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Bioactive Carotenoid, Fucoxanthin as Chemotaxonomic Marker and Antioxidative Agent from the Marine Bacillariophycean Microalga *Hantzschia marina*

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Abstract – Allenic and epoxy carotenoid, fucoxanthin (**1**) was isolated from the marine bacillariophycean microalga *Hantzschia marina* and the structure was assigned on the basis of comprehensive spectroscopic analyses. Fucoxanthin was detected only from diatom among three families (green algae, diatom and blue-green algae) of the marine microalgae tested. Fucoxanthin showed free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxynitrite ($ONOO^-$) with IC_{50} values of 32 μM and 60 μM, respectively.

Key words – Marine microalgae; diatom; Bacillariophyceae; *Hantzschia marina*; carotenoid; fucoxanthin; chemotaxonomic marker; radical scavenging activity; antioxidant; DPPH; peroxynitrite

Introduction

The phylogenetic breadth of microalgae is reflected in an equally broad biochemical diversity of pigments, photosynthetic storage products, cell walls and mucilages, fatty acids and lipids, oils, sterols and hydrocarbons, and bioactive compounds, including secondary metabolites (Metting Jr, 1996).

As part of our search for new biologically active marine natural products from cultured marine microalgae, we initiated a survey for free radical scavengers using DPPH, focusing on the development of an antiaging agent. Our initial screening process yielded several extracts of the marine microalgae with the scavenging activity of DPPH (Choi *et al.*, 2000) and peroxynitrite ($ONOO^-$) (Soung *et al.*, 1999). Here, we report the isolation of the carotenoid from the marine diatom *Hantzschia marina*.

Experimental

General – Optical rotation was determined on a Perkin Elmer model 341 polarimeter. IR spectrum was recorded on a Bruker FT-IR model IFS-88 spectrometer. ^1H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using TMS or solvent peaks as reference standard. Mass spectrum was obtained on a JEOL JMS-700 spectrometer. UV/visible spectrum was measured on a Hitachi U-2001 UV/Vis spectrometer.

Culture – Bacillariophycean microalga *Hantzschia marina* (strain #, KMCC B-37) was obtained from Korea Marine Microalgae Culture Center, Institute of Fisheries Science, Pukyong National University. The strain was cultured for 28 days at 23°C in a f/2 medium with aeration (filtered air, 0.3 L/min) under cool-white fluorescent illumination of 5000 lux. The f/2 medium composed of $NaNO_3$ (150 mg), NaH_2PO_4 (8.69 mg), Ferric EDTA (10.0 mg), $MnCl_2$ (0.22 mg), $CoCl_2$ (0.11 mg), $CuSO_4 \cdot 5\ H_2O$ (0.0196 mg), $ZnSO_4 \cdot 7\ H_2O$ (0.044 mg), $Na_2SiO_3 \cdot 9\ H_2O$ (50.0 mg), $Na_2MoO_4 \cdot 2\ H_2O$ (0.012 mg), vitamin B_{12} (1.0 μg), biotin (10.0 μg), thiamine·HCl (0.2 mg) per seawater (1 L). After 4 weeks, the alga was harvested by centrifugation at 10,000 g and by filtration with filterpaper from the 20 liter culture, and lyophilized.

Isolation of fucoxanthin (1) – The lyophilized alga

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(11.0 g) was extracted with CH_2Cl_2-MeOH (1:1) at r.t. and concentrated under reduced pressure to yield an extract (4.1 g). From the extract (1.0 g), 7 fractions were obtained over a silica gel column by eluting with *n*-hexane-EtOAc (100 % → 0 %). The fraction 4 (60 mg) containing **1** showed scavenging activity against DPPH and was further chromatographed on Si gel eluting with *n*-hexane/EtOAc (100% → 0%). The active fraction eluting with *n*-hexane/EtOAc (1:1) was subjected to semipreparative reversed-phase HPLC (YMC, ODS-A, 10×250 mm, 1 ml/min, detection at 445 nm) using MeOH to afford **1** (22 mg).

