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Synthesis of enantiopure 2-carba-cyclic phosphatidic acid and effects of its chirality on biological functions

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ABSTRACT

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator, which has a quite unique cyclic phosphate ring at *sn*-2 and *sn*-3 positions of the glycerol backbone. We have designed and chemically synthesized several metabolically stabilized derivatives of cPA. 2-Carba-cPA (2ccPA) is one of the synthesized compounds in which the phosphate oxygen was replaced with a methylene group at the *sn*-2 position, and it showed much more potent biological activities than natural cPA. Here, we developed a new method of 2ccPA enantiomeric synthesis. And we examined the effects of 2ccPA enantiomers on autotaxin (ATX) activity, cancer cell invasion and nociceptive reflex. As well as racemic-2ccPA, both enantiomers showed inhibitory effects on ATX activity, cancer cell invasion and nociceptive reflex. As their effects were not significantly different from each other, the chirality of 2ccPA may not be critical for these biological functions of 2ccPA.
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1. Introduction

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator. This bioactive lipid was originally isolated and identified from myxoamoebae of a true slime mold, *Physarum polycephalum* [1]. At present, cPA has been found in a wide range of organisms, from slime molds to humans [2,3]. The chemical formula of cPA is similar to lysophosphatidic acid (LPA), but cPA has a quite unique structure with a cyclic phosphate ring at *sn*-2 and *sn*-3 positions of the glycerol backbone, and a chiral carbon atom is assigned an *R* designation [4]. These features of cPA may provide the difference of biological functions of cPA and LPA. For example, LPA stimulates cell proliferation and induces cancer cell invasion and metastasis [5]. The intraplantar injection of LPA into the hind limb of mice induces nociceptive flexor responses [6], and intrathecal injection of LPA induces neuropathic pain in mice [7]. In contrast, cPA inhibits autotaxin (ATX) activity, cancer cell invasion and metastasis [8,9]. And recently, we found that cPA suppressed nociceptive responses by primary afferent C-fiber [10].

As the cyclic phosphate ring of cPA would be opened by hydrolysis resulted in the formation of LPA [11], we have synthesized several metabolically stabilized carba derivatives of cPA (ccPA) [11,12]. 2-Carba-cPA (2ccPA) is the compound in which one of the phosphate oxygen is replaced with a methylene group at the *sn*-2 position. We have revealed that 2ccPA well conserved numerous biological functions of cPA, and the inhibition of cancer cell invasion and metastasis [11] and nociceptive reflex suppression [10] by 2ccPA were much more potent than those of natural cPA. These results suggested that 2ccPA could be used as a therapeutic compound for cancer and pain. Meanwhile, these data were obtained by using a racemic-2ccPA, due to the difficulty of enantiopure 2ccPA synthesis. As it is well known that each enantiomer shows same or different biological activities in many cases, the determination of biological activities of each enantiopure 2ccPA is strongly required for further 2ccPA studies.

In this report, we newly developed the method of 2ccPA enantiomer synthesis. And we clarified the effects of each enantiomer on ATX activity, cancer cell invasion and nociceptive reflex.

2. Materials and methods

2.1. Synthesis of (R)-2ccPA (Fig. 1a) and (S)-2ccPA (Fig. 1b)

(2*R*,3*E*)-2-(Hydroxymethyl)-5-methylhex-3-enyl acetate (**2**) [13].

To a solution of (3*E*)-2-(hydroxymethyl)-5-methylhex-3-enol (**1**, 36.9 g) in vinyl acetate (500 ml) was added molecular sieves 3A

Abbreviations: cPA, Cyclic phosphatidic acid; LPA, Lysophosphatidic acid; ATX, Autotaxin; 2ccPA, 2-carba-cyclic phosphatidic acid

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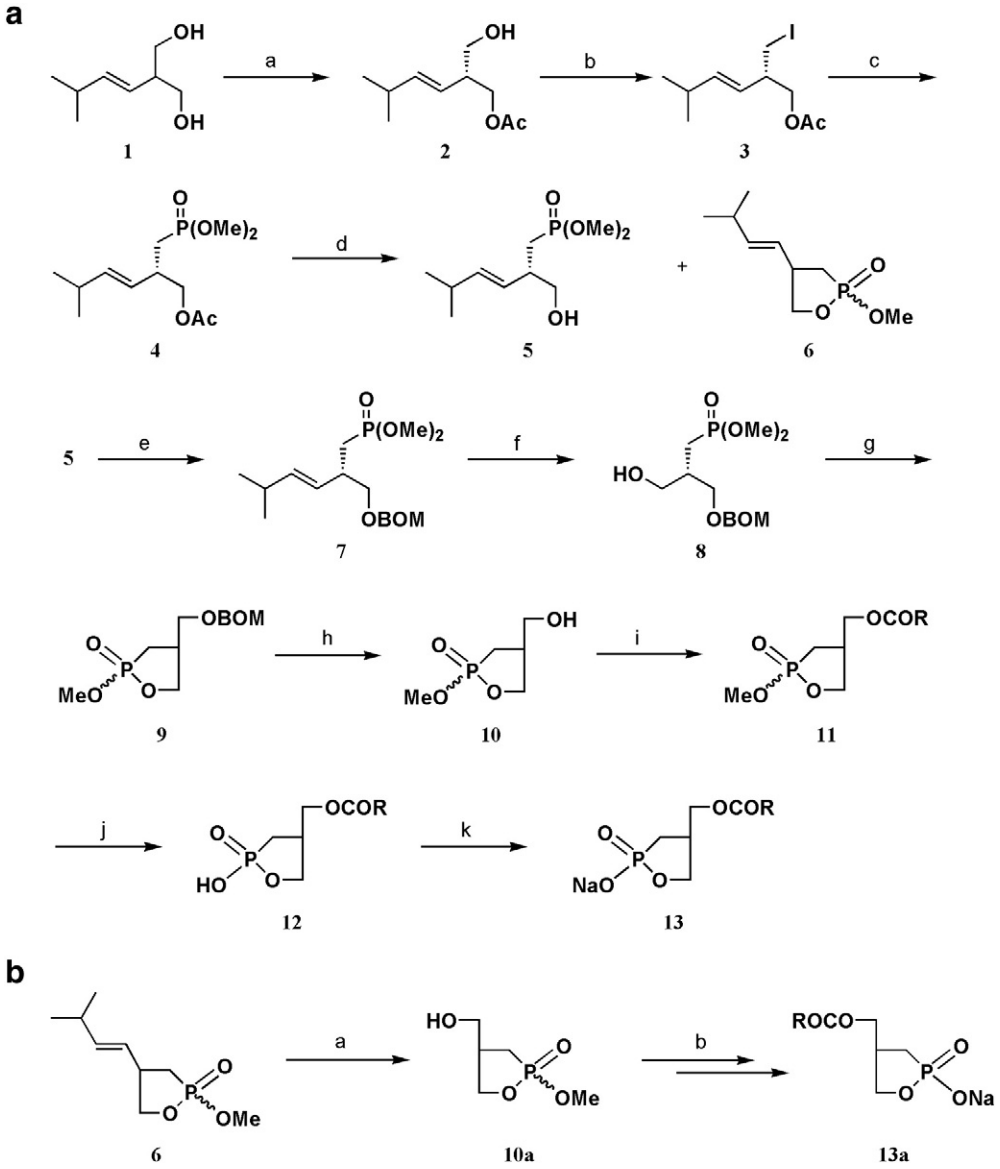

