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ORIGINAL ARTICLE

Hydroxy-3,4-dehydro-apo-8′-lycopene and methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate, novel C_{30} carotenoids produced by a mutant of marine bacterium *Halobacillus halophilus*

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We performed the chemical mutagenesis of *Halobacillus halophilus* (the producer of a C_{30} carotenoid, methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate) to isolate novel carotenoids that are biosynthetic intermediates of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate. As a result, we isolated two novel C_{30} carotenoids, hydroxy-3,4-dehydro-apo-8′-lycopene and methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate, which were biosynthesized through a novel 8′-apo C_{30} pathway. These carotenoids showed antioxidative activity in the $^1O_2$ suppression model.
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Keywords: antioxidative activity; chemical mutagenesis; *Halobacillus halophilus*; hydroxy-3,4-dehydro-apo-8′-lycopene; methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate

INTRODUCTION

Some species of bacteria, yeasts and fungi, as well as algae and higher plants, can synthesize a large number of carotenoids, and some marine bacteria can produce dicyclic, monocyclic or acyclic carotenoids.[1,2] More than 750 carotenoids with different molecular structures have been isolated from natural sources.[2] Evaluating the pharmaceutical potential of such various carotenoid pigments could be an exciting field of medical research; however, the carotenoid species so far studied for this purpose have been restricted to a small number, including dicyclic carotenoids such as β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin, astaxanthin and fucoxanthin, and an acyclic carotenoid, lycopene.[3–6] With the exception of those carotenoids that can be isolated from a species of higher plants or be chemically synthesized, it has been difficult to find natural sources that can supply sufficient amounts of these rare carotenoids.

In previous studies, we have performed the analysis of novel or rare types of carotenoids from new colored marine bacteria, which were classified as belonging to a new species by 16S rRNA analysis.[7–9] These studies have reported the isolation of rare carotenoids, (*3R*)-saproxanthin and myxol, from Flavobacteriaceae,[7] and novel glyco-C_{30} carotenoids (diapolycopenedioic acid xylosyl esters A, B, and C from *Rubritalea squalenifaciens*[8] and methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate from *Planococcus maritimus*[9]).

We found that a moderately halophilic bacterium *Halobacillus halophilus* (formerly called *Sporosarcina halophila*) produced methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate, which is the same C_{30} carotenoid recently isolated from *P. maritimus*.[9] Because methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate has a unique structure and antioxidative activity, we searched for its biosynthetic intermediates to investigate the biosynthetic pathway and to find novel antioxidative structures. For this study, we performed chemical mutagenesis with *H. halophilus*, and obtained mutant OC1, which shows a light orange phenotype (parent strain: deep orange). From OC1, we isolated two novel carotenoids, hydroxy-3,4-dehydro-apo-8′-lycopene and methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate. In this report, we describe the isolation, structural determination and the antioxidative ($^1O_2$ suppressive) activity of these compounds.

RESULTS

Culture of the bacteria and isolation of pigments

OC1 was inoculated into 100 ml of the seed medium (Marine Broth 2216; Difco, Becton-Dickinson, Heidelberg, Germany) in a 500 ml Erlenmeyer flask, and cultured at 30 °C for 24 h on a rotary shaker (150 r.p.m.). Two milliliters of the seed culture was inoculated into 100 ml portions of the production medium (=seed medium), and cultivation was carried out at 30 °C for 24 h on a rotary shaker (150 r.p.m.). The OD_{490} of the culture reached 4.0 at the end of fermentation.

OC1 cells were isolated from 16 l of culture by centrifugation at 13 000 *g*. After removing the supernatant, we suspended the

