Characterization and Antioxidative Activities of Rare C₅₀ Carotenoids-Sarcinaxanthin, Sarcinaxanthin Monoglucoside, and Sarcinaxanthin Diglucoside-Obtained from *Micrococcus yunnanensis*

Ayako Osawa¹, Yoko Ishii¹, Nao Sasamura¹, Marie Morita¹, Hiroaki Kasai², Takashi Maoka³ and Kazutoshi Shindo^{1*}

¹ Department of Food and Nutrition, Japan Women's University (2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, JAPAN)

² Marine Biosciences Kamaishi Research Laboratory, Kitasato University (3-75-1 Heita, Kamaishi, Iwate 026-0001, JAPAN)

³ Research Institute for Production Development, Division of Food Function and Chemistry (15 Shimogamo-morimoto-cho, Sakyo-ku, Kyoto 606-0805, JAPAN)

Abstract: While screening for antioxidative carotenoids from marine bacteria, we isolated and identified sarcinaxanthin and its glucosylated compounds (sarcinaxanthin monoglucoside and sarcinaxanthin diglucoside) from a moderately halophilic bacterium-*Micrococcus yunnanensis* strain AOY-1. In the singlet oxygen (¹O₂) quenching model, the IC₅₀ values of the antioxidative activities of these carotenoids were as follows: sarcinaxanthin, 57 μM; sarcinaxanthin monoglucoside, 54 μM; and sarcinaxanthin diglucoside, 74 μM. In addition, the complete proton nuclear magnetic resonance (¹H NMR) assignments of sarcinaxanthin monoglucoside pentaacetate and sarcinaxanthin diglucoside octaacetate, and fast atom bombardment mass spectrometry/mass spectrometry (FAB-MS/MS) analyses of sarcinaxanthin and sarcinaxanthin monoglucoside are reported for the first time.

Key words: antioxidative activity, sarcinaxanthin, sarcinaxanthin monoglucoside, sarcinaxanthin diglucoside, *Micrococcus yunnanensis*

1 INTRODUCTION

Evaluating the pharmaceutical potential of various carotenoid pigments is an exciting field of medical research. However, the carotenoid species studied thus far in medical research are limited; they include dicyclic carotenoids, such as β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin, astaxanthin, and fucoxanthin, and an acyclic carotenoid, lycopene¹⁻⁴⁾. Carotenoids are widely distributed in marine organisms; it is hypothesized that they play an important role as antioxidants and protect these organisms from injury caused by free radicals and active oxygen species such as ¹O₂, an important active oxygen species⁵⁻⁷⁾. Thus, we began screening pigmented marine bacteria for novel or rare antioxidative carotenoids that possess ¹O₂-quenching activity. We isolated and identified 3 antioxidative carotenoids from *Micrococcus yunnanensis*¹⁰⁾: sarcinaxanthin⁸⁾, sarcinaxanthin monoglucoside,

and sarcinaxanthin diglucoside⁹⁾. In this report, we describe the isolation, identification, proton nuclear magnetic resonance (¹H NMR) assignments, and the singlet oxygen-quenching activity of these compounds.

2 EXPERIMENTAL PROCEDURES

2.1 Isolation

Strain AOY-1 was isolated from a leaf piece of hard coral growing on Akajima Island, Okinawa, Japan. Isolation and enrichment of strain AOY-1 was performed on 1/10 marine agar 2216 (Difco). To prepare this medium, 3.74 g marine broth 2216, 15 g agar, 700 mL artificial seawater¹¹⁾, and 300 mL distilled water were mixed and autoclaved. A piece of freshly collected hard coral (approximately 1 cm³) was vigorously crushed with a glass bar in 5 mL sterile artificial

*Correspondence to: Kazutoshi Shindo, Department of Food and Nutrition, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, JAPAN

E-mail: kshindo@fc.jwu.ac.jp

Accepted June 1, 2010 (received for review April 20, 2010)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
<http://www.jstage.jst.go.jp/browse/jos/>

seawater and left to stand in order to let the solid particles settle. The supernatant was further diluted to 1×10^{-1} using sterile artificial seawater; a 50 μL aliquot of diluted supernatant was spread plated on 1/10 marine agar 2216. The enrichment agar medium was incubated for 2 weeks at room temperature. The resulting yellow colony was transferred twice onto the same medium in order to purify the isolate and was used in this study.

