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Determination of Stereochemistry of Bacteriochlorophyll g_F and 8^1-Hydroxy-chlorophyll a_F from *Heliobacterium modesticaldum*¶

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ABSTRACT

The reaction center complex of heliobacteria contains three kinds of chlorophyll pigments, bacteriochlorophyll g_F (BChl g_F), its 13^2-epimer BChl g'_F and 8^1-hydroxy-chlorophyll a_F (8^1-OH-Chl a_F). Because the full stereochemistry of these naturally occurring chlorophyllous pigments has remained unknown, we determined the stereochemistry of both BChl g_F and 8^1-OH-Chl a_F extracted from *Heliobacterium modesticaldum*. The configurations of the specific functional groups at ring-B as well as those at ring-D and -E were investigated by use of nuclear Overhauser effect correlations in their ^{1}H-NMR spectra and circular dichroism spectra, as well as by modified Mosher's method in their chemical modification: (1) *E*-configuration was confirmed for the 8-ethylidene group at ring-B in BChl g_F, (2) *R*-configuration was identified for the 1-hydroxyethyl group at ring-B in 8^1-OH-Chl a_F and (3) 13^2-(*R*)-, 17-(*S*)- and 18-(*S*)-configurations at ring-D and -E in both BChl g_F and 8^1-OH-Chl a_F were confirmed. These stereochemistries enabled us to discuss their biosynthesis and to propose possible routes for preparation of ethylidene and 1-hydroxyethyl groups at the 8-position.

INTRODUCTION

Bacteriochlorophylls (BChls) are major pigments in photosynthetic bacteria. They function as light-harvesting and energy-migrating pigments in the antenna complexes and as electron transfer pigments in the reaction centers. The molecular structures of BChl, including the stereochemistry of the specific functional groups, have been extensively studied for the purpose of constructing photosynthetic apparatuses and elucidating photosynthetic mechanisms, as well as biosynthetic pathways of BChl molecules (1).

The BChl pigment in both the reaction center (2) and antenna complexes (3) in purple bacteria is generally a single molecular structure, as in either BChl *a* or *b*, which is dependent on the species; their metal-free derivatives, bacteriopheophytins (BPhes), as the primary electron-accepting pigment are also present in the reaction center. On the other hand, heliobacteria contain three kinds of (B)Chl: BChl g_F (4,5), its 13^2-epimer BChl g'_F (6) and 8^1-hydroxy-chlorophyll a_F (8^1-OH-Chl a_F [7], see Fig. 1a). BChl g_F is a major BChl pigment in the reaction center complex in heliobacteria, and two other special derivatives, BChl g'_F(6) and 8^1-OH-Chl a_F (7), have been found in its reaction center. The molar ratio of BChl g_F to BChl g'_F to 8^1-OH-Chl a_F in the reaction center complex of *Heliobacterium* (*Hbt.*) *chlorum* was reported to be 36–40:2:2 per unit (6,7), and the electron transfer reaction center is assumed to contain two BChl g'_F as the special pair (P798) (6), two BChl g_F as the accessory pigment and two 8^1-OH-Chl a_F as the primary acceptor (7). The molecular structure of BChl g_F is closely related to that of BChl b_P from *Blastochloris* (*Blc.*) *viridis*: both are characterized by an ethylidene group at the 8-position (4,8). BChl g_F has a vinyl at the 3-position and a farnesyl (C15, designated with subscript F) group in the 17-propionate (4,5), whereas BChl b_P has the 3-acetyl and the phytyl (C20, designated with subscript P) groups (8,9).

