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Diapolycopenedioic Acid Xylosyl Esters A, B, and C, Novel Antioxidative Glyco-C_{30}-carotenoic Acids Produced by a New Marine Bacterium *Rubritalea squalenifaciens*

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Abstract Novel acyl glyco-carotenoic acids, diapolycopenedioic acid xylosyl esters A, B, and C, were isolated as red pigments by using chromatographic methods from a marine bacterium, *Rubritalea squalenifaciens*, belonging to subdivision 1 of *Verrucomicrobia*. The structures of these diapolycopenedioic acid xylosyl esters were determined to be 4-[2-*O*-acyl-β-D-xylopyranosyl] hydrogen 4,4′-diapo-Ψ,Ψ-carotene-4,4′-dioate by spectroscopic analysis. Diapolycopenedioic acid xylosyl ester A showed potent antioxidative activity in a $^1O_2$ suppression model.

Keywords *Rubritalea squalenifaciens*, diapolycopenedioic acid xylosyl ester, antioxidative activity

Introduction

Some species of bacteria, yeasts, and fungi, as well as algae and higher plants, synthesize a large number of carotenoids with different molecular structures [1]. Several species of marine bacteria have been reported to produce dicyclic, monocyclic, or acyclic carotenoids [2]. More than 750 carotenoids have been isolated from natural sources [1]. Evaluating the pharmaceutical potential of various carotenoids with different structures has been an auspicious field of medical research. However, the carotenoids studied so far for this purpose have been restricted to a small number of examples, 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 these carotenoids that can be isolated from higher plants or synthesized chemically, it has been difficult to find natural sources supplying sufficient amounts of carotenoids for detailed analysis.

Recently, we have initiated the analysis of novel or rare types of carotenoids from a new colored marine bacterium, which was classified as belonging to a new species by 16S rRNA analyses [7]. *Rubritalea squalenifaciens* (strain HOact23^T; MBIC08254^T) is a rare marine bacterium belonging to subdivision 1 of *Verrucomicrobia*, a Gram-negative, heterotrophic mesophile, which was isolated from the marine sponge *Halichondria okdai*. Its taxonomic and biochemical characteristics have been reported previously [8]. From this bacterium, we isolated novel acyl glyco-carotenoic acids, diapolycopenedioic acid xylosyl esters A, B, and C. In the previous report [9], we announced the isolation and structural determination of the major pigment produced by *R. squalenifaciens* (corresponding to diapolycopenedioic acid xylosyl ester A in this report). In this report, we describe the isolation and structural determination (including the absolute configurations) of

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diapolycopenedioic acid xylosyl esters A, B, and C, and their antioxidative activity.

Material and Methods

Spectroscopic Analysis

^{1}H- and ^{13}C- NMR spectra were measured at 500 MHz with a Varian UNITY Inova 500. The HRESI-MS and FAB-MS were recorded with a Jeol JMS-T100LP and a JEOL JMS-HX100A mass spectrometer, respectively. Optical rotation was determined using a Rudolph Research Analytical AUTOPOL IV. UV spectra were recorded with a Hitachi U-3200 spectrophotometer.

Preparation of Methyl D-Xylosides, and Methanolysis of Diapolycopenedioic Acid Xylosyl Ester A

D-Xylose (50 mg, Sigma) was dissolved in 10 ml of 10% HCl - MeOH, and heated under reflux for 6 hours. After removal of the solvent *in vacuo*, the reaction mixture was chromatographed by silica gel column chromatography (silica gel 60, Merck) using CH_2Cl_2 - MeOH (5 : 1) to give the methyl D-xylosides (48.6 mg). Methanolysis of diapolycopenedioic acid xylosyl ester A (26.5 mg) was performed in the same way to give the methyl-xylosides (1.6 mg).

