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Nitrocapsanthin and Nitrofucoxanthin, Respective Products of Capsanthin and Fucoxanthin Reaction with Peroxynitrite

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ABSTRACT: The in vitro reactivity of capsanthin (**1**) and fucoxanthin (**2**) with peroxynitrite was investigated, and the reaction products produced by scavenging with peroxynitrite were analyzed. (14′*Z*)-Nitrocapsanthin (**3**) and 12-nitrocapsanthin (**4**) were isolated from the products of the reaction of capsanthin with peroxynitrite. Similarly, (14*Z*)-15-nitrofucoxanthin (**5**), (11*Z*)-11-nitrofucoxanthin (**6**), and (14*Z*,9′*Z*)-15-nitrofucoxanthin (**7**) were obtained from the reaction of peroxynitrite reaction with fucoxanthin. Capsanthin and fucoxanthin inhibited the nitration of tyrosine by peroxynitrite. Furthermore, nitrocapsanthins (**3** and **4**) and nitrofucoxanthins (**5** and **6**) exhibited an inhibitory effect on Epstein−Barr virus early antigen activation in Raji cells and an antiproliferative effect on human pancreatic carcinoma. Moreover, nitrocapsanthins (**3** and **4**) inhibited carcinogensis of mouse skin tumors initiated by 7,12-dimethylbenz[a]anthracene (DMBN).

KEYWORDS: Capsanthin, fucoxanthin, nitrocapsanthin, nitrofucoxanthin, reaction with peroxynitrite

INTRODUCTION

Carotenoids show an excellent capacity to quench singlet oxygen ($^1O_2$) and inhibit lipid peroxidation. However, they cannot scavenge superoxide anion radicals (O_2^-), hydroxy radicals ($OH^•$), or hydrogen peroxide (H_2O_2). On the other hand, there have been few reports on scavenging and/or the reaction of reactive nitrogen species with carotenoids.[1] Peroxynitrite ($ONOO^-$), which is one of the reactive nitrogen species, is formed from superoxide (O_2^-) and nitric oxide ($NO^•$) in vivo and is a highly reactive oxidant that causes nitration of the aromatic ring of free tyrosine and protein tyrosine residues. Furthermore, peroxynitrite was found to induce various forms of oxidative damage such as low-density lipoprotein (LDL) oxidation, lipid peroxidation, and DNA strand breakage.[2]

It has been reported[3] that β-carotene is an effective scavenger of nitrogen dioxide (NO_2), peroxynitrous acid (ONOOH), and peroxynitrite and that the nitrogen atoms derived from nitrogen dioxide were tightly bound to the β-carotene molecule.[3] This suggested that carotenoids scavenge reactive nitrogen species. Thus, we have studied the reaction of carotenoids such as β-carotene, astaxanthin, and lutein with peroxynitrite[4−6] to clarify the mechanism whereby reactive nitrogen species are scavenged by carotenoids and reported the formation of nitrocarotenoids by this reaction.

Capsanthin (**1**), a major carotenoid in red paprika, has excellent antioxidative[7−9] and anticancer activities.[10−12] Fucoxanthin (**2**), a major carotenoid in brown algae, has also been noted to exhibit biological activities such as radical scavenging and singlet oxygen quenching[13] and anticarcinogenic,[14,15] antidiabetic,[16] and antiobesity[17] potential. In this study, we investigated the products of the reaction of capsanthin (**1**) and fucoxanthin (**2**) with peroxynitrite (Figure 1). Furthermore, the inhibitory effect of the nitration of tyrosine with capsanthin and fucoxanthin and the antitumor-promoting activity of nitrocapsanthins and nitrofucoxanthins were studied.

MATERIALS AND METHODS

General Experimental Procedures. UV−visible (UV/vis) spectra were recorded with a Hitachi U-2001 in diethyl ether (Et_2O). Positive ion fast atom bombardment−mass spectrometry (FAB-MS) spectra were recorded using a JEOL JMS-700 110A mass spectrometer with *m*-nitrobenzyl alcohol as a matrix. The ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Varian UNITY INOVA 500 spectrometer in $CDCl_3$ with TMS as an internal standard. High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD with a Shimadzu SPD-6AV spectrophotometer.

Reagents. Capsanthin (**1**) and fucoxanthin (**2**) were prepared from paprika and brown algae, respectively, according to the method described previously.[9,11,13] Peroxynitrite, 12-*O*-tetradecanoylphorbol-13-acetate

