Fucoxanthin Derivatives: Synthesis and their Chemical Properties

Shiro Komba*, Eiichi Kotake-Nara and Sachiko Machida

Abstract: Novel fucoxanthin derivatives that could change the size of mixed micelles were synthesized. The mixed micelles under consideration consist of a bile acid and some additives. To change the affinity against a bile acid, we designed the synthesis of a fucoxanthin-lithocholic acid complex. Lithocholic acid is one of the bile acids. The 3-OH on lithocholic acid was protected by a levulinyl group, and the protected lithocholic acid was selectively coupled via an ester linkage to the 3-OH on fucoxanthin to obtain levulinyl-protected lithocholyl fucoxanthin (LevLF). The levulinyl group was then selectively deprotected using hydrazine to obtain a lithocholyl fucoxanthin (LF). The average sizes of the micelles that contained these compounds (fucoxanthin, LevLF, and LF) with a bile acid (sodium taurocholate) were measured. The LevLF induced larger micelles than fucoxanthin or LF. Interestingly, the addition of 1-oleoyl-rac-glycerol induced a more efficient change in the micelle size. The large micelles grew larger, and the small micelles became smaller.

Key words: fucoxanthin, fucoxanthin derivatives, levulinyl protected lithocholyl fucoxanthin, lithocholyl fucoxanthin

1 INTRODUCTION

Fucoxanthin is a xanthophyll-type carotenoid found in brown macroalgae (Eisenia bicyclus, Laminaria japonica, Petalonia binghamiae, Sargassum binderi, Sargassum duplicatum, Sargassum fusiforme, Sargassum plagiophyllum, Scytosiphon lomentaria, Turbinaria turbinata, and Undaria pinnatifida) and in microalgae (Chaetoceros gracilis, Cylindrotheca closterium, Isochrysis aff. galbana, Nitzschia sp., Odontella aurita, and Phaeodactylum tricornutum). As a component of the photochemical system II, it participates in photosynthesis. The structural characteristics of fucoxanthin are an allene bond, a polyene chain, and an epoxy group. In addition, it exhibits anticarcinogenic, anti-obesity, anti-inflammatory, antiangiogenic, and antioxidantive activities. Recently oral administration of fucoxanthin in humans was investigated by Asai et al. and Hashimoto et al. Asai et al. demonstrated the presence of fucoxanthinol in the plasma of all participants after one week of a daily intake of stirred wakame (Undaria pinnatifida). They served stirred wakame containing 6.1 mg fucoxanthin per day for one week, and found 0.8 nM concentrated fucoxanthinol in human plasma 4 h after a single intake. From these results, the bioavailability of fucoxanthinol seems to be lower than that of other dietary carotenoids such as β-carotene, lutein, and astaxanthin. The ab-
The optical rotations were determined in chloroform on a ODS-80T RP-18 GP 250-4.6 column. The powdered dry wakame from China purchased in Japan was milled using a Beckman Coulter spectrophotometer (DU 650). Cells were homogenized using a Taitec probe-type sonicator (VP-5S).

2.3 Purification of fucoxanthin (3)

Fucoxanthin (3) was purified from dry wakame. The efficiency of extraction solvents was examined. We compared ethanol and the mixed solvent chloroform/methanol/water (5/4/1) for the efficiency of fucoxanthin (3) extraction. The fucoxanthin (3) content was determined by the area under the peak on analytical reverse-phase HPLC (condition A) detected at 450 nm. After 1 h and 15 h for ethanol extraction, the extracted fucoxanthin (3) was 0.12 and 0.17 mg/g dry wakame, respectively. On the other hand, in the case of the mixed solvent chloroform/methanol/water (5/4/1) extraction, the extracted fucoxanthin (3) was 0.91 and 1.2 mg/g dry wakame, respectively. Based on these observed extraction efficiencies we used the mixed solvent chloroform/methanol/water (5/4/1) for fucoxanthin (3) extraction. Dry wakame from China purchased in Japan was milled. The powdered dry wakame (427 g) in chloroform/methanol/water (500 mL/400 mL/100 mL) was stirred at room temperature for 64 h in the absence of light. The insoluble materials were filtered off by using celite, and washed with chloroform/methanol/water (5/4/1) several times. Then, the combined filtrate and washings were concentrated. Chromatography of the residue on silica gel with ethyl acetate/toluene (1/5) gave fucoxanthin (3, 464 mg, 1.09 mg/g dry wakame). The 1H-NMR and 13C-NMR spectra were identical to those previous reported.

