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Methyl Glucosyl-3,4-dehydro-apo-8′-lycopenoate, a Novel Antioxidative Glyco-C_{30}-carotenoic Acid Produced by a Marine Bacterium *Planococcus maritimus*

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Abstract *Planococcus maritimus* strain iso-3 was isolated from an intertidal sediment sample from the Clyde estuary in the UK. A novel red pigment glyco-carotenoic acid ester, methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate has been isolated from this marine bacterium using chromatographic methods. The structure of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate was determined to be methyl 1-(β-D-glucopyranosyloxy)-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten-8′-oate by the degradation experiment and the spectroscopic analyses. The methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate showed potent antioxidative activity in the $^1O_2$ suppression model.

Keywords *Planococcus maritimus*, methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate, antioxidative activity

Introduction

More than 750 carotenoids with different molecular structures have been isolated from natural sources [1]. It has also been reported that 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]. 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 for supplying sufficient amounts of these rare carotenoids.

Recently we have performed the analysis of novel or rare types of carotenoids from new colored marine bacteria, which were classified to belong to a new species by the 16S rRNA analyses [7, 8]. These studies have reported the isolation of rare carotenoids, (3*R*)-saproxanthin and myxol, from *Flavobacteriaceae* [7], and novel carotenoids glyco-C_{30}-carotenoic acids (diapolycopenedioic acid xylosyl esters A, B, and C) from *Rubritalea squalenifaciens* [8]. This time, we have found a novel carotenoioic acid ester produced by a marine bacterium *Planococcus maritimus*, and describe its structural determination (including the

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absolute configurations) as methyl glucosyl-3,4-dehydro-apo-8'-lycopenoate, its antioxidative activity included.

Materials and Methods

Culture of Bacteria and Taxonomical Analysis

Strain iso-3 was isolated as an pigmented colony on Marine Agar 2216 (MA) (Difco) from an intertidal sediment sample from the shoreline of the Clyde estuary in the UK. Previously these areas have been heavily industrialized. The culture was passed through several rounds of purification to obtain a reproducible homogenous sample. Taxonomical characteristics were analyzed as described in Katsuta *et al.*, 2005. Sequence alignment and similarity search of the 16S rRNA gene sequence were conducted using the Ribosomal database Project [9]. Similarity search was done by using the blastn [10] as well. Phylogenetic tree was constructed by Neighbor-joining method based on the evolutionary distances calculated based on Kimura 2-parameter by using MEGA4 software [11]. Cells were harvested after 24 hours cultivation by using MB at 30°C for menaquinone, fatty acid and the guanine and cytosine contents of the genomic DNA analyses. Menaquinones and fatty acids were analyzed as described previously [12]. Chromosomal DNA was isolated and purified according to the method described previously [13]. DNA base compositions were determined by the HPLC method described by Tamaoka and Komagata [14].

Spectroscopic Analysis

The ^{1}H- and ^{13}C-NMR spectra were measured at 400 MHz with a Bruker AMX400. The HRAPCI-MS was recorded with a Jeol JMS-T100LP. Optical rotation was determined using a Rudolph Research Analytical AUTOPOL IV. UV spectrum was recorded with a Hitachi U-3200 spectrophotometer.

Acid Hydrolysis of Methyl Glucosyl-3,4-dehydro-apo-8′-lycopenoate

Methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (12.2 mg) was suspended in 5.0 ml of 1.0 N HCl, and heated in reflux for 6 hours. After removal of the solvent *in vacuo*, the reaction mixture was chromatographed on silica gel column chromatography (silica60, Merck) using CH_2Cl_2 : MeOH (2 : 1) to give glucose (2.3 mg).

$^{1}O_2$ Suppresion Experiment

The $^{1}O_2$ quenching activity of glucosyl-3,4-dehydro-apo-8′-lycopenoate was examined by measuring methylene blue-sensitized photooxidation of linoleic acid [15]. Fourty microliters 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 micro glass vials (5.0 ml). The vials were tightly closed with a screw cap with a septam, and the mixtures were illuminated at 7,000 lux at 22°C for 3 hours in a corrugated cardboard. 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 [16]. 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

