

# Rare carotenoids, (3*R*)-saproxanthin and (3*R*,2'*S*)-myxol, isolated from novel marine bacteria (*Flavobacteriaceae*) and their antioxidative activities

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Received: 13 September 2006 / Revised: 17 November 2006 / Accepted: 17 November 2006 / Published online: 11 January 2007  
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**Abstract** We isolated three orange or yellow pigment-producing marine bacteria, strains 04OKA-13-27 (MBIC08261), 04OKA-17-12 (MBIC08260), and YM6-073 (MBIC06409), off the coast of Okinawa Prefecture in Japan. These strains were classified as novel species of the family *Flavobacteriaceae* based on their 16S rRNA gene sequence. They were cultured, and the major carotenoids produced were purified by chromatographic methods. Their structures were determined by spectral data to be (3*R*)-saproxanthin (strain 04OKA-13-27), (3*R*,2'*S*)-myxol (strain YM6-073), and (3*R*,3'*R*)-zeaxanthin (strains YM6-073 and 04OKA-17-12). Saproxanthin and myxol, which are monocyclic carotenoids rarely found in nature, demonstrated significant antioxidative activities against lipid peroxidation in the rat brain homogenate model and a neuro-protective effect from L-glutamate toxicity.

**Electronic supplementary material** Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00253-006-0774-y> and is accessible for authorized users.

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## Introduction

Some species of bacteria, yeasts, and fungi, as well as algae and higher plants, synthesize a large number of carotenoids with different molecular structures (Britton et al. 2004). Several species of marine bacteria have been reported to produce dicyclic, monocyclic, or acyclic carotenoids (Goodwin 1980). The Marine Biotechnology Institute (MBI) has identified structurally novel or rare carotenoids from marine bacteria belonging to  $\alpha$ -*Proteobacteria*. These carotenoids have included astaxanthin glucoside from *Paracoccus* sp. N81106 (re-classified from *Agrobacterium aurantiacum*; MBIC01143; Yokoyama et al. 1995), 2-hydroxyastaxanthin from *Brevundimonas* sp. SD212 (MBIC03018; Yokoyama et al. 1996b), and 4-ketoxanthin 3'-sulfate from *Erythrobacter* sp. PC6 (re-classified from *Flavobacterium* sp. PC-6; MBIC02351; Yokoyama et al. 1996a), these being xanthophylls derived from  $\beta$ -carotene. Recently, we have initiated the analysis of novel and/or rare types of carotenoids from new colored marine bacteria. The new isolates, 04OKA-13-27 (MBIC08261), 04OKA-17-12 (MBIC08260), and YM6-073 (MBIC06409), belonging to the family *Flavobacteriaceae* were selected for use in this study. The carotenoids produced by these isolates were purified by chromatographic methods and their structures subsequently determined by spectroscopic analyses [diode array detector (DAD) high-performance liquid chromatography (HPLC), field desorption mass spectrometry (FD-MS), proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ), and circular dichroism (CD)].

The generation of free radicals has been suggested to play a major role in the progression of a wide range of pathological

disturbances, including myocardial and cerebral ischemia (Traystman et al. 1991), atherosclerosis (Palinski et al. 1989), renal failure (Erdogan et al. 2002), inflammation (Cheeseman and Forni 1988), and rheumatoid arthritis (Bodamyali et al. 2004). Subsequent peroxidative disintegration of cells and organelle membranes has previously been implicated in various pathological processes (Mylonas and Kouretas 1999). Carotenoid pigments, which have been proven to possess strong antioxidative activities, have attracted greater attention due to their beneficial effects on human health, e.g., their potential in the prevention of such diseases as cancer and cardiovascular complaints (van den Berg et al. 2000). More than 700 carotenoids have been isolated from natural sources (Britton et al. 2004), and evaluating the pharmaceutical potential of various carotenoids with different structures could be an exciting field of medical research. However, the carotenoid species so far studied for this purpose have been restricted to a small number, including  $\beta$ -carotene and its derivatives, zeaxanthin,  $\beta$ -cryptoxanthin, canthaxanthin, and astaxanthin, and the other dicyclic carotenoids,  $\alpha$ -carotene and lutein, and the acyclic carotenoid, lycopene. With the exception of those carotenoids that can be isolated from a species of higher plants or be chemically synthesized, it has been difficult to find natural sources for supplying sufficient amounts of carotenoids. This study shows that the monocyclic carotenoids that are very rarely found in nature, saproxanthin and myxol, are respectively produced by the marine bacteria, strains 04OKA-13-27 and YM6-073. The antioxidative activities of these isolated carotenoids are evaluated for their inhibitory activity against lipid peroxidation induced by free radicals in a rat brain homogenate (Kato et al. 1993) and for their neuroprotective effect against L-glutamate toxicity on the neuronal hybridoma cell line, N18-RE-105 (Shindo et al. 2004).

