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ARTICLE

Carotenoids in Clams, *Ruditapes philippinarum* and *Meretrix petechialis*

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Carotenoids were investigated in two species of clams, *Rudiapes philippinarum* and *Meretrix petechialis*. Fucoxanthin 3-esters and fucoxanthinol 3-esters were found to be major components, along with crassostreaxanthin A 3-acetate, crassostreaxanthin A, crassostreaxanthin B 3-acetate, crassostreaxanthin B, halocynthiaxanthin 3′-acetate, halocynthiaxanthin, alloxanthin, diatoxanthin, diadinoxanthin, and heteroxanthin. Fatty acids esterified with fucoxanthin and fucoxanthinol were identified as C24:6, C22:5, C22:6, C20:5, C20:0, C20:1, C18:0, C18:1, C16:0, C16:1, and C14:0 from fast atom bombardment−mass spectrometry (FAB−MS) data. Crassostreaxanthin A 3-acetate and crassostreaxanthin B 3-acetate were first isolated and completely characterized by spectroscopic data. Furthermore, possible metabolic pathways of fucoxanthin in clams were proposed.

KEYWORDS: Carotenoids; clams; *Rudiapes philippinarum*; *Meretrix petechialis*; fucoxanthin esters; fucoxanthinol esters

INTRODUCTION

Marine shellfish contain carotenoids, which show great structural diversity (*1*, *2*). Some marine carotenoids showed biological functions, such as antioxidative, anti-tumor, anti-carcinogenic, and immune enhancement activities (*3*).

Some edible clams are bright orange or yellow color because of the presence of carotenoids. However, there have been only few reports on the carotenoids in clams (*4*−*6*). Short-neck clam, *Rudiapes philippinarum*, and hard clam, *Meretrix petechiali*, belonging to the family Veneridae, are important seafood in Japan. During the course of carotenoid studies on shellfish (*7*−*12*), there has been a particular focus on these two species. In the present investigation, 16 carotenoids isolated from these clams were characterized by spectroscopic methods with ultraviolet/visible (UV/vis), ^{1}H nuclear magnetic resonance (NMR), and fast atom bombardment−mass spectrometry (FAB−MS) data. Here, we describe the carotenoids in these two species of clams.

MATERIALS AND METHODS

Apparatus. The UV/vis spectra were recorded with a Shimadzu UV-240 spectrophotometer in diethyl ether (Et_2O). The positive-ion FAB−MS spectra were recorded using a JEOL JMS-HX 110A mass spectrometer, with *m*-nitrobenzyl alcohol as a matrix. The ^{1}H NMR (500 MHz) spectra were measured with a Varian UNITY INOVA 500 spectrometer in $CDCl_3$, with tetramethylsilane (TMS) as an internal standard. Preparative high-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD with a Shimadzu SPD-6AV spectrophotometer set at 450 nm. The column used was a 250 × 10 mm inner diameter, 10 μm LiChrospher RP-18 (e) (Cica-Merck, Darmstadt, Germany). Preparative gel permeation chromatography (GPC) was performed on a LC-908 HPLC system using a 600 × 20 mm inner diameter, 16 μm JAIGEL 2H column (Japan Analytical Industry Co., Ltd., Tokyo, Japan), with an RI-5 detector (Japan Analytical Industry Co., Ltd., Tokyo, Japan) and $CHCl_3$ as the eluting solvent at the flow rate of 3.8 mL/min.

Animal Materials. Short-neck clam, *R. philippinarum* (Veneridae), and hard clam, *M. petechialis* (Veneridae), were purchased at a local fish market in Kyoto city in February and March.

Quantification of Carotenoids. The total carotenoid content and the amount of carotenoids eluted by column chromatography were calculated using the extinction coefficient of $E^{1\%}_{cm} = 2500$ at λ_{max} (450 nm) and 1600 in the case of fucoxanthin derivatives (*13*). In the HPLC analysis, the relative amounts of individual carotenoids were calculated from the peak area detected at 450 nm.