$^{1}O_2$ Suppression Experiment

The $^{1}O_2$ quenching activity of diapolycopenedioic acid xylosyl ester A was examined by measuring methylene blue-sensitized photooxidation of linoleic acid [10]. Fourty μl of 0.05 mM methylene blue, 10 μl of 2.4 M linoleic acid, with or without 40 μl of carotenoid (final concentration, 1～100 μM) (each dissolved in EtOH) were added to glass micro vials (5.0 ml). The vials were tightly closed with a screw cap with a septum, and the mixtures were illuminated at 7,000 lux at 22°C for 3 hours in a corrugated cardboard support. Then, 50 μl of the reaction mixture was removed and diluted to 1.5 ml with EtOH, and the absorbance at 235 nm was measured to estimate the formation of conjugated dienes [11]. The value in the absence of carotenoid was determined and the $^{1}O_2$ repression activity was calculated relative to this reference value. The activity is indicated as the IC_{50} value representing the concentration at which 50% inhibition was observed.

Results

Taxonomy of the Producing Strain

A novel marine bacterium *Rubritalea squalenifaciens* (strain HOact23^{T}), was isolated from a homogenate of the marine sponge *Halichondria okadai*, which had been collected from the Miura peninsula (Kanagawa, Japan). Taxonomic analysis of the bacterium was reported previously [8]. The results were summarized as follows.

The strain was a non-motile, rod-shaped (0.44～0.53×0.65～0.79 mm) bacterium, which produced squalene and carotenoid pigments. The cell-wall peptidoglycan contained meso-diaminopimelic acid, glutamic acid and alanine. The genomic DNA G+C content was 52.4 mol%. The major fatty acids were iso-$C_{14:0}$ (43.1%), iso-$C_{16:0}$ (20.6%) and anteiso-$C_{15:0}$ (18.1%), and the major isoprenoid quinone was MK-9 (90.8%). Based on 16S rRNA gene sequence data, the strain formed a distinct group within subdivision 1 in the phylum '*Verrucomicrobia*'. The bacterium showed a range of phenotypic properties which distinguished it from its closest relative, *Rubritalea marina* Pol012^{T} (94.3% 16S rRNA gene sequence similarity). On the basis of polyphasic taxonomic evidence, it was concluded that strain HOact23^{T} should be classified as a novel species in the genus Rubritalea. The name proposed for the new taxon is *Rubritalea squalenifaciens* sp. nov., with the type strain HOact23^{T} (=MBIC08254^{T}=DSM 18772^{T}).

Fermentation and Isolation

R. squalenifaciens was inoculated into 100 ml of the seed medium (1.0% starch, 0.4% yeast extract, and 0.2% peptone in seawater) in a 500-ml Erlenmeyer flask, and cultured at 30°C for 2 days on a rotary shaker (150 rpm). Two ml of the seed culture was inoculated into 100 ml portions of the production medium (=seed medium), and the fermentation was carried out at 30°C for 2 days on a rotary shaker (150 rpm). The OD490 of the culture reached 4.2 at the end of fermentation.

The cells of *R. squalenifaciens* were isolated from 42 liters of culture by centrifugation at 13,000 *g*. After removing the supernatant, the red pigment in the cells was extracted 3 times with CH_2Cl_2 - MeOH (1 : 1). The extracts were combined, and concentrated to a small volume in vacuo, and partitioned between EtOAc/H_2O without adjusting pH. The EtOAc layer was evaporated to dryness and subjected to silica gel chromatography, using CH_2Cl_2 - MeOH (20 : 1). The red-colored fractions were collected and concentrated to dryness to give a red oil (204.5 mg). This red oil was subjected to preparative silica gel HPLC (YMC-Pack SIL column, 20×250 mm) and separated with CH_2Cl_2 - MeOH (15 : 1) as a solvent. The red-colored fractions were collected and evaporated to give a red powder (30.3 mg). This crude powder was subjected to preparative ODS HPLC (Senshu Pak PEGASIL ODS column, 20×250 mm), and separated with MeOH as a