## Materials and methods

### Survey of pigment-producing marine bacteria

Samples of algae and hard corals were collected from Majyanohama, Akajima, Kerama Islands in Okinawa Prefecture of Japan. A sediment sample was collected from Maeda–Misaki in Okinawa Prefecture of Japan.

Strain 04OKA-13-27 (MBIC08261) was isolated from the dense mats of filamentous algae, which were collected in March 2004 from within the territory of damselfish (*Stegastes nigricans*). The algae (1 cm<sup>3</sup>) were homogenized with a glass rod in 5 ml of sterile seawater. The homogenate was diluted ten times with sterile seawater and was used to isolate the bacterium on a 1/10 MA + Ca medium [3.74 g of 2216 marine broth (Difco, Detroit, MI, USA), 750 ml of filtered seawater, and 250 ml of distilled water with 1%

CaCO<sub>3</sub> containing 1.5% agar] after being cultivated for 3–7 days. Strain 04OKA-17-12 (MBIC08260) was isolated from hard coral (*Acropora nobilis* Dana 1846), which had been collected in March 2004 from 2–5 m below the surface of the sea. The coral was cut into small pieces (1 cm<sup>3</sup>), ground with a mortar and pestle, and transferred into 5 ml of sterile seawater. The bacterium was then isolated on 2216 marine broth (Difco) containing 1.5% agar after being cultivated for 3–7 days. Strain YM6-073 (MBIC06409) was isolated from the sediment sample collected in July 2003 from 0.1 m below the surface of the sea by cultivating for 30 days on an HSV medium (see “Electronic Supporting Material”).

### 16S rRNA gene sequence determination and analysis

Genomic DNA was extracted by using the InstaGene matrix (BioRad Laboratories, Hercules, CA, USA). PCR-mediated amplification of the 16S rRNA gene was carried out by using universal primers 27F and 1492R (Lane 1991). The PCR products were purified by Montage PCR<sub>μ</sub>96 (Millipore, Bedford, MA, USA) and sequenced by BigDye terminator cycle sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was determined with a 3730 capillary sequencer (Applied Biosystems) after cleaning up the products of the DNA sequencing reaction with Montage SEQ<sub>96</sub> (Millipore). The closely related sequences were searched by using the BlastN program (Altschul et al. 1997) in the DNA Data Bank of Japan (DDBJ) and Classifier in RDP-II database release 9 (Cole et al. 2005). The 16S rRNA gene sequences were aligned by using ClustalX (Thompson et al. 1997) with the related 16S rRNA gene sequences in the RDP-II database. The aligned sequence was used to construct a phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987) based on genetic distances calculated from the Kimura two-parameter model (Kimura 1980) by Mega v. 3.1 (Kumar et al. 2004). The robustness of the topology was evaluated by the neighbor-joining method through 1,000 bootstrap replications (Felsenstein 1985).

### Cultivation of the biological materials

Each isolated marine bacterium was cultured for 3 days in 100 ml of marine broth (Difco) in 500-ml Sakaguchi flasks at 30°C on a rotary shaker at 100 rpm. The cells grown from 2 l of the culture were collected by centrifugation, the total yield of the cells being about 20–25 g.

### Purification of the pigments

Pigments were extracted from the wet cell pellets with 300 ml of 90% methanol by using an ultra sonicator several

times, and the methanol was removed by evaporation. Distilled water (150 ml) was then added, and the pigments were extracted twice with ethyl acetate (100 ml) to complete the pigment recovery. The ethyl acetate fraction was dried overnight over anhydrous sodium sulfate and concentrated to dryness under reduced pressure. The resulting residue was loaded into a column of silica gel 60 (20×200 mm, Merck, Germany), the major pigments being eluted with 2:1, v/v hexane/ethyl acetate (04OKA-13-27 and YM6-073) and 3:2, v/v hexane/ethyl acetate (3:2, v/v; 04OKA-17-12). These pigments were further purified by preparative silica gel high-performance thin layer chromatography (HPTLC; Merck) developed with dichloromethane/methanol (10:1, v/v), and finally by HPLC in a preparative  $\mu$ Bondapak C18 column (10×250 mm, Waters, Milford, MS, USA), eluting with methanol in the case of the 04OKA-13-27 and YM6-073 pigments and with 95% methanol for the 04OKA-17-12 pigment at a flow rate of 3 ml/min.

