NOTE

New Acetylenic Carotenoid 6'-Epimonadoxanthin from the Rosary Goby *Gymnogobius castaneus*

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Abstract: Carotenoids of the rosary goby *Gymnogobius castaneus* (juzukakehaze in Japanese) were investigated. A new acetylenic carotenoid, (3R,3'R,6'S)-7,8-didehydro- β,ϵ -carotene-3,3'-diol (1), named 6'-epimonadoxanthin, was isolated along with 18 known carotenoids. The structure of 6'-epimonadoxanthin was determined by UV-VIS, ESI-TOF MS, $^1\text{H-NMR}$, and CD spectral data. The metabolic pathway from diatoxanthin to 6'-epimonadoxanthin is also discussed.

Key words: carotenoid, 6'-epimonadoxanthin, rosary goby, *Gymnogobius castaneus*

1 INTRODUCTION

Gobies are small fish of less than 10 cm in length that belong to the family Gobiidae (Order Perciformes), and inhabit brackish and freshwater environments. Tunaxanthin, lutein, zeaxanthin, alloxanthin, diatoxanthin, and tri-hydroxy- β -carotene have been reported as characteristic carotenoids in gobies by Matsuno *et al.*^{1,2)}. Furthermore, Tsushima *et al.*³⁾ isolated the novel *syn*-epoxy carotenoids diadinoxanthin B and antheraxanthin B, and an acetylenic tri-hydroxy carotenoid, gobiuxanthin, from the freshwater goby *Rhinogobius brunneus*.

The rosary goby *Gymnogobius castaneus* (juzukakehaze in Japanese) is a small goby (4~6 cm body length) that inhabits the middle stretches of rivers, lakes, and lagoons in North Eastern Japan. Its tail fin is bright orange in color. This goby is edible and is consumed boiled in soy sauce as 'tukudani' or fried.

In this study, carotenoids of *G. castaneus* were investigated. As a result, a new acetylenic carotenoid, 6'-epimonadoxanthin (1), was isolated along with 18 known carotenoids. This report describes the isolation and structural elucidation of this new carotenoid from the rosary goby *G. castaneus*. Furthermore, the metabolic pathway from diatoxanthin to this new carotenoid (1) is discussed.

2 EXPERIMENTALS

2.1 Apparatus

UV-Visible (UV-VIS) absorption spectra were recorded with a Hitachi U-2001 spectrophotometer (Hitachi Field Navigator, Tokyo, Japan) in diethyl ether (Et_2O). LC/MS analysis of carotenoids was performed using a Waters Xevo G2S Q ToF mass spectrometer (Waters Corporation, Milford, CT, USA) equipped with an Acquity UPLC system. Electrospray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from m/z 100 to 1,500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120°C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured using a Q-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V and used a photodiode array detector (PDA) to record UV-VIS absorption spectra from 200 to 600 nm. Acquity 1.7 μm BEH UPLC C18 column (Waters Corporation, Milford, CT, USA) and MeOH are used as stationary phase with a mobile phase at a flow rate of 0.4 mL/min for the HPLC system. $^1\text{H-NMR}$ (500 MHz) spectrum was measured with a Varian Unity Inova 500 spectrometer (Varian Corporation, Palo Alto, California USA) in CDCl_3 with TMS as an internal standard. Because of small amount of carotenoid sample (about 30 μg), $^1\text{H-NMR}$ was measured using a SHIGEMI micro tube (sample solution volume 200 μL) (Shigemi Co., Ltd. Hachioji, Tokyo, Japan). The CD spectrum was recorded in Et_2O at room temperature with a Jasco J-500C spectropolarimeter (JASCO Corporation, Hachioji, Tokyo, Japan).

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Preparative HPLC was performed with a Hitachi L-6000 HPLC intelligent pump and Hitachi L-4250 UV-VIS detector (Hitach Field Navigator, Tokyo, Japan) set at 450 nm. The column used were 250 × 4.6 mm i.d., 5 µm Cosmosil 5SL-II and Cosmosil 5C18-II (Nacalai Tesque, Kyoto, Japan).

2.2 Animal Material

The rosary goby *G. castaneus*, collected in Aomori prefecture at October 2014, were purchased from a local fish market.

2.3 Extraction, Isolation, and Identification of Carotenoids

One kg of fresh fish (whole body) was extracted with acetone at room temperature. The acetone extract was partitioned with *n*-hexane/Et₂O (1:1) and water. The *n*-hexane/Et₂O (1:1) layer was evaporated to dryness. The residue contained large amount of lipids and carotenoids were existed as fatty acid esterified form¹⁻⁴⁾. Thus, the residue was saponified with 5% KOH/MeOH (10 mL) at room temperature for 2 hr. Then, carotenoid was extracted with *n*-hexane/Et₂O (1:1) and washed with water. After evaporating of extract solution, individual carotenoids were separated by according to the methods described by Tsushima *et al.*³⁾.