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cells in 1.0 M NaOH at 40 °C for 30 min and sonicated them for 5 min to digest the cell wall. After removing the NaOH solution by centrifugation at 13 000 *g*, we extracted the orange pigment in the digested cells three times with dichloromethane (CH_2Cl_2)–MeOH (1:1). The extracts were combined, concentrated to a small volume *in vacuo* and partitioned between ethyl acetate (EtOAc)/H_2O without adjusting pH. The EtOAc layer was evaporated to dryness (307.1 mg) and subjected to silica gel chromatography (Silica Gel 60; KANTO CHEMICAL, Tokyo, Japan) using n-hexane–EtOAc (3:1). In this chromatography, two orange fractions (fraction I and II) were obtained, collected and concentrated to dryness to give an orange oil (fraction I, 5.9 mg) and an orange powder (fraction II, 1.8 mg). The orange oil (fraction I) was washed with n-hexane, and the precipitate was subjected to preparative silica gel HPLC (Senshu Pak SIL column, 10×250 mm; Senshu Scientific, Tokyo, Japan) and developed with n-hexane–acetone (7:2) as a solvent (flow rate 3 ml min^{-1}). The light orange component (retention time (Rt) 8.28 min) was collected and concentrated to give pure hydroxy-3,4-dehydro-apo-8′-lycopene (**1**, 1.5 mg). The orange powder (fraction II) was also washed with n-hexane and the precipitant was subjected to preparative silica gel HPLC (Senshu Pak SIL column, 10×250 mm) and developed with n-hexane–acetone (3:1) as a solvent (flow rate 3 ml min^{-1}). The deep orange component (Rt 11.2 min) was collected and concentrated to give pure methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate (**2**, 0.3 mg).

Structural elucidation

Compound **2** was dissolved in CH_2Cl_2 and analyzed by positive ion high-resolution electrospray ionization mass spectrometry. The

Table 1 ^{1}H and ^{13}C NMR data for hydroxy-3,4-dehydro-apo-8′-lycopene (1), methyl hydroxyl-3,4-dehydro-apo-8′-lycopenoate (2) and methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate tetraacetate[9] in $CDCl_3$

