Isolation and Structure Elucidation of Carotenoid Glycosides from the Thermoacidophilic Archaea *Sulfolobus shibatae*

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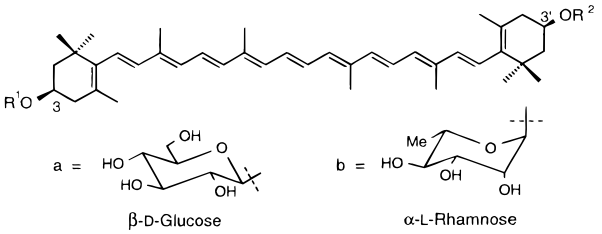
Seven zeaxanthin glycosides **1**−**7**, including *(Z)*-isomers, were isolated and characterized from the extreme thermoacidophilic Archaea *Sulfolobus shibatae*. Structure elucidation was based on spectroscopic data interpretation. This represents the first investigation of the carotenoid composition in the genus *Sulfolobus*. The (*all-E*)-isomers **1**, **2**, and **4** have been found before in other organisms, but never in Archaea. In addition, this is the first report on naturally occurring (*Z*)-isomers of zeaxanthin glycosides.

The genus *Sulfolobus* belongs to the thermoacidophilic Archaeae, which are—from the point of view of evolution—very ancient organisms. Because of the "carotenoid−hostile" growth conditions and their special evolutionary position, it was considered that a study of the carotenoid composition would be of great interest. *Sulfolobus* has been investigated at the Max Planck Institute in Martinsried (Munich, Germany) to obtain a detailed phenotypic description.[1] It was observed that a mutant of *Sulfolobus shibatae* showed a photoregulated production of carotenoids, but no further investigations on the carotenoid composition were carried out. In the present paper, we report on the isolation and structure elucidation of the carotenoids in this mutant of *S. shibatae*. This represents the first detailed investigation of carotenoids in the genus *Sulfolobus*.

The *S. shibatae* mutant, showing a high carotenoid production on irradiation with light, was cultivated in a 250-L fermentation vessel. The cultivation was carried out at 80 °C and pH 3 in a mineral medium to which were added fresh yeast extract and sucrose for 4 days. During this period the medium was stirred, ventilated with a mixture of 5% CO_2 and 95% compressed air, and illuminated with powerful light sources. After harvesting 344 g of fresh cell material, the cells were lyophilized. Afterwards, the dry powder was carefully extracted with different solvents in a purpose-built vacuum Soxhlet apparatus. The colored polar fractions were combined and used for further investigation. Part of the remaining colorless lipids were removed by precipitation from MeOH at −21 °C. The extract was further purified by reversed-phase chromatography on a Lobar column, and the major part of the carotenoids was eluted with MeOH. The high polarity of the carotenoids gave an indication of the presence of either carotenoid glycosides or glycosyl esters.

Separation of the carotenoids was carried out by HPLC with photodiode-array-detection using a C_{18} column. The chromatogram (Figure 1) showed at least 11 different carotenoids, from which the seven most significant compounds (**1**−**7**) were isolated and characterized. On the basis of UV-vis, CD, and mass spectra



1 (all-*E*, 3*R*,3'*R*)-Zeaxanthin-di-β-D-glucopyranoside (R^1=a, R^2=a)

2 (all-*E*, 3*R*,3'*R*)-Zeaxanthin-di-α-L-rhamnopyranoside (R^1=b, R^2=b)

3 (9*Z*, 3*R*,3'*R*)-Zeaxanthin- di-α-L-rhamnopyranoside (R^1=b, R^2=b)

4 (all-*E*, 3*R*,3'*R*)-Zeaxanthin-α-L-rhamnopyranoside (R^1=b, R^2=H)

5 (9*Z*, 3*R*,3'*R*)-Zeaxanthin-α-L-rhamnopyranoside (R^1=b, R^2=H)

6 (13*Z*, 3*R*,3'*R*)-Zeaxanthin-α-L-rhamnopyranoside (R^1=b, R^2=H)

7 (15*Z*, 3*R*,3'*R*)-Zeaxanthin-α-L-rhamnopyranoside (R^1=b, R^2=H)

and especially on the 1D and 2D ^{1}H-NMR investigations, **1**−**7** were identified as zeaxanthin glycosides.

