Supporting Information

Preparation of apoastaxanthinals and evaluation of their anti-inflammatory action against lipopolysaccharide-stimulated macrophages and adipocytes

Naoki Takatani[†], Fumiaki Beppu[†], Yumiko Yamano[‡], Takashi Maoka[§], Kazuo Miyashita[†], Masashi Hosokawa[†,*]

[†] Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

[‡] Comprehensive Education and Research Center, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

[§] Research Institute for Production and Development, 15 Shimogamo-morimoto-cho, Sakyo-ku, Kyoto 606-0805, Japan

***Corresponding author**

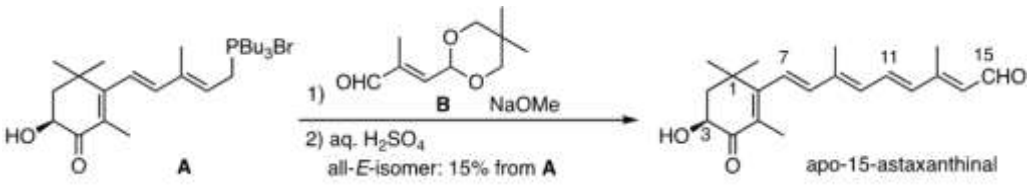
Masashi Hosokawa

E-mail: hoso@fish.hokudai.ac.jp

Tel & Fax: +81-138-40-5530

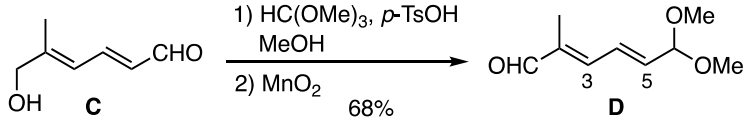
Contents

Figure S1. Synthesis of apo-15-astaxanthinal



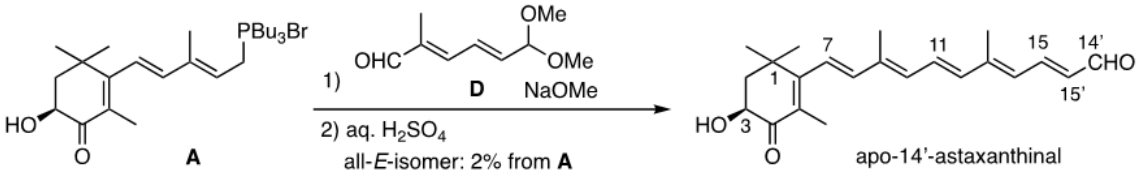
NaOMe (5.18 M in MeOH; 0.29 mL, 1.50 mmol) was added to a stirred solution of phosphonium salt **A** (Yamano et al., 2001) (520 mg, 1.00 mmol) and acetal-aldehyde **B** (Martin et al., 1999) (184 mg, 1.00 mmol) in CH_2Cl_2 (7 mL) at room temperature (rt). After being stirred at rt for 10 min, the mixture was quenched by addition of saturated aq. NH_4Cl and extracted with AcOEt. The extracts were washed with brine and evaporated. H_2SO_4 (1.5 M in H_2O: 0.33 mL, 0.50 mmol) was added to a stirred solution of the resulting residue in THF (10 mL), MeOH (1 mL) and H_2O (1 mL) at rt and stirring was continued for a further 30 min. After being quenched by addition of saturated aq. $NaHCO_3$, the mixture was extracted with AcOEt. The extracts were washed with brine, dried over Na_2SO_4 and evaporated to give a residue, which was purified by flash silica gel column chromatography (AcOEt–*n*-hexane, 3:5 to MeOH-AcOEt–*n*-hexane, 0.1:5:5) to provide an isomeric mixture of condensed products (109 mg, 34% from **A**). This was then purified by preparative HPLC (COSMOSIL 5CN-MS 20 × 250 mm; AcOEt–*n*-hexane, 2:8) to give all-*E*-apo-15-astaxanthinal (48 mg, 15% from **A**) as a yellow viscous oil: UV (EtOH) λ 236, 292, 378 nm; IR ($CHCl_3$) ν 3497 (OH), 1658 (conj. C=O), 1609 and 1585 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.20 and 1.33 (each 3H, s, 1-*gem*-Me), 1.83 (3H, br t, *J* 13, 2-H_{ax}), 1.93 (3H, d, *J* 0.5, 5-Me), 2.06 (3H, d, *J* 0.5, 9-Me), 2.17 (1H, dd, *J* 5.5, 13, 2-H_{eq}), 2.34 (3H, d, *J* 1.5, 13-Me), 3.66 (1H, br s, OH), 4.34 (1H, br dd, *J* 5.5, 14, 3-H), 6.01 (1H, br d, *J* 8, 14-H), 6.32 (1H, br d, *J* 11.5, 10-H), 6.34 (1H, br d, *J* 16, 7-H), 6.40 (1H, d, *J* 16, 8-H), 6.46 (1H, d, *J* 15, 12-H), 7.11 (1H, dd, *J* 11.5, 15, 11-H), 10.13 (1H, d, *J* 8, CHO); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.85 (9-Me), 13.11 (13-Me), 13.89 (5-Me), 26.05 and 30.63 (1-*gem*-Me), 36.78 (C1), 45.36 (C2), 69.22 (C3), 125.93 (C7), 127.46 (C5), 129.97 (C14), 131.43 (C11), 133.13 (C10), 136.95 (C12), 139.13 (C9), 141.19 (C8), 135.91 (C13), 161.56 (C6), 191.09 (CHO), 200.42 (C4) ; HRMS (ESI) *m/z* calcd for $C_{20}H_{26}O_3Na$ $[M+Na]^+$ 337.1774, found 337.1776.

