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REGULAR PAPER

Isolation and characterization of a new bacteriochlorophyll-*c* bearing a neopentyl substituent at the 8-position from the *bciD*-deletion mutant of the brown-colored green sulfur bacterium *Chlorobaculum limnaeum*

Tadashi Mizoguchi · Jiro Harada · Yusuke Tsukatani · Hitoshi Tamiaki

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Abstract We recently constructed the mutant of the brown-colored green sulfur bacterium *Chlorobaculum limnaeum* lacking BciD which was responsible for formation of a formyl group at the 7-position in bacteriochlorophyll(BChl)-*e* biosynthesis. This mutant exclusively gave BChl-*c*, but not BChl-*e*, as the chlorosome pigments (Harada et al. in PLoS One 8(4):e60026, 2013). By the mutation, the homolog and epimer composition of the pigment was drastically altered. The methylation at the 8^2-position in the mutant cells proceeded to create BChl-*c* carrying large alkyl substituents at this position. Correspondingly, the content of BChls-*c* having the (*S*)-configuration at the chiral 3^1-position remarkably increased and accounted for 80.6 % of the total BChl-*c*. Based on the alteration of the pigment composition in the mutant cells, a new BChl-*c* bearing the bulkiest, triple 8^2-methylated neopentyl substituent at the 8-position ([N,E]BChl-*c*) was identified. The molecular structure of [N,E]BChl-*c* was fully determined by its NMR, mass, and circular dichroism spectra. The newly identified [N,E]BChl-*c* was epimerically pure at the chiral 3^1-position and its stereochemistry was determined to be an (*S*)-configuration by modified Mosher's method. Further, the effects of the $C8^2$-methylation on the optical absorption properties of monomeric BChls-*c* were investigated. The Soret but not Qy absorption bands shifted to longer wavelengths by the extra methylation (at most 1.4 nm). The $C8^2$-methylation induced a slight but apparent effect on absorption properties of BChls-*c* in their monomeric states.

Keywords Bacteriochlorophyll-*c* · Bacteriochlorophyll-*e* · Methylation · Chlorosomes · Green sulfur bacteria · Ultra-fast HPLC

Abbreviations

APCI	Atmospheric pressure chemical ionization
BChl	Bacteriochlorophyll
BPhe	Bacteriopheophytin
Cba.	*Chlorobaculum*
CD	Circular dichroism
Chls	Chlorophylls
LCMS	Liquid chromatography mass spectrometry
MTPA	α-Methoxy-α-(trifluoromethyl)phenylacetyl
NOE	Nuclear Overhauser effect
PDA	Photodiode array
R[E,E]	(3^1R)-8-ethyl-12-ethyl
R[E,M]	(3^1R)-8-ethyl-12-methyl
R[I,E]	(3^1R)-8-isobutyl-12-ethyl
R[P,E]	(3^1R)-8-propyl-12-ethyl
ROESY	Rotating frame Overhauser enhancement spectroscopy
S[E,E]	(3^1S)-8-ethyl-12-ethyl
S[I,E]	(3^1S)-8-isobutyl-12-ethyl

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T. Mizoguchi · Y. Tsukatani · H. Tamiaki (✉)
Graduate School of Life Sciences, Ritsumeikan University, Kusatsu 525-8577, Japan
e-mail: tamiaki@fc.ritsumei.ac.jp

J. Harada
Department of Medical Biochemistry, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan

Y. Tsukatani
PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

Present Address:
Y. Tsukatani
Earth-Life Science Institute (ELSI), Tokyo Institute of Technology, Meguro-ku, Tokyo 152-8550, Japan

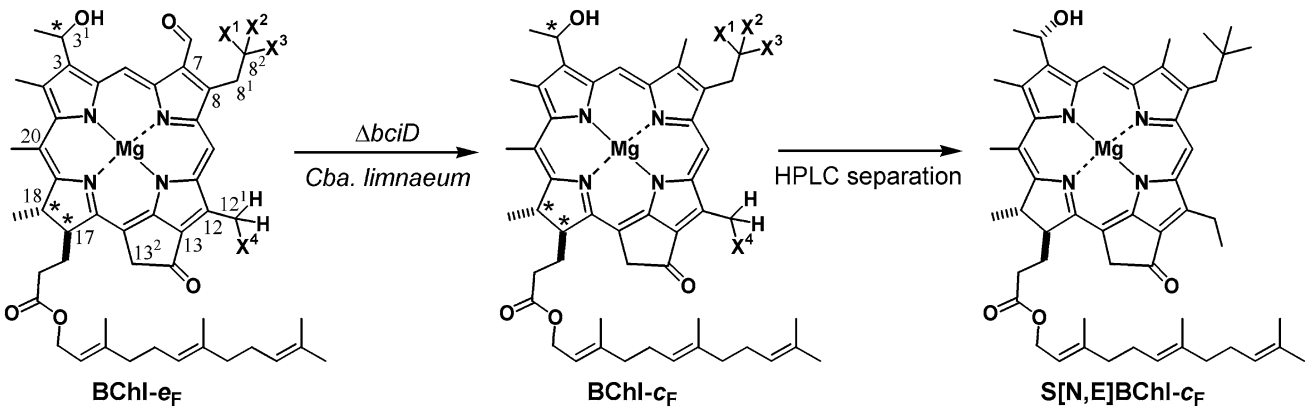
S[N,E] (3^1S)-8-neopentyl-12-ethyl
S[P,E] (3^1S)-8-propyl-12-ethyl
THF Tetrahydrofuran
TOF Time of flight

