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Pharmacological evaluation of a novel cyclic phosphatidic acid derivative 3-*S*-cyclic phosphatidic acid (3-*S*-cPA)

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ABSTRACT

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator possessing cyclic phosphate ring, which is necessary for its specific biological activities. To stabilize cyclic phosphate ring of cPA, we synthesized a series of cPA derivatives. We have shown that racemic 3-*S*-cPA, with a phosphate oxygen atom replaced with a sulfur atom at the *sn*-3, was a more effective autotaxin (ATX) inhibitor than cPA. In this study, we showed that racemic 3-*S*-cPA also had potent biological activities such as inhibition of cancer cell migration, suppression of the nociceptive reflex, and attenuation of ischemia-induced delayed neuronal cell death in the hippocampal CA1. Moreover, we synthesized both enantiomers of palmitoleoyl derivative of 3-*S*-cPA, and found that the chirality of 3-*S*-cPA is not involved in ATX inhibition. Based on these findings, racemic 3-*S*-cPA is suggested as an effective therapeutic compound like cPA.

1. Introduction

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator, which was originally isolated from the myxoamoebae of a true slime mold, *Physarum polycephalum*, in 1992.[1] At present, cPA has been found in a wide range of organisms, from slime molds to humans.[2,3] cPA has potent biological activities, such as inhibition of autotaxin (ATX),[4,5] invasion and metastasis of cancer cells,[4,5] suppression of nociceptive responses by primary afferent C-fiber,[6] and attenuation of ischemia-induced delayed neuronal cell death in rat hippocampal CA1 regions.[7] Therefore, cPA is considered to be a promising candidate for developing an effective therapeutic agent for some diseases, including cancer and neurodegeneration.

cPA has a unique structure, composed of a cyclic phosphate ring at the *sn*-2 and the *sn*-3 positions of the glycerol backbone, which is required for some biological activities.[8] 2-*O*-carba-cPA (2ccPA) and 3-*O*-carba-cPA (3ccPA) are derivatives of cPA in which one of the phosphate oxygen atoms, either at the *sn*-2 or the *sn*-3 position, is replaced with a methylene group in order to protect the cyclic phosphate ester moiety from hydrolysis. These carba-cPAs, as well as cPA, are potent inhibitors of ATX, cancer cell invasion and metastasis, and transient ischemia-caused neuronal damage in the hippocampal CA1 region.[7,9,10] In addition, 2ccPA also suppresses the nociceptive reflex similarly to cPA.[6] Some studies have shown that replacing a phosphate oxygen atom with a sulfur atom results in increased stability to nuclease-mediated and base-catalyzed hydrolysis in nucleic acids.[11] Therefore, we have prepared the sulfur analogs of cPA, that is, racemic 3-*S*-cPA **1a–d** with fatty acid moieties of 16:1, 18:0, 18:1 or 16:0 (Fig. 1), and investigated their action toward ATX.[12] Our results showed that they have a similar inhibitory effect on ATX as naturally occurring cPA, and we presume that numerous biological functions are conserved between 3-*S*-cPA and cPA.

In this study, we examined the effects of racemic 3-*S*-cPA on the following biological activities, (1) inhibition of cancer cell migration, (2) suppression of the nociceptive reflex, and (3) attenuation of ischemia-induced delayed neuronal cell death. Subsequently, to determine the biological activities of enantiopure 3-*S*-cPA, we synthesized both enantiomers of palmitoleoyl derivative of 3-*S*-cPA **1a** and studied their effects on ATX activity.

2. Chemistry

We have already synthesized racemic 3-S-cPA **1a–d**.[12] According to the synthetic method of 3-*S*-cPA which has been previously

Abbreviation: cPA, cyclic phosphatidic acid; LPA, lysophosphatidic acid; ATX, autotaxin; 2ccPA, 2-*O*-carba-cyclic phosphatidic acid; 3ccPA, 3-*O*-carba-cyclic phosphatidic acid.

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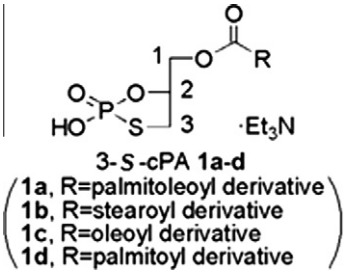


Figure 1. Structure of 3-*S*-cPA **1a–d**.

