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NEW CAROTENOID GLUCOSIDES, ASTAXANTHIN GLUCOSIDE AND ADONIXANTHIN GLUCOSIDE, ISOLATED FROM THE ASTAXANTHIN-PRODUCING MARINE BACTERIUM, *AGROBACTERIUM AURANTIACUM*

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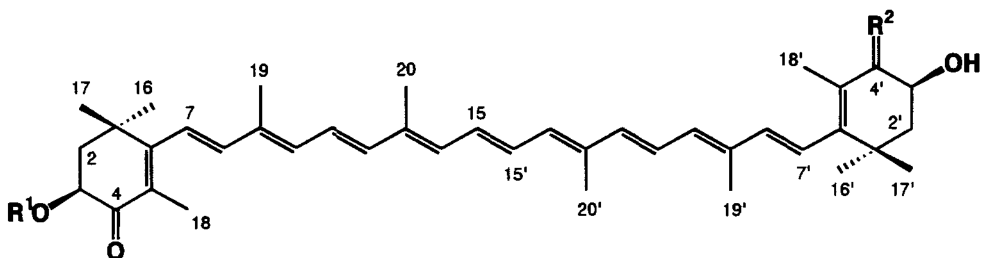
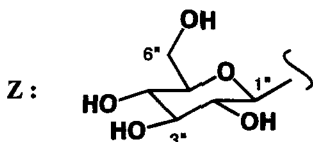
ABSTRACT.—Two new carotenoid glycosides, (3*S*,3′*S*)-astaxanthin-β-D-glucoside [**3**] and (3*S*,3′*R*)-adonixanthin-β-D-glucoside [**4**], were isolated from the astaxanthin-producing marine bacterium *Agrobacterium aurantiacum*, and their structures were determined by spectral means.

Astaxanthin is a highly oxidized carotenoid which is found commonly in marine animal tissues. This carotenoid has been developed successfully as an agent for the pigmentation of cultured fish and shellfish (1–3). We recently reported that the marine bacterium *Agrobacterium aurantiacum* (Rhizobiaceae) (4) produced (3*S*,3′*S*)-astaxanthin [**1**] and (3*S*,3′*R*)-adonixanthin [**2**] (5), and proposed a biosynthetic pathway for astaxanthin by identification of the presumed precursors of **1** (6). We describe herein the isolation and determination of two new carotenoid glycosides from the polar fractions of Me_2CO extracts of *A. aurantiacum*.

The astaxanthin-producing bacterium *A. aurantiacum* was mass-cultured in media A and B containing 1% and 0.2% glucose, respectively. The concentrated Me_2CO extract from the cultured cells in medium A was transferred into EtOAc from an aqueous NaCl solution. The organic layer was dried and fractionated by Si gel cc with *n*-hexane-Me_2CO (7:3) and CH_2Cl_2-MeOH (9:1) as eluents. Known carotenoids such as **1** and **2** were eluted in the *n*-hexane-Me_2CO (7:3) fraction. The CH_2Cl_2-MeOH (9:1) fraction was further purified by reversed-phase hplc (95% aqueous MeOH) to afford astaxanthin-β-D-glucoside [**3**]. Adonixanthin-β-D-glucoside [**4**] was isolated from cells cultured in medium B by the same procedure.

By tlc analysis with *n*-hexane-Me_2CO (7:3), **3** and **4** did not migrate, but **1** and **2** showed R_f values of 0.45 and 0.42, respectively. When CH_2Cl_2-MeOH (9:1) was used as solvent, the R_f values of **3** (0.32) and **4** (0.26) were similar to that of myxoxanthophyll (0.25), a typical cyanobacterial carotenoid monoglycoside (7). Using reversed-phase hplc analysis, the





1 R^1=H, R^2=O
2 R^1=H, R^2=H_2
3 R^1=Z, R^2=O
4 R^1=Z, R^2=H_2

R_t values of **3** and **4** were 3.9 min and 4.9 min, respectively, which were much smaller than those of **1** (6.3 min) and **2** (8.3 min). By analysis of these chromatographic observations and the data discussed below, **3** and **4** seemed to be carotenoids with sugars or other polar functional groups.

The uv-vis maxima of **3** (487 nm) and **4** (476 nm) in C_6H_6 were compatible with those of **1** and **2**, respectively (5). By hrfabms, the molecular formulas of **3** and **4** were established as $C_{46}H_{62}O_9$ and $C_{46}H_{64}O_8$, compatible with a **1**-hexoside and a **2**-hexoside, respectively.