2.4 Synthesis of fucoxanthin derivatives

2.4.1 Synthesis of 3-O-(4-oxopentanoyl)-lithocholic acid (2)

To a solution of lithocholic acid (1; 100 mg, 0.266 mmol) in pyridine/dichloromethane (1 mL/1 mL) was added levulinic anhydride (114 mg, 0.532 mmol) and 4-(dimethylamino)pyridine (32 mg, 0.26 mmol) at room temperature (Fig. 1). The mixture was stirred at room temperature for 2 h and then diluted with chloroform, washed with 2 M HCl, and the organic phase was concentrated in vacuo. Chromatography of the residue on silica gel with ethyl acetate/n-hexane (1/2) containing 0.1% acetic acid afforded 2 (90.7 mg, 72%), \( [\alpha]_D^1 + 39 (c = 1.8, \text{chloroform}) \). 1H-NMR (400 MHz, CDCl3): \( \delta = 0.64 (s, 3H, CH-18), 0.92 (s, 3H, CH-19), 0.92 (d, 3H, J = 6.2 Hz, CH-21), 0.97-1.98 (m, 26H), 2.19 (s, 3H, CH3-COOH), 2.21-2.43 (m, 2H, CH2-23), 2.54 (t, 2H, J = 6.5 Hz, CH3-COOH), 2.74 (t,
Fig. 1 Synthesis of fucoxanthin derivatives. AcOH: acetic acid, CH$_2$Cl$_2$: dichloromethane, DIC: N,N'-diisopropylcarbodiimide, DMAP: 4-(dimethylamino)pyridine, NH$_2$NH$_2$H$_2$O: hydrazine monohydrate, pyr.: pyridine, r.t.: room temperature.

2H, $J = 6.5$ Hz, CH$_3$(COCH$_2$CH$_2$COO), 4.71 (m, 1H, CH-3). $^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ = 12.04 (CH$_3$-18), 18.24 (CH$_2$-19), 31.04 (CH$_2$-23), 32.17 (CH$_2$-2 or 4), 34.57 (C-10), 35.01 (CH$_3$-1), 35.29 (CH$_2$-20), 35.78 (CH-8), 38.03 (CH$_2$(COCH$_2$CH$_2$COO), 40.12 (CH$_2$-12), 40.41 (CH-9), 41.90 (CH-5), 42.73 (C-13), 55.97 (CH-17), 56.45 (CH-14), 74.75 (CH-3), 172.33 (CH$_2$(COCH$_2$CH$_2$COO), 180.26 (C-24), 206.92 (CH$_2$(COCH$_2$CH$_2$COO). ESI-FT-MS: Calcd. for C$_{30}$H$_{46}$O$_{13}$Na$^+$ (M + Na)$^+$: 497.3237, found m/z: 497.3238.

2.4.2 Synthesis of 3-O-[3-O-(4-oxopentanoyl)-lithocholyl]-fucoxanthin (4)

To a solution of purified fucoxanthin (3; 157 mg, 0.238 mmol) and compound 2 (509 mg, 1.07 mol) in dichloromethane (20 mL) was added N,N'-diisopropylcarbodiimide (1.1 mL, 7.0 mmol) and 4-(dimethylamino)pyridine (175 mg, 1.43 mmol) at 0°C. The mixture was stirred at 0°C for 3 h, quenched with methanol (20 mL), and then concentrated in vacuo. Chromatography of the residue on silica gel with ethyl acetate/toluene (1/3) afforded 4 (234 mg, 88%). $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ = 0.64 (s, 3H, LC CH$_3$-18), 0.91 (d, 3H, $J = 6.8$ Hz, LC CH$_3$-21), 0.92 (s, 3H, LC CH$_3$-19), 0.96 (s, 3H, Fx CH$_3$-17), 1.01-2.39 (m, 36H, LC CH-5, 8, 9, 14, 17, 20 and LC 15CH$_3$), 1.07 (s, 6H, Fx CH$_3$-16, 17), 1.22 (s, 3H, Fx CH$_3$-18), 1.35 (s, 3H, Fx CH$_3$-18'), 1.38 (s, 3H, Fx CH$_3$-16'), 1.82 (s, 3H, Fx CH$_3$-19'), 1.95 (s, 3H, Fx CH$_3$-19), 1.99 (s, 6H, Fx CH$_3$-20, 20'), 2.04 (s, 3H, Ac), 2.19 (s, 3H, CH$_3$(COCH$_2$CH$_2$COO), 2.56 (t, 2H, $J = 6.5$ Hz, CH$_2$(COCH$_2$CH$_2$COO), 2.61 (d, 1H, $J = 18.4$ Hz, Fx CH-7a), 2.74 (t, 2H, $J = 6.5$ Hz, CH$_3$(COCH$_2$CH$_2$COO), 3.66 (d, 1H, $J = 18.4$ Hz, Fx CH-7b), 4.70 (m, 1H, LC CH-3), 4.88 (m, 1H, Fx CH-3), 5.38 (m, 1H, Fx CH-3'), 6.05 (s, 1H, Fx CH-8'), 6.13 (d, 1H, $J = 11.4$ Hz, Fx CH-10'), 6.27 (d, 1H, $J = 11.4$ Hz, Fx CH-1'), 6.35 (d, 1H, $J = 14.9$ Hz, Fx CH-12), 6.41 (d, 1H, $J = 11.4$ Hz, Fx CH-14), 6.57 (dd, 1H, $J = 12.0$ Hz, Fx CH-11), 6.60 (broad t, 1H, $J = 13.1$ Hz, Fx CH-11'), 6.63 (t, 1H, $J = 11.9$ Hz, Fx CH-15), 6.67 (d, 1H, $J = 14.9$ Hz, Fx CH-12), 6.75 (dd, 1H, $J = 11.7$ Hz, $J = 14.0$ Hz, Fx CH-15), 7.14 (d, 1H, $J = 11.2$ Hz, Fx CH-10). $^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ = 11.81 (Fx CH$_3$-19), 12.05 (LC CH$_3$-18), 12.74 (Fx CH$_3$-20 or 20'), 12.90 (Fx CH$_3$-20 or 20'), 13.99 (Fx CH$_3$-19'), 18.28 (LC CH$_3$-21), 20.84 (LC CH$_3$-17), 20.95 (Fx CH$_3$-18), 21.39 (CH$_3$(COO)), 23.33 (LC CH$_3$-19), 24.19 (LC CH$_3$-15), 24.77 (Fx CH$_3$-16), 26.33 (LC CH$_3$-7), 26.61 (LC CH$_3$-2 or 4), 27.02 (LC CH$_3$-6), 27.81 (Fx CH$_3$-17), 28.18 (LC

