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

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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 defferent 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.[11] Recent investigations revealed that a selective inhibitor of SphK2 attenuates experimental osteoarthritis.[12] Accordingly, SphKs could be promising drug targets for cancer chemotherapy and inflammatory diseases. So far, a number of sphingosine kinase inhibitors have been reported.[13]

Pachastrissamine, a naturally occurring anhydrophytosphingosine derivative, was isolated from the Okinawan marine sponge, *Pachastrissa* sp. (Figure 2).[14] 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.[15] Rao and co-workers also revealed that pachastrissamine enantiomer is less cytotoxic than pachastrissamine.[16] 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.[17] 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 4*S*-configuration.[18] 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 OsO_4.[19] The diol **11** was converted into the corresponding bis-tosylate **12** with TsCl, Et_3N and $Me_3N{\cdot}HCl$. Treatment of **12** with TsOH successfully constructed the expected tetrahydrofuran ring. Cleavage of the tosyl group with Mg in MeOH gave the (2*R*,3*R*,4*R*)-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 $BF_3{\cdot}OEt_2$ afforded **17**. Protection of the carbamate nitrogen of **17** with Boc_2O 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, (2*R*,3*S*,4*R*)-isomer **7** and (2*S*,3*S*,4*R*)-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-*threo*-sphingosines.[23–25] 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.[23–26] Whereas the *cis*-olefin-containing hydrocarbon is indispensable for SphK inhibition by B-5354c,[27] 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,[28, 29] little is known about its isoform selectivity. The individual PKC isoform is involved in different cellular process,[30] 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 PKCι is an oncogenic protein required for the transformed growth and tumorigenesis of human cancer cells.[31] Pachastrissamines **1**, **7** and **8** exerted complete inhibition of PKCζ and PKCι 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 PKCι 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 (PKCα, β1, β2, γ), novel PKCs (PKCδ, ε,

θ, η), and atypical PKCs (PKCζ, ι), whereas the structural features of the regulatory domain vary.[32] 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.[26,33] In contrast, atypical PKCs, including distinct nucleotide binding loop and ATP binding pocket, bind phosphatidylinositol 3,4,5-trisphosphate (PIP_3) 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,[34] PKC-isoform selectivity of pachastrissamines could be derived from the binding to the site in the domain.[35]

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, PKCι) 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 δ (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.[18] 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 $C_{15}H_{31}PPh_3Br$ (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_4Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et_2O. 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; $[\alpha]^{25}_D$ –59.4 (*c* 0.43, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 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-[(1*R*,2*S*)-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 OsO_4 (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 $Na_2S_2O_3$ at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et_2O and washed with brine, 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 (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; $[\alpha]^{25}_{D}$ +8.87 (*c* 1.02, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ0.88 (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 $C_{26}H_{51}NO_5$: 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-[(1*R*,2*S*)-1,2-Bis(tosyloxy)hexadecyl]-2,2-dimethyloxazolidine-3-carboxylate (12). To a stirred solution of **11** (194 mg, 0.424 mmol) in CH_2Cl_2 (850 μL) were added Et_3N (588 μL, 4.24 mmol), TsCl (404 mg, 2.12 mmol) and Me_3N·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 NH_4Cl at 0 °C and the whole was extracted with CH_2Cl_2. The extract was washed with saturated NH_4Cl and brine, 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 (10:1 to 7:1) to give **12** as a colorless oil (285 mg, 88% yield); $[\alpha]^{25}_{D}$ +23.9 (*c* 0.68, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{40}H_{64}NO_9S_2$ (MH^+) 766.4017, found 766.4015.

4.1.5. (2*R*,3*R*,4*R*)-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 $TsOH·H_2O$ (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 $CHCl_3$–MeOH–28% NH_4OH (95:4:1) to give **13** as a white solid (140 mg, 90% yield)**;** mp 65–66 °C; $[\alpha]^{25}_D$ –18.6 (*c* 0.28, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{25}H_{44}NO_4S$ (MH^+) 454.2986, found 454.2993.

4.1.6. (2*R*,3*R*,4*R*)-4-Amino-2-tetradecyltetrahydrofuran-3-ol (5). To a stirred mixture of **13** (23 mg, 0.0551mmol) 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 $CHCl_3$–MeOH–28% NH_4OH (95:4:1) to give **5** as a white solid (13 mg, 79% yield); mp 95–96 °C; $[\alpha]^{25}_D$ –9.61 (*c* 0.61, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{18}H_{37}NO_2$: C, 72.19; H, 12.45; N, 4.68. Found: C, 72.10; H, 12.63; N, 4.65.