Fucoxanthin (1): bright orange solid; $[\alpha]_D$ +18° (*c* 0.2, $CHCl_3$); IR (KBr): 3438, 2361, 2332, 1723, 1654, 1605, 1251, 1030 cm^{-1}; UV (MeOH): 468 nm (ε 51,000) (sh), 446 (56,000), 332 (17,000), 267 (24,000); LREIMS *m/z* 658[(M^+), rel. int. 3], 640 [(M^+-H_2O), 15], 580 [(M^+-AcOH), 25], 562 [(580-H_2O), 3], 313 (2), 287 (24), 263 (24), 247 (55), 237 (36), 221 (100), 207 (50); See Table 1 for NMR spectral data of fucoxanthin (**1**).

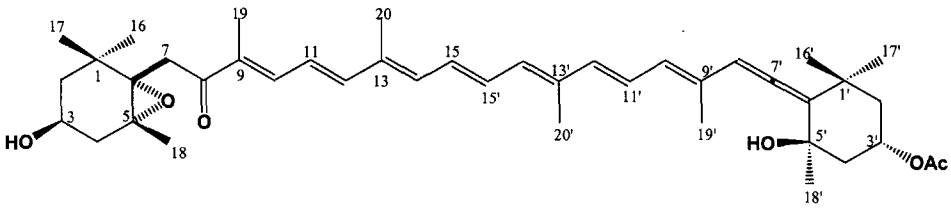
Distribution of fucoxanthin in the marine microalgae – The cultures (20 L) of 30 strains (Choi *et al.*, 2000) of the marine microalgae were harvested and extracted, as described above. From each extract, fucoxanthin was isolated, and analyzed by TLC [Merck, $60F_{254}$ precoated plate, *n*-hexane-EtOAc (1:1), R_f = 0.4] and HPLC (the same condition, as described above, t_R = 12.0 min).

Free radical (DPPH) scavenging assay (Choi *et al.*, 2000) – Samples to be tested were dissolved in MeOH and the solution (160 μl) was dispensed into wells of a 96-well microtiter tray. 40 μl of the DPPH solution in MeOH (1.5×10^{-4} M) was added to each well. The mixture was shaken and left to stand for 30 min, and the absorbance of the resulting solution was measured at 520 nm with microplate reader (Packard Co., Spectra Count™). The scavenging activity on DPPH radical was expressed as IC_{50}, which is the concentration of the tested compound required to give a 50% decrease of the absorbance from that of the blank solution [consisting of MeOH (160 μl) and DPPH solution (40 μl)].

Peroxynitrite scavenging assay (Soung *et al.*, 1999) – DHR (dihydrorhodamine) 123 (5 mM) in DMF, which was purged with nitrogen, was stored at −80 °C as a stock solution. DHR 123 diluted from the stock was placed in ice without exposure to light prior to the study. The buffer used was the mixture of 90 mM NaCl, 50 mM Na_3PO_4, and 5 mM KCl at pH 7.4, including 100 M diethylenetriaminepentaacetic acid (DTPA), each prepared with high quality deionized water and purged with nitrogen. The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured after 5 min (1 hr) with or without treatment of authentic peroxynitrite (SIN-1). Oxidation of DHR 123 by SIN-1 gradually increased. However, DHR 123 was oxidized rapidly by authentic peroxynitrite, and its final fluorescent intensity was unchanged over time. The fluorescent intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500, Bio-Tex Instruments) at the excitation wavelength of 480 nm and the emission wavelength of 525 nm, respectively. The effects were expressed as the % inhibition of oxidation of DHR 123.

Results and Discussion

Fucoxanthin (**1**), $[\alpha]_D$ +18°, was isolated as a bright orange solid. The IR spectrum of **1** showed the presence of the hydroxyl (3438 cm^{-1}), sp-hybrid carbon (allenic) (2361, 2332 cm^{-1}), ester (1723, 1251 cm^{-1}), and polyene (1654, 1605 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **1** revealed signals assignable to polyene having acetyl, conjugated ketone, two quaternary geminal dimethyls, two quaternary geminal methyls of oxygen, four olefinic methyls, and allene functionalities (Table 1). The physicochemical features outlined above suggested that **1** was a carotenoid in which one of the hydroxyl groups was acetylated. This suggestion was further supported by the UV/



fucoxanthin (**1**)