Comparison of the UV-vis spectra of the seven isolated compounds revealed a great similarity, both in the absorption maxima and in their fine structure. For compounds **1**, **2**, and **4** the absorption maxima at 478 and 450 nm and the fine structural details were in agreement with data published for (*all-E*)-zeaxanthin.[2] In contrast, in compounds **3** and **5**−**7**, the absorption maxima were slightly shifted toward shorter wavelengths (470−474 nm and 444−448 nm) with a decreased fine structural information, which is characteristic of carotenoid *(Z)*-isomers. This inference was supported by the additional absorption at 335−339 nm, the so-called *cis*-peak. The intensities of the *cis*-peak indicated the presence of the (9*Z*)-isomer for **3** and **5**, the (13*Z*)-isomer for **6,** and the (15*Z*)-isomer for **7**.

By LCMS compounds **1**−**7** all showed characteristic [M^+ − 92] and [M^+ − 106] fragments of carotenoids, which are due to the loss of toluene and xylene from the polyene chain.[3] For compound **1** the molecular ion was observed at *m*/*z* 892, which is consistent with a molecular formula of $C_{52}H_{76}O_{12}$ ($C_{40}H_{56}O_2$ + 2 $C_6H_{10}O_5$). This corresponds to a dihexoside of a dihydroxycarotenoid, a notation supported by [M^+ − 162] and [M^+ − 162 − 162] fragments corresponding to the loss of one and two hexoses, respectively, and the fragment at *m*/*z* 162.

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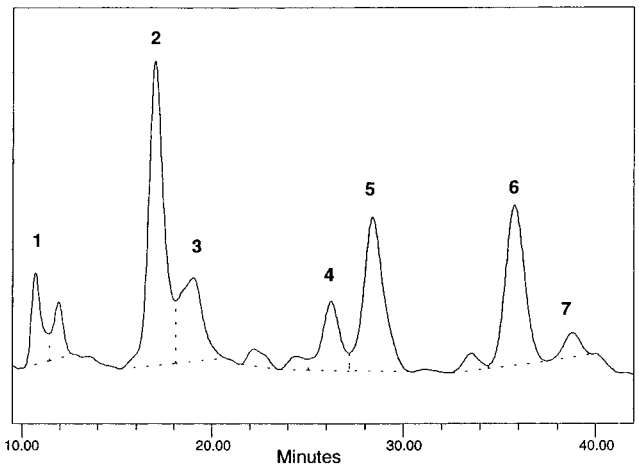

Figure 1. HPLC chromatogram of the extract of *S. shibatae*.

The mass spectra of **2** and **3** both exhibited a molecular ion at *m/z* 860, which is consistent with an elemental formula of $C_{52}H_{76}O_{10}$. Both compounds were therefore assigned as dideoxyhexosides, as supported by prominent $[M^+ - 146]$ and $[M^+ - 146 - 146]$ fragment peaks, corresponding to the loss of one and two deoxyhexose moieties, respectively. The mass spectra of **4**−**7** were all very similar, all showing a molecular ion at *m/z* 714 that corresponds to an elemental formula of $C_{46}H_{66}O_6$ and a $[M^+ - 146]$ fragment, suggesting they were all deoxyhexosides.

The interpretation of the 1D and 2D ^{1}H-NMR spectra and the comparison with literature data[4−6] revealed that the carotenoid units of compounds **1**−**7** contained two β-end groups, which were hydroxylated or glycosylated at the C-3 and C-3′ positions, respectively. Compounds **1**−**7** were therefore identified as monoglycosides or diglycosides of zeaxanthin.[2−7] The assignment of signals in the olefinic region of the spectra of **1**, **2**, and **4** revealed an (*E*)-configuration for all of the double bonds, which meant that the aglycon of these compounds could be identified as (*all-E*)-zeaxanthin. The NMR spectra of **3**, **5**, and **6** showed in the olefinic region between 5.8 and 6.8 ppm the doubling of chemical shifts, when compared to those of **1**, **2**, and **4**.

Compounds **3** and **5** showed identical signals in the olefinic region of their ^{1}H-NMR spectra. Each H-8 signal was shifted 0.54 ppm towards a lower field when compared to the (*all-E*)-isomer. Simultaneously, a shift of 0.11 ppm in each H-11 signal towards a lower field was observed, while the H-10 and H-12 resonances were shifted towards a higher field by 0.1 and 0.05 ppm, respectively. These data are characteristic for (9*Z*)-isomers of carotenoids.[4] Therefore, the aglycon of **3** and **5** was confirmed as (9*Z*)-zeaxanthin, in each case.