Figure S2. Synthesis of (2*E*,4*E*)-6,6-Dimethoxy-2-methylhexa-2,4-dienal



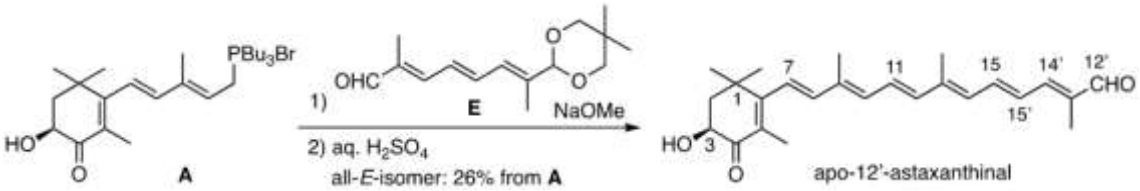
To a solution of hydroxy-aldehyde **C** (Barrero et al., 2011) (960 mg, 7.6 mmol) and $HC(OMe)_3$ (1.25 mL, 11.4 mmol) in MeOH (10 mL) was added *p*-TsOH•H_2O (29 mg, 0.15 mmol) at 0 °C. After being stirred at 0 °C for 10 min, the mixture was quenched by addition of saturated aq. $NaHCO_3$ and extracted with AcOEt. The extracts were washed with brine, dried over Na_2SO_4 and evaporated. The resulting crude acetal was dissolved in ether (10 mL) and *n*-hexane (10 mL), and $NaHCO_3$ (100 mg) and MnO_2 (5.0 g) were added to it. After being stirred at rt for 2 h, the mixture was filtered through a pad of Celite and washed with AcOEt. The filtrate was evaporated to give a residue, which was purified by flash CC (acetone–*n*-hexane, 1:2) to provide acetal-aldehyde **D** (876 mg, 68% for 2 steps) as a colorless oil: UV (EtOH) λ 269 nm; IR ($CHCl_3$) ν 1680 (conj. C=O), 1644 and 1608 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.88 (3H, s, 2-Me), 3.37 (6H, s, OMe × 2), 4.98 (1H, d, *J* 4, 6-H), 6.12 (1H, m, 5-H), 6.80–6.92 (2H, m, 3-H and 4-H), 9.49 (1H, s, CHO); ^{13}C NMR (75 MHz, $CDCl_3$) δ 9.52, 52.71 (C × 2), 101.31, 128.16, 138.10, 139.26, 146.62, 194.99; HRMS (ESI) *m/z* calcd for $C_9H_{15}O_3$ $[M+H]^+$ 171.1016, found 171.1018.