#### Spectroscopic analysis of the carotenoids

The absorption spectra were measured with an MCPD-3600 photodiode array detector (Otsuka Electronics, Japan) attached to HPLC apparatus equipped with an analytical  $\mu$ Bondapak C18 column (8×100 mm, RCM type; Waters) and eluted with methanol at a flow rate of 1.8 ml/min. The CD spectra of the carotenoids were measured at 22°C with a J-600 spectropolarimeter (Jasco, Japan) in diethyl ether/2-pentane/ethanol (5:5:2, by vol). The relative molecular masses were measured by FD-MS, using an M-2500 double-focusing gas chromatograph–mass spectrometer (Hitachi, Japan) equipped with field-desorption apparatus. The  $^1\text{H-NMR}$  spectra in  $\text{CDCl}_3$  at 18°C were measured at 400 MHz with an AMX400 NMR spectrometer (Bruker, Germany).

#### Inhibitory activity against lipid peroxidation in a rat brain homogenate

A rat brain homogenate was prepared according to the method of Kubo et al. (1984) with some modifications. In brief, the frozen rat brain (Wistar, 8 weeks, male) was purchased from Funakoshi (Japan). After defrosting the brain in an ice-cold 100 mM phosphate buffer at pH 7.4, 0.4 g of the brain was immediately mixed for 30 s with 15 ml of the ice-cold phosphate buffer in a Teflon homogenizer. The reaction mixture for the assay consisted of 0.2 ml of the homogenate, 0.6 ml of a 100 mM phosphate buffer, 0.1 ml of 1 mM sodium ascorbate and 0.05 ml of a carotenoid solution dissolved in methanol. The mixture was incubated at 37°C for 1 h under reciprocal agitation. Malondialdehyde (MDA) was stoichiometrically

formed in the reaction mixture according to the concentration of the lipid peroxides. MDA thus formed was allowed to react with thiobarbituric acid for spectrophotometric quantification at 532 nm. The percentage inhibition was calculated as follows:  $[1-(T-B)/(C-B)]\times 100$  (%), in which  $T$ ,  $C$ , and  $B$  are respectively the  $A_{532}$  readings of the treated carotenoid, the control (peroxidation with no reagent), and the zero-time control (no peroxidation).

#### Inhibitory activity against glutamate toxicity in N18-RE-105 cells

N18-RE-105 cells (mouse neuroblastoma clone N18TG-2× Fisher rat 18-day embryonic neural retina; Malouf et al. 1984) were maintained at 37°C in 25-cm<sup>2</sup> tissue culture flasks in 90% Dulbecco's modified Eagle's medium containing HAT (0.1 mM hypoxanthine, 40  $\mu$ M aminopterin, and 0.14 mM thymidine) and 10% fetal calf serum under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were plated in 96-well microplates at a density of 2,000 cells per well with 100  $\mu$ l of the medium. After culturing for 24 h, the medium was replaced with one containing 10 mM L-glutamate and/or reagents. The cytotoxicity was quantified after 48 h of treatment by an automated colorimetric assay based on the production of dark blue formazan crystals by living cells incubated with the tetrazolium salt, MTS. The MTS activity was measured by using a commercial kit (Promega, Madison, WI, USA). The percentage cell survival was calculated as follows:  $(T-C)/(B-C)\times 100$  (%), in which  $T$ ,  $C$ , and  $B$  are respectively the absorbance values at 492 nm of the carotenoid (+) plus L-glutamate (+), no carotenoid (-), and L-glutamate (+) and neither the carotenoid (-) nor L-glutamate (-), respectively. The EC<sub>50</sub> value is the reagent concentration necessary to reduce glutamate-induced cell death by 50% (Miyamoto et al. 1989).

## Results

#### Classification of the marine bacteria

The three marine bacteria, which had been collected off the coast of Okinawa Prefecture, were classified on the basis of the 16S rRNA gene sequences. The lengths of the determined sequences were 1,467 bp for strain 04OKA-13-27 (MBIC08261), 1,463 bp for strain 04OKA-17-12 (MBIC08260), and 1,465 bp for strain YM6-073 (MBIC06409). A similarity search in the databases of DDBJ and RNA Database Project II (RDPII) showed the 16S rRNA gene sequences of strains 04OKA-13-27, 04OKA-17-12, and YM6-073 to respectively be 96.5% (1,408 bp/1,459 bp) similar to *Stanielletta latercula* ATCC

23177<sup>T</sup>, 95.5% (1,324 bp/1,386 bp) similar to *Gaetbulimicrobium brevivittae* strain SMK-19<sup>T</sup>, and 94.2% (1,306 bp/1,386 bp) similar to *Robiginitalea biformata* strain HTCC2501<sup>T</sup>. A neighbor-joining tree was constructed (Fig. 1) to deduce the phylogenetic relationship between these strains with already known species in the family *Flavobacteriaceae*. The result shows that the three bacterial strains should be classified as novel species of the family *Flavobacteriaceae*.

