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2′-Methyl and 1′-xylosyl derivatives of 2′-hydroxyflexixanthin are major carotenoids of *Hymenobacter* species

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ABSTRACT

Bacteria related to the genus *Hymenobacter* are colored intensely red due to their carotenoid pigmentation. Using high-resolution mass spectrometry and comparison to the literature reports, we previously isolated and presumptively identified seven structurally related carotenoids from representative *Hymenobacter*-like strains. After further analysis using one- and two-dimensional ^{1}H NMR, UV–vis, and circular dichroism spectroscopies, we determined that the major carotenoid present in these strains is 2′-hydroxyflexixanthin, as proposed previously by others in a related organism. Furthermore, we have identified three novel, related carotenoids abundant in these strains as 1′-xylosyl-2′-hydroxyflexixanthin, 2′-methoxyflexixanthin, and 3-deoxy-2′-methoxyflexixanthin.

1. Introduction

Bacteria of the genus *Hymenobacter* (Flavobacteria, Bacteroidetes) are often isolated from dry environments subject to intense oxidative stress, including dry soils,[2,12,13,22,25,35,37] aerosols,[8,9] and irradiated meat.[10] Habitation of these high-radiation and high-desiccation environments requires efficient antioxidative defense mechanisms. Accordingly, all isolated *Hymenobacter* strains are colored bright red-pink due to the presence of carotenoids, natural pigments with notable biotechnological application as natural colorants[21] and nutritional supplements.[11,24] Carotenoid antioxidant function is well established, especially in *Deinococcus*,[32,33,36] a genus that has been repeatedly co-detected with *Hymenobacter*.[12,22,25] Carotenoids likely play an important antioxidative role in both genera.

We have previously isolated 10 *Hymenobacter*-related strains from Victoria Upper Glacier, Antarctica glacial ice.[14] In these strains and nine additional reference *Hymenobacter* species we detected and isolated seven chemically distinct carotenoids using high-performance liquid chromatography (HPLC), with the exact carotenoid composition varying between strains.[14] Based on in-line HPLC UV–vis spectra, high-resolution mass spectrometry (MS), and comparison to previous synthetic work[6] claiming to represent the major carotenoids of an unidentified "*Taxeobacter*" (now *Hymenobacter*[8]) strain, these carotenoids were proposed to be 2′-hydroxyflexixanthin, its pentosyl-, hexosyl-, and methyl- derivatives and related non-ketolated precursors. Because the previous synthetic work[6] did not present the isolation and characterization of 2′-hydroxyflexixanthin from its natural source, the identification of this pigment as the major carotenoid in '*Taxeobacter*' (now *Hymenobacter*) is considered unsubstantiated.[7] Here we report full characterization of the four most abundant carotenoids isolated from *Hymenobacter*-like strains VUG-A42aa and VUG-A141a using ^{1}H NMR, circular dichroism (CD), and UV–vis spectroscopies and previously generated high-resolution MS data.[14]

High resolution MS and in-line HPLC UV–vis spectroscopy previously suggested that the dominant carotenoid in all surveyed *Hymenobacter* strains (**1**; previously designated carotenoid 5[14]) is a monocyclic ketocarotenoid containing 12 double bonds and having a molecular formula of $C_{40}H_{54}O_4$. UV–vis spectroscopy of purified **1** shows a broad peak with a central absorbance maximum at 494 nm and minimal spectral fine structure (Supplementary data Fig. S1), consistent with this proposed structure. The ^{1}H NMR spectra of **1** (Table 1, Supplementary data Figs. S2 and S3) are consistent with those previously reported for 2′-hydroxyflexixanthin,[3] with important diagnostic peaks at δ1.76 (H-2; doublet of doublets split by H-3 and OH-3; see Fig. 1 for numbering scheme), δ4.28 (H-3; doublet of doublets split by H-2$_{ax}$ and H-2$_{eq}$), and δ3.96 (H-2′; linked in the 2D-TOCSY to H-3′ and H-4′); the latter shift clearly suggests hydroxylation at the 2′-position by comparison with the NMR structure of flexixanthin.[3] The CD spectrum of **1** (Supplementary data Fig. S4) clearly indicates *S* stereochemistry at the 2′-hydroxyl position by comparison to the previously published data.[3,23] No CD signal, however, was observed for any of the 2′-methoxy carotenoids identified in this study (see below). We therefore hesitate to apply the additivity hypothesis for carotenoids[4] to the 3-hydroxyl group of **1** and consider the stereochemistry at this position undetermined. Based on this evidence, we identify **1** as 2′*S*-hydroxyflexixanthin (Fig. 1), as previously