The minor components BChl g'_F and 8^1-OH-Chl a_F are biosynthesized from BChl g_F or its precursors (10). Thus, it is important to determine the configuration of these (B)Chl derivatives; however to our best knowledge no report is available on their stereochemistry except for ambiguous speculation (*vide infra*). Brockmann and Lipinski (4) speculated on the stereochemistry of BChl *g*, comparing the circular dichroism (CD) and ^{1}H-NMR spectra of its chemically modified products with those of structurally well-defined chlorophylls (Chls) as follows. The stereochemistry of the 7-, 17- and 18-positions in BChl *g* was tentatively assigned as *R*-, *S*- and *S*-configurations, respectively, on the basis of the CD signs of Chl *a* and BChl *b*. The stereochemistry of the 8- and 13^2-positions was also proposed to be *E*- and *R*-configurations, respectively, from their proton chemical shifts. By mass spectrometry, Michalski *et al.* (5) established the molecular structure of BChl *g* as possessing a farnesyl group as the 17-propionate but did not determine any stereochemistry of BChl g_F.

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Abbreviations: 8^1-OH-Chl, 8^1-hydroxy-chlorophyll; Abs, absorbance; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; *Blc.*, *Blastochloris*; CD, circular dichroism; Chl, chlorophyll; COSY, correlation spectroscopy; FAB-MS, fast atom bombardment mass; *Hba.*, *Heliobacillus*; *Hbt.*, *Heliobacterium*; MTPA, α-methoxy-α-(trifluoromethyl)-phenylacetyl; NOE, nuclear Overhauser effect; Phe $a_{F\ (P)}$, pheophytin *a* [subscript F (P) means farnesyl (phytyl) ester]; Phe a_M, methyl pheophorbide *a*; ROESY, rotating-frame Overhauser effect spectroscopy; TMS, tetramethylsilane.

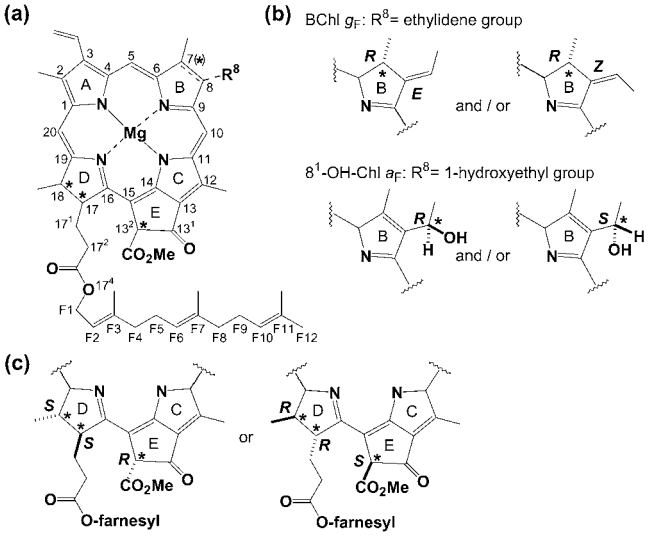


Figure 1. Expected molecular structures of BChl g_F ($*C^7H–C^8=CHCH_3$) and 8^1-OH–Chl a_F ($C^7=C^8–*CH(OH)CH_3$) from *Hbt. modesticaldum* (**a**). Their partial structure is shown at ring-B (**b**) and at ring-D and -E (**c**). The numbers on carbon atoms are given by IUPAC-IUB nomenclature. Optically active carbon atoms are indicated by asterisks.

Here, we first report the stereochemistry of BChl g_F and 8^1-OH-Chl a_F extracted from *Hbt. modesticaldum* without ambiguity. On the basis of the present stereochemical findings, we discuss the structural relationship between BChl g_F and 8^1-OH-Chl a_F, especially for the configurational correlation between the ethylidene group of BChl g_F and the 1-hydroxyethyl group of 8^1-OH-Chl a_F, and we also propose possible biosynthetic routes for introduction of the 8-ethylidene and 8-(1-hydroxyethyl) groups (5).

