NOTE

Isolation and Identification of 4, 4'-Diapolycopene-4,4'-dioic Acid Produced by *Bacillus firmus* GB1 and its Singlet Oxygen Quenching Activity

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Abstract: We succeeded in purifying the carotenoid produced by *Bacillus firmus* GB1 using chromatography on polystyrenic synthetic adsorbents, and identified it as 4,4'-diapolycopene-4,4'-dioic acid by HRESI-MS and NMR analyses of it and its dimethylester. In addition, we clarified the singlet oxygen (¹O₂) quenching activities of 4,4'-diapolycopene-4,4'-dioic acid and its methyl esters (mono and dimethyl ester). Their IC₅₀ values were 5.8 μM, 6.0 μM, and 6.2 μM, respectively. To our knowledge, this is the first report concerning the isolation and antioxidative activity of 4,4'-diapolycopene-4,4'-dioic acid, a product of the C30 carotenoid biosynthesis pathway.

Key words: 4,4'-diapolycopene-4,4'-dioic acid, *Bacillus firmus* GB1, polystyrenic synthetic adsorbent, ¹O₂ quenching activity

1 INTRODUCTION

Carotenoids are terpenoids possessing a chain of 30, 40 or 50 carbons. More than 750 carotenoids with different molecular structures have been isolated from natural sources¹⁾. Evaluating the pharmaceutical potential of various carotenoid pigments could be an exciting field of medical research; however, the carotenoid species so far studied for medical research 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, lycopene²⁻⁵⁾. Thus, we have started to screen for novel or rare antioxidative carotenoids from pigmented rare bacteria.

Bacillus firmus GB1 is a microorganism isolated from the human ileum and identified to be a novel *Bacillus* species by 16S rRNA analyses⁶⁾. *B. firmus* GB1 produce a deep pink pigment, which was speculated to be a C30 carotenoid due to its characteristic UV-Vis absorption and the biosynthetic genes found in *B. firmus* GB1⁷⁾, however the isolation and detailed structural analyses by nuclear magnetic resonance (NMR) of this pigment were not successful in previous studies due to its inconsistent retention behav-

iors on silica gel and ODS HPLC. In this study, it was found that this pigment gave a sharp elution peak on polystyrenic synthetic adsorbents (HP20SS and CHP20/C10) and it could be purified through an isolation scheme including these resins. It was also identified as 4,4'-diapolycopene dioic acid by high resolution electrospray ionization mass spectrometry (HRESI-MS) and NMR analyses of it and its dimethyl ester. Although the production of 4,4'-diapolycopene-4,4'-dioic acid by bacteria has been reported in previous studies⁸⁾, 4,4'-diapolycopene-4,4'-dioic acid itself has not been isolated before as a free acid, therefore, this is the first report on the isolation of 4,4'-diapolycopene-4,4'-dioic acid without modification from the producing bacteria. In addition, we also report the complete ¹H and ¹³C NMR assignments of 4,4'-diapolycopene-4,4'-dioic acid and its methyl esters (monomethyl ester and dimethyl ester) for the first time.

We investigated the singlet oxygen (¹O₂) quenching activities of 4,4'-diapolycopene-4,4'-dioic acid and its methyl esters (monomethyl ester and dimethyl ester), since potent ¹O₂ quenching activities were reported on some carotenoids^{9, 10)}. The tested carotenoids showed potent antioxidative activities.

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2 EXPERIMENTAL

2.1 Cultivation

The -80°C frozen culture of *B. firmus* GB1 (1 mL) was inoculated into 200 mL LB medium (tryptone 10.0 g, yeast extract 5.0 g, and NaCl 7.0 g in 1L) in a 500 mL Erlenmeyer flask and cultured at 30°C for 48 h on a rotary shaker (150 rpm). Each 2 mL of the seed culture was inoculated into 200 mL LB medium in a 500 mL Erlenmeyer flask and cultured at 30°C for 48 h on a rotary shaker (150 rpm). The OD_{490} was 9.3 after the 48 h cultivation.

2.2 HPLC

HPLC was carried out using a Hitachi L-7100 intelligent pump and L-7400 DAD detector. The maximum absorbance was measured in the range of 200–600 nm.