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Table 1
^{1}H NMR spectral assignments in CD_2Cl_2, referenced to the major solvent peak defined as δ5.31, for compounds **1** and **2**, isolated from strain VUG-A141a, and **4** and **5**, isolated from strain VUG-A42aa, determined using 1D–^{1}H NMR and 2D–^{1}H-TOCSY spectra (Supplementary data Figures S2, S3, and S5–S9)

Proton location	Compound							
	1		**2**		**4**		**5**	
	δ (ppm)	Multiplicity and *J* (Hz)	δ (ppm)	Multiplicity and *J* (Hz)	δ (ppm)	Multiplicity and *J* (Hz)	δ (ppm)	Multiplicity and *J* (Hz)
H-2	1.76	dd, 6.5, 6.5	1.75	dd, 6.6, 6.6	1.76	dd, 6.2, 6.2	1.84	t, 6.9
H-3	4.28	dd, 6.2, 13.9	4.19	dd, 5.9, 14.0	4.28	dd, 9.7, 20.4	2.45	t, 9.1
H-7	6.11	d, 10.3	6.19	d, 11.3	6.21	d, 17.2	6.21	d, 11.5
H-8	6.32	m	6.44	d, 16.2	6.52	d, 16.2	6.37	d, 17.2
H-10	6.20	d, 10.7	6.23	d, 15.0	6.33	d, 17.9	6.26	d, 15.9
H-11	6.62	t, 15.7	6.62	dd, 11.4, 15.0	6.63	t, 13.8	6.63	t, 15.8
H-12	6.39	d, 16.6	6.39	d, 9.9	6.40	d, 17.2	6.40	d, 19.6
H-14	6.29	m	6.26	m	6.30	m	6.30	m
H-15	6.95	m	6.94	m	6.94	m	6.94	m
H-15′	6.99	m	6.99	dd, 9.2, 15.8	6.98	m	6.98	m
H-14′	6.35	m	6.24	m	6.35	m	6.33	m
H-12′	6.45	d, 12.8	6.45	d, 14.8	6.45	d, 17.7	6.44	d, 15.3
H-11′	6.68	t, 10.75	6.67	m	6.68	t, 15.1	6.68	t, 16.4
H-10′	6.30	d, 9.28	6.32	m	6.29	m	6.36	m
H-8′	6.30	d, 8.42	6.30	m	6.26	d, 16.8	6.28	d, 13.9
H-7′	6.84	dd, 11.7, 26.5	6.84	m	6.85	dd, 11.5, 22.4	6.84	dd, 11.5, 14.9
H-6′	6.15	m	6.11	d, 12.1	6.16	d, 10.9	6.12	m
H-4′	6.37	d, 16.71	6.37	d, 16.3	6.33	d, 11.9	6.33	m
H-3′	5.71	t, 7.73	5.64	dd, 7.0, 15.8	5.53	t, 14.0	5.53	dd, 8.5, 16.5
H-2′	3.96	m	4.08	d, 7.0	3.41	m	3.40	d, 14.61
H-16	1.13	s	1.18	s	1.10	s	1.10	s
H-17	1.15	s	1.19	s	1.12	s	1.11	s
H-18	1.91	s	1.90	s	1.91	s	1.93	s
H-19	1.98	s	1.99	s	1.98	s	1.98	s
H-20	2.00	s	1.98	s	2.00	s	1.99	s
H-20′	2.00	s	1.98	s	2.00	s	2.00	s
H-19′	1.98	s	1.98	s	1.98	s	1.93	s
H-18′	1.92	s	1.90	s	1.93	s	1.25	s
H-17′	1.30	s	1.25	s	1.30	s	1.18	s
H-16′	1.19	s	1.23	s	1.18	s	2.11	s
2-OH	2.11	m	2.11	m	2.11	m	—	
1′-OH	2.11	m		—	2.11	m	2.11	m
2′-OH	2.11	m	2.11	m	—			
2′-Methyl		—		—	3.28	m	3.41	d, 5.57
Sugar								
H-1″		—	4.53	d, 7.36		—		—
H-2″		—	3.30	dd, 7.36, 8.72		—		—
H-3″		—	3.48	dd, 8.69, 8.69		—		—
H-4″		—	3.67	ddd, 5.34, 8.61, 9.55		—		—
H-5″a		—	3.29	dd, 9.92, 11.60		—		—
H-5″b		—	3.99	dd, 5.18, 11.77		—		—