J. Oleo Sci. 64, (9) 1009-1018 (2015)
To a solution of compound 4 (144 mg, 0.129 mmol) in pyridine (5 mL) was added a 1 M solution of hydrazine monohydrate in pyridine/acidic acid (3/2)20 to (5 mL) at room temperature. The mixture was stirred at room temperature for 20 min and then diluted with ethyl acetate, washed with 2 M HCl, and the organic phase was concentrated in vacuo. Chromatography of the residue on silica gel with ethyl acetate/toluene (1/3) afforded 5 (93.6 mg, 71%).

\[ \text{H-NMR (400 MHz, CDCl3):} \delta = 0.64 (s, 3H, LC CH-19), 0.91 (d, 3H, J = 6.0 Hz, LC CH-21), 0.92 (s, 3H, LC CH-19), 0.96 (s, 3H, LC CH-17), 1.00-2.83 (m, 36H, LC CH-5, 8, 9, 14, 17, 20 and LC CH-16), 1.22 (s, 3H, LC CH-18), 1.35 (s, 3H, LC CH-18), 1.38 (s, 3H, LC CH-16), 1.81 (s, 3H, LC CH-19), 1.94 (s, 3H, LC CH-19), 1.99 (s, 6H, LC CH-20, 20'), 2.04 (s, 3H, Ac), 2.61 (d, 1H, J = 18.5 Hz, LC CH-7a), 3.62 (m, 1H, LC CH-3), 3.66 (d, 1H, J = 18.4 Hz, LC CH-7b), 4.87 (m, 1H, LC CH-3), 5.38 (m, 1H, LC CH-3'), 6.05 (s, 1H, LC CH-8), 6.13 (d, 1H, J = 10.8 Hz, LC CH-10), 6.27 (d, 1H, J = 11.5 Hz, LC CH-14), 6.35 (d, 1H, J = 15.0 Hz, LC CH-12), 6.41 (d, 1H, J = 11.4 Hz, LC CH-14), 6.57 (dd, 1H, J = 12.0 Hz, J = 15.0 Hz, LC CH-11), 6.60 (broad d, 1H, J = 13.7 Hz, LC CH-11), 6.63 (t, 1H, J = 11.8 Hz, LC CH-15), 6.67 (d, 1H, J = 14.9 Hz, LC CH-12), 6.75 (dd, 1H, J = 11.7 Hz, J = 14.0 Hz, LC CH-15), 7.14 (d, 1H, J = 10.8 Hz, LC CH-10). \]

