Isolation of Stereoisomeric Epoxy Carotenoids and New Acetylenic Carotenoid from the Common Freshwater Goby *Rhinogobius brunneus*

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Stereoisomeric epoxy carotenoids with 3,5-*cis* configuration, diadinoxanthin B [(3*S*,5*S*,6*R*,3′*R*)-diadinoxanthin] (**1**) and antheraxanthin B [(3*S*,5*S*,6*R*,3′*R*)-antheraxanthin] (**2**), along with diadinoxanthin A [(3*S*,5*R*,6*S*,3′*R*)-diadinoxanthin] and antheraxanthin A [(3*S*,5*R*,6*S*,3′*R*)-antheraxanthin], were isolated from the common freshwater goby *Rhinogobius brunneus*. This is the first example in nature of 3,5-*cis* carotenoid epoxides. Furthermore, a new acetylenic triol carotenoid, gobiusxanthin (**3**), was obtained, and its structure was determined to be 7,8-didehydro-*β*, *ϵ*-carotene-3,3′,6′-triol by chemical and spectral data.

Naturally occurring epoxy carotenoids such as violaxanthin, antheraxanthin, and diadinoxanthin possess the 3*S*,5*R*,6*S* configuration (3,5-*trans*-type), and other optical stereoisomers have not been reported.[1] On the other hand, tunaxanthin,[2] lutein,[3] zeaxanthin,[4] and astaxanthin[5,6] from fish have been found to be mixtures of optical stereoisomers. In the course of our stereochemical studies of carotenoids in fish, epoxy carotenoids having the 3*S*,5*S*,6*R* configuration (3,5-*cis* type), namely diadinoxanthin B [(3*S*,5*S*,6*R*,3′*R*)-diadinoxanthin] (**1**) and antheraxanthin B [(3*S*,5*S*,6*R*,3′*R*)-antheraxanthin] (**2**), along with diadinoxanthin A [(3*S*,5*R*,6*S*,3′*R*)-diadinoxanthin] and antheraxanthin A [(3*S*,5*R*,6*S*,3′*R*)-antheraxanthin], were recently isolated from the common freshwater goby *Rhinogobius brunneus* Temminck et Schlegel) (Gobbiidae), as shown in Figure 1. Furthermore, a new acetylenic carotenoid triol, gobiusxanthin [7,8-didehydro-*β*, *ϵ*-carotene-3,3′,6′-triol] (**3**) was obtained along with 33 known carotenoids. The purpose of the present paper is to report the results of the isolation, identification, and structure determination of new carotenoids in a freshwater goby.

Results and Discussion

Common freshwater gobies, *Rhinogobius brunneus* (whole bodies) (1000 specimens, 1100 g) were extracted with Me_2CO. After transfer to ether−*n*-hexane (1:1) and washing with water, the carotenoid extracts were concentrated below 40 °C. The crude carotenoids were saponified with 10% KOH/MeOH at 37 °C for 3 h, and the unsaponifiable portion was subjected to column chromatography on Si gel and preparative HPLC on Sumichiral OA-2000 to yield diadinoxanthin B (**1**, 1.2 mg), antheraxanthin B (**2**, 1.4 mg), and gobiusxanthin (**3**, 0.6 mg).

Carotenoid **1** showed a molecular ion peak at *m*/*z* 582.4088 in HREIMS, corresponding to the formula $C_{40}H_{54}O_3$ and vis maxima at 420, 445, and 475 nm (Et_2O). A hypsochromic shift of 20 nm in the vis spectrum by treatment with HCl indicated the presence of a 5,6-epoxy-end group in the molecule.[7] Acetylation of **1** gave a diacetate having a molecular ion at *m*/*z* 666. The ^{1}H NMR data for **1** indicated the presence of the partial structures of alloxanthin (H-2′ to H-20′)[8] and 3,5(3′,5′)-*cis*-type violaxanthin (H-2 to H-20).[8] All-*E* geometry of the polyene chain was confirmed by NOE data as shown in Figure 1. The constitution of 5,6-epoxy-5,6-dihydro-7′,8′-didehydro-*β*,*β*-carotene-3,3′-diol for **1** was confirmed by DQF−COSY