	1		*2*		*Methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate tetraacetate*	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		71.0 (C)		71.0 (C)		78.8 (C)
2	2.31 (2H, d, 7.4)	47.5 (CH_2)	2.32 (2H, d, 7.4)	47.5 (CH_2)	2.24 (1H, dd, 7.9, 13.8)	45.9 (CH_2)
					2.42 (1H, dd, 7.4, 13.8)	
3	5.76 (1H, ddd, 7.4, 7.4, 15.7)	124.5 (CH)	5.78 (1H, ddd, 7.4, 7.4, 15.7)	124.6 (CH)	5.72 (1H, ddd, 7.4, 7.9, 15.1)	125.2 (CH)
4	6.21 (1H, d, 15.7)	138.8 (CH)	6.36 (1H, d, 15.2)	138.8 (CH)	6.13 (1H, d, 15.1)	137.9 (CH)
5		135.0 (C)		135.1 (C)		135.5 (C)
6	6.14 (1H, d, 11.4)	131.4 (CH)	6.14 (1H, d, 10.9)	131.2 (CH)	6.08 (1H, d, 11.3)	130.9 (CH)
7	6.61 (1H, dd, 11.4, 15.2)	124.9 (CH)	6.64 (1H, dd, 10.9, 15.2)	125.1 (CH)	6.63 (1H, dd, 11.3, 14.4)	125.0 (CH)
8	6.35 (1H, d, 15.2)	137.8 (CH)	6.36 (1H, d, 15.2)	137.6 (CH)	6.35 (1H, d, 14.4)	137.4 (CH)
9		136.2 (C)		136.3 (C)		136.2 (C)
10	6.28 (1H, d, 10.3)	132.5 (CH)	6.25 (1H, d, 10.3)	132.5 (CH)	6.26 (1H, d, 9.7)	132.5 (CH)
11	6.63 (1H, dd, 10.3, 15.2)	124.9 (CH)	6.64 (1H, dd, 10.3, 15.2)	124.7 (CH)	6.65 (1H, dd, 9.7, 15.2)	124.6 (CH)
12	6.35 (1H, d, 15.2)	137.3 (CH)	6.35 (1H, d, 15.2)	139.1 (CH)	6.49 (1H, d, 15.2)	139.9 (CH)
13		136.3 (C)		137.0 (C)		137.0 (C)
14	6.28 (1H, d, 10.3)	132.7 (CH)	6.29 (1H, d, 9.8)	134.1 (CH)	6.28 (1H, d, 9.7)	134.1 (CH)
15	6.63 (1H, m)	130.0 (CH)	6.63 (1H, m)	130.8 (CH)	6.63 (1H, dd, 9.7, 15.0)	130.8 (CH)
16	1.24 (3H, s)	29.2 (CH_3)	1.26 (3H, s)	29.5 (CH_3)	1.17 (3H, s)	26.6 (CH_3)
17	1.24 (3H, s)	29.2 (CH_3)	1.26 (3H, s)	29.5 (CH_3)	1.19 (3H, s)	25.6 (CH_3)
18	1.93 (3H, s)	13.0 (CH_3)	1.94 (3H, s)	13.0 (CH_3)	1.91 (3H, s)	13.0 (CH_3)
19	1.97 (3H, s)	12.8 (CH_3)	1.98 (3H, s)	12.8 (CH_3)	1.98 (3H, s)	12.8 (CH_3)
20	1.97 (3H, s)	12.8 (CH_3)	1.98 (3H, s)	12.8 (CH_3)	2.01 (3H, s)	12.8 (CH_3)
8′	1.82 (3H, s)	26.3 (CH_3)		169.0 (C)		169.1 (C)
9′		135.8 (C)		125.1 (C)		125.7 (C)
10′	5.94 (1H, d, 10.8)	126.1 (CH)	7.30 (1H, d, 12.3)	139.1 (CH)	7.30 (1H, d, 11.3)	139.1 (CH)
11′	6.47 (1H, dd, 10.8, 15.9)	124.7 (CH)	6.50 (1H, dd, 12.3, 15.0)	122.9 (CH)	6.51 (1H, dd, 11.3, 15.1)	122.9 (CH)
12′	6.21 (1H, d, 15.9)	135.0 (CH)	6.66 (1H, d, 15.0)	144.2 (CH)	6.65 (1H, d, 15.1)	144.2 (CH)
13′		136.6 (C)		135.2 (C)		135.1 (C)
14′	6.18 (1H, d, 12.0)	131.4 (CH)	6.32 (1H, d, 11.9)	136.3 (CH)	6.35 (1H, d, 11.4)	136.0 (CH)
15′	6.63 (1H, m)	130.2 (CH)	6.63 (1H, m)	129.9 (CH)	6.63 (1H, dd, 11.4, 15.0)	129.9 (CH)
19′	1.82 (3H, s)	18.6 (CH_3)	2.00 (3H, s)	12.8 (CH_3)	2.01 (3H, s)	12.8 (CH_3)
20′	1.97 (3H, s)	13.0 (CH_3)	2.00 (3H, s)	12.8 (CH_3)	2.00 (3H, s)	12.8 (CH_3)
COOCH3			3.77 (3H, s)	51.8 (CH_3)	3.77 (3H, s)	51.7 (CH_3)
1″					4.69 (1H, d, 7.8)	93.4 (CH)
2″					4.98 (1H, dd, 7.8, 9.5)	71.6 (CH)
3″					5.24 (1H, dd, 9.0, 9.5)	73.1 (CH)
4″					5.04 (1H, dd, 9.0, 10.0)	68.9 (CH)
5″					3.68 (1H, ddd, 1.5, 5.8, 10.0)	71.6 (CH)
6″					4.10 (1H, dd, 1.5, 12.1)	62.5 (CH_2)
					4.22 (1H, dd, 5.8, 12.1)	

^{13}C signals of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate tetraacetate were observed at δ 20.6–20.7 (CH_3) and δ 169.0–170.6 (C=O).