MATERIALS AND METHODS

General methods. HPLC was performed with a Shimadzu LC-10AD liquid chromatograph equipped with a Shimadzu SPD-M10A diode-array detector (Shimadzu Ltd., Kyoto, Japan). Fast atom bombardment mass (FAB-MS) spectra were recorded with a JEOL GCmate II spectrometer (JEOL Ltd., Akishima, Japan); *m*-nitrobenzyl alcohol was used as a matrix. ^{1}H-NMR spectra in $CDCl_3$ (99.8% CEA, Gif-Sur-Yvette, France) were recorded at room temperature with a JEOL JNM-A400 Fourier transform NMR spectrometer; tetramethylsilane (TMS) was used as an internal standard. Spectra from ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{1}H rotating-frame Overhauser effect spectroscopy (ROESY; τ_m = 250 ms) were recorded to aid in assignment of the proton signals. Visible absorption and circular dichroism (CD) spectra were measured with a Hitachi U-3500 spectrophotometer (Hitachi Ltd., Tokyo, Japan) and a JASCO J-720W spectropolarimeter (JASCO Ltd., Hachioji, Japan), respectively. All solvents were used without further purification except dry pyridine for preparation of Mosher's esters.

Hbt. modesticaldum was grown in a pyruvate–yeast extract medium (11) at 45°C instead of the optimal temperature of 50–52°C for 24 h to avoid accumulation of appreciable amounts of lysed cell (12). The wet cells were extracted with a mixture of methanol and acetone (1:10 vol/vol) under nitrogen, then the solution was evaporated in a rotary evaporator to dryness. The residue was dissolved in acetone, treated with aqueous diluted HCl (30 m*M*) (13) and poured into aqueous 4% $NaHCO_3$. After evaporation, resulting BPhe g_F and 8^1-hydroxy-pheophytin a_F (8^1-OH-Phe a_F) were isolated by the following HPLC conditions: normal phase column (SiO_2); cosmosil 5SL-II 6.0 mm $\phi \times 250$ mm (Nacalai Tesque Inc., Kyoto, Japan); eluent, hexane:2-propanol:methanol = 100:2.0:0.2 (vol/vol) for analysis and hexane:acetone = 4:1 (vol/vol) for preparation; flow rate, 1.5 mL/min for analysis and 3.0 mL/min for preparation. Methyl pheophorbide *a* (Phe a_M) as an authentic sample was prepared from the demetallation and transesterification of Chl a_P extracted from cyanobacterium, *Spirulina geitleri* as reported previously (14).

Isomerization of BPhe g_F *to "Phe* a_F*"*. Aqueous HCl (4.6 M, 500 μL) was added to 10 mL of an acetone solution of BPhe g_F (absorbance at 748 nm, Abs[λ = 748 nm] = 2.7/cm) (10). The mixture was stirred at room temperature for 30 min in the dark, poured in H_2O, extracted with $CHCl_3$, washed with H_2O three times, dried over Na_2SO_4 and filtered. After evaporation, the residue was purified by the above described HPLC scheme to give pure "pheophytin a_F" (Phe a_F).

Transesterification of "Phe a_F*" to "Phe* a_M*"*. An ice-chilled methanolic sulfuric acid solution (6.4 M, 1.0 mL) was added to a 2.0 mL methanol solution of "Phe a_F" (Abs[λ = 666 nm] = 3.5/cm) (8,15,16). The mixture was stirred at room temperature for 90 min in the dark, poured into ice water, extracted with CH_2Cl_2, washed with H_2O twice, dried over Na_2SO_4 and filtered. After evaporation, the residue was purified by HPLC to give pure "Phe a_M."

Preparation of Mosher's ester of 8^1*-OH-Phe* a_F. Commercially available and optically active α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, 10 μL, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) was added in the dark to 100 μL of a dry pyridine solution of 8^1-OH-Phe a_F (*ca* 0.38 mg) (17). After stirring at room temperature for 1 h, *N*,*N*-dimethyl-1,3-propandiamine (15 μL; Nacalai Tesque) was added. The mixture was stirred for 15 min, poured into aqueous 4% $NaHCO_3$, extracted with CH_2Cl_2, washed with H_2O twice, dried over Na_2SO_4 and filtered. After evaporation, the residue was purified by HPLC to give the corresponding desired ester.