2.2 Phylogenetic identification

The 16S rRNA gene was amplified from extracted DNA using universal bacterial primers specific to that gene: 27F and 1492R⁽¹²⁾. A neighbor-joining tree⁽¹³⁾ was constructed based on the nucleotide sequences of the 16S rRNA genes of closely related species of micrococci using MEGA3.1 software⁽¹⁴⁾. The reliability of the phylogenetic tree was evaluated based on the bootstrap probability⁽¹⁵⁾.

2.3 Cultivation

Yellow bacteria AOY-1 were inoculated on to a 100 mL medium (nutrient broth containing 1.0 M NaCl) in a 500 mL Erlenmeyer flask and cultured at 30°C for 24 h on a rotary shaker (150 rpm). The OD₄₃₀ of the culture reached 8.0 by the end of fermentation.

2.4 Isolation of carotenoids

Bacterial cells (32 L) were collected by centrifugation at 13,000 g. After removing the supernatant, the cells were suspended in 0.5 M NaOH at room temperature for 10 min and sonicated for 5 min to digest the cell walls. After removing the NaOH solution by centrifugation, the yellow pigment in the digested cells was extracted 3 times with $\text{CH}_2\text{Cl}_2\text{:MeOH}$ (1:1). The extracts were combined, concentrated to a small volume *in vacuo*, and partitioned between $\text{EtOAc/H}_2\text{O}$ without adjusting the pH. The EtOAc layer was evaporated to dryness (574.6 mg) and subjected to preparative ODS HPLC using a Senshu Pak ODS column (10 $\times$ 250 mm) (Senshu Scientific Co. Ltd.). Development was set at a flow rate of 3 mL/min with solvent A (MeOH) for 10 min, followed by a linear gradient from solvent A to solvent B ($\text{CH}_2\text{Cl}_2\text{:MeOH}$ = 1:6) for 15 min, and finally with solvent B for 15 min; the maximum absorbance being monitored was in the range of 200–600 nm. The yellow compound was collected at retention time (Rt) 7.47 min, Rt 12.45 min, and Rt 19.75 min, and concentrated to dryness to give pure **1** (1.1 mg), **2** (2.6 mg), and **3** (1.6 mg), respectively.

2.5 Spectroscopic analysis

The ^1H and ^{13}C NMR spectra for sarcinaxanthin were measured at 500 MHz using a Varian UNITY INOVA 500 spectrometer, and the ^1H NMR spectra for the other compounds were measured at 400 MHz using a Bruker AMX400. HRFAB-MS and HRESI-MS were recorded with

JEOL JMS-HX100A and JMS-T100LP mass spectrometers respectively. Positive ion FAB-MS/MS spectra were recorded using a JEOL JMS-HX/HX 110A four-sector tandem mass spectrometer with *m*-nitrobenzyl alcohol as a matrix^(16, 17). UV-VIS spectra were recorded using a Hitachi U-3200.

2.6 Acetylation of carotenoids

The carotenoid [sarcinaxanthin monoglucoside (1.5 mg) and sarcinaxanthin diglucoside (2.0 mg)] was dissolved in 3 mL acetic anhydride and 3 mL dried pyridine, and stirred for 18 h at room temperature under dark conditions. After removing the solvents *in vacuo*, the peracetylated carotenoid was applied to silica gel (Silica Gel 60; Merck) column chromatography (1 $\times$ 20 cm) with hexane- EtOAc (5:2) to give pure carotenoid [sarcinaxanthin monoglucoside pentaacetate (1.8 mg) and sarcinaxanthin diglucoside octaacetate (2.2 mg)].