The reddish residue was subjected to silica gel 60 (Nacalai Tesque, Kyoto, Japan) column chromatography (200 × 17 mm). The fraction (Fr.) eluted with Et₂O/*n*-hexane (1:9 v/v, Fr. 1) was subjected to preparative HPLC on Cosmosil 5C18-II with a methanol at a flow rate of 2.0 mL/min to yield α -carotene (retention time; rt. 34.0 min, yield 1 µg) and β -carotene (rt. 35.5 min, yield 35 µg). The fraction eluted with Et₂O/*n*-hexane (5:5 v/v, Fr. 2) was subjected to preparative HPLC on Cosmosil 5C18-II with a methanol at a flow rate of 2.0 mL/min to yield canthaxanthin (rt. 11.6 min, yield 12 µg), α -cryptoxanthin (rt. 17.0 min, yield 30 µg), β -cryptoxanthin (rt. 18.5 min, yield 55 µg), and echinenone (rt. 27.9 min, yield 10 µg). The fraction eluted with Et₂O/*n*-hexane (8:2 v/v, Fr. 3) was subjected to preparative HPLC on Cosmosil 5SL-II with acetone/*n*-hexane (25:75 v/v) to yield 3'-hydroxyechinenone (rt. 10.5 min, yield 10 µg), and astacene (rt. 20.0 min, yield 50 µg). The fraction eluted with acetone/*n*-hexane (1:1 v/v) was subjected to preparative HPLC on Cosmosil 5 SL-II with acetone/*n*-hexane (2:8 v/v) to yield tunaxanthins (rt. 14.0 min), luteins (rt. 14.6 min), zeaxanthin (rt. 15.5 min, yield 50 µg), a new carotenoid (rt. 16.5 min, yield 30 µg), diatoxanthin (rt. 17.0 min, yield 70 µg), and alloxanthin (rt. 18.8 min, yield 90 µg). Tunaxanthins fraction was further separated by HPLC on Cosmosil 5C18-II with methanol to yield (3*R*,6*S*,3'*R*,6'*S*)-tunaxanthin (rt. 15.2 min, yield 20 µg), (3*S*,6*S*,3'*R*,6'*S*)-tunaxanthin (rt. 16.2 min, yield 18 µg), and (3*S*,6*S*,3'*S*,6'*S*)-tunaxanthin (rt. 16.8 min, yield 7 µg). Luteins fraction was further separated by HPLC on Cosmosil 5C18-II with methanol to yield (3*R*,3'*R*,6'*S*)-lutein

(rt. 13.2 min, yield 30 µg) and (3*R*,3'*S*,6'*S*)-lutein (rt. 16.2 min, yield 18 µg). The fraction eluted with acetone was subjected to preparative HPLC on Cosmosil 5 SL-II with acetone/*n*-hexane (3:7, v/v) to yield β -carotene-3,4,3'-triol (rt. 20.5 min, yield 20 µg) and pectenol (rt. 22.5 min, yield 30 µg). Identification of individual carotenoids were accomplished by UV-VIS, ESI TOF MS, and comparison of retention time with authentic samples. Furthermore, (3*S*,6*S*,3'*R*,6'*S*)-tunaxanthin, (3*S*,6*R*,3'*R*,6'*S*)-tunaxanthin, (3*R*,3'*S*,6'*S*)-lutein, (3*R*,3'*R*,6'*S*)-lutein, zeaxanthin, diatoxanthin, alloxanthin, and pectenol were analyzed by ¹H-NMR and CD spectral data⁴⁾.

2.4 Quantitative Analysis of Carotenoids

The total carotenoid content and the amount of carotenoids eluted by column chromatography were calculated using the extinction coefficient of E_{cm}^{1%} = 2,500 at λ_{max}⁴⁾. In the HPLC analysis, the relative amounts of individual carotenoids were calculated from the peak area detected at 450 nm.

