingolipid Cer binds to the atypical C1 domain, PKC-isoform selectivity of pachastrissamines could be derived from the binding to the site in the domain.

3. Conclusions

In conclusion, we identified pachastrissamine (jaspine B) as a novel SphK1 and SphK2 inhibitor. Several non-natural stereoisomers are more potent SphK inhibitors than the naturally occurring pachastrissamine. The distinct chiral tetrahydrofuran scaffold with accessory groups could facilitate favorable interactions with both enzymes. In addition, the inhibitory activities of these SphK inhibitors against atypical PKCs (PKCζ, PKCτ) were revealed, suggesting that SphKs and PKCs represent alternative molecular targets of pachastrissamines to induce apoptotic process. As such, these pachastrissamine stereoisomers obtained by our unique divergent synthesis could be promising leads to design sphingolipid-based kinase inhibitors. Further optimization of these inhibitors (especially for the alkyl side-chains) and investigations to elucidate the biological impact of these inhibitors are underway.

4. Experimental

4.1. Synthesis
4.1.1. General Methods. $^1$H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer at 400 or 500 MHz frequency, and chemical shifts are reported in $\delta$ (ppm) relative to TMS (in CDCl$_3$) as internal standard. $^1$H NMR spectra are tabulated as follows: chemical shift, multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, br s = broad singlet, m = multiplet), number of protons, and coupling constant(s). All $^1$H NMR spectra were in agreement with those of the enantiomers of our previous report. Optical rotations were measured with a JASCO P-1020 polarimeter. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. The purity of the compounds was determined by $^1$H NMR and elemental analysis (>95%).

4.1.2. tert-Butyl (S,Z)-4-(Hexadec-1-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate (10). To a stirred solution of C$_{15}$H$_{31}$PPh$_3$Br (53.3 g, 96.3 mmol) in THF (350 mL) was added LHMDS (1.0 M in THF; 88.0 mL, 88.0 mmol) at 0 °C, and the mixture was stirred for 0.5 h at room temperature. To the resulting dark red solution was added dropwise Garner’s aldehyde 9 (9.60 g, 41.9 mmol) in THF (70.0 mL) at −78 °C. The mixture was stirred for 30 min at this temperature and then 8 h at room temperature. The mixture was quenched by addition of saturated NH$_4$Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et$_2$O. The extract was washed with brine, and dried over MgSO$_4$. The filtrate was concentrated under reduced pressure followed by rapid filtration through a short pad of silica gel with n-hexane–EtOAc (10:1) to give a crude mixture. Further purification by flash chromatography over silica gel with n-hexane–EtOAc (100:1 to 80:1) gave 10 (15.4 g, 87%) as a colorless oil; $[\alpha]_{D}^{25} = -59.4$ (c 0.43, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.88 (t, J = 6.9 Hz, 3H), 1.22–1.33 (m, 24H), 1.36–1.62 (m, 15H), 1.98–2.18 (m, 2H), 3.64 (dd, J = 8.6, 4.0 Hz, 1H), 4.05 (dd, J = 8.6, 6.3 Hz, 1H), 4.66 (m, 1H), 5.35–5.54 (m, 2H); HRMS (FAB) calcd for C$_{26}$H$_{50}$NO$_3$ (MH$^+$) 424.3785, found 424.3784.

4.1.3. tert-Butyl (R)-4-[(1R,2S)-1,2-Dihydroxyhexadecyl]-2,2-dimethyloxazolidine-3-carboxylate (11). To the stirred solution of 10 (6.7 g, 15.8 mmol) and N-methylmorpholine N-oxide
(2.8 g, 23.7 mmol) in t-BuOH (40 mL) and water (40 mL) was added OsO₄ (2.5 w/v% in t-BuOH, 8.0 mL, 0.791 mmol) at 0 °C, and the mixture was stirred for 9.0 h at room temperature. The mixture was quenched by addition of saturated Na₂S₂O₅ at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et₂O and washed with brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (5:1) to give 11 (4.5 g, 62% yield) and its C3/C4-stereoisomer (1.4g, 19% yield) as white solids. Recrystallization from n-hexane–EtOAc gave pure 11 as colorless crystals; mp 55–56 °C; [α]²⁵'D +8.87 (c 1.02, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.19–1.33 (m, 24H), 1.47–1.52 (m, 15H), 1.58–1.63 (m, 2H), 3.14–3.32 (m, 2H), 3.53–3.67 (m, 2H), 4.00 (m, 1H), 4.11–4.25 (m, 2H); Anal. Calcd for C₂₆H₅₁NO₅: C, 68.23; H, 11.23; N, 3.06. Found: C, 67.95; H, 11.38; N, 3.08.