2.7 Singlet oxygen quenching activity

$^1\text{O}_2$ -quenching activity was examined by measuring methylene blue-sensitized photooxidation of linoleic acid⁽¹⁸⁾. Forty microliters of 0.05 mM methylene blue and 10 μL of 2.4 M linoleic acid with or without 40 μL carotenoid (final concentration 1–100 μM , each dissolved in EtOH) were added to micro glass vials (5.0 mL). The vials were tightly closed with a screw cap and a septum, and the mixtures were illuminated at 7,000 lux at 22°C for 3 h in a corrugated cardboard. Then, 50 μL of the reaction mixture was removed and diluted to a final volume of 1.5 mL with EtOH . The absorbance at 235 nm was measured to estimate the formation of conjugated dienes⁽¹⁹⁾. The value of the absence of carotenoid was determined; $^1\text{O}_2$ -repression activity was calculated relative to this reference value. Activity is indicated as the IC_{50} value, which represents the concentration at which 50% inhibition was observed.

3 RESULTS AND DISCUSSION

3.1 Isolation and identification of bacteria

A yellow bacterium designated as strain AOY-1 was selected from 65 bacterial strains isolated from hard coral. Because the hard coral specimen was carefully washed with sterile artificial seawater, the isolated bacteria were probably tightly associated with the surface or certain organs of the hard coral.

A 1,465-nucleotide sequence of the 16S ribosomal RNA (rRNA) of strain AOY-1 was determined. On the basis of the 16S rRNA gene sequence analysis, strain AOY-1 was found to be closely related to the genus *Micrococcus*. The nucleotide sequence of the 16S rRNA gene of strain AOY-1 had a 99.6% similarity to that of *Micrococcus yunnanensis* YIM 65004^T, followed by *M. luteus* DSM 20030^T

Fig. 1 Phylogenetic tree showing the phylogenetic relationship of strain AOY-1 and all known cultivated species of the genus *Micrococcus* based on 16S rRNA gene sequences. Accession numbers of 16S rRNA gene sequence are in parentheses. Numbers at nodes are percentage bootstrap values derived from 1000 replications. Bar, 0.5% estimated difference in nucleotide sequence.

Fig. 2 Structures of carotenoids produced by *Micrococcus yunnanensis* [sarcinaxanthin (1), sarcinaxanthin monoglucoside (2), and sarcinaxanthin diglucoside (3)], and peracetylated derivative of sarcinaxanthin monoglucoside (4) and sarcinaxanthin diglucoside (5).

(99.4%), and *M. endophyticus* YIM 56238^T (99.1%); all other confirmed species of the genus *Micrococcus* were more distantly related (97.1-98.5%). The phylogenetic re-

lationship between strain AOY-1 and micrococci is shown in Fig. 1; strain AOY-1 is grouped with *M. yunnanensis* and *M. luteus*. On the basis of the phylogenetic analysis

and the 16S rRNA gene similarity, strain AOY-1 was identified as *M. yunnanensis*. *M. yunnanensis* is closely related to *M. luteus* (identity of 16S rRNA gene, 99.71%); however, the differences in phenotypic and genetic characteristics were sufficient to differentiate *M. yunnanensis* from *M. luteus*¹⁰. The original strain of *M. yunnanensis* was isolated from the sterilized surface of eggplant roots. This is the first report on carotenoid production by a marine strain of *M. yunnanensis*.

Thus far, the production of sarcinaxanthin has been reported in 7 bacterial strains: *Flavobacterium dehydrogenans*²⁰, *F. marinotypicum*²¹, *M. luteus*⁹, *Corynebacterium poissetiae*²², *C. erythrogenes*²³, *Cellulomonas biazotea*²⁴, and *Staphylococcus aureus*²⁵. Two strains that were formerly identified as *Flavobacterium* have been reclassified as follows: *Agromyces mediolanus* (basonym, *F. dehydrogenans*) and *Microbacterium maritypicum* (basonym, *F. marinotypicum*); because of this, all sarcinaxanthin-producing bacteria are classified as gram-positive bacteria. The production of sarcinaxanthin mono- and diglucosides is therefore limited to 2 closely related species of the genus *Micrococcus* (*M. luteus*⁹ and *M. yunnanensis*¹⁰).