Classification of Isolated Marine Bacteria

Strain iso-3 was isolated as a orange-pigmented colony observed from the microbial analysis of a sample derived from the intertidal sediment sampled from the Clyde estuary, UK. Morphological, physiological and biochemical characteristics are given in Table 1. The strain iso-3 could grow over a broad temperature range (4～41°C) and NaCl concentration of the medium (0～10%). These growth characteristics were similar to those of *Planococcus maritimus* [17]. The results of blastn search suggested the strain iso-3 was affiliated with genus *Planococcus* of family *Planococcaceae*, order *Bacillales*, class *Bacilli*, phylum *Firmicutes* based on 16S rRNA gene sequence. Phylogenetic tree of family *Planococcaceae* based on 16S rRNA gene is shown in Fig. 1. The 16S rRNA gene sequence of strain iso-3 was the most similar to that of type strain of *Planococcus maritimus* (99.5 as a similarity score, and 96.4 as an s_ab score, from the Sequencematch analysis of RDP). In conclusion, iso-3 could be identified as *Planococcus maritimus*.

Culture of the Bacteria and Isolation of Pigments

P. maritimus was inoculated into 100 ml of the seed medium (Marine Broth 2216, Difco) in a 500-ml Erlenmeyer flask, and cultured at 30°C for 1 days on a rotary shaker (150 rpm). Two milliliters 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 1 day on a rotary shaker (150 rpm). The OD_{490} of the culture reached 4.2 at the end of fermentation.

The cells of *P. maritimus* were isolated from 18 liters of

Table 1 Phenotypic properties of strain iso-3

	Strain iso-3
Charcteristics	
Growth at temperature (°C)	4～41
Growth at NaCl (%)	0～10
Biolog profile	
glycogen	−
tween 40	−
tween 80	−
N-acetyl-D-glucosamine	−
glucose	+
arabinose	−
galactose	−
lactose	−
D-mannitol	+
D-sorbitol	+
hydroxybutyric acid	+
α-keto valeric acid	+
D-malic acid	+
pyrubic acid	+
succinic acid	−
glycerol	+
API ZYM profile	
Positive reactions	Alkaline phosphatase, Leucine alylamidase, Valine alylamidase, Cystine alylamidase, Acid phophatase, Naphtol-AS-BI-phosphohydratase, α-glucosidase
Weakly positive reactions	Esterase (C4), Esterase lipase (C8), Chimotrypsin
Negative reactions	Lipase (C4), Trypsin, α-galactosidase, β-galactosidase, β-glucronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase
Major fatty acids	anteiso-C15:0, C16:1 ω7c alcohol
Predominant	MK-8, MK-7
DNA G+C content	47.8

+, positive, −, negative.

culture by centrifugation at 13,000 *g*. After removing the supernatant, the cells was suspended in 1.0 N NaOH and sonicated for 5 minutes to digest the cell wall. After removing the NaOH solution by centrifugation at 13,000 *g*, the digested cells were 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 (10 : 1). The red-colored fractions were collected and concentrated to dryness to give a red oil (13.9 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 (10 : 1) as a solvent. The red-colored fractions were collected and evaporated to give a red powder (10.5 mg). This crude powder was subjected to preparative ODS HPLC (Senshu Pak PEGASIL ODS column, 20×250 mm), and separated with 96% MeOH as a solvent (flow rate 8.0 ml/minute). The red component (Rt 21.0 minutes) was collected and concentrated to give pure methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (**1**, 2.5 mg).

Structural Elucidation

Compound **1** was dissolved in CH_2Cl_2 - MeOH (1 : 1) and analyzed by positive ion HRAPCI-MS. The $(M+H)^+$ peak was observed at *m/z* 625.37330 (calcd: 625.37404), and the molecular formula of **1** was determined to be $C_{37}H_{52}O_8$. Although **1** was slightly soluble in $CDCl_3$, the ^{1}H-NMR (Table 2) and ^{1}H-^{1}H DQF COSY spectra of **1** were measured and analyzed. The analyses showed the presence of a β-hexose {H-1″ [δ 4.69 (J=7.8 Hz)]} and a methoxy group [H-21′ (δ 3.77)] in **1**.