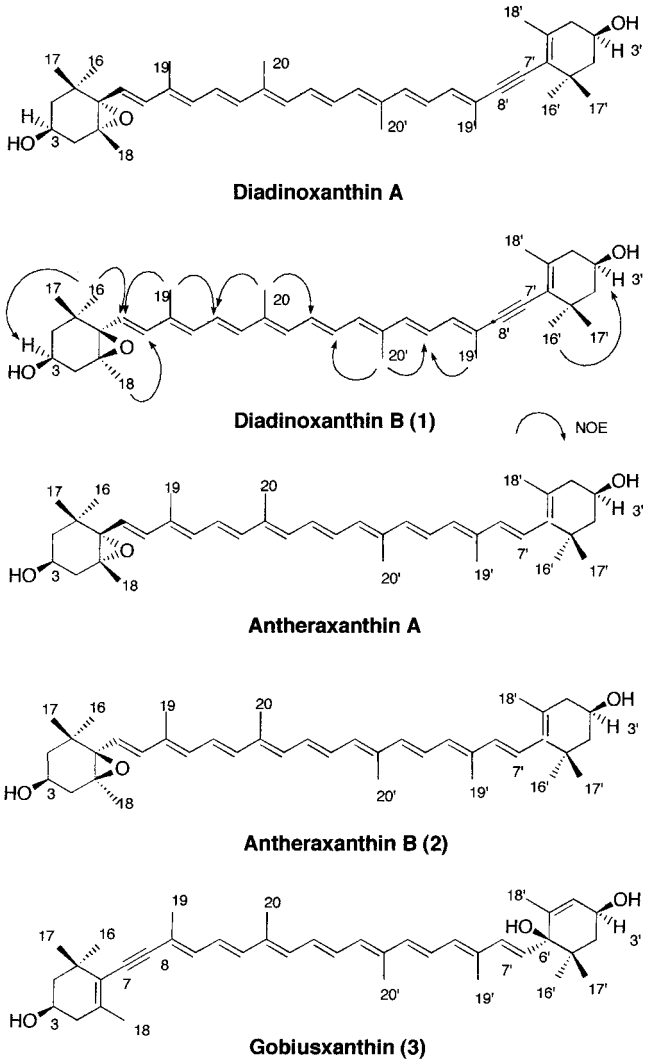

Figure 1. Structures of diadinoxanthins A and B (**1**), antheraxanthins A and B (**2**), and gobiusxanthin (**3**).