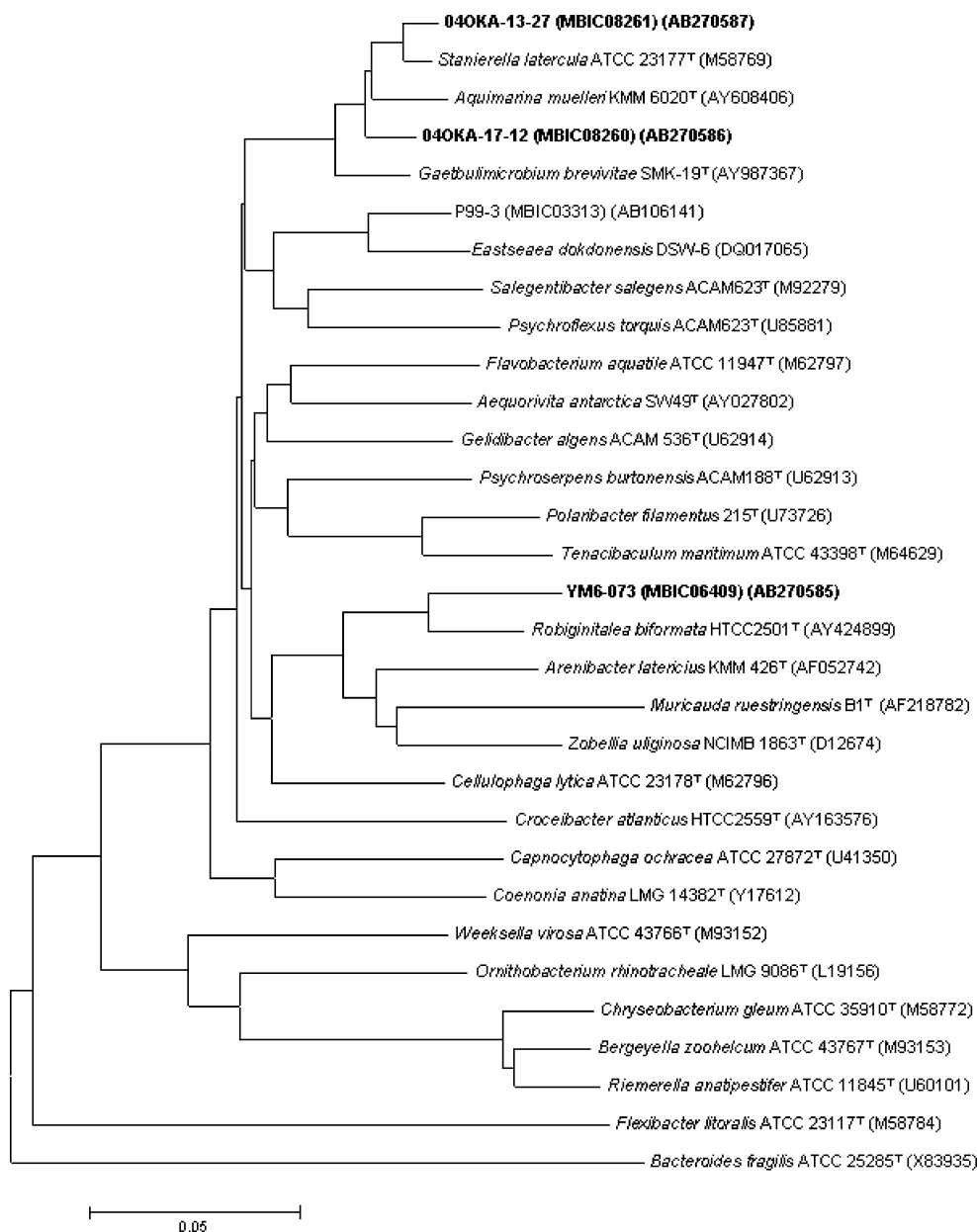
#### Identification of the pigment produced by bacterial strain 04OKA-13-27