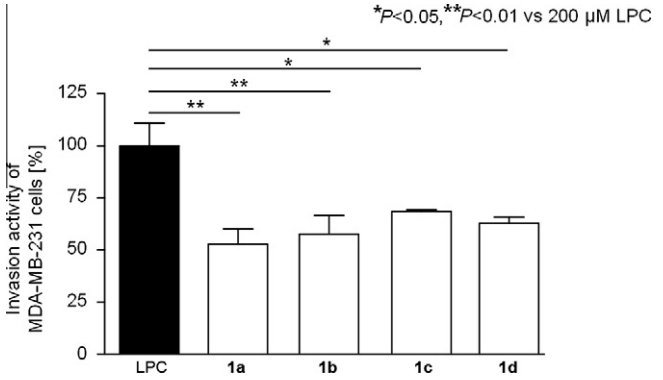


Figure 2. Effect of racemic 3-*S*-cPA on MDA-MB-231 cell migration: Maximal migration activity in the presence of LPC is represented by 100%. Each column indicates the mean ± S.E. of triplicate wells and is representative of at least three independent experiments with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from LPC as determined by one-way ANOVA and Dunnett's test.

reported by us, both enantiomers of palmitoleoyl derivative of 3-*S*-cPA **1a** were synthesized starting from enantiopure glycidol **2** (Scheme 1). First, commercially available (*R*)-(+)-glycidol **2** was esterified with palmitoleic acid using WSC and DMAP. Then, epoxide **3a** was cleaved with HSCN in acetic acid, and the resulting alcohol **4a** was reacted with salicylchlorophosphite,[13] followed by hydrolysis with TEAB buffer to give *H*-phosphonate **5a**. Finally, the treatment of **5a** with trimethylsilyl chloride formed bistrimethylsilyl phosphite **6a** in situ, which underwent a simultaneous intramolecular Arbuzov reaction to give cyclic thiophosphate **7a**. After the hydrolysis of **7a** in acetonitrile, (*R*)-(+)-3-*S*-cPA **1a** was obtained. In the same manner, (*S*)-(−)-3-*S*-cPA **1a** was synthesized from (*S*)-(−)-glycidol **2**.

3. Results and discussion

3.1. Effect of racemic 3-*S*-cPA on MDA-MB-231 cell migration

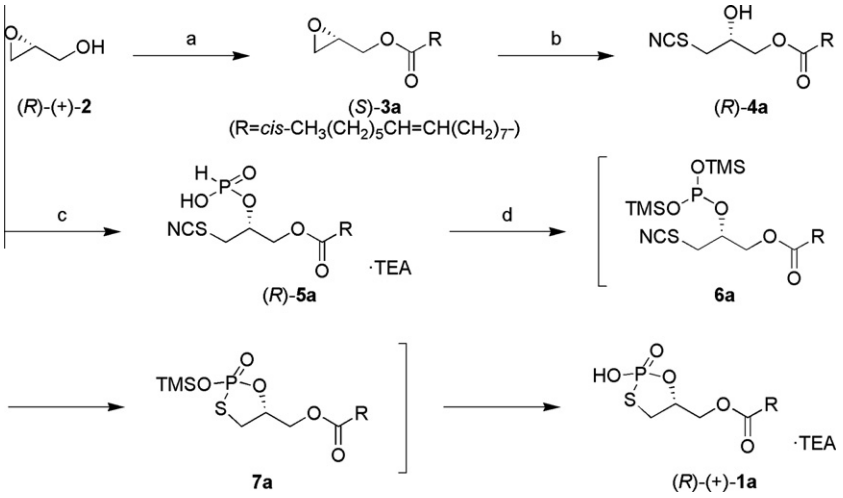
ATX is a cell motility-stimulating factor that synthesizes lysophosphatidic acid (LPA) by hydrolysis lysophosphatidylcholine (LPC).[14–16] We have previously shown previously that cPA and its derivatives suppress synthesis of LPA via inhibition of ATX activity, and as a result, also inhibit cancer cell invasion and metastasis.[9,10,17,18] As shown in Figure 2, cell migration was inhibited by racemic 3-*S*-cPAs with different fatty acids (**1a**, **b**, **c** and **d**), to the same extent (>40%). These results are consistent with those previously published demonstrating that the nature of the acyl chain of racemic 3-*S*-cPA dose not significantly influences the inhibition of the ATX activity.[12]

3.2. Effect of racemic 3-*S*-cPA on somato-somatic C-reflex

We have already reported that cPA and 2ccPA suppressed the nociceptive reflex via C-fibers.[6,17] In this study, we examined whether racemic 3-*S*-cPA **1a** suppresses the nociceptive reflex induced by the activation of C-fibers. Single shock stimulation of the myelinated A- and unmyelinated C-afferent fibers of the saphenous nerve (at 10 V with 0.5 ms pulse duration) produced two distinct A- and C-somatic reflex components in the hind limb electromyogram (EMG), that is, a short latency (about 10 ms) A-reflex and a long latency (about 40 ms) C-reflex. Intravenous injection of 0.2 mg/kg racemic 3-*S*-cPA **1a** depressed the C-reflex, but not A-reflex. After injection of racemic 3-*S*-cPA **1a**, the C-reflex reached 87 ± 5% of the control (Fig. 3). The effective dose of racemic 3-*S*-cPA is comparable to that of racemic 2ccPA (0.2 mg/kg, iv; 83 ± 2.5% of the control); however, the effective dose of racemic 2ccPA was almost 10-fold less than that of natural cPA.[6] Therefore racemic 3-*S*-cPA and 2ccPA are more potent inhibitors of the C-reflex than the naturally occurring cPA.