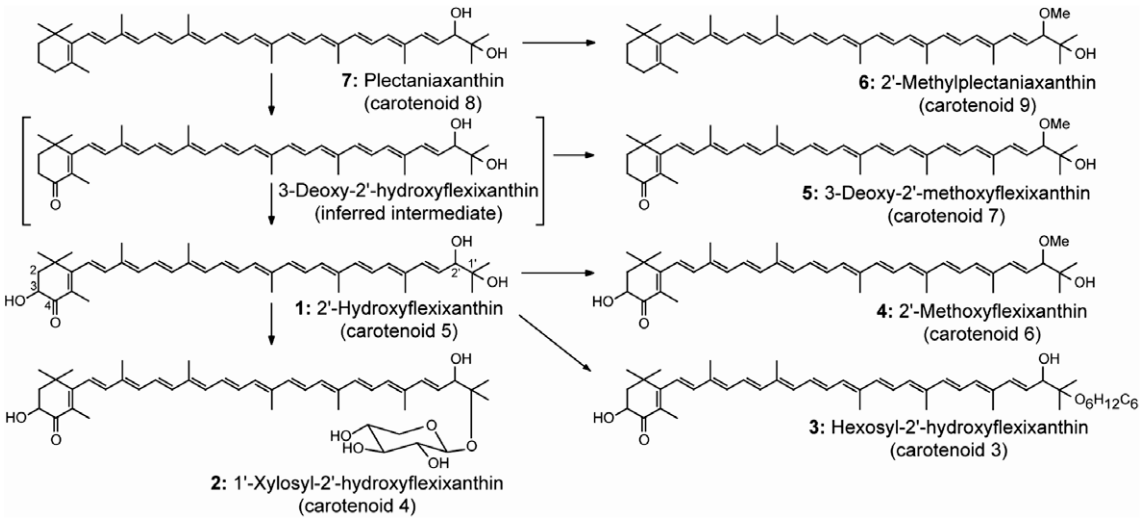

Figure 1. The major carotenoids in *Hymenobacter* and their proposed biosynthetic relationships. Compounds described in this study are numbered, with their previous designations[14] indicated in parentheses. 3-Deoxy-2′-hydroxyflexixanthin, an inferred intermediate compound not detected in this study, is indicated in square brackets. Carbon numbers mentioned in the text are indicated for compound **1**.

reported (albeit without full substantiation[7]) from a *Taxeobacter* (now *Hymenobacter*) species.[14]

Our previous analyses suggested the presence of pentosyl (**2**; molecular formula: $C_{45}H_{62}O_8$; previously designated carotenoid 4[14]) and hexosyl (**3**; molecular formula: $C_{46}H_{64}O_9$; previously designated carotenoid 3[14]) derivatives of **1** in strain VUG-A141a. The latter was present only in small amounts and was not analyzed further. Both the UV–vis and ^{1}H NMR spectra (Supplementary data Figs. S1, S5, and S6) of **2** are nearly identical to those of **1**, excepting ^{1}H NMR peaks for **2** corresponding to a β-linked xylose (compare Table 1 with ^{1}H NMR assignments for β-glucosylated and acyl-β-glucosylated monocyclic carotenoids;[17,18] xylose has the same stereochemistry as glucose excepting the C6 substituent; see also Shindo et al.[26]). Because TOCSY cross-correlations were present for both 2- and 2′-hydroxyl protons, xylosylation is presumed to be at the 1′-hydroxyl group, consistent with the position of glycosylation in other flavobacterial monocyclic carotenoids.[17,18] The CD spectrum of **2** (not shown) showed only a weak signal and was not comparable to that of **1** or to similar previously published spectra,[4,23] likely due to the presence of the glycosyl moiety; the stereochemistry of the 2′-hydroxyl group of **2** therefore remains undetermined. We identify **2** as 1′-β-xylosyl-2′-hydroxyflexixanthin (Fig. 1). This is only the second report of a xylosylated carotenoid[26] and the first in which the xylose moiety is not otherwise modified.