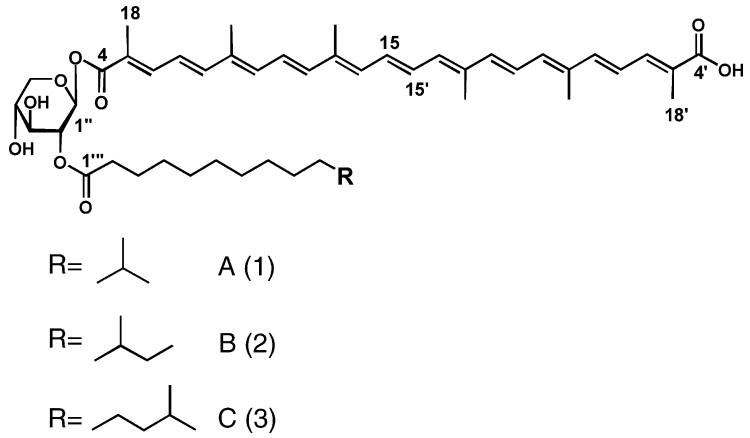

Fig. 1 The structures of diapolycopenedioic acid xylosyl esters A (**1**), B (**2**), and C (**3**).
The numbering of carbon atoms is according to the rule described in Carotenoid Handbook [1].

solvent (flow rate 8.0 ml/minute). More than five red components could be separated by this chromatography. The main component (Rt 16.0 minutes) was collected and concentrated to give pure diapolycopenedioic acid xylosyl ester A (**1**, 10.2 mg). The second main (Rt 19.0 minutes) and the third main (Rt 21.1 minutes) components were also collected and concentrated to give diapolycopenedioic acid xylosyl ester B (**2**, 3.0 mg) and C (**3**, 2.2 mg), respectively.

Structural Elucidation

Compound **1** was dissolved in CH_2Cl_2 - MeOH (1 : 1) and analyzed by HRESI-MS. The $(M+Na)^+$ peak was observed strongly at *m*/*z* 825.49308 (calcd: 825.49175), and the molecular formula of **1** was determined to be $C_{49}H_{70}O_9$. Since **1** was slightly soluble in $CDCl_3$ or CD_3OD, the ^{1}H-NMR (Table 1) and ^{1}H-^{1}H DQF COSY spectra of **1** were measured and analysed in $CDCl_3$ - CD_3OD (5 : 1). The analyses clearly showed the presence of β-xylose {H-1″ [δ 5.65 (J=8.3 Hz)], H-2″ [δ 5.01 (J=8.3, 8.9 Hz)], H-3″ [δ 3.62 (J=8.2, 8.9 Hz)], H-4″ [δ 3.73 (J=4.7, 8.2, 9.0 Hz)], H-5″ [δ 3.44 (J=9.0, 11.3 Hz) and δ 4.06 (J=4.7, 11.3 Hz)]} in **1**, and the chemical shifts of H-1″ and H-2″ implied the presence of an ester linkage at C-1″ and C-2″.

The acetylation of **1** with Ac_2O in dry pyridine gave diacetyl derivative **4** [FAB-MS *m*/*z* 886.5 (M^+)]. Since 4 was highly soluble in $CDCl_3$, further structural studies were performed on **4**. The ^{1}H-NMR data for **4** in $CDCl_3$ (Table 1) showed 6 methyl singlets, 2 methyl doublets, 11 sp^3 methylenes, 5 sp^3 methines, and 16 sp^2 methines, apart from the signals derived from the two acetyl groups. The ^{13}C-NMR (Table 1) and DEPT experiments revealed 8 methyls, 11 sp^3 methylenes, 5 sp^3 methines, 16 sp^2 methines, and 9 sp^2 quaternary carbons, apart from the signals derived from the acetyl groups. The sp^2 quaternary carbons observed at δ 164.1～δ 172.2 were estimated to be ester or carboxylic acid carbons.

Analyses of the ^{1}H-^{1}H COSY and HSQC spectra of **4** reconfirmed the presence of β-xylose ($J_{1'',2''}$=7.0 Hz) in **4**. The downfield shifts of H-3″ (δ 5.26) and H-4″ (δ 5.04) in xylose confirmed that these positions were acetylated in **4**. The analyses also showed the presence of a 12-methyltridecanoyl group in **4** {H-13‴ and H-14‴ [δ 0.86 (d, J=6.5 Hz), 6H], H-12‴ [δ 1.50 (m), ^{1}H], H-11‴ [δ 1.13 (m), 2H], H-4‴～H-10‴ (δ 1.18～1.26, 14H), H-3‴ [δ 1.55 (m), 2H], H-2‴ [δ 2.28 (t, J=7.5 Hz), 2H]}. The ^{1}H-^{13}C long range coupling observed from H-2″ (δ 5.17) and H-2‴ (δ 2.28) to C-1‴ (δ 172.2) in the HMBC spectrum (Fig. 2) proved the attachment of the 12-methyltridecanoyl group at C-2″.