RESULTS AND DISCUSSION

Molecular structures of BChl g_F and 8^1-OH-Chl a_F from *Hbt. modesticaldum*

Figure 1 shows the molecular structure of BChl g_F and 8^1-OH-Chl a_F in which F denotes the farnesyl group at the 17^4-position. Optically active carbon atoms are indicated by asterisks. BChl g_F is characterized by the ethylidene group at the 8-position (4), which was also seen in BChl b_P from *Blc. viridis* (8,9). The configurations of the ethylidene group shown in the upper part of Fig. 1b and at the chiral 13^2-, 17- and 18-positions shown in Fig. 1c had been undetermined. 8^1-OH-Chl a_F is also characterized by the 1-hydroxyethyl group at the 8-position (7). The absolute configurations of the 1-hydroxyethyl group shown in the lower part of Fig. 1b and at the 13^2-, 17- and 18-positions shown in Fig. 1c had also remained unsolved. Replacement of the central Mg^{2+} in these (B)Chls by two protons gives the corresponding (B)Phe derivatives: BPhe g_F and 8^1-OH-Phe a_F.

HPLC of extracted pigments from *Hbt. modesticaldum*

Figure 2 shows a set of representative HPLC analyses of pigments extracted from the cells of *Hbt. modesticaldum* (panel a) and after acid treatment (panel b). In Fig. 2a, five peaks were resolved at the region before 23 min of retention time. On the basis of their visible spectra, each peak was assigned as peak 1 = a carotenoid, peaks 2 and 3 = BPhe *g* species and peaks 4 and 5 = BChl *g* species. An additional small peak was observed at 116 min (peak 6) shown in the insert of Fig. 2a. This highly polar peak has a visible spectrum similar to Chl *a* and is assigned as 8^1-OH-Chl a_F (7).

The stereoisomers at the 13^2-position exhibit a general order of normal phase HPLC elution: Chl *a* (Phe *a*) from higher plants and BChl *a* (BPhe *a*) from purple bacteria possessing the 13^2-(*R*)-configuration tend to elute more slowly than the corresponding 13^2-(*S*)-epimer, Chl *a*′ and BChl *a*′, respectively (13,18). Furthermore, nonprimed types, 13^2-(*R*)-isomers are always the major component, and primed types, 13^2-(*S*)-isomers are minor in higher plants, algae and cyanobacteria (19,20) as well as in green sulfur bacteria (18). As in the case of *Hbt. modesticaldum*, one can predict that a set of stereoisomers at the 13^2-position should appear in the general