Isolation of Carotenoids. Isolation procedures of carotenoids in *R. philippinarum* and *M. petechialis* were as follows. The edible part of *R. philippinarum* (140 g, about 50 specimens) was extracted with acetone (Me_2CO) at room temperature. The Me_2CO extract was partitioned between Et_2O and aqueous NaCl. The organic layer was dried over Na_2SO_4, then concentrated to dryness, and subjected to silica gel column chromatography (300 × 20 mm). The fraction eluted with 200 mL of ether/hexane (1:9, v/v) was subjected to preparative HPLC on ODS, LiChrospher RP-18 (e) with CH_3CN at the flow rate of 2.0 mL/min to yield β-carotene. The fraction eluted with 400 mL of ether from silica gel column chromatography contained fucoxanthin 3-esters and large amounts of steroid and lipid. To remove these lipid contaminants, this fraction was subjected to GPC on JAIGEL 2H with $CHCl_3$ as an eluting solvent. The individual fucoxanthin 3-esters were separated by HPLC on ODS with $CHCl_3$/CH_3CN (3:9, v/v) at the flow rate of 2.0 mL/min. The fraction eluted with 400 mL

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of acetone/ether (4:6, v/v) from silica gel column chromatography was further purified by GPC on JAIGEL 2H with $CHCl_3$, affording fucoxanthinol 3-esters. Individual fucoxanthinol 3-esters were separated by HPLC on ODS with $CHCl_3/CH_3CN$ (3:9, v/v). The fraction eluted with 400 mL of acetone/ether (6:4, v/v) from silica gel column chromatography was further purified by HPLC on ODS with $CHCl_3/CH_3CN$ (3:9, v/v), affording crassostreaxanthin A 3-acetate, crassostreaxanthin B 3-acetate, and halocynthiaxanthin 3′-acetate. The fraction eluted with acetone/ether (8:2, v/v) from silica gel column was further purified by preparative HPLC on ODS with $CHCl_3/CH_3CN$ (1:9, v/v) at the flow rate of 2.0 mL/min to yield crassostreaxanthin A, diatoxanthin, crassostreaxanthin B, diadinoxanthin, and diatoxanthin 3,6-epoxide. The fraction eluted with 400 mL of acetone from silica gel column chromatography was subjected to preparative HPLC on ODS with $CHCl_3/CH_3CN$ (1:9, v/v) at a flow rate of 2.0 mL/min to yield fucoxanthinol, fucoxanthin, heteroxanthin, and halocynthiaxanthin.

Carotenoids of *M. petechialis* were similarly isolated according to the methods described above.

Identification and Characterization of Carotenoids. β-Carotene (**1**), fucoxanthin (**2**), fucoxanthinol (**4**), crassostreaxanthin A (**6**), crassostreaxanthin B (**8**), halocynthiaxanthin (**10**), halocynthiaxanthin 3′-acetate (**11**), alloxanthin (**12**), diatoxanthin (**13**), diatoxanthin 3,6-epoxide (**14**), diadinoxanthin (**15**), and heteroxanthin (**16**) were identified on the basis of UV/vis, ^{1}H NMR, and FAB−MS data (*7*−*11*) and chromatographic behavior with our authentic samples obtained from the oyster *Crassostrea gigas* (*7*, *8*), *Corubicla japonica* (*9*, *10*), and *Mactra chinensis* (*11*).

Fucoxanthin 3-Esters (*3*). UV/vis and ^{1}H NMR data were the same as previous published values (*11*). FAB−MS: *m*/*z* 996.6852 $[M]^+$ (calcd $C_{66}H_{92}O_7$, 996.6843) fucoxanthin tetracosahexaenoate (C24:6), *m*/*z* 970.0669 $[M]^+$ (calcd $C_{64}H_{90}O_7$, 970.6687) docosapentaenoate (C22:5), *m*/*z* 968.6542 $[M]^+$ (calcd $C_{64}H_{88}O_7$, 968.6548) docosahexaenoate (C22:6), *m*/*z* 952.7160 $[M]^+$ (calcd $C_{62}H_{96}O_7$, 952.7156) eicosanonate (C20:0), *m*/*z* 950.6980 $[M]^+$ (calcd $C_{62}H_{94}O_7$, 950.7000) eicosenoate (C20:1), *m*/*z* 942.6349 $[M]^+$ (calcd $C_{62}H_{86}O_7$, 924.6372) eicosapentaenonate (C20:5), *m*/*z* 924.6860 $[M]^+$ (calcd $C_{60}H_{92}O_7$, 924.6843) octadecanate (C18:0), *m*/*z* 922.6690 $[M]^+$ (calcd $C_{60}H_{90}O_7$, 922.6687) octadesenate (C18:1), *m*/*z* 910.6692 $[M]^+$ (calcd $C_{59}H_{90}O_7$, 910.6687) heptadecanoate (C17:0), *m*/*z* 896.6495 $[M]^+$ (calcd $C_{58}H_{88}O_7$, 896.6530) hexadecanate (C16:0), *m*/*z* 894.6395 $[M]^+$ (calcd $C_{58}H_{86}O_7$, 894.6373) hexadecenoate (C16:1), *m*/*z* 868.6245 $[M]^+$ (calcd $C_{56}H_{84}O_7$, 868.6217) tetradecanoate (C14:0).