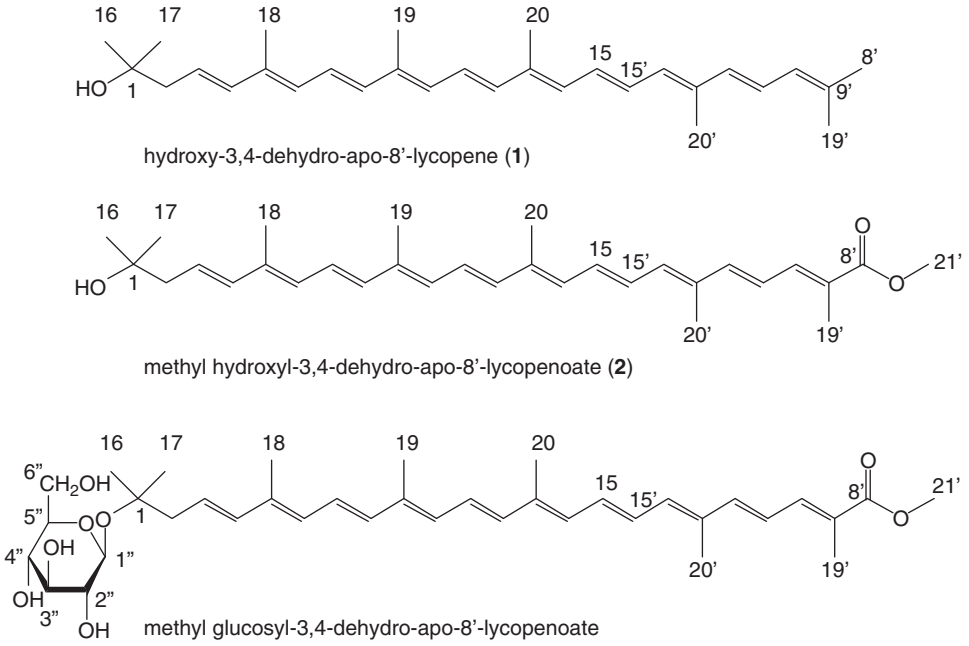

Figure 1 The structure of carotenoids **1** and **2** produced by mutant OC1 (a mutant of *Halobacillus halophilus*), and methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate produced by *H. halophilus*.

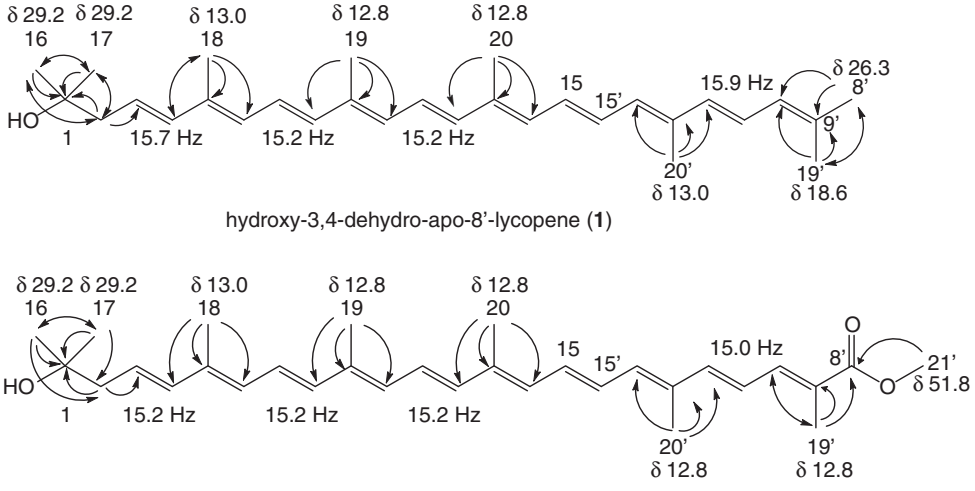

Figure 2 Key ^{1}H-^{13}C long-range couplings, *J* values and δ_C values observed in the NMR analyses of compounds **1** and **2**.

$(M+Na)^+$ peak was observed at *m/z* 485.30331 (calcd: 485.30316), and the molecular formula of **2** was determined to be $C_{31}H_{42}O_3$.

The ^{1}H NMR spectrum for **2** in $CDCl_3$ showed 8 methyl (Me) singlets, 1 sp^3 methylenes and 15 sp^2 methines (Table 1), and the ^{13}C NMR spectrum for **2** revealed 8 Me, 1 sp^3 methylenes, 15 sp^2 methines, 1 sp^3 quanternary carbons and 6 sp^2 quaternary carbons (Table 1). These spectra were closely similar to that of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate tetraacetate (Table 1). Although the signals due to glucose in methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate were not observed in **2**. Because the molecular formula of **2** was methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate—$C_6H_{11}O_5$, it was proposed to be a deglucosyl derivative of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate. The difference of the ^{13}C chemical shift in C-1 (δ_C 71.0 in **2** and δ_C 78.8 in methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate tetraacetate) supported this structure. Thus, the structure of **2** was determined to be methyl 1-hydroxy-3,4-didehydro-1,2-dihydro-apo-8′-lycopenoate (Figure 1). The key ^{1}H-^{13}C long-range couplings, *J* values and δ_C values observed in the NMR analyses of **2** are shown in Figure 2.