Fig. 1. a. synthesis of (*R*)-2ccPA (**13**). Reagents and conditions: (a) PPL, MS 3A, Vinyl acetate, rt, 24 h, 52%; (b) I_2, PPh_3, imidazole, THF, rt, 12 h, 98%; (c) $HP(O)(OMe)_2$, Cs_2CO_3, TBAI, DMF, rt, 24 h, 66%; (d) K_2CO_3, MeOH, −20 °C, 2 h, **5** 64% and **6** 8%; (e) BOMCl, $^iPr_2NEt$, CH_2Cl_2, rt, 4.5 h, 94%; (f) O_3, MeOH, $CHCl_3$, −78 °C, 3.5 h, then sodium borohydride, rt, 1 h, 80%; (g) Et_3N, toluene, 80 °C, 36 h, 39%; (h) H_2, $Pd(OH)_2$, THF, rt, 6 days, 64%; (i) palmitoleic chloride, Et_3N, 4-DMAP, rt, 19 h, 75%; (j) TMSBr, CH_2Cl_2, −20 °C, 75%; (k) 0.01 N NaOH aq, extraction, 43%. (**R** = $(CH_2)_7CH=CH(CH_2)_5CH_3$). b. Synthesis of (*S*)-2ccPA (**13a**). Reagents and conditions: (a) O_3, MeOH, $CHCl_3$, −78 °C, 1 h, then PPh_3, $NaBH_4$, 0 °C, 1 h, 88%. (b) These reagents and conditions follow the same procedures for **9**–**13**. (**R** = $(CH_2)_7CH=CH(CH_2)_5CH_3$).

(18.5 g) and PPL (porcine pancreas lipase, 18.5 g) at room temperature. The reaction mixture was stirred for 24 h and then filtered through Celite and concentrated in vacuo. The residue was purified by chromatography on silica gel (hexane/ethyl acetate = 20/1 to 1/1) to afford **2** (24.6 g, 52%) as a colorless oil. Enantiomeric excess was determined to be >99% ee by chiral HPLC (DAICEL OD, hexane: *i*-PrOH = 50:1, flow rate 1.0 mL/min, monitoring 220 nm, Rt; 12.41 min for **2**, and 15.96 min for *ent*-**2**).

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.61 (dd, 1H, J = 15.6, 6.6 Hz), 5.24 (ddd, 1H, J = 15.6, 8.2, 1.2 Hz), 4.19 (dd, 1H, J = 11.2, 5.6 Hz), 4.07 (1H, dd, J = 11.2, 7.2 Hz), 3.46–3.70 (m, 2H), 2.52 (m, 1H), 2.29 (m, 1H), 2.07 (s, 3H), 0.98 (d, 6H, J = 6.6 Hz); $[\alpha]_D^{23}$ +27.8 (*c* 2.35, $CHCl_3$); HRMS (ESI) calcd for $C_{10}H_{18}O_3Na$ $[M+Na]^+$ 209.1153, found 209.1154.

(2 *S*,3*E*)-2-(Iodomethyl)-5-methylhex-3-enyl acetate (**3**).

To a solution of **2** (95.4 g) in cold THF (500 ml) was added triphenylphosphine (161 g), imidazole (41.8 g) and iodine (156 g). The reaction mixture was stirred at room temperature. After 12 h, the reaction mixture was quenched with saturated $Na_2S_2O_3$ aqueous solution and extracted with ethyl acetate. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (hexane) to afford **3** (149 g, 98%) as a yellow oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.56 (dd, 1H, J = 15.6, 6.8 Hz), 5.18 (ddd, 1H, J = 15.6, 8.2, 1.2 Hz), 4.14 (dd, 1H, J = 11.0, 5.6 Hz), 3.99 (1H, dd, J = 11.0, 7.2 Hz), 3.20–3.25 (m, 2H), 2.45 (m, 1H), 2.28 (m, 1H), 2.06 (s, 3H), 0.99(d, 3H, J = 6.8 Hz), 0.98 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 9.3, 20.9, 22.3, 22.3, 31.2, 42.8, 66.6, 124.8, 144.8, 170.8; $[\alpha]_D^{24}$ +4.51 (*c* 1.04, $CHCl_3$); HRMS (ESI) calcd for $C_{10}H_{17}IO_2Na$ $[M+Na]^+$ 319.0171, found 319.0170.