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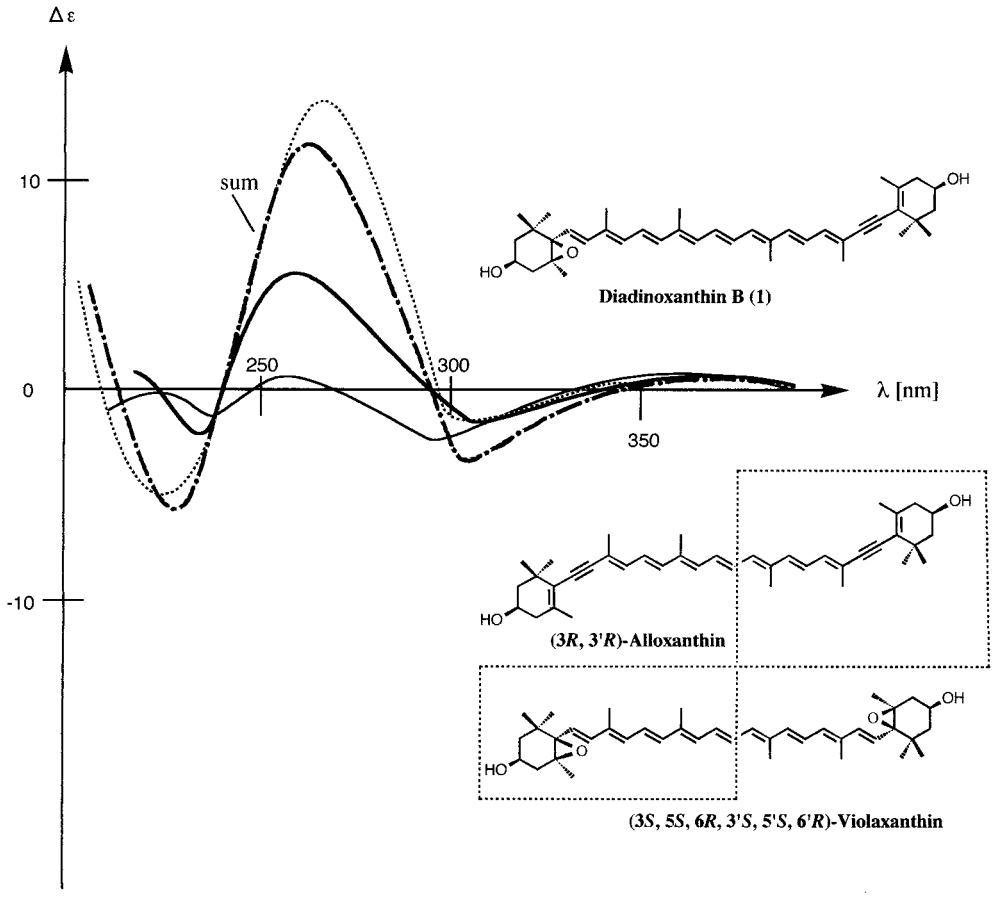

Figure 2. CD spectra (3*R*,3′*R*)-alloxanthin (1/2 intensity) —, (3*S*,5*S*,6*R*,3′*S*,5′*S*,6′*R*)-violaxanthin (1/2 intensity) ···, sum of both spectra —·—, and spectrum of diadinoxanthin B (**1**) — (in EPA) at 20 °C.

experiments. The relative stereochemistry of the 3-hydroxy-5,6-epoxy-end group was deduced by NOESY experiments. NOEs between δ 1.16 [CH_3-16 (α)] and δ 3.88 (H-3), between δ 1.16 [CH_3-16 (α)] and δ 5.83 (H-7) and between δ 1.19 [CH_3-18 (α)] and δ 6.30 (H-8) were compatible with the 3,5-*cis*-type epoxy end group (Figure 1). The absolute configuration at C-5, C-6, and C-3′ of **1** was postulated from CD spectral data. Compound **1** has two end groups, one 3-hydroxy-5,6-epoxy end group and one 3-hydroxy-7,8-didehydro-β end group, as shown in Figure 2. According to the additivity rules of CD spectra of dichiral carotenoids,[9] the absolute configuration of **1** can be deduced by comparison of the CD spectrum of diadinoxanthin B with that calculated for 1/2 (3*S*,5*S*,6*R*,3′*S*,5′*S*,6′*R*)-violaxanthin,[9,10] and 1/2 (3*R*,3′*R*)-alloxanthin,[9] as shown in Figure 2. This result showed that **1** has the same configuration of the epoxy group (C-5 and C-6) as (3*S*,5*S*,6*R*,3′*S*,5′*S*,6′*R*)-violaxanthin and the same chirality at C-3′ as (3*R*,3′*R*)-alloxanthin. Based on the result of the relative stereochemistry of 3-OH and epoxy groups described above, chirality at C-3 in **1** was assigned to be *S*. Therefore, the absolute configuration of **1** was postulated to be 3*S*,5*S*,6*R*,3′*R*.