By means of extensive nmr studies including ^1H-^1H COSY, HSQC, and HMBC, **3** (see Tables 1 and 2 for data) appeared to consist of carotenoid and sugar moieties. One end-group of the

TABLE 1. ^1H-Nmr Data for Astaxanthin-β-D-glucoside [**3**] and Adonixanthin-β-D-glucoside [**4**].[a]

Proton(s)	Compound	
	3[b]	**4**[b]
H_2-2	2.04 (t, 14)	2.04 (m)
	2.15 (dd, 6,14)	2.15 (dd, 6,14)
H-3	4.40 (dd, 6,14)	4.40 (dd, 6,14)
H_3-16	1.35 (s)	1.35 (s)
H_3-17	1.23 (s)	1.23 (s)
H_3-18	1.91 (s)	1.91 (s)
H_2-2′	1.81 (t, 13)	1.48 (t, 12)
	2.16 (dd, 6,13)	1.77 (ddd, 2,4,12)
H-3′	4.32 (ddd, 2,6,13)	4.00 (m)
H_2-4′	—	2.05 (m)
	—	2.39 (ddd, 2,6,17)
H_3-16′	1.32 (s)	1.08 (s)
H_3-17′	12.1 (s)	1.08 (s)
H_3-18′	1.94 (s)	1.74 (s)
H-7	6.21 (d, 16)	6.20 (d, 16)
H-7′	6.22 (d, 16)	6.12 (s)
H-8	6.44 (d, 16)	6.44 (d, 16)
H-8′	6.44 (d, 16)	6.12 (s)
H-10	6.30 (m)	6.30 (m)
H-10′	6.30 (m)	6.16 (d, 11)
H-11	6.65 (m)	6.65 (m)
H-11′	6.65 (m)	6.67 (m)
H-12	6.45 (d, 15)	6.45 (d, 15)
H-12′	6.45 (d, 15)	6.36 (d, 15)
H-14	6.30 (m)	6.30 (m)
H-14′	6.30 (m)	6.26 (d, 11)
H-15	6.67 (m)	6.67 (m)
H-15′	6.67 (m)	6.66 (m)
H_3-19,20,19′,20′	1.99–2.01 (s)	1.98–2.00 (s)
H-1″	4.57 (d, 8)	4.57 (d, 8)
H-2″	3.45 (dd, 8,9)	3.45 (dd, 8,9)
H-3″	3.61 (m)	3.61 (m)
H-4″	3.61 (m)	3.61 (m)
H-5″	3.41 (m)	3.41 (m)
H_2-6″	3.83 (m)	3.83 (m)
	3.93 (m)	3.93 (m)

[a]Data recorded in $CDCl_3$ at 500 MHz.
[b]Multiplicity and J values in Hz are given in parentheses.

TABLE 2. ^{13}C-Nmr Data for Astaxanthin-β-D-glucoside [3].[a]

Carbon	δ (mult.)	Carbon	δ (mult.)
1	37.4 (s)	1′	36.8 (s)
2	44.7 (t)	2′	45.5 (t)
3	77.6 (d)	3′	69.2 (d)
4	199.3 (s)	4′	200.4 (s)
5	127.9 (s)	5′	126.9 (s)
6	162.4 (s)	6′	162.2 (s)
7	123.0 (d)	7′	123.3 (d)
8	142.7 (d)	8′	142.3 (d)
9	134.4 (s)	9′	134.6 (s)
10	135.5 (d)	10′	135.1 (d)
11	124.5 (d)[b]	11′	124.7 (d)[b]
12	140.0 (d)	12′	139.7 (d)
13	136.8 (s)[c]	13′	136.7 (s)[c]
14	134.0 (d)	14′	133.8 (d)
15	130.6 (d)	15′	130.8 (d)
16	26.6 (q)	16′	26.2 (q)
17	30.6 (q)	17′	30.8 (q)
18	14.1 (q)[d]	18′	14.0 (q)[d]
19	12.59 (q)[e]	19′	12.58 (q)[e]
20	12.84 (q)[f]	20′	12.83 (q)[f]
1″	104.9 (d)		
2″	74.3 (d)		
3″	77.2 (d)		
4″	70.3 (d)		
5″	75.7 (d)		
6″	62.7 (t)		

[a]Data were recorded in $CDCl_3$ at 125 MHz and multiplicities were determined by an HSQC experiment.
[b–f]These assignments may be interchangeable.

