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4-Ketoantheraxanthin, a novel carotenoid produced by the combination of the bacterial enzyme β-carotene ketolase CrtW and endogenous carotenoid biosynthetic enzymes in higher plants

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Abstract

Higher plants do not ordinarily possess ketocarotenoids due to the absence of a carotenoid ketolase enzyme. We expressed genes coding for marine-bacterial enzymes β-carotene ketolase (CrtW) and β-carotene hydroxylase (CrtZ) in tobacco plants (*Nicotiana tabacum*) by transplastomic engineering. A novel carotenoid, 4-ketoantheraxanthin, was isolated from the leaves of the tobacco transformants. The structure of 4-ketoantheraxanthin was determined to be (3*S*,3′*S*,5′*R*,6′*S*)-5′,6′-epoxy-3,3′-dihydroxy-β,β-caroten-4-one by analysis of the MS, NMR, and CD data. This carotenoid was considered to be synthesized by a 4-ketolation reaction by CrtW of antheraxanthin that had been synthesized by the endogenous carotenoid biosynthetic enzymes present in higher plants and CrtZ. 4-Ketoantheraxanthin was also shown to have potent antioxidative activity against a $^1O_2$ suppression model.
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Carotenoid pigments, which possess colors ranging from light yellow through orange to deep red, are biosynthesized in all photosynthetic bacteria, cyanobacteria, algae, and higher plants, and also in some non-photosynthetic bacteria, yeasts, and fungi. More than 750 different carotenoids have been isolated from natural sources.[1] A number of studies on carotenoids in relation to their health benefits have revealed that each carotenoid has characteristic, individual activities,[2] for example, β-cryptoxanthin has been shown to be associated with a reduction in the risk of lung cancer,[3,4] and lutein and zeaxanthin are thought to protect against the development or progression of age-related macular degeneration and other eye diseases.[5,6] Astaxanthin has been shown to inhibit the oxidation of low-density lipoprotein[7] and have preventative effects against cancer.[8,9] However, the carotenoid species studied so far for this purpose have been restricted to a small number of examples, including the above-mentioned carotenoids. With the exception of those carotenoids that can be isolated from the species of higher plants or synthesized chemically, it has been difficult to find natural sources to supply sufficient amounts of carotenoids. Metabolic engineering (pathway engineering), using a variety of carotenoid biosynthesis genes, should be one of the most powerful methods to generate large quantities of structurally diverse carotenoids. β-Carotene ketolase (CrtW) and β-carotene hydroxylase (CrtZ) are important enzymes that catalyze the conversion of β-carotene to astaxanthin by 4,4′-ketolation

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and 3,3′-hydroxylation reactions, respectively.[10] Recently, we introduced the genes coding for CrtW and CrtZ from a marine bacterium *Brevundimonas* sp. strain SD212[11] into tobacco plants (*Nicotiana tabacum*) by transplastomic engineering (plastid-transformation engineering) and expressed them successfully.[12] The tobacco transformants were able to accumulate large amounts of astaxanthin in the leaves corresponding to 5.44 mg g^{-1} DW (in line ZW-9) or 74% of the total carotenoids. In the present study, we elucidated that these enzymes synthesized a novel carotenoid, 4-ketoantheraxanthin, in a considerable amount and which was also shown to have antioxidative activity. The combination of the foreign bacterial enzyme CrtW and the endogenous carotenoid biosynthesis enzymes for synthesizing antheraxanthin in tobacco plants was able to produce this novel carotenoid.

The freeze-dried leaves of the tobacco (*N. tabacum* L. cv Xanthi) ransformants (line ZW-9) (44.28 g) were ground in a mill. The resulting powder was extracted twice with 1 L each of CH_2Cl_2–MeOH (1:1). The extracts were combined and concentrated to a small volume in vacuo and partitioned with EtOAc/H_2O without adjusting pH. The EtOAc layer was evaporated to dryness and subjected to silica gel chromatography, using *n*-hexane–EtOAc (1:1). The red-colored fractions were collected and concentrated to dryness to give a red oil (150.3 mg). This red oil was subjected to preparative ODS HPLC (Senshu Pak PEGASIL ODS column, 20 × 250 mm), and separated with CH_3CN–CH_2Cl_2 (8:2) as a solvent (flow rate 8.0 ml/min). Three red components could be separated by this chromatography. The main red component [eluted at 11.0 min, 15.9 mg] was identified as (3*S*,3′*S*)-astaxanthin[13] by direct comparison with an authentic sample. The red compound eluted at 12.1 min (6.7 mg) was identified as fritschiellaxanthin[14] from ^{1}H NMR and HRESI-MS spectral data. The red compound eluted at 9.8 min (6.5 mg) was further purified by preparative silica gel HPLC (YMC-Pack SIL column, 20 × 250 mm) using hexane–acetone (7:3) to give a pure compound (**1**, 3.2 mg).[15]

Compound **1** was dissolved in CH_2Cl_2 and analyzed by HRESI-MS. The intense $(M+Na)^+$ peak was observed at *m*/*z* 621.39235 ($C_{40}H_{54}O_4Na$, calcd for 621.39198) in the positive-ion mode. Thus, the molecular formula of **1** was deduced to be $C_{40}H_{54}O_4$.