The molecular formula of **2** was determined to be $C_{40}H_{56}O_3$ by HREIMS measurement (M^+, *m*/*z* 584.4220). Carotenoid **2** showed vis maxima at 420, 445, and 475 nm (Et_2O) and at 405, 427, and 453 nm after addition of HCl. This carotenoid also provided a diacetate by acetylation. ^{1}H NMR spectral assignments for **2** were consistent with a (3*S*,5*S*,6*R*,3′*R*)-antheraxanthin synthesized by Märki-Fischer et al.[11] Furthermore, the CD spectrum of **2** was also in good agreement with that of (3*S*,5*S*,6*R*,3′*R*)-antheraxanthin.[11] (Figure 3).

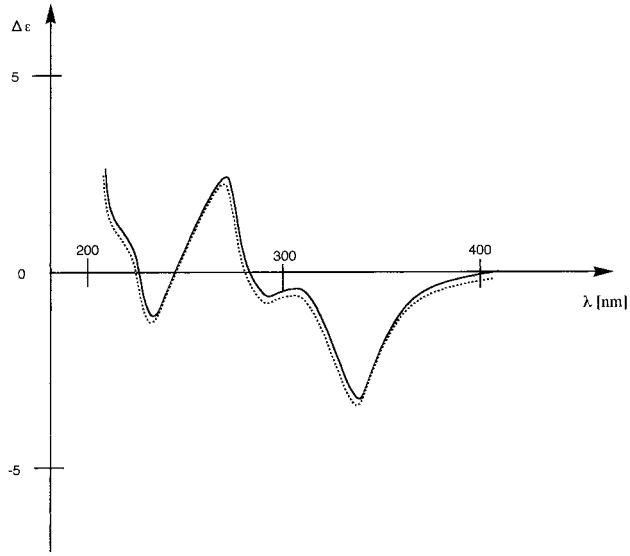

Figure 3. CD spectra of antheraxanthin B (**2**) — and synthetic (3*S*,5*S*,6*R*,3′*R*)-antheraxanthin ··· (in EPA) at 20 °C.

The absolute configurations of violaxanthin and antheraxanthin were 3*S*,5*R*,6*S*(3,5-*trans* type), and other stereoisomers have not been reported previously.[1] In the present study, we have isolated epoxy carotenoids having the 3*S*,5*S*,6*R* configuration (3,5-*cis* type), (3*S*,5*S*,6*R*,3′*R*)-diadinoxanthin (diadinoxanthin B) (**1**), and (3*S*,5*S*,6*R*,3′*R*)-antheraxanthin (antheraxanthin B) (**2**) from the common freshwater goby. This is the first report on the occurrence of **1** and **2** in nature. We proposed the name diadinoxanthins A and B and antheraxanthins A and B for the

