NOTE

Astaxanthin dirhamnoside, a new astaxanthin derivative produced by a radio-tolerant bacterium, *Sphingomonas astaxanthinifaciens*

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Astaxanthin (2) (Figure 1) is a red carotenoid renowned for a high antioxidant activity.¹ Owing to the protective function as well as the profitable effect on organoleptic properties, the use of this carotenoid benefits consumer acceptance of commercial products. Currently, it is extensively used as feed additives for fish, lobsters and chickens. Astaxanthin is also used as a nutraceutical and medicinal ingredient against degenerative diseases, such as cancer, skin-related illness and heart diseases.^{2–4} An astaxanthin-containing microalga has provided a new strategy in treatment against *Helicobacter pylori* infection.⁵ Thus, this carotenoid species provides wide application in agricultural, medicinal and pharmaceutical industries.³

We previously reported the isolation of carotenoid-producing bacteria from Misasa (Tottori, Japan), a region known for high radioactivity content, and their marked phylogenetic diversity.⁶ The frequent isolation of carotenoid producers made us assume that there is some correlation between productivity of the protective pigment and persistence in a radioactive environment of microorganisms.⁶ Recently, we taxonomically characterized one of the radio-tolerant isolates (strain TDMA-17), and validated it to be *Sphingomonas astaxanthinifaciens*, a novel species of the gram-negative bacterial genus *Sphingomonas*.⁷

The amount of carotenoids produced by *S. astaxanthinifaciens* was significantly high (2.8 mg g⁻¹ dry cells); interestingly, the HPLC analysis showed that the red pigment fraction of this strain contained astaxanthin (24.6%) and dihydroxyastaxanthin (7.5%) as minor components and an unknown substance (carotenoid 1) as a major component (58.4%).⁷ The unknown component was assumed to be an astaxanthin derivative, as it showed a similar absorption maximum as astaxanthin at around 470 nm. In this study, we isolated the major carotenoid 1 produced by *S. astaxanthinifaciens*, and determined its chemical structure.

To isolate the major carotenoid pigment, *S. astaxanthinifaciens* NBRC102146 was cultured in PE medium (containing (g l⁻¹): Bactopeptone (Difco, Detroit, MI, USA), 4; Bactoyeast extract (Difco), 2; NaCl, 5; fish meat extract, (Kyokuto, Tokyo, Japan) 2; MgSO₄, 2 and maltose 10 (pH 7.2)) (chemicals were purchased from Kokusan, Tokyo, Japan, if not indicated otherwise) shaken at 180 rpm for 2 days at 37 °C. The cells of *S. astaxanthinifaciens* were harvested by centrifugation at 9000×g, suspended in five times volume of methanol, and shaken at 180 rpm for 30 min at 28 °C and filtrated. This extraction process was repeated three times. Then, the filtrates were combined, dried up by evaporation and dissolved in CHCl₃. The CHCl₃ solution was then applied to Purif-pack silica-gel chromatography (Moritex, Tokyo, Japan). After washing the column with CHCl₃, the pigment was eluted with CHCl₃/methanol (9:1). The eluate was dried up, dissolved in an adequate volume of CHCl₃/methanol (9:1) and applied onto a preparative C18-TLC (Whatman, Maidstone, UK) developed with CHCl₃/methanol (4:1) to isolate the final principle. By this process, ~56 mg carotenoid 1 and 4.7 mg astaxanthin were obtained. All procedures were performed under dim light.

The UV-vis absorption spectra in the HPLC eluent of MeOH were measured with a Shimadzu SPD-M10AVP photodiode array spectrophotometer (Shimadzu, Kyoto, Japan). NMR spectra were recorded on a Varian Unity INOVA 500 (Varian, Palo Alto, CA, USA) or JEOL JMN-600 spectrometer (JEOL, Tokyo, Japan). Electrospray ionization-time of flight mass (ESI-TOFMS) spectra were recorded using a JEOL JMS-T100LC AccuTOF (JEOL). IR and CD spectra were measured with an FT/IR 4000 spectrometer (JASCO, Tokyo, Japan) and J-720 spectropolarimeter (JASCO), respectively. The analytical data of carotenoid 1 were as follows: Vis in ether/2-methylbutane/ethanol (5:5:1) λ_{max} (ε) 471 (130 000); CD in ether/2-methylbutane/ethanol

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Figure 1 Chemical structure of astaxanthin dirhamnoside (**1**) and astaxanthin (**2**).