Introduction

Green photosynthetic bacteria have unique light-harvesting complexes called "chlorosomes" (Orf and Blankenship 2013). Chlorosomes are unique in their composite bacteriochlorophyll(BChl)-*c*, *d*, *e*, or *f* pigments, depending on the bacterial species (Olson 1998; Blankenship and Matsuura 2003; Harada et al. 2012; Vogl et al. 2012). These pigments are called "chlorosomal chlorophylls (Chls)" and are characterized by the presence of a 3-(1-hydroxyethyl) group and by the lack of a 13^2-methoxycarbonyl group in a molecule (Scheme 1). In green sulfur bacteria, chlorosomal Chls dominantly take a farnesyl (C_{15}) moiety as their ester group in the 17-propionate residues instead of the usual phytyl (C_{20}) found in most naturally occurring Chl pigments (Scheer 2006; Tamiaki et al. 2007). Based on such structural features, chlorosomal Chls spontaneously form well-ordered self-aggregates through coordination and hydrogen bonds: Mg···3^1-O–H···O=C-13 (Miyatake and Tamiaki 2005; Balaban et al. 2005). The aggregates constitute the core part of chlorosomes and play key roles in harvesting light energy and transferring it efficiently to reaction centers. Thus, chlorosomal Chls are of major supramolecular, photochemical, and fundamental interest, and a number of comprehensive reviews concerning chlorosomes and self-aggregative pigments have been published (Olson 1998; Matsuura and Blankenship 2003; Miyatake and Tamiaki 2005; Balaban et al. 2005; Frigaard et al. 2006; Tamiaki and Kunieda 2011; Orf and Blankenship 2013).

In addition to the above structural features, chlorosomal Chls are biosynthesized as a mixture of diastereomers originating from the chiral 3^1-position as well as homologs bearing different degrees of methylation at the 8^2- and 12^1-positions (see Scheme 1). Interestingly, with a decrease of irradiated light intensity to culture green photosynthetic bacteria, the red-most absorption (Qy) bands in self-aggregates of chlorosomal Chls shifted to longer wavelengths (>10 nm). Corresponding to the red-shifts, the content of chlorosomal Chls with a higher degree of the $C8^2$-methylation increased (Bobe et al. 1990; Huster and Smith 1990; Borrego and Garcia-Gil 1995; Tamiaki 2005). As methylation progressed, the stereochemistry at the 3^1-position tended to change from an (*R*)- to (*S*)-configuration. Thus, the structural variation induced by the extra methylation of chlorosomal Chls is assumed to be crucial for regulating absorption properties of green photosynthetic bacteria. The shifts of the absorption depending on the methylation were confirmed by construction of a mutant lacking BchQ and BchR, which catalyzed the methylation at the 8^2- and 12^1-positions, respectively, of a green sulfur bacterium *Chlorobaculum* (*Cba.*) *tepidum*; the mutant gave a ~15 nm blue-shifted absorption band in comparison with that of the corresponding wild type (Chew et al. 2007).

As the alkyl substituent at the 8-position of chlorosomal Chls, an ethyl, propyl, or isobutyl group was identified for BChls-*c*, *d*, *e*, and *f* (Olson 1998; Scheer 2006; Harada et al. 2012). Additionally, most (triple) methylated neopentyl group was found in BChl-*d* and BChl-*e* (Smith et al. 1983a; Otte et al. 1993; Borrego et al. 1999); although later investigation of the pigment composition of green sulfur bacteria producing BChl-*e* (referred to as brown-colored green sulfur bacteria) questioned the presence of BChl-*e* with a neopentyl group (Glaeser et al. 2002). BChl-*c* with



Scheme 1 Biosynthesis of BChl-*c* in the brown-colored green sulfur bacterium *Cba. limnaeum* by the Δ*bciD* mutation. X^1, X^2, X^3, and X^4 = H or CH_3

a neopentyl group has never been identified in green photosynthetic bacteria.

Here, we investigated the presence of BChl-*e* and BChl-*c* molecules carrying a neopentyl group at the 8-position in green sulfur bacteria; using ultra-fast HPLC in order to efficiently detect a trace chlorosomal Chl. For this purpose, we focused our attention on brown-colored green sulfur bacteria, because the organisms were reported to dominate in extremely low light environments among various green sulfur bacteria (Overmann et al. 1992). Such low light-adapted bacteria potentially have the ability to create much 8^2-methylated BChl as their photoactive pigment. In addition to this idea, we recently constructed a mutant lacking BciD, which was responsible for formation of a C7-formyl group in BChl-*e*, of the brown-colored green sulfur bacterium *Cba. limnaeum* (Harada et al. 2013). The mutant could exclusively produce BChl-*c* but not BChl-*e* as its chlorosomal Chls, and was found to use a new BChl-*c* bearing a neopentyl group.

Materials and methods

Bacterial strains and their cultivation conditions

Construction of the *bciD*-deletion mutant of *Cba. limnaeum* RK-j-1 (as a wild type) was reported previously (Harada et al. 2013). The green sulfur bacterium *Cba. tepidum* ATCC49652 was used as a reference of BChl-*c*. The wild type of *Cba. limnaeum* and its *bciD*-deletion mutant (at 30 °C) and *Cba. tepidum* (at 45 °C) were anaerobically cultured with the light intensity of 30 μE/s/m^2 as described (Harada et al. 2008), and were harvested in their late stationary phases.