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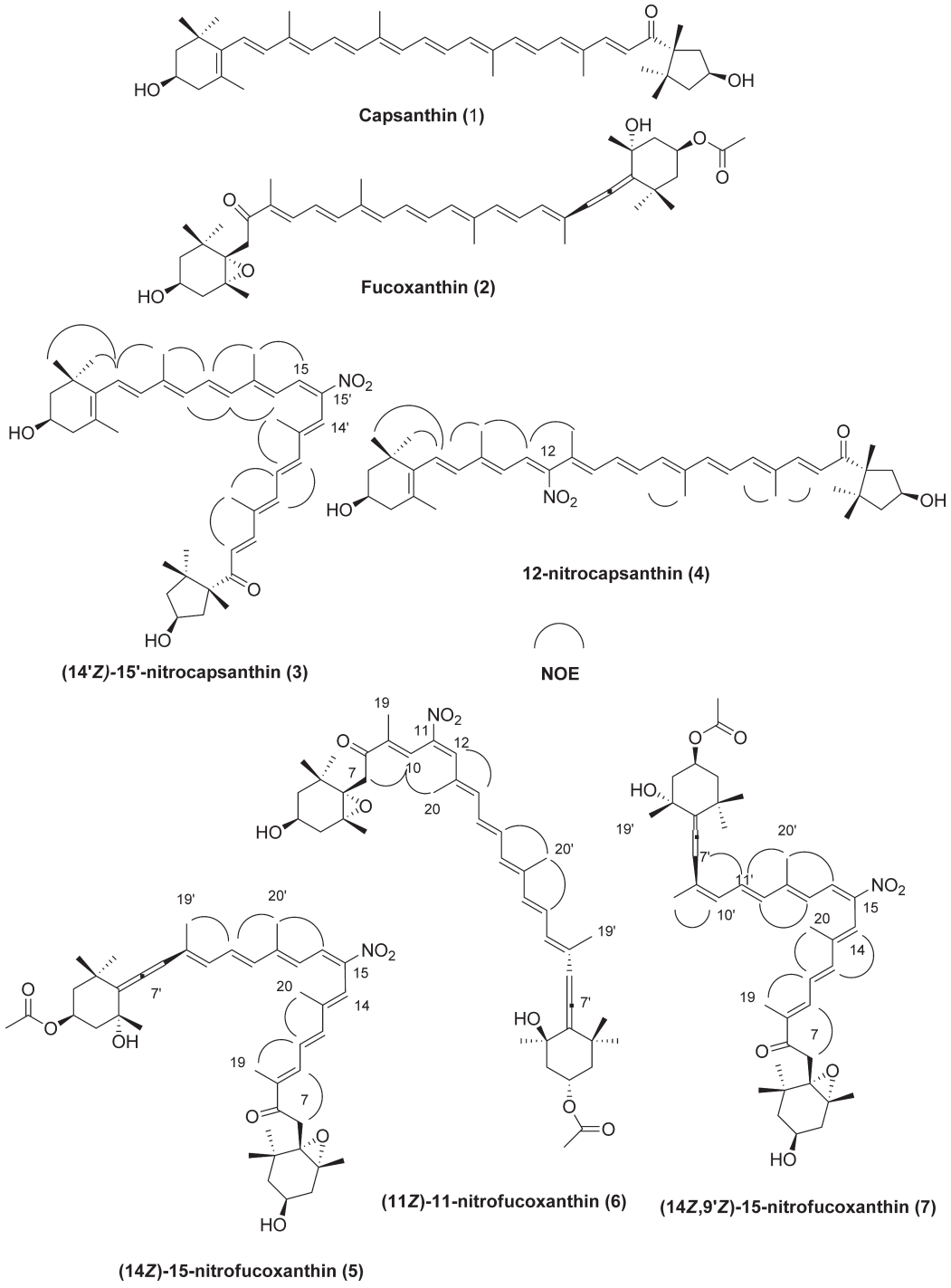

Figure 1. Structures of carotenoids.

(TPA), 7,12-dimethylbenz[a]anthracene (DMBA), and L-tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO).

Reaction of Capsanthin and Fucoxanthin with Peroxynitrite. According to the previous methods,[4−6] capsanthin or fucoxanthin (400 mg) was dissolved in 50 mL of tetrahydrofuran (THF). To this, trifluoroacetic acid (TFA) was added to make up the final concentration to 0.2%, before the addition of 16 mL of peroxynitrite in NaOH solution (final concentration, 6.8 mM). Then, the solution was allowed to react for 1 min. To the above mixture, 300 mL of $CHCl_3$ and 300 mL of H_2O were added so as to separate the reaction products into organic and aqueous phases. The whole procedure was performed three times. The organic layer was dried over anhydrous sodium sulfate and concentrated. This organic concentrate was then subjected to HPLC. For HPLC separation, a 250 mm × 4.6 mm i.d., 5 μm, Develosil C30-UG-5 column (Nomura Chemical Co., Ltd., Seto, Japan) was used and kept at 40 °C. The mobile phase used was a MeCN/H_2O (82:18 v/v), at a flow rate of 1 mL/min. A more specific separation procedure was performed using a 250 mm × 4.6 mm i.d., 5 μm, TSK gel ODS 100Z column (Tosoh Corp., Tokyo, Japan), kept at 40 °C with a mobile phase of MeCN/H_2O (75:25 v/v), at a flow rate of 1 mL/min.