4.1.4. tert-Butyl (R)-4-[(1R,2S)-1,2-Bis(tosyloxy)hexadecyl]-2,2-dimethyloxazolidine-3-carboxylate (12). To a stirred solution of 11 (194 mg, 0.424 mmol) in CH₂Cl₂ (850 μL) were added Et₃N (588 μL, 4.24 mmol), TsCl (404 mg, 2.12 mmol) and Me₃N·HCl (41 mg, 0.424 mmol) at room temperature. After stirring for 1 d at this temperature, the mixture was quenched by addition of saturated NH₄Cl at 0 °C and the whole was extracted with CH₂Cl₂. The extract was washed with saturated NH₄Cl and brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (10:1 to 7:1) to give 12 as a colorless oil (285 mg, 88% yield); [α]²⁵'D +23.9 (c 0.68, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.08–1.33 (m, 26H), 1.43–1.54 (m, 15H), 2.41 (s, 3H), 2.43 (s, 3H), 3.84 (dd, J = 9.2, 6.9 Hz, 1H), 3.92 (dd, J = 9.2, 2.9 Hz, 1H), 4.05 (m, 1H), 4.63 (m, 1H), 5.21 (m, 1H), 7.26 (d, J = 8.0 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H), 7.73 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 8.0 Hz, 2H); HRMS (FAB) calcd for C₄₀H₆₄NO₉S₂ (MH⁺) 766.4017, found 766.4015.
4.1.5. (2R,3R,4R)-4-Amino-2-tetradecyltetrahydrofuran-3-yl 4-Methylbenzenesulfonate (13).

To a stirred solution of 12 (262 mg, 0.342 mmol) in MeOH (11 mL) was added TsOH·H₂O (65 mg, 0.342 mmol) at 70 °C. After stirring for 8 h at this temperature, the mixture was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with CHCl₃–MeOH–28% NH₄OH (95:4:1) to give 13 as a white solid (140 mg, 90% yield); mp 65–66 °C; [α]²⁵ D −18.6 (c 0.28, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.02–1.32 (m, 24H), 1.39–1.63 (m, 4H), 2.46 (s, 3H), 3.43 (dd, J = 8.6, 8.6 Hz, 1H), 3.72 (m, 1H), 3.88 (ddd, J = 4.6, 4.6, 4.0 Hz, 1H), 3.99 (dd, J = 8.6, 8.6 Hz, 1H), 4.85 (dd, J = 4.6, 4.6 Hz, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.83 (d, J = 8.0 Hz, 2H); HRMS (FAB) calcd for C₂₅H₄₄NO₄S (MH⁺) 454.2986, found 454.2993.

4.1.6. (2R,3R,4R)-4-Amino-2-tetradecyltetrahydrofuran-3-ol (5). To a stirred mixture of 13 (23 mg, 0.0551 mmol) in MeOH (1.1 mL) was added Mg (13 mg, 0.0551 mmol) at room temperature. After stirring for 1.5 h at this temperature, silica gel was added to the solution and the mixture was concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel with CHCl₃–MeOH–28% NH₄OH (95:4:1) to give 5 as a white solid (13 mg, 79% yield); mp 95–96 °C; [α]²⁵ D −9.61 (c 0.61, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.20–1.49 (m, 24H), 1.49–1.59 (br s, 2H), 1.60–1.70 (m, 2H), 3.52 (dd, J = 8.5, 7.1 Hz, 1H), 3.66 (m, 1H), 3.73 (ddd, J = 7.1, 7.1, 3.4 Hz, 1H), 3.87 (dd, J = 4.6, 3.4 Hz, 1H), 3.92 (dd, J = 8.5, 7.3 Hz, 1H). Anal. Calcd for C₁₈H₃₇NO₂: C, 72.19; H, 12.45; N, 4.68. Found: C, 72.10; H, 12.63; N, 4.65.