3.3. Effect of racemic 3-*S*-cPA on delayed neuronal cell death

Recently, we have reported the effects of cPA and 2ccPA on ischemia-induced delayed neuronal cell death in the rat hippocampal



Scheme 1. Reagents and conditions: (a) Palmitoleic acid, WSC, DMAP, CH_2Cl_2, rt; (b) HSCN, AcOH, 72% in two steps; (c) salicylchlorophosphite, pyridine, rt, then TEAB buffer, 62%; (d) TMSCl, Et_3N, pyridine, rt, then H_2O, MeCN, 81%.

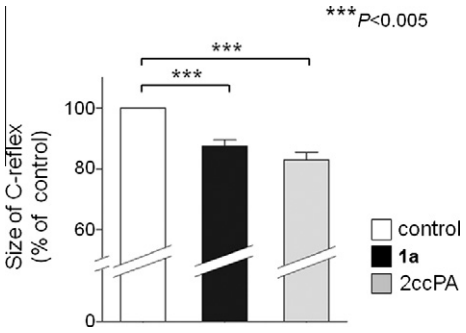


Figure 3. Effect of racemic 3-*S*-cPA on somato-somatic C-reflex: The mean size of the C-reflex 0–15 min before each injection is expressed as 100%. All subsequent reflexes are expressed as percentages of the control values. Each column represents the mean ± S.E. $^{***}P < 0.005$, significantly different from control as determined by paired *t*-test.

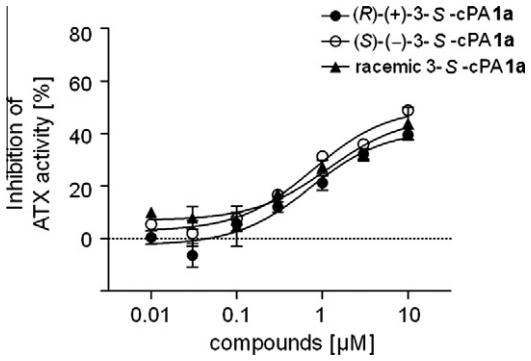


Figure 5. Effect of chirality of 3-*S*-cPA on ATX activity: The catalytic activity of ATX in FBS was examined in the presence of (*R*)-(+)-3-*S*-cPA **1a** (closed circles), (*S*)-(−)-3-*S*-cPA **1a** (open circles), and racemic 3-*S*-cPA **1a** (closed triangles). Each data was calculated as percent inhibition, by comparison with the respective controls without any compounds, denoted as 100%. Each data point represents the mean ± S.E. of the triplicate wells and is representative of at least three independent experiments.

CA1 region.[7] cPA and 2ccPA significantly attenuates neuronal damage and decreased glial fibrillary acidic protein (GFAP) accumulation. In the present study, we examined whether racemic 3-*S*-cPA **1a** attenuates neuronal damage in the hippocampal CA1 region and decreased GFAP accumulation. Figure 4 shows representative histological pictures of the rat hippocampal CA1 region from coronal sections taken 5 days after transient occlusion for 8 min. Rats were treated with either 0.2% fatty acid free bovine serum albumin (BSA) vehicle (Fig. 4Aa–c) or 180 $\mu g \cdot kg^{-1} \cdot 5\ days^{-1}$ of racemic 3-*S*-cPA **1a** (Fig. 4Ba–c). The majority of cells in the CA1 region exposed to the vehicle were histologically damaged, that is, they showed pyknosis and shrinkage of cell bodies (Fig. 4Ab). Comparatively, the racemic 3-*S*-cPA **1a** treatment induced attenuated neuronal damage in the hippocampal CA1 region following transient occlusion (Fig. 4Bb). Transient ischemia induced an increase in anti-GFAP antibody immunoreactivity as reported previously.[19] Treatment with racemic 3-*S*-cPA **1a** decreased anti-GFAP antibody immunoreactivity compared to the vehicle-treated rats (Fig. 4Ac, Bc). These results demonstrate that 3-*S*-cPA, as well as cPA and 2ccPA, has a potent neuroprotective effect.

Subcutaneous administration of racemic 3-*S*-cPA **1a** showed neuroprotection, as well as cPA and 2ccPA. We suggest the low molecular weight (507.71 Da) and high lipophilicity (Log*P* = 5.96) of racemic 3-*S*-cPA **1a** may allow it to penetrate the blood–brain barrier BBB, and our results demonstrate that 3-*S*-cPA, in addition to cPA and 2ccPA, has potential therapeutic advantages.