(2 *S*,3*E*)-Dimethyl 2-(acetoxymethyl)-5-methylhex-3-enylphosphonate (**4**).

To a solution of dimethyl phosphite (13 ml), Cs_2CO_3 (49.5 g) and tetrabutylammonium iodide (5.4 g) in DMF (100 ml) was added a solution of **3** (21.4 g) in DMF (50 ml), and stirred for 24 h at room temperature. The reaction mixture was quenched with water (the solution was became clear), and extracted with ethyl acetate. The

combined organic layer was washed with water and brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (hexane/ethyl acetate = 2/1) to afford **4** (13.3 g, 66%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.55 (dd, 1H, J = 15.4, 6.8 Hz), 5.23 (ddd, 1H, J = 15.4, 8.6, 1.0 Hz), 3.90–4.15 (m, 2H), 3.73 (d, 3H, J = 1.1 Hz), 3.71 (d, 3H, J = 1.1 Hz), 2.76 (m, 1H), 2.26 (m, 1H), 2.05 (s, 3H), 1.93–2.00 (m, 1H), 1.74–1.81 (m, 1H), 0.97 (d, 6H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 20.9, 22.3, 22.3, 27.1, 30.9, 36.6, 52.2, 52.2, 67.2, 125.7, 125.8, 170.8; $[\alpha]_D^{24}$ +5.49 (*c* 1.01, $CHCl_3$); HRMS (ESI) calcd for $C_{12}H_{23}O_5PNa$ $[M+Na]^+$ 301.1180, found 301.1180.

(2 *S*,3*E*)-Dimethyl 2-(hydroxymethyl)-5-methylhex-3-enylphosphonate (**5**) and (*E*)-6-methylpent-4-ene-2-methoxy-2λ^5-[1,2]oxaphospholane 2-oxide (**6**).

To a solution of **4** (13.3 g) in MeOH was added K_2CO_3 (9.9 g) at −20 °C, and stirred for 2 h. The reaction mixture was quenched with saturated NH_4Cl aqueous solution and extracted with chloroform. The combined organic layer was washed with brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (ethyl acetate) to afford **5** (7.25 g, 64%) as a colorless oil and **6** (0.75 g, 8%) as a colorless oil.

Compound **5**: ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.55 (dd, 1H, J = 15.4, 6.6 Hz), 5.26 (ddd, 1H, J = 15.4, 8.2, 0.8 Hz), 3.74 (dd, 6H, J = 11.0, 6.0 Hz), 3.54–3.59 (m, 2H), 2.66 (brs, 1H), 2.57–2.66 (m, 1H), 2.23–2.31 (m, 1H), 1.82–1.95 (m, 1H), 1.74–1.81 (m, 1H), 0.98 (d, 3H, J = 6.8 Hz), 0.97 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 22.4, 22.4, 27.1, 30.9, 39.9, 52.3, 52.4, 66.3, 126.7, 126.9; $[\alpha]_D^{23}$ +4.5 (*c* 0.99, $CHCl_3$); HRMS (ESI) calcd for $C_{10}H_{21}O_4PNa$ $[M+Na]^+$ 259.1075, found 259.1082.

Compound **6**: ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.56 (dd, 1H, J = 15.4, 6.6 Hz), 5.22 (m, 1H), 4.10–4.28 (m, 1H), 3.79 (ddd, 3H, J = 11.0, 2.0, 0.6 Hz), 3.60–3.82 (m, 1H), 3.04–3.22 (m, 1H), 2.22–2.30 (m, 1H), 2.01–2.17 (m, 1H), 1.60–1.73 (m, 1H), 0.97 (d, 6H, J = 6.8 Hz); HRMS (ESI) calcd for $C_9H_{17}O_3PNa$ $[M+Na]^+$ 227.0807, found 227.0814.

(2 *S*,3*E*)-Dimethyl 2-(benzyloxymethoxymethyl)-5-methylhex-3-enylphosphonate (**7**).

To a solution of **5** (7.17 g) in dichloromethane (60 ml) was added diisopropylethylamine (7.2 ml) at 0 °C under argon atmosphere. Benzylchloromethyl ether (5.8 ml) was added to the solution and, the reaction mixture was allowed to warm up to room temperature and stirred for 4.5 h. The reaction mixture was quenched with saturated NH_4Cl aqueous solution and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (hexane/ethyl acetate = 3/1) to afford **7** (10.2 g, 94%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 7.28–7.36 (m, 5H), 5.36 (dd, 1H, J = 15.4, 6.4 Hz), 5.31 (ddd, 1H, J = 15.6, 8.2, 1.2 Hz), 4.75 (s, 2H), 4.59 (s, 2H), 3.70 (d, 6H, J = 10.8 Hz), 3.57 (ddd, 1H, J = 9.4, 6.8, 2.0 Hz), 3.48 (dd, 1H, J = 9.4, 6.8 Hz), 2.68–2.73 (m, 1H), 2.24–2.29 (m, 1H), 2.06–2.13 (m, 1H), 1.72–1.76 (m, 1H), 0.98 (d, 3H, J = 6.8 Hz), 0.97 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 22.3, 22.4, 26.4, 27.8, 30.9, 37.4, 52.1, 69.4, 71.6, 94.6, 126.7, 126.8, 127.7, 127.8, 128.4, 128.4, 137.8, 139.6; $[\alpha]_D^{23}$ +21.0 (*c* 1.03, $CHCl_3$); HRMS (ESI) calcd for $C_{18}H_{29}O_5PNa$ $[M+Na]^+$ 379.1650, found 379.1660.