4.1.7. tert-Butyl [(2R,3R,4S)-1,3,4-trihydroxyoctadecan-2-yl]carbamate (14). To a stirred solution of 11 (2.42 g, 5.29 mmol) in MeOH (176 mL) was added TsOH·H₂O (101 mg, 0.529 mmol) at 0 °C, and the mixture was stirred for 10 h at room temperature. The mixture was quenched by addition of Et₃N at 0 °C and concentrated under reduced pressure, followed by flash chromatography over silica gel with n-hexane–EtOAc (1:1) to give 14 as a white solid (1.93 g, 87%
yield); mp 85–86 °C; [α]_{25}^{D} = –6.61 (c 1.61, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.21–1.39 (m, 24H), 1.45 (s, 9H), 1.47–1.55 (m, 2H), 2.42 (d, J = 4.0 Hz, 1H), 2.98 (m, 1H), 3.29 (d, J = 6.9 Hz, 1H), 3.62 (m, 1H), 3.68 (m, 1H), 3.77 (m, 1H), 3.85 (m, 1H), 3.92 (m, 1H), 5.30 (m, 1H); HRMS (FAB) calcd for C₂₃H₄₈NO₅ (MH⁺) 418.3527, found 418.3533.

4.1.8. tert-Butyl [(3R,4R,5S)-4-Hydroxy-5-tetradecyltetrahydrofuran-3-yl]carbamate (15).
To a stirred mixture of 14 (90 mg, 0.216 mmol) and Et₃N (108 μL, 0.778 mmol) in CH₂Cl₂ (7.2 mL) were added TsCl (74 mg, 0.389 mmol) and DMAP (2.4 mg, 0.0214 mmol) at 0 °C, and the mixture was stirred for 18 h at room temperature. The mixture was quenched by addition of saturated NH₄Cl at 0 °C, and the whole was extracted with CH₂Cl₂. The extract was washed with brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (5:1 to 3:1) followed by recrystallization from n-hexane–EtOAc to give 15 (76 mg, 88% yield) as colorless crystals.; mp 80–81 °C; [α]_{25}^{D} = –7.76 (c 0.29, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.22–1.34 (m, 24H), 1.46 (s, 9H), 1.52–1.57 (m, 2H), 2.10 (m, 1H), 3.51 (m, 1H), 3.71 (m, 1H), 3.94 (m, 1H), 4.11–4.17 (m, 2H), 4.95 (m, 1H); HRMS (FAB) calcd for C₂₃H₄₆NO₄ (MH⁺) 400.3421, found 400.3413.

4.1.9. (2S,3R,4R)-4-Amino-2-tetradecyltetrahydrofuran-3-ol (6). To a stirred solution of 15 (45 mg, 0.113 mmol) in CH₂Cl₂ (800 μL) was added TFA (800 μL) at 0 °C, and the mixture was stirred for 30 min at room temperature. The mixture was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with CHCl₃–MeOH–28% NH₄OH (95:4:1) followed by recrystallization from n-hexane–EtOAc to give 6 as colorless crystals (31 mg, 92% yield); mp 104–105 °C; [α]_{25}^{D} = –8.78 (c 0.75, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.19–1.34 (m, 24H), 1.34–2.37 (m, 5H), 3.40 (dd, J = 8.6, 6.9 Hz, 1H), 3.46 (m, 1H), 3.57–3.66 (m, 2H), 4.13 (dd, J = 8.6, 6.3 Hz, 1H). Anal. Calcd for C₇₈H₄₁NO₂: C, 71.12; H, 12.43; N, 4.61. Found: C, 71.39; H, 12.39; N, 4.59.
4.1.10. **tert-Butyl** [(2R,3R,4S)-3,4-Dihydroxy-1-(triisopropylsilyloxy)octadecan-2-yl]carbamate (16). To a stirred solution of 14 (1.74 g, 4.17 mmol) in DMF (42 mL) were added imidazole (1.14 g, 16.7 mmol) and TIPSCI (3.53 mL, 16.7 mmol) at 0 °C, and the mixture was stirred for 1 h at room temperature. The mixture was quenched by addition of MeOH at 0 °C, and concentrated under reduced pressure. The residue was diluted with CH₂Cl₂, washed with saturated NH₄Cl and brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (8:1) to give 16 as a colorless oil (2.15 g, 90% yield); [α]²⁵ ice = −23.5 (c 0.25, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.04–1.09 (m, 18H), 1.10–1.18 (m, 2H), 1.18–1.38 (m, 24H), 1.44 (s, 9H), 1.47–1.75 (m, 3H), 2.55 (d, J = 5.7 Hz, 1H), 3.24 (m, 1H), 3.58 (m, 1H), 3.63 (m, 1H), 3.86 (m, 1H), 4.05 (m, 1H), 5.24 (d, J = 8.0 Hz, 1H); HRMS (FAB) calcd for C₃₂H₆₈NO₅Si (MH⁺) 574.4861, found 574.4855.