The ^{1}H NMR data for **1** in $CDCl_3$ showed 10 singlet methyls, 3 sp^3 methylenes, 2 sp^3 methines, and 14 sp^2 methines. The ^{13}C NMR and DEPT experiments revealed 10 methyls, 3 sp^3 methylenes, 2 sp^3 methines, 14 sp^2 methines, 4 sp^3 quaternary carbons, and 6 sp^2 quaternary carbons. The sp^3 quaternary carbons observed at δ 67.0 and δ 70.3 were estimated to be epoxide carbons, and the sp^2 quaternary carbon observed at δ 200.4 was judged to be a ketone carbon. The complete assignments of direct ^{1}H–^{13}C connections were established by an HMQC experiment.

Further structural analyses of **1** were performed by ^{1}H–^{1}H COSY and HMBC experiments. The ^{1}H–^{1}H vicinal spin network of H-2 (δ 1.83 and δ 2.15)–H-3 (δ 4.32) and the long range couplings from H-16 (δ 1.21) and H-17 (δ 1.32) to C-1 (δ 36.8), C-2 (δ 45.4), and C-6 (δ 162.3) and from H-18 (δ 1.95) to C-6, C-5 (δ 126.8), and C-4 (δ 200.4) revealed a 3-hydroxy-4-keto β-end group in **1** (Fig. 2). The ^{1}H–^{1}H vicinal spin network of H-2′ (δ 1.23 and δ 1.66)–H-3′ (δ 3.91)–H-4′ (δ 1.66 and δ 2.37) and the long range couplings from H-16′ (δ 0.98) and H-17′ (δ 1.15) to C-1′ (δ 35.4), C-2′ (δ 47.2), and C-6 (δ 70.3) and from H-18′ (δ 1.19) to C-6′, C-5′ (δ 67.0), and C-4′ (δ 41.0) also revealed a 3-hydroxy-5,6-epoxy-5,6-dihydro β-end group in **1** (Fig. 2). The structure of the polyene moiety (C-7–C-7′) was established by the ^{1}H–^{1}H vicinal spin networks and ^{1}H–^{13}C long range couplings from the singlet methyls (Fig. 2). The linkages of the polyene moiety to both end groups were established by the ^{1}H–^{13}C long range couplings from H-8 (δ 6.43) to C-6 and from H-8′ (δ 6.29) to C-6′ (Fig. 2). Thus, the total structure of **1** was clarified.

The relative stereochemistries of both end groups were determined by the comparison of the ^{1}H and ^{13}C NMR data with those of astaxanthin[16] and violaxanthin[16] as shown in Figure 1. The double bonds in the polyene structure ($\Delta^{7,8}$, $\Delta^{9,10}$, $\Delta^{11,12}$, $\Delta^{13,14}$, $\Delta^{7',8'}$, $\Delta^{9',10'}$, $\Delta^{11',12'}$, $\Delta^{13',14'}$, and $\Delta^{15,15'}$) were determined to be all *E* configuration by the J_{HH} values and ^{13}C chemical shifts of the singlet methyls (Fig. 2).

The absolute configurations of **1** were analyzed by CD spectrum. The CD spectrum showed almost the same Cotton effect as that of antheraxanthin[17] except for the longer wavelength shift (20 nm), which was attributed to introducing the carbonyl group at C-4 in antheraxanthin. Thus, the 3*S*,3′*S*,5′*R*,6′*S* configurations in **1** were confirmed.

All the above observations allowed the structure of **1** to be determined as that shown in Figure 1. The IUPAC-IUB semisystematic name of **1** is (3*S*,3′*S*,5′*R*,6′*S*)-5′,6′-epoxy-3,3′-dihydroxy-β,β-caroten-4-one.

Since the antioxidative activities of some carotenoids have been reported previously,[18] the in vitro inhibitory effect of **1** on linoleic acid oxidation by methyleneblue[19]

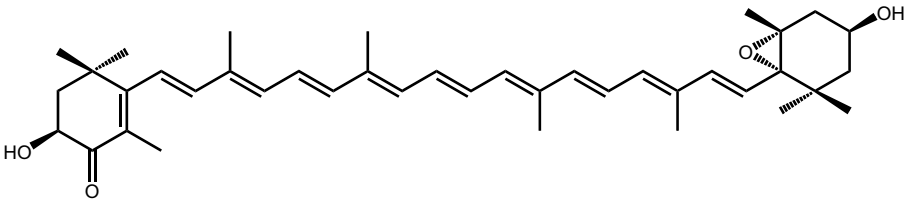

Fig. 1. Structure of 4-ketoantheraxanthin.