3.2 Identification of carotenoids

Compound **1** was analyzed by HRFAB-MS (M^+ calcd, 704.5532; found 704.5519; $C_{50}H_{72}O_2$) and identified as sarcinaxanthin (Fig. 2) by comparison with previously reported spectral data²⁶, the 1H and ^{13}C NMR spectra are shown in Table 1. In general, FAB-MS provides a non-fragmented spectrum. On the other hand, the FAB MS/MS technique generates several fragmented ions that are derived from structural features. Thus, the FAB MS/MS spectrum of **1** was also measured for its characterization of this compound (Fig. 3). Compound **1** exhibited characteristic product ions in this spectrum at the following: m/z 686 [$M-H_2O$]⁺, m/z 618 [$M-86$]⁺ (elimination of side-chain isoprene unit $C_5H_{10}O$ unit), m/z 612 [$M-92$]⁺ (elimination of toluene from polyene chain)¹⁶, and m/z 496 [$M-208$]⁺ (attributed to cleavage between C-6 and C-7). This information was important in identifying **1** as sarcinaxanthin using MS analyses.

The UV-VIS spectra of **2** and **3** in CH_3OH were similar to that of **1** [λ_{max} (MeOH) 410, 433, 457, (%III/II 25.0)], indicating they possess the same aglycone. HRESI-MS analyses of **2** and **3** confirmed their molecular formulas to be $C_{56}H_{82}O_7$ [**2**, ($M+Na$)⁺ calcd, 889.59582; found 889.59270] and $C_{62}H_{92}O_{12}$ [**3**, ($M+Na$)⁺ calcd, 1051.64864; found, 1051.65088] respectively. Considering the molecular formulas of **2** (**1** + $C_6H_{12}O_6-H_2O$) and **3** (**1** + $2C_6H_{12}O_6-2H_2O$), they were speculated to be sarcinaxanthin monoglucoside⁹ and sarcinaxanthin diglucoside⁹ respectively. The characteristic product ions at m/z 848 [$M-H_2O$]⁺, m/z 780 [$M-86$]⁺ (elimination of side-chain isoprene unit $C_5H_{10}O$ unit), m/z

Fig. 3 FAB MS/MS spectra of sarcinaxanthin (**1**) and sarcinaxanthin monoglucoside (**2**).

774 [$M-92$]⁺ (elimination of toluene from polyene chain), m/z 687 [M -glucose + H]⁺, m/z 658 [$M-208$]⁺ (attributed to cleavage between C-6' and C-7'), and m/z 495 [$M-371$]⁺ (attributed to cleavage between C-6 and C-7) observed in the FAB MS/MS analysis of **2** (Fig. 3) also support this speculation. To clarify the structures of **2** and **3**, they were individually acetylated with Ac_2O in dry pyridine to give the pentaacetate [**4**, ESI-MS m/z 1099 ($M+Na$)⁺] and octaacetate derivative [**5**, ESI-MS m/z 1387 ($M+Na$)⁺], respectively. Each acetylated derivative was analyzed by 1H NMR and 1H - 1H COSY spectra. In these analyses, the large vicinal coupling constants observed in the sugar signals ($J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ = 7.7–9.9 Hz) indicate that the sugar(s) are β -glucose; the complete assignments of the aglycone signals indicate that they are sarcinaxanthin. Thus, **2** and **3** were identified as sarcinaxanthin monoglucoside- and sarcinaxanthin diglucoside, respectively. The 1H NMR assignments of these peracetylated derivatives are listed in Table 1.

Although **2** and **3** were not soluble in $CDCl_3$, the acetylated samples (**4** and **5**) were highly soluble. Since the reported NMR data (1H and ^{13}C chemical shifts) of sarcinaxan-

Table 1 ^1H NMR data for sarcinaxanthin monoglucoside penta-acetate (4) and sarcinaxanthin octa-acetate (5), and ^1H and ^{13}C NMR data for sarcinaxanthin (1) in CDCl_3 .