\[ ^{13}C-NMR (100 MHz, CDCl3):} \delta = 11.81 (LC CH-19), 12.05 (LC CH-21), 12.74 (LC CH-20 or 20'), 12.89 (LC CH-20 or 20'), 13.99 (LC CH-19), 18.27 (LC CH-21), 20.83 (LC CH-11), 20.95 (LC CH-18), 21.39 (LC CH-COOO), 23.38 (LC CH-19), 24.21 (LC CH-15), 24.77 (LC CH-16), 26.42 (LC CH-7), 27.20 (LC CH-6), 27.80 (LC CH-17), 28.18 (LC CH-16), 29.18 (LC CH-16), 30.57 (LC CH-2), 30.99 (LC CH-22), 31.25 (LC CH-18'), 31.60 (LC CH-23), 32.07 (LC CH-17'), 34.58 (LC C-10), 35.23 (LC CH-20 or Fx C-1'), 35.30 (LC CH-20 or Fx C-1'), 35.76 (LC CH-8 or Fx C-1), 36.47 (LC CH-4), 37.77 (LC CH-2 or 4), 40.17 (LC CH-12), 40.44 (LC CH-9), 40.68 (LC CH-7), 42.12 (LC CH-5), 42.75 (LC CH-13), 43.06 (LC CH-2 or 4), 45.24 (LC CH-2'), 45.43 (LC CH-2'), 55.98 (LC CH-17), 56.50 (LC CH-14), 65.84 (Fx C-5), 67.08 (Fx C-6), 67.37 (Fx CH-3), 68.02 (Fx CH-3'), 71.88 (LC CH-3), 72.65 (Fx C-5'), 103.36 (Fx CH-8'), 117.51 (Fx C-6'), 123.35 (Fx CH-11), 125.67 (Fx CH-11'), 128.51 (Fx CH-10'), 129.40 (Fx CH-15), 132.15 (Fx CH-14'), 132.51 (Fx CH-15' and Fx C-9'), 134.52 (Fx C-9), 135.38 (Fx C-13), 136.62 (Fx CH-14), 137.09 (Fx CH-12'), 138.06 (Fx C-13'), 139.02 (Fx CH-10), 145.00 (Fx CH-12), 170.38 (CH2COO), 172.29 (CH2COCH2COO), 173.71 (LC CH-24), 197.60 (Fx CH-8'), 202.34 (Fx CH-7'), 206.78 (CH2COCH2COO). ESI-FT-MS: Calcd. for C40H60O9Na+ (M + Na)+: 1137.7365, found m/z: 1137.7366.

2.4.3 Synthesis of 3-O-lithocholyloxyfucoxanthin (5)

Water (200 μL) was added to each of the dried residues. The final concentrations of each component are shown in Table 1. All of the mixtures were clearly dissolved in water, filtered through 0.2 μm pore size filters, and the average diameter of the micelles was measured by DLS. All experiments were done in dim light. The results are shown in Table 1.

2.6 Enzymatic hydrolysis assay

The following three types of solutions of micelles in water as described in "2.5 Measurements of the size of micelles" were used for an enzyme assay: 1: 2 mM sodium taurocholate and 100 μM fucoxanthin (3) in ethanol (20 μL), 2: 1 mM 1-oleoyl-rac-glycerol in ethanol (20 μL), and 3: 1 mM 1-oleoyl-rac-glycerol in ethanol (20 μL) and 1 mM fucoxanthin (3) in ethanol (20 μL) and 100 μM LevLF (4) in ethanol (200 μL), 8: 1 mM 1-oleoyl-rac-glycerol in ethanol (20 μL) and 100 μM LevLF (4) in ethanol (200 μL). To a solution of each type of micelle (10 μL) was added phosphate buffered saline (PBS) (60 μL) and to each, one of the following three types of enzymes, 1: 180 units/mL esterase from porcine liver in PBS (30 μL), 2: 180 units/mL lipase from porcine pancreas in PBS (30 μL), 3: 180 units/mL cholesterol esterase from Pseudomonas sp. in PBS (30 μL). After 62 h at 37°C, the reaction mixtures were analyzed by analytical reverse-phase HPLC (condition A) detected at 450 nm. The eluates
**Table 1** Measurements of micelle size by DLS and FWHM<sup>a</sup> by spectrophotometer.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sodium taurocholate (mM)</th>
<th>1-Oleoyl-rac-glycerol (μM)</th>
<th>Fucoxanthin (3) (μM)</th>
<th>LevLF (4) (μM)</th>
<th>LF (5) (μM)</th>
<th>Average diameter of micelles (nm)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (nm)</th>
<th>FWHM&lt;sup&gt;b&lt;/sup&gt; (nm)</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>2</td>
<td>100</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>2</td>
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<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>111</td>
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<tr>
<td>8</td>
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<td>100</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
<td>438</td>
<td>115</td>
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</table>

<sup>a</sup>FWHM: Full width at half maximum.  <sup>b</sup>Fucoxanthin (3) in EtOH (λ<sub>max</sub>: 449 nm, FWHM: 90.5 nm), LevLF (4) in EtOH (λ<sub>max</sub>: 448 nm, FWHM: 92.5 nm), LF (5) in EtOH (λ<sub>max</sub>: 450 nm, FWHM: 92.0 nm).  
<sup>c</sup>: Not containing.  
<sup>d</sup>N.D.: No data.