The acetylation of **1** with Ac_2O in dry pyridine gave

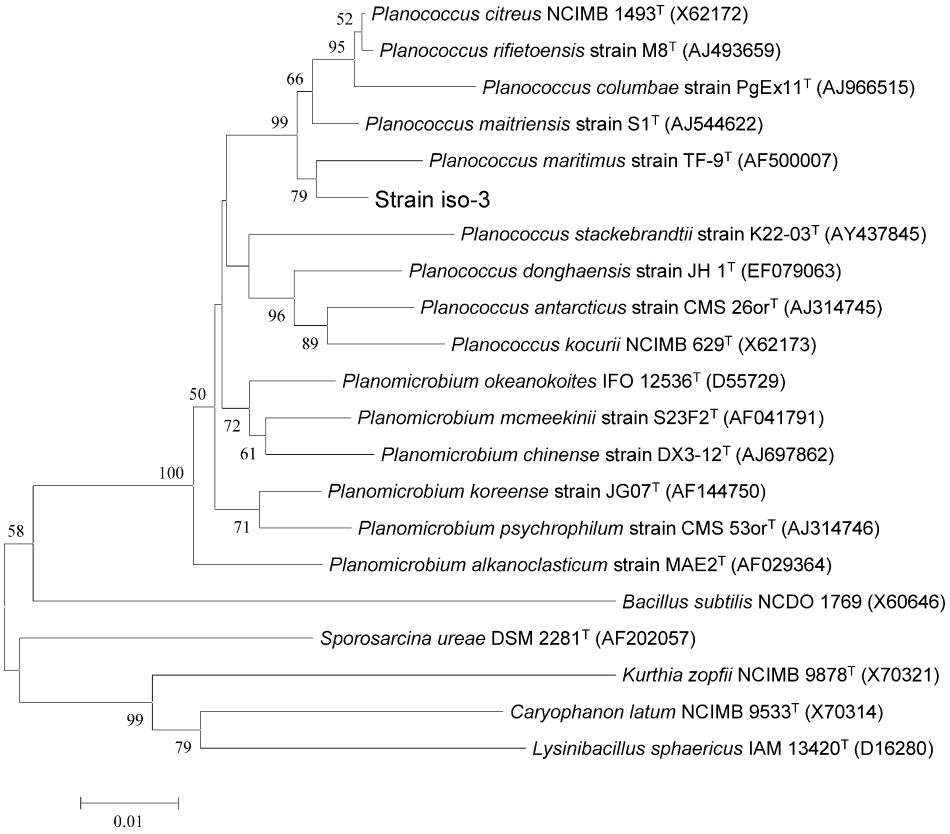

Fig. 1 Neighbour-joining tree based on 16S rDNA sequences showing the positions of strain iso-3, *Planococcus* species, *Planomicrobium* species and representatives of other taxa.

tetraacetyl derivative **2** [APCI-MS *m/z* 815.4 $(M+Na)^+$]. Since **2** was highly soluble in $CDCl_3$, further structural studies were performed on **2**. The ^{1}H-NMR data for **2** in $CDCl_3$ (Table 2) showed 8 methyl singlets, 2 sp^3 methylenes, 5 sp^3 methines, and 15 sp^2 methines, apart from the signals derived from the four acetyl groups. The ^{13}C-NMR (Table 1) and DEPT experiments revealed 8 methyls, 2 sp^3 methylenes, 5 sp^3 methines, 1 sp^3 quaternary carbon, 15 sp^2 methines, and 6 sp^2 quaternary carbons, apart from the signals derived from the acetyl groups. The sp^2 quaternary carbons observed at δ 169.1 was estimated to be an ester or a carboxylic acid carbon.

Analyses of the ^{1}H-^{1}H COSY and HMQC spectra of **2** confirmed the presence of β-glucose {H-1″ [δ 4.69 (J=7.8 Hz)], H-2″ [δ 4.98 (J=7.8, 9.5 Hz)], H-3″ [δ 5.24 (J=9.0, 9.5 Hz)], H-4″ [δ 5.04 (J=9.0, 10.0 Hz)], H-5″ [δ 3.68 (J=1.5, 5.8, 10.0 Hz)], H-6″ [δ 4.10 (J=1.5, 12.1 Hz) and δ 4.22 (J=5.8, 12.1 Hz)] in **2**. The downfield shifts of H-2″, H-3″, H-4″ and H-6″ in glucose confirmed that these positions were acetylated in **2**.