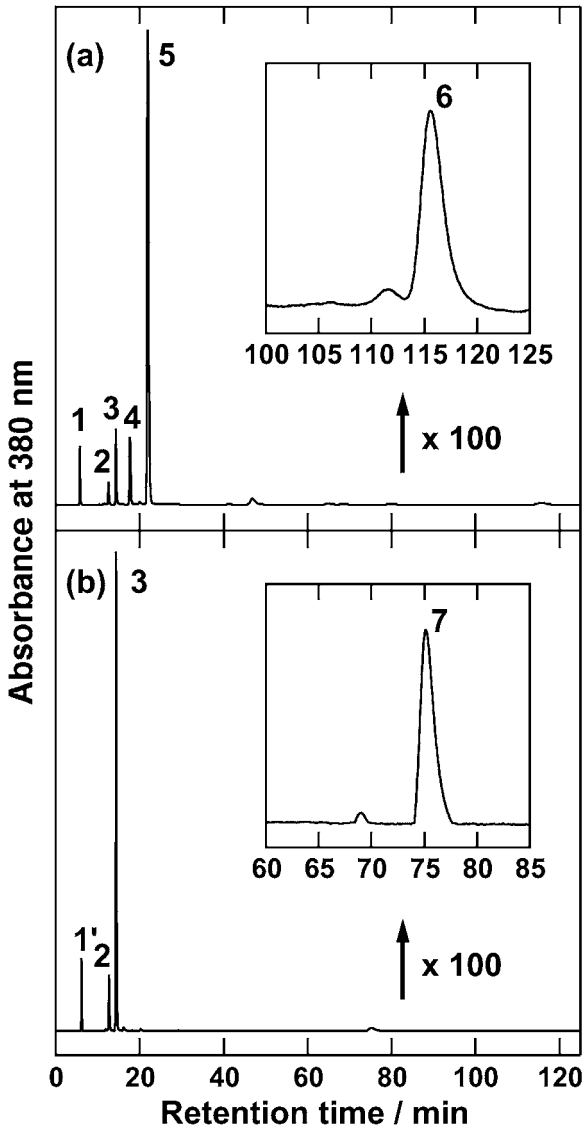


Figure 2. A set of representative HPLC profiles of pigments extracted from the cells of *Hbt. modesticaldum* (**a**) and after acid treatment (**b**) (column, cosmosil 5SL-II, 6.0 mm $\phi \times 250$ mm; eluent, hexane:2-propanol:methanol = 100:2.0:0.2, vol/vol).

elution order (6). Thus, peaks 2 and 4 were assigned to the primed-type pigments, and peaks 3 and 5 to nonprimed-type pigments: peak 2 = BPhe g'_F, peak 3 = BPhe g_F, peak 4 = BChl g'_F and peak 5 = BChl g_F. This assignment of each peak is consistent with reported data (6,7).

Table 1. FAB-MS spectral data of BPhe g_F and 8^1-OH-Phe a_F from *Hbt. modesticaldum* and synthetic (*R*)/(*S*)-MTPA esters of 8^1-OH-Phe a_F

		Molecular ion peak	
	Chemical formula	Observed	Calculated
BPhe g_F	$C_{50}H_{60}N_4O_5$	796.4	796.5
8^1-OH-Phe a_F	$C_{50}H_{60}N_4O_6$	812.6	812.5
(*R*)-MTPA ester	$C_{60}H_{67}F_3N_4O_8$	1028.5	1028.5
(*S*)-MTPA ester	$C_{60}H_{67}F_3N_4O_8$	1028.5	1028.5

Table 2. ^{1}H-chemical shifts (δs in ppm) in $CDCl_3$ (TMS as an internal standard)

Protons	BPhe g_F	8^1-OH-Phe a_F	(*R*)-MTPA ester	(*S*)-MTPA ester	Δ*
10-H	8.780	10.075	9.804	9.612	−0.192
5-H	8.416	9.438	9.485	9.462	−0.023
20-H	8.393	8.588	8.620	8.615	−0.005
3^1-H	7.877	8.014	8.017	8.015	−0.002
8^1-H	7.082	6.247	7.392	7.292	−0.100
13^2-H	5.992	6.272	6.280	6.279	−0.001
3^2-H	6.151	6.256	6.268	6.267	−0.001
18-H	4.311	4.470	4.476	4.480	+0.004
17-H	3.910	4.225	4.230	4.238	+0.008
13^2-CO_2Me	3.812	3.888	3.899	3.906	+0.007
MTPA-OMe	—	—	3.382	3.593	+0.211
12-Me	3.350	3.695	3.494	3.479	−0.015
2-Me	3.251	3.415	3.426	3.425	−0.001
7-Me	1.821	3.346	3.364	3.325	−0.039
17^2-H_2	∼2.50†	∼2.56†	∼2.56†	∼2.55†	∼−0.01
17^1-H_2	∼2.28†	∼2.26†	∼2.27†	∼2.26†	∼−0.01
8^1-Me	2.262	2.138	2.208	2.293	+0.085
18-Me	1.685	1.813	1.820	1.835	+0.015
NH	‡	0.381	0.236	0.212	−0.024
NH	‡	−1.740	−1.814	−1.812	+0.002
MTPA-H_o	—	—	7.456	∼7.26§	∼−0.20
MTPA-H_m	—	—	6.743	7.027	+0.284
MTPA-H_p	—	—	7.145	6.867	−0.278

*Δ = δ[(*S*)-MTPA ester] − δ[(*R*)-MTPA ester].
†Values were obtained from the contour plots of 2D spectra because of the multiple splitting of signals.
‡Values were not obtained because of the broadening of signals.
§Value was obtained from the contour plots of the 2D spectrum because of overlap with the solvent peak ($CHCl_3$).