Fucoxanthinol 3-Ester (*5*). UV/vis and ^{1}H NMR data were the same as previous published values (*11*). FAB−MS: *m*/*z* 954.6724 $[M^+]$ (calcd for $C_{64}H_{90}O_6$, 954.6737) fucoxanthinol tetracosahexaenoate (C24:6), *m*/*z* 928.6575 $[M^+]$ (calcd $C_{62}H_{88}O_6$, 928.6581) docosapentaenoate (C22:5), *m*/*z* 926.6429 $[M^+]$ (calcd $C_{62}H_{86}O_6$, 926.6425) docosahexaenoate (C22:6), *m*/*z* 910.7015 $[M^+]$ (calcd $C_{60}H_{94}O_6$, 910.705) eicosanonate (C20:0), *m*/*z* 908.6920 $[M^+]$ (calcd $C_{60}H_{92}O_6$, 908.6894) eicosenoate (C20:1), *m*/*z* 900.6248 $[M^+]$ (calcd $C_{60}H_{84}O_6$, 900.6268) eicosapentaenonate (C20:5), *m*/*z* 882.6729 $[M^+]$ (calcd $C_{58}H_{90}O_6$, 882.6738) octadecanate (C18:0), *m*/*z* 880.6583 $[M^+]$ (calcd $C_{58}H_{88}O_6$, 880.6581) octadesenate (C18:1), *m*/*z* 868.6558 $[M^+]$ (calcd $C_{57}H_{88}O_6$, 868.6581) heptadecanoate (C17:0), *m*/*z* 854.6436 $[M^+]$ (calcd $C_{56}H_{86}O_6$, 854.6425) hexadecanate (C16:0), *m*/*z* 852.6280 $[M^+]$ (calcd $C_{56}H_{84}O_6$, 852.6268) hexadecenoate (C16:1), *m*/*z* 826.6125 $[M^+]$ (calcd $C_{54}H_{82}O_6$, 826.6123) tetradecanoate (C14:0).

Crassostreaxanthin A 3-Acetate (*7*). UV/vis: 450 and 470 nm (Et_2O). High-resolution (HR) FAB−MS: *m*/*z* 640.4125 $[M^+]$ (calcd for $C_{42}H_{56}O_5$, 640.4128). ^{1}H NMR ($CDCl_3$) δ: 0.99 (3H, d, *J* = 7 Hz, H-18′), 1.10 (3H, s, H-17′), 1.18 (3H, s, H-16), 1.20 (3H, s H-17), 1.31 (1H, ddd, *J* = 12, 11, 10 Hz, H-4′α), 1.57 (1H, dd, *J* = 12.5, 12.5 Hz, H-2*β*), 1.83 (1H, ddd, *J* = 12.5, 4, 1.5 Hz, H-2α), 1.92 (3H, s, H-18), 1.93 (1H, s, H-19′), 1.95 (1H, s, H-20), 1.99 (1H, s, H-20′), 2.01 (1H, s, H-19), 2.04 (1H, s, Ac), 2.14 (1H, dd, *J* = 18, 9 Hz, H-4*β*), 2.14 (1H, s, H-16′), 2.17 (1H, ddd, *J* = 12, 7, 5 Hz, H-4′*β*), 2.32 (1H, ddq, *J* = 11, 7, 7 Hz, H-5′), 2.49 (1H, ddd, *J* = 18, 5.5, 1.5 Hz, H-4α), 2.52 (1H, dd, *J* = 15, 5 Hz, H-2′ α), 2.69 (1H, dd, *J* = 15, 7 Hz, H-2′ *β*), 2.86 (1H, d, *J* = 13.5 Hz, H-7′), 2.93 (1H, d, *J* = 13.5, H-7′), 4.21 (1H, m, H-3′), 5.04 (1H, m, H-3), 6.24 (1H, d, *J* = 11.5 Hz, H-14), 6.37 (1H, d, *J* = 15 Hz, H-12), 6.42 (1H, d, *J* = 10 Hz, H-14′), 6.47 (1H, d, *J* = 11.5 Hz, H-10), 6.56 (1H, d, *J* = 15, 11.5 Hz, H-11), 6.59 (1H, dd, *J* = 15, 11 Hz, H-11′), 6.65 (1H, dd, *J* = 14, 10 Hz, H-15′), 6.68 (1H, d, *J* = 15 Hz, H-12′), 6.74 (1H, dd, *J* = 14, 11 Hz, H-15), 7.26 (1H, d, *J* = 11 Hz, H-10′).