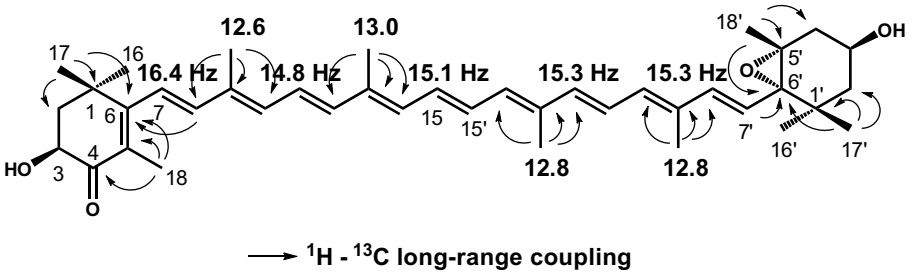

Fig. 2. Key ^{1}H–^{13}C long range couplings, $^{3}J_{HH}$ values, and δ_C values observed in the NMR analyses of 4-ketoantheraxanthin.

was examined. The IC_{50} value of **1** was 50.8 μM (>100 μM for β-carotene, 20.9 μM for astaxanthin, and 48.6 μM for fritschiellaxanthin) indicating that **1** possessed potent antioxidative activity. The introduction of the keto function at C-4 in the β-end group was reported to enhance the antioxidative activity.[20] Therefore, the production of novel carotenoids using CrtW may be a good approach to synthesize antioxidative carotenoids.

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15. *Physical data for compound* **1**: UV–vis (MeOH) λ_{max}: 447.3 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.98 (s, 3H, H-16′), 1.15 (s, 3H, H-17′), 1.19 (s, 3H, H-18′), 1.21 (s, 3H, H-17), 1.23 (dd, 1H, $J = 13.0$, 14.0 Hz, H-2′ax), 1.32 (s, 3H, H-16), 1.66 (ddd, 1H, $J = 1.5$, 3.5, 14.0 Hz, H-2′eq), 1.66 (dd, 1H, $J = 8.5$, 14.0 Hz, H-4′ax), 1.83 (dd, 1H, J=12.8, 13.8 Hz, H-2ax), 1.93 (s, 3H, H-19′), 1.95 (s, 3H, H-18), 1.97 (s, 3H, H-20′), 1.98 (s, 3H, H-20), 2.00 (s, 3H, H-19), 2.15 (dd, 1H, $J = 5.8$, 12.8 Hz, H-2eq), 2.37 (ddd, 1H, $J = 1.5$, 4.7, 14.1 Hz, H-4′eq), 3.91 (m, 1H, H-3′), 4.32 (dd, 1H, $J = 5.8$, 13.8 Hz, H-3), 5.89 (d, 1H, $J = 15.3$ Hz, H-7′), 6.21 (d, 1H, $J = 10.7$ Hz, H-10′), 6.21 (d, 1H, $J = 16.4$ Hz, H-7), 6.29 (d, $J = 15.3$ Hz, H-8′), 6.30 (d, 1H, $J =$ 11.8 Hz, H-14), 6.30 (d, 1H, $J = 11.8$ Hz, H-10), 6.30 (d, 1H, $J = 11.8$ Hz, H-14′), 6.38 (d, 1H, $J = 15.3$ Hz, H-12′), 6.43 (d, 1H, $J = 16.4$ Hz, H-8), 6.45 (d, 1H, $J = 14.8$ Hz, H-12), 6.62 (dd, 1H, $J = 10.7$, 15.3 Hz, H-11′), 6.65 (m, 1H, H-15′), 6.66 (dd, 1H, $J = 11.8$, 14.8 Hz, H-11), 6.67 (m, 1H, H-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 12.6 (C-19), 12.8 (C-19′), 12.8 (C-20′), 13.0 (C-20), 14.0 (C-18), 20.0 (C-18′), 24.9 (C-16′), 26.2 (C-16), 29.8 (C-17′), 30.7 (C-17), 35.4 (C-1′), 36.8 (C-1), 41.0 (C-4′), 45.4 (C-2), 47.2 (C-2′), 64.3 (C-3′), 67.0 (C-5′), 69.2 (C-3), 70.3 (C-6′), 123.2 (C-7), 124.0 (C-7′), 124.3 (C-11′), 125.0 (C-11), 126.8 (C-5), 130.0 (C-15′), 130.9 (C-15), 132.2 (C-14′), 132.7 (C-10′), 134.0 (C-14), 134.4 (C-9′), 134.5 (C-9), 135.3 (C-10), 136.2 (C-13′), 136.9 (C-13), 137.3 (C-8′), 138.1 (C-12′), 139.9 (C-12), 142.4 (C-8), 162.3 (C-6), 200.4 (C-4); HRESI-MS m/z 621.38817 [$(M+Na)^+$, $C_{40}H_{54}O_4Na$, calcd for 621.39198]; CD (Et_2O) λ nm ($\Delta\varepsilon$): 227 (−5.5), 240 (0), 255 (+7.0), 296 (−18.0), 330 (0), 358 (+2.0).
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