carotenoid moiety was identified as a 3-substituted-3-hydroxy-4-keto-β end-group, and the other was identified as a 3-hydroxy-4-keto-β end-group similar to that of **1** (see Tables 1 and 2) (8). Furthermore, the characteristic polyene signals of a β-carotene type carotenoid were observed: four allylic methyl carbons, four quaternary carbons, and fourteen olefinic methine carbons (see Table 2) (8). The sugar moiety was identified as β-D-glucopyranose by the assignments of ^{1}H-nmr signals using C_6D_6 as the solvent (δ 4.51, 1H, d, J=8 Hz, H-1″; δ 3.73, 1H, dd, J=8 and 9 Hz, H-2″; δ 3.62, 1H, t, J=9 Hz, H-3″; δ 3.56, 1H, t, J=9 Hz, H-4″; δ 3.35, 1H, m, H-5″; δ 3.83 and 3.89, 2H, m, H_2-6″) and ^{13}C-nmr signals (see Table 2) (9). From the chemical shift of C-1″ (δ_C 104.9), the coupling constant of the anomeric proton [δ_H ($CDCl_3$) 4.57, 1H, d, J=8 Hz, H-1″], and the correlation between H-1″ and H-3 by NOESY, C-1″ of the glucose was found to be linked to C-3 of the carotenoid molecule by a β-glycosidic linkage. The cd curve, especially the minimum Cotton effect at 314 nm (Δε −18.5), was quite compatible with that of the (3*S*,3′*S*)-form of **1** (5,10). Therefore, the configurations at C-3 and C-3′ of **3** were determined as 3*S*,3′*S*, whose configurations were in turn the same as those of **1** in this bacterial strain as reported previously (5). As a result, **3** was determined as (3*S*,3′*S*)-astaxanthin-β-D-glucoside.

Similarly, the ^{1}H-nmr data of **4**, isolated from mass culture in medium B, were similar to those of **3**, except for one end-group. This was identified as a 3β-hydroxy end-group like that of **2** (see Table 1) (8). Thus, the carotenoid moiety

of **4** was determined to be (3*S*,3′*R*)-3-substituted-adonixanthin by assignments of the ^{1}H-nmr signals and from the cd spectrum (5,10). The sugar moiety was identified as β-D-glucopyranose by essentially the same ^{1}H-nmr data as **3** around δ 3.4–4.7 in $CDCl_3$. Thus, **4** was determined as (3*S*,3′*R*)-adonixanthin-β-D-glucoside.

Bioactivities of astaxanthin [**1**] such as acting as an antioxidant (11) and enhancement of antibody production (12) have been reported recently. It seems that astaxanthin glucoside [**3**] may possess interesting bioactivities, as **3** is more hydrophilic than **1**.

Recently, Misawa *et al.* established the carotenoid biosynthetic pathway of zeaxanthin-β-D-diglucoside produced by the soil bacterium *Erwinia uredovora* at the level of the biosynthetic gene (13). They reported that the *crtX* gene coded for the enzyme zeaxanthin-glucosyltransferase. We expect that *A. aurantiacum* has a glucosyltransferase which is coded for by a *crtX*-like gene. No **1**- and **2**-diglucosides were detected in the present study. It seems that the glucosyltransferase in *A. aurantiacum* selectively catalyzes the glucosylation of one 3-hydroxy-4-keto-β end-group only, or that **1** and **2** are glycosylated selectively by virtue of their position across the membrane (14) by an enzyme which may exist on only one side of the membrane in contact with only one end-group of the carotenoid molecule.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv-vis spectra in C_6H_6 were recorded with a Shimadzu UV-160A spectrophotometer, and cd with a Jasco J-600 spectropolarimeter, using a mixture of Et_2O, isopentane, and EtOH (5:5:2) as the solvent. Nmr experiments were carried out on a Varian Unity 500 nmr spectrometer using $CDCl_3$ or C_6D_6 as the solvent [residual $CHCl_3$ (δ_H 7.26) and C_6HD_5 (δ_H 7.12) as internal standards for ^{1}H and $CDCl_3$ (δ_C 77.0) as the internal standard for ^{13}C nmr]. Hrfabms were measured with a JEOL JMS-SX102 mass spectrometer. Tlc analyses were performed using precoated Si gel 60 F_{254} plates (Merck) with *n*-hexane-Me_2CO (7:3) or CH_2Cl_2-MeOH (9:1) as eluent. Hplc analysis was carried out on a TSK gel ODS-80Ts column (4.6×150 mm, Tosoh) with H_2O-MeOH (5:95) eluent at a 1 ml/min flow rate and monitored at 470 nm.