HPLC analysis of chlorosomal Chls

Chlorosomal Chls were extracted from the harvested cells of the wild type and its *bciD*-deletion mutant of *Cba. limnaeum*, together with *Cba. tepidum* as a reference for BChl-*c*, using a mixture of methanol and acetone (1:1, v/v). The supernatant after centrifugation at 2,000*g* (for ~60 s) was collected. Extracts were evaporated to dryness by a stream of N_2 gas, dissolved in a small amount of 25 % acetone in acetonitrile, and subjected to the following two HPLC systems—conventional HPLC and ultra-fast HPLC. Conventional HPLC was performed using a Shimadzu Prominence liquid chromatograph system comprising a CBM-20A communications bus module, an SPD-M20A photodiode array (PDA) detector, an LC-20AD pump, a DGU-20A$_3$ degasser, and a CTO-20AC column oven (Shimadzu, Kyoto, Japan). Reverse-phase HPLC was performed as follows: column, Cosmosil 5C18-AR-II (5.0 μm, 4.6 × 150 mm, Nacalai Tesque Inc., Kyoto); eluent, acetonitrile/acetone/water = 65/15/20 (v/v/v); flow rate, 1.0 mL/min; and range of detection wavelength by PDA, 350-800 nm. Ultra-fast HPLC was performed using a Shimadzu Nexera X2 system comprising a CBM-20A communications bus module, an SPD-M30A PDA detector equipped with a high sensitive flow cell, an LC-30AD pump, a DGU-20A$_{5R}$ degasser, and a SIL-30AC autosampler (Shimadzu). Reverse-phase HPLC was performed as follows: column, Shim-pack XR-ODS III (1.6 μm, 2.0 × 75 mm, Shimadzu GLC Ltd., Tokyo, Japan); eluent, acetonitrile/water = 90/10 (v/v); flow rate, 0.4 mL/min; and range of detection wavelength by PDA, 300–700 nm. Liquid chromatography mass spectrometry (LCMS) was performed under the above conventional and ultra-fast HPLC conditions, using a Shimadzu LCMS-2010EV or LCMS-8040 system comprising a quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) probe as described (Mizoguchi et al. 2006). All solvents used for the analytical HPLC were of HPLC grade quality and were purchased from Nacalai Tesque Inc. and Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation and structural determination of a new BChl-*c*

A homologous mixture of BChl-*c* was extracted from 3 L cultures of the *bciD*-deletion mutant of *Cba. limnaeum* by basically the same method as mentioned above. A new BChl-*c*, (3^1*S*)-8-neopentyl-12-ethyl-(S[N,E])BChl-c_F, was isolated from the mixture by the following HPLC: column, Cosmosil 5C18-AR-II (10 × 250 mm); eluent, 2 % (v/v) H_2O in methanol; and flow rate, 2.0 mL/min (S[N,E]BChl-c_F was eluted at the retention time of 22 min). The 600 MHz ^{1}H-NMR spectrum of the isolated pigment was recorded in tetrahydrofuran(THF)-d_8 (Euriso-top, Saclay, France) using a JEOL ECA-600 NMR spectrometer (JEOL, Tokyo); the residual proton at the 3-position of THF (δ_H = 1.72 ppm) was used as an internal standard. A set of assignments of ^{1}H signals was obtained by correlation spectroscopy and rotating frame Overhauser enhancement spectroscopy (ROESY) spectra (τ_m = 400 ms). The accurate molecular mass spectrum was obtained using a time-of-flight (TOF) mass spectrometer (micrOTOF-II; Bruker Daltonik, Bremen, Germany) coupled with an APCI probe. The APCI conditions were set as follows: drying gas flow, 3.0 L/min; nebulizer pressure, 1,600 hPa; drying gas temperature, 200 °C; vaporizer temperature, 350 °C; capillary voltage (positive), 4,000 V; and corona current, 216 nA. The electronic absorption and circular dichroism (CD) spectra were measured in Et_2O at room temperature, using a Hitachi U-3500 spectrophotometer (Hitachi High-

Technologies Corp., Tokyo) and a Jasco J-720 W spectropolarimeter (Jasco Corp., Tokyo), respectively. The absorption spectra were recorded at ±0.2 nm resolution after calibration by a 656.1-nm line from a deuterium lamp.

S[N,E]BChl-c_F

VIS (Et_2O) λ_{max}/nm 660.2 (relative absorbance, 0.62), 624.8 (0.10) and 433.4 (1.00); δ_H/ppm 9.88 (1H, s, 5-H), 9.56 (1H, s, 10-H), 6.32 (1H, dq, $J = 2.4$, 6.9 Hz, 3^1-H), 5.20 (1H, t, $J = 6.9$ Hz, f2-H), 5.10, 5.05 (each 1H, d, $J = 19.5$ Hz, 13^2-H), 5.03 (2H, t, $J = 7.5$ Hz, f6-H, f10-H), 4.82 (1H, d, $J = 1.8$ Hz, 3^1-OH), 4.68 (1H, q, $J = 7.2$ Hz, 18-H), 4.47 (2H, m, f1-H_2), 4.18 (1H, m, 17-H), 4.04 (2H, q, $J = 7.8$ Hz, 12^1-H_2), 3.89 (3H, s, 20-CH_3), 3.72 (2H, s, 8^1-H_2), 3.38 (3H, s, 2-CH_3), 3.31 (3H, s, 7-CH_3), 2.47 (2H, m, 17^2-H_2), 2.18, 2.04 (each 1H, m, 17^1-H_2), 1.99 (3H, d, $J = 6.0$ Hz, 3^1-CH_3), 1.90 (3H, t, $J = 7.5$ Hz, 12^1-CH_3), 1.61 (3H, s, f3-CH_3), 1.60 (3H, s, f12-H_3), 1.51, 1.49 (each 3H, s, f7-CH_3, f11-CH_3), 1.50 (3H, d, $J = 7.2$ Hz, 18-CH_3), 1.24 (9H, s, 8^1-$C(CH_3)_3$), 2.05–1.85 (8H, m, f4-, f5-, f8-, f9-H_2); HRMS (APCI) found: *m/z* 849.5170, calcd for $C_{53}H_{69}N_4O_4Mg$: 849.5169 ($[MH]^+$).