Strain VUG-A42aa possesses two 12-conjugated double bond-containing ketocarotenoids in addition to 2′-hydroxyflexixanthin: **4** (previously designated carotenoid 6[14]), with a molecular formula of $C_{41}H_{56}O_4$, now presumed to be methyl-2′-hydroxyflexixanthin and **5** (previously designated carotenoid 7[14]), with a molecular formula of $C_{41}H_{56}O_3$, chemically similar to **4** but lacking a hydroxyl group. In both cases ^{1}H NMR spectra (Table 1, Supplementary data Figs. S7–S9) suggest methylation at the 2′-hydroxyl group, based on the lack of interactions in the TOCSY spectrum between protons H-2′ and H-3′ and the hydroxyl hydrogen (δ2.11; see also Bircher and Pfander[6]). This is consistent with the lack of CD signal for either **4** or **5** (data not shown), presumably from masking or abolishment of stereochemistry at this position due to methylation. The presence of a peak at δ2.45 instead of δ4.28 (as with **1** and **4**) in the ^{1}H NMR spectrum of **5** also indicated the absence of a 2-hydroxyl group. The novel methyl-carotenoids **4** and **5** are therefore identified as 2′-methoxyflexixanthin and 3-deoxy-2′-methoxyflexixanthin, respectively (Fig. 1).

Previously determined UV–vis spectra and molecular formulae inferred from mass spectrometry[14] suggested the presence of two non-ketolated carotenoids in strain VUG-A42aa, one of which (**6**; molecular formula: $C_{41}H_{58}O_2$; previously designated carotenoid 9[14]) was presumed to be a methyl derivative of the other (**7**; molecular formula: $C_{40}H_{56}O_2$; previously labeled carotenoid 8[14]). Compound **6** was present only in trace amounts and was not analyzed further. Unfortunately, replicate extracts of **7** yielded a ^{1}H NMR spectrum consistently contaminated by an unknown glycosyl moiety (Supplementary data Figs. S10 and S11). Clearly present in this spectrum, however, were H-2′, H-3′, and H-4′ signals similar to those determined for **1** and TOCSY cross-correlated peaks at δ1.31, δ1.59, and δ2.30 (annotated as H-4, H-3, and H-2, respectively). The similarity of these signals to those determined by others[19] and the lack of a 2′-hydroxyflexixanthin-like H-3 peak suggest that **7** is most likely plectaniaxanthin; **6** is most likely 2′-methylplectaniaxanthin by analogy to **4** and **5** (Fig. 1).

The structural similarity of the carotenoids identified in this study suggests a metabolic relationship between them, as indicated in Figure 1. The accumulation of **7** in relatively small amounts in strain VUG-A42aa[14] suggests that it is a biosynthetic precursor for **1**, which is likely in turn a precursor of **4** in VUG-A42aa and of **2** and **3** in VUG-A141a. The reactions leading to carotenoids similar to **7** have been at least partially elucidated in other Bacteroidetes,[29–31] Cyanobacteria[20,28] and *Deinococcus radiodurans*.[32,33,36] Compound **5** is a likely methylation product of the presumed intermediate between **7** and **1**, 3-deoxy-2′-hydroxyflexixanthin. It is most parsimonious to suppose the presence of a single methyltransferase having broad specificity leading to **4**, **5,** and **6**, although our data cannot preclude the existence of multiple dedicated enzymes. Further determination of this pathway will be assisted by the genome sequence for the 2′-hydroxyflexixanthin-producing *Hymenobacter roseosalivarius*, undergoing sequencing at the Joint Genome Institute (http://www.jgi.doe.gov/sequencing/statusreporter/psr.php?projectid=98064, accessed February 2009).