BACTERIAL MATERIAL.—*Agrobacterium aurantiacum* was isolated from the surface of the sea near Okinawa in Japan (4), and has been deposited in the National Institute of Bioscience and Human Technology (Tsukuba, Japan) with the accession number "FERM P-14023." The bacterium was cultured in a 500-ml Erlenmyer flask containing 300 ml of medium at 20° for 4 days on a rotary shaker at 100 rpm, by employing modified Marine Broth medium [500 ml of sea water, 500 ml of distilled H_2O, 5 g of Bacto-peptone (Difco), 1 g of Bacto-yeast extract (Difco), 0.04 g of iron (III) phosphate *n*-hydrate, 0.1 g of sodium acetate trihydrate] (3) containing 1% (medium A) and 0.2% (medium B) of glucose.

EXTRACTION AND ISOLATION.—Cultured cells were gathered by centrifugation from media A (15 liters) and B (18 liters) and were extracted with Me_2CO. The concentrated extracts were partitioned between EtOAc and aqueous NaCl solution. The organic layers were dried over Na_2SO_4, and concentrated to dryness. Each crude carotenoid from medium A or B was applied to Si gel 60 (Merck) cc with a mixture of *n*-hexane/Me_2CO, and CH_2Cl_2/MeOH as the eluent. Then both eluents with CH_2Cl_2-MeOH (9:1) were independently purified by reversed-phase hplc (TSK gel ODS-80Ts, 7.8×300 mm, Tosoh) with H_2O-MeOH (5:95) to afford **3** (2.3 mg) and **4** (ca. 0.6 mg). The total carotenoids comprised 0.17% and 0.14% of dry wt cell in media A and B, respectively. By reversed-phase hplc analysis with gradient elution (6), **3** and **4** constituted 10.5% and 3.7%, respectively, of the total carotenoids.

(3S,3′S)-Astaxanthin-β-D-*glucoside* [**3**].—Vis λ max (C_6H_6) 487 nm; hrfabms *m/z* $[M+H]^+$ 759.4474 (calcd for $C_{46}H_{63}O_9$, 759.4472); cd (Δε) 314 (−18.5), 274 (+10.1), 242 (−12.0) nm; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2, respectively.

(3S,3′R)-Adonixanthin-β-D-*glucoside* [**4**].—Vis λ max (C_6H_6) 476 nm; hrfabms *m/z* $[M+H]^+$ 745.4689 (calcd for $C_{46}H_{65}O_8$, 745.4679); cd (Δε) 301 (−21.4), 260 (+17.4), 234 (−14.2) nm; ^{1}H-nmr data are shown in Table 1.

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LITERATURE CITED

1. T. Matsuno, in: "Carotenoids: Chemistry and Biology." Ed. by N.I. Krinsky, M.M. Mathews-Roth, and R.F. Taylor, Plenum

Press, New York, 1989, pp. 59–74.
2. W. Miki, K. Yamaguchi, and S. Konosu, *Comp. Biochem. Physiol.,* **71B**, 7 (1982).
3. K. Bernhard, in: "Carotenoids: Chemistry and Biology." Ed. by N.I. Krinsky, M.M. Mathews-Roth, and R.F. Taylor, Plenum Press, New York, 1989, pp. 337–363.
4. H. Izumida, K. Adachi, M. Nishijima, M. Endo, and W. Miki, *J. Mar. Biotechnol.,* **2**, 115 (1995).
5. A. Yokoyama, H. Izumida, and W. Miki, *Biosci. Biotech. Biochem.,* **58**, 1842 (1994).
6. A. Yokoyama and W. Miki, *FEMS Microbiol. Lett.,* **128**, 139 (1995).
7. S. Hertzberg and S. Liaaen-Jensen, *Phytochemistry,* **8**, 1259 (1969).
8. G. Englert, in: "Carotenoids." Ed. by G. Britton, S. Liaaen-Jensen, and H. Pfander, Birkhäuser Verlag, Basel, 1995, Vol. 1B, Chap. 6, pp. 147–260.
9. L.M.M. Valente, A.A.L. Gunatilaka, D.G.I. Kingston, and A.C. Pinto, *J. Nat. Prod.,* **57**, 1560 (1994).
10. R. Buchecker and K. Noack, in: "Carotenoids." Ed. by G. Britton, S. Liaaen-Jensen, and H. Pfander, Birkhäuser Verlag, Basel, 1995, Vol. 1B, Chap. 3, pp. 63–116.
11. W. Miki, *Pure Appl. Chem.,* **63**, 141 (1991).
12. H. Jyonouchi, L. Zhang, and Y. Tomita, *Nutr. Cancer,* **19**, 269 (1993).
13. N. Misawa, M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima, *J. Bacteriol.,* **172**, 6704 (1990).
14. G. Ourisson and Y. Nakatani, in: "Carotenoids: Chemistry and Biochemistry." Ed. by N.I. Krinsky, M. M. Mathews-Roth, and R.F. Taylor, Plenum Press, New York, 1989, pp. 237–245.

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