Preparation of Mosher's esters

The isolated [N,E]BChl-c_F (ca. 0.5 mg/3L cultures) was dissolved in Et_2O and was treated with aqueous diluted HCl (0.34 M) to yield the corresponding demetalized bacteriopheophytin(BPhe)-*c*, [N,E]BPhe-c_F. The mixture was poured into aqueous 4 % $NaHCO_3$ and was extracted with Et_2O. After evaporation, the resulting [N,E]BPhe-c_F was used for Mosher's reactions without further purification. The purity of [N,E]BPhe-c_F thus obtained was confirmed by the following normal-phase HPLC [see Fig. S1(a)] and was adequate for the further reaction: column, Cosmosil 5SL-II (5.0 μm, 6.0 × 250 mm, Nacalai Tesque Inc.); eluent, 20 % acetone in hexane; and flow rate, 1.0 mL/min. To a dry pyridine solution (100 μL) of [N,E]BPhe-c_F (ca. 0.2 mg), commercially available and optically active MTPA-Cl [α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, ~10 mg, Tokyo Kasei Kogyo Co. Ltd., Tokyo] was added in the dark (Tamiaki et al. 2004; Mizoguchi et al. 2005). After stirring at room temperature for 1 h, *N,N*-dimethyl-1,3-propandiamine (15 μL, Nacalai Tesque Inc.) was added. The mixture was stirred for 15 min, poured into aqueous 4 % $NaHCO_3$, extracted with Et_2O, and washed with H_2O twice. After evaporation, the residue was purified by the above normal-phase HPLC [see Fig. S1(a)]. The ^{1}H-NMR spectra of the resultant MTPA esters were recorded in $CDCl_3$ (Merck KGaA, Darmstadt, Germany) at room temperature using a 3 mm ϕ semi-micro NMR tube (Wilmad LabGlass, New Jersey, USA); the residual proton of $CDCl_3$ was used as an internal standard ($\delta_H = 7.26$ ppm).

S[N,E]BPhe-c_F

VIS (Et_2O) λ_{max}/nm 667.2 (relative absorbance, 0.51), 548.0 (0.15), 516.0 (0.10) and 412.6 (1.00); MS (APCI) found: *m/z* 827.40 and 623.30, calcd for $C_{53}H_{71}N_4O_4$: 827.55 ($[MH]^+$) and $C_{38}H_{47}N_4O_4$: 623.36 ([M–farnesyl + H]$^+$).

(R)-MTPA ester of S[N,E]BPhe-c_F

VIS (Et_2O) λ_{max}/nm 672.4 (relative absorbance, 0.62), 548.2 (0.16), 517.4 (0.10) and 412.8 (1.00); MS (APCI) found: *m/z* 1,043.30, 809.35 and 605.20, calcd for $C_{63}H_{78}F_3N_4O_6$: 1,043.59 ($[MH]^+$), $C_{53}H_{69}N_4O_3$: 809.54 ([M–(MTPA-OH) + H]$^+$) and $C_{38}H_{45}N_4O_3$: 605.35 ([M–(MTPA-OH)–farnesyl + H]$^+$).

(S)-MTPA ester of S[N,E]BPhe-c_F

VIS (Et_2O) λ_{max}/nm 672.4 (relative absorbance, 0.61), 548.0 (0.15), 517.4 (0.10) and 412.8 (1.00); MS (APCI) found: *m/z* 1,043.30, 809.35 and 605.25, calcd for $C_{63}H_{78}F_3N_4O_6$: 1,043.59 ($[MH]^+$), $C_{53}H_{69}N_4O_3$: 809.54 ([M–(MTPA-OH) + H]$^+$) and $C_{38}H_{45}N_4O_3$: 605.35 ([M–(MTPA-OH)–farnesyl + H]$^+$).

Results and discussion

Ultra-fast HPLC analysis of BChl-*e* in *Cba. limnaeum*

The HPLC separation of a mixture of chlorosomal Chls composed of diastereomers at the 3^1-position and homologs possessing various alkyl substituents at the 8- and 12-positions has been demonstrated for BChl-*c* (Balaban et al. 1995; Mizoguchi et al. 2000; Ishii et al. 2000), BChl-*d* (Smith and Goff 1985; Mizoguchi et al. 2002), and their derivatives (Tamiaki et al. 1998, 2004). However, the separation for BChl-*e*, especially for the diastereomers, was quite limited. For example, Steensgaard et al. (2000) demonstrated a normal-phase HPLC separation of diastereomeric BChl-e_F after the first fractionation of each homolog by reverse-phase HPLC. Saga et al. (2001) reported the separation using reverse-phase HPLC; however, it took about 150 min to achieve sufficient separation. In our previous report, we demonstrated the diastereomeric separation of BChl-*e* by conventional reverse-phase HPLC (Tamiaki et al. 2011; Harada et al. 2013; Tsukatani et al.

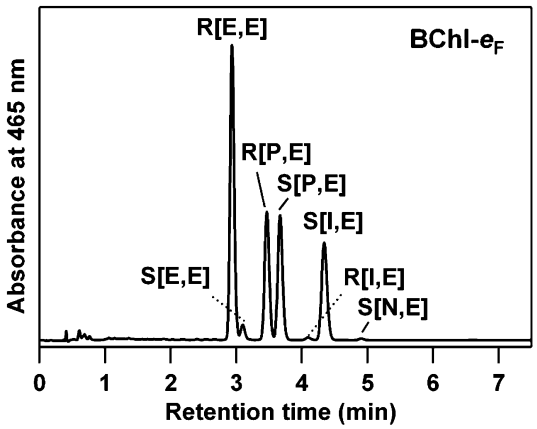


Fig. 1 An ultra-fast HPLC profile monitored at 465 nm of the pigments extracted from the wild type of *Cba. limnaeum* containing BChl-*e*

2013). Here, we further improved the separation using ultra-fast HPLC in order to shorten the elution time and permit detection of a trace component.