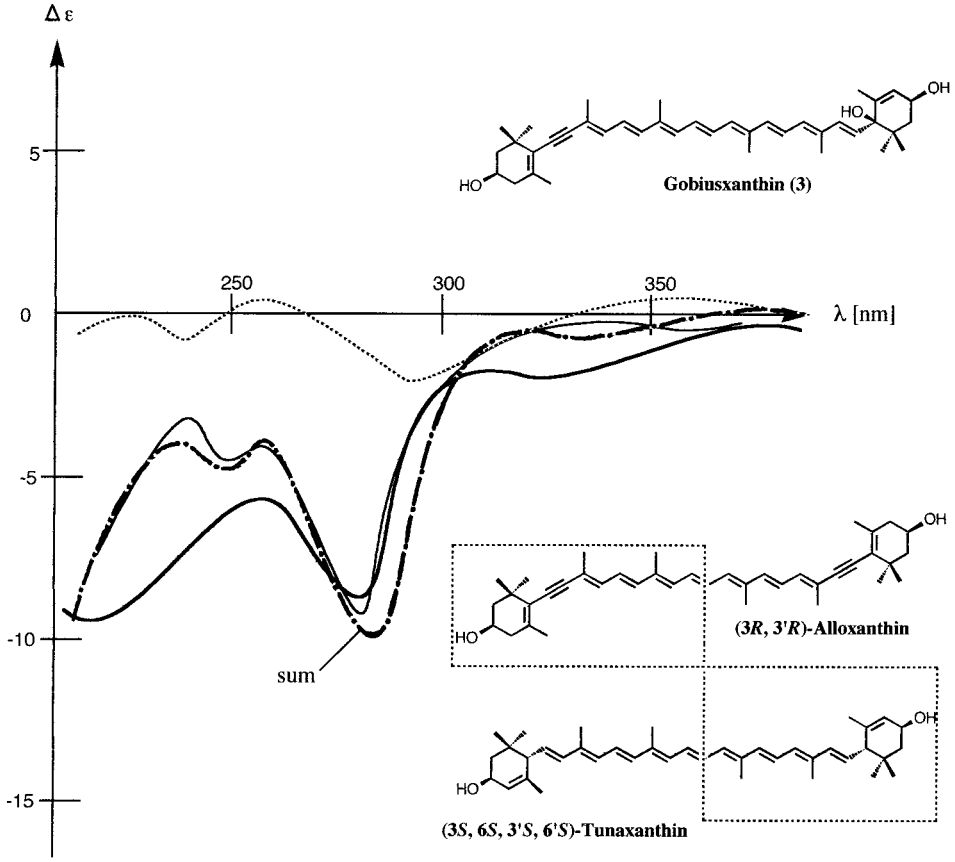

Figure 4. CD spectra (3*R*,3*R*′)-alloxanthin (1/2 intensity) ···, (3*S*,6*S*,3′*S*,6′*S*)-tunaxanthin (1/2 intensity) —, sum of both spectra —·—, and spectrum of gobiusxanthin (**3**) — (in EPA) at 20 °C.

stereoisomers of diadinoxanthin and antheraxanthin, respectively, as shown in Figure 1.

The molecular formula of **3** was determined to be $C_{40}H_{54}O_3$ by HREIMS measurement (M^+, *m*/*z* 582.4085). The presence of three hydroxyl groups was consistent with the formation of a diacetate and tri-trimethylsilyl ether. The ^{1}H NMR chemical shifts and coupling constants of the partial structure of alloxanthin part (H-2 to H-20)[8] in **3** were identical with the correponding data of all-*E*-alloxanthin (Table 1). Furthermore, the characteristic ^{1}H NMR signals at δ 0.94 (3H, s), 1.02 (3H, s), 1.68 (3H, s), 1.92 (3H, s), 1.97 (3H, s), 4.24 (1H, m), 5.63 (1H, d), and 5.64 (1H, s) indicated the presence of a salmoxanthin moiety (H-2′ to H-20′)[12,13] (Table 1). On the basis of the evidence described above, we assigned the 7,8-didehydro-β,ϵ-carotene-3,3′,6′-triol structure. The absolute configuration of **3** was postulated from CD data (Figure 4). 7,8-Didehydro-β,ϵ-carotene-3,3′,6′-triol (**3**) has two end groups, one 3-hydroxy-7,8-didehydro-β-end group and one 3,6-dihydroxy-ϵ-end group. According to the additivity rule of CD spectra,[9] the CD spectrum of **3** was similar to that of the calculated CD of 1/2 (3*R*,3′*R*)-alloxanthin (having a 3-hydroxy-7,8-didehydro-β-end group) and 1/2 (3*S*,6*S*,3′*S*,6′*S*)-tunaxanthin (tunaxanthin A)[9] (having a 3-hydroxy-ϵ-end group) as shown in Figure 4. From the results described above, it seemed that **3** has the same chiralities at C-3 as that of (3*R*,3′*R*)-alloxanthin and the C-3′ and C-6′ as those of tunaxanthin A. Thus, the structure of **3** has been postulated to be (3*R*,3′*S*,6′*R*)-7,8-didehydro-β,ϵ-carotene-3,3′,6′-triol. This was named as gobiusxanthin.