2.4.1 6'-Epiminoxanthin

Yield: 30 µg. UV-VIS: 423, 445, 474 nm. ESI TOF MS: *m/z* 556.4130 (M⁺, calcd. for C₄₀H₅₄O₂, 556.4124), *m/z* 589.4014 (M + Na⁺, calcd. for C₄₀H₅₄O₂Na, 589.4021). ¹H-NMR δ (CDCl₃): 0.86 (3H, s, CH₃-16'), 0.94 (3H, s, CH₃-17'), 1.15 (3H, s, CH₃-16), 1.20 (3H, s, CH₃-17), 1.39 (1H, dd, *J* = 13, 10 Hz H-2'α), 1.46 (1H, dd, *J* = 12, 12 Hz H-2β), 1.65 (1H, overlapped with CH₃-18', H-2'β), 1.65 (3H, s, CH₃-18'), 1.84 (1H, ddd, *J* = 12, 3, 1.5 Hz, H-2α), 1.91 (3H, s, CH₃-19'), 1.93 (3H, s, CH₃-18), 1.96 (3H, s, CH₃-20'), 1.97 (3H, s, CH₃-20), 2.01 (3H, s, CH₃-19), 2.07 (1H, dd, *J* = 18, 10 Hz, H-4β), 2.16 (1H, d, *J* = 10 Hz, H-6'), 2.43 (1H, ddd, *J* = 18, 5, 1.5 Hz, H-4α), 3.99 (1H, m, H-3), 4.23 (1H, m, H-3'), 5.48 (1H, br.s, H-4'), 5.54 (1H, dd, *J* = 15, 9 Hz, H-7'), 6.13, (d, *J* = 15.5 Hz, H-8'), 6.14 (1H, d, *J* = 11 Hz, H-10'), 6.25 (1H, d, *J* = 11 Hz, H-14'), 6.29 (1H, d, *J* = 11 Hz, H-14), 6.34 (1H, d, *J* = 15 Hz, H-12'), 6.35 (1H, d, *J* = 15 Hz, H-12), 6.46 (1H, d, *J* = 11 Hz, H-10), 6.51 (1H, dd, *J* = 15, 11 Hz, H-11), 6.61 (1H, dd, *J* = 15, 11 Hz, H-11'), 6.64 (2H, overlapped, H-15, 15'). ¹H-¹H COSY correlations; H₂-2-H-3-H₂-4, CH₃-19-H-10-H-11-H-12, CH₃-20-H-14-H-15-H-15'-H-14'-CH₃-20', CH₃-19'-H-10'-H-11'-H-12', H-6'-H-7'-H-8', and H₂-2'-H-3'-H-4', CD: nm (Δε) 230 (0), 245 (-6.0), 265 (-7.0), 290 (0), 328 (-3.0), 350 (0).

3 RESULTS AND DISCUSSION

3.1 Carotenoids of the rosary goby *G. castaneus*

The 18 known carotenoids and a new carotenoid (Fig. 1 and Table 1) were found in the rosary goby *G. castaneus*. As in other gobies¹⁻³⁾, tunaxanthins, luteins, diatoxanthin, and alloxanthin were found to be major components.

Fig. 1 Structures of identified 18 known carotenoids the rosary goby *Gymnogobius castaneus*.

Table 1 Carotenoid content and percentage composition of the rosary goby *Gymnogobius castaneus*.

Total Carotenoid	4.18 µg/g
Composition	%
β-Carotene	5.8
α-Carotene	0.2
Echinenone	1.9
β-Cryptoxanthin	8.8
α-Cryptoxanthin	5.4
Canthaxanthin	1.9
3'-Hydroxy-echinenone	1.5
Astaxanthin*	7.9
(3S,6S,3'S,6'S)-Tunaxanthin	1.1
(3S,6S,3'R,6'S)-Tunaxanthin	2.8
(3R,6S,3'R,6'S)-Tunaxanthin	3.0
(3R,3'S,6'S)-Lutein	7.8
(3R,3'R,6'S)-Lutein	4.5
6'-Epimonadoxanthin (1)	4.5
Zeaxanthin	7.9
Diatoxanthin	10.6
Alloxanthin	14.4
Pectenol	4.5
β, β-Carotene-3,4,3' -triol	3.0
Others	2.5