Compound **1** was dissolved in CH_2Cl_2 and analyzed by positive-ion high-resolution fast atom bombardment mass spectrometry. The M^+ peak was observed at *m/z* 418.3242 (calcd: 418.3236), and the molecular formula of **1** was determined to be $C_{30}H_{42}O$.

The ^{1}H NMR spectrum of **1** in $CDCl_3$ showed 8 Me singlets, 1 sp^3 methylenes and 15 sp^2 methines, and the ^{13}C NMR spectrum of **1** revealed 8 Me, 1 sp^3 methylenes, 15 sp^2 methines, 1 sp^3 quanternary carbons and 5 sp^2 quaternary carbons (Table 1). These NMR spectra

were quite similar to those of **2**, whereas the signals of a carbonyl carbon (C-8′ (δ_C 169.0)) and methoxy signals (H-21′ (δ_H 3.77) and C-21′ (δ_C 51.8)) were not observed in **1**, and one singlet Me signal (H-8′ (δ_H 1.82) and C-8′ (δ_C 26.3)) was observed only in **1**. Considering these observations and the molecular formula of **1** (**2**— CO_2), **1** was proposed to be C-8′ deoxygenated derivative of **2**. This was confirmed by the observation of ^1H-^{13}C long-range coupling from H-8′ and H-19′ (δ_H 1.82) to C-9′ (δ_C 135.8) and C-10′ (δ_C 126.1) in the heteronuclear multiple bond correlation experiment of **1** (Figure 2). Thus, the structure of **1** was determined to be 1-hydroxy-3,4-didehydro-1,2-dihydro-apo-8′-lycopene (Figure 1). The key ^1H-^{13}C long-range couplings, *J* values and δ_C values observed in the NMR analyses of **1** are shown in Figure 2.

Table 2 Physicochemical properties of hydroxy-3,4-dehydro-apo-8′-lycopene (1) and methyl hydroxyl-3,4-dehydro-apo-8′-lycopenoate (2)

	1	*2*
Appearance	Light orange powder	Deep orange powder
Molecular formula	$C_{30}H_{42}O$	$C_{31}H_{42}O_3$
HR-MS (*m/z*)	FAB-MS	ESI-MS
Found	418.3242 M^+	485.30331 $(M+Na)^+$
Calcd	418.3236	485.30316
UV δ_{max}^{MeOH}	277, 426, 450, 480	287, 437, 465, 492
	% III/II=72	% III/II=16
Rf value[a]	0.42	0.25

[a]Silica gel TLC (Merck Art. 1.05715, n-hexane–EtOAc=4:1).

The IUPAC-IUB semisystematic names of **1** and **2** are 1-hydroxy-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten, and methyl 1-hydroxy-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten-8′-oate, respectively, which are both novel carotenoids. The physicochemical properties of **1** and **2** are summarized in Table 2.

Antioxidative activity

We examined the $^1O_2$ suppression activity of **1** and **2**, and the IC_{50} values were 79 and 44 μM, respectively. We also examined the $^1O_2$ suppression activities of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate, astaxanthin and β-carotene. Their IC_{50} values were 5.1, 8.9 and >100 μM, respectively.

DISCUSSION

In this study, we found two novel carotenoids, hydroxy-3,4-dehydro-apo-8′-lycopene (**1**) and methyl hydroxy-3,4-dehydro-apo-8′-lycopenoate (**2**) in a pigment mutant OC1 that was obtained by mutagenesis of *H. halophilus*. The parent strain, the carotenoid gene cluster of which was recently elucidated,[10] produces methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate, whereas both **1** and **2** were deglucosylated carotenoids; therefore, OC1 should have a mutation in a gene encoding a glucosylase, which is responsible for the glucosylation of **1**. The prominent candidate gene for glucosidation is orf-AT in the carotenogenic gene cluster of *H. halophilus*.[10] Because **1** was the major carotenoid in OC1, it determines its light orange color.