In conclusion, we have isolated two stereoisomeric epoxy carotenoids having the 3,5-*cis* configuration, diadinoxanthin B and antheraxanthin B, and a new acetylenic triol carotenoid, gobiusxanthin. These compounds were the characteristic carotenoids in the common freshwater goby.

Experimental Section

General Experimental Procedures. Vis spectra were recorded on a Shimadzu UV-240 spectrophotometer. Concentrations were calculated using $E^{1\%}_{1cm} = 2500$ at λ_{max} (in Et_2O). EIMS were recorded on a JEOL GCMATE (BU20) mass spectrometer with a direct inlet system with ionization energy of 70 eV. CD spectra were recorded on a JASCO J-500 C spectropolarimeter in EPA [Et_2O/isopentane/EtOH (5:5:2)] solution at 20 °C. ^{1}H NMR spectra were recorded in $CDCl_3$ with TMS as standard, with a Varian XL-300 (300 MHz) or Varian UNITY INOVA 500 (500 MHz) instrument. All 2D spectra were recorded with a Varian UNITY INOVA 500 without spinning in 0.15 mL $CDCl_3$. DQF−COSY and NOESY were acquired using the standard Varian pulse programs. HPLC was carried out on a Shimadzu LC-6A instrument with a chiral column Sumichiral OA-2000 (300 × 8.0 nm i.d., 5 mm, Sumika Chemical Analysis Service, Ltd.).

Animal Materials. Common freshwater gobies *R. brunneus* (1000 specimens, 1100 g), were collected from the Yura River, Kyoto Prefecture, in March 1998.

Extraction and Isolation of Carotenoids. Carotenoids were extracted repeatedly with Me_2CO from common freshwater gobies (whole bodies) at room temperature until the residue was colorless. The carotenoids were then transferred to ether/*n*-hexane (1:1) by addition of water. The upper layer was washed with water and dried over Na_2SO_4. The extract was concentrated below 40 °C. The crude carotenoids were separated by column chromatography on Si gel. Each fraction was saponified with 10%

Table 1. ^{1}H NMR Data (500 MHz) in $CDCl_3$ for Gobiusxanthin (**3**)[a]

position		
H-2α,2′α	1.84 ddd (12, 3, 1.5)	1.84 dd (14, 5.5)
H-2β,2′β	1.45 dd (12, 12)	1.66 dd (14, 7)
H-3,3′	4.00 m	4.24 m
H-4α,4′α	2.43 ddd (18, 6, 1.5)	5.64 br s
H-4β,4′β	2.07 dd (18, 10)	
H-7,7′		5.63 d (15.5)
H-8,8′		6.38 d (15.5)
H-10,10′	6.45 d (10)	6.22 d (11.5)
H-11,11′	6.54 dd(15, 11)	6.62 d (15, 11.5)
H-12,12′	6.38 d (15)	6.37 d (15)
H-14,14′	6.29 m	6.26 m
H-15,15′	6.64 m	6.64 m
H-16,16′	1.15 s	0.94 s
H-17,17′	1.20 s	1.02 s
H-18,18′	1.93 s	1.68 br s
H-19,19′	2.01 s	1.92 s
H-20,20′	1.99 s	1.97 s

[a] δ mult (J in Hz).

KOH/MeOH or hydrolyzed with lipase,[14] and the unsaponifiable portions were submitted to further purification by preparative HPLC. Compounds **1** (1.2 mg), **2** (1.4 mg), and **3** (0.6 mg) were eluted with Me_2CO from a Si gel column and were further purified by HPLC on Sumichiral OA-2000 with *n*-hexane/$CHCl_3$/EtOH (48:16:2.0).