	4	5	1	
position	δ_{H} (mult. J in Hz)	δ_{H} (mult. J in Hz)	δ_{H} (mult. J in Hz)	δ_{C}
C (1)				39.2
CH (2)	1.26 (1H, m)	1.24 (1H, m)	1.28 (1H, m)	48.4
CH ₂ (3)	1.18 (1H, m)	1.19 (1H, m)	1.18 (1H, m)	28.9
	1.71 (1H, m)	1.71 (1H, m)	1.71 (1H, m)	
CH ₂ (4)	2.02 (1H, m)	2.04 (1H, m)	2.05 (1H, m)	36.3
	2.35 (1H, m)	2.35 (1H, m)	2.35 (1H, m)	
C (5)				150.4
CH (6)	2.48 (1H, d, 10.0)	2.49 (1H, d, 10.0)	2.48 (1H, d, 9.5)	58.4
CH (7)	5.82 (1H, dd, 10.0, 15.0)	5.82 (1H, dd, 10.0, 15.0)	5.83 (1H, dd, 9.5, 15.5)	128.3
CH (8)	6.12 (1H, d, 15.0)	6.13 (1H, d, 15.0)	6.12 (1H, d, 15.5)	137.5
C (9)				135.4
CH (10)	6.12 (1H, d, 12.0)	6.12 (1H, d, 10.8)	6.12 (1H, d, 12.0)	130.7
CH (11)	6.62 (1H, dd, 12.0, 14.9)	6.62 (1H, dd, 10.8, 15.0)	6.62 (1H, dd, 12.0, 15.0)	124.9
CH (12)	6.34 (1H, d, 14.9)	6.34 (1H, d, 15.0)	6.34 (1H, d, 15.0)	137.3
C (13)				136.4
CH (14)	6.25 (1H, d, 7.3)	6.24 (1H, d, 8.8)	6.24 (1H, d, 8.0)	132.4
CH (15)	6.62 (1H, m)	6.63 (1H, m)	6.62 (1H, m)	130.0
CH ₃ (16)	0.96 (3H, s)	0.96 (3H, s)	0.95 (3H, s)	27.6
CH ₃ (17)	0.72 (3H, s)	0.73 (3H, s)	0.73 (3H, s)	15.2
CH ₂ (18)	4.53 (1H, s)	4.54 (1H, s)	4.53 (1H, s)	108.1
	4.76 (1H, s)	4.77 (1H, s)	4.75 (1H, s)	
CH ₃ (19)	1.98 (3H, s)	1.98 (3H, s)	1.98 (3H, s)	13.2
CH ₃ (20)	1.97 (3H, s)	1.97 (3H, s)	1.97 (3H, s)	12.8
C (1')				39.2
CH (2')	1.26 (1H, m)	1.24 (1H, m)	1.28 (1H, m)	48.4
CH ₂ (3')	1.18 (1H, m)	1.19 (1H, m)	1.18 (1H, m)	28.9
	1.74 (1H, m)	1.71 (1H, m)	1.71 (1H, m)	
CH ₂ (4')	2.02 (1H, m)	2.04 (1H, m)	2.05 (1H, m)	36.3
	2.35 (1H, m)	2.35 (1H, m)	2.35 (1H, m)	
C (5')				150.4
CH (6')	2.48 (1H, d, 9.2)	2.49 (1H, d, 10.0)	2.48 (1H, d, 9.5)	58.4
CH (7')	5.82 (1H, dd, 9.2, 15.0)	5.82 (1H, dd, 10.0, 15.0)	5.83 (1H, dd, 9.5, 15.5)	128.3
CH (8')	6.12 (1H, d, 15.0)	6.13 (1H, d, 15.0)	6.12 (1H, d, 15.5)	137.5
C (9')				135.4
CH (10')	6.12 (1H, d, 12.0)	6.12 (1H, d, 10.8)	6.12 (1H, d, 12.0)	130.7
CH (11')	6.62 (1H, dd, 12.0, 14.9)	6.62 (1H, dd, 10.8, 15.0)	6.62 (1H, d, 12.0, 15.0)	124.9
CH (12')	6.34 (1H, d, 14.9)	6.34 (1H, d, 15.0)	6.34 (1H, d, 15.0)	137.3
C (13')				136.4
CH (14')	6.25 (1H, d, 7.3)	6.24 (1H, d, 8.8)	6.24 (1H, d, 8.0)	132.4
CH (15')	6.62 (1H, m)	6.63 (1H, m)	6.62 (1H, m)	130.0
CH ₃ (16')	0.95 (3H, s)	0.96 (3H, s)	0.95 (3H, s)	27.6
CH ₃ (17')	0.72 (3H, s)	0.73 (3H, s)	0.73 (3H, s)	15.2
CH ₂ (18')	4.53 (1H, s)	4.54 (1H, s)	4.53 (1H, s)	108.1
	4.76 (1H, s)	4.77 (1H, s)	4.75 (1H, s)	
CH ₃ (19')	1.98 (3H, s)	1.98 (3H, s)	1.98 (3H, s)	13.2
CH ₃ (20')	1.97 (3H, s)	1.97 (3H, s)	1.97 (3H, s)	12.8
CH ₂ (1'')	1.71 (1H, m)	1.74 (1H, m)	1.72 (1H, m)	28.4
	2.25 (1H, m)	2.25 (1H, m)	2.24 (1H, dd, 5.5, 14.0)	
CH (2'')	5.42 (1H, dd, 6.2, 7.5)	5.41 (1H, dd, 6.3, 6.8)	5.43 (1H, m)	126.1