In the olefinic region of **6**, a shift of H-12 signal of 0.52 ppm towards a lower field was highly significant, while H-14 and H-15′ were shifted by 0.14 and 0.1 ppm towards higher field, respectively, when compared to the signals of the (*all-E*)-isomer. In contrast, the H-15 and H-10 signals were shifted towards a lower field by 0.15 and 0.05 ppm, respectively. These data are typical for (13*Z*)-isomers of carotenoids,[4] and in consequence the aglycon of **6** was identified as (13*Z*)-zeaxanthin.

Using the cross peaks in the COSY spectra in the region from 3.5 to 5.5 ppm, it was possible to identify the carbohydrate moieties of **1** as β-glucopyranose and those of **2**−**6** as α-rhamnopyranose (α-6-deoxymannopyranose), which was consistent with the MS data. Thus, the carbohydrate unit of **1** was identified by starting with the doublet at 4.48 ppm, which was assigned to the H-1 anomeric proton and which coupled with H-2 (^3J = 10 Hz). The value of the coupling constant indicated that these two protons were diaxially oriented. In addition, a series of six consecutive proton signals with ^3J = 10 was seen in the COSY spectrum. Based on the detailed analysis of the spectra, the carbohydrate moiety of **1** could be identified as β-glucopyranoside.[8,9]

The rhamnose residue found in all of **2**−**6** was identified by beginning with the doublet at 4.93 ppm of the H-1 anomeric proton, of the carbohydrate, which coupled to H-2 (^3J = 3 Hz). This finding confirmed that this signal originated from the anomeric proton and that both protons were in equatorial positions. From the coupling of H-2 to H-3 (^3J = 5 Hz), an axial position for H-3 could be deduced. The coupling constants of the following protons in sequence with $^3J \approx 10$ Hz proved the axial positions of H-4 and H-5 as expected.[9] The H-5 signal coupled with the C-6 methyl protons at 1.23 ppm, which is consistent with a 6-deoxyhexose. On the basis of these data, the carbohydrate portion of **2**−**6** could be identified as α-rhamnopyranose (6-deoxy-α-mannopyranose).

The small amount of **7** isolated prevented any NMR investigations. However, its UV-vis spectrum, MS data, and chromatographic behavior give an indication that it was a monorhamnoside of zeaxanthin. The increased intensity of the *cis*-peak at 337 nm of **7** compared to **6**, with otherwise the same spectroscopic behavior, gave the strong indication that (15*Z*)-zeaxanthin was the aglycon of **7**.

The determination of the absolute configuration at C-3 and C-3′ in zeaxanthin and the establishment of the D- or L-series of the carbohydrates were both carried out by CD. The CD data of the (*all-E*)-isomers, **1**, **2**, and **4** were in accordance with those of (3*R*,3′*R*)-zeaxanthin.[6,10] The *(Z)*-isomers **3** and **5**−**7** showed the expected inversion of signals compared to the (all-*E*)-isomer. The CD spectra of the *(Z)*-isomers were, as for the *cis*-peak in the UV-vis spectrum seen to be more intense the closer to the middle of the polyene chain the *(Z)*-bond was located.[10] For the determination of the absolute configuration of the carbohydrates, they were cleaved from the carotenoid by mild methanolysis and subsequently perbenzylated. The CD spectra of the modified carbohydrates in the region from 200 to 250 nm were compared to those of authentic samples and to literature spectral data.[11] The spectrum of β-glucose in **1** belongs to the D-series because it was in accordance with published data for β-D-glucose,[11] while the α-rhamnose of **2**−**7** showed the same spectrum as α-L-rhamnose[11] and therefore belongs to the L-series.

The present report represents the first investigation of the carotenoid composition in the genus *Sulfolobus* and has presented evidence that glycosides of zeaxanthin are the major carotenoids in a mutant of *Sulfolobus shibatae*. The (*all-E*)-isomers **1**, **2**, and **4** have been found before in bacteria, algae, and other organism, but never in an Archaea.[12] To the best of our knowledge, this is the first report of naturally occurring *(Z)*-isomers of zeaxanthin glycosides.