2.3 Spectroscopic analysis

^1H , ^{13}C , and 2D NMR spectra were measured with a Bruker AVANCE400. Chemical shifts were referenced to tetramethylsilane. Data processing was performed using Top Spin-NMR software (version 3.0) (Bruker BioSpin). HRESI-MS and high resolution atmospheric pressure chemical ionization mass spectrometer (HRAPCI-MS) was recorded with a JEOL JMS-T100LP mass spectrometer. UV-VIS spectra were recorded with a Hitachi U-3200.

2.4 Isolation of 4,4'-diapolycopene-4,4'-dioic acid

B. firmus GB1 cells from 10 L culture were collected by centrifugation at 13,000 g. After removing the supernatant, the deep pink pigment in the cells was extracted 6 times with MeOH (50 mL) by sonication for 5 min. The extracts were combined (300 mL) and added *n*-hexane (300 mL) to partition between *n*-hexane/MeOH. The MeOH (lower) layer was concentrated to a small volume, and partitioned between *t*-butyl methyl ether-MeOH (500 mL: 100 mL)/ H_2O (600 mL) without adjusting the pH. The *t*-butyl methyl ether-MeOH (upper) layer was evaporated to dryness to give a brown oil (474.2 mg). This oil was dissolved in *n*-hexane-EtOH (1: 3) 0.5 mL and applied to HP20SS (Mitsubishi Chemical Co.) column chromatography [20 $\times$ 300 mm, solvent: *n*-hexane-EtOH (1: 3)], and developed with the same solvent.

The deep pink fraction was collected and concentrated to dryness to give a deep pink oil (98.5 mg). This oil was subjected to preparative ODS HPLC (Develosil C30-UG-5, 10 $\times$ 250 mm, Nomura Chemical Co.) and developed at a flow rate of 3 mL/min with solvent A [*t*-butyl methyl ether-MeOH (1:9)] for 5 min, then by linear gradient from solvent A to solvent B [*t*-butyl methyl ether-MeOH- H_2O (70:26:4)] for 6 min, and finally with solvent B for 14 min.

The deep pink peak (Rt 12–14 min) was collected and evaporated to dryness (31.2 mg), and further subjected to a preparative polystyrenic HPLC column (MCI gel CHP20/C10, 4.6 $\times$ 250 mm; Mitsubishi Chemical Co.) and developed

at a flow rate of 1 mL/min with solvent C (CH_2Cl_2) for 5 min, then by a linear gradient from solvent C to solvent D [CH_2Cl_2 -MeOH (5:1)] for 10 min, and finally with solvent D for 15 min. The deep pink peak eluted at 15.8 min was collected and concentrated to give pure 4,4'-diapolycopene-4,4'-dioic acid.

2.5 Methylation of 4,4'-diapolycopene dioic acid

The *B. firmus* GB1 cells (17 L) were extracted with MeOH (360 mL) and partitioned between *n*-hexane (360 mL)/MeOH. The MeOH layer was concentrated to dryness (1.62 g), and re-dissolved in 20 mL CH_2Cl_2 . The diazomethane solution (100 mL Et_2O) prepared by adding 5 g of 1-methyl-3-nitro-1-nitrosoguanidine (Tokyo Chemical Industry Co., Ltd.) to an Et_2O (120 mL)/3 N KOH (50 mL) bilayer, was added to the CH_2Cl_2 solution, and the solution was stirred for 10 min at room temperature under dark conditions. After removing the solvent *in vacuo*, the residue was dissolved in EtOAc (500 mL), and partitioned between EtOAc/ H_2O (500 mL) without adjusting the pH. The EtOAc layer was concentrated to dryness (842.6 mg), and applied to HW40 gel chromatography (20 $\times$ 40 mm) with CH_2Cl_2 -MeOH (1:1). Two red fractions {fr. 8–13 (A) and fr. 15–17 (B)} were collected separately and concentrated to dryness (A: 215.1 mg, B: 153.1 mg). Fraction A was applied to silica gel (Silica Gel 60; Merck) column chromatography [20 $\times$ 160 mm, solvent: *n*-hexane-EtOAc (20:1)] and developed with *n*-hexane-EtOAc first with a 20:1 mixture (350 mL) and then with a 10:1 mixture (220 mL). The red fraction eluted by *n*-hexane-EtOAc (10:1) was collected and concentrated to give pure 4,4'-diapolycopene-4,4'-dioic acid dimethyl ester (2, 2.6 mg). Fraction B was applied to silica gel column chromatography [15 $\times$ 120 mm, solvent: *n*-hexane-EtOAc (3:1)] and developed with the same solvent. The red fraction was collected and concentrated to give pure 4,4'-diapolycopene-4,4'-dioic acid monomethyl ester (3, 0.9 mg).