3.4. Effect of chirality of 3-*S*-cPA on ATX activity

We synthesized both enantiomers of palmitoleoyl derivative of 3-*S*-cPA **1a** and studied their effects on ATX activity.[12] ATX activity

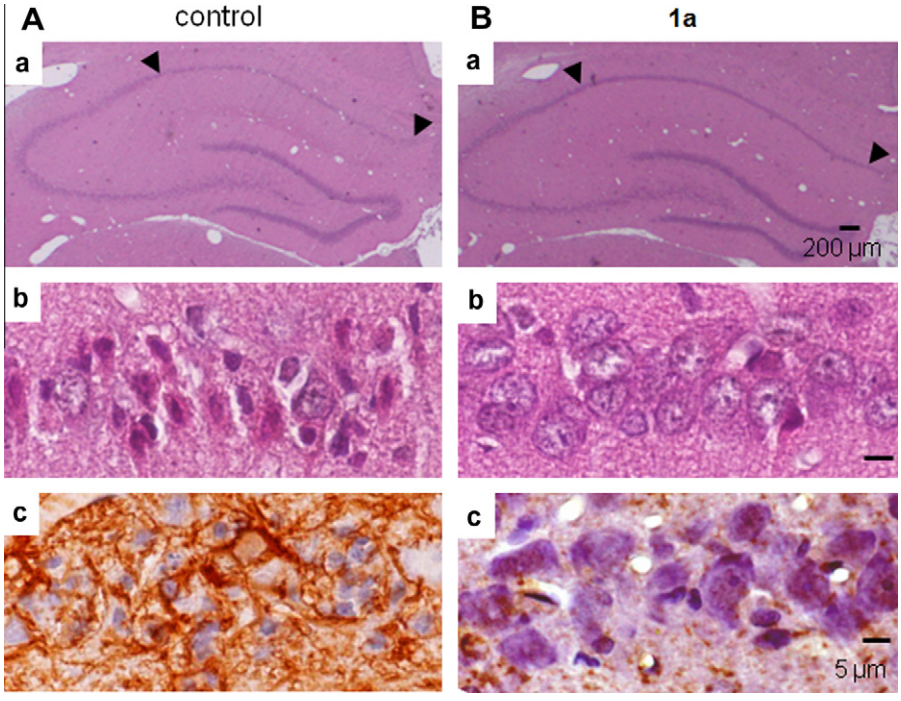


Figure 4. Effect of racemic 3-*S*-cPA on delayed neuronal death: Representative histological pictures of neurons and astrocytes in the hippocampal CA1 region of the rats treated with vehicle (0.2% BSA, (A) and racemic 3-*S*-cPA **1a** (180 $\mu g \cdot kg^{-1} \cdot 5\ days^{-1}$; (B) samples were acquired by coronal section on the 5th day after 8 min occlusion, and stained with HE (a, b) and anti-GFAP antibody (c). Arrowheads show the medial and lateral borders of the CA1 region.

was measured using the FS-3 substrate, which fluoresces upon cleavage by ATX. The catalytic activity of ATX in fetal bovine serum (FBS) was significantly inhibited by (*R*)-(+)-3-*S*-cPA **1a**, (*S*)-(−)-3-*S*-cPA **1a** and racemic 3-*S*-cPA **1a** (Fig. 5). The ~40% inhibitory effect of (*R*)-(+)-3-*S*-cPA **1a** on ATX occurred at 10 μM. The dose-response relationship of ATX inhibition showed no significant difference between (*R*)-(+)-3-*S*-cPA **1a** and (*S*)-(−)-3-*S*-cPA **1a**, indicating that the chirality of 3-*S*-cPA **1a** did not affect the degree of ATX inhibition. This result was consistent with the previous report that no stereoselective differences were observed between the enantiopure 2ccPA and 3ccPA with respect to inhibition of ATX.[17,18,20]

4. Conclusion

In this study, we clarified the effect of 3-*S*-cPA on the following biological functions, which are known to be specific biological activities of cPA: (1) inhibition of cancer cell migration, (2) suppression of the nociceptive reflex, and (3) attenuation of ischemia-induced delayed neuronal cell death. We showed that both enantiomers of 3-*S*-cPA inhibit ATX as efficiently as cPA and 2ccPA. Based on these findings, racemic 3-*S*-cPA is suggested to be an effective therapeutic compound for disorders such as cancer and neurodegeneration.

5. Experimental

5.1. Chemistry

All non-aqueous reactions were performed under an atmosphere of dry argon in oven-dried glassware. Triethylamine (TEA) and pyridine were distilled from calcium hydride. Other reagents were used without further purification. Flash column chromatography was performed with PSQ 100B (Fuji Silysia Co., Ltd, Japan). Reversed-phase chromatography was performed with Cosmosil 140C_{18}-PREP (Nacalai Tesque, Inc, Japan). Solvents for chromatographies are listed as volume/volume ratios. Analytical thin layer chromatography was performed using commercial silica gel plates (E. Merck, Silica Gel 60 F_{254}). Infrared spectra (FT-IR) were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer. Absorbance frequencies are recorded in reciprocal centimeters (cm^{-1}). High resolution mass spectra (HRMS) were obtained from Applied Biosystems mass spectrometer (API QSTAR pulsar i) for electrospray ionization (ESI). Melting points were recorded on Yanaco MP-3S. ^{1}H NMR spectra were acquired at 400 MHz on a JEOL JNM-LD400 spectrometer. Solvent for NMR is used chloroform-*d*. Chemical shifts are reported in delta (δ) units in parts per million (ppm) relative to the singlet (7.26 ppm) for chloroform-*d*. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; sept, septet; m, multiplet and br, broad. Coupling constants are recorded in Hertz (Hz). ^{13}C NMR spectra were acquired at 100 MHz on a JEOL JNM-LD400 spectrometer. Chemical shifts are reported in ppm relative to the central line of the triplet at 77.0 ppm for chloroform-*d*.