(*S*)-(2-Benzyloxymethoxymethyl-3-hydroxypropyl)-phosphonic acid dimethyl ester (**8**).

To a stirred solution of **7** (9.37 g) in dichloromethane (90 ml) and methanol (50 ml) was bubbled O_3 at −78 °C until the color of solution turned blue. After 3.5 h, the reaction mixture was treated sodium borohydride (6.0 g), allowed room temperature and stirred for 1 h. The reaction mixture was quenched with saturated NH_4Cl aqueous solution and extracted with dichloromethane. The combined organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (methanol/chloroform = 1/30 to 1/20) to afford **8** (6.72 g, 80%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 7.28–7.48 (m, 5H), 4.76 (s, 2H), 4.60 (s, 2H), 3.75 (d, 3H, J = 10.8 Hz), 3.75 (d, 3H, J = 10.8 Hz), 3.69–3.73 (m, 2H), 3.64 (d, 2H, J = 5.7 Hz), 2.97 (br, 1H), 2.20–2.27 (m, 1H), 1.82–1.96 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 23.3, 24.7, 36.5, 52.5, 64.2, 64.3, 69.8, 69.9, 94.9, 127.8, 127.8, 128.5, 128.5, 137.7; $[\alpha]_D^{23}$ +1.4 (*c* 1.00, $CHCl_3$); HRMS (ESI) calcd for $C_{14}H_{23}O_6PNa$ $[M+Na]^+$ 341.1131, found 341.1129.

(*R*)-4-Benzyloxymethoxymethyl-2-methoxy-[1,2]oxaphospholane 2-oxide (**9**).

To a stirred solution of **8** (12.4 g) in toluene (80 ml) was added triethylamine (6.6 ml) at 80 °C. After 36 h, the reaction mixture was concentrated in vacuo. The residue was purified by chromatography on silica gel (methanol/chloroform = 1/50) to afford **9** (4.42 g, 39%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 7.29–7.38 (m, 5H), 4.75 (s, 2H), 4.56 (s, 2H), 4.16–4.33 (m, 1H), 3.82–4.01 (m, 1H), 3.79 (d, 1.5H, J = 6.6 Hz), 3.77 (d, 1.5H, J = 6.6 Hz), 3.55–3.63 (m, 2H), 2.76–2.90 (m, 1H), 1.95–2.05 (m, 1H), 1.69–1.76 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 21.1, 37.1, 52.4, 67.7, 68.9, 69.6, 94.6, 127.6, 127.6, 128.3, 129.5, 129.5, 137.4; IR (neat): 2952, 2904, 1454, 1270, 1053, 996, 859, 824, 742, 701, 581 cm^{-1}; HRMS (ESI) calcd for $C_{13}H_{19}O_5PNa$ $[M+Na]^+$ 309.0862, found 309.0876.

(*R*)-(2-Methoxy-2-oxo-2λ^5-[1,2]oxaphospholan-4-yl)-methanol (**10**).

To a solution of **9** (7.87 g) in THF (140 ml) was added 20% Pd $(OH)_2$/C at room temperature. The reaction mixture was stirred 6 days under hydrogen atmosphere and then filtered through Celite and concentrated in vacuo. The residue was purified by chromatography on silica gel (methanol/chloroform = 1/10) to afford **10** (2.91 g, 64%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 4.17–4.36 (m, 1H), 3.89–4.11 (m, 1H), 3.80 (d, 1.5H, J = 3.6 Hz), 3.78 (d, 1.5H, J = 3.6 Hz), 3.67–3.73 (m, 2H), 2.70–2.85 (m, 1H), 1.96–2.06 (m, 1H), 1.71–1.90 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 20.9, 39.2, 52.6, 62.3, 69.1; IR (neat): 3387, 2955, 1464, 1414, 1258, 1049, 989, 862, 824 cm^{-1}; HRMS (ESI) calcd for $C_5H_{11}O_4PNa$ $[M+Na]^+$ 189.0293, found 189.0282.

(*R*)-Palmitoleic acid 2-methoxy-2-oxo-2λ^5-[1,2]oxaphospholan-4-ylmethyl ester (**11**).

To a stirred solution of **10** (439 mg) in dichloromethane (6.6 mL) was added palmitoleic chloride (936 mg), triethylamine (0.5 ml) and 4-DMAP (64.5 mg) at 0 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 19 h. The reaction mixture was quenched with brine and extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (hexane/ethyl acetate = 1/2 to 1/4) to afford **11**(792 mg, 75%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.26–5.33 (m, 2H), 4.23–4.29 (m, 0.5H), 4.02–4.18 (m, 2H), 3.90–3.95 (m, 0.5H), 3.77–3.80 (m, 1H), 3.76 (dd, 3H, J = 11.2, 3.6 Hz), 3.67–3.73 (m, 2H), 2.80–2.91 (m, 1H), 2.27 (t, 2H, J = 7.2 Hz), 2.00–2.07 (m, 1H), 1.94–1.99 (m, 4H), 1.62–1.71 (m, 1H), 1.56–1.58 (m, 2H), 1.28–1.30 (br, 16H), 0.88 (t, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 14.1, 20.7, 22.3, 22.6, 24.8, 27.1, 27.2, 29.0, 29.1, 29.1, 29.6, 29.7, 31.7, 34.0, 36.3, 52.8, 64.0, 68.5, 129.7, 130.0, 173.4; IR (neat): 2926, 2854, 1739, 1465, 1272, 1175, 1049, 1005, 860, 823 cm^{-1}; HRMS (ESI) calcd for $C_{21}H_{39}O_5P$ $[M^+]$ 402.2535, found 402.2541.