At this point, the unassigned ^{1}H and ^{13}C signals were 8 methyl singlets (including 1 methoxy), 1 sp^3 methylene, 1 sp^3 quaternary carbon, 15 sp^2 methines, 5 sp^2 quaternary carbons, and 1 carbonyl carbon. These carbons were proposed to constitute an aglycone. The ^{1}H-^{13}C long range couplings from H-19′ (δ 2.01) and H-methoxy (δ 3.77) to C-8′ (δ 169.1) showed that one end of the aglycone is a methyl ester (Fig. 3). The other end structure was determined to be 1-*O*-3,4-didehydro-1,2-dihydro-ψ-end group by the observation of ^{1}H-^{13}C long range couplings from H-16 (δ 1.17) and H-17 (δ 1.19) to C-1 (δ 78.8) and C-2 (δ 45.9). In addition, detailed analyses of the ^{1}H-^{1}H COSY spectrum and ^{1}H-^{13}C long-range couplings from the methyl singlets [H-18 (δ 1.91), H-19 (δ 1.98), H-20 (δ 2.01), H-19′ (δ 2.01), and H-20′ (δ 2.00)] in the HMBC spectrum (Fig. 3) established the assignment and connectivity of all the remaining ^{1}H and ^{13}C signals in aglycone as shown in Fig. 3. All double bonds in the aglycone 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. 3). These results confirmed the aglycone of **2** to be methyl 1-hydroxy-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten-8′-oate. The UV-VIS spectrum of **1** was closely similar to that of methyl hexosyl-3,4-dehydro-apo-8′-lycopenoate [18], which possess the same aglycone.

Table 2 ^{1}H-NMR data for methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (**1**) and ^{1}H- and ^{13}C-NMR data for tetra-acetate of **1** (**2**) in $CDCl_3$

Position	**1**	**2**	
	δ_H	δ_H	δ_C
1			78.8 (s)
2	2.37 (m)	2.24 (1H, dd, 7.9, 13.8)	45.9 (t)
	2.40 (m)	2.42 (1H, dd, 7.4, 13.8)	
3	5.74 (1H, ddd, 6.9, 7.8, 15.3)	5.72 (1H, ddd, 7.4, 7.9, 15.1)	125.2 (d)
4	6.19 (1H, d, 15.3)	6.13 (1H, d, 15.1)	137.9 (d)
5			135.5 (s)
6	6.13 (1H, d, 11.5)	6.08 (1H, d 11.3)	130.9 (d)
7	6.63 (1H, dd, 11.5, 16.0)	6.63 (1H, dd, 11.3, 14.4)	125.0 (d)
8	6.35 (1H, d, 16.0)	6.35 (1H, d, 14.4)	137.4 (d)
9			136.2 (s)
10	6.26 (1H, d, 10.1)	6.26 (1H, d, 9.7)	132.5 (d)
11	6.65 (1H, d, 10.1, 14.9)	6.65 (1H, dd, 9.7, 15.2)	124.6 (d)
12	6.45 (1H, d, 14.9)	6.49 (1H, d, 15.2)	139.9 (d)
13			137.0 (s)
14	6.29 (1H, d, 11.3)	6.28 (1H, d, 9.7)	134.1 (d)
15	6.63 (1H, m)	6.63 (1H, dd, 9.7, 15.0)	130.8 (d)
16	1.29 (3H, s)	1.17 (3H, s)	26.6 (q)
17	1.30 (3H, s)	1.19 (3H, s)	25.6 (q)
18	1.92 (3H, s)	1.91 (3H, s)	13.0 (q)
19	1.98 (3H, s)[a]	1.98 (3H, s)	12.8 (q)
20	2.00 (3H, s)[a]	2.01 (3H, s)	12.8 (q)
8′			169.1 (s)
9′			125.7 (s)
10′	7.28 (1H, d, 11.5)	7.30 (1H, d, 11.3)	139.1 (d)
11′	6.51 (1H, dd, 11.5, 14.3)	6.51 (1H, dd, 11.3, 15.1)	122.9 (d)
12′	6.62 (1H, d, 14.3)	6.65 (1H, d, 15.1)	144.2 (d)
13′			135.1 (s)
14′	6.25 (1H, d, 10.6)	6.35 (1H, d, 11.4)	136.0 (d)
15′	6.63 (1H, m)	6.63 (1H, dd, 11.4, 15.0)	129.9 (d)
19′	1.98 (3H, s)[a]	2.01 (3H, s)	12.8 (q)
20′	2.00 (3H, s)[a]	2.00 (3H, s)	12.8 (q)
$COO\underline{CH_3}$	3.77 (3H, s)	3.77 (3H, s)	51.7 (q)
1″	4.52 (1H, d, 7.4)	4.69 (1H, d, 7.8)	93.4 (d)
2″	3.33 (1H, dd, 7.4, 8.0)	4.98 (1H, dd, 7.8, 9.5)	71.6 (d)
3″	3.58 (1H, m)	5.24 (1H, dd, 9.0, 9.5)	73.1 (d)
4″	3.41 (1H, m)	5.04 (1H, dd, 9.0, 10.0)	68.9 (d)
5″	3.77 (1H, m)	3.68 (1H, ddd, 1.5, 5.8, 10.0)	71.6 (d)
6″	3.77 (1H, m)	4.10 (1H, dd, 1.5, 12.1)	62.5 (t)
	3.89 (1H, m)	4.22 (1H, dd, 5.8, 12.1)	

[a] Interchangeable.