*(14′Z)-15′-Nitrocapsanthin (**3**).* UV/vis λ max (Et_2O) nm 359, 456. HR FAB MS *m/z* 630.4159 [M + H^+] (calcd for $C_{40}H_{56}O_5N$, 630.4161). ^1H NMR and ^{13}C NMR (Table 1).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for (14′Z)-15′-Nitrocapsanthin (3) and 12-Nitrocapsanthin (4) and ^{1}H (500 MHz) Data for (14Z)-15-Nitrofucoxanthin (5), (11Z)-11-Nitrofucoxanthin (6), and (14Z,9′Z)-15-Nitrofucoxanthin (7) in $CDCl_3$[a]

	3			4			5			6			7	
no.	δ ^{13}C	δ ^{1}H	mult (J in Hz)	δ ^{13}C	δ ^{1}H	mult (J in Hz)	δ ^{13}C	δ ^{1}H	mult (J in Hz)	δ ^{13}C	δ ^{1}H	mult (J in Hz)	δ ^{1}H	mult (J in Hz)
1	37.1			37.1			35.8			35.8				
2	48.3	α 1.77	overlapped	48.3	α 1.77	overlapped	47.1	α 1.50	overlapped	47.2	α 1.50	overlapped	α 1.50	overlapped
		β1.48	dd (12, 12)		β 1.48	dd (12, 12)		β ~1.36	overlapped		β ~1.36	overlapped	β ~1.36	overlapped
3	64.9	4.00	m	64.8	4.00	m	64.2	3.82	m	64.1	3.82	m	3.82	m
4	42.5	α 2.39	dd (17, 6)	42.6	α 2.39	dd (17, 6)	41.6	α 2.35	dd (15, 6)	41.5	α 2.35	dd (15, 6)	α 2.35	dd (15, 6)
		β 2.06	dd (17, 9)		β 2.04	dd (17, 9)		β 1.79	dd (15, 9)		β 1.79	dd (15, 9)	β 1.79	dd (15, 9)
5	126.9			127.4			66.1			66.1				
6	137.5			137.6			66.9			66.7				
7	126.9	6.13	d (16)	132.3	6.46	d (15)	41.0	2.61	d (18)	41.4	2.67	d (18)	2.61	d (18)
								3.67	d (18)		3.73	d (18)	3.67	d (18)
8	128.1	6.24	d (16)	137.7	6.20	d (15)	198.1			197.8				
9	139.2			148.6			137.2			143.1				
10	130.9	6.16	d (10)	124.0	6.15	d (11)	127.7	7.16	d (11)	128.3	7.31	s	7.16	d (11)
11	130.7	6.95	dd (15, 11)	129.4	7.99	d (11)	126.4	6.74	dd (15, 11)	141.6			6.74	dd (15, 11)
12	136.0	6.41	d (15)	152.4			144.7	6.82	d (15)	141.8	7.91	s	6.82	d (15)
13	149.9			128.4			141.6			129.7				
14	125.4	5.96	d (12)	136.0	6.25	d (11)	123.0	6.49	s	146.1	6.80	d (11)	6.49	s
15	130.3	8.09	d (12)	132.0	6.65	m	150.3			127.5	6.57	dd (14.11)		
16	28.7	1.07	s	28.8	1.08	s	25.0	0.97	s	24.9	0.98	s	0.97	s
17	30.2	1.07	s	30.2	1.08	s	28.1	1.05	s	28.1	1.05	s	1.05	s
18	21.6	1.73	s	21.7	1.74	s	21.1	1.22	s	21.1	1.22	s	1.23	s
19	13.7	2.02	s	13.5	2.13	s	12.0	1.97	s	13.9	1.72	s	1.96	s
20	14.1	2.18	s	16.4	2.00	s	15.2	1.80	s	14.8	1.86	s	1.80	s
1′	44.0			44.0			35.1			35.1				
2′	50.8	α 2.00	overlapped	50.8	α 2.00	m	45.3	α 2.00	overlapped	45.4	α 2.00	overlapped	α 1.99	overlapped
2′		β 1.71	overlapped		β 1.71	m		β 1.41	dd (15, 15)		β 1.41	dd (15, 15)	β 1.40	overlapped
3′	70.3	4.52	m	70.3	4.51	m	67.8	5.38	m	67.9	5.38	m	5.38	m
4′	45.2	α 2.96	dd (15, 8)	45.3	α 2.96	m	45.2	α 2.29	dd (15, 6)	45.2	α 2.29	dd (15, 6)	α 2.29	dd (15, 6)
		β 1.49	overlapped		β 1.49	overlapped		β 1.51	overlapped		β 1.51	overlapped	β 1.51	overlapped
5′	59.0			59.0			72.6			72.7				
6′	203.0			202.9			117.8			117.6				
7′	122.0	6.51	d (15)	121.3	6.47	d (15)	203.0			202.6				
8′	146.4	7.33	d (15)	146.7	7.33	d (15)	103.2	6.05	s	103.3	6.06	s	6.59	s
9′	140.1			134.4			134.1			134.1				
10′	130.9	6.57	d (11)	140.2	6.53	d (11)	127.7	6.13	d (11)	128.7	6.14	d (11)	6.01	d (11)
11′	138.0	6.75	dd (15, 11)	125.0	6.67	dd (15, 11)	130.8	6.88	dd (15, 11)	127.0	6.69	dd (15, 11)	7.00	dd (15, 11)
12′	139.8	6.65	d (15)	141.4	6.55	d (15)	136.8	6.38	d (15)	136.5	6.36	d (15)	6.33	d (15)
13′	142.1			137.2			135.7			140.9				
14′	121.4	6.41	s	133.9	6.36	d (11)	125.1	5.93	d (12)	131.4	6.28	d (11)	5.93	d (12)
15′	145.1			129.8	6.67	m	131.2	8.12	d (12)	137.4	6.92	dd (14, 11)	8.12	d (12)
16′	25.1	1.38	s	25.8	1.37	s	29.2	1.39	s	29.2	1.39	s	1.37	s
17′	25.8	1.22	s	25.1	1.21	s	32.0	1.07	s	32.0	1.07	s	1.09	s
18′	21.2	0.85	s	21.2	0.85	s	31.2	1.35	s	31.2	1.35	s	1.40	s
19′	13.0	1.98	s	12.9	1.97	s	11.7	1.86	s	14.1	1.83	s	1.84	s
20′	15.2	1.77	s	12.8	1.98	s	14.2	2.18	s	13.1	2.02	s	2.18	s
CH_3–							21.4	2.04	s	21.4	2.05	s	2.04	s
CO							170.4			170.4				