	4	5	1	
position	δ_{H} (mult. J in Hz)	δ_{H} (mult. J in Hz)	δ_{H} (mult. J in Hz)	δ_{C}
C (3'')				135.2
CH ₃ (3'')	1.59 (3H, s)	1.59 (3H, s)	1.67 (3H, s)	13.8
CH ₂ (4'')	4.00 (1H, d, 11.6)	4.00 (1H, d, 11.8)	4.03 (2H, s)	69.1
	4.20 (1H, d, 11.6)	4.20 (1H, d, 11.8)		
CH ₂ (1''')	1.77 (1H, m)	1.74 (1H, m)	1.72 (1H, m)	28.4
	2.25 (1H, m)	2.25 (1H, m)	2.24 (1H, dd, 5.5, 14.0)	
CH (2''')	5.47 (1H, dd, 6.6, 6.5)	5.41 (1H, dd, 6.3, 6.8)	5.43 (1H, m)	126.1
C (3''')				135.2
C (3''')	1.64 (3H, s)	1.59 (3H, s)	1.67 (3H, s)	13.8
CH ₂ (4''')	4.47 (2H, s)	4.00 (1H, d, 11.8)	4.03 (2H, s)	69.1
		4.20 (1H, d, 11.8)		
CH (1''''')	4.50 (1H, d, 7.9)	4.50 (1H, d, 7.7)		
CH (2''''')	5.03 (1H, dd, 7.9, 9.3)	5.03 (1H, dd, 7.7, 9.4)		
CH (3''''')	5.21 (1H, dd, 9.3, 9.6)	5.21 (1H, dd, 9.4, 9.9)		
CH (4''''')	5.10 (1H, dd, 9.4, 9.6)	5.10 (1H, dd, 9.2, 9.9)		
CH (5''''')	3.66 (1H, ddd, 2.2, 4.7, 9.5)	3.66 (1H, ddd, 2.1, 4.5, 9.2)		
CH (6''''')	4.14 (1H, dd, 2.2, 12.2)	4.15 (1H, dd, 2.1, 12.0)		
	4.27 (1H, dd, 4.7, 12.2)	4.27 (1H, dd, 4.5, 12.0)		
CH (1''''')		4.50 (1H, d, 7.7)		
CH (2''''')		5.03 (1H, dd, 7.7, 9.4)		
CH (3''''')		5.21 (1H, dd, 9.4, 9.9)		
CH (4''''')		5.10 (1H, dd, 9.2, 9.9)		
CH (5''''')		3.66 (1H, ddd, 2.1, 4.5, 9.2)		
CH (6''''')		4.15 (1H, dd, 2.1, 12.0)		
		4.27 (1H, dd, 4.5, 12.0)		

CH₃ signals derived from acetates were observed at δ 1.24–2.10 in **4** and **5**.

s: singlet, d: doublet, dd: double doublet, m: multiplet (mult. J in Hz)

thin(**1**) were recorded in CDCl₃, **4** and **5** were useful for clarifying their aglycones as **1**. The ¹H signals of the aglycone moiety were very similar among **1**, **4**, and **5**—except H-4'' and H-4'''. H-4'' and H-4''' in **1** were both observed at δ 4.03 (singlet); H-4'' at δ 4.00 (J = 11.6 Hz) and δ 4.20 (J = 11.6 Hz); H-4''' at δ 4.47 (singlet) in **4**; and both H-4'' and H-4''' in **5** at δ 4.00 (J = 11.8 Hz) and δ 4.20 (J = 11.8 Hz). H-4'' in **4**, and H-4'' and H-4''' in **5** were observed as AB type (nonequivalent) signals by the attachment of glucose at C-4''('). The downfield shift of H-4''' in **4** compared to that of **1** is reasonably explained by acetylation at C-4''. Compounds **4** and **5** were also useful for identifying the sugar (s) as glucose, because no overlap in the ¹H signals of the sugar (s) was observed.