Figure 1 shows a representative ultra-fast HPLC profile of the pigments extracted from the wild type of *Cba. limnaeum* containing BChl-*e*. In the profile, a pair of diastereomers at the chiral 3^1-position as well as homologs carrying different degrees of methylation at the 8^2-position were completely separated within 5 min. The assignment of each peak was obtained by on-line APCI mass spectra (Table S1) as well as absorption spectra, and was consistent with the literature (Steensgaard et al. 2000; Saga et al. 2001): (3^1R)-8-ethyl-12-ethyl-(R[E,E]), (3^1S)-8-ethyl-12-ethyl-(S[E,E]), (3^1R)-8-propyl-12-ethyl-(R[P,E]), (3^1S)-8-propyl-12-ethyl-(S[P,E]), (3^1R)-8-isobutyl-12-ethyl-(R[I,E]), and (3^1S)-8-isobutyl-12-ethyl-(S[I,E])BChl-e_F in the order of elution. Notably, a trace component (0.4 % of the total BChl-e_F content by peak areas of HPLC) was observed at the retention time of 4.9 min. The peak could not be detected by conventional HPLC (data not shown) due to its broadening during prolonged elution. This trace component exhibited the molecular ion peak at $m/z = 863.60$, which was consistent with the calculated mass number of [N,E]BChl-e_F ($C_{53}H_{67}N_4O_5Mg$: 863.50). One of the fragment ion peaks ($[M1 + H]^+$ in Table S1) was observed at $m/z = 659.50$ produced by the loss of the esterifying alcohol in the 17-propionate residue, indicating that the pigment had a C_{15} farnesyl group: 863.60–659.50 = 204.10 = $C_{15}H_{25}$–H. Moreover, another fragment ion peak was observed at $m/z = 845.40$ due to the mass number loss of 18 ($[M2 + H]^+$ in Table S1); supporting the presence of a hydroxy group in the pigment. According to the previous report on BChl-*d* which possessed a neopentyl group at the 8-position (Smith et al. 1983a), the stereochemistry at the 3^1-position is most likely to take an (*S*)-configuration. The BChl-*e* pigment carrying a methyl group at the 12-position, (3^1R)-8-ethyl-12-methyl-(R[E,M])BChl-e_F, was not identified in this study; although its presence has been reported in other species of brown-colored green sulfur bacteria (Glaeser et al. 2002).

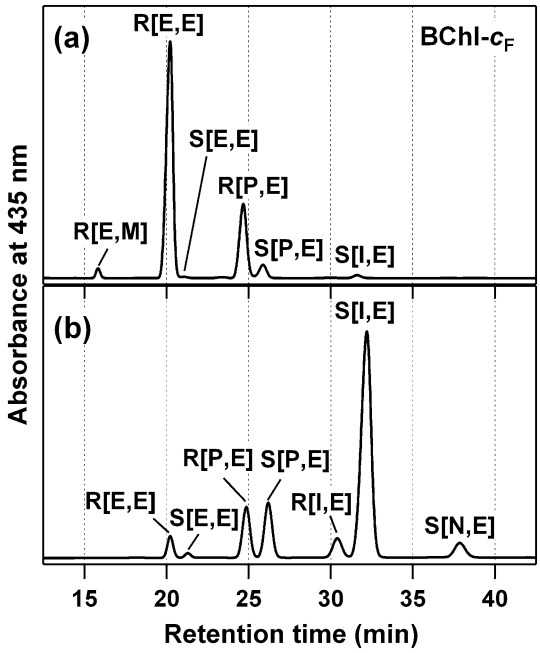


Fig. 2 HPLC profiles monitored at 435 nm of the pigments extracted from the cells of *Cba. tepidum* (**a**) and the *bciD*-deletion mutant of *Cba. limnaeum* (**b**)

HPLC analysis of BChl-*c* in the *bciD*-deletion mutant

Figure 2 shows conventional reverse-phase HPLC profiles monitored at 435 nm for the pigments extracted from the cells of *Cba. tepidum* (a) and the *bciD*-deletion mutant of *Cba. limnaeum* (b). Six BChl-*c* components for control *Cba. tepidum* and seven for the mutant were detected. According to the previous reports on accumulation of several BChl-*c* components in *Cba. tepidum*, the six peaks were assigned to R[E,M], R[E,E], S[E,E], R[P,E], S[P,E], and S[I,E] forms of farnesylated BChl-*c* in the order of elution (Balaban et al. 1995; Mizoguchi et al. 2000; Ishii et al. 2000). The assignment was confirmed by on-line APCI-mass spectrometry (data not shown). Similarly, the pigments in the mutant cells were assigned to R[E,E], S[E,E], R[P,E], S[P,E], R[I,E], and S[I,E] forms of BChl-*c* in the order of elution. In addition to these six BChl-*c* components, the other peak was apparent at the retention time of 37.3 min. The pigment exhibited the Soret and Qy absorption at 433.4 and 660.2 nm, respectively, in Et_2O; showing the absorption properties similar to authentic BChl-*c* from *Cba. tepidum* as shown in Fig. 3(a). From electronic absorption properties and

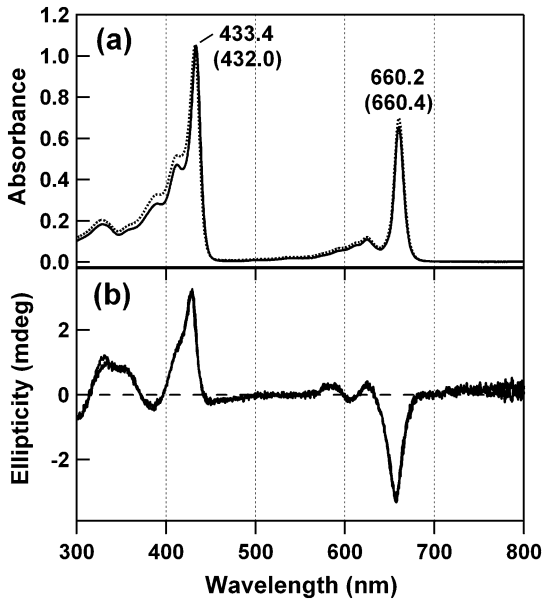


Fig. 3 Electronic absorption (**a**) and CD (**b**) spectra of S[N,E]BChl-c_F from the *bciD*-deletion mutant of *Cba. limnaeum* (*solid*) and R[E,E]BChl-c_F isolated from *Cba. tepidum* (dotted) in Et_2O. The λ_{max} of R[E,E]BChl-c_F is shown in *parenthesis*

elution order on reverse-phase HPLC, the newly accumulated BChl-*c* pigment in the *bciD*-deletion mutant of *Cba. limnaeum* was expected to be the C8-neopentyl homolog of BChl-*c*.