It can be speculated that these glycosides may act in *Sulfolobus* as membrane reinforcers, as previously proposed for bacteria.[12,13] For this role C_{50}-carotenoids with polar end groups, such as bacterioruberin or

bisanhydrobacterioruberin, have been proposed in particular. The shorter aglycon of **1**−**7**, compared to the C_{50}-carotenoids, may be compensated by the presence of the carbohydrate moieties, and therefore, the isolated zeaxanthin glycosides **1**−**7** may also be postulated as membrane reinforcers.

Experimental Section

General Experimental Procedures. UV-vis spectra were obtained on spectrophotometer Perkin-Elmer 554 and Waters PDA 996. CD spectra were recorded on a Dichrograph II (Jobin-Yvon). NMR spectra were recorded on Bruker AM 400 (400 MHz) and DRX 500 (500 MHz) spectrometers. The Lobar apparatus consisted of a Sepapress PCP-150/75 pressure ceramic pump, a Merck Lobar Lichroprep RP-8 column, 40−63 μm, size B (310−25) and a Uvikon LCD 725 detector. HPLC analysis was carried out on an apparatus consisting of a Waters 600E Multisolvent Delivery System, a Laboc Gastorr GT-104 (4-channel online degasser), an injector Rheodyne 7725i; a photodiodearray detector Waters PDA 996; data-processing by Millennium 2010 Software; and a C_{18} column Spherisorb 5 ODS-2 (250 × 4.6 mm). LC-MS analysis was perfomed with an apparatus consisting of two Altex 110 pumps with a gradient-controller, an injector Rheodyne 7710, a Kontron 725 UV-vis detector and a VG Platform, Fisons Instruments.

Microorganism. *S. shibatae* was provided by Dr. D. Grogan as a mutant of an isolate from a hot spring in Beppu (Japan) and has been deposited in the German collection of microorganisms and cell cultures in Braunschweig (Germany) under the accession number DSM no. 5389. The cultivation was carried out in a 250-L fermentation vessel using a basal medium. This medium contained per 10 L of distilled H_2O 13.0 g of $(NH_4)_2SO_4$, 2.8 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 0.7 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $FeSO_4 \cdot 7H_2O$ and prepared from solutions of 1 g/100 mL of H_2O 1.8 mL of $MnCl_2 \cdot 4H_2O$, 4.5 mL of $Na_2B_4O_7 \cdot 10H_2O$, 220 μL of $ZnSO_4 \cdot 7H_2O$, 50 μL of $CuCl_2 \cdot 2H_2O$, 30 μL of $NaMoO_4 \cdot 2H_2O$, 30 μL of $VOSO_4 \cdot 5H_2O$, and 10 μL of $CoSO_4 \cdot 7H_2O$. To this mixture was added 1 g fresh yeast extract and sucrose 1 g/L of medium. The temperature inside the vessel was 80 °C, and the pH was adjusted to 3.5 with H_2SO_4. The well-stirred medium was ventilated with 95% compressed air and 5% CO_2 and illuminated with high pressure Hg light bulbs (125 W). After 4 days of cultivation the optical density of the medium rose from 0.07 to 1.5, and the cell material was harvested still in the exponential growth phase. By continuous centrifugation, 344 g of wet cells were separated from the medium, which were immediately frozen in liquid N_2 and stored at −21 °C.