4.1.7. *tert*-Butyl [(2*R*,3*R*,4*S*)-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 $TsOH·H_2O$ (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 Et_3N 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; $[\alpha]^{25}_{D}$ –6.61 (*c* 1.61, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{23}H_{48}NO_5$ (MH^+) 418.3527, found 418.3533.

4.1.8. *tert*-Butyl [(3*R*,4*R*,5*S*)-4-Hydroxy-5-tetradecyltetrahydrofuran-3-yl]carbamate (15). To a stirred mixture of **14** (90 mg, 0.216 mmol) and Et_3N (108 μL, 0.778 mmol) in CH_2Cl_2 (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 NH_4Cl at 0 °C, and the whole was extracted with CH_2Cl_2. The extract was washed with brine, 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 (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; $[\alpha]^{25}_{D}$ –7.76 (*c* 0.29, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{23}H_{46}NO_4$ (MH^+) 400.3421, found 400.3413.

4.1.9. (2*S*,3*R*,4*R*)-4-Amino-2-tetradecyltetrahydrofuran-3-ol (6). To a stirred solution of **15** (45 mg, 0.113 mmol) in CH_2Cl_2 (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 $CHCl_3$–MeOH–28% NH_4OH (95:4:1) followed by recrystallization from *n*-hexane–EtOAc to give **6** as colorless crystals (31 mg, 92% yield); mp 104–105 °C; $[\alpha]^{25}_{D}$ –8.78 (*c* 0.75, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{18}H_{37}NO_2$: C, 71.12; H, 12.43; N, 4.61. Found: C, 71.39; H, 12.39; N, 4.59.

4.1.10. *tert*-Butyl [(2*R*,3*R*,4*S*)-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 TIPSCl (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 CH_2Cl_2, washed with saturated NH_4Cl and brine, 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 (8:1) to give **16** as a colorless oil (2.15 g, 90% yield); $[\alpha]^{25}_D$ –23.5 (*c* 0.25, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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), 3.89 (m, 1H), 4.05 (m, 1H), 5.24 (d, *J* = 8.0 Hz, 1H); HRMS (FAB) calcd for $C_{32}H_{68}NO_5Si$ (MH^+) 574.4861, found 574.4855.

4.1.11. (*S*)-1-{(4*R*,5*S*)-2-Oxo-4-[(triisopropylsilyloxy)methyl]oxazolidin-5-yl}pentadecyl Acetate (17). To a stirred solution of **16** (1.56 g, 2.72 mmol) in CH_2Cl_2 (270 mL) were added $MeC(OMe)_3$ (2.0 mL, 16.3 mmol) and $BF_3{\cdot}OEt_2$ (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); $[\alpha]^{25}_D$ +25.5 (*c* 0.66, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{30}H_{60}NO_5Si$ (MH^+) 542.4235, found 542.4240.

4.1.12. *tert*-Butyl [(2*R*,3*S*,4*S*)-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 Et_3N (194 μL, 1.40 mmol), Boc_2O (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 NH_4Cl at 0 °C. The mixture was concentrated under reduced pressure, and the residue was extracted with Et_2O. The extract was washed with brine, and dried over $MgSO_4$. 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 NH_4Cl, and concentrated under reduced pressure. The residue was extracted with EtOAc, washed with brine, 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 (4:1) to give **18** as a colorless oil (562 mg, 70% yield); $[\alpha]^{25}_{D}$ –20.6 (*c* 3.21, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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 $C_{32}H_{68}NO_5Si$ (MH^+) 574.4861, found 574.4858.

4.1.13. (2*R*,3*S*,4*S*)-2-[(*tert*-Butoxycarbonyl)amino]-1-(triisopropylsilyloxy)octadecane-3,4-diyl Bis(4-methylbenzenesulfonate) (19). To a stirred solution of **18** (255 mg, 0.444 mmol) in CH_2Cl_2 (1.0 mL) were added Et_3N (613 μL, 4.44 mmol), TsCl (423 mg, 2.22 mmol), and Me_3N·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 NH_4Cl at 0 °C, and the whole was extracted with CH_2Cl_2, 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 (10:1) to give **19** as a colorless oil (373 mg, 95% yield); $[\alpha]^{25}_{D}$ –18.8 (*c* 0.95, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) 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), 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 $C_{46}H_{79}NNaO_9S_2Si$ (MNa^+) 904.4863, found 904.4863.

4.1.14. (2*R*,3*S*,4*R*)-4-[(*tert*-Butoxycarbonyl)amino]-2-tetradecyltetrahydrofuran-3-yl 4-Methylbenzenesulfonate (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_4Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with Et_2O, 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); $[\alpha]^{25}_D$ +1.48 (*c* 0.056, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 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 $C_{30}H_{50}NO_6S$ ($[M–H]^-$) 552.3364, found 552.3370.