3.3 Singlet oxygen-quenching activity

First, we examined the ¹O₂-quenching activity of astaxanthin, which is reported to possess potent activity⁵⁾, as a positive control. The IC₅₀ value of astaxanthin was 8.9 μ M. We also examined the ¹O₂-quenching activity of β -carotene, which is widely distributed in nature. The IC₅₀ value of β -carotene was >100 μ M. Finally, we examined the ¹O₂-quenching activity of **1**, **2**, and **3**: their IC₅₀ values were 57,

54, and 74 μ M, respectively. Shimidzu *et al.* report that an increased number of C=C or C=O conjugated double bonds enhance ¹O₂-quenching activity²⁷⁾. Although β -carotene possesses 11 conjugated double bonds, it exhibits less ¹O₂-quenching activity than **1**, **2**, or **3** (9 conjugated double bonds); therefore, the activities of **1**, **2**, and **3** may be enhanced by exomethylene and/or dimethylallyl structures attached to the γ -ionone ring. Since the producing organism (*M. yunnanensis*) is often isolated from surface seawater in tropical areas, **1**, **2**, and **3** might contribute to the tolerance of micrococci to environmental stresses such as UV irradiation.

4 CONCLUSION

We isolated sarcinaxanthin(**1**), sarcinaxanthin monoglucoside(**2**), and sarcinaxanthin diglucoside(**3**) from a marine bacterium, *M. yunnanensis*. Although the production of **1**, **2**, and **3** by *Micrococcus* species has been reported previously⁹⁾, this is the first study reporting their antioxidative activities. We also report the complete ¹H NMR assignments of peracetylated **2** and **3**, and the FAB MS/MS analyses

of 1 and 2 for the first time. Further examinations of the antioxidative activities of these carotenoids are being carried out.