Figure S3. Synthesis of apo-14'-astaxanthinal



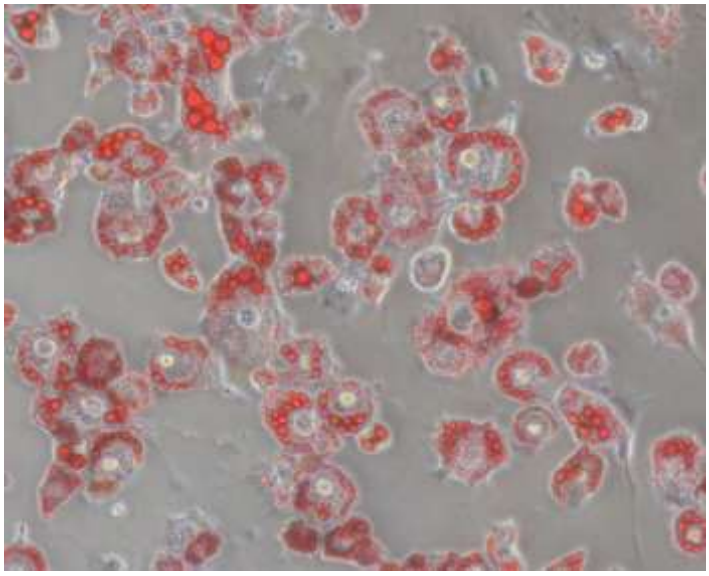
In the same manner as described for the preparation of apo-15-astaxanthinal, phosphonium salt **A** (520 mg, 1.00 mmol) was condensed with acetal-aldehyde **D** (170 mg, 1.00 mmol) and subsequently hydrolyzed with acid. The resulting crude products were purified by flash silica gel column chromatography (AcOEt–*n*-hexane–CH_2Cl_2, 1.5:4:4 to 2:2:4) and then preparative HPLC (COSMOSIL 5CN-MS 20 × 250 mm; AcOEt–*n*-hexane, 2:8) to give all-*E*-apo-14'-astaxanthinal (6.5 mg, 2% from **A**) as an orange foam. The 1H NMR spectral data were identical with those reported (Etoh et al., 2012) : UV-VIS (EtOH) λ 312, 405 nm; IR ($CHCl_3$) ν 3504 (OH), 1668 (conj. C=O), 1603, 1587 and 1573 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.21 and 1.33 (each 3H, s, 1-*gem*-Me), 1.82 (1H, br t, *J* 13.5, 2-H_{ax}), 1.93 (3H, d, *J* 0.5, 5-Me), 2.04 (3H, br s, 9-Me), 2.12 (3H, d, *J* 0.5, 13-Me), 2.17 (1H, dd, *J* 5, 13, 2-H_{eq}), 3.67 (1H, d *J* 1.5, OH), 4.33 (1H, br dd, *J* 5, 13.5, 3-H), 6.21 (1H, dd, *J* 8, 15, 15'-H), 6.30 (1H, br d, *J* 16, 7-H), 6.30 (1H, br d, *J* 11.5, 10-H), 6.40 (1H, br d, *J* 12, 14-H), 6.41 (1H, d, *J* 16, 8-H), 6.47 (1H, d, *J* 15, 12-H), 6.90 (1H, dd, *J* 11.5, 15, 11-H), 7.51 (1H, dd, *J* 12,15, 15-H), 9.63 (1H, d, *J* 8, CHO) ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.77 (9-Me), 13.29 (13-Me), 13.96 (5-Me), 26.11 and 30.69 (1-*gem*-Me), 36.82 (C1), 45.40 (C2), 69.24 (C3), 125.00 (C7), 127.25 (C5), 128.86 (C11), 129.80 (C14), 131.57 (C15'), 133.96 (C10), 137.45 (C9), 137.95 (C12), 141.62 (C8), 145.93 (C13), 147.11 (C15), 161.94 (C6), 193.50 (CHO), 200.45 (C4); HRMS (ESI) *m/z* calcd for $C_{22}H_{29}O_3$ $[M+H]^+$ 341.2111, found 341.2019.