2.6 Singlet oxygen quenching activity

$^1\text{O}_2$ quenching activity was examined by measuring methylene blue-sensitized photooxidation of linoleic acid⁹⁾. Eighty μL of 0.025 mM methylene blue in EtOH and 280 μL of 100 mM linoleic acid in EtOH with or without 40 μL carotenoid [10 μM , 100 μM , and 1000 μM in EtOH (final concentration 1 μM , 10 μM and 100 μM)] 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 7,000 lux at 22°C for 3 h in corrugated cardboard. Then, 100 μL of the reaction mixture was removed and diluted to 3.0 mL with EtOH, and absorbance at 235 nm was measured to estimate the formation of conjugated dienes¹⁰⁾. The value in the absence of carotenoid was determined and $^1\text{O}_2$ quenching activity was calculated relative to this reference value. Activity is indicated as the IC_{50} (μM) repre-

senting the concentration at which 50% inhibition was observed. The IC_{50} value was calculated by averaging the data from triplicated experiments.

3 RESULTS

3.1 Isolation of pigments

B. firmus GB1 cells from 10 L culture were collected by centrifugation, and extracted with MeOH by sonication. The extracts were partitioned between *n*-hexane/MeOH, and the concentrated MeOH layer was further partitioned between *t*-butyl methyl ether-MeOH/H₂O without adjusting the pH. The *t*-butyl methyl ether-MeOH layer was evaporated to dryness and purified by subsequent chromatography of HP20SS column chromatography, ODS column HPLC, and polystyrenic column HPLC to give pure 4,4'-diapolycopene-4,4'-dioic acid (**1**, 1.0 mg).

3.2 Structural elucidation

First, **1** was dissolved in MeOH and analyzed by HRAPCI-MS and HRESI-MS in positive and negative modes, and the peak due to molecular ion was not observed in any experiment. The ¹H NMR and ¹H-¹H DQF COSY analyses of **1** in CDCl₃-CD₃OD (2:1) revealed 6 methyl singlet signals (δ 1.97-2.00) characteristic of acyclic carotenoid, and 2 downfield shifted *sp*² methine signals (δ 7.34), suggesting that are located at β position of carbonyl functions. The signals due to glycosyl units were not observed in the ¹H NMR spectrum. Considering these observations and the high polarity of **1**, **1** was proposed to be an acyclic carotenoid possessing carboxylic acid functions.

Since the ¹H NMR signals of **1** were very broad, methylation of **1** by diazomethane was performed for further structural analyses. From the MeOH extract of 17 L culture of GB1 cells, pure dimethyl ester (**2**, 2.6 mg) and monomethyl ester (**3**, 0.9 mg) were obtained by EtOAc extraction, HW40 column chromatography, and silica gel column chromatography.

Compound **2** was dissolved in MeOH and analyzed by positive ion HRESI-MS. The (M + Na)⁺ peak was observed at m/z 511.28400 [calcd. for 511.28243 (C₃₂H₄₀O₄Na)], and the molecular formula of **2** was determined to be C₃₂H₄₀O₄ [1 (C₃₀H₃₆O₄) + 28 (CH₃-H) $\times$ 2]. The ¹H NMR spectrum of **2** in CDCl₃ gave sharp signals and clearly showed 6 singlet methyl groups attached at the olefin structure (δ 2.00 $\times$ 4, δ 2.02 $\times$ 2), 2 methoxy methyl (δ 3.77 $\times$ 2), 14 *sp*² methine (δ 6.32- 6.67), and 2 *sp*² methines groups (δ 7.30 (H-6) $\times$ 2] (Table 1). These ¹H NMR signals showed good agreement with those of 4,4'-diapolycopene-4,4'-dioic acid dimethyl ester, which were partially reported in the previous report⁸⁾. The ¹³C NMR, ¹H-¹H DQF COSY, HMQC and HMBC analyses of **2** also confirmed that **2** was 4,4'-diapolycopene-4,4'-dioic acid dimethyl ester. The key long-range

couplings observed in the HMBC experiment on **2** are shown in Fig. 1, and the ¹H and ¹³C NMR data for **2**, which have not been reported previously are listed in Table 1.