(*R*)-Palmitoleic acid 2-hydroxy-2-oxo-2λ^5-[1,2]oxaphospholan-4-ylmethyl ester (**12**).

To a stirred solution of **11** (959 mg) in dichloromethane (12 ml) was added bromotriethylsilane (0.95 ml) at −20 °C. After 1 h, the reaction mixture was allowed to warm up to 0 °C and stirred for 1 h. The reaction mixture was concentrated in vacuo. The residue was purified by chromatography on silica gel (methanol/chloroform = 1/10) to afford **11**(792 mg, 75%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$ + 1 drop CD_3OD) δ_H: 5.31–5.38 (m, 2H), 3.91–4.31 (m, 4H), 2.85–2.95 (m, 1H), 2.31 (t, 2H, J = 7.6 Hz), 2.05–2.12

(m, 1H), 1.99–2.03 (m, 4H), 1.70–1.78 (m, 1H), 1.59–1.63 (m, 2H), 1.26–1.30 (br, 16H), 0.88 (t, 3H, $J=6.8$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 1.0, 14.1, 22.6, 24.9, 27.1, 27.2, 28.9, 29.0, 29.1, 29.2, 29.3, 29.7, 29.8, 31.7, 34.1, 59.7, 61.3, 129.7, 129.9, 174.0; ^{31}P NMR (400 MHz, CD_3OD) δ_P: 48.75.

(*R*)-2ccPA (**13**).

A solution of **12** (900 mg) in diethyl ether (250 ml) was extracted with 0.01 mol/L sodium hydroxide aqueous solution 6 times (total volume, 250 ml). The aqueous layer was freeze-dried and the residue was purified by chromatography on silica gel (methanol/chloroform/water = 80/400/1). The purified compound was dissolved water and freeze-dried to afford **13** (428 mg, 43%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$ + 1 drop CD_3OD) δ_H: 5.30–5.40 (m, 2H), 3.96–4.34 (m, 3H), 3.66–3.78 (m, 1H), 2.73–2.87 (m, 1H), 2.29 (t, 2H, $J=7.4$ Hz), 1.98–2.05 (m, 4H), 1.77–1.87 (m, 1H), 1.57–1.65 (m, 2H), 1.37–1.48 (m, 1H), 1.24–1.37 (br, 16H), 0.87 (t, 3H, $J=6.8$ Hz) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 14.4, 23.7, 26.0, 28.1, 28.1, 30.0, 30.1, 30.2, 30.2, 30.8, 30.8, 32.9, 34.9, 38.6, 38.6, 66.6, 67.8, 130.8, 130.9, 175.3; ^{31}P NMR (400 MHz, CD_3OD) δ_P: 42.64; $[\alpha]_D^{23}$ -12.3 (*c* 0.71, $CHCl_3$).

(*S*)-4-Benzyloxymethoxymethyl-2-methoxy-[1,2]oxaphospholane 2-oxide (**10a**).

To a stirred solution of **6** (114.7 mg) in dichloromethane (2.8 ml) and methanol (4.7 ml) was bubbled O_3 at −78 °C until the color of solution turned blue. After 1 h, the reaction mixture was treated triphenylphosphine (162.0 mg) and allowed to 0 °C. The reaction mixture was treated sodium borohydride (63.7 mg) and stirred for 1 h. The reaction mixture was quenched with acetic acid (0.1 ml) and concentrated in vacuo. The residue was purified by chromatography on silica gel (methanol/chloroform = 1/30) to afford **10a** (81.8 mg, 88%) as a colorless oil.

^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.56 (dd, 1H, $J=15.4$, 6.8 Hz), 5.22 (m, 1H), 4.10–4.28 (m, 1H), 3.79 (ddd, 3H, $J=11.2$, 2.0, 0.6 Hz), 3.60–3.82 (m, 1H), 3.04–3.22 (m, 1H), 2.22–2.30 (m, 1H), 2.01–2.17 (m, 1H), 1.60–1.73 (m, 1H), 0.97 (d, 6H, $J=6.8$ Hz); HRMS (ESI) calcd for $C_5H_{11}O_4PNa$ $[M+Na]^+$ 189.0293, found 189.0282.

(*S*)-2ccPA (**13a**).

13a was prepared from **10a** following the same procedures for **10**–**13**.

$[\alpha]_D^{23}$ +13.6 (*c* 0.72, $CHCl_3$).

2.2. Culture of MDA-MB-231

Human breast cancer MDA-MB-231 cells (a generous gift from Dr. Gabor Tigyi, University of Tennessee) were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, FL). And cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Collection and concentration of conditioned medium (CCM)

MDA-MB-231 cells were grown until 90% confluence. The cells were washed 3 times with phosphate buffered saline (PBS). The cells were incubated for 12 h in serum-free DMEM, and then the conditioned medium was centrifuged at 200×*g* for 5 min to remove cellular debris. Subsequently, the conditioned medium was concentrated to 10-fold on a 100 kDa cutoff filter (Millipore, Cork, Ireland).