*Identified as astacene

3.2 Structure of the new acetylenic carotenoid (1)

A new acetylenic carotenoid (1) was obtained as a minor component (30 µg from 1 kg of fish, 4.6 % of the total carotenoid amount). This carotenoid exhibited absorption maxima at 423, 445, and 474 nm. The molecular formula of **1** was determined to be $C_{40}H_{54}O_2$ by ESI-TOF MS data. 1H -NMR signals of **1** indicated the presence of a 7,8-didehydro-3-hydroxy-β-end group (H-2 to H-4, CH_3 -16 to CH_3 -19)⁵⁾, a 3,6-*cis*-3-hydroxy-ε-end group (H-2' to H-6', CH_3 -16' to CH_3 -18')⁵⁾, and an all-*trans*-polyene chain attached by an acetylenic bond at C-7 (H10 to H-15, H-7' to H-15', CH_3 -19, 20, 19', 20')⁵⁾. This assignment was confirmed by 1H - 1H COSY. Thus, the structure of this compound was determined to be 7,8-didehydro-β,ε-carotene-3,3'-diol with a 3,6-*cis* configuration (Fig. 2). The absolute configuration of **1** was analyzed by CD spectral data. The CD spectrum of **1** is shown in Fig. 3. The additivity rule of CD was used to determine the chirality of carotenoids with different end groups on both sides of the polyene chain^{4,6)}. The CD spectrum of this compound was similar with the additive CD spectra of (3*R*,3'*R*)-alloxanthin and (3*R*,6*S*,3'*R*,6'*S*)-tunaxanthin, as shown in Fig. 3. Thus, the absolute configuration of this compound was determined as (3*R*,3'*R*,6'*S*). This new carotenoid, (3*R*,3'*R*,6'*S*)-7,8-didehydro-β,ε-carotene-3,3'-diol (**1**), corresponded with the 6'-epimer of monadoxanthin [(3*R*,3'*R*,6'*R*)-7,8-didehydro-β,ε-carotene-3,3'-diol] reported by Pennington *et al.*⁷⁾. Therefore, this carotenoid was named 6'-epimonadoxanthin.

Fig. 2 Structure of a new carotenoid (1) in the rosary goby *Gymnogobius castaneus*.

Fig. 3 CD spectrum of (3R,3'R,6'S)-6'-epimonadoxanthin (1) (—), (3R, 3'R)-alloxanthin (1/2 intensity) (---), and (3R,6S,3'R,6'S)-tunaxanthin (1/2 intensity) (••••) in ether at room temperature and additive spectrum of (3R, 3'R)-alloxanthin and (3R,6S,3'R,6'S)-tunaxanthin (1/2 intensity) (—).

3.3 Biosynthesis of 6'-epimonadoxanthin (1) in the rosary goby

It was previously reported that carotenoids with 3-hydroxy-ε-end groups in animals are formed from carotenoids with 3-hydroxy-β-end groups via carotenoids with 3-oxo-ε-end groups^{8,9)}. Namely, the 3-hydroxy-β-end group in carotenoids is converted to a 3-oxo-ε-end group by oxidation of the hydroxy group at C-3 with transition of the double bond from C5-C6 to C4-C5. Then, the carbonyl group at C3 in the 3-oxo-ε-end group is reduced to a hydroxy group to form the 3-hydroxy-ε-end group^{8,9)}. Thus, (3R,3'S,6'S)- and (3R,3'R,6'S)-lutein in fish were formed from zeaxanthin through (3R,6'S)-3-hydroxy-β, ε-caroten-3'-one, as shown in Fig. 4 (right column)⁸⁻¹¹⁾. Similarly, 6'-epimonadoxanthin (1) was speculated to be formed from diatoxanthin through (3R,6'S)-3-hydroxy-7,8-didehydro-β,ε-caroten-3'-one, as shown in Fig. 4 (left column). (3R,6'S)-3-Hydroxy-7,8-didehydro-β,ε-caroten-3'-one, which has not been reported yet in nature, was considered to be a possible key intermediate of this metabolic conversion. This compound has a 3-oxo-ε-end group. It is well known that carotenoids with 3-oxo-ε-end groups are very unstable in alkaline medium¹²⁾. Therefore, this compound was decomposed during the saponification procedure and was not detected in the rosary goby in the present investigation. Furthermore, the corresponding 3'-epimer of 1, (3R,3'S,6'S)-7,8-didehydro-β,ε-caroten-3,3'-

diol (2), may have been formed through this metabolic pathway, as shown in Fig. 4 (left column). Indeed, a very minor carotenoid (HPLC retention time; 16.8 min) with absorption maxima at 423, 445, and 474 nm, and molecular formula C₄₀H₅₄O₂ was observed. This carotenoid may have been (3R,3'S,6'S)-7,8-didehydro-β,ε-caroten-3,3'-diol (2). However, because of the available sample amount, further structural characterization of 2 was not possible.

4 Conclusion

Diatoxanthin, which originated from diatom, is widely distributed among freshwater fish^{1-3, 11)}. However, there are a few reports on the metabolites of diatoxanthin in freshwater fish³⁾. In the present investigation, a new metabolite of diatoxanthin, named 6'-epimonadoxanthin (1), was isolated from the rosary goby *G. castaneus*.

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Fig. 4 Possible metabolic pathways of diatoxanthin to 6'-epimonadoxanthin(1) (left column) and zeaxanthin to (3R,3'S,6'S)- and (3R,3'R,6'S)-lutein (right column) in the rosary goby *Gymnogobius castaneus*.

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