It should be pointed out that the identified intermediates **1** and **2** as well as methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate are 8′-apo derivatives with asymmetrical arrangement of the Me groups (Figure 3), unlike the 4,4′-diapo derivatives, which are typically

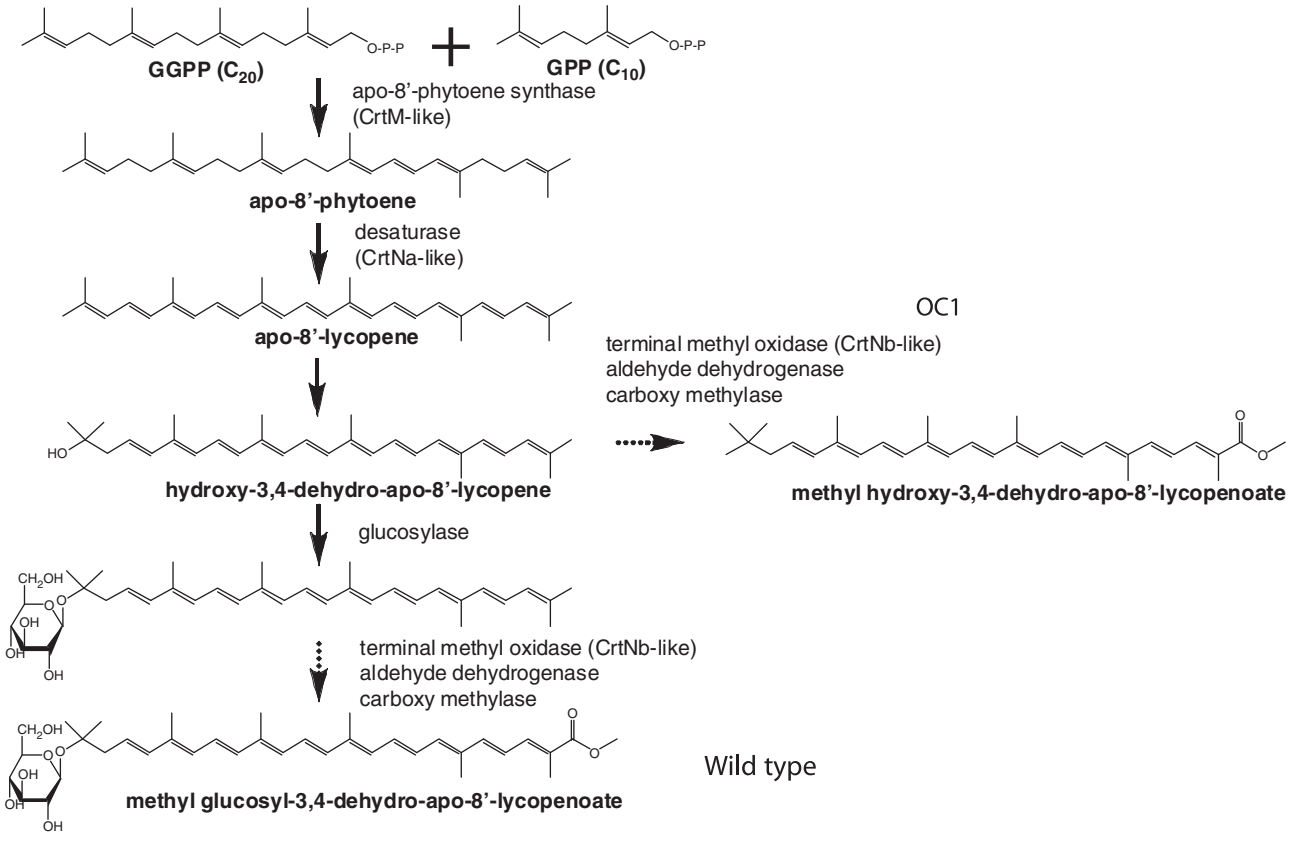

Figure 3 Proposed biosynthetic pathway of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate in *Halobacillus halophilus* DSM 2266.

synthesized with two molecules of farnesyl diphosphate.[10,11] In addition, the identification of intermediate **1** without terminal end-group oxidation excludes the possibility that apo-8′ products are derived by cleavage of C_{40} carotenoids at position 8′. Therefore, we propose that the C_{30} pathway in *H. halophilus* initially starts with apo-8′ phytoene by condensation of C_{20} geranylgeranyl pyrophosphate (GGPP) with C_{10} geranyl pyrophosphate (GPP). This postulated novel apo-8′ C_{30} biosynthetic pathway is outlined in Figure 3.