Fig. 2 The deduced products of enzymatic hydrolysis of fucoxanthin (3) and fucoxanthin derivatives (4 and 5).

producing the observed peaks were collected and analyzed by ESI-FT-MS to deduce the structures (Fig. 2). In addition, the yields were calculated from the area under the peak. The results are shown in Table 2. Compound 6, ESI-FT-MS: Calcd. for C₆₈H₇₀O₉Na⁺ (M + Na)<sup>+</sup>: 1055.7334, found m/z: 1055.7338. Compound 8, ESI-FT-MS: Calcd. for C₆₉H₇₁O₁₈⁺ (M + H – H₂O)<sup>+</sup>: 957.6972, found m/z: 957.6972.

2.7 Cellular uptake of micellar fucoxanthin derivatives

2.7.1 Cell culture

Caco-2 cells (American Type Culture Collection, Rockville, MD) were maintained in 10-cm dishes (Corning Inc., Corning, NY) containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 40,000 U/L penicillin, 40 mg/L streptomycin and 0.1 mM nonessential amino acids. Cells were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were grown by passaging twice per week. To obtain differentiated Caco-2 cell monolayers, the cells at passages of 33–60 were seeded into 24-well plates at a density of 5 × 10<sup>³</sup> cells per well containing 0.5 mL of the culture medium, and the medium was replaced with fresh medium twice per week for 22 days.

2.7.2 Cellular uptake

Fucoxanthin derivatives were delivered to Caco-2 cells as the mixed micelles referred to in the previously described method<sup>20</sup>. Briefly, the final mixed micelles concentration of each component in the serum-free DMEM medium was as follows: 2 mM sodium taurocholate, 100 μM 1-oleoyl-rac-glycerol, 33.3 μM oleic acid, 50 μM 1-palmitoyl-sn-glycero-3-phosphocholine, and 1.0 μM each carotenoids (fucoxanthin (3), LevLF (4), or LF (5)). The differentiated monolayers of the Caco-2 cells in a 24-well plate were supplemented with 0.5 mL of the each mixed micelles. After incubation in the cell culture section for 2 h, the monolayers were washed with PBS containing 10 mM sodium taurocholate and then washed twice with PBS. They were homogenized in 1 mL of PBS containing 0.01 mL of 0.2 mM α-tocopherol/methanol as an antioxidant, with a probe-type sonicator. An aliquot of each homogenized cell was taken to determine the protein content.<sup>27</sup> To extract the carotenoids, 0.8 mL ethanol, 0.8 mL of ethyl acetate, and 0.8 mL of n-hexane were added to these homogenized cell (0.8 mL), and the solution was agitated with a Vortex Mixer after each addition. The resultant upper phase of n-hexane-ethyl acetate was withdrawn. The lower phase was similarly extracted with 0.8 mL of ethyl acetate and 0.8 mL of n-hexane. The combined extract was dried
in a centrifugal evaporator. The extract was then dissolved in 0.2 mL of dimethyl sulfoxide/methanol/water (2/7/1) for fucoxanthin (3) or dimethyl sulfoxide/methanol (2/7) for LevLF (4) and LF (5). The residues were analyzed by analytical reverse-phase HPLC condition B. The results are shown in Fig. 3. The data were tested for homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, the data were analyzed by one-way ANOVA, followed by the Tukey-Kramer test. P-values < 0.05 were considered significant.

3 RESULTS AND DISCUSSION

3.1 Synthesis of fucoxanthin derivatives

We designed novel fucoxanthin derivatives to change the affinity for bile acid. Lithocholic acid, one of the bile acids, was selected due to its high hydrophobicity. It contains only one hydroxyl group, while other bile acids, cholic acid, chenodeoxycholic acid, and deoxycholic acid, contain three, two, and two hydroxyl group, respectively. There was a case in which the compounds with increased hydrophobicity also induced high absorption \(^2\). We designed lithocholic acid-linked fucoxanthin via ester linkage (compounds 4 and 5) with the aim of releasing fucoxanthin (or fucoxanthinol) by intracellular hydrolase enzyme. The difference between compounds 4 and 5 is the hydrophobicity. The difference in hydrophobicity induces the difference in affinity for bile acid. The 3-OH of lithocholic acid (1) was protected by the levulinyl group using levulinic anhydride to synthesize levulinyl-protected lithocholic acid (2) in 72% yield (Fig. 1). A significant \(^1\)H NMR signal of compound 2 was H-3 (δ 4.71). The levulinyl group has two roles. It provides hydrophobicity and an orthogonal protecting group for the hydroxyl group. It could be selectively removed by hydrazine without influencing the other ester group. The purified fucoxanthin (3) from wakame was coupled with protected lithocholic acid (2) by \(N,N'\)-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)pyridine (DMAP) in dichloromethane to obtain lev-lithocholyl-fucoxanthin (4: LevLF) in 88% yield. The coupling position was confirmed.