Structural determination of the new BChl-*c* homolog

The new BChl-*c* homolog gave the molecular ion peak at $m/z = 849.40$ (Table S2) by on-line APCI-mass spectrometry, which was consistent with the value of the protonated [N,E]BChl-c_F (calcd for 849.52). The two characteristic fragment ion peaks as seen in the above BChl-*e* were observed at $m/z = 645.25$ ($[M1 + H]^+$) and 831.40 ($[M2 + H]^+$) (see Table S2). These peaks indicated that the pigment had C_{15} farnesyl and hydroxy groups in a molecule.

Figure 4 shows the ^{1}H-NMR spectrum of the new BChl-*c* in THF-d_8 at room temperature. We can find two singlet proton signals originating from a characteristic neopentyl group in the pigment: chemical shifts, $\delta_H = 3.72$ (8^1-H_2) and 1.24 [8^1-$C(CH_3)_3$] ppm. These chemical shifts were well consistent with those of methyl bacteriopheophorbide-*d* derived from BChl-*d* in *Cba. vibrioforme* (3.76 ppm for 8^1-H_2 and 1.25 ppm for 8^1-$C(CH_3)_3$ in $CDCl_3$) (Smith et al. 1983a). In order to determine the molecular structure, especially for location of the neopentyl group, we employed 2D-NMR spectroscopy. The inset molecular structure of Fig. 4 summarizes the observed nuclear Overhauser effect (NOE) correlations in the ROESY spectrum using arrows. These NOE correlations established the molecular structure of [N,E]BChl-c_F, starting from the 3^1-proton which was definitely assigned. Additionally, the presence of an ethyl group at the 12-position in the pigment was also confirmed by the ^{1}H-NMR spectrum: $\delta_H = 4.04$ (12^1-H_2) and 1.90 (12^1-CH_3) ppm. Moreover, the accurate molecular mass spectrum of [N,E]BChl-c_F was obtained by a TOF mass spectrometer coupled with an APCI probe as shown in Fig. S2. The evidence obtained from NMR and mass measurements was fully consistent with the molecular formulation of [N,E]BChl-c_F as shown in the right drawing of Scheme 1.

Stereochemistry at the 3^1-position in [N,E]BChl-c_F

According to the ^{1}H-NMR spectrum of isolated [N,E]BChl-c_F, it was a single diastereomer (lack of satellite signals as shown in the inset panel of Fig. 4). After demetalation of [N,E]BChl-c_F, 3^1-epimerically pure [N,E]BPhe-c_F was treated with commercially available (+)-MTPA-Cl in dry pyridine to afford the corresponding Mosher's ester, (*R*)-MTPA ester (see Scheme 2). Similarly, reaction of [N,E]BPhe-c_F with (–)-MTPA-Cl gave the corresponding (*S*)-MTPA ester. The resulting (*R*)- and (*S*)-MTPA esters were characterized using both ^{1}H-NMR and mass spectra (Table 1 and Fig. S3).

Modified Mosher's method was reported to be effective for determination of epimerically pure secondary alcohols including Chl pigments (Ohtani et al. 1991; Kelly 1999; Tamiaki et al. 2004; Mizoguchi et al. 2005). The method is based on different shielding effects of a phenyl ring in diastereomeric (*R*)- or (*S*)-MTPA ester on NMR spectroscopy. When the 3^1-proton of BChl-*c* as well as carbonyl oxygen and trifluoromethyl carbon atoms of MTPA lie in the same side on the planar conformation H–C3^1–O–C(O)–C–C(F_3) as shown in the right drawing of Scheme 2, the *syn*/*anti*-orientation of the 3^1-methyl group of BChl-*c* to the phenyl group of MTPA is opposite in (*R*)- and (*S*)-MTPA esters. Table 1 lists the ^{1}H-chemical shifts (δ_Hs) of (*R*)- and (*S*)-MTPA esters together with their differences, $\Delta = \delta_H$[(*S*)-MTPA ester] $- \delta_H$[(*R*)-MTPA ester]. The δ_Hs of 3^1-methyl group in (*R*)- and (*S*)-MTPA esters were measured at 2.35 and 2.29 ppm, respectively, in $CDCl_3$. The value in Δ (=2.29–2.35) = −0.06 ppm was negative. In contrast, almost all the Δs on the peripheral positions of the chlorin π-system gave positive values; which tended to decrease with an increase of the distance from the 3^1-chiral position. According to modified Mosher's rule (Ohtani et al. 1991), the present secondary alcohol was assigned to an (*S*)-configuration at the 3^1-position. Moreover, the Δ value in the methoxy group of MTPA substituents was negative, −0.08 ppm, indicating that epimerically pure [N,E]BPhe-c_F has a (3^1S)-configuration on the basis of another modified Mosher's rule (Kelly 1999).