4.1.11. **(S)-1-[(4R,5S)-2-Oxo-4-[(triisopropylsilyloxy)methyl]oxazolidin-5-yl]pentadecyl Acetate** (17). To a stirred solution of 16 (1.56 g, 2.72 mmol) in CH₂Cl₂ (270 mL) were added MeC(OMe)₃ (2.0 mL, 16.3 mmol) and BF₃·OEt₂ (67 µL, 0.544 mmol) at 0 °C, and the mixture was stirred for 1.5 h at room temperature. The mixture was quenched by addition of MeOH at 0 °C, and concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (4:1) to give 17 as a colorless oil (1.28 g, 87% yield); [α]²⁵ ice = +25.5 (c 0.66, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.03–1.16 (d, J = 5.7 Hz, 18H), 1.08–1.14 (m, 3H), 1.22–1.37 (m, 24H), 1.63–1.75 (m, 2H), 2.10 (s, 3H), 3.61–3.63 (m, 1H), 3.67–3.73 (m, 2H), 4.41 (dd, J = 4.6, 3.4 Hz, 1H), 5.00 (ddd, J = 6.9, 6.9, 3.4 Hz, 1H), 5.28 (m, 1H); HRMS (FAB) calcd for C₃₀H₆₀NO₅Si (MH⁺) 542.4235, found 542.4240.

4.1.12. **tert-Butyl** [(2R,3S,4S)-3,4-Dihydroxy-1-(triisopropylsilyloxy)octadecan-2-yl]carbamate (18). To a stirred solution of 17 (760 mg, 1.40 mmol) in THF (14 mL) were added Et₃N (194 µL, 1.40 mmol), Boc₂O (428 mg, 1.96 mmol), and DMAP (343 mg, 2.81 mmol) at 0 °C,
and the mixture was stirred 2 h at room temperature. The mixture was quenched by addition of saturated NH₄Cl at 0 °C. The mixture was concentrated under reduced pressure, and the residue was extracted with Et₂O. The extract was washed with brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was dissolved in MeOH (7.0 mL). NaOMe (1.13 g, 21.0 mmol) was added to this solution under stirring at 0 °C, and the mixture was stirred for 10 min at room temperature. The mixture was quenched by addition of saturated NH₄Cl, and concentrated under reduced pressure. The residue was extracted with EtOAc, washed with brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (4:1) to give 18 as a colorless oil (562 mg, 70% yield); [α]²⁵D –20.6 (c 3.21, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.07 (d, J = 5.7 Hz, 18H), 1.08–1.14 (m, 3H), 1.14–1.42 (m, 24H), 1.44 (s, 9H), 1.45–1.61 (m, 2H), 2.55 (m, 1H), 3.46 (m, 1H), 3.60 (m, 1H), 3.66–3.79 (m, 2H), 3.79–3.99 (m, 2H), 5.15 (d, J = 8.0 Hz, 1H); HRMS (FAB) calcd for C₃₂H₆₈NO₅Si (MH⁺) 574.4861, found 574.4858.

4.1.13. (2R,3S,4S)-2-[(tert-Butoxycarbonyl)amino]-1-(triisopropylsilyloxy)octadecane-3,4-diy1 Bis(4-methylbenzenesulfonate) (19). To a stirred solution of 18 (255 mg, 0.444 mmol) in CH₂Cl₂ (1.0 mL) were added Et₃N (613 µL, 4.44 mmol), TsCl (423 mg, 2.22 mmol), and Me₃N·HCl (42 mg, 0.444 mmol) at room temperature, and the mixture was stirred 4 h at room temperature. The mixture was quenched by addition of saturated NH₄Cl at 0 °C, and the whole was extracted with CH₂Cl₂, dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (10:1) to give 19 as a colorless oil (373 mg, 95% yield); [α]²⁵D –18.8 (c 0.95, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 0.91–1.11 (m, 26H), 1.11–1.36 (m, 18H), 1.41 (s, 9H), 1.54 (m, 3H), 2.44 (s, 3H), 2.45 (s, 3H), 3.46 (dd, J = 10.3, 6.3 Hz, 1H), 3.56 (dd, J = 10.3, 4.6 Hz, 1H), 4.01 (m, 1H), 4.65 (m, 1H), 4.79 (d, J = 9.7 Hz, 1H), 5.03 (dd, J = 4.6, 3.4 Hz, 1H), 7.31 (d, J = 8.3 Hz, 2H), 10.3–10.7 (m, 2H).
2H), 7.34 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 8.3 Hz, 2H), 7.85 (d, J = 8.3 Hz, 2H); HRMS (FAB) calcd for C_{46}H_{79}N_{19}O_{9}S_{2}Si (M^{+}) 904.4863, found 904.4863.