(5:5:1) $\Delta\epsilon_{381}=1.5$, $\Delta\epsilon_{351}=0$, $\Delta\epsilon_{310}=-18.1$, $\Delta\epsilon_{282}=0$, $\Delta\epsilon_{274}=6.5$, $\Delta\epsilon_{253}=0$, $\Delta\epsilon_{245}=-15.0$; ^1H NMR (DMSO- d_6 , 500 MHz) and ^{13}C NMR (125 MHz), see Table 1; ESI-TOFMS m/z 889 ($\text{M}+\text{H}$) $^+$, 911 ($\text{M}+\text{Na}$) $^+$; HRESI-TOFMS m/z 911.4878 ($\text{M}+\text{Na}$) $^+$ (calcd for $\text{C}_{52}\text{H}_{72}\text{NaO}_{12}$, 911.4921); IR (nujol) 3337, 1668, 1039 cm^{-1} . The ^1H and ^{13}C NMR data of **1** in DMSO- d_6 (Table 1) indicated the presence of astaxanthin as the carotenoid moiety and two rhamnose residues. The values of $^3J_{\text{H}-1''(\text{H}-1'''),\text{H}-2''(\text{H}-2''')}$ (1 Hz) ^{8} and $^1J_{\text{C}-1''(\text{C}-1'''),\text{H}-1''(\text{H}-1''')}$ (171 Hz) ^{9} of rhamnose moieties indicated the α -glycoside.

To determine the absolute configuration of rhamnose moieties of **1**, methyl 2,3,4-tribenzoyl- α -rhamnopyranoside was derived from **1** and its CD spectrum was compared with that of authentic D-rhamnose. Astaxanthin dirhamnoside **1** (7.2 mg) was hydrolyzed in a sealed tube with 4 M HCl at 105 $^{\circ}\text{C}$ for 3 h. The reaction solution was evaporated to remove HCl and lyophilized. The obtained residue (5.2 mg) was treated with 5% HCl-methanol (3 ml) at room temperature for 1 h and the reaction solution was applied on a Dowex-1 (OH^- type; Wako Pure Chemicals, Osaka, Japan) column. The passed-through solution from the column was evaporated and benzoylated with benzoyl chloride (0.5 ml) in dry pyridine (4 ml) containing 4-dimethylamino-pyridine (2 mg). Water was put into the reaction solution and extracted with ethyl acetate. The ethyl acetate solution was washed with sat. NaCl, sat. NaHCO_3 and water, successively, and dried with anhydrous Na_2SO_4 and evaporated. The residue was loaded on a silica gel column (Wakogel C-200, 40 g) (Wako) packed with *n*-hexane and eluted stepwise with *n*-hexane-ethyl acetate (95:5, 1.5 l) and ethyl acetate (500 ml). The ethyl acetate fraction (26.5 mg) was washed with sat. NaCl, sat. NaHCO_3 and water, successively, and evaporated. The obtained residue (3.3 mg) was purified by HPLC on a 250 $\times$ 20 mm i.d. Capcell-Pak C₁₈ column with a gradient of 60–100% acetonitrile in water in 15 min and then isocratic elution of 100% acetonitrile at a flow rate of 5 ml min^{-1} to obtain methyl 2,3,4-tribenzoyl- α -rhamnopyranoside (retention time: 26.7 min; yield: 40 μg).

Authentic methyl 2,3,4-tribenzoyl- α -D-rhamnopyranoside (2.5 mg) was prepared from α -D-rhamnose (25.0 mg) by 5% HCl-methanol treatment and benzoylation using the same method mentioned above. The spectral data of authentic methyl 2,3,4-tribenzoyl- α -D-rhamnopyranoside: ESI-TOFMS m/z 513 ($\text{M}+\text{Na}$) $^+$; CD in acetonitrile $\Delta\epsilon_{238}=7.3$, $\Delta\epsilon_{227}=0$, $\Delta\epsilon_{221}=-2.3$; δ_{H} (CDCl_3 , 600 MHz): 7.2–8.2 (15H, Bz), 5.92 (1H, d, $J=4$ Hz, H-2), 5.63 (1H, t, $J=10$ Hz, H-4), 5.55 (1H, dd, $J=10, 4$ Hz, H-3), 4.79 (1H, s, H-1), 3.85 (1H, m, H-5), 3.57 (3H, s, OCH_3), 1.45 (3H, d, $J=6$ Hz, H-6). As the retention time on HPLC and ESI-TOFMS, and CD spectra of the derivative from **1** were identical with those of authentic methyl 2,3,4-tribenzoyl- α -D-rhamnopyranoside, the configuration of the rhamnose moieties of **1** was determined as D. The CD spectrum of **2** isolated from the cells of