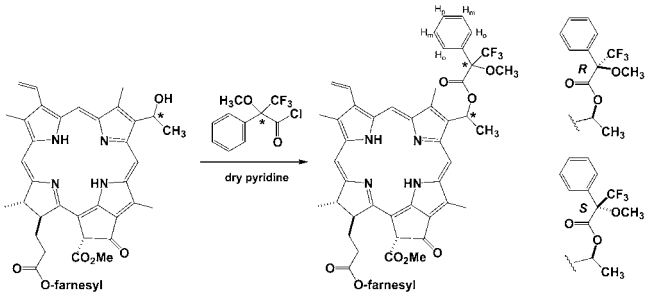
Acid treatment of extracted pigments from *Hbt. modesticaldum*

Because BChl g_F was reported to be quite unstable (4), we demetallated (B)Chls from *Hbt. modesticaldum* to get more stable metal-free (B)Phes. When the intact extract was treated with an aqueous diluted HCl solution, BChl g'_F/g_F (peaks 4 and 5), seen in Fig. 2a, were completely eliminated, with increases of the corresponding BPhe g'_F/g_F (peaks 2 and 3) (6). The metal-free 8^1-OH-Phe a_F is also seen at 75 min (peak 7) in the insert of Fig. 2b. Compared with Figs. 2a, b, no significant epimerization at the 13^2-position was observed under the present experimental conditions: this means that the configurations of metal-free (B)Phes obtained by the acid treatment retain those of the original (B)Chls. Therefore, we

Table 3. Observed ^{1}H–^{1}H NOE correlations of BPhe g_F and 8^1-OH-Phe a_F from *Hbt. modesticaldum* in $CDCl_3$

(B)Chl	Observed NOE correlations		
BPhe g_F*	8^1-H	⇔	10-H
	8^1-CH_3	⇔	7-H
	8^1-CH_3	⇔	7-CH_3
BPhe g_F†	13^2-H	⇔	17^1-H / 17^2-H
	18-H	⇔	17^1-H / 17^2-H
	18-CH_3	⇔	17-H
8^1-OH-Phe a_F†	13^2-H	⇔	17^1-H / 17^2-H
	18-H	⇔	17^1-H / 17^2-H
	18-CH_3	⇔	17-H

*NOE correlations around ring-B.
†NOE correlations around ring-D and -E.



Scheme 1. Synthesis of (*R*)- and (*S*)-Mosher's esters of 8^1-OH-Phe a_F.

analyzed the resulting (B)Phes for the stereochemical determination of the intact (B)Chls. Both BPhe g_F and 8^1-OH-Phe a_F were characterized by means of FAB-MS spectrometry and ^{1}H-NMR spectroscopy. Table 1 summarizes the results of mass spectrometry, and Table 2 shows the ^{1}H chemical shift of the HPLC-isolated BPhe g_F (peak 3) and 8^1-OH-Phe a_F (peak 7) in $CDCl_3$.