Table 2  Enzymatic hydrolysis assays.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Enzyme (a)</th>
<th>Products (yield) (b)</th>
</tr>
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<td>Fucoxanthin (3)</td>
<td>Esterase (a)</td>
<td>Fucoxanthinol (6, 84%)</td>
</tr>
<tr>
<td>2</td>
<td>Fucoxanthin (3)</td>
<td>Lipase (b)</td>
<td>Fucoxanthinol (6, 51%)</td>
</tr>
<tr>
<td>3</td>
<td>Fucoxanthin (3)</td>
<td>Cholesterol esterase (c)</td>
<td>Fucoxanthinol (6, 79%)</td>
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<tr>
<td>4</td>
<td>LevLF (4)</td>
<td>Esterase (a)</td>
<td>no react</td>
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<tr>
<td>5</td>
<td>LevLF (4)</td>
<td>Lipase (b)</td>
<td>LF-OH (8, 4%), LF (5, 17%)</td>
</tr>
<tr>
<td>6</td>
<td>LevLF (4)</td>
<td>Cholesterol esterase (c)</td>
<td>Fucoxanthinol (6, 2%), LF-OH (8, 5%), LevLF-OH (7, 55%)</td>
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<td>7</td>
<td>LF (5)</td>
<td>Esterase (a)</td>
<td>LF-OH (8, 0.5%)</td>
</tr>
<tr>
<td>8</td>
<td>LF (5)</td>
<td>Lipase (b)</td>
<td>LF-OH (8, 6%)</td>
</tr>
<tr>
<td>9</td>
<td>LF (5)</td>
<td>Cholesterol esterase (c)</td>
<td>LF-OH (8, 68%)</td>
</tr>
</tbody>
</table>

\(a\) Esterase: Esterase from porcine liver. \(b\) Lipase: Lipase from porcine pancreas. \(c\) Cholesterol esterase: Cholesterol esterase from \(Pseudomonas\) sp. \(d\) Products were deduced by ESI-FT-MS and the yields were determined by HPLC.

Fig. 3  Comparison of the uptake of carotenoids by Caco-2 cells. Differentiated Caco-2 cell monolayers (22-day-old) were incubated in serum-free DMEM containing micelles composed of 2 mM sodium taurocholate, 100 μM 1-oleoyl-rac-glycerol, 33.3 μM oleic acid, 50 μM 1-palmitoyl-sn-glycero-3-phosphocholine, and 1.0 μM each carotenoids for 2 h. The bars showed, from left to right, amounts of fucoxanthin (3) plus fucoxanthinol (6), LevLF (4) plus LevLF-OH (7), and LF (5) plus LF-OH (8), respectively. The data represent the mean ± standard deviation of three wells. Replicate experiments demonstrated a similar trend. The values not sharing a common letter are significantly different by the Tukey-Kramer test \(p < 0.05\).
Fucoxanthin derivatives

J. Oleo Sci. 64, (9) 1009-1018 (2015)

by \(^1\text{H}\) and \(^{13}\text{C}\) NMR signals of \(\text{LevLF}(4)\); H-3(\(\delta \) 4.88) and C-3(\(\delta \) 67.38) of fucoxanthin residue, compared with the signals of fucoxanthin (3); H-3(\(\delta \) 3.82) and C-3(\(\delta \) 64.18). The levulinyl protecting group of compound 4 was selectively removed using 1 M solution of hydrazine monohydrate in pyridine/acetic acid (3/2) for 20 min to obtain lithocholyl-fucoxanthinol \(\text{LF}(5)\) in 71 % yield. A significant \(^1\text{H}\) NMR signal of \(\text{LF}(5)\) was H-3(\(\delta \) 3.62) of the lithocholic acid residue. These compounds were characterized with \(^1\text{H}\) NMR, \(^{13}\text{C}\) NMR, HSQC NMR, HMBC NMR, and ESI-FT-MS. The maximum absorption wavelengths (\(\lambda_{\text{max}}\)) for these compounds 4 and 5 were determined to be 448 nm and 450 nm, respectively, and the molecular absorption coefficients (\(\varepsilon\)) in ethanol for them were also determined to be 50400 and 69400, respectively.