Table 1 ^1H and ^{13}C assignments of astaxanthin dirhamnoside^a

C no.	δ_{C}	δ_{H}
1, 1'	36.7	
2, 2'	44.26	1.89, 1.87
3, 3'	71.33	4.36 dd (13, 5.5)
4, 4'	197.80	
5, 5'	127.41	
6, 6'	159.95	
7, 7'	123.81	6.28 d (16)
8, 8'	141.58	6.50 d (16)
9, 9'	134.98	
10, 10'	134.92	6.42 d (12)
11, 11'	125.15	6.71 dd (15, 12)
12, 12'	139.29	6.49 d (15)
13, 13'	136.62	
14, 14'	133.76	6.40 dd (8, 3)
15, 15'	130.98	6.76 dd (8, 3)
16, 16'	26.06	1.28 s
17, 17'	30.09	1.17 s
18, 18'	13.78	1.79 s
19, 19'	12.37	1.99 s
20, 20'	12.62	1.96 s
1'', 1'''	100.29	4.92 d (1)
2'', 2'''	70.25	3.75 ($J_{2,3}=3.6$ Hz) ^b
2''-OH, 2'''-OH		4.84 d (4)
3'', 3'''	70.49	3.43 ($J_{3,4}=9.5$ Hz) ^b
3''-OH, 3'''-OH	70.4	4.58 d (6)
4'', 4'''	71.94	3.20 ($J_{4,5}=9.5$ Hz) ^b
4''-OH, 4'''-OH		4.78 d (5)
5'', 5'''	68.98	3.44
6'', 6'''	17.9	1.14 d (6)

Coupling constants in Hertz are given in parentheses.

^aSpectra were obtained in DMSO- d_6 .

^bObtained by decoupling experiments.

S. astaxanthinifaciens indicated the (3*S*,3'*S*) configuration of **2**, strongly suggesting the (3*S*,3'*S*) configuration of **1**. This was confirmed by the CD spectrum of **1** having the minimum or maximum Cotton effects at 310 ($\Delta\epsilon$ −18.1), 274 ($\Delta\epsilon$ +6.5) and 245 nm ($\Delta\epsilon$ −15.0), which was compatible with (3*S*,3'*S*)-**2** or (3*S*,3'*S*)-**2** monoglucoside (314 ($\Delta\epsilon$ −18.5), 274 ($\Delta\epsilon$ +10.1) and 242 nm ($\Delta\epsilon$ −12.0)).¹⁰ Therefore, the structure of the major carotenoid of *S. astaxanthinifaciens* was assigned as (3*S*,3'*S*)-3,3'-bis(α -D-rhamnopyranosyloxy)- β , β -carotene-4,4'-dione (**1**) (Figure 1).

Antioxidant activity of compound **1** was measured by the inhibition of methyl linoleate hydroperoxide production because of the reaction of methyl linoleate with 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN).¹¹ Reaction mixture (containing (μl): methyl linoleate, 50; ethanol, 450; 0.15 mM AMVN, 125 and 1 mM carotenoid in CHCl_3 , 125) was incubated at 37 $^{\circ}\text{C}$ for 4 h with reciprocal shaking at 80 rpm under dark conditions. The reactant was analyzed by normal phase HPLC using a Shodex SIL 5B column (4.6 $\times$ 250 mm; Showa Denko, Tokyo, Japan) (mobile phase, *n*-hexane/2-propanol (99:1); flow rate, 1.0 ml min^{-1} ; $\lambda=235$ nm), and measured for the content of the four kinds of hydroperoxides. The antioxidant activity was expressed as a percentage of inhibition of hydroperoxide production because of the addition of carotenoids. The result showed that 1 mM of astaxanthin, astaxanthin dirhamnoside and β -carotene inhibited methyl linoleate hydroperoxide production at 27, 20 and 16%, respectively (a representative result; though the inhibitory values fluctuated, similar results were obtained in analyses repeated three times). This indicated that

compound **1** shows a distinct antioxidant activity, which is slightly lower than that of astaxanthin.

Compound **1** was also studied for solubility in a water-based solvent by a spectroscopic measurement. First, carotenoid **1** and astaxanthin were dissolved in 100% dimethyl sulfoxide (DMSO), acetone and methanol at 20 mM. The solutions were then added with water at various ratios. After removing the insoluble pigment particles by filtration (pore size, 0.1 µm; Millipore, Billerica, MA, USA), each solution was measured for absorption at 475 nm. Similarly, the absorption of the above 20 mM solution and its diluted series was measured and used as standards to quantify the amount of solubilized pigment. Astaxanthin dirhamnoside was dissolved in solutions of H₂O–DMSO (99:1), H₂O–acetone (99:1) and H₂O–methanol (99:1) at 13, 6.2 and 5.4 µM, respectively. Solubility of astaxanthin in these water-containing solvents was below the detectable level (<0.1%) (appearance of various solutions is shown in Supplementary Figure S1).

The known carotenoid rhamnosides to date include myxocoxanthin rhamnoside from myxobacterium,¹² and oscillol 2,2'-dirhamnoside¹³ and myxol 2'-rhamnoside¹⁴ from blue-green algae. Although these known molecular species were produced as minor components, astaxanthin dirhamnoside (**1**) identified in this study was a major carotenoid of *S. astaxanthinifaciens*. The unique properties of astaxanthin dirhamnoside may expand the application of this kind of carotenoids.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)