At this point, the unassigned ^{1}H and ^{13}C signals were 6 methyl singlets, 16 sp^2 methines, 6 sp^2 quarternary carbons, and 2 carbonyl carbons. These carbons were proposed to constitute an aglycon. Detailed analyses of the ^{1}H-^{1}H COSY spectrum and ^{1}H-^{13}C long-range couplings from the singlet methyls in the HMBC spectrum (Fig. 2) established the assignment and connectivity of all these unassigned ^{1}H and ^{13}C signals, as shown in Fig. 2, and the ^{1}H-^{13}C long range couplings from H-18 (δ 1.98) to C-4 (δ 166.3), and from H-18′ (δ 2.02) to C-4′ (δ 164.1) showed the presence of a carbonyl group at both ends of the aglycon (Fig. 2). All double bonds in the aglycon were determined to be *E* configuration by the *J* values of the sp^2 methines and ^{13}C chemical shifts of the singlet methyls (Fig. 2). These results confirmed the aglycon of **4** to be diapolycopenedioic acid [12]. The UV-VIS spectrum of **1** was closely similar to that of diapolycopenedioic acid.

The HMBC experiment showed a long range coupling from H-1″ (δ 5.78) to C-4 (δ 166.3), and the ester linkage

Table 1 ^{1}H-NMR data for diapolycopenedioic acid xylosyl esters A (**1**), B (**2**), and C (**3**) in $CDCl_3$-CD_3OD (5 : 1), and ^{1}H- and ^{13}C-NMR data for diacetyl diapolycopenedioic acid xylosyl ester A (**4**) in $CDCl_3$