5.1.1. Thiocyanate (*R*)-4a

To a solution of glycidol (*R*)-(+)-2 (534 mg, 7.21 mmol) and palmitoleic acid (1.83 g, 7.21 mmol) in CH_2Cl_2 (24 mL) was added WSC (1.38 g, 7.21 mmol) and DMAP (88 mg, 0.72 mmol) at room temperature, then the resulting mixture was stirred at that temperature for 5 h. The reaction mixture was quenched with aqueous citric acid solution. The aqueous layer was extracted with $CHCl_3$ and combined organic layer was washed with a saturated aqueous solution of $NaHCO_3$ and brine, dried over $MgSO_4$, filtrated and concentrated under reduced pressure. The crude epoxide (*S*)-**3a** was used in the next reaction without further purification.

To a solution of crude epoxide (*S*)-**3a** in AcOH (18 mL) was added HSCN (1 M in 70% AcOH aq, 18 mL) at room temperature, then the resulting mixture was stirred at that temperature for 20 min. The reaction mixture was quenched with a saturated aqueous solution of $NaHCO_3$. The aqueous layer was extracted with EtOAc. Combined organic layer was washed with brine, dried over $MgSO_4$, filtrated and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (Hexane/EtOAc = 5/1) to afford the thiocyanate (*R*)-**4a** (1.92 g, 72%, two steps). Colorless oil: TLC, R_f = 0.50 (Hexane/EtOAc = 2/1); IR (neat) 3463, 3017, 2926, 2855, 2158, 1733, 1458, 1415, 1379, 1215, 1167, 1111, 1016 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39–5.30 (m, 2H), 4.30–4.16 (m, 3H), 3.17 (dd, *J* = 13.5, 4.3, 1H), 3.04 (dd, *J* = 13.5, 7.1, 1H), 2.37 (t, *J* = 7.6, 2H), 2.05–1.98 (m, 4H), 1.68–1.59 (m, 2H), 1.37–1.25 (m, 16H), 0.88 (t, *J* = 6.8, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 130.01, 129.66, 112.0, 68.6, 65.8, 37.2, 34.0, 31.7, 29.69, 29.64, 29.09, 29.04, 28.95, 27.19, 27.11, 24.8, 22.6, 14.1; $[\alpha]_D$ +12.3 (*c* 1.00, $CHCl_3$); HRMS (ESI) Calcd for $C_{20}H_{35}NO_3NaS$ $[M+Na]^+$ 392.2235, Found 392.2324.

5.1.2. *H*-phosphonate (*R*)-5a

To a solution of thiocyanate (*R*)-**4a** (425 mg, 1.15 mmol) in pyridine (11.5 mL) was added salicylchlorophophite (233 mg, 1.15 mmol) at room temperature, then the resulting mixture was stirred at that temperature for 2.5 h. The reaction mixture was quenched with triethylammonium bicarbonate buffer and concentrated under reduced pressure. The residue was purified by reversed-phase chromatography (H_2O/MeOH = 1/2) to afford the *H*-phosphonate (*R*)-**5a** (381 mg, 62%). Colorless oil: TLC, R_f = 0.46 ($CHCl_3$/MeOH/H_2O = 60/20/3); IR (neat) 2925, 2854, 2615, 2349, 2155, 1737, 1456, 1391, 1359, 1218, 1167, 1117, 1056 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.2 (br s, 1H), 6.96 (d, *J* = 644, 1H), 5.39–5.30 (m, 2H), 4.74–4.66 (m, 1H), 4.32 (dd, *J* = 11.7, 5.1, 1H), 4.27 (dd, *J* = 11.7, 5.8, 1H), 3.37 (dd, *J* = 13.6, 4.5, 1H), 3.22 (dd, *J* = 13.6, 6.3, 1H), 3.08 (qd, *J* = 7.4, 4.4, 6H), 2.33 (t, *J* = 7.7, 2H), 2.04–1.98 (m, 4H), 1.65–1.57 (m, 2H), 1.34 (t, *J* = 7.4, 9H) 1.42–1.21 (m, 16H), 0.88 (t, *J* = 7.0, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 129.88, 129.62, 112.2, 69.8, 64.1, 45.6, 36.7, 33.92, 31.65, 29.60, 29.57, 29.04, 28.99, 28.85, 27.09, 27.05, 24.7, 22.5, 14.0, 8.5; $[\alpha]_D$ +13.9 (*c* 1.00, $CHCl_3$); LRMS (ESI) Calcd for $C_{20}H_{35}NO_5PS$ $[M\text{-}Et_3NH]^-$ 432.1974, Found 432.2409.