4.1.14. \((2R,3S,4R)-4-[(\text{\textit{tert}}-\text{Butoxycarbonyl})\text{amino}]2\text{-tetracyclitetrahydrofuran-3-yl} \text{ 4-Methylbenzenesulfonylate (20)}\). To a stirred solution of 19 (172 mg, 0.195 mmol) in THF (3.9 mL) was added TBAF (1.0 M in THF; 390 µL, 0.390 mmol) at 0 °C, and the mixture was stirred for 2 h at room temperature. The mixture was quenched by addition of saturated NH_{4}Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et_{2}O, and dried over MgSO_{4}. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with \(n\)-hexane–EtOAc (7:1) to give 20 as a colorless oil (71 mg, 65% yield); [\(\alpha\)]_{D}^{25} +1.48 (c 0.056, CHCl_{3}); ^{1}H NMR (500 MHz, CDCl_{3}) \(\delta\) 0.88 (t, J = 6.9 Hz, 3H), 1.10–1.36 (m, 26 H), 1.43 (s, 9H), 2.45 (s, 3H), 3.73 (dd, J = 9.7, 2.9 Hz, 1H), 3.78 (dd, J = 9.7, 5.2 Hz, 1H), 3.93 (m, 1H), 4.07 (m, 1H), 4.43 (m, 1H), 4.74 (m, 1H), 7.53 (d, J = 8.0 Hz, 2H), 7.83 (d, J = 8.0 Hz, 2H); HRMS (FAB) calcd for C_{30}H_{50}NO_{6}S ([M–H]^-) 552.3364, found 552.3370.

4.1.15. \((2R,3S,4R)-4\text{-Amino-2-tetracyclitetrahydrofuran-3-ol (7)}\). To a stirred solution of 20 (48 mg, 0.087 mmol) in MeOH (2.9 mL) was added Mg (21 mg, 0.87 mmol) at room temperature, and the mixture was stirred for 45 min at this temperature. The mixture was concentrated under reduced pressure, and the residue was dissolved with EtOAc, washed with H_{2}O, and dried over MgSO_{4}. The filtrate was concentrated under reduced pressure to give a white solid, which was dissolved in CH_{2}Cl_{2} (1.5 mL). TFA (1.5 mL) was added to the mixture at 0 °C. After stirring for 15 min at room temperature, the mixture was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with CHCl_{3}–MeOH–28% NH_{4}OH (95:4:1) to give 7 as a white solid (23 mg, 88% yield); [\(\alpha\)]_{D}^{25} +1.48 (c 0.056, CHCl_{3}); ^{1}H NMR (500 MHz, CDCl_{3}) \(\delta\) 0.88 (t, J = 6.9 Hz, 3H), 1.20–1.42 (m, 24H), 1.42–1.67 (m, 2H), 2.14–2.42 (m, 2H), 3.33 (m, 1H), 3.53–3.57 (m, 1H), 3.57–3.63 (m, 2H), 4.00 (dd, J = 9.2, 6.3 Hz, 1H). Anal. Calcd for C_{18}H_{37}NO_{2}: C, 72.19; H, 12.45; N, 4.68. Found: C, 72.29; H, 12.16; N, 4.61.
4.1.16. tert-Butyl [(2R,3S,4S)-1,3,4-Trihydroxyoctadecan-2-yl]carbamate (21). To a stirred solution of 18 (87 mg, 0.152 mmol) in THF (3.0 mL) was added TBAF (1.0 M in THF; 304 µL, 0.304 mmol) at 0 °C, and the mixture was stirred for 20 min at room temperature. The mixture was quenched by addition of saturated NH₄Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with EtOAc, dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (1:1 to 1:2) to give 21 as a white solid (59 mg, 93% yield); mp 68–70 °C; [α]²⁵_D −4.33 (c 1.14, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.20–1.39 (m, 24H), 1.45 (s, 9H), 2.29 (m, 1H), 2.65 (m, 1H), 2.99 (m, 1H), 3.61–3.68 (m, 2H), 3.69–3.77 (m, 2H), 3.89 (m, 1H), 5.27 (m, 1H); HRMS (FAB) calcd for C_{23}H_{46}NO_{5} ([M–H]⁻) 416.3381, found 416.3391.