Extraction and Isolation. The frozen cells were lyophilized for 4 days at −70 °C. The extraction of the cells was carried out in a specially developed vacuum Soxhlet apparatus. A 500 mL round-bottomed flask at the base of the apparatus, which contained the solvent, was heated electrically. On the flask a Soxhlet apparatus was mounted, containing the cells for extraction, and above this were placed two water-cooled condensers. The whole apparatus was closed with a connector to the water-stream vacuum, which was controlled by a pressure controller. This arrangement allowed a fast, continuous, and careful extraction of the pigments from *S. shibatea*. For each extraction the flask was filled with ca. 300 mL of solvent, and a 15−20 g portion of cell material was placed into the Soxhlet apparatus. A vacuum of 200 mbar was applied, and then the pressure was adjusted so that the solvent boiled at 35 °C and the vapors condensed on the middle of the second condenser. As soon as the solvent in the reservoir above the flask were no longer colored, the colored solvent was replaced with 300 mL of a solvent of higher polarity. The first extraction was carried out with hexane, and this fraction was discarded. Then followed three extractions with Me_2CO, EtOAc, and MeOH. The yellow extracts were immediately evaporated *in vacuo* (< 30 °C) to dryness and stored at −21 °C. The three yellow fractions were combined, dissolved in 50 mL of MeOH, and stored for several hours at −21 °C. The solution was filtered over Celite to remove the precipitated colorless lipids. This procedure was repeated until no more precipitation could be observed. The purified extract was dissolved in 10 mL of MeOH and 4 mL of toluene and separated by column chromatography (Lobar apparatus). A C_8 column was used as stationary phase, and the solvent pressure with MeOH as mobile phase was adjusted to ca. 8 bar (flow = 5.5 mL/min). For each injection, 2 mL of extract could be loaded onto the column. After 18 min the polar components were eluted from the column, while some toluene-soluble components remained on the column, which were eluted after the last separation with toluene. The HPLC separation of the MeOH fraction was carried out using a reversed-phase system with a C_{18} column (Spherisorb 5-ODS-2, 250 × 4.6 mm), A:B = 90:10 v/v (A = MeOH−H_2O, 9:1; B = MeOH−EtOAc, 8:2) at a flow rate of 1 mL/min. The seven most abundant peaks (compounds **1**−**7**) with retention times between 11 and 39 min were collected individually. In order to remove impurities **1**−**7** were again subjected to HPLC on the same C_{18} column with 100% MeOH as solvent. For the most polar fractions 2−7% H_2O was added to shorten retention times. By this procedure, 1.6 mg of **1**, 9.8 mg of **2**, 4.2 mg of **3**, 3.1 mg of **4**, 4.8 mg of **5**, 5.6 mg of **6**, and 0.4 mg **of 7** were isolated.

(*all-E*,3*R*,3′*R*)-Zeaxanthin di-*β*-D-glucopyranoside (1): UV-vis λ_{max} 477, 449 (90% A + 10% B), 474, 447 nm (EPA); CD (EPA, −180 °C), 206.5 (25.7), 225.5 (−10.6), 251.0 (23.1), 289 (−13.2), 353.5 (6.4), 438.5 (−1.4), 449 (−3.7), 468 (−3.1), 483 (−0.9), 498 (−3.6) nm; ^{1}H NMR (500 MHz, MeOH-d_4), aglycon 1.07 (6H, s, CH_3-16, CH_3-16′), 1.08 (6H, s, CH_3-17, CH_3-17′), 1.48 (2H, m, H_{eq}-2, H_{eq}-2′), 1.77 (2H, m, H_{ax}-2, H_{ax}-2′), 1.74 (6H, s, CH_3-18, CH_3-18′), 1.97 (6H, s, CH_3-19, CH_3-19′), 1.98 (6H, s, CH_3-20, CH_3-20′), 2.04 (2H, m, H_{ax}-4, H_{ax}-4′), 2.39 (2H, m, H_{eq}-4, H_{eq}-4′), 4.05 (2H, m, br, H_{ax}-3, H_{ax}-3′), 6.10 (2H, d, H-7, H-7′), 6.14 (2H, d, H-8, H-8′), 6.15 (2H, d, H-10, H-10′), 6.25 (2H, m, H-14, H-14′), 6.36 (2H, d, H-12, H-12′), 6.63 (2H, m, H-15, H-15′), 6.64 (2H, dd, H-11, H-11′); sugar moiety 1.23 (2H, d, H_{ax}-6, H_{ax}-6′), 3.38 (1H, m, H_{ax}-5, H_{ax}-5′), 4.48 (1H, d, H_{ax}-1, H_{ax}-1′), 3.56 (2H, m, H_{ax}-4, H_{ax}-4′), 3.31 (2H, dd, H_{ax}-2, H_{ax}-2′), 3.59 (2H, dd, H_{ax}-3, H_{ax}-3′); EIMS 892 (M^+, 12), 772 (6), 754 (8), 730 (M^+−162, 9), 712 (11), 694 (3), 638 (4), 620 (4), 610 (9), 592 (8), 568 (M^+−162−162, 78), 550 (78), 532 (14), 476 (12), 458 (9), 416 (5), 359 (3), 327 (2), 209 (18), 162 (44), 157 (60), 145 (74), 119 (100), 106 (92), 92 (95).