3.2 Measurements of micelles size

The micelle size is one of the important factors affecting absorption by intestinal epithelial cells. The average size of micelles that contain these compounds (fucoxanthin (3), \(\text{LevLF}(4)\), and \(\text{LF}(5)\)) with a bile acid (sodium taurocholate) were measured by DLS (Table 1). In addition, we verified the effect of 1-oleoyl-rac-glycerol as an additive for micelles. As the results show in Table 1, comparing with single micelle of sodium taurocholate (44 nm), the mixed micelles of each fucoxanthin (3, 33 nm) and \(\text{LF}(5)\) (33 nm) led to smaller micelles (entry 3 and 5), whereas \(\text{LevLF}(4)\) (59 nm) led to larger micelles (entry 4). Interestingly, the addition of 1-oleoyl-rac-glycerol induced a more efficient change in the micelle size. The large micelles grew larger, and the small micelles became smaller. Triple-mixed micelles with \(\text{LevLF}(4)\), sodium taurocholate, and 1-oleoyl-rac-glycerol formed the largest micelle with a diameter of 68 nm. On the other hand, triple-mixed micelles using \(\text{LF}(5)\), sodium taurocholate, and 1-oleoyl-rac-glycerol made the smallest micelles with diameters as low as 12 nm. We succeeded in inducing different micelle sizes merely based on whether the 3-OH of \(\text{LF}(5)\) was protected or not by the levulinyl group. The interaction between these compounds (fucoxanthin (3), \(\text{LevLF}(4)\), and \(\text{LF}(5)\)) and a bile acid (sodium taurocholate) was confirmed by the measurements of full width at half maximum (FWHM) \(^{32}\) in Table 1. FWHM values of the micelles (entries 3 to 8) increased in comparison with the ethanol solutions of each compound (3, 4, and 5), indicating these compounds formed a complex in the micellar inside.

3.3 Enzymatic hydrolysis assay

The enzymatic hydrolysis of fucoxanthin (3), \(\text{LevLF}(4)\), and \(\text{LF}(5)\) was investigated to determine the fate of these compounds after absorption by intestinal epithelial cells. The metabolic fate of fucoxanthin (3) in mice revealed its conversion into two metabolites, fucoxanthinol (6) and amarouciaxanthin A. By using PC-3 cells, fucoxanthin (3), fucoxanthinol (6), and amarouciaxanthin A demonstrated \(IC_50\) values of 3.0 \(\mu\)M, 2.0 \(\mu\)M, and 4.6 \(\mu\)M, respectively, indicating that fucoxanthinol (6) was the most active form \(^{39}\). We designed \(\text{LevLF}(4)\) and \(\text{LF}(5)\) consisting of ester linkages to be able to release fucoxanthinol (6) by esterase. A compound that could release fucoxanthinol (6) has the possibility of being a bioactive compound. We tested three types of enzymes; esterase from porcine liver, lipase from porcine pancreas, and cholesterol esterase from Pseudomonas sp. As the results show in Table 2, fucoxanthin (3) was hydrolyzed to fucoxanthinol (6) by esterase and cholesterol esterase as almost the same rate. On the other hand, \(\text{LevLF}(4)\) and \(\text{LF}(5)\) was effectively hydrolyzed by cholesterol esterase. Interestingly, \(\text{LevLF}(4)\) was the only one to have hydrolyzed by an enzyme the ester linkage between lithocholic acid and fucoxanthin. From the products ratio, the hydrolysis of \(\text{LevLF}(4)\) was projected in the following order; at the first step, the acetyl group of \(\text{LevLF}(4)\) was hydrolyzed to \(\text{LevLF-OH}(7)\). In the second step, \(\text{LevLF-OH}(7)\) was hydrolyzed to fucoxanthanol (6) or \(\text{LF-OH}(8)\). The levulinyl group of \(\text{LevLF}(4)\) was important for enzymatic hydrolysis of ester linkage between lithocholic acid and fucoxanthin because without the levulinyl protecting group, no fucoxanthinol production was induced (entries 7 to 9).