Figure S4. Synthesis of apo-12'-astaxanthinal



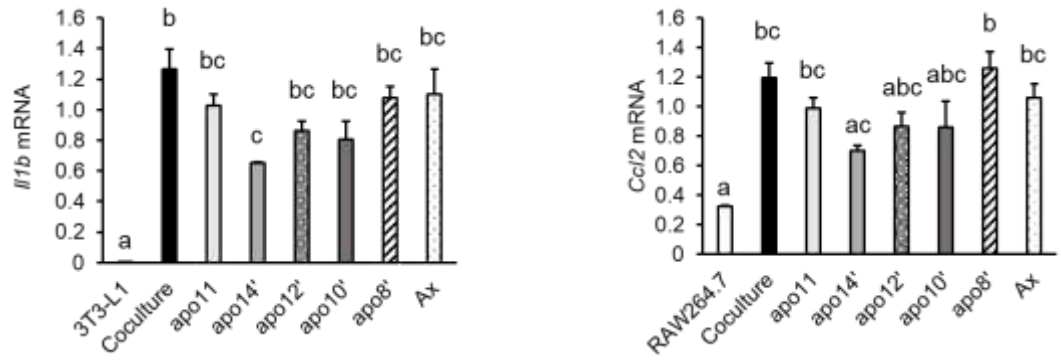
In the same manner as described for the preparation of apo-15-astaxanthinal, phosphonium salt **A** (540 mg, 1.05 mmol) was condensed with acetal-aldehyde **E** (Bernhard et al., 1998) (250 mg, 1.00 mmol) and subsequently hydrolyzed with acid. The resulting crude products were purified by flash silica gel column chromatography (AcOEt–*n*-hexane–CH_2Cl_2, 1.5:4:4 to 2:3:4) to provide all-*E*-apo-12'-astaxanthinal (103 mg, 26% from **A**) as red-orange solids: UV-VIS (EtOH) λ 431 nm; IR ($CHCl_3$) ν 3496 (OH), 1660 (conj. C=O), 1611, 1594, 1579 and 1547 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.21 and 1.33 (each 3H, s, 1-*gem*-Me), 1.82 (1H, br t, *J* = 13.5, 2-H_{ax}), 1.89 (3H, br s, 13'-Me), 1.94 (3H, br s, 5-Me), 2.02 (3H, br s, 9-Me), 2.06 (3H, br s,13-Me), 2.16 (1H, dd, *J* 5.5, 12.5, 2-H_{eq}), 3.68 (1H, d, *J* 2, OH), 4.33 (1H, ddd, *J* 2, 5.5, 14, 3-H), 6.26 (1H, br d, *J* 16, 7-H), 6.31 (1H, br d, *J* 11.5, 10-H), 6.35 (1H, br d, *J* 12, 14-H), 6.42 (1H, d, *J* 16, 8-H), 6.46 (1H, d, *J* 15, 12-H), 6.72 (1H, dd, *J* 11.5, 14.5, 15'-H), 6.78 (1H, dd, *J* 11.5, 15, 11-H), 6.96 (1H, br d, *J* 11.5, 14'-H), 7.03 (1H, dd, *J* 12, 14.5, 15-H), 9.47 (1H, s, CHO); ^{13}C NMR (125 MHz, $CDCl_3$) δ 9.63 (13'-Me), 12.66 (9-Me), 13.03 (13-Me), 13.95 (5-Me), 26.11 and 30.70 (1-*gem*-Me), 36.79 (C1), 45.38 (C2), 69.21 (C3), 124.19 (C7), 126.77 (C11), 127.03 (C5), 128.18 (C15'), 132.19 (C14), 134.54 (C10), 136.06 (C9), 137.30 (C15), 137.38 (C13'), 138.82 (C12), 141.12 (C13), 141.94 (C8), 148.53 (C14'), 162.02 (C6), 194.44 (CHO), 200.43 (C4); HRMS (ESI) *m/z* calcd for $C_{25}H_{33}O_3Na$ $[M+Na]^+$ 403.2244, found 403.2249.

Figure S5. Pictures of differentiated 3T3-L1 adipocytes at Day 10.



3T3-L1 preadipocytes (ATCC CL-173, passage number 4) were cultured in DMEM with 10% FBS containing 100 μg/mL streptomycin and 100 U/mL penicillin. To differentiate, 3T3-L1 cells reaching confluence (on day 2) were replaced in flesh DMEM with 1 μM dexamethasone, 500 μM IBMX, and 10 μg/mL insulin. After incubated for two days (on day 4), the media were replaced to flesh DMEM with 5 μg/mL insulin. The insulin-containing media were changed every two days. On day 10, the cells were stained by Oil Red O.

Figure S6. mRNA expression data of inflammatory cytokines in co-culturing of RAW264.7 macrophages and 3T3-L1 adipocytes



Contact coculture of RAW264.7 and 3T3-L1 cells (day 10) in the presence or absence of carotenoids (5 μM for 24 h). *Il1b* and *Ccl2* mRNA levels were evaluated by quantitative PCR methods. *Actb* was used as the endogenous control. Data are represented as the mean ± SEM (n=3) with different letters ($p < 0.05$). Apo11: apo-11-astaxanthinal. Apo14′: apo-14′-astaxanthinal. Apo12′: apo-12′-astaxanthinal. Apo10′: apo-10′-astaxanthinal. Apo8′: apo-8′-astaxanthinal. Ax: astaxanthin.