Table 1. ^{1}H- (δ, mult, *J*) and ^{13}C-NMR (δ, mult) data for fucoxanthin (**1**)[1,2]

C#	δ_H	δ_C	C#	δ_H	δ_C
1		35.2 (s)	1'		35.8 (s)
2	1.24(m), 1.46(m)	47.1 (t)	2'	1.35(m), 1.93(m)	45.5 (t)
3	3.80(m)	64.4 (d)	3'	5.37(m)	68.0 (d)
4	1.77(m), 2.29(m)	41.7 (t)	4'	1.46(m), 2.29(m)	45.3 (t)
5		66.2 (s)	5'		72.7 (s)
6		67.2 (s)	6'		117.5 (s)
7	2.58, 3.64(each d,18.3)	40.8 (t)	7'		202.4 (s)
8		197.9 (s)	8'	6.04(s)	103.4 (d)
9		134.5 (s)	9'		132.5 (s)
10	7.14(d, 10.7)	139.1 (d)	10'	6.11(d, 11.5)	128.5 (d)
11	6.59(m)	123.4 (d)	11'	6.59(m)	125.7 (d)
12	6.59(m)	145.0 (d)	12'	6.33(d, 15.1)	137.1 (d)
13		135.4 (s)	13'		138.1 (s)
14	6.39(d, 11.5)	136.6 (d)	14'	6.73(dd, 14.2, 11.7)	132.5 (d)
15	6.25(d, 11.5)	132.2 (d)	15'	6.59(m)	129.4 (d)
16	0.94(s)	28.1 (q)	16'	1.33(s)	31.3 (q)
17	1.02(s)	25.1 (q)	17'	1.05(s)	32.1 (q)
18	1.20(s)	21.4 (q)	18'	1.37(s)	29.2 (q)
19	1.93(s)	11.8 (q)	19'	1.80(s)	14.0 (q)
20	1.98(s)	12.8 (q)	20'	1.98(s)	12.9 (q)
			Ac		170.4 (s)
			Ac	2.03(s)	21.4 (q)

[1]Recorded in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C). Chemical shifts are relative to internal TMS (δ = 0 ppm) and $CDCl_3$ (δ = 77.0 ppm).
[2]Assignments aided by DEPT, COSY, HMQC and HMBC.

visible spectrum [468 nm (ε 51,000) (sh), 446 (56,000), 332 (17,000), 267 (24,000)]. From detailed comparison of the data for **1** with those of fucoxanthin, **1** was in agreement with an authentic fucoxanthin in all aspects (Palermo *et al.*, 1991; Yan *et al.*, 1999). Based on the above evidence, **1** was determined as fucoxanthin.

To our knowledge, this is the first isolation from *Hantzschia marina*. We have examined the distribution of fucoxanthin (1) in the marine microalgae. Fucoxanthin (1) was detected only from diatom among the family of the marine microalgae, Chlorophyceae (green alga), Bacillariophyceae (diatom) and Cyanophyceae (blue-green algae) from the TLC and HPLC examinations. Therefore, the fucoxanthin (1) is a good chemotaxonomic marker for three families (30 strains) tested (Choi *et al.*, 2000).

Fucoxanthin (**1**) showed a radical scavenging activity against DPPH and peroxynitrite ($ONOO^-$) with IC_{50} values of 32 μM and 60 μM, respectively.

The antioxidant properties of carotenoids, including fucoxanthin, have received attention due to their application in clinical, nutritional and industrial fields (Metting Jr, 1996). The presence of fucoxanthin has been reported in several marine organisms, e.g. diatom (Boczar *et al.*, 1989), red algi (Palermo *et al.*, 1991), and brown alga (Yan *et al.*, 1999), and it exhibited diverse biological activities such as antioxidant (Yan *et al.*, 1999), anticarcinogenesis (Nishino, 1995), and antimutagenesis (Nishino, 1998). Furthermore, because fucoxanthin is a major antioxidant in a common edible brown seaweed, *Hizikia fusiformis* (Yan *et al.*, 1999), it seems very worthwhile to explore the industrial utilization of fucoxanthin from the marine microalgae.

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