4.1.17. tert-Butyl [(3R,4S,5S)-4-Hydroxy-5-tetradecyltetrahydrofuran-3-yl]carbamate (22). To a stirred mixture of 21 (42 mg, 0.100 mmol) and Et₃N (111 µL, 0.800 mmol) in CH₂Cl₂ (3.3 mL) were added TsCl (76 mg, 0.400 mmol) and Me₃N·HCl (10 mg, 0.100 mmol) at −78 °C, and the mixture was stirred for 4 h at this temperature. The mixture was quenched by addition of EtOH at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et₂O, washed with saturated NH₄Cl and brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was dissolved in MeOH (3.3 mL). K₂CO₃ (41 mg, 0.300 mmol) was added to the stirred mixture at 0 °C, and the mixture was stirred for 30 min at room temperature. The mixture was quenched by addition of saturated NH₄Cl, and concentrated under reduced pressure. The residue was extracted with EtOAc, washed with brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure to give an oily residue, which was purified by flash chromatography over silica gel with n-hexane–EtOAc (5:1 to 3:1) to give 22 as a white solid (21 mg, 53% yield); mp 105–107 °C; [α]²⁵_D +26.3 (c 0.13, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, J = 6.9 Hz, 3H), 1.24–1.35 (m, 24H), 1.45 (s, 9H), 1.59–1.66 (m, 2H), 2.27 (m, 1H), 3.44
(dd, J = 9.7, 4.0, 1H), 3.81 (m, 1H), 4.00 (m, 1H), 4.07 (m, 1H), 4.24 (m, 1H), 4.62 (m, 1H); HRMS (FAB) calcd for C_{23}H_{44}NO_{5} ([M–H]−): 398.3276, found 398.3275.

4.1.18. **(2S,3S,4R)-4-Amino-2-tetradecyltetrahydrofuran-3-ol** (8). By a procedure identical with that described for synthesis of 6 from 15, 22 (30 mg, 0.075 mmol) was converted into 8 as a white solid (22 mg, 98%); mp 80–82 °C; [α]_{D}^{25} +2.33 (c 0.20, CHCl_{3}); ^{1}H NMR (500 MHz, CDCl_{3}) δ 0.88 (t, J = 6.9 Hz, 3H), 1.21–1.40 (m, 24H), 1.40–1.69 (m, 5H), 3.39 (dd, J = 9.7, 3.4 Hz, 1H), 3.47 (m, 1H), 3.81 (m, 1H), 3.90 (m, 1H), 4.21 (dd, J = 9.7, 6.3 Hz, 1H). Anal. Calcd for C_{18}H_{37}NO_{2}·0.25H_{2}O: C, 71.12; H, 12.43; N, 4.61. Found: C, 71.19; H, 12.45; N, 4.40.

4.2. Sphingosine Kinase Assay

SphK inhibitory activities were evaluated by the off-chip mobility shift assay by the QuickScout® service from Carna Bioscience (Kobe, Japan). SphK1(1-384) and SphK2(1-618) were expressed as N-terminal GST-fusion proteins using a baculovirus expression system. They were purified using glutathione sepharose chromatography. Each chemical in DMSO at different concentrations was diluted fourfold with reaction buffer [20 mM HEPES (pH 7.5), 0.01% Triton X-100, 2 mM DTT]. For SphK reactions, a combination of the compound, 1 µM Sph, 5 mM MgCl_{2}, ATP (25 µM for SphK1; 600 µM for SphK2) in reaction buffer (20 µL) were incubated with each SphK in 384-well plates at room temperature for 1 h (n = 4). The reaction was terminated by addition of 60 µL of termination buffer (Carna Biosciences). Substrate and product were separated by electrophoretic means using the LabChip3000 system. The kinase reaction was evaluated by the product ratio, which was calculated from the peak heights of the substrate (S) and product (P): [P/(P+S)]. Inhibition data were calculated by comparing with no-enzyme controls for 100% inhibition and no-inhibitor reactions for 0% inhibition. IC_{50} values were calculated using GraphPad Prism 4 software (GraphPad Software, Incorporated, La Jolla, CA, USA).
4.3. Protein Kinase C (PKC) Assay