(*all-E*,3*R*,3′*R*)-Zeaxanthin di-α-L-rhamnopyranoside (2): UV-vis λ_{max} 478, 450 (90% A + 10% B), 474, 447 nm (EPA); CD (EPA, −180 °C), 206.5 (26.6), 225.5 (−14.3), 250.5 (19.4), 289 (−19.1), 352.5 (3.9), 466.5 (−3.5), 482 (−0.9), 498.5 (−4.2) nm; ^{1}H NMR (400 MHz, MeOH-d_4), aglycon same as for (**1**); sugar moiety 1.23 (6H, d, CH_3-6, CH_3-6′), 3.92 (2H, m, H_{ax}-5, H_{ax}-5′), 4.93 (2H, d, H_{eq}-1, H_{eq}-1′), 5.07 (2H, m, H_{ax}-4, H_{ax}-4′), 5.21 (2H, dd, H_{ax}-2, H_{ax}-2′), 5.32 (2H, dd, H_{ax}-3, H_{ax}-3′); EIMS 860 (M^+, 100), 714 (M^+−146, 28), 696 (40), 600 (6), 584 (8), 568 (M^+−146−146, 12), 550 (28), 534 (16), 432 (62), 413 (90), 396 (62), 374 (88), 292 (14), 279 (42), 234 (46), 163 (8), 146 (6),119 (16), 106 (12), 92 (4).

(9*Z*,3*R*,3′*R*)-Zeaxanthin di-α-L-rhamnopyranoside (3): UV-vis λ_{max} 474, 442, 343 (90% A + 10% B), 474, 442, 337 nm (EPA); CD (EPA, −180 °C), 223 (9.5), 249 (−9.6), 290 (9.6), 352 (−6.5), 467 (−3.7), 477.5 (−1.7), 490 (−4.0) nm; ^{1}H NMR (400 MHz, MeOH-d_4), aglycon 1.07 (6H, s, CH_3-16, CH_3-16′), 1.08 (6H, s, CH_3-17, CH_3-17′), 1.48 (2H, m, H_{eq}-2, H_{eq}-2′), 1.77 (2H, m, H_{ax}-2, H_{ax}-2′), 1.74 (6H, s, CH_3-18, CH_3-18′), 1.97 (6H, s, CH_3-19, CH_3-19′), 1.98 (6H, s, CH_3-20, CH_3-20′), 2.04 (2H, m, H_{ax}-4, H_{ax}-4′), 2.39 (2H, m, H_{eq}-4, H_{eq}-4′), 4.05 (2H, m, br, H_{ax}-3, H_{ax}-3′), 6.05 (1H, d, H-10), 6.10 (2H, d, H-7, H-7′), 6.14 (1H, d, H-8), 6.15 (1H, d, H-10), 6.25 (2H, m, H-14, H-14′), 6.31 (1H, d, H-12), 6.36 (1H, d, H-12′), 6.63 (2H, m, H-15, H-15′), 6.64 (1H, dd, H-11′), 6.68 (1H, d, H-8′), 6.74 (1H, H-11); sugar moiety, same as for (**2**); EIMS 860 (M^+, 100), 714 (M^+−146, 28), 696 (40), 600 (6), 584 (8), 568 (M^+−146−146, 12), 550 (28), 534 (16), 432 (62), 413 (90), 396 (62), 374 (88), 292 (14), 279 (42), 234 (46), 163 (10), 146 (6),119 (16), 106 (12), 92 (4).

(*all-E*,3*R*,3′*R*)-Zeaxanthin α-L-rhamnopyranoside (4): UV-vis λ_{max} 478, 450 (90% A + 10% B), 474, 447 nm (EPA); CD (EPA, −180 °C), 206.5 (26.6), 225.5 (−14.3), 250.5 (19.4), 289 (−19.1), 352.5 (3.9), 466.5 (−3.5), 482 (−0.9), 498.5 (−4.2) nm; ^{1}H NMR (500 MHz, MeOH-d_4), aglycon, same as for (**1**); sugar moiety 1.23 (3H, d, CH_3-6), 3.92 (1H, m, H_{ax}-5), 4.93 (1H, d, H_{eq}-1), 5.07 (1H, m, H_{ax}-4), 5.21 (1H, dd, H_{ax}-2), 5.32 (1H, dd, H_{ax}-3); EIMS 714 (M^+, 100), 698 (18), 640 (6), 600 (32), 584 (16), 568 (M^+−146, 12), 550 (10), 475 (54), 458 (33), 432 (12), 425 (38), 282 (6), 267 (4), 234 (4), 163 (10), 119 (25), 106 (12), 92 (4).