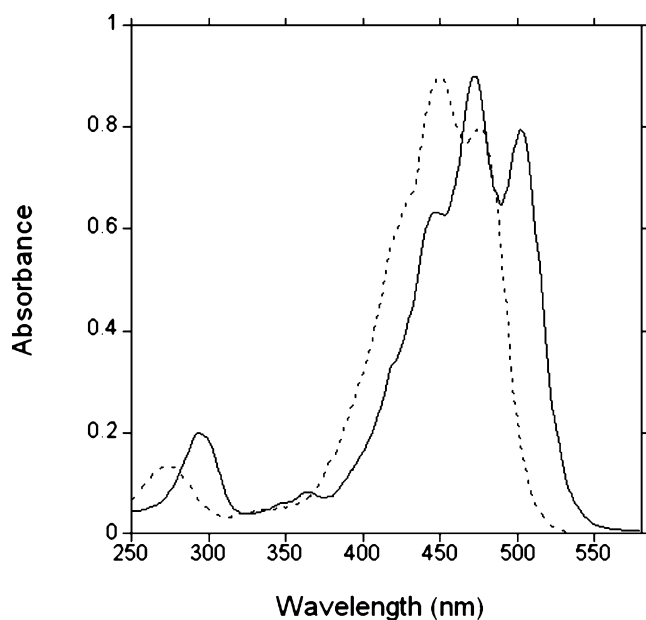
The colonies of marine bacterial strain 04OKA-13-27 were red. The elution profile of HPLC for the organic solvent-

soluble pigment showed one major carotenoid peak that was eluted at 4.5 min. A yield of about 0.3 mg of the purified carotenoid was obtained from 2 l of the culture after extraction and isolation by chromatographic methods.

The absorption maxima of this carotenoid were at 294, 363, 445, 471, and 501 nm, and the spectral fine structure of %III/II, which is the ratio of the peak heights of the longest and the medium wavelength absorption bands from the trough between the two peaks (Takaichi and Shimada 1992), was 46 in methanol (Fig. 2, solid line). These results indicate that the carotenoid was a derivative of  $\gamma$ -carotene with 12 conjugated double bonds (Takaichi and Shimada 1992). The relative molecular mass ( $M^+$ ) was observed at 568 m/z, and the <sup>1</sup>H-NMR spectrum (Table 1) is compatible with that of saxropoxanthin (Englert 1995). The CD spectrum

**Fig. 1** Phylogenetic tree based on the neighbor-joining method and using the 16S rRNA sequence to demonstrate the phylogenetic position of the carotenoid-producing strains in the family *Flavobacteriaceae*. Estimated distances were obtained from the Kimura 2 model parameters. Branch points supported by bootstrap resampling are indicated by *solid circles* (values > 90%) and *open circles* (values > 75%). The *scale bar* indicates 0.05 changes per nucleotide. EMBL/GenBank/DDBJ accession numbers are listed in *parentheses*





**Fig. 2** Absorption spectra of some carotenoids. Shown are saproxanthin from bacterial strain 04OKA-13-27 (MBIC08261; solid line) and myxol from strain YM6-073 (MBIC06409; solid line) and zeaxanthin (broken line) from strain 04OKA-17-12 (MBIC08260) and strain YM6-073 (MBIC06409) in methanol

is compatible with that of (3*R*)-bacteriorubixanthin (Takaichi et al. 1988), indicating a 3*R* configuration (Fig. 3). These data enabled the carotenoid to be identified as (3*R*)-saproxanthin. The International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC–IUB) semi-systematic name of (3*R*)-saproxanthin is (3*R*)-3',4'-didehydro-1',2'-dihydro- $\beta$ , $\psi$ -carotene-3,1'-diol.

Identification of the pigments produced by bacterial strain YM6-073

The colonies of marine bacterial strain YM6-073 were red. The elution profile of HPLC for the pigments showed two major carotenoid peaks eluted at 3.6 and 4.2 min. The polar and less polar carotenoids were separated from each other by chromatographic methods to respectively yield about 0.3 and 0.5 mg of the purified compounds from 2 l of the culture.