3.4 Cellular uptake of micellar fucoxanthin derivatives

To evaluate the efficiency of intestinal absorption for fucoxanthin derivatives, we used Caco-2 cells as a model of the intestinal epithelial cells. Fucoxanthin derivatives (\(\text{LevLF}(4)\) and \(\text{LF}(5)\)) were compared with fucoxanthin (3) for Caco-2 cell uptake. As the results show in Fig. 3, unfortunately no advantage for uptake was observed as a result of the chemical modification. The fucoxanthin (3) -treated cells formed fucoxanthanol (6), which is a hydrolysis of fucoxanthin (3). The amount of fucoxanthinol (6) in the cells was one-half that of the fucoxanthin (3). Similarly, the \(\text{LevLF}(4)\) -treated cells could possibly form fucoxanthin (3), fucoxanthinol (6), \(\text{LF}(5)\), \(\text{LF-OH}(8)\), and \(\text{LevLF-OH}(7)\). On the other hands, \(\text{LF}(5)\)-treated cells could possibly form fucoxanthin (3), fucoxanthinol (6), \(\text{LF-OH}(8)\). However, only \(\text{LevLF-OH}(7)\) and \(\text{LF-OH}(8)\) as a hydrolysat from \(\text{LevLF}(4)\) and \(\text{LF}(5)\) was observed, respectively. In addition, the ratio of hydrolysat amount to the total uptake amounts was approximately 5%. These results suggested that the cholesterol esterase activity for the hydrolysis of \(\text{LevLF}(4)\) and \(\text{LF}(5)\) was low in the Caco-2 cells used in the present study as previously reported \(^{40}\).

4 CONCLUSION

We designed and successfully synthesized novel fucoxanthin derivatives to effect a change in their affinity to the cells.
We demonstrated the dependency on structural differences in micelle sizes. Depending on the presence or absence of the hydroxyl-protecting group, the micelles of different micelle sizes are induced. From the enzymatic hydrolysis assay, LevLF(4) could be hydrolyzed by the enzyme to release fucoxanthinol (6), which is the active form of fucoxanthin (3). In addition, this result showed the potential of LevLF(4) as a bio-active compound to release the fucoxanthinol (6) inside the cell. Unfortunately, from the Caco-2 cell uptake assay, no advantage was observed resulting from the chemical modification of fucoxanthin for absorption efficiency, unless different micelle sizes are induced and hydrophobicity is increased. From our results, the micelle size may not influence absorption efficiency for Caco-2 cells. However, from the Pa (probability “to be active”) values of PASS (Prediction of Activity Spectra for Substances) online approach (www.pharmaexpert.ru/PASSOnline), these fucoxanthin derivatives may have an advantage compared with fucoxanthin in their chemopreventive activity, anticarcinogenic activity, and proliferative diseases treatment activity (Table 3). Continuous studies of these fucoxanthin derivatives are in progress and will be reported elsewhere.

ACKNOWLEDGMENT

We thank Dr. M. Kameyama and her staff for the ESI-FT-MS measurements and Dr. Nishimoto for performing the enzyme assays.

Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/jos.64.10.5650/jos.e.15039

Table 3  Pa (probability “to be active”) values for predicted biological activities* of compounds.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Apoptosis agonist</th>
<th>Chemopreventive</th>
<th>Antineoplastic</th>
<th>Anticarcinogenic</th>
<th>Free radical scavenger</th>
<th>Antioxidant</th>
<th>Proliferative diseases treatment</th>
<th>Antiobesity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fucoxanthin (3)</td>
<td>0.990</td>
<td>0.986</td>
<td>0.980</td>
<td>0.974</td>
<td>0.968</td>
<td>0.967</td>
<td>0.966</td>
<td>0.960</td>
</tr>
<tr>
<td>2</td>
<td>Fucoxanthinol (6)</td>
<td>0.994b</td>
<td>0.976</td>
<td>0.986b</td>
<td>0.948</td>
<td>0.953</td>
<td>0.975b</td>
<td>0.950</td>
<td>0.982b</td>
</tr>
<tr>
<td>3</td>
<td>LevLF (4)</td>
<td>0.964</td>
<td>0.997b</td>
<td>0.954</td>
<td>0.981b</td>
<td>0.758</td>
<td>0.880</td>
<td>0.990b</td>
<td>0.848</td>
</tr>
<tr>
<td>4</td>
<td>LF (5)</td>
<td>0.967</td>
<td>0.998b</td>
<td>0.953</td>
<td>0.991b</td>
<td>0.758</td>
<td>0.885</td>
<td>0.993b</td>
<td>0.872</td>
</tr>
<tr>
<td>5</td>
<td>LevLF-OH (7)</td>
<td>0.964</td>
<td>0.997b</td>
<td>0.950</td>
<td>0.981b</td>
<td>0.734</td>
<td>0.879</td>
<td>0.990b</td>
<td>0.960</td>
</tr>
<tr>
<td>6</td>
<td>LF-OH (8)</td>
<td>0.968</td>
<td>0.997b</td>
<td>0.951</td>
<td>0.987b</td>
<td>0.731</td>
<td>0.892</td>
<td>0.992b</td>
<td>0.891</td>
</tr>
</tbody>
</table>

* Pa value was calculated by the PASS (Prediction of Activity Spectra for Substances) online approach (www.pharmaexpert.ru/PASSOnline). Pa > 0.7 is shown. b More active compound than fucoxanthin is marked.

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