[a] s, singlet; d, doublet; m, multiplet.

*12-Nitrocapsanthin (**4**).* UV/vis λ max (Et_2O) nm 346, 456. HR FAB MS m/z 630.4168 [M + H$^+$] (calcd for $C_{40}H_{56}O_5N$, 630.4161). ^{1}H NMR and ^{13}C NMR (Table 1).

*(14Z)-15-Nitrofucoxanthin (**5**).* UV/vis λ max (Et_2O) nm 314, 451. HR FAB MS m/z 704.4169 [M + H]$^+$ (calcd for $C_{42}H_{58}O_8N$, 704.4171). ^{1}H NMR and ^{13}C NMR (Table 1).

*(11Z)-11-Nitrofucoxanthin (**6**).* UV/vis λ max (Et_2O) nm 334, 456. HR FAB MS m/z 704.4168 [M + H]$^+$ (calcd for $C_{42}H_{58}O_8N$, 704.4171). ^{1}H NMR and ^{13}C NMR (Table 1).

*(14Z,9′Z)-15-Nitrofucoxanthin (**7**).* UV/vis λ max (Et_2O) nm 334, 453. HR FAB MS m/z 704.4167 [M + H]$^+$ (calcd for $C_{42}H_{58}O_8N$, 704.4171). ^{1}H NMR (Table 1).

Inhibition of Nitration of Tyrosine with Peroxynitrite by Carotenoids. Capsanthin or fucoxanthin (0.2 mg) was dissolved in 1 mL of methanol (MeOH). L-Tyrosine (0.36 mg) was added to this solution. In the control group, L-tyrosine (0.36 mg) was dissolved in 1 mL of MeOH. Then, 20 μL of peroxynitrite (final concentration, 6.8 mM) was added to the solution. The same procedure was carried out for γ-tocopherol (0.15 mg), which was used as a positive control for this assay. The solution was subsequently allowed to react for 5 min at room temperature. After that time, the content of nitrotyrosine, a product of the reaction between tyrosine and peroxynitrite, was quantified using HPLC. The following HPLC systems were employed for the quantification of nitrotyrosine. Column: Develosil ODS-UG-5 (20.0 mm × 250 i.d. mm; mobile phase, phosphate buffer (50 mM)/MeOH (97:3 v/v); flow rate, 1 mL/min; column temp, 40 °C; detection, 250 nm. Inhibition of the nitrotyrosine generated was measured by comparing absorbance of the sample solution (in MeOH) and ascorbic acid solution (in H_2O).

In Vitro Antitumor-Promoting Activity. *In Vitro Epstein–Barr Virus (EVA) Early Antigen Activation Induction Effect.* EBV genome-carrying lymphoblastoid cells (Raji cells) derived from Burkitt's lymphoma were cultivated in RPMI-1640 medium with 10% fetal bovine serum (FBS). The Raji cells were incubated for 48 h at 37 °C in a medium containing *n*-butyric acid (4 nmol), TPA (32 pmol), and various amounts of test compounds. Smears were made from the cell suspension, and we employed an indirect immunofluorescence technique. Details of the in vitro assay on EBV-EA induction have been reported previously.[11,18]