Compound **3** was dissolved in MeOH and analyzed by negative ion HRESI-MS, and the (M-Na)⁻ peak was observed at m/z 473.27143 [calcd. for 473.26906 (C₃₁H₃₇O₄)]. The molecular formula of **3** was determined to be C₃₂H₄₀O₄ [1 (C₃₀H₃₆O₄) + 14 (CH₃ - H)], and **3** was speculated to be 4,4'-diapolycopene-4,4'-dioic acid monomethyl ester. The ¹H NMR spectrum of **3** in CDCl₃ was quite similar to that of **2**, while the integration value of methoxy signal (δ 3.77) was reduced by 1/2, and H-6 (δ 7.38) and H-6' (δ 7.30), and H-18 (δ 2.00) and H-18' (δ 2.02) in **3** were observed at different chemical shifts due to its asymmetric structure. Thus, we determined **3** to be 4,4'-diapolycopene-4,4'-dioic acid monomethyl ester. The key ¹H-¹³C long-range couplings observed in the HMBC experiment on **3** are shown in Fig. 1, and the ¹H and ¹³C NMR data for **3**, which have not been reported previously are listed in Table 1.

Since **2** and **3** were identified as 4,4'-diapolycopene-4,4'-dioic acid dimethyl ester and monomethyl ester, respectively, **1** was determined to be 4,4'-diapolycopene-4,4'-dioic acid. The ¹H data for **1**, which have not been reported previously, are listed in Table 1.

3.3 Singlet oxygen quenching activity of carotenoids

We examined the ¹O₂ quenching activity of astaxanthin, which was reported to possess potent activity¹¹⁾, as a positive control. The IC_{50} value of astaxanthin was 9.3 μ M. The ¹O₂ quenching activity of **1**, **2** and **3** was evaluated, and their IC_{50} values were 5.8 μ M, 6.2 μ M and 6.0 μ M, respectively.

4 DISCUSSION

In this study, 4,4'-diapolycopene-4,4'-dioic acid (**1**) could be successfully isolated and the ¹H and/or ¹³C NMR signals of **1**, 4,4'-diapolycopene-4,4'-dioic acid dimethyl ester (**2**) and monomethyl ester (**3**) were assigned for the first time. As **1** and its related compounds [norbixin (6,6'-diapolycopene-6,6'-dioic acid)¹²⁾ and crocetin (8,8'-diapolycopene-8,8'-dioic acid)¹³⁾] from the producing plants were not isolated in previous studies successfully, all these compounds may show inconsistent retention behaviors on silica gel and ODS chromatography. We succeeded in purifying **1** by using polystyrenic synthetic adsorbents, named HP20SS, and a CHP20/C10 packed column (both Mitsubishi Chemical Co.) for separation. These resins adsorb compounds by hydrophobic interaction between the compounds and the planar surface of the aromatic-based polystyrene materials, and the surface of the polystyrenic synthetic adsorbents is different from that of silica gel and ODS materials. Since **1** possesses linear and planar struc-

Table 1 ^1H NMR Data for 4,4'-diapolycopene dioic acid (**1**) in CDCl_3 - CD_3OD (2:1), and ^1H and ^{13}C NMR Data for 4,4'-diapolycopene dioic acid dimethyl ester (**2**) and 4,4'-diapolycopene dioic acid monomethyl ester (**3**) in CDCl_3 .