The following carotenoids were identified from *R. brunneus*: β,β-carotene; β-echinenone; (2*R*)- and (2*S*)-β,β-caroten-2-ol;[15] (3′*R*,6′*S*)- and (3′*S*,6′*S*)-β,ε-caroten-3′-ol,[16] β-cryptoxanthin; canthaxanthin; tunaxanthins A, B, C, G, and H,[17] luteins A, D, and F,[18] (3*R*,3′*R*)-, (3*R*,3′*S*; *meso*)-, and (3*S*,3′*S*)-zeaxanthin;[19] diatoxanthin; alloxanthin; (3*R*,3′*R*)-, (3*R*,3′*S*; *meso*)-, and (3*S*,3′*S*)-astaxanthin;[20] 4-ketoluteins A, D, and F;[21] and (3*S*,3′*R*)-4-ketozeaxanthin;[22] based on the vis, MS, and CD spectral data. Thirty-three known carotenoids and two new carotenoids were isolated from *R. brunneus* as shown in Table 2. Tunaxanthin (21% of the total carotenoids), lutein (24%), and zeaxanthin (18%) were major carotenoids.

Diadinoxanthin A: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; CD (EPA) 204 ($\Delta\epsilon$ −5.0), 238 (+2.0), 284 (−8.5), 367 (+2.5) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.98 (3H, s, CH_3-16), 1.15 (6H, s, CH_3-17, -16′), 1.19 (3H, s, CH_3-18), 1.20 (3H, s, CH_3-17′), 1.93 (6H, s, CH_3-19, -18′), 1.97 (6H, s, CH_3-20, -20′), 2.01 (3H, s, CH_3-19′), 3.91 (1H, m, H-3), 4.00 (1H, m, H-3′), 5.88 (1H, d, J = 15.5 Hz, H-7), 6.29 (1H, d, J = 15.5 Hz, H-8) 6.20−6.64 (11H, m, olefinic); EIMS (70 eV) *m*/*z* 582 [M^+] (31), 564 [M^+−18] (3), and 502 [M^+−80] (19); HREIMS *m*/*z* 582.4071(calcd for $C_{40}H_{54}O_3$, 582.4073).

Diadinoxanthin B (1): UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; CD see Figure 2; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.01 (3H, s, CH_3-17), 1.15 (3H, s, CH_3-16′), 1.16 (3H, s, CH_3-16), 1.19 (3H, s, CH_3-18), 1.20 (3H, s, CH_3-17′), 1.92 (3H, s, CH_3-19), 1.93 (3H, s, CH_3-18′), 1.97 (6H, s, CH_3-20, -20′), 2.01 (3H, s, CH_3-19′), 3.88 (1H, m, H-3), 4.00 (1H, m, H-3′), 5.83 (1H, d, J = 15.5 Hz, H-7), 6.30 (1H, d, J = 15.5 Hz, H-8) 6.20−6.64 (11H, m, olefinic); EIMS (70 eV) *m*/*z* 582 [M^+] (100), 564 [M^+−18] (5), and 502 [M^+−80] (20); HREIMS *m*/*z* 582.4088 (calcd for $C_{40}H_{54}O_3$, 582.4073).

Diadinoxanthin B (1) diacetate: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; HREIMS *m*/*z* 666.4288 (calcd for $C_{44}H_{58}O_5$, 666.4284).

Antheraxanthin A: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; CD (EPA) 216 ($\Delta\epsilon$ −13.2), 239 (+10.3), 275 (−21.1), 335 (+5.7) nm; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.98 (3H, s, CH_3-16), 1.07 (6H, s, CH_3-17′, -16′), 1.15 (3H, s, CH_3-17), 1.19 (3H, s, CH_3-18), 1.74 (3H, s, CH_3-18′), 1.93 (3H, s, CH_3-19), 1.97 (9H, s, CH_3-19′, -20, -20′), 3.91 (1H, m, H-3), 4.00 (1H, m, H-3′), 5.88 (1H, d, J = 15.5 Hz, H-7), 6.12 (2H, br, s, H-7′, -8′), 6.29 (1H, d, J = 15.5 Hz, H-8), 6.15−6.64 (11H, m, olefinic); EIMS (70 eV) *m*/*z* 584 [M^+] (60), 504 [M^+−80] (30), 492 [M^+−92] (6); HREIMS *m*/*z* 584.4233 (calcd for $C_{40}H_{56}O_3$, 584.4230).