5.1.3. 3-*S*-cPA (*R*)-(+)-1a

To a solution of *H*-phosphonate (*R*)-**5a** (62.0 mg, 0.116 mmol) in pyridine (1.2 mL) and TEA (0.58 mmol) was added TMSCl (63 mg, 0.58 mmol) at room temperature, then the resulting mixture was stirred at that temperature for 4 h. The reaction mixture was concentrated under reduced pressure. To the obtained residue was added MeCN/H_2O (v/v = 99/1). The reaction mixture was concentrated under reduced pressure. The residue was purified by silica-gel column chromatography ($CHCl_3$/MeOH = 30/1, containing trace amount of TEA) to afford the triethylammonium salt of 3-*S*-cPA (*R*)-(+)-**1a** (47.7 mg, 81%). Colorless oil: TLC, R_f = 0.46 ($CHCl_3$/MeOH/H_2O = 60/20/3); IR (neat) 3409, 2978, 2925, 2854, 2602, 2496, 1738, 1475, 1444, 1397, 1383, 1364, 1171, 1071, 1035 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.1 (br s, 1H), 5.39–5.30 (m, 2H), 4.55–4.47 (m, 1H), 4.29 (dd, *J* = 11.5, 5.9, 1H), 4.23 (dd, *J* = 11.5, 5.6, 1H), 3.35–3.24 (m, 2H), 3.10 (qd, *J* = 7.2, 4.6, 6H), 2.32 (t, *J* = 7.7, 2H), 2.05–1.95 (m, 4H), 1.65–1.57 (m, 2H), 1.35 (t, *J* = 7.2, 9H), 1.39–1.25 (m, 16H), 0.88 (t, *J* = 6.8, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4, 130.01, 129.76, 74.5, 64.5, 45.8, 36.5, 34.1, 31.8, 29.74, 29.71, 29.17, 29.12, 29.11, 28.98, 27.23, 27.17, 24.8, 22.7, 14.1, 8.6; $[\alpha]_D$ +3.76 (*c* 0.14, $CHCl_3$); LRMS (ESI) Calcd for $C_{19}H_{34}O_5PS$ $[M\text{-}Et_3NH]^-$ 405.1865, Found 405.2510.

5.1.4. 3-*S*-cPA (*S*)-(−)-1a

3-*S*-cPA (*S*)-(−)-**1a** was prepared from glycidol (*S*)-(−)-**2** following the same procedures for 3-*S*-cPA (*R*)-(+)-**1a**. $[\alpha]_D$ −4.98 (*c* 1.00, $CHCl_3$).

5.2. Biological assays

5.2.1. Migration assay

The migration assay was performed as reported previously with some modifications.[17] Migration of the cells across the 8-μm pore-sized membrane was assessed using Chemo Tx System (Neuro Probe) according to the manufacturer's protocol. Briefly, human breast cancer MDA-MB-231 cells were stained with calcein-AM (Dojindo, Kumamoto, Japan), and the cell suspension was centrifuged and washed with phosphate-buffered saline (PBS). After washing, the cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) containing 0.1% fatty acid free bovine serum albumin (BSA; Sigma–Aldrich, MO) (4×10^5 cells/mL). To the lower well, 200 μM lysophosphatidylcholine (LPC) and 10 μM racemic 3-*S*-cPA (**1a–d**) in DMEM containing 0.1% BSA were added. In addition, 25 μL of cell suspension was added to each upper filter well. Plates were incubated at 37 °C for 8 h, and the medium was carefully removed from the upper filter. Then, the upper filter was carefully washed with PBS containing 2 mM ethylendiaminetetraacetic acid (EDTA), and gently wiped with a cotton swab to remove the non-invaded cells. The filter was then separated from the microplate, and the fluorescence of the migrated cells on each lower well was measured (emission (em)/excitation (ex): 485/530 nm) with a Cyto Fluor series 4000 micro-plate reader (Applied Biosystems, Foster City, CA). Data were calculated as percent inhibition in comparison to the respective controls without any compounds, and are shown as the mean ± S.E. of triplicate wells.