4.1.15. (2*R*,3*S*,4*R*)-4-Amino-2-tetradecyltetrahydrofuran-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 diluted with EtOAc, washed with H_2O, and dried over $MgSO_4$. The filtrate was concentrated under reduced pressure to give a white solid, which was dissolved in CH_2Cl_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_4OH (95:4:1) to give **7** as a white solid (23 mg, 88% yield); $[\alpha]^{25}_D$ +1.48 (*c* 0.056, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 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 [(2*R*,3*S*,4*S*)-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 NH_4Cl at 0 °C, and concentrated under reduced pressure. The residue was extracted with EtOAc, 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 (1:1 to 1:2) to give **21** as a white solid (59 mg, 93% yield); mp 68–70 °C; $[\alpha]^{25}_D$ –4.33 (*c* 1.14, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J = 6.9 Hz, 3H), 1.20–1.39 (m, 26H), 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 [(3*R*,4*S*,5*S*)-4-Hydroxy-5-tetradecyltetrahydrofuran-3-yl]carbamate (22). To a stirred mixture of **21** (42 mg, 0.100 mmol) and Et_3N (111 μL, 0.800 mmol) in CH_2Cl_2 (3.3 mL) were added TsCl (76 mg, 0.400 mmol) and Me_3N·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 Et_2O, washed with saturated NH_4Cl and brine, and dried over $MgSO_4$. The filtrate was concentrated under reduced pressure to give an oily residue, which was dissolved in MeOH (3.3 mL). K_2CO_3 (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 NH_4Cl, and concentrated under reduced pressure. The residue was extracted with EtOAc, washed with brine, 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 (5:1 to 3:1) to give **22** as a white solid (21 mg, 53% yield); mp 105–107 °C; $[\alpha]^{25}_D$ +26.3 (*c* 0.13, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 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. (2*S*,3*S*,4*R*)-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; $[\alpha]^{25}_D$ +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 $C_{18}H_{37}NO_2 \cdot 0.25H_2O$: 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), PKCε(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 PKCθ 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 $MgCl_2$, and 50 μM $CaCl_2$ (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.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.061.

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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

	IC_{50} [a]	
compound	SphK1	SphK2
1 (pachastrissamine)	4.6	6.6
2	3.9	15.8
3	2.1	6.1
4	3.0	2.2
5	2.7	10.5
6	2.1	6.2
7	0.59	1.8
8	0.94	0.48
DMS[b]	2.8	13.7

[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.

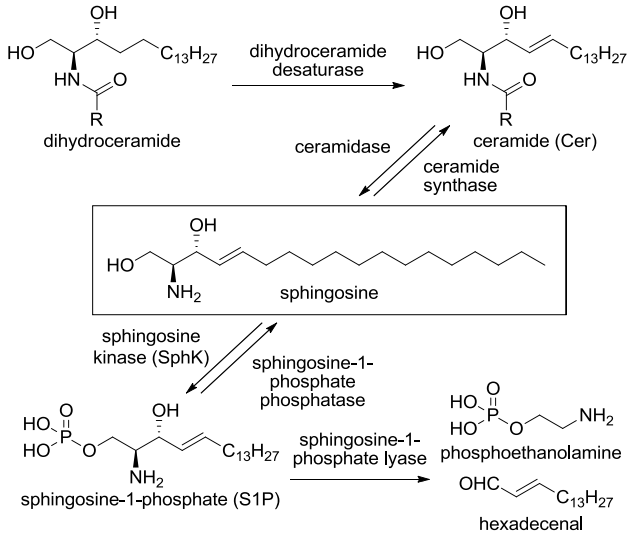


Figure 2. Structures of pachastrissamine stereoisomers.