	1	2	3		
Position	δ_{H}	δ_{H} (multi, J (Hz))	δ_{C}	δ_{H} (multi, J (Hz))	δ_{C}
4			169.1		164.1
-O-CH ₃		3.77 (s, 3H)	51.6		
5			125.5		124.3
6	7.34 (d, 11.0)	7.30 (d, 11.9)	138.9	7.38 (d, 11.2)	140.9
7	6.4-6.6	6.51 (dd, 11.9, 15.5)	122.9	6.54 (dd, 11.2, 15.8)	122.7
8	6.6-6.8	6.62 (d, 15.5)	146.7	6.68 (d, 15.8)	144.3
9			135.3		135.2
10	6.3-6.4	6.37 (d, 11.0)	136.9	6.38 (d, 11.2)	136.0
11	6.6-6.8	6.65 (dd, 11.0, 15.0)	124.7	6.64 (dd, 11.2, 15.2)	124.7
12	6.4-6.6	6.44 (d, 15.0)	139.7	6.43 (d, 15.2)	140.0
13			135.6		135.2
14	6.3-6.4	6.32 (d, 11.0)	133.7	6.32 (d, 11.0)	134.1
15	6.6-6.8	6.67 (dd, 11.0, 15.0)	130.6	6.67 (dd, 11.0, 15.5)	130.7
18	1.97-2.00	2.02 (s, 3H)	12.7	2.00 (s, 3H)	12.8
19	1.97-2.00	2.00 (s, 3H)	12.7	2.00 (s, 3H)	12.8
20	1.97-2.00	2.00 (s, 3H)	12.7	2.00 (s, 3H)	12.9
4'			169.1		168.3
-O-CH ₃		3.77 (s, 3H)	51.6	3.77 (s, 3H)	51.6
5'			125.5		124.0
6'	7.34 (d, 11.0)	7.30 (d, 11.9)	138.9	7.30 (d, 11.9)	138.7
7'	6.4-6.6	6.51 (dd, 11.9, 15.5)	122.9	6.51 (dd, 11.9, 15.8)	122.9
8'	6.6-6.8	6.62 (d, 15.5)	146.7	6.61 (d, 15.8)	144.4
9'			135.3		135.2
10'	6.3-6.4	6.37 (d, 11.0)	136.9	6.38 (d, 11.2)	136.0
11'	6.6-6.8	6.65 (dd, 11.0, 15.0)	124.7	6.64 (dd, 11.2, 15.2)	124.7
12'	6.4-6.6	6.44 (d, 15.0)	139.7	6.43 (d, 15.2)	140.0
13'			135.6		135.2
14'	6.3-6.4	6.32 (d, 11.0)	133.7	6.32 (d, 11.0)	134.1
15'	6.6-6.8	6.67 (dd, 11.0, 15.0)	130.6	6.67 (dd, 11.0, 15.5)	130.7
18'	1.97-2.00	2.02 (s, 3H)	12.7	2.02 (s, 3H)	12.7
19'	1.97-2.00	2.00 (s, 3H)	12.7	2.00 (s, 3H)	12.7
20'	1.97-2.00	2.00 (s, 3H)	12.7	2.00 (s, 3H)	12.7

tures, hydrophobic interactions between **1** and the polystyrenic synthetic adsorbent will be strong. Furthermore, **1** has many conjugated double bonds that can create π interactions with polystyrenic synthetic adsorbents. Strong and consistent retentivity of **1** on polystyrenic synthetic adsorbents may be attributed to the structural and π interaction effects mentioned above.

In the $^1\text{O}_2$ quenching experiments (methylene-blue method), **1**, **2** and **3** showed potent antioxidative activities (IC_{50} 5.8 μM , 6.2 μM and 6.0 μM , respectively) as astaxanthin (9.3 μM). Shimidzu *et al.* reported that an increased number of C=C conjugated double bonds and the presence of a C=O unit enhance $^1\text{O}_2$ quenching activity¹⁴⁾. In addition, it has been reported that both acyclic carotenoid

Fig. 1 The structure of 4,4'-diapolycopene dioic acid (1), its dimethyl ester (2) and monomethyl ester (3). The ^1H - ^{13}C key long-range couplings of 2 and 3 are shown as arrows.

and cyclic carotenoid possessing the same number of conjugated double bonds showed the same potent activity^{15, 16}. Therefore, the almost identical activities of 1, 2, 3 and astaxanthin may be explainable by their same C=C conjugated double bond numbers and C=O units. In addition, the almost identical activities of 1, 2, 3 may show that carboxylic and ester functions do not affect $^1\text{O}_2$ quenching activity.

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