Crassostreaxanthin B 3-Acetate (*9*). UV/vis: 450 and 470 nm (Et_2O). HR FAB−MS: *m*/*z* 640.4123 $[M^+]$ (calcd for $C_{42}H_{56}O_5$, 640.4128). ^{1}H NMR ($CDCl_3$) δ: 1.18 (3H, s, H-16), 1.20 (3H, s H-17), 1.57 (1H, dd, *J* = 12.5, 12.5 Hz, H-2*β*), 1.64 (1H, d, *J* = 1.5 Hz, H-17′), 1.68 (1H, d, *J* = 1.5 Hz, H-18′), 1.83 (1H, ddd, *J* = 12.5, 4, 1.5 Hz, H-2α), 1.92 (3H, s, H-18), 1.95 (3H, s, H-19′), 1.98 (3H, s, H-19), 1.99 (3H, s, H-20′), 2.01 (1H, s, H-19), 2.05 (3H, s, Ac), 2.14 (1H, dd, *J* = 18, 9 Hz, H-4*β*), 2.20 (1H, dd, *J* = 13, 6 Hz, H-4′), 2.22 (3H, s, H-16′), 2.44 (1H, dd, *J* = 13, 8 Hz, H-4′), 2.49 (1H, ddd, *J* = 18, 5.5, 1.5 Hz, H-4α), 2.65 (2H, m, H-2′), 3.51 (1H, d, *J* = 17 Hz, H-7′), 3.57 (1H, d, *J* = 17 Hz, H-7′), 4.20 (1H, m, H-3′), 5.04 (1H, m, H-3), 6.29 (1H, d, *J* = 11.5 Hz, H-14), 6.37 (1H, d, *J* = 15 Hz, H-12), 6.40 (1H, d, *J* = 11.5 Hz, H-14′), 6.47 (1H, d, *J* = 11.5 Hz, H-10), 6.56 (1H, dd, *J* = 15, 11.5 Hz, H-11), 6.62 (1H, dd, *J* = 15, 11.5 Hz, H-11′), 6.65 (1H, dd, *J* = 14, 11.5 Hz, H-15), 6.66 (1H, d, *J* = 15 Hz, H-12′), 6.74 (1H, dd, *J* = 14, 11.5 Hz, H-15′), 7.20 (1H, d, *J* = 10 Hz, H-10′).

Table 1. Carotenoid Content and Composition of Two Species of Clams

	R. philippinarum (mg/100 g of edible part)	*M. petechialis* (mg/100 g of edible part)
total carotenoid content	1.15	0.53
Carotenoid Compositions	(%)	(%)
β-carotene (**1**)	1.5	2.5
fucoxanthin (**2**)	10.9	8.9
fucoxanthin 3-esters (**3**)	9.5	11.5
fucoxanthinol (**4**)	3.9	10.0
fucoxanthinol 3-esters (**5**)	11.2	12.3
crassostreaxanthin A (**6**)	2.3	5.5
crassostreaxanthin A 3-acetate (**7**)	5.3	6.5
crassostreaxanthin B (**8**)	19.8	9.0
crassostreaxanthin B 3-acetate (**9**)	4.6	5.5
halocynthiaxanthin (**10**)	5.8	5.5
halocynthiaxanthin 3′-acetate (**11**)	6.2	8.5
alloxanthin (**12**)	4.0	2.0
diatoxanthin (**13**)	3.0	3.5
diatoxanthin 3,6-epoxide (**14**)	2.2	2.0
diadinoxanthin (**15**)	2.5	1.5
heteroxanthin (**16**)	5.0	4.2
unidentified carotenoids	2.3	1.1

RESULTS AND DISCUSSION

Carotenoid content and composition in *R. philippinarum* and *M. petechialis* are shown in **Table 1**.