Position	**1**	**2**	**3**	**4**	
	δ_H	δ_H	δ_H	δ_H	δ_C
4					166.3 (s)
5					123.9 (s)
6	7.33 (1H, d, 11.0)	7.33 (1H, d, 11.2)	7.35 (1H, d, 11.0)	7.34 (1H, d, 11.5)	143.2 (d)
7	6.50 (1H, m)	6.50 (1H, m)	6.50 (1H, m)	6.52 (1H, dd, 11.5, 14.7)	122.6 (d)[a]
8	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.67 (1H, d, 14.7)	146.7 (d)
9					135.3 (s)
10	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.39 (1H, d, 12.0)	136.9 (d)
11	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.66 (1H, dd, 12.0, 14.7)	124.8 (d)[b]
12	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.49 (1H, d, 14.7)	140.3 (d)[c]
13					137.1 (s)[d]
14	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.35 (1H, d, 10.5)	134.3 (d)[e]
15	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.70 (1H, dd, 10.5, 15.0)	130.8 (d)[f]
18	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98 (3H, s)	12.6 (q)
19	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	2.00 (3H, s)	12.8 (q)
20	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	2.00 (3H, s)	12.8 (q)
4′					164.1 (s)
5′					124.4 (s)
6′	7.33 (1H, d, 11.0)	7.33 (1H, d, 11.2)	7.35 (1H, d, 11.0)	7.34 (1H, d, 11.5)	141.5 (d)
7′	6.50 (1H)	6.50 (1H)	6.50 (1H)	6.52 (1H, dd, 11.5, 14.7)	122.7 (d)[a]
8′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.73 (1H, d, 14.7)	143.6 (d)
9′					135.2 (s)
10′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.42 (1H, d, 12.0)	137.7 (d)
11′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.66 (1H, dd, 12.0, 14.7)	124.9 (d)[b]
12′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.49 (1H, d, 14.7)	140.9 (d)[c]
13′					137.1 (s)[d]
14′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.35 (1H, 10.5)	134.6 (d)[e]
15′	6.30~6.80 (1H)	6.30~6.80 (1H)	6.30~6.80 (1H)	6.70 (1H, dd, 10.5, 15.0)	131.1 (d)[f]
18′	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	2.02 (3H, s)	12.8 (q)
19′	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	2.01 (3H, s)	12.8 (q)
20′	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	1.98~2.07 (3H, s)	2.00 (3H, s)	12.8 (q)
1″	5.65 (1H, d, 8.3)	5.62 (1H, d, 8.5)	5.62 (1H, d, 8.5)	5.78 (1H, d, 7.0)	92.3 (d)
2″	5.01 (1H, dd, 8.3, 8.9)	5.02 (1H, dd, 8.5, 8.5)	5.02 (1H, dd, 8.5, 8.5)	5.17 (1H, d, 7.0, 8.5)	69.1 (d)
3″	3.62 (1H, dd, 8.2, 8.9)	3.61 (1H, dd, 8.5, 8.9)	3.62 (1H, dd, 8.5, 8.9)	5.26 (1H, dd, 8.5, 8.5)	70.9 (d)
4″	3.73 (1H, ddd, 4.7, 8.2, 9.0)	3.72 (1H, ddd, 4.7, 8.5, 9.0)	3.72 (1H, ddd, 4.7, 8.5, 9.0)	5.01 (1H, ddd, 5.5, 8.5, 8.5)	68.4 (d)
5″	3.44 (1H, dd, 9.0, 11.3)	3.42 (1H, dd, 9.0, 11.3)	3.42 (1H, dd 9.0, 11.3)	3.57 (1H, dd, 8.5, 12.0)	62.8 (t)
	4.06 (1H, dd, 4.7, 11.3)	4.03 (1H, dd, 4.7, 11.3)	4.03 (1H, dd, 4.7, 11.3)	4.17 (1H, dd, 5.5, 12.0)	
1‴					172.2 (s)
2‴	2.32 (2H, t, 7.3)	2.33 (2H, t, 7.2)	2.33 (2H, t, 7.2)	2.28 (2H, t, 7.5)	34.1 (t)
3‴	1.57 (2H, m)	1.57 (2H, m)	1.57 (2H, m)	1.55 (2H, m)	24.9 (t)
4‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.3 (t)
5‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	27.4 (t)*
6‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.0 (t)*
7‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.4 (t)*
8‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.7 (t)*
9‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.7 (t)*
10‴	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.18~1.26 (2H, m)	29.9 (t)*
11‴	1.12 (2H, m)	1.20~1.30 (2H, m)	1.20~1.30 (2H, m)	1.13 (2H, m)	39.0 (t)
12‴	1.50 (1H, m)	1.12 (2H, m)	1.20~1.30 (2H, m)	1.50 (1H, m)	28.0 (t)

Table 1 Continued

Position	1	2	3	4	
	δ_H	δ_H	δ_H	δ_H	δ_C
13‴	0.85 (3H, d, 6.3)	1.12 (1H, m), 1.38 (1H, m)	1.20∼1.30 (2H, m)	0.86 (3H, d, 6.5)	22.7 (q)
14‴	0.85 (3H, d, 6.3)	0.83 (3H, t, 6.7)	1.50 (1H, m)	0.86 (3H, d, 6.5)	22.7 (q)
15‴		0.86 (3H, d, 6.3)	0.85 (3H, d, 6.3)		
16‴			0.85 (3H, d, 6.3)		

[a, b, c, d, e, f] interchangeable, * unassigned.

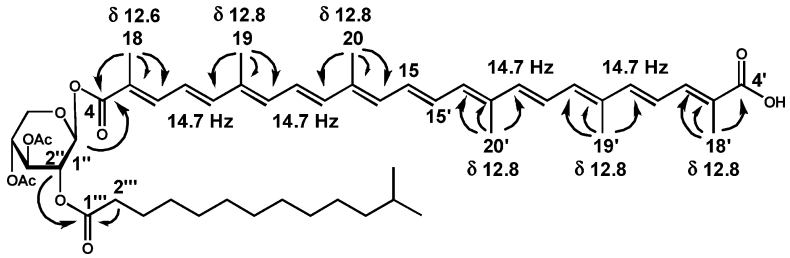

Fig. 2 Key ^{1}H-^{13}C long range couplings, *J* values, and δ_C values observed in the NMR analysis of diacetyl diapolycopenedioic acid xylosyl ester A (**4**).

of β-xylose and the aglycon was proved. Considering the molecular formula of **4**, there was a free carboxylic acid moiety at C-4′. All of the above observations allowed the structure of **1** to be determined as that shown in Fig. 1.