Antiproliferative Effects of Human Pancreatic Carcinoma (MIA PaCa-2). The MIA PaCa-2 (PaCa) cell line was established in 1975 from pancreatic tumor tissue of a 65 year old Caucasian male obtained from DS Pharma Biochemical Co. (Osaka, Japan). The cells were cultured in suspension at 37 °C in DMEM medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 in air. Nunclon 3.5 cm cell culture dishes from Nunc Co. (Kamstrupvej, Denmark) were used. PaCa cancer cells in the exponential growth phase were seeded and incubated with different concentrations of the carotenoids. For antiproliferation evaluation, the PaCa cells were incubated with the various concentrations of the test substances, and the effect on the cells was assessed after 3 days. For determination of the antiproliferation effects on surviving cells, the cell counting method was used. At the end of each experiment, the visible round-form cells were counted within a fixed range by macroscopic observation. All results are expressed as the number of round-form cells as a percentage of all visible cells.[19]

In Vivo Two-Stage Carcinogensis Assay for Nitrocapsanthins on Mouse Skin Papillomas Initiated by DMBA and Promoted by TPA. The animals (specific pathogen-free female ICR 6 week old mice) were divided into three experimental groups, each with 10 mice. The backs of the mice were shaved with surgical clippers, and they were treated topically with DMBA (100 μg, 390 nmol) in acetone (0.1 mL) as the initiator. One week after initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) to the skin. Group 1 (control group) received TPA treatment alone, and groups II and III received the topical application of 12-nitrocapsanthin (**4**) (85 nmol) and (14′Z)-15′-nitrocapsanthin (**3**) (85 nmol) in acetone, respectively, 1 h before the TPA treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

Experiments involving mice were conducted in accordance with Kanazawa University, Institute for Experimental Animals and use Committee Guidelines under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

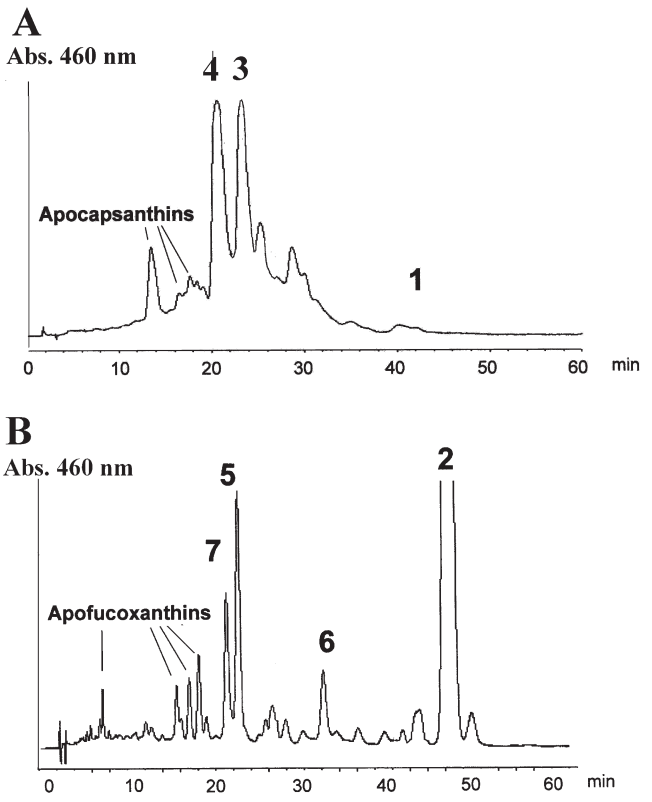

Figure 2. (A) HPLC of products of reaction of capsanthin with peroxynitrite. Peaks: 1, capsanthin; 3, (14′Z)-15′-nitrocapsanthin; and 4, 12-nitrocapsanthin. (B) HPLC of products of reaction of fucoxanthin with peroxynitrite. Peaks: 2, fucoxanthin; 5, (14Z)-15-nitrofucoxanthin; 6, (11Z)-11-nitrofucoxanthin; and 7, (14Z,9′Z)-15-nitrofucoxanthin.

RESULTS AND DISCUSSION

Structure of Products of Capsanthin and Fucoxanthin Reaction with Peroxynitrite. Capsanthin (**1**) was reacted with peroxynitrite, and the reaction products were analyzed by HPLC, as shown in Figure 2A. Two major reaction products, compounds **3** and **4**, were obtained.