Carotenoid production is widespread in the Flavobacteria (which includes the genus *Hymenobacter*), with carotenoid biosynthetic gene homologs present in all currently available genome sequences excepting the insect symbiont *Candidatus* 'Sulcia muelleri' (Integrated Microbial Genome database version 2.7 [last updated December 2008]; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Many, but not all, Flavobacteria produce highly oxygenated monocyclic carotenoids,[1,17,18,27,29,34] some of which possess antioxidant activity surpassing that of oxygenated bicyclic carotenoids such as zeaxanthin.[27] While the exact reason for the current extent of flavobacterial structural diversity remains unknown, possibilities may include fine-scale tuning of electrochemical activity or membrane fluidity. The presence of these potent antioxidants in *Hymenobacter* very likely facilitates their survival in the high-radiation environments in which they have most commonly been detected.

2. Experimental

2.1. General experimental procedures

UV–vis absorption spectra of all purified carotenoids were determined in HPLC-grade methanol and HPLC-grade chloroform (Fisher) using an Ultrospec 3100 pro spectrometer (Biochrom). Absorption at wavelengths between 200 and 800 nm was recorded at 1 nm intervals. CD spectra of all purified carotenoids were determined at room temperature in 95% ethanol (Fisher) using an Olis DSM 17 spectrometer. Polarization was recorded at 1 nm intervals between 195 and 600 nm.

Both one-dimensional ^{1}H and two-dimensional ^{1}H,^{1}H total correlation spectroscopy[5,15,16] (TOCSY) NMR spectra were collected at 800 MHz (Oxford 18.8 T) on a Varian Inova console controlled by a Sun Blade (Solaris) host computer. Samples were dissolved in 600 μL of methylene chloride (D2—99.9%; Cambridge Isotope Laboratories Inc.), placed in 5 mm Wilmad 535-PP-9 constricted tubes and stoppered because flame sealing of these tubes often caused abrupt sample degradation. Samples were either prepared just prior to data acquisition or stored in the dark at −20 °C until run at 25 °C. The TOCSY pulse sequence used was the standard BioPack (Varian Inc.) version 2008-08-19 and experimental details are provided in Supplementary data Table S1. All experiments were conducted in a linear-uniform fashion with sweep widths of 11990 and 8000 Hz for the directly and indirectly detected dimensions, respectively. All experiments were processed and analyzed using Varian software VNMRJ 1.1D native Macintosh on a G5 PowerPC with OS X 10.5×. Each spectrum was referenced to the major CD_2Cl_2 peak (defined as δ5.31). When processing the data a Pi/3 shifted cosine squared apodization was applied prior to zero filling the acquired data to a total data size of twice the acquired data.

2.2. Biological material and isolation procedure

The isolation and phylogenetic position of strains VUG-A141a and VUG-A42aa within the genus *Hymenobacter* have been

described elsewhere.[14] To generate biomass for carotenoid extraction, both strains were grown on R2A agar plates (Difco) in the dark at 18 °C for 1 week because growth in liquid media is poor or absent.[14] For each strain, biomass harvested from 45 replicate plates was extracted twice using 100 mL of 80:20 methanol–acetone (HPLC grade; Fisher) at 65 °C for 5 min and gravity filtering the extract each time through Whatman #1 filter paper which was finally washed twice with 25 mL 80:20 methanol–acetone. For each sample the resulting filtrate was pooled, dried by rotary evaporation, and dissolved in a minimal volume of methanol. Carotenoids were purified by preparative HPLC as described previously[14] until only one peak was detected by the in-line UV–vis photo diode array (PDA) detector at 478 nm. In-line UV–vis spectroscopy suggested that each collected peak contained nearly entirely the all-*trans* isomer, with *cis*-carotenoids excluded from analysis for simplicity.