PKC inhibitory activities were evaluated using the off-chip mobility shift assay by the QuickScout® service from Carna Bioscience (Kobe, Japan). N-terminal GST-fusion protein were employed for the assays: PKCα(1-672), PKCβ1(1-671), PKCβ2(1-673), PKCγ(1-697), PKCδ(1-676), PKCe(1-737), PKCζ(1-592), PKCη(1-683), PKCθ(1-706), and PKCι(1-587). These were expressed using the baculovirus expression system and purified by glutathione sepharose chromatography. Each chemical in DMSO at different concentrations was diluted fourfold with reaction buffer [20 mM HEPES (pH 7.5), 0.01% Triton X-100, 2 mM DTT]. For the kinase reactions except for PKCζ and PKCι, phosphatidylserine (50 μg/mL) and diacyl glycerol (5 μg/mL) were added. A combination of the compound, 1 μM PKC0 substrate (N-FL), ATP (25 μM for PKCα/β1/γ/η/θ/ι; 10 μM for PKCβ2/δ/ε; 5 μM for PKCζ), 5 mM MgCl₂, and 50 μM CaCl₂ (for PKCα/β1/β2/γ) in reaction buffer (20 μL) were incubated with each PKC in 384-well plates at room temperature for 1 h (n = 2). The reaction was terminated by addition of 60 μL of termination buffer (Carna Biosciences). Substrate and product were separated by electrophoretic means using the LabChip3000 system. The kinase reaction was evaluated by the product ratio, which was calculated from the peak heights of the substrate (S) and product (P) peptides: [P/(P+S)]. Inhibition data were calculated by comparing with no-enzyme controls for 100% inhibition and no-inhibitor reactions for 0% inhibition.

Acknowledgements

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Supplementary data


References and footnotes

1 For a review, see: Fyrst, H.; Saba, J. D. Nat. Chem. Biol. 2010, 6, 489.


35 Although we assumed that pachastrissamines could mimic the structure of Cer, the potential inhibition of atypical PKCs by binding to the catalytic domain cannot be ruled out.
Table 1. Inhibitory activity of pachastrissamine and its stereoisomers against sphingosine kinases

<table>
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<th>compound</th>
<th>IC$_{50}^{a}$</th>
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<th>SphK2</th>
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<tr>
<td>2</td>
<td>3.9</td>
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<tr>
<td>5</td>
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<tr>
<td>7</td>
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<td>1.8</td>
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<tr>
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<tr>
<td>DMS$^b$</td>
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<td>13.7</td>
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</tbody>
</table>

$^a$ IC$_{50}$ values are the concentrations for 50 % inhibition of the sphingosine phosphorylation by SphK1 or SphK2. The data were derived from the dose-response curves generated from triplicate data points. $^b$ N,N-dimethylsphingosine.

Figure 1. Metabolism of sphingolipids in mammalian cells.
Figure 2. Structures of pachastrissamine stereoisomers.

4S-isomers synthesized from L-serine

\[
\begin{array}{ll}
\text{pachastrissamine (jaspine B, 1)} \\
\text{(2R,3S,4S)-isomer (2) (2S,3R,4S)-isomer (3) (2R,3R,4S)-isomer (4)}
\end{array}
\]

4R-isomers synthesized from D-serine

\[
\begin{array}{ll}
\text{(2R,3R,4R)-isomer (5) (2S,3R,4R)-isomer (6)} \\
\text{(2R,3S,4R)-isomer (7) (2S,3S,4R)-isomer (8)}
\end{array}
\]

Figure 3. Inhibitory activity of pachastrissamine stereoisomers against PKC\(\zeta\) (A) and PKC\(\tau\) (B).

Values are reported as percentages of maximum activity of PKCs from duplicate data points.

Graphical Table of Contents

8 stereoisomers of pachastrissamine