2.4. ATX inhibition assay

ATX inhibition activity of compounds ((*R*)-2ccPA 16:1, (*S*)-2ccPA 16:1 and racemic-2ccPA 16:1) was measured using FS-3 (Echelon Biosciences, Inc., UT), which fluoresces upon cleavage by ATX. FBS or CCM from MDA-MB-231 cells was used as the source of ATX. FBS or CCM and various concentrations of compounds in 10 μM fatty acid free bovine serum albumin (BSA; Sigma-Aldrich, MO) were mixed with 1 μM of FS-3 in assay buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 140 mM NaCl, 50 mM Tris–HCl, pH 8.0). The fluorescence intensity (em/ex: 485/530 nm) of each well was measured by Cyto Flour series 4000 micro plate reader (Applied Biosystems, Foster City, CA) at 0 time and after 2 h incubation at 37 °C. Data were normalized to vehicle control after subtraction of 0 time value and expressed as percent inhibition activity of compounds. All data were reported as mean ± S.E. of triplicate wells.

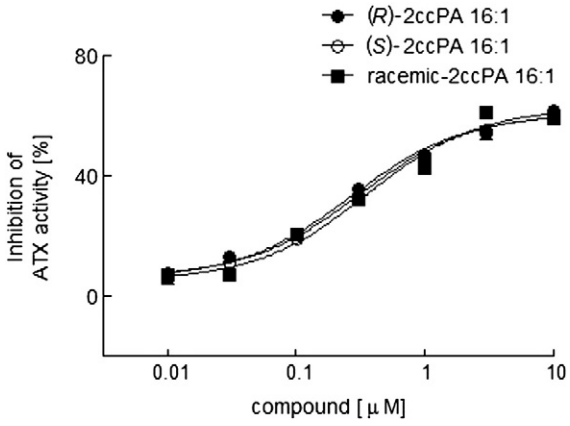

Fig. 2. Effects of chirality of 2ccPA on ATX activity. The catalytic activity of ATX in CCM was examined in the presence of (*R*)-2ccPA 16:1 (closed circle), (*S*)-2ccPA 16:1 (open circle) and racemic-2ccPA 16:1 (closed square). Each data point represents the mean ± S.E. of triplicate wells and is representative of at least three independent experiments with similar results. Each data was calculated as percent inhibition in comparison respective controls of 100% without any compounds.

2.5. Invasion assay

Invasion of the cells across 8 μm pore size membrane was assessed using Chemo Tx System (Neuro Probe) according to the manufacturer's protocol. Briefly, cells were stained with calcein-AM (Dojindo, Kumamoto, Japan), and the cell suspension was centrifuged and washed with PBS. After washing, the cells were resuspended in DMEM containing 0.1% BSA (4×10^5 cells/ml). The 200 μM lysophosphatidylcholine (LPC)

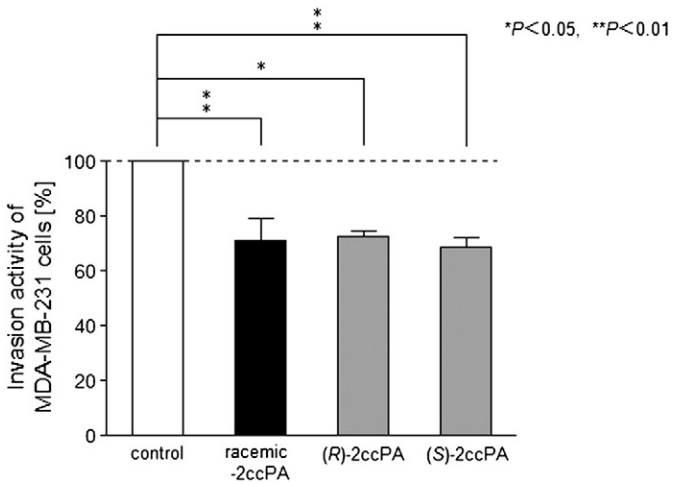

Fig. 3. Effects of chirality of 2ccPA on MDA-MB-231 cell invasion. The effect of (*R*)-2ccPA 16:1, (*S*)-2ccPA 16:1 and racemic-2ccPA 16:1 on MDA-MB-231 cell in vitro invasion was examined by Boyden-chamber modified assay. The MDA-MB-231 cells were seeded on upper chamber and 200 μM of LPC and 3 μM of compounds were added into lower well. 100% represents maximal migration activity in the presence of 200 μM LPC. Each column represents 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 control by 1 way ANOVA.