Antheraxanthin B (2): UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; CD see Figure 3; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.01 (3H, s, CH_3-17), 1.07 (6H, s, CH_3-17′, -16′), 1.16 (3H, s, CH_3-16), 1.19 (3H, s, CH_3-18), 1.74 (3H, s, CH_3-18′), 1.92 (3H, s, CH_3-19), 1.97 (9H, s, CH_3-19′, -20, -20′), 3.88 (1H, m, H-3), 4.00 (1H, m, H-3′), 5.83 (1H, d, J = 15.5 Hz, H-7), 6.12 (2H, br, s, H-7′, -8′), 6.30 (1H, d, J = 15.5 Hz, H-8), 6.15−6.64 (11H, m, olefinic); EIMS (70 eV) *m*/*z* 584 [M^+] (60), 504 [M^+−80] (30), 492 [M^+−92] (6); HREIMS *m*/*z* 584.4220 (calcd for $C_{40}H_{56}O_3$, 584.4230).

Antheraxanthin B (2) diacetate: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; HREIMS *m*/*z* 668.4435 (calcd for $C_{44}H_{60}O_5$, 668.4440).

Gobiusxanthin (3): UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; CD, see Figure 4; ^{1}H NMR ($CDCl_3$, 500 MHz), see Table 1, EIMS (70 eV) *m*/*z* 582 [M]$^+$ (31), 564 [M^+−18] (7), and 526 [M^+−56] (3); HREIMS *m*/*z* 582.4085 (calcd for $C_{40}H_{54}O_3$, 582.4073).

Gobiusxanthin (3) diacetate: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; HREIMS *m*/*z* 666.4276 (calcd for $C_{44}H_{58}O_5$, 666.4284).

Gobiusxanthin (3) tri-trimethylsilyl ether: UV−vis λ_{max} (Et_2O) 420, 445, and 475 nm; HREIMS *m*/*z* 798.5250 (calcd for $C_{49}H_{78}O_3Si_3$ 798.5259).

Table 2. Amount and Percent Composition of Individual Carotenoids in the Common Freshwater Goby

total carotenoid contents (mg/100 g body wt)	2.6	
composition (%)		
β,β-carotene	+	
β-echinenone	+	
(2*R*)-β,β-caroten-2-ol	2	3
(2*S*)-β,β-caroten-2-ol	1	
(3′*R*,6′*S*)-β,ε-caroten-3′-ol	1	1
(3′*S*,6′*S*)-β,ε-caroten-3′-ol	+	
canthaxanthin	+	
β-cryptoxanthin	1	
tunaxanthin A	1	21
tunaxanthin B	16	
tunaxanthin C	2	
tunaxanthin G	1	
tunaxanthin H	1	
lutein A	4	24
lutein D	3	
lutein F	17	
(3*R*,3′*R*)-zeaxanthin	13	18
(3*R*,3′*S; meso*)-zeaxanthin	4	
(3*S*,3′*S*)-zeaxanthin	1	
diatoxanthin	3	
alloxanthin	2	
β,ε-carotene-3,4,3′-triol	+	
β,β-carotene-3,4,3′-triol	1	
diadinoxanthin A	3	7
diadinoxanthin B (**1**)	4	
antheraxanthin A	1	5
antheraxanthin B (**2**)	4	
gobiusxanthin (**3**)	2	
(3*R*,3′*R*)-astaxanthin	1	5
(3*R*,3′*S; meso*)-astaxanthin	+	
(3*S*,3′*S*)-astaxanthin	4	
4-ketolutein A	1	4
4-ketolutein D	+	
4-ketolutein F	3	
(3*S*,3′*R*)−4-ketozeaxanthin	1	
unidentified carotenoids	2	

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