Compound **2** was dissolved in CH_2Cl_2 - MeOH (1 : 1) and analyzed by HRESI-MS(+). The $(M+Na)^+$ peak was apparent at *m*/*z* 839.50847 (calcd: 839.50740), and the molecular formula of **2** was determined to be $C_{50}H_{72}O_9$ (**1**+CH_2).

The ^{1}H-NMR spectrum of **2** in $CDCl_3$ - CD_3OD (5 : 1) (Table 1) was almost identical to that of **1**, and the presence of the same aglycone (diapolycopenedioic acid) and β-xylose in **2** was confirmed. A triplet methyl (δ 0.83) and a doublet methyl (δ 0.86) was observed in the ^{1}H-NMR of **2**, and a vicinal spin network of δ 0.83 (methyl) - δ 1.50 (methine) - δ 1.12∼1.38 (methylene) - δ 0.86 (methyl) was observed in the ^{1}H-^{1}H DQF COSY spectrum of **2**. Considering these observations and the molecular formula of **2**, the fatty acid in **2** was confirmed to be 12-methyltetradecanoic acid. Thus, the structure of **2** was thus determined as shown in Fig. 1.

Compound **3** was dissolved in CH_2Cl_2 - MeOH (1 : 1) and analyzed by HRESI-MS(+). The $(M+Na)^+$ peak was apparent at *m*/*z* 853.52466 (calcd: 853.52546), and the molecular formula of **3** was determined to be $C_{51}H_{74}O_9$ (**1**+C_2H_4).

The ^{1}H-NMR spectrum of **3** in $CDCl_3$ - CD_3OD (5 : 1) (Table 1) was also almost identical to that of **1**, and the preservation of the aglycone and β-xylose moiety in **3** was confirmend. In addition, the doublet methyl signal (δ 0.86, 6H) in **1** was also observed in **3** (δ 0.86, 6H). Thus, the fatty acid in **3** was confirmed to be 14-methylpentadecanoic acid, and the structure of **3** was determined as shown in Fig. 1.

In an attempt to assign the absolute configuration, **1** was methanolyzed in HCl - MeOH, and one newly observed spot [RF 0.3 in silica gel TLC {CH_2Cl_2 - MeOH (5 : 1)}, the spot was detected by anisaldehyde-sulphuric acid.] was purified by silica gel column chromatography. The NMR spectroscopic analyses {^{1}H- and ^{13}C-NMR including 2D-NMR (^{1}H - ^{1}H DQF COSY, HMQC, HMBC, and NOESY)} showed that this spot was composed of methyl α-xylofuranoside [13]: methyl β-xylopyranoside (3 : 1). The $[\alpha]_D^{23}$ value of this mixture showed +62.8 (*c* 0.1, MeOH). D-Xylose was treated in the same way, and the product was purified by silica gel column chromatography. The ^{1}H-NMR analysis showed that the same product was obtained, and the $[\alpha]_D^{23}$ value of the product was +79.2 (*c* 1.0, MeOH). From these results, the sugar in diapolycopenedioic acid xylosyl esters was clarified to be D-xylose. The ^{1}H- and ^{13}C-NMR data for methyl α-furanoxyloside and methyl β-pyranoxyloside are listed in Table 2.

The IUPAC-IUB semisystematic names of **1**, **2**, and **3**

Table 2 The ^{1}H- and ^{13}C-NMR data for methyl α-D-xylofuranoside and methyl β-D-xylopyranoside in CD_3OD.