Compound **3** showed absorption maxima at 359 and 456 nm. Its molecular formula was determined as $C_{40}H_{55}O_5N$ by HRFAB-MS, and it demonstrated a NO_2-substituted capsanthin structure. This structure was also characterized from ^{1}H and ^{13}C NMR including 2D NMR experiments. The partial structure of the end group and the polyene chain of compound **3** were characterized by ^{1}H NMR and ^{13}C NMR including ^{1}H−^{1}H correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments. The downfield shift of the ^{13}C NMR

signal at C-15′ (δ 145.1, quaternary carbon) along with disappearance of a methine proton at the C-15′ position in ^{1}H NMR as compared with capsanthin clearly indicated that a nitro group was attached to the C-15 position of capsanthin. Furthermore, the change in the coupling pattern and the downfield shifts of the ^{1}H NMR signals at H-15 (δ 8.09) and H-14′ (δ 6.41) as compared with capsanthin supported the substitution position of the nitro group at C-15′. The steric structure was confirmed by NOESY correlations between CH_3-16/17 and H-7, CH_3-19 and H-7/11, CH_3-20 and H-11/15, CH_3-19′ and H-7′/11′, and CH_3-20′ and H-11′/14. Therefore, the structure of **3** was determined to be (14′*Z*)-15′-nitrocapsanthin (**3**), as shown in Figure 1. A high intensity *cis* peak in the UV/vis spectrum (about 80% A_B/A_{II}) of **3** was also in agreement with this structure.[20]

Compound **4** showed absorption maxima at 346 and 456 nm. Its molecular formula was also determined to be $C_{40}H_{55}O_5N$ by HRFAB-MS, compatible with the formula of nitrocapsanthin. The ^{1}H and ^{13}C NMR signals of **4** were assigned based on ^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC experiments. The appearance of a quaternary carbon at δ 152.4 (C-12) in the ^{13}C NMR spectrum, along with the disappearance of a methine proton at the C-12 position in ^{1}H NMR as compared with capsanthin clearly indicated that a nitro group was attached to the C-12 position of capsanthin. Furthermore, the change in the coupling pattern from double doublet to doublet and the downfield shifts of the ^{1}H NMR signals at H-11 (δ 7.99) as compared with capsanthin also supported the substitution position of the nitro group at C-12. The *trans* geometry of the polyene chain in (**4**) was determined based on NOESY data, as shown in Figure 1. Disappearance of the *cis* peak in UV/vis spectrum also supports this geometry.[20] Therefore, the structure of **4** was determined to be 12-nitrocapsanthin (Figure 1). Employing a similar method to that described above, fucoxanthin (**2**) was reacted with peroxynitrite, and the reaction products were analyzed by HPLC, as shown in Figure 2B.

Compound **5** showed absorption maxima at 314 and 451 nm. Its molecular formula was determined to be $C_{42}H_{57}O_8N$ by HRFAB-MS, and it demonstrated an NO_2-substituted fucoxanthin structure. This structure was also characterized from ^{1}H and ^{13}C NMR including 2D NMR experiments. The partial structure of the end group and the polyene chain of compound **5** was characterized by ^{1}H NMR including ^{1}H−^{1}H COSY and NOESY experiments. The disappearance of a methine proton at the C-15 position and in ^{1}H NMR and the change in the coupling pattern from double doublet to doublet at H-15′ as compared with fucoxanthin clearly indicated that a nitro group was attached to the C-15 position of fucoxanthin. The marked downfield shift of quaternary carbon at C-15 (δ 150.3) was in agreement with this structure. The steric structure was confirmed by NOESY correlations between H-7 and H-11, CH_3-19 and H-11, CH_3-20 and H-11, CH_3-19′ and H-11′, and CH_3-20′ and H-11′/15′, as shown in Figure 1. Therefore, the structure of **5** was determined to be (14*Z*)-15-nitrofucoxanthin (**5**). A high-intensity *cis* peak in the UV/vis spectrum (about 80% A_B/A_{II}) of **5** was also in agreement with this structure.[20]

Compound **6** showed absorption maxima at 334 and 456 nm. Its molecular formula was determined to be $C_{42}H_{57}O_8N$ by HRFAB-MS, and it demonstrated an NO_2-substituted fucoxanthin structure. This structure was also characterized from ^{1}H and ^{13}C NMR including 2D NMR experiments. The disappearance of a methine proton at the C-11 position and in ^{1}H NMR and the change in the coupling pattern from double to singlet at H-10 (δ 7.31) and H-12 (δ 7.91) as compared with fucoxanthin clearly indicated that a nitro group was attached to the C-11 position of fucoxanthin. The marked downfield shift of quaternary carbon at C-11 (δ 141.6) was in agreement with this structure. NOESY correlations between CH_3-20 and H-10 and between H-7 and H-10 indicated the (11*Z*) configuration. Therefore, the structure of **6** was determined to be 11-*cis*-11-nitrofucoxanthin (**6**), as shown in Figure 1.

Compound **7** also showed a molecular formula of $C_{42}H_{57}O_8N$ by HRFAB-MS. ^{1}H NMR was similar to that of (14*Z*)-15-nitrofucoxanthin (**3**) except for H-7′, H-10′, and H-11′. The marked downfield shift of H-7′ (0.54 ppm) as compared with that of **5** suggested that this compound was a 9′-*cis* isomer of **3**. NOESY correlations between H-19′ and H-10′ and between H-7′ and H-11′ were in agreement with this structure. Therefore, **7** was determined to be (14*Z*,9′*Z*)-15-nitrofucoxanthin (Figure 1).