The total carotenid contents in *R. philippinarum* and *M. petechialis* were found to be 1.15 and 0.5 mg/100 g, respectively, in the edible parts (wet weight). The following 16 carotenoids: β-carotene (**1**), fucoxanthin (**2**), fucoxanthin 3-ester (**3**), fucoxanthinol (**4**), fucoxanthinol 3-ester (**5**), crassostreaxanthin A (**6**), crassostreaxanthin A 3-acetate (**7**), crassostreaxanthin B (**8**), crassostreaxanthin B 3-acetate (**9**), halocynthiaxanthin (**10**), halocynthiaxanthin 3′-acetate, (**11**), alloxanthin (**12**), diatoxanthin (**13**), diatoxanthin 3,6-epoxide (**14**), diadinoxanthin (**15**), and heteroxanthin (**16**), were identified in both clams (**Figure 1**). Among them, fucoaxnthin and fucoxanthinol including their fatty acid esters were found to be major components. Fatty acids esterified with fucoxanthin and fucoxanthinol were identified as C24:6, C22:5, C22:6, C20:5, C20:0, C20:1, C18:0, C18:1, C17:0, C16:0, C16:1, and C14:0 from FAB−MS data. ^{1}H NMR revealed that the hydroxy groups at C-3 in fucoxanthin and fucoxanthinol were acylated, and fatty acids esterified with fucoxanthin and fucoxanthinol were normal type (*11*). The carotenoid composition in both cases was similar to that of *M. chinensis* (*11*), belonging to the family Mactridae.

In general, animals do not synthesize carotenoids *de novo*, and those found in animals are either directly accumulated from food or partly modified through metabolic reactions. The major food