The absorption maxima of the polar carotenoid were at 294, 365, 445, 472, 489, and 502 nm, and the spectral fine structure of %III/II was 58 in methanol (Fig. 2). This absorption spectrum is compatible with that of saproxanthin from strain 04OKA-13-27. The relative molecular mass ( $M^+$ ) was observed at 584 m/z. The  $^1\text{H-NMR}$  spectrum (Table 1) is compatible with that of myxol (Yokoyama and Miki 1995; Takaichi et al. 2001), and the CD spectrum is also compatible with that of (3*R*,2'*S*)-myxol (Takaichi et al. 2001). The polar carotenoid was therefore identified to be (3*R*,2'*S*)-myxol (Fig. 3). The IUPAC–IUB semi-systematic

**Table 1**  $^1\text{H-NMR}$  data for the three carotenoids in  $\text{CDCl}_3^a$

Proton	(3 <i>R</i> )- Saproxanthin from 04OKA- 13-27	(3 <i>R</i> ,2' <i>S</i> )- Myxol from YM6-073	(3 <i>R</i> ,3' <i>R</i> )-Zeaxanthin from 04OKA-17-12 and YM6-073
H <sub>3</sub> -16	1.07 s	1.07 s	1.07 s
H <sub>3</sub> -17	1.07 s	1.07 s	1.07 s
H <sub>3</sub> -18	1.74 s	1.74 s	1.74 s
H <sub>3</sub> -19	1.97 s	1.97 s	1.97 s
H <sub>3</sub> -20	1.97 s	1.97 s	1.97 s
H $\alpha$ -2	1.78	1.77 d (12.0)	1.77
H $\beta$ -2	1.48	1.48 dd (11.6, 11.6)	1.48
Hax-3	4.00 m	4.00 m	4.00 m
HO-3	1.38	1.38 d (5)	
H $\alpha$ -4	2.39	2.39 dd (5.6, 17.7)	2.40
H $\beta$ -4	2.06	2.05	2.05
H <sub>3</sub> -16'	1.24 s		
H <sub>3</sub> -17'	1.24 s		
H <sub>3</sub> -18'	1.93 s		
H <sub>3</sub> -19'	1.98 s		
H <sub>3</sub> -20'	1.98 s		
H <sub>2</sub> -2'	2.32 d (7.9)		
H-3'	5.76		
H-4'	6.20		
H <sub>3</sub> -16'		1.24 s	
H <sub>3</sub> -17'		1.18 s	
H <sub>3</sub> -18'		1.93 s	
H <sub>3</sub> -19'		1.99 s	
H <sub>3</sub> -20'		1.97 s	
HO-1'		2.05 s	
H-2'		4.00 m	
HO-2'		2.05	
H-3'		5.71 dd (7.3, 15.3)	
H-4'		6.38	

<sup>a</sup>  $\delta$  (ppm), multiplicity (d, doublet; m, multiplet; s, singlet), and coupling constants (Hz).

name is (3*R*,2'*S*)-3',4'-didehydro-1',2'-dihydro- $\beta$ , $\psi$ -carotene-3,1',2'-triol.

The absorption maxima of the less polar carotenoid were 276, (345), (425), 449, 466, and 475 nm, and the spectral fine structure of %III/II were 25 in methanol (Fig. 2, broken line). These results indicate that the carotenoid was a derivative of  $\beta$ -carotene (Takaichi and Shimada 1992). The relative molecular mass ( $M^+$ ) was observed at 568 m/z. The  $^1\text{H-NMR}$  spectrum (Table 1) is compatible with that of zeaxanthin, and the CD spectrum is also compatible with that of (3*R*,3'*R*)-zeaxanthin (Takaichi et al. 1990). The structure of the carotenoid was therefore identified to be that of (3*R*,3'*R*)-zeaxanthin (Fig. 3). The IUPAC–IUB semi-systematic name is (3*R*,3'*R*)- $\beta$ , $\beta$ -carotene-3,3'-diol.

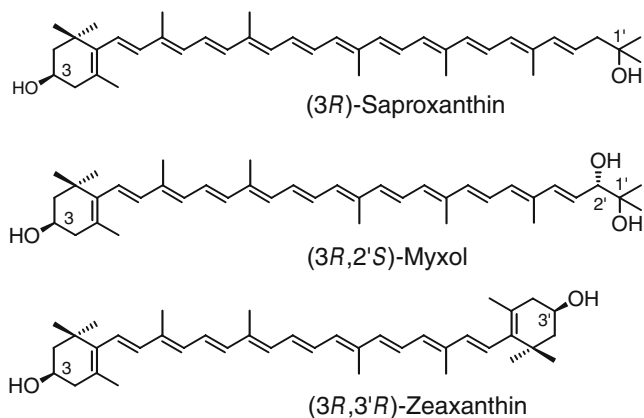


Fig. 3 Structures of the identified carotenoids

#### Identification of the pigment produced by bacterial strain 04OKA-17-12

The colonies of marine bacterial strain 04OKA-17-12 were yellow. The elution profile of HPLC for the pigments showed one major carotenoid peak that was eluted at 4.2 min. The yield of the purified carotenoid was about 0.3 mg from 2 l of the culture.