Other minor reaction products were obtained. Because of the small amount of samples, complete structural characterization could not be accomplished. From the partial assignment of ^{1}H NMR, they may be 12- and 12′-nitrofucoxanthins.

Inhibition of Nitration of Tyrosine with Peroxynitrite by Carotenoids. Capsanthin inhibited the nitration of tyrosine by peroxynitrite. Capsanthin suppressed the formation of nitrotyrosine to about 10.5% of that of the control group. This effect was almost the same as that of γ-tocopherol (9.2%). A similar result was also obtained in the case of fucoxanthin (11.5%). These results indicate that capsanthin and fucoxanthin are able to capture peroxynitrite to form nitrocarotenoids and inhibit the nitration of tyrosine.

It was reported that phenolic compounds such as *p*-coumaric acid and pelargonidin could scavenge peroxynitrite by the formation of nitro-*p*-coumaric acid and nitro-pelargonidin, respectively, and protect against the nitration of tyrosine.[21−23] Similar results were reported for tocopherols.[24,25] Our investigations also indicated that carotenoids could take up peroxynitrite through the formation of nitrocarotenoids.

Antitumor-Promoting Activity of Nitrocapsanthins and Nitrofucoxanthin. It is well-known that peroxynitrite and nitric oxide are strong initiators of carcinogenesis.[26,27] Previously, we reported that paprika carotenoids such as capsanthin inhibited the carcinogenesis initiated by peroxynitrite and promoted with TPA using a two-stages mouse carcinogenesis model.[12] This result suggested that capsanthin inhibited peroxynitrite-initiated carcinogenesis by the uptake of peroxynitrite and nitrocarotenoids themselves and that this might have an anticarcinogenic effect.

Thus, antitumor-promoting activities of nitrocapsanthins and nitrofucoxanthin were investigated. The in vitro antitumor-promoting activity of nitrocapsanthins (**3** and **4**) and nitrofucoxanthins (**5** and **6**) was examined using the Epstein−Barr virus (EBV) activation assay in Raji cells. The results are shown in Table 2. Nitrocapsanthins (**3** and **4**) and nitrofucoxanthins (**5** and **6**) showed inhibitory effects on the EBV-EA induction of Raji cells without significant cytotoxicity (more than 60% viability of Raji cells) in this assay. Among them, nitrofucoxanthins (**5** and **6**) showed slightly higher activity than fucoxanthin (**2**). Furthermore, nitocapsanthins (**3** and **4**) and nitrofucoxanthins (**5** and **6**) inhibited the proliferation of human pancreatic cancer cells, as shown in Table 3. These results showed that nitrocarotenoids themselves have anticarcinogenic activity.

Table 2. Relative Rate of EBV-EA Activation[a] with Respect to the Positive Control (100%) in the Presence of Carotenoids (1−6)

concn (mol ratio/TPA)[b]	1000	500	100	10	IC_{50}
compds	values				
capsanthin (**1**)	5.5 (>60)[c]	29.4	78.1	100	310
12-nitrocapsanthin (**4**)	7.4 (>60)	30.9	79.7	100	346
(14′Z)-15′-nitrocapsanthin (**3**)	4.6 (>60)	26.9	76.8	100	305
fucoxanthin (**2**)	3.1 (>60)	26.8	76.6	100	296
(11Z)-11-nitrofucoxanthin (**6**)	1.3 (>60)	23.6	72.0	95.0	275
(14Z)-15-nitrofucoxanthin (**5**)	2.6 (>60)	25.0	74.2	100	282

[a] Values represent the percentage relative to the positive control values (100%). [b] TPA concentration was 20 ng (32 pmol)/mol. [c] Values in parentheses are percentage viability of Raji cells. There are significant differences ($P < 0.01$) in inhibitory capacity in treatment groups of compounds **1**−**5** as compared with the control group.

Table 3. Relative Ratio of Antiproliferative Effects on Human Pancreastic Carcinoma (MIA PaVa-2) with Respect to the Positive Control (100%) in the Presence of Carotenoids (1−6)[a]

	% to control group	
concn	1 mM	0.1 mM
compds	values	
capsanthin (**1**)	90	100
12-nitrocapsanthin (**4**)	90	100
(14′Z)-15′-nitrocapsanthin (**3**)	80	100
fucoxanthin (**2**)	70	90
(11Z)-11-nitrofucoxanthin (**6**)	60	90
(14Z)-15-nitrofucoxanthin (**5**)	80	100

[a] There are significant differences ($P < 0.01$) in inhibitory capacity in the treatment groups of compounds **1**−**6** at 1 mM as compared with the control group.