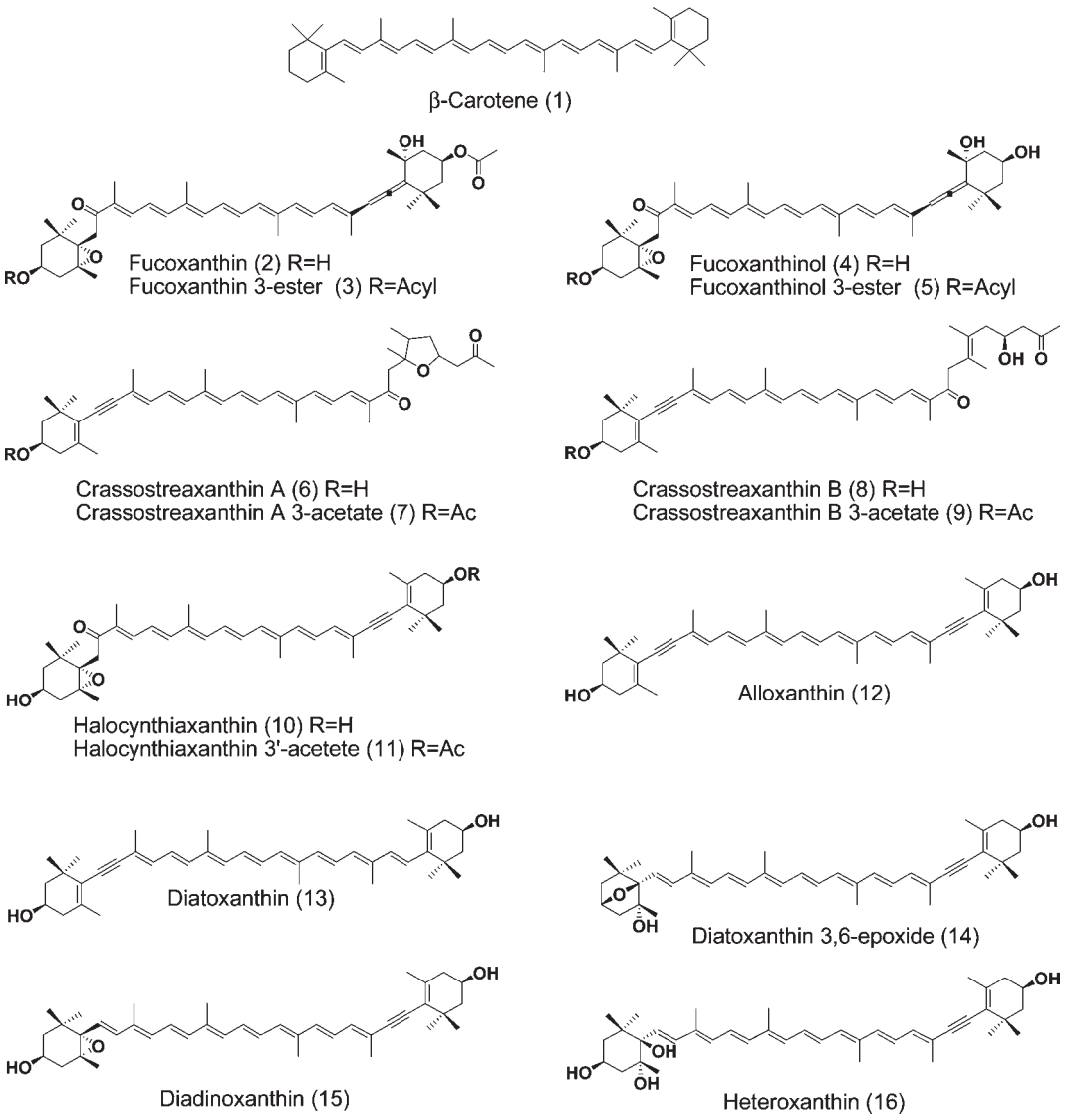

Figure 1. Identified carotenoids in two species of clams.

sources of the clam are microalgae, such as diatoms, which contain fucoxanthin as a major carotenoid (*2*). Fucoxanthin accumulated from dietary microalgae is metabolized to fucoxanthinol and halocynthiaxanthin in digestive organs (*1*, *2*, *11*). Some proportion of fucoxanthin and fucoxanthinol is esterified with fatty acids and deposited in the muscles and gonads of clams (*11*). Vershinin (*14*) reported that carotenoids in mollusca are dissolved in a lipid matrix and not bound to proteins and that their most likey function is in the stabilization of the fluidity of cell membranes. Therefore, it may be rationalized that esterification of fucoxanthin and fucoxanthinol with fatty acids increases solubility and stability in the lipid matrix in clams.

Crassostreaxanthin A and B, which have unique structural end groups, were first isolated from the oyster *C. gigas*, and their structures were determined to be 3′,6′-epoxy-3-hydroxy-6′-methyl-7,8-didehydro-1′,2′,5′,6′,7′,8′-hexahydro-16′-nor-β,ψ-carotene-1′,8′-dione and 3,3′-dihydroxy-7,8-didehydro-1′,2′,7′,8′-tetrahydro-6′-ethyl-16′-nor-β,ψ-carotene-1′,8′-dione, respectively (*7*). From the structural similarity, they are also assumed to be metabolites of halocynthiaxanthin (**Figure 2**). Furthermore, Tode et al. (*15*, *16*) demonstrated that the crassostreaxanthin B could be converted from halocynthiaxanthin by biomimetic chemical reactions.

In the present study, crassostreaxanthin A 3-acetete and crassostreaxanthin B 3-acetete were first isolated and could be completely characterized with reference to UV/vis, FAB−MS, and ^{1}H NMR data. They were also assumed to be metabolites of halicynthiaxanthin 3′-acetate. From the results of the present investigation, the possible metabolic pathways of fucoxanthin in clams are proposed in **Figure 2**.

Acetylenic carotenoids, alloxanthin, diatoxanthin, diadinoxanthin, and heteroxanthin, also accumulate from dietary microalgae (*2*). Diatoxanthin 3,6-epoxide was assumed to be a metabolite of diatoxanthin in clams, and 3,6-epoxy carotenoids are now known to be found in several shellfish (*8*−*11*).

Recently, fucoxanthin and its metabolites were noted to feature biological activities, such as radical scavenging and singlet oxygen quenching (*17*), and anti-carcinogenesis (*18*, *19*), anti-diabetic (*20*), and anti-obesity (*21*) potential. Furthermore, acetylenic carotenoids, alloxanthin and diatoxanthin, have been reported to have anti-carcinogenesis (*22*, *23*) and anti-inflammatory activities (*24*). Clams accumulate microalgal carotenoids, such as fucoxanthin, diatoxanthin, and alloxanthin, in their bodies. Therefore, they are good dietary sources of carotenoids originating from microalgae for humans.