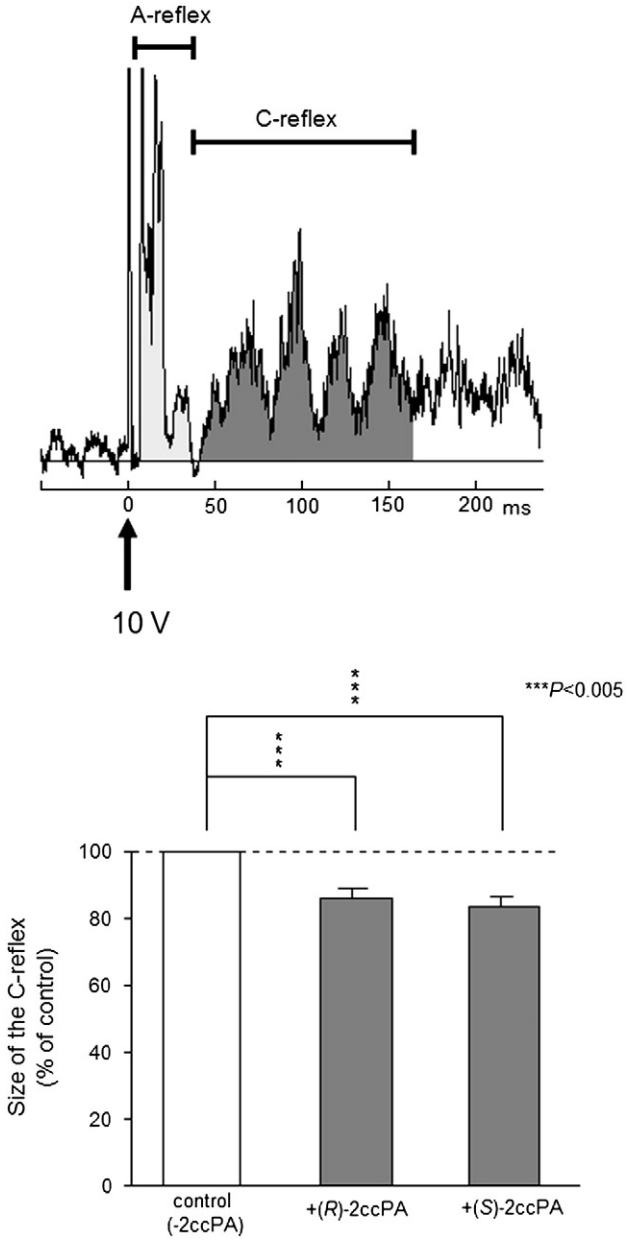

Fig. 4. Effects of chirality of 2ccPA on somato-somatic C-reflexes. In the top of figure, the specimen record of A- and C-reflexes (average of 50 trials) was elicited by single electrical stimulation of a saphenous nerve using stimulus strength of 10 V. In the bottom of figure, the graph summarizes the size of the C-reflex 0–12.5 min after i.v. injection of compounds at a dose of 0.2 mg/kg ($n=4$). The mean size (area under the evoked responses) of the C-reflex 0–15 min before each injection was expressed as 100%. All subsequent reflexes were expressed as percentages of the control values. Each column represents the mean ± S.E. ***$P<0.005$, significantly different from control by 1 way ANOVA.

and 3 μM compounds ((*R*)-2ccPA 16:1, (*S*)-2ccPA 16:1 and racemic-2ccPA 16:1) in DMEM containing 0.1% BSA were added to lower well. And 25 μl of cell suspension were added to each upper filter well. Plates were incubated for 8 h at 37 °C, and the medium was carefully removed from the upper filter. Then the upper filter was carefully washed with PBS containing 2 mM EDTA, and gently wiped with a cotton swab to remove the non-invaded cells. Then the filter was separated from the microplate, and measured the fluorescence (em/ex: 485/530 nm) of invaded cells on the bottom of each well by fluorescence plate reader (Cyto Flour series 4000). Data were calculated as percent inhibition in comparison to respective controls without any compounds, and are presented as the mean and S.E. of triplicate wells.

2.6. Recording of spinal somato-somatic reflex

Male Wistar rats ($n=4$; weighing 320–360 g) were anesthetized by pentobarbital (50 mg/kg, i.p.). A juglar vein was catheterized for i.v. administration of compounds ((*R*)-2ccPA 16:1 or (*S*)-2ccPA 16:1, 0.2 mg/kg). A juglar artery was catheterized to record 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 about 3.0%. Body temperature was kept about 37.5 °C using an automatically regulated heating pad and lamp (Animal blanket controller ATB-1100, Nihon koden, 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 thigh skin was cut at level of thigh and covered with warm paraffin oil. The central cut end 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 koden, Tokyo, Japan). Electromyogram (EMG) was recorded from the right leg muscles by inserting silver electrodes using the AC preamplifier (Bioelectric amplifier MEG-1200, Nihon koden, Tokyo, Japan, time constant set at 0.01 s). The amplified EMG signals were digitized (micro 1401, Cambridge Electronic Design, Cambridge, UK) and rectified for later processing (Spike 2 version 5, Cambridge Electronic Design, Cambridge, UK). With single shocks, reflex responses of EMG activity were averaged for approximately 50 trials at 3 intervals. The size of the reflex response was measured as the area under the evoked response and expressed as % of the control size preceding drug injection. All data were reported as mean ± S.E.

2.7. Statistical analysis

All values are given as mean ± S.E. The data were statistically analyzed by Student's *t*-test, one way ANOVA and nonlinear regression. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of chirality of 2ccPA on ATX activity

ATX activity was measured using FS-3 which fluoresces upon cleavage by ATX. The catalytic activity of ATX in CCM from MDA-MB-231 cells was significantly inhibited by (*R*)-2ccPA 16:1, (*S*)-2ccPA 16:1 and racemic-2ccPA 16:1 (Fig. 2). In case of (*R*)-2ccPA 16:1, it inhibited ATX activity as low concentration as 10 nM. At 10 μM, an inhibitory effect of (*R*)-2ccPA 16:1 on ATX arose over 60%. And, the catalytic activity of ATX in FBS was also significantly inhibited by (*R*)-2ccPA 16:1, (*S*)-2ccPA 16:1 and racemic-2ccPA 16:1 (data not shown). The inhibitory activity of (*R*)-2ccPA was similar to (*S*)-2ccPA and racemic-2ccPA, indicating that the chirality of 2ccPA did not affect the degree of ATX inhibition. Additionally, it has been reported that no stereoselective differences were found between (*R*)-3ccPA and (*S*)-3ccPA toward the inhibition of ATX [14].