The absorption spectrum (Fig. 2, broken line) and the retention time on HPLC of the carotenoid are compatible with those of zeaxanthin from strain YM6-073. The relative molecular mass ( $M^+$ ) was also observed at 568  $m/z$ , and the  $^1\text{H-NMR}$  spectrum (Table 1) and CD spectrum are compatible with those of (3R,3'R)-zeaxanthin. The carotenoid was therefore identified to be (3R,3'R)-zeaxanthin (Fig. 3).

#### Antioxidative activities of the carotenoids

The hydroxyl radicals (OH) produced by Fenton's reaction (Walling 1975) in the presence of  $\text{Fe}^{2+}$  have been suggested to initiate lipid peroxidation and lead to subsequent disintegration of the cell membrane. The rat brain homogenate model has been accepted as being adequate to reflect this lipid peroxidation damage (Kato et al. 1993). The inhibitory activities against lipid peroxidation in the rat brain homogenate by saproxanthin, myxol, and zeaxanthin were evaluated (Fig. 4). The  $\text{IC}_{50}$  values were 2.1, 6.2, and 13.5  $\mu\text{M}$ , respectively (10.9  $\mu\text{M}$  for  $\beta$ -carotene), indicating these carotenoids to be more active than flunarizine ( $\text{IC}_{50}$  of 55.0  $\mu\text{M}$ ), which is a brain protective calcium antagonist with free radical-scavenging activity (Kubo et al. 1984). These carotenoids can therefore be expected to be useful for alleviating tissue damage resulting from the generation of free radicals and subsequent peroxidative disintegration of the cell membrane.

It is widely accepted that L-glutamate, which acts as a neurotransmitter in most areas of the central nervous

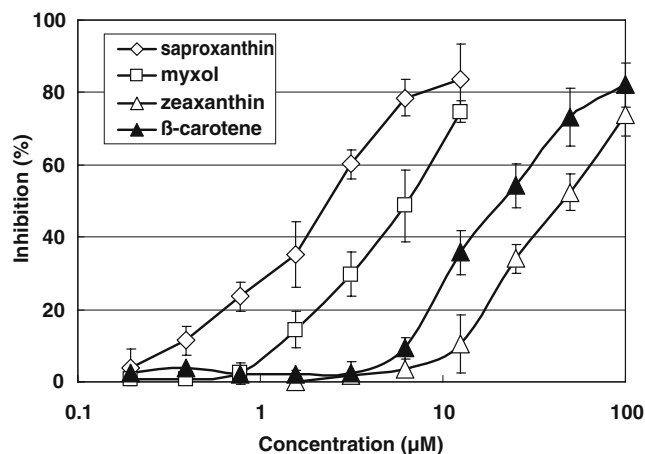


Fig. 4 Inhibition of the lipid peroxidation activity of carotenoids

system, induces delayed neuronal cell death after an ischemic assault (Malouf et al. 1984; Choi 1990). Thus, substances that inhibit L-glutamate toxicity can be expected to prevent or ameliorate the brain damage caused by brain ischemia. The toxicity of L-glutamate in N18-RE-105 cells has reportedly been mediated by the inhibition of cysteine uptake, which consequently suppresses synthesis of the intracellular reducing agent, glutathione (Murphy et al. 1990). Thus, such antioxidants as  $\alpha$ -tocopherol ( $\text{EC}_{50}$  of 57 nM) suppress the toxic effects of L-glutamate in N18-RE-105 cells by scavenging the oxygen radicals in place of glutathione. Saproxanthin, myxol, and zeaxanthin were tested for their inhibitory activities against L-glutamate toxicity in N18-RE-105 cells by the evaluation system described by Murphy et al. (1990; Fig. 5). The respective  $\text{EC}_{50}$  values were 3.1, 8.1, and  $>500$   $\mu\text{M}$  ( $\beta$ -carotene  $>100$   $\mu\text{M}$ ). These results show that saproxanthin and myxol possessed potent neuro-protective activity and may be useful for use against cerebral ischemic disease.

