Although the biological activities of n-3 highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) have been well documented in humans1–4 and fish, 5,6 very little is known about the metabolism of HUFA in fish. This is possibly due to the difficulty of using radioactive tracers in feeding experiments of aquatic animals. In the present study we attempted to prepare EPA and phosphatidylcholine (PC) containing a stable isotope, [13C]. We describe the preparation of [13C] EPA and [13C] 1-myristoyl-2-eicosapentenoic phosphatidylcholine ([13C] EPA-PC) containing EPA moiety at the C-2 position by using Nannochloropsis oculate followed by chemical synthesis. As a result, we successfully prepared enough [13C] EPA and [13C] EPA-PC to be applied to rearing experiments with aquatic animals. This paper presents and discusses the study’s results.

To prepare [13C] EPA, N. oculate was cultured in artificial seawater7 (2.7 L/3 L Erlenmeyer flask) with [13C] CO₂ and fertilizers under fluorescent light (3200 lx; approximately 9000 cd) at 25°C for 7 days. The initial density of N. oculate was 6–8 × 10⁶ cells/mL. The fertilizers used were 150 mg/L of ammonium sulfate, 15 mg/L of superphosphate, which included 17% soluble phosphoric acid, 10 mg/L of urea, and 30 mg/L of Clewat-32. Before the inoculation of N. oculate, the culture medium was bubbled with [13C] CO₂, which was formed by adding 5 mL of lactic acid to 1.6 g of [13C] BaCO₃ in a 30-mL flask, and introduced repeatedly into the medium by an air pump. When cell density reached 8–12 × 10⁸ cells/mL, the cells were harvested by centrifugation.

The isolation and identification of reaction products were conducted using medium-pressure liquid chromatography (MPLC), gas–liquid chromatography (GC), GC–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), and ¹H nuclear magnetic resonance spectrometry (NMR). The instruments were operated under the following conditions: the MPLC column was ODS (LP60 C18; Wako Pure Chemical Inc., Osaka, Japan) and an acetonitrile–methanol (9:1 v/v) solvent system was used; GC used a capillary column of Omegawax™ 320 (30 m × 0.32 mm, 0.25 μm film thickness) with a column temperature of 280°C; and the GC-MS was an Hitachi M-80 mass spectrometer and an M-8063 gas chromatograph on a cross-linked fused silica capillary column of Supelcowax (30 m × 0.25 mm i.d., 0.25 μm film thickness). The MS spectrometer was operated in the total ion monitoring mode with electron ionization (ionization energy of 20 eV). The LC-MS was an Hitachi M-1200AP mass spectrometer and the high-performance liquid chromatograph was a LaChrom L-7100 with a skimmer cone voltage of 150 V and a mass collection range of 10–100 m/z. A sample of [13C] EPA-PC was dissolved in chloroform–methanol (1:1 v/v) containing NaCl, loaded onto the column, and eluted with methanol. Sample ions were generated using an N₂ nebulization-assisted electrospray with a source temperature of 100°C. The NMR spectra were recorded using a Bruker ARX-300 NMR spectrometer in chloroform D or dimethylsulfoxide D₆.

[13C] EPA was isolated as follows. The mixture of fatty acids ([13C] FA) was isolated from N. oculate cells by the extraction of lipids with chloroform followed by saponification with methanolic potassium hydroxide in the usual manner. An aliquot of
[13C] FA was converted to fatty acid methylesters (FA-ME) for GC and GC-MS spectrometry. The [13C] FA were converted to phenacyl esters with phenacyl bromide and subjected to MPLC. Isolated [13C] EPA phenacyl ester was converted to [13C] EPA methylester by transmethylation with 14% boron trifluoride in methanol, followed by purification by silica gel-silver ion chromatography. The synthesis of [13C] EPA was done to six [13C] atoms were 13.2%, 13.2%, 18.4%, 13.2%, 1.0%, and 0.2%, respectively. Hydrolysis of the [13C] EPA methylester gave [13C] FA were converted to fatty acid methylesters. From the relative intensity of the NMR spectra of [13C] EPA methylester by transmethylation with 1,1-carbonyl-bis-[1H]-imidazole (64 mg, 1.2 mM) in tetrahydrofuran anhydride (10 mL) for 1 h at room temperature. Second, sodium imidazole was also formed by stirring imidazole (310 mg, 3.0 mM) in dimethylsulfoxide (3.0 mL) and sodium hydride (120 mg) for 1 h. [13C] EPA-PC was synthesized by stirring [13C] EPA imidazole, myristoyl lyso-phosphatidylcholine (Lyso-PC; 102 mg, 0.22 mM), sodium imidazole (0.11 mL), and pyridine (0.19 mL) in tetrahydrofuran (15 mL) for 3 h at room temperature. From the reaction mixture, the [13C] EPA-PC fraction was extracted with chloroform–methanol–water (65 : 25 : 2 v/v) after removal of imidazole by purification by silica gel column chromatography with chloroform–methanol–water (65 : 25 : 2 v/v) and Amberlite MB-3 to the [13C] EPA-PC fraction dissolved in 95% ethanol. These synthesis and hydrolysis procedures were done under nitrogen gas flow.