The ^{13}C signals of tetra acetates in **2** were observed at δ 20.6~20.7 (CH_3) and δ 169.0~170.6 (C=O).

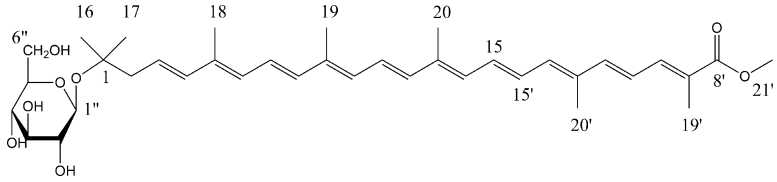

Fig. 2 The structure of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (**1**).

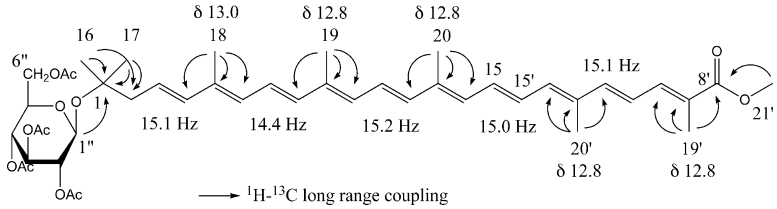

Fig. 3 Key ^{1}H-^{13}C long range couplings, *J* values, and δ_C values observed in the NMR analysis of **2**.

The HMBC experiment also showed a ^{1}H-^{13}C long range coupling from H-1″ (δ 4.69) to C-1 (δ 78.8), and the linkage of β-glucose at C-1 was proved. All of the above observations allowed the structure of **1** to be determined as that shown in Fig. 2.

In an attempt to assign the absolute configuration, **1** was hydrolyzed in 1.0 N HCl, and one newly observed spot {Rf 0.3 in silica gel TLC [CH_2Cl_2 - MeOH (2 : 1)], the spot was detected by anisaldehyde-sulphuric acid.} was purified by silica gel column chromatography. This spot was confirmed to be glucose by ^{1}H-NMR analysis. Since the $[\alpha]_D^{20}$ value of this compound was +52.3° (*c* 0.2, H_2O), the sugar in **1** was clarified to be D-glucose [19].

The IUPAC-IUB semisystematic name of **1** is methyl 1-(β-D-glucopyranosyloxy-3,4-didehydro-1,2-dihydro-8′-apo-y-caroten-8′-oate. The physico-chemical properties for **1** was summarized in Table 3.

Antioxidative Activity

We examined the $^1O_2$ suppression activity of **1**. Compound **1** showed $^1O_2$ suppression activity with an IC_{50} of 5.1 μM. The $^1O_2$ supression activities of astaxanthin and β-carotene were also examined, and the IC_{50} values were 8.9 μM and >100 μM, respectively.

Discussion

The methyl hexosyl-3,4-dehydro-apo-8′-lycopenoate (**3**) [18] from halophilic cocci strain SE20-4 was previously reported as a compound related to **1**. The aglycon of **1** was identical to **3**. While, the sugars of both compounds were different. Sugar of **3** was reported to be mannose using paper chromatography analysis followed by acid hydrolysis [20]. In the present study, sugar part of **1** was determined to be D-glucose by chemical and spectroscopic evidences. Several carotenoids including β-glucose have also been reported [21, 22], while **1** is the first example as a C_{30} carotenoic acid ester including β-D-glucose through glycoside linkage.

The $^1O_2$ suppression activity of **1** was examined. **1** possessed more potent antioxidative activity compared to astaxanthin and β-carotene. In the previous studies, keto function conjugated polyene structure was shown to enhance the $^1O_2$ suppression activity [16, 23]. Since **1** contains ester carbonyl at one end of the aglycone, this may be involved in the potent antioxidative activity. Further examinations on the antioxidative activities of **1** are in progress.

Table 3 Physico-chemical properties of methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate (**1**)

Appearance	Red powder
Molecular formula	$C_{37}H_{52}O_8$
HRAPCI-MS (*m/z*)	
Found	625.37330 $(M+H)^+$
Calcd.	625.37404
UV λ_{max} (MeOH)	458.3 (ε 133,000)
Rf value[a]	0.30

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

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