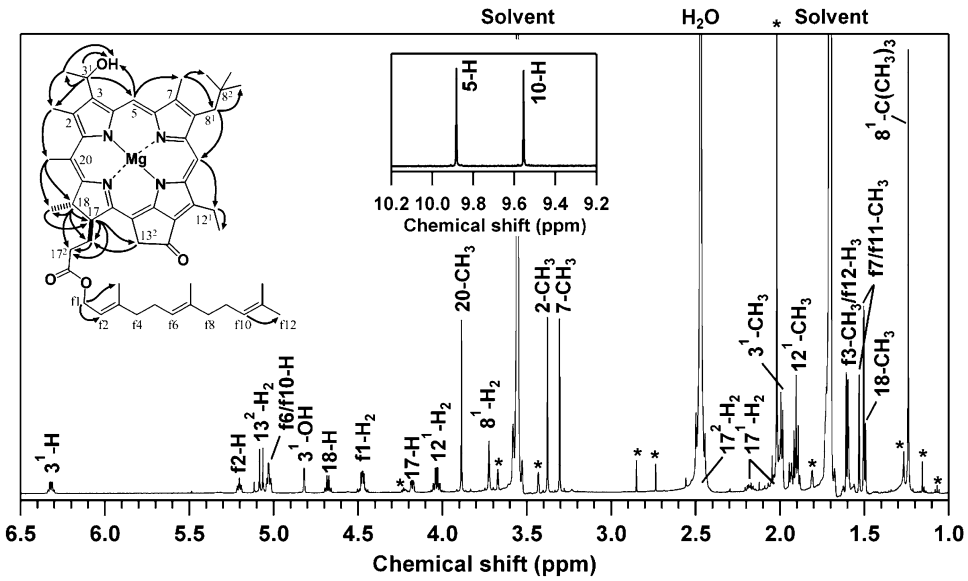
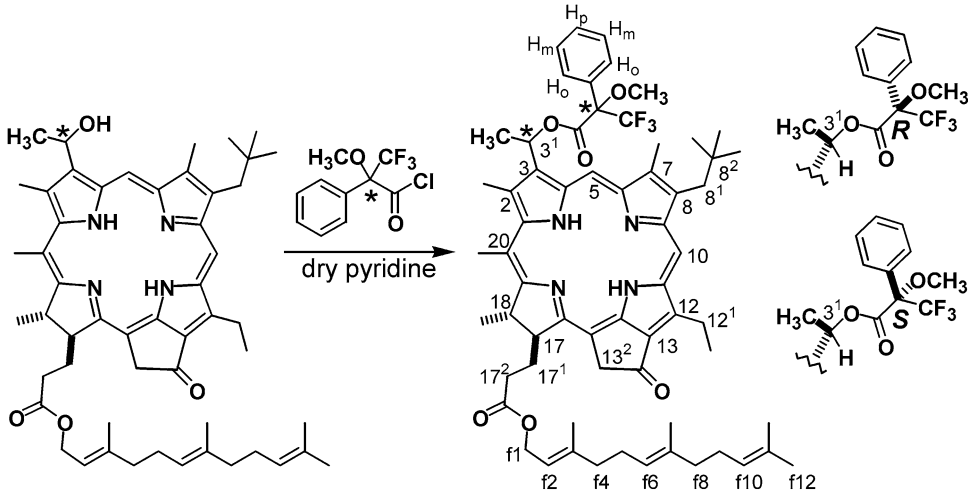


Fig. 4 ^{1}H-NMR spectrum of isolated S[N,E]BChl-c_F from the *bciD*-deletion mutant of *Cba. limnaeum* in THF-d_8 at room temperature. Observed NOE correlations obtained by the ROESY spectrum are indicated by *arrows* in the *inset* molecular structure. *Inset panel* shows the *meso*-proton region. Impurities are indicated by *asterisks*



Scheme 2 Synthesis of (*R*)- and (*S*)-Mosher's esters of [N,E]BPhe-c_F

Stereochemistry at the 17- and 18-positions in S[N,E]BChl-c_F

To confirm the configurations at the chiral 17- and 18-positions, we used NOE correlations and CD spectra. The *syn*-orientations of 17-CH_2CH_2 and 18-H (or 17-H and 18-CH_3) were confirmed based on the NOE correlations as shown in the inset molecular structure of Fig. 4: 17-H ↔ 18-CH_3, 17^1-H_2 ↔ 18-H, and 17^2-H_2 ↔ 18-H. From these NOE correlations, the stereochemistry was established to be (17*S*)- and (18*S*)-configurations or their mirror image (17*R*)- and (18*R*)-configurations. Figure 3(b) compares the CD spectrum of S[N,E]BChl-c_F with that of structurally well-defined R[E,E]BChl-c_F from *Cba. tepidum* [with (17*S*)- and (18*S*)-configurations] in Et_2O. These pigments exhibited identical CD spectra in the visible region: negative and positive CD signals at the Qy and Soret regions, respectively. The results indicated that the two BChls-*c* had the same stereochemistry at the chlorin chromophore, so the absolute configurations at the 17- and 18-positions of newly identified S[N,E]BChl-c_F were confirmed as (17*S*)- and (18*S*)-configurations as generally seen in Chl pigments.

Effects of the 8^2-methylation upon absorption properties of monomeric BChls-*c*

Triply 8^2-methylated S[N,E]BChl-c_F gave a slight red-shift of the Soret absorption in Et_2O, compared to less methylated R[E,E]BChl-c_F as shown in Fig. 3(a). However, almost no shift of the Qy absorption upon the 8^2-methylation was observed. Using R[P,E], S[P,E], and S[I,E]BChl-c_F, slight but successive red-shifts of the Soret absorption upon the methylation were confirmed in Et_2O: R[E,E] (432.0 nm) →

Table 1 ^{1}H-chemical shifts (δ_Hs in ppm) of the (*R*)- and (*S*)-MTPA esters of [N,E]BPhe-c_F in $CDCl_3$ at room temperature