In conclusion, 16 carotenoids were identified from the clams, *R. philippinarum* and *M. petechialis*. Fucoxanthin 3-esters and fucoxanthinol 3-etsers were found to be major carotenoids in both clams. Furthermore, crassostreaxanthin A 3-acetate and

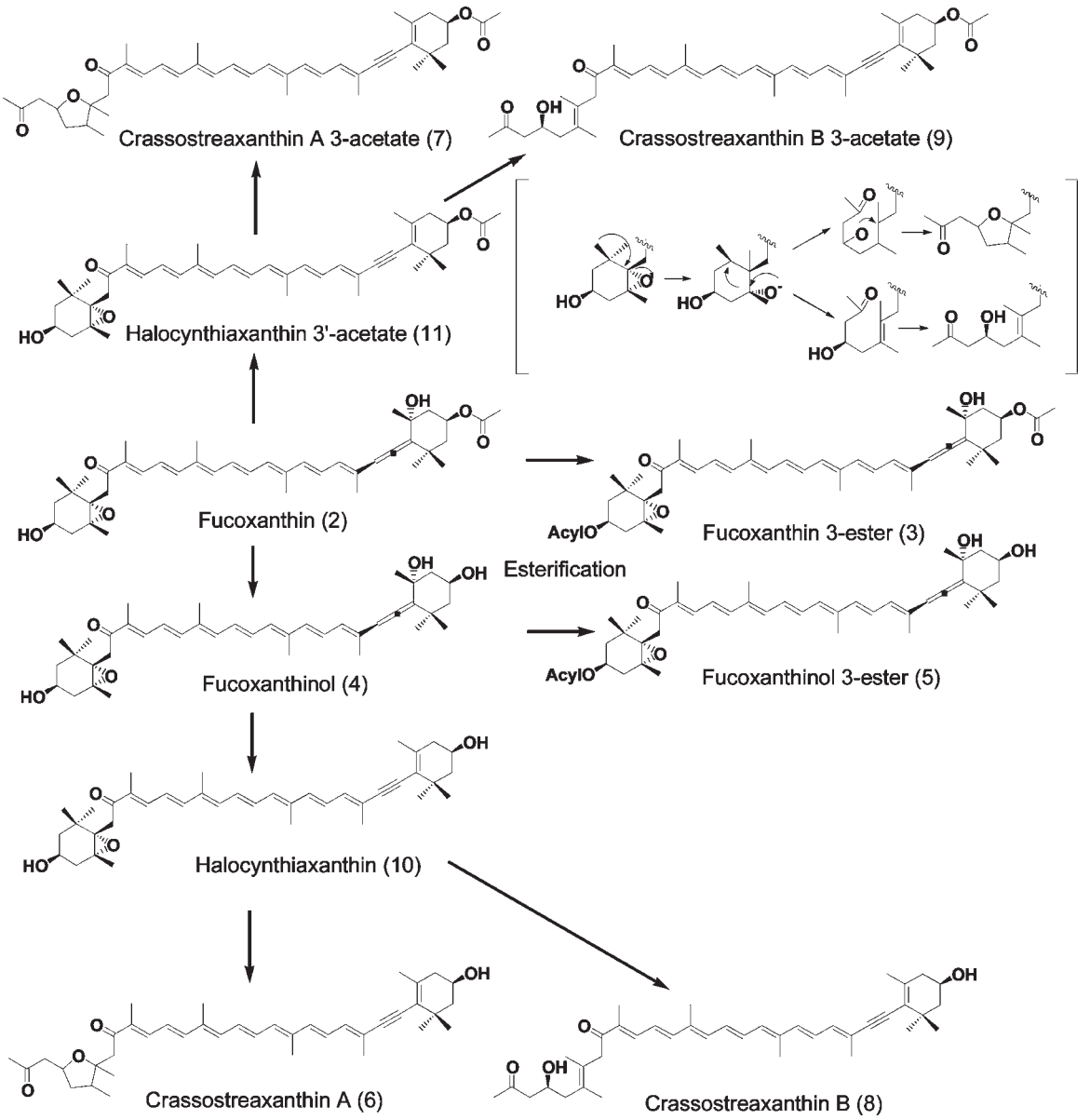

Figure 2. Possible metabolic pathways of fucoxanthin in clams.

crassestreaxanthin B 3-acetate were completely characterized by UV/vis, ^{1}H NMR, and FAB−MS data. Possible metabolic pathways of fucoxanthin in clams were proposed. Clams are good dietary sources of fucoxanthin and its metabolites, which have several biological activities, for humans.

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