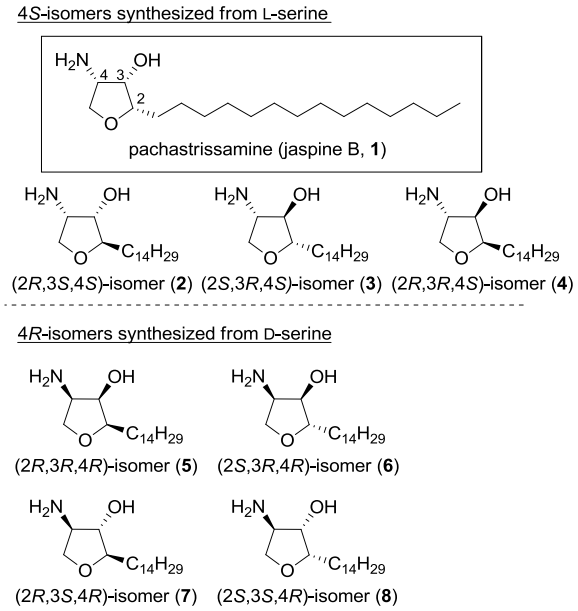
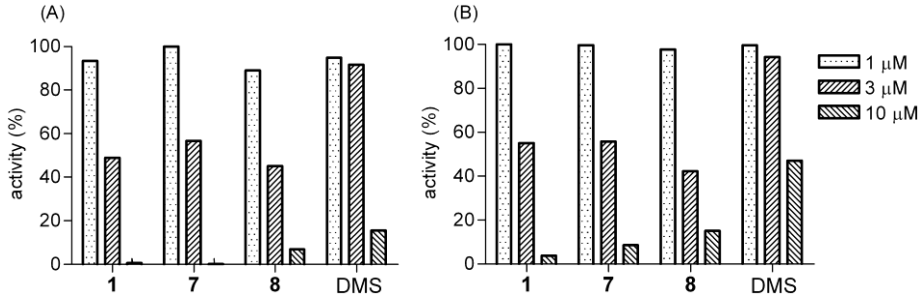
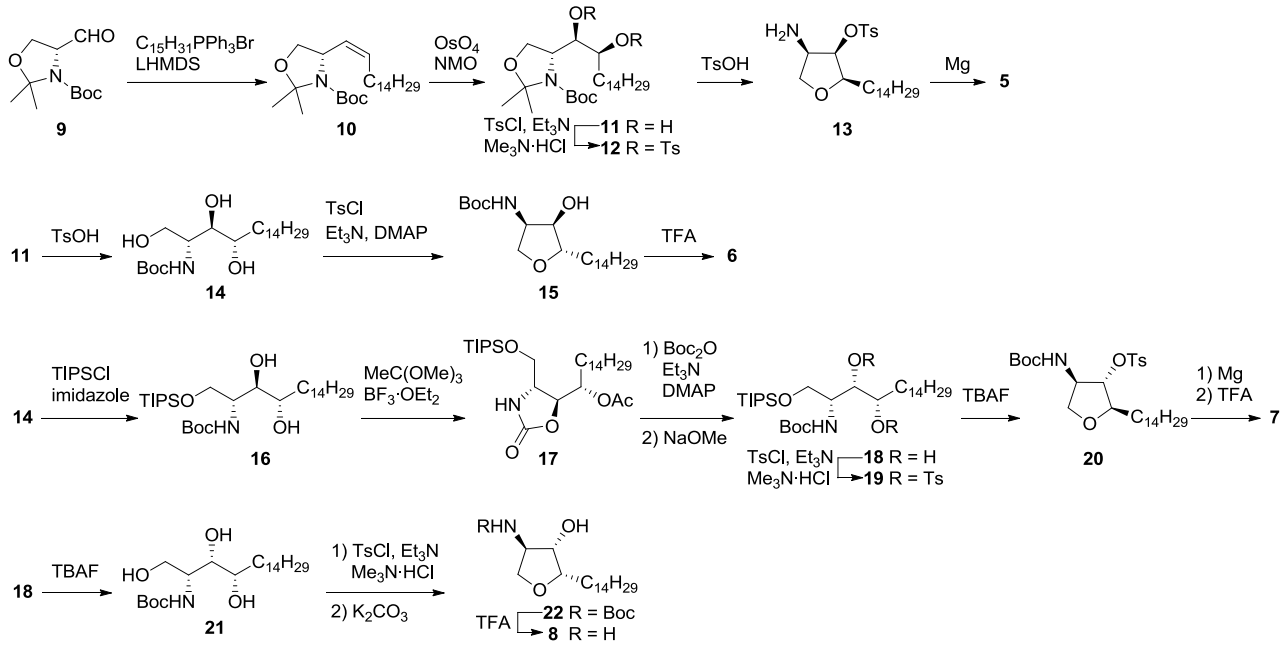


Figure 3. Inhibitory activity of pachastrissamine stereoisomers against PKCζ (A) and PKCι (B). Values are reported as percentages of maximum activity of PKCs from duplicate data points.



Scheme 1. Stereoselective synthesis of pachastrissamine stereoisomers **5**–**8**.



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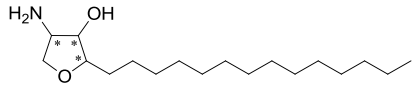

8 stereoisomers of pachastrissamine