3. Spectral data

3.1. 2′-*S*-Hydroxyflexixanthin ((2′*S*)3,1′,2′-trihydroxy-β,ψ-caroten-4-one) (1)

Major carotenoid of *Hymenobacter* str. VUG-A141a; UV–vis (MeOH): λ_{max} 452 (sh), 479, 508 nm (%III/II = 0), see Supplementary data Figure S1; UV–vis ($CHCl_3$): λ_{max} 464 (sh), 492, 520 (sh) (%III/II = 0); CD: see Supplementary data Figure S4; ^{1}H NMR ($CDCl_2$, 800 MHz): see Table 1 and Supplementary data Figures S2 and S3. High resolution MALDI (sodiated, from Klassen and Foght[14]): *m*/*z* 621.39161 ($C_{40}H_{54}O_4Na$).

3.2. 1′-Xylosyl-2′-hydroxyflexixanthin (1′-β-xylosyl-3,2′-dihydroxy-β,ψ-caroten-4-one) (2)

Major carotenoid of *Hymenobacter* str. VUG-A141a; UV–vis (MeOH): λ_{max} 452 (sh), 479, 502.5 nm (%III/II = 5.1), see Supplementary data Figure S1; UV–vis ($CHCl_3$): λ_{max} 464 (sh), 494, 522 (sh) (%III/II = 0); ^{1}H NMR ($CDCl_2$, 800 MHz): see Table 1 and Supplementary data Figures S5 and S6. High resolution MALDI (from Klassen and Foght[14]): *m*/*z* 730.44392 ($C_{45}H_{62}O_8$).

3.3. Hexosyl-2′-hydroxyflexixanthin (hexosyl-3,1′,2′-trihydroxy-β,ψ-caroten-4-one) (3)

Minor carotenoid of *Hymenobacter* str. VUG-A141a, see Klassen and Foght[14] for characterization.

3.4. 2′-Methoxyflexixanthin (2′-methoxy-3,1′-dihydroxy-β,ψ-caroten-4-one) (4)

Major carotenoid of *Hymenobacter* str. VUG-A42aa; UV–vis (MeOH): λ_{max} 453 (sh), 478, 504 nm (%III/II = 9.1), see Supplementary data Figure S1; UV–vis ($CHCl_3$): λ_{max} 464 (sh), 492.5, 522 (sh) (%III/II = 0); ^{1}H NMR ($CDCl_2$, 800 MHz): see Table 1 and Supplementary data Figure S7. The 1D–^{1}H NMR spectrum was unfortunately overwritten during data analysis and is therefore not shown. High resolution MALDI (sodiated, from Klassen and Foght[14]): *m*/*z* 635.40708 ($C_{41}H_{56}O_4Na$).

3.5. 3-Deoxy-2′-methoxyflexixanthin (2′-methoxy-1′-hydroxy-β,ψ-caroten-4-one) (5)

Major carotenoid of *Hymenobacter* str. VUG-A42aa; UV–vis (MeOH): λ_{max} 452 (sh), 479, 508 nm (%III/II = 0), see Supplementary data Figure S1; UV–vis ($CHCl_3$): λ_{max} 464 (sh), 492, 520 (sh) (%III/II = 0); ^{1}H NMR ($CDCl_2$, 800 MHz): see Table 1 and Supplementary data Figures S8 and S9. High resolution MALDI (sodiated, from Klassen and Foght[14]): *m*/*z* 596.42240 ($C_{41}H_{56}O_3$).

3.6. 2′-Methylplectanixanthin (2′-methoxy-1′-hydroxy-β,ψ-carotene) (6)

Minor carotenoid of *Hymenobacter* str. VUG-A42aa, see Klassen and Foght[14] for characterization.

3.7. Plectaniaxanthin (1′,2′-dihydroxyl-β,ψ-carotene) (7)

Minor carotenoid of *Hymenobacter* str. VUG-A141a, see Klassen and Foght[14] for chemical characterization. Isolated as an impure preparation. ^{1}H NMR ($CDCl_2$, 800 MHz): see Supplementary data Figures S10 and S11 and highlighted signals in the text.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.03.117.

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