(9*Z*,3*R*,3′*R*)-Zeaxanthin α-L-rhamnopyranoside (5): UV-vis λ_{max} 472, 446, 335 (90% A + 10% B), 471, 444, 337 nm (EPA); CD (EPA, −180 °C), 223 (9.5), 249 (−9.6), 290 (9.6), 352 (−6.5), 467 (−3.7), 477.5 (−1.7), 490 (−4.0) nm; ^{1}H NMR (500 MHz, MeOH-d_4), aglycon, same as for (**3**); sugar moiety same as for (**4**); EIMS 714 (M^+, 100), 698 (18), 640 (6), 600 (32), 584 (16), 568 (M^+ − 146, 12), 550 (10), 475 (54), 458 (33), 432 (12), 425 (38), 282 (6), 267 (4), 234 (4), 163 (10), 119 (25), 106 (12), 92 (4).

(13*Z*,3*R*,3′*R*)-Zeaxanthin α-L-rhamnopyranoside (6): UV-vis λ_{max} 470, 444, 339 (90% A + 10% B), 468, 442, 337 nm (EPA); ^{1}H NMR (500 MHz, MeOH-d_4), aglycon 1.07 (6H, s, CH_3-16, CH_3-16′), 1.08 (6H, s, CH_3-17, CH_3-17′), 1.48 (2H, m, H_{eq}-2, H_{eq}-2′), 1.77 (2H, m, H_{ax}-2, H_{ax}-2′), 1.74 (6H, s, CH_3-18, CH_3-18′), 1.97 (6H, s, CH_3-19, CH_3-19′), 1.98 (6H, s, CH_3-20, CH_3-20′), 2.04 (2H, m, H_{ax}-4, H_{ax}-4′), 2.39 (2H, m, H_{eq}-4, H_{eq}-4′), 4.05 (2H, m, br, H_{ax}-3, H_{ax}-3′), 6.10 (2H, d, H-7, H-7′), 6.11 (1H, m, H-14), 6.14 (2H, d, H-8, H-8′), 6.15 (1H, d, H-10′), 6.20 (1H, d, H-10), 6.25 (1H, m, H-14′), 6.36 (1H, d, H-12′), 6.53 (1H, m, H-15′), 6.64 (2H, dd, H-11, H-11′), 6.68 (1H, m, H-15), 6.88 (1H, d, H-12); sugar moiety, same as for (**4**); EIMS 714 (M^+, 100), 698 (18), 640 (6), 600 (32), 584 (16), 568 (M^+ − 146, 12), 550 (10), 475 (54), 458 (33), 432 (12), 425 (38), 282 (6), 267 (4), 234 (4), 163 (10), 119 (25), 106 (12), 92 (4).

(15*Z*,3*R*,3′*R*)-Zeaxanthin α-L-rhamnopyranoside (7): UV-vis λ_{max} 472, 448, 337 (90% A + 10% B), 472, 447, 337 nm (EPA); CD (EPA, −180 °C), 211 (3.7), 218.5 (2.1), 232 .5 (4.5), 247 (3.5), 271.5 (6.2), 351 (−7.1), 443 (-0.4), 456 (0.5), 471.5 (−0.6), 500.5 (−1.0) nm; EIMS 714 (M^+, 100), 698 (18), 640 (6), 600 (32), 584 (16), 568 (M^+ − 146, 12), 550 (10), 475 (54), 458 (33), 432 (12), 425 (38), 282 (6), 267 (4), 234 (4), 163 (10), 119 (25), 106 (12), 92 (4).

Determination of Carbohydrate Configuration. For establishment of the configuration of the glycosides, **1** and **2** were subjected to a mild methanolysis in 0.5 mL of 0.1 M methanolic HCl at room temperature overnight. The solvent was removed under Ar gas. To each dried sample was added 0.5 mL of freshly prepared benzoic anhydride (5% benzoic anhydride and 10% (dimethylamino)pyridine in pyridine). The reaction was carried out overnight at room temperature, and the solvent removed again under Ar. The sample was dissolved in EtOAc and subjected to preparative TLC (Si 60, hexane−EtOAc, 1:2). The perbenzylated glycosides were eluted from the TLC plates, desorbed with EtOAc, filtered, and evaporated *in vacuo* to dryness.

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