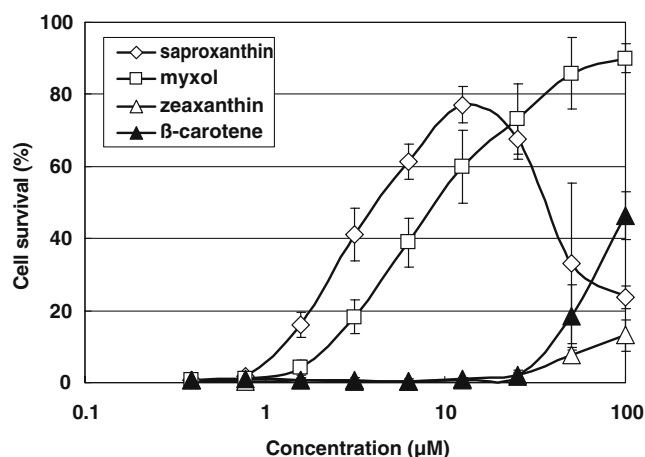


Fig. 5 Neuroprotective activities of the carotenoids. The abscissa shows the survival ratio (%) of N18-RE-108 cells. Saproxanthin demonstrated cytotoxicity against the cells at  $>20$   $\mu\text{M}$

## Discussion

(3*R*)-saproxanthin has only previously been found from *Saprospira grandis* (*Saprospiraceae*; Aasen and Liaaen-Jensen 1966). Hence, marine bacterial strain 04OKA-13-27 is the second species to produce saproxanthin. (3*R*,2'*S*)-Myxol has only previously been found in marine bacterial strain P99-3 (MBIC03313) belonging to the family *Flavobacteriaceae* (Fig. 1; Yokoyama and Miki 1995) and in cyanobacterium *Anabaena variabilis* ATCC 29413 (Takaichi et al. 2006). Hence, marine bacterial strain YM6-073 is the third species to produce myxol. On the other hand, myxol 2'-glycosides are widely distributed in cyanobacteria such as myxol 2'-dimethyl-fucoside from *Synechocystis* sp. PCC 6803 (Takaichi et al. 2001). It is interesting that myxol has only been found in marine bacteria belonging to the family *Flavobacteriaceae*, apart from in cyanobacteria. If 2'-hydroxylase works on saproxanthin, this carotenoid is capable of being converted into myxol (Fig. 3). The saproxanthin-producing bacterial strain 04OKA-13-27, which phylogenetically belongs to a cluster different from a cluster containing strain P99-3 (Fig. 1), seems not to possess a gene encoding 2'-hydroxylase that strains P99-3 and YM6-073 should possess. Phylogenetic position of strain YM6-073 is distant from those of strains P99-3 and 04OKA-13-27 (Fig. 1). Thus, monocyclic carotenoids such as saproxanthin and myxol that contain  $\gamma$ -carotene skeleton may widely be present in bacteria belonging to the family *Flavobacteriaceae*. The *crtYm* gene coding for lycopene  $\beta$ -monocyclase, which converts lycopene into  $\gamma$ -carotene, has already been isolated from bacterial strain P99-3 (Teramoto et al. 2003).

The hydroxyl groups of saproxanthin and myxol are thought to influence the orientation of carotenoids in the membrane (Woodall et al. 1997). They are very likely to be anchored in the head-group region of the phospholipids that form the bilayer. In respect of the orientation in the membrane, the hydroxyl group(s) at the acyclic terminal seem very likely to play the major role, compared with the hydroxyl group at  $\beta$ -end group (Albrecht et al. 2000). They have also reported that carotenoids possessing hydroxyl groups at both acyclic terminals or with no hydroxyl groups were less antioxidative. Such integration of saproxanthin or myxol should lead to the reinforcement and stabilization of biological membranes, which may decrease their permeability for oxygen (Subczynski et al. 1991) and may enhance protection against radical-induced peroxidation. The superior antioxidative activities of saproxanthin and myxol that were demonstrated in the two bioassays used, when compared with those of zeaxanthin and  $\beta$ -carotene, seem logical for the foregoing reason.

In conclusion, we isolated three novel marine bacteria belonging to the family *Flavobacteriaceae*. Two rare

carotenoids, (3*R*)-saproxanthin and (3*R*,2'*S*)-myxol, were identified from these strains and proved to possess strong antioxidative activities. These rare carotenoids need to be evaluated for their potential as development materials for pharmaceuticals or foods preventing such diseases as cancer and cardiovascular complains. The result of the present work also suggests that analyses of carotenoids in marine bacteria belonging to *Flavobacteriaceae* would be promising for finding diverse rare carotenoids.

**Acknowledgement** The hard coral used was identified by Hiroki Taniguchi of Akajima Marine Science Laboratory, to whom we are grateful. This work was supported in part by the Biotechnology and Medical Technology Development Department of the New Energy and Industrial Technology Development Organization of Japan (NEDO).

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