Furthermore, the inhibitory effects of nitrocapsanthins on two-stages mouse skin carcinogenesis were investigated. The incidence (%) of papilloma-bearing mice and the average number of papillomas per mouse are presented and compared with a control group in Figure 3. When 12-nitrocapsanthin (**4**) (85 nmol) and (14′Z)-15′-nitrocapsanthin (**3**) (85 nmol) were applied before each TPA treatment, they markedly delayed the formation of papillomas and reduced the number per mouse, as shown in Figure 3. In the positive control group, the first papillomas appeared within 5 weeks of promotion, and in groups treated with 12-nitrocapsanthin (**4**) and (14′Z)-15′-nitrocapsanthin (**3**), the first papillomas appeared at 6 and 8 weeks, respectively. After 11 weeks of promotion, the control group showed a 100% incidence of papillomas, while in groups treated with 12-nitrocapsanthin (**4**) and (14′Z)-15′-nitrocapsanthin (**3**), only 50 and 30% of mice bore papillomas, respectively. After 20 weeks of promotion, 7.9 papillomas were found per mouse in the control group, whereas only 5.9 and 4.1 papillomas were found per mouse in the groups treated with 12-nitrocapsanthin (**4**) (85 nmol) and (14′Z)-15′-nitrocapsanthin (**3**), respectively. These results indicate that nitrocapsanthins (**3** and **4**) also had

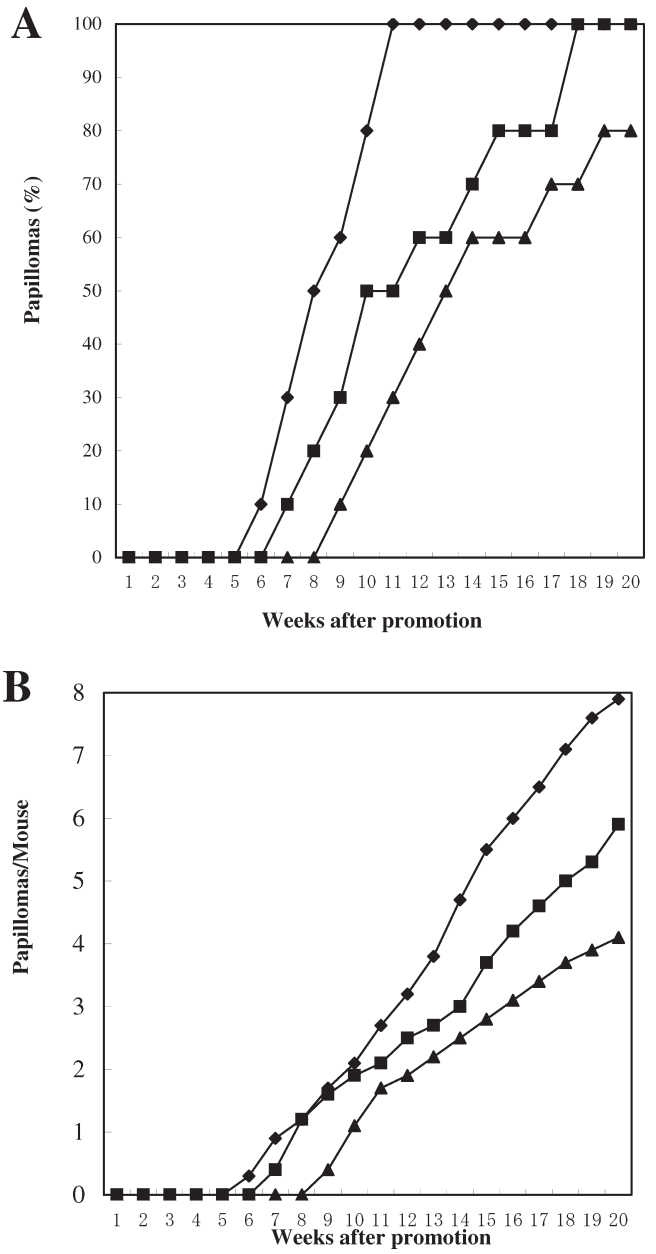

Figure 3. Inhibitory effects of casanthin on DMBA-induced mouse skin carcinogenesis. (A) Percentage of mice bearing papillomas and (B) average numbers of papillomas per mouse. Group I, DMBA (390 nmol) + TPA (1.7 nmol) (●). Group II, 12-nitrocapsanthin (**4**) (85 nmol) + peroxynitrite (390 nmol) + TPA (1.7 nmol) (■). Group III, (14′Z)-15′-nitrocapsanthin (**3**) (85 nmol) + peroxynitrite (390 nmol) + TPA (1.7 nmol) (▲).

inhibitory effects on two stages of mouse skin carcinogenesis as well as capsanthin.[11]

In conclusion, capsanthin and fucoxanthin could take up peroxynitrite through the formation of nitrocapsanthins and nitrofucoxanthins, respectively, and inhibit the nitration of tyrosine by peroxynitrite. Furthermore, nitrocapsanthins and nitrofucoxanthins exhibited antitumor-promoting activity. Therefore, carotenoids such as capsanthin and fucoxanthin may have the potential to reduce the risk of disease induced by reactive nitrogen species. Paprika and sea weeds, which contain capsanthin (**1**) and fucoxanthin (**2**) as major carotenoids,

respectively, might be valuable foods as prevention for active nitrogen-induced disease.

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