3.2. Effects of chirality of 2ccPA 16:1 on MDA-MB-231 cell invasion

After 8 h incubation of invasion assay plate at 37 °C, MDA-MB-231 cells were invaded to bottom membrane through 8 μm pore. It was due to the hydrolysis of LPC in bottom well by secreted ATX from the cells of upper well, which caused formation of the bioactive lipid LPA. In the presence of 2ccPAs, invasion of MDA-MB-231 cells was inhibited as

shown in Fig. 3. The inhibitory activity of (*R*)-2ccPA was almost the same as that of (*S*)-2ccPA and racemic-2ccPA. Similar to the ATX inhibition, the chirality of 2ccPA did not affect the inhibitory activity of cancer cell invasion.

3.3. Effects of chirality of 2ccPA on somato-somatic C-reflexes in anesthetized rats

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 hindlimb EMG, a short latency (about 10 ms) A-reflex and a long latency (about 40 ms) C-reflex (the top of Fig. 4). Injection of 0.2 mg/kg, i.v. of compounds ((*R*)-2ccPA 16:1 or (*S*)-2ccPA 16:1) depressed the C-reflex, but not A-reflex. In most experiments, 2ccPA induced depression of the C-reflex components started several min after the injection and reached their maxima in less than 12 min, and then gradually returned to the control within 30 min.

The bottom of Fig. 4 summarized the effects of i.v. injection of compounds ((*R*)-2ccPA 16:1 and (*S*)-2ccPA 16:1) on C-reflex tested in 4 rats. The responses were measured 0–12.5 min after i.v. injection of compounds after reaching the maximum effects. The size of C-reflex response was measured as the area under the evoked response. The averaged responses expressed as % of the control size preceding drug injection. After injection of (*R*)-2ccPA 16:1, the C-reflex reached 86 ± 3% of the control. After injection of (*S*)-2ccPA 16:1, the C-reflex reached 84 ± 3% of the control. These results indicated that the chirality of 2ccPA did not affect their inhibitory activities of C-reflexes suppression.

4. Discussion

Cyclic-PA is a natural occurring bioactive lipid, and the chiral carbon atom is assigned an *R* designation. We have already reported that 2ccPA and 3ccPA have inhibitory activities on ATX, cancer cell invasion and metastasis, as well as cPA. However, these data were obtained by using a racemic-2ccPA and (*S*)-3ccPA , those were easy to synthesize chemically [11]. As it is well known that each enantiomer shows same or different biological activities, the biological activities of each enantiopure cPA derivatives are required to be demonstrated.

In this study, we focus on 2ccPA to investigate the effects of enantiopure derivatives on some biological functions, because of 2ccPA was shown to be the most potent ATX inhibitor [12]. Here, we newly developed the method of 2ccPA enantiomer synthesis. And we clarified the effects of each enantiomer on ATX activity, cancer cell invasion and nociceptive reflex.

ATX has lysophospholipase D (lyso PLD) activity, which produces LPA from lysophosphatidylcholine (LPC) by hydrolysis [15,16]. Many cancer cells secrete ATX [17,18], and it causes the synthesis of LPA [5,17,18]. In consequence, LPA stimulates cancer cell invasion and metastasis [18]. Therefore, it is considered that 2ccPA suppressed synthesis of LPA via inhibition of ATX activity, and as a result, cancer cell invasion was inhibited. In this study, we did not detect significant difference between (*R*)-2ccPA and (*S*)-2ccPA on the inhibitory activities on ATX and cancer cell invasion.

It has been reported that LPA induces neuropathic pain [6,7]. The neuropathic pain is suggested to develop aberrant myelination (splitting, detachment and loss of myelin), and LPA decreases in gene expression and protein production of the myelin-associated proteins, and as a result, demyelination was induced [19,20]. Moreover, LPA induces the loss of C-fiber responses and the down-regulation of spinal substance P signal [21]. It induces that sprouting A-fiber of LPA-induced demyelination to C-fiber or ephaptic crosstalk between different types of fibers, and in consequence, abnormal sensory perceptions might be caused by getting cross at the level of sensory fibers or at the level of pain transmission in the spinal dorsal horn [19]. From these findings, it is suggested that 2ccPA may suppress the synthesis of LPA by inhibition of ATX activity, and as a result, neuropathic pain may be inhibited. On the other hand, it has been clarified that 2ccPA suppressed nociceptive reflex via C-fiber [10]. While the mechanisms of 2ccPA suppressing pain are still unknown, we did not detect significance to anti-nociceptive effects by (*R*)-2ccPA and (*S*)-2ccPA.

Until now, eight kinds of LPA receptor have been reported. Among these receptors, LPA_1 and LPA_3 are suggested to be involved in cancer cell invasion [22-24] and LPA-induced neuropathic pain [25-27]. It has been reported that LPA_1 and LPA_3 did not have stereospecificity for LPA [28]. Recently, it has been reported that (*S*)-3ccPA was slightly more efficacious in the activation on LPA_5 compared to the (*R*)-3ccPA [14]. It needs to investigate stereoselectivity of LPA receptors toward to 2ccPA. In the case of 2ccPA, it has been reported that it can activate LPA_1, LPA_3 and LPA_5 receptors, but the ability is less than that of LPA [29]. In this study, however, we did not detect the significant difference between 2ccPA enantiomers to the inhibition of ATX activity, cancer cell invasion and C-fiber suppression. Therefore it is suggested that target molecules, which related to 2ccPA on inhibition of cancer cell invasion and pain relief, did not have stereoselectivity for each enantiomers.

In conclusion, our study shows the method of enantiopure synthesis of 2ccPA, and it suggests that the chirality of 2ccPA is not involved in 2ccPA biological functions such as ATX inhibition, cancer cell invasion and anti-nociceptive effects. Based on these findings, racemic 2ccPA is considered to be utilized as an effective compound for therapeutic applications for cancer and pain.

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