5.2.2. ATX inhibition assay

The autotaxin (ATX) inhibition assay was performed according to a previously described method[17] with some modifications. ATX inhibition activities of (*R*)-(+)-3-*S*-cPA **1a**, (*S*)-(−)-3-*S*-cPA **1a** and racemic 3-*S*-cPA **1a** were measured using the FS-3 substrate (Echelon Biosciences, Inc., UT), which fluoresces upon cleavage by ATX. Fetal bovine serum (FBS; Biowest, FL) was used as the ATX source and was mixed with various concentrations of compounds in 0.1% BSA and 1 μM FS-3 in the assay buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 140 mM NaCl, 50 mM tris (hydroxymethyl) aminomethane (Tris)–HCl, pH 8.0). The fluorescence intensity (em/ex: 485/530 nm) of each well was measured by a Cyto Flour series 4000 micro plate reader prior to and after 2 h incubation at 37 °C. Data were normalized to the vehicle control after subtraction the before-incubation value and expressed as percent inhibition. All data were shown as mean ± S.E. of triplicate wells.

5.2.3. Animals

The experiments were performed with adult male Wistar rats (body weight, 310–370 g). The rats were provided by the animal breeding farm at the Tokyo Metropolitan Institute of Gerontology. This study was approved by the Animal Committee of the Institution.

5.2.4. Recording of spinal somato-somatic reflex

The recording and induction of spinal somato-somatic reflexes was completed according to previously described procedures[17] with some modifications. The animals were anesthetized by pentobarbital (somunopentyl, 50 mg/kg, intraperitoneal (ip); Kyoritsu Seiyaku Corporation, Tokyo, Japan). A juglar vein was catheterized for intravenous (iv) administration of racemic 3-*S*-cPA **1a** (0.2 mg/kg) or 2ccPA (0.2 mg/kg). A juglar artery was catheterized to record the arterial blood pressure and heart rate (Rekuchicoda WT-685 G, Nihon Kohden, Tokyo, Japan). The ventilation was monitored with a gas analyzer (Respirator SN-480-7, Sinano seisakuzyo, Tokyo, Japan) and adjusted to maintain an end-tidal CO_2 level at ∼3.0%. Body temperature was maintained at ∼37.5 °C by using an automatically regulated heating pad and lamp (Animal blanket controller ATB-1100, Nihon Kohden, Tokyo, Japan). The spinal cord was completely transected at the upper thoracic level.

With the rat in a supine position, a branch of the right saphenous nerve innervating the thigh skin was cut at the thigh level and covered with warm paraffin oil. The central cut end of the segment of the nerve was placed on bipolar platinum iridium wire electrodes for electrical stimulation. Single square pulse stimuli of 10 V with 0.5 ms duration were delivered every 3 s by a digital electrical stimulator (Nihon Kohden, Tokyo, Japan). Electromyogram (EMG) was recorded from the right leg muscles by inserting the silver electrodes using the AC preamplifier (Bioelectric amplifier MEG-1200, time set constant at 0.01 s; Nihon Kohden, Tokyo, Japan). The amplified EMG signals were digitized (micro 1401, Cambridge Electronic Design, Cambridge, UK). The EMG activity of the reflex responses induced by single shocks, and was averaged for approximately 50 trials at 3-s intervals. The size of the reflex response was measured as the area under the evoked response and expressed as the percentage of the control size preceding drug injection. All data were reported as mean ± S.E.

5.2.5. Transient cerebral ischemia

Transient cerebral ischemia was induced according to the methods previously described[7] with some modifications. The animals were anesthetized by halothane (3.5% during the induction of anesthesia; 1.5% during the surgery and experiments) and an antibiotic (viccllin, 50 mg/kg, intranasal) was administered. An osmotic pump (Mini-Osmotic Pump Model 2001; Alzet, Cupertino, CA) containing the vehicle (0.2% fatty acid free BSA) or racemic 3-*S*-cPA **1a** solution was placed under the abdominal skin, and the solution was continuously administer for 5 days to yield a concentration of 180 $\mu g \cdot kg^{-1} \cdot 5\ days^{-1}$ at a speed of 1 μL/h. The pump was filled with solutions according to the manufacturer's procedure, and was implanted subcutaneously at 35–60 min before the onset of occlusion. Then, the trachea was incubated, and respiration was maintained using an artificial respirator (SN-480-7). Rectal temperature and temporal muscle temperature were monitored and maintained at approximately 37.5 °C by using a feedback-controlled heating pad and lamp (ATB-1100). Vertebral arteries on both sides were permanently ligated, and common carotid arteries on both sides were transiently occluded for 8 min. Halothane anesthesia was immediately removed at the end of transient ischemia, and the respirator was disconnected after 15 min.

Five days after transient ischemia, the rat was anesthetized by pentobarbital and perfused transcardially with heparinized saline followed by 10% formalin in 0.1 M PBS at pH 7.4. The rats were left in situ at 4 °C for 2 h. Then, the brains were removed, coronally cut into 3 mm thick slices, and the specimens were embedded in paraffin. Cross-sections of the brain were cut at 6 μm thickness. Brain sections were stained with hematoxylin and eosin (HE), or immunohistochemically stained with anti-glial fibrillary acidic protein (GFAP) rabbit antibody (Dako Japan Inc., Tokyo, Japan) to identify the astrocytes. The dorsal hippocampus, approximately 3.3 mm posterior to the bregma, was selected as the representative area for CA1 neurons.