References

- Nishino, H.; Tokuda, H.; Murakoshi, M.; Satomi, Y.; Masuda, M.; Onozuka, M.; Yamaguchi, S.; Takayasu, J.; Tsuruta, J.; Okuda, M.; Khachik, F.; Narisawa, T.; Takasuka, N.; Yano, M. Cancer prevention by natural carotenoids. *Biofactors* **13**, 89-94 (2000).
- Iwamoto, T.; Hosoda, K.; Hirano, R.; Kurata, H.; Matsumoto, A.; Miki, W.; Kamiyama, M.; Itakura, H.; Yamamoto, S.; Kondo, K. Inhibition of low-density lipoprotein oxidation by astaxanthin. *J. Atheroscler. Thromb.* **7**, 216-222 (2000).
- Naito, Y.; Uchiyama, K.; Aoi, W.; Hasegawa, G.; Nakamura, N.; Yoshida, N.; Maoka, T.; Takahashi, J.; Yoshikawa, T. Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. *Biofactors* **20**, 49-59 (2004).
- Hosokawa, M.; Kudo, M.; Maeda, H.; Kohno, H.; Tanaka, T.; Miyashita, K. Fucoxanthin induces apoptosis and enhances the antiproliferative effect of the PPAR gamma ligand, troglitazone, on colon cancer cells. *Biochim. Biophys. Acta* **1675**, 113-120 (2004).
- Miki, W. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **63**, 141-146 (1991).
- Miki, W.; Otaki, N.; Shimidzu, N.; Yokoyama, A. Carotenoids as free radical scavengers in marine animals. *J. mar. Biotechnol.* **2**, 35-37 (1994).
- Miki, W.; Otaki, N.; Yokoyama, A.; Izumida, H.; Shimidzu, N. Okadaxanthin, a novel C₅₀-carotenoid from a bacterium, *Pseudomonas* sp. KK10206C associated with marine sponge, *Halichondria okadai*. *Experientia* **50**, 684-686 (1994).
- Takeda, Y.; Ota, T. A new bacterial carotenoid, sarcinaxanthin. *Z. physiol. Chem.* **268**, I-II (1941).
- Hretzberg, S.; Liaaen-Jensen, S. Bacterial carotenoids. LIII. C₅₀-Carotenoids. 19. Absolute configuration of sarcinaxanthin and sarcinaxanthin mono-β-D-glucoside. Isolation of sarcinaxanthin diglycoside. *Acta. Chem. Scand.* **B31**, 215-18 (1977).
- Zhao, G.-Z.; Li, J.; Qin, S.; Zhou, Y.-Q.; Zhu, W.-Y.; Jiang, C.-L.; Xu, L.-H.; Li, W.-J. *Micrococcus yunnanensis* sp. nov, a novel actinobacterium isolated from surface-sterilized *Polyspora axillaris* roots. *Int. J. Syst. Evol. Microbiol.* **59**, 2383-2387 (2009).
- Lyman, J.; Fleming, R. H. Composition of sea water. *J. Mar. Res.* **3**, 134-146 (1940).
- Weisburg, W. G.; Barns, S. M.; Pelletier, D. A.; Lane, D. J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697-703 (1991).
- Saitou, N.; Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425 (1987).
- Kumar, S.; Tamura, K.; Nei, M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinformatics.* **5**, 150-163 (2004).
- Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* **39**, 783-791 (1985).
- Akimoto, N.; Maoka, T.; Fujiwara, Y.; Hashimoto, K.; Analysis of carotenoids by FAB CID-MS/MS (in Japanese). *J. Mass Spectrom. Soc. Jpn.* **48**, 32-41 (2000).
- Maoka, T.; Fujiwara, Y.; Hashimoto, K.; Akimoto, N. Characterization of epoxy carotenoids by fast atom bombardment collision-induced dissociation MS/MS. *Lipids* **39**, 179-183 (2004).
- Kobayashi, M.; Sakamoto, Y. Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. *Biotechnol. Lett.* **21**, 265-269 (1999).
- Hirayama, O.; Nakamura, K.; Hamada, S.; Kobayashi, Y. Singlet oxygen quenching ability of naturally occurring carotenoids. *Lipids* **29**, 149-150 (1994).
- Liaaen-Jensen, S.; Weeks, O. B.; Strang, R. H. C.; Thirkell, D. Identity of the C₅₀-Carotenoid Dehydrogenans-P439 and sarcinaxanthin. *Nature* **214**, 379-380 (1967).
- Courington, D. P.; Goodwin, T. W. A survey of the pigments of A number of chromogenic marine bacteria, with special reference to the carotenoids. *J. Bacteriol.* **70**, 568-571 (1955).
- Swift, I. E.; Milborrow, B. V. Stereochemistry of β-, γ-, and ε-ring formation in bacterial C₅₀ carotenoids. *J. Biol. Chem.* **256**, 11607-11611 (1981).
- Hodgkiss, W.; Liston, J.; Goodwin, T. W.; Jamikorn, M. The isolation and description of two marine microorganisms with special reference to their pigment production. *J. Gen. Microbiol.* **11**, 438-450 (1954).
- Weeks, O. B.; Montes, A. R.; Andrewes, A. G. Structure of the principal carotenoid pigment of *Cellulomonas biazotes*. *J. Bacteriol.* **141**, 1272-1278 (1980).
- Marshall, J. H.; Wilmoth, G. J. Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. *J. Bacteriol.* **147**, 900-913 (1981).
- Lanz, M.; Bartels, B.; Pfander, H. C₄₅- and C₅₀-carotenoids. Part 7. Total synthesis of (all-E,2R,6R,2' R,6' R)- and (all-E,2R,6S,2' R,6' S)-2,2'-bis(4-hydroxy-3-methylbut-2-enyl)-γ, γ-carotene (sarcinaxanthin). *Helv. Chim. Acta.* **80**, 804-817 (1997).
- Shimidzu, N.; Goto, M.; Miki, W. Carotenoids as singlet oxygen quenchers in marine organisms. *Fish Sci.* **62**, 134-137 (1996).