In the later biosynthesis steps toward methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate, glucosylated **1** seems to be oxidized to the corresponding aldehyde in the terminal Me group with CrtNb-like enzyme, as described by Tao *et al*,[11] and then oxidized to the corresponding carboxylic acid with another oxidase, which may be encoded by gene CrtNc.[10] Due to the ratio of about 5:1 between **1** and **2**, it can be assumed that in the parent strain, compound **1** is the preferred substrate for the glucosylase and that terminal oxidation to a carboxylic group typically proceeds with glucosyl-3,4-dehydro-apo-8′-lycopene as the original substrate for the CrtNb-like enzyme (Figure 3).

Both **1** and **2** possessed $^1O_2$ suppression (antioxidative) activity (IC_{50} 79 and 44 μM, respectively), but their activities were not so potent as methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (IC_{50} 5.1 μM). This may be explained by the lower hydrophobicity of **1** and **2**, as reported by Shimidzu *et al*.[12] Shimidzu *et al*.[12] also reported that the increasing number of C=C or C=O double bonds conjugated enhanced $^1O_2$ suppression activity. Compound **2** may possess more potent $^1O_2$ suppression activity than **1** for this reason. Further examinations of the antioxidative activities of these carotenoids are in progress.

EXPERIMENTAL SECTION

Taxonomy of the strain

H. halophilus is a moderately halophilic bacterium. Taxonomic analysis of this bacterium was reported previously.[13] For our studies, we used the type strain DSM 2266 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Random mutagenesis

Mutagenesis of *H. halophilus* was carried out with cells resuspended in minimal G10 medium (glucose 10 g, NH_4Cl 2 g, $FeSO_4{\cdot}7H_2O$ 10 mg, Tris base 12 g, K_2HPO_4 0.49 g, yeast extract 0.1 g, DSM 141 vitamin solution 1 ml and DSM 79 artificial seawater 250 ml in 750 ml tap water) in the presence of 1.0 M NaCl. To 2 ml of the cell suspension, we added and mixed 20 μl ethyl methane sulfonate (Sigma-Aldrich, Steinheim, Germany) vigorously. After 20 min of incubation at 30 °C, we washed cells twice in G10 medium, diluted 50-fold and grew them in broth (Marine Broth 2216; Difco) overnight. Different dilutions were plated on nonselective agar plates. Colonies were visually screened for white and yellow/orange pigmentation differing from the deep orange color of the parent strain. For further study, we selected a light orange colony named OC1.

Spectroscopic analysis

The ^1H and ^{13}C NMR spectra were measured at 400 MHz with a Bruker AMX400 (Bruker BioSpin, Rheinstetten, Germany). High-resolution electrospray ionization mass spectrometry and high-resolution fast atom bombardment mass spectrometry were recorded with a JEOL JMS-T100LP and a JEOL JMS-HX100A mass spectrometer (JEOL, Tokyo, Japan), respectively. UV spectrum was recorded with a Hitachi U-3200 spectrophotometer (Hitachi, Tokyo, Japan).

$^1O_2$ suppression experiment

$^1O_2$ quenching activity was examined by measuring methylene blue-sensitized photooxidation of linoleic acid.[14] Forty microliters of 0.05 mM methylene blue, 10 μl of 2.4 M linoleic acid with or without 40 μl carotenoid (final concentration 1–100 μM, each dissolved in EtOH) were added to micro-glass vials (5.0 ml). The vials were tightly closed with a screw cap and a septum, and the mixtures were illuminated at 7000 lux at 22 °C for 3 h in corrugated cardboard. Then, 50 μl of the reaction mixture was removed and diluted to 1.5 ml with EtOH, and absorbance at 235 nm was measured to estimate the formation of conjugates dienes.[15] The value in the absence of carotenoid was determined and $^1O_2$ repression activity was calculated relative to this reference value. Activity is indicated as the IC_{50} value representing the concentration at which 50% inhibition was observed.

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