5.2.6. Statistical analysis

All values are given as the mean ± S.E. The data were statistically analyzed by paired *t*-test, one-way ANOVA, Dunnett's test,

or nonlinear regression. A *P*-value of <0.05, <0.01 or <0.005 was considered statistically significant.

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References and notes

1. Murakami-Murofushi, K.; Shioda, M.; Kaji, K.; Yoshida, S.; Murofushi, H. *J. Biol. Chem.* **1992**, *267*, 21512.
2. Takahashi, Y.; Shimada, Y.; Shioda, M.; Yoshida, S.; Murofushi, H.; Murakami-Murofushi, K. *Cell Struct. Funct.* **1993**, *18*, 135.
3. Kobayashi, T.; Tanaka-Ishii, R.; Taguchi, R.; Ikezawa, H.; Murakami-Murofushi, K. *Life Sci.* **1999**, *65*, 2185.
4. Murakami-Murofushi, K.; Uchiyama, A.; Fujiwara, Y.; Kobayashi, T.; Kobayashi, S.; Mukai, M.; Murofushi, H.; Tigyi, G. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2002**, *1582*, 1.
5. Fujiwara, Y. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2008**, *1781*, 519.
6. Kakiuchi, Y.; Nagai, J.; Gotoh, M.; Hotta, H.; Murofushi, H.; Ogawa, T.; Ueda, H.; Murakami-Murofushi, K. *Mol. Pain* **2011**, 7.
7. Gotoh, M.; Hotta, H.; Murakami-Murofushi, K. *Eur. J. Pharmacol.* **2010**, *649*, 206.
8. Kobayashi, S.; Tokunoh, R.; Shibasaki, M.; Shinagawa, R.; Murakami-Murofushi, K. *Tetrahedron Lett.* **1993**, *34*, 4047.
9. Baker, D. L.; Fujiwara, Y.; Pigg, K. R.; Tsukahara, R.; Kobayashi, S.; Murofushi, H.; Uchiyama, A.; Murakami-Murofushi, K.; Koh, E.; Bandle, R. W.; Byun, H.; Bittman, R.; Fan, D.; Murph, M.; Mills, G. B.; Tigyi, G. *J. Biol. Chem.* **2006**, *281*, 22786.
10. Uchiyama, A.; Mukai, M.; Fujiwara, Y.; Kobayashi, S.; Kawai, N.; Murofushi, H.; Inoue, M.; Enoki, S.; Tanaka, Y.; Niki, T.; Kobayashi, T.; Tigyi, G.; Murakami-Murofushi, K. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2007**, *1771*, 103.
11. Inoue, N.; Minakawa, N.; Matsuda, A. *Nucleic Acids Res.* **2006**, *34*, 3476.
12. Tanaka, R.; Kato, M.; Suzuki, T.; Nakazaki, A.; Nozaki, E.; Gotoh, M.; Murakami-Murofushi, K.; Kobayashi, S. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4180.
13. Young, R. W. *J. Am. Chem. Soc.* **1952**, *74*, 1672.
14. Umezu-Goto, M.; Kishi, Y.; Taira, A.; Hama, K.; Dohmae, N.; Takio, K.; Yamori, T.; Mills, G. B.; Inoue, K.; Aoki, J.; Arai, H. *J. Cell Biol.* **2002**, *158*, 227.
15. Tokumura, A.; Majima, E.; Kariya, Y.; Tominaga, K.; Kogure, K.; Yasuda, K.; Fukuzawa, K. *J. Biol. Chem.* **2002**, *277*, 39436.
16. Gotoh, M.; Fujiwara, Y.; Yue, J.; Liu, J.; Lee, S.; Fells, J.; Uchiyama, A.; Murakami-Murofushi, K.; Kennel, S.; Wall, J.; Patil, R.; Gupte, R.; Balazs, L.; Miller, D. D.; Tigyi, G. J. *Biochem. Soc. Trans.* **2012**, *40*, 31.
17. Nozaki, E.; Gotoh, M.; Hotta, H.; Hanazawa, S.; Kobayashi, S.; Murakami-Murofushi, K. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2011**, *1811*, 271.
18. Nozaki, E.; Gotoh, M.; Hanazawa, S.; Mori, H.; Kobayashi, S.; Murakami-Murofushi, K. *Cytologia* **2011**, *76*, 73.
19. Kagitani, F.; Uchida, S.; Hotta, H.; Sato, A. *Jpn. J. Physiol.* **2000**, *50*, 585.
20. Gupte, R.; Siddam, A.; Lu, Y.; Li, W.; Fujiwara, Y.; Panupinthu, N.; Pham, T. C.; Baker, D. L.; Parrill, A. L.; Gotoh, M.; Murakami-Murofushi, K.; Kobayashi, S.; Mills, G. B.; Tigyi, G.; Miller, D. D. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7525.