The culture of N. oculate with [13C] CO2 was conducted 15 times. The harvest contained 20:5n-3 (26.9–37.7% of total FA), 16:1, and 16:0 as the major FA (Table 1). Approximately 268 mg of N. oculate dry cells contained 65.4 ± 14.2 mg of lipids (mean ± SD). From 654 mg of total lipids, 450 mg of FA and 118 mg of EPA were isolated. The GC-MS of the EPA-ME fraction gave the molecular ions ranging from 316 m/z to 322 m/z (at m/z 316, 317, 318, 319, 320, 321, and 322), indicating that the fraction was a mixture of unlabeled and labeled EPA-ME with one to six [13C] atoms, but it was too complex to analyse the fragmentation. From the relative intensity of these molecular ion peaks, the composition ratio of unlabeled and labeled EPA-ME with one to six [13C] atoms were 13.2%, 13.2%, 18.4%, 13.2%, 21.1%, 18.4%, and 2.4%, respectively. Approximately 1.5% of [13C] atoms of total [13C] atoms added to the culture medium was recovered in the [13C] EPA-ME fraction. The NMR spectra of [13C] EPA-ME in chloroform D agreed with the data obtained in a previous report.

### Table 1 Fatty acid composition of Nannochloropsis oculate cultured for 7 days at 24°C water temperature

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mean ± SD</th>
</tr>
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<tbody>
<tr>
<td>14:0</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>16:0</td>
<td>17.6 ± 1.7</td>
</tr>
<tr>
<td>16:1</td>
<td>27.1 ± 3.4</td>
</tr>
<tr>
<td>18:0</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>20:0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>20:2(n-6)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>20:3(n-3)</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>20:4(n-3)</td>
<td>5.8 ± 1.3</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>32.9 ± 3.8</td>
</tr>
</tbody>
</table>

[13C] EPA-PC (150 mg) was synthesized from [13C] EPA with a yield of 56%. The LC-MS demonstrated main peaks at 774–780 m/z, as the addition of sodium [M+23]⁺, indicating that the [13C] EPA moieties were mixtures of unlabeled and labeled EPA-PC with one to six [13C] atoms. In the NMR spectra of [13C] EPA-PC in dimethylsulfoxide D₆, the following protons were detected: CH₃CH₂ (0.90 p.p.m., t, 3H), CH₃CH₂ (1.00 p.p.m., t, 3H), CH₃(CH₂)₉CH₂ (1.28 p.p.m., s, 20H), CH₃CH₂CH=CH (1.60 p.p.m., m, 2H), CH₃CH₂CH=CH (1.71 p.p.m., m, 4H), CH₃CH=CH=CH, and =CHCH₂CH₂ (2.11 p.p.m., m, 4H), CH₃CO₂ (2.31 p.p.m., m, 2H), CH₂CH₂CH=CH (2.86 p.p.m., m, 16H), NCH₃ (3.40 p.p.m., s, 9H), CH₃CH₃N (3.85, t, 2H), PO(CH₂)CH₂ (4.00 p.p.m., s, 2H), CH₂CH₂PO₂ and CH₂CH₂OCO (4.16 p.p.m., m, 2H); and 4.42 p.p.m., m, 2H), CH (5.24 p.p.m., s, 1H), and CH₃CH=CHCH₂ (5.40 p.p.m., m, 10H). These data supported the formation of [13C] EPA-PC from [13C] EPA.

The present study described the biosynthesis of [13C] EPA by incubating N. oculate with [13C] CO₂ and the successful synthesis of [13C] EPA-PC from [13C] EPA. Both [13C] EPA and [13C] EPA-PC obtained in the present study have been utilized successfully in the metabolic studies of fish, the results of which are yet to be published. However, the low yield of [13C] EPA by the method described here may warrant further investigation to improve the introduction of [13C] atoms into the EPA molecule by N. oculate.

### REFERENCES