Protons	(*R*)-MTPA ester	(*S*)-MTPA ester	Δ[a]
5-H (*s*[b])	9.57	9.73	+0.16
10-H (*s*)	9.56	9.57	+0.01
3^1-H (*q*)	7.60 (7.8[c])	7.64 (6.6)	+0.04
MTPA-H_o (*d*)	7.19 (8.4)	7.40 (7.2)	+0.21
MTPA-H_p (*t*)	6.87 (7.5)	7.10 (7.5)	+0.23
MTPA-H_m (*t*)	6.71 (7.5)	6.95 (7.8)	+0.24
13^2-H_2 (*d*)	5.29 (19.2)	5.29 (19.2)	±0.00
	5.23 (19.2)	5.24 (19.2)	+0.01
f2-H (*t*)	5.24 (6.6)	5.23 (7.2)	−0.01
f6/f10-H (*t*)	5.01 (7.5)	5.01 (7.5)	±0.00
18-H (*q*)	4.60 (7.8)	4.60 (7.2)	±0.00
f1-H_2 (*d*)	4.53 (6.6)	4.53 (6.6)	±0.00
17-H (*m*)	4.22	4.22	±0.00
12^1-H_2 (*q*)	4.11 (7.8)	4.11 (7.8)	±0.00
20-CH_3 (*s*)	3.86	3.91	+0.05
MTPA-OCH_3 (*s*)	3.62	3.54	−0.08
8^1-H_2 (*s*)	3.60	3.61	+0.01
2-CH_3 (*s*)	3.43	3.26	−0.17
7-CH_3 (*s*)	3.07	3.05	–0.02
17^2-H_2 (*m*)	~2.53	~2.52	−0.01
3^1-CH_3 (*d*)	2.35 (7.2)	2.29 (6.6)	−0.06
17^1-H_2 (*m*)	~2.19	~2.18	−0.01
12^1-CH_3 (*t*)	1.94 (7.8)	1.94 (7.5)	±0.00
f3-CH_3 (*s*)	1.63	1.63	±0.00
f12-H_3 (*s*)	1.60	1.60	±0.00
f7-CH_3 (*s*)[d]	1.52	1.52	±0.00
f11-CH_3 (*s*)[d]	1.50	1.51	+0.01
18-CH_3 (*d*)	1.49 (7.8)	1.50 (6.6)	+0.01
8^1-$C(CH_3)_3$ (*s*)	1.26	1.24	−0.02
NH (*s*)	~0.47	~0.50	+0.03
NH (*s*)	−1.82	−1.78	+0.04
f4-, f5-, f8-, f9-H_2[e]	1.82–2.05	1.82–2.05	n.d.[f]

[a] $\Delta = \delta_H$[(*S*)-MTPA ester] − δ_H[(*R*)-MTPA ester]

[b] Multiplicity: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet

[c] Coupling constants in Hz

[d] The assignment is interchangeable

[e] These protons are not differentiated

[f] Not determined

R[P,E]/S[P,E] (432.4 nm) → S[I,E] (432.6 nm) → S[N,E] (433.4 nm). The results indicate that the extra 8^2-methylated substituents on the Qx axis of BChl-*c* molecules slightly affected their chlorin π-systems due to the successive addition of electron-donating methyl groups through two C–C single bonds. An alternative explanation for the red-shifts is a distortion of the chlorin macrocycles due to the steric hindrance between the 7-CH_3 and bulky C8-substituents. Consequently, the 8^2-methylation induced a slight but apparent effect upon absorption properties of monomeric BChls-*c*: at most 1.4 nm by triple 8^2-methylation. In contrast, a pair of diastereomers of BChls-*c* gave identical absorption spectra in Et_2O, indicating that the (3^1*R*/*S*)-stereochemistry did not affect absorption properties of the monomeric pigments: the Soret/Qy absorption maxima of R[P,E] and S[P,E]BChl-c_F were both observed at 432.4/660.6 as an example.

Alteration of pigment composition by the Δ*bciD* mutation

The Δ*bciD* mutant of *Cba. limnaeum* did not synthesize BChl-*e* pigments, but exclusively accumulated BChl-*c* species as in *Cba. tepidum*. By the mutation, the pigment composition was drastically altered in terms of methylation degrees at the 8^2-position and (*R*)- or (*S*)-stereochemistry at the 3^1-position. The ratio of (3^1*R*)- and (3^1*S*)-diastereomers of BChl species in the mutant was determined to be 19.4/80.6 (*S*-rich) by peak areas of HPLC, while that in the wild type showed the ratio of 56.8/43.2 (*R*-rich). The homolog composition of ethyl, propyl, isobutyl, and neopentyl groups at the 8-position in the mutant showed the ratio of 4.4/22.9/68.2/4.5 and was quite different from the wild type (36.7/44.7/18.2/0.4). These results indicate that the methylation ability of BchQ ($C8^2$-methyltransferase) and the hydration function of BchF/V (C3-vinyl-hydratase) (Chew et al. 2004) are affected by the mutation in BciD. The BchQ enzyme in the present *Cba. limnaeum* would have a higher activity against C7-methyl substrates than C7-formyl ones. Smith et al. (1983b) proposed that the C3-vinyl group in substrates possessing large alkyl substituents at the 8-position rotates 180° during its hydration, compared to that in substrates with a less methylated ethyl group. Thus, the hydration of the C3-vinyl groups would lead to chiral centers with the opposite configuration. The present stereochemical characterization of a new BChl-*c* carrying a large neopentyl group supports this hypothesis.

With a decrease of irradiated light intensity on green sulfur bacteria, the content of BChl homologs with a higher degree of methylation increased and concomitantly the Qy absorption maximum in vivo shifted to longer wavelengths (Bobe et al. 1990; Huster and Smith 1990; Borrego and Garcia-Gil 1995). Possibly, the variation of the alkyl side chains induced by the 8^2-methylation represents a mechanism of low-light adaptation; since BChl homologs with red-shifted Qy bands may facilitate the channeling of excitation energy toward reaction centers, thereby increasing the energy transfer efficiency in chlorosomes. Additionally, the present study revealed that the 8^2-methylation on BChl-*c* induced a slight but apparent red-shift of the Soret absorption at the monomeric state. The shift would serve as an increase in light-harvesting ability of the pigment. The differences in the pigment composition

between the wild type and its Δ*bciD* mutant of *Cba. limnaeum* may, thus, reflect differences in the capability of their low-light adaptation.

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