No.	Methyl α-D-xylofuranoside		Methyl β-D-xylopyranoside	
	δ_H	δ_C	δ_H	δ_C
1	4.60 (1H, d, 3.7)	101.5 (d)	4.10 (1H, d, 8.0)	106.1 (d)
1-OCH_3	3.38 (3H, s)	55.6 (q)	3.48 (3H, s)	57.2 (q)
2	3.39 (1H, m)	73.6 (d)	3.13 (1H, d, 8.0, 8.0)	74.8 (d)
3	3.53 (1H, m)	75.2 (d)	3.28 (1H, m)	77.8 (d)
4	3.45 (1H, m)	71.5 (d)	3.45 (1H, m)	71.2 (d)
5	3.44 (1H, m)	62.8 (t)	3.18 (1H, dd, 10.0, 12.0)	66.9 (t)
	3.52 (1H, m)		3.84 (1H, dd, 5.0, 12.0)	

Table 3 Physico-chemical properties of diapolycopenedioic acid xylosyl esters A (**1**), B (**2**), and C (**3**).

	1	**2**	**3**
Appearance	Red powder	Red powder	Red powder
Molecular formula	$C_{49}H_{70}O_9$	$C_{50}H_{72}O_9$	$C_{51}H_{74}O_9$
HRESI-MS (*m/z*)			
Found	825.49308 $(M+Na)^+$	839.50847 $(M+Na)^+$	853.52466 $(M+Na)^+$
Calcd.	825.49175	839.50740	853.52546
UV λ_{max} (MeOH)	312, 470, 490, 518	312, 470, 490, 518	312, 470, 490, 518
	%III/II=30	%III/II=30	%III/II=30
Rf value[a]	0.30	0.30	0.30

[a] Silica gel TLC (Merck Art. 1.05715), CH_2Cl_2 : MeOH=10 : 1.

are 4-[2-*O*-(12-methyltridecanoyl)-β-D-xylopyranosyl] hydrogen 4,4′-diapo-ψ,ψ-carotene-4,4′-dioate, 4-[2-*O*-(12-methyltetradecanoyl)-β-D-xylopyranosyl] hydrogen 4,4′-diapo-ψ,ψ-carotene-4,4′-dioate, and 4-[2-*O*-(14-methylpentadecanoyl)-β-D-xylopyranosyl] hydrogen 4,4′-diapo-ψ,ψ-carotene-4,4′-dioate, respectively. The physico-chemical properties for **1**, **2**, and **3** are summarized in Table 3.

Antioxidative Activity

To examine the antioxidative activity of diapolycopenedioic acid xylosyl esters, we tested the $^{1}O_2$ suppression activity of **1** as the representative member of these compounds. Compound **1** showed $^{1}O_2$ suppression activity with an IC_{50} of 5.1 μM. The $^{1}O_2$ suppression activities of astaxanthin and β-carotene were also examined, and the IC_{50} values were 8.9 μM and higher than 100 μM, respectively.

Discussion

Diapolycopenedioic acid glucosyl ester [12] from *Methylobacterium rhodinum* ATCC 14821 (formerly *Pseudomonas rhodos*) has been reported previously as a compound related to diapolycopenedioic acid xylosyl esters. The aglycons of the compounds identified in this study were identical, while the diapolycopenedioic acid glucosyl ester possessed a β-glucose ester at C-4 of diapolycopenedioic acid. To our knowledge, diapolycopenedioic acid xylosyl esters are the first reported carotenoids to include D-xylose. The acyl chain attachment at C-2 in D-xylose is also a structural feature of diapolycopenedioic acid xylosyl esters. Several carotenoids including C-6 acyl glucose have been reported previously [12, 14], while diapolycopenedioic acid xylosyl esters are the first examples of a carotenoid including a sugar acylated at C-2. The fatty acids contained in diapolycopenedioic acid xylosyl esters were identical to those of major fatty acids produced by *R. squalenifaciens*.

The $^{1}O_2$ suppression activity of **1** was examined. **1** possessed more potent antioxidative activity than astaxanthin or β-carotene. In previous studies, the keto group conjugated with the polyene structure was shown to enhance the $^{1}O_2$ suppression activity [11, 15]. Since

diapolycopenedioic acid xylosyl esters contain carboxylic acids at both ends of the aglycone, they may be involved in the potent antioxidative activity. Further examinations of the antioxidative activities of diapolycopenedioic acid xylosyl esters are in progress.

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