Stereochemistry at ring-B in BPhe g_F and 8^1-OH-Phe a_F

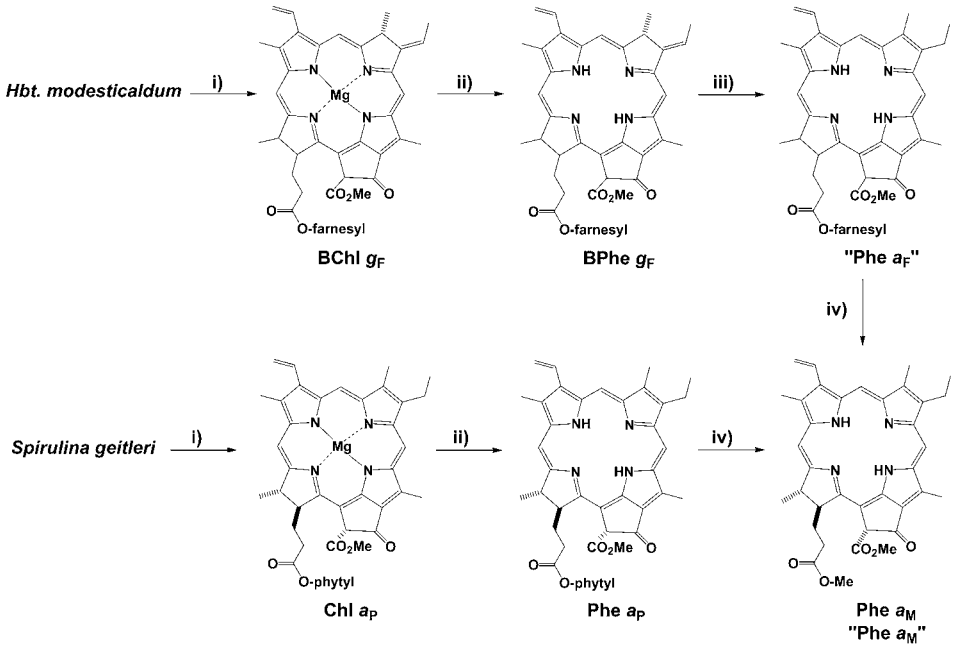
Configuration of the ethylidene group in BPhe g_F *with* ^1H–^1H *NOE correlations*. The assignments of the ^{1}H signals in BPhe g_F were obtained with COSY and ROESY spectra. After the assignments were established, their nuclear Overhauser effect (NOE) correlations were used to determine the configurations of the 8-ethylidene group at ring-B. The two configurations of the ethylidene group are shown in the upper part of Fig. 1b. Table 3 lists a set of NOE correlations around the ring-B found for BPhe g_F dissolved in $CDCl_3$. The apparent NOE correlations between 8^1-H and 10-H, as well as 8^1-CH_3 and 7-H/7-CH_3, clearly indicate the *E*-configuration for the tri-substituted double bond at the 8-ethylidene group of BPhe g_F. The result is compatible with the stereochemistry of the 8-ethylidene group in the structurally related BChl b_P (9). By analogy with the stereochemistry of BChl *a* (16), the *R*-configuration is expected for the 7-position of BPhe g_F.

Stereochemistry of the 1-hydroxyethyl group in 8^1*-OH-Phe* a_F *with modified Mosher's method*. According to the ^{1}H-NMR spectrum of isolated 8^1-OH-Phe a_F (lack of satellite signals), it was a single stereoisomer. Epimerically pure 8^1-OH-Phe a_F was treated with commercially available (+)-MTPA-Cl in dry pyridine to afford the corresponding Mosher's ester, (*R*)-MTPA ester (see Scheme 1). Similarly, reaction of 8^1-OH-Phe a_F with (−)-MTPA-Cl gave the corresponding (*S*)-MTPA ester. The resulting MTPA esters were characterized by both FAB-MS (Table 1) and ^{1}H-NMR spectra (Table 2).

Recently, modified Mosher's method was reported to be effective for determination of epimerically pure secondary alcohols, including Chl derivatives (17,21,22). Table 2 lists the ^{1}H-chemical shifts (δs) of the (*R*)-MTPA and (*S*)-MTPA esters together with their differences, $\Delta = \delta[(S)\text{-MTPA ester}] - \delta[(R)\text{-MTPA ester}]$. The δs of the 8^1-methyl group in (*R*)- and (*S*)-MTPA esters were measured in $CDCl_3$ to be 2.208 and 2.293 ppm, respectively. The Δ value (=2.293 − 2.208 = +0.085 ppm) was positive. In contrast, all the Δs at the assigned protons on the peripheral positions of the chlorin π-system gave negative values, which tended to increase with an increase of the distance from the 8^1-chiral position. According to modified Mosher's rule (21), the secondary alcohol was assigned to the *R*-configuration at the 8^1-position. Moreover, the Δ value in the methoxy group of the MTPA esters was positive, +0.211 ppm, indicating that epimerically pure 8^1-OH-Phe a_F has the *R*-configuration at the 8^1-position on the basis of the other modified Mosher's rule (22).

Stereochemistry at ring-D and -E in BPhe g_F and 8^1-OH-Phe a_F

Configurations of the 13^2*-, 17- and 18-positions in BPhe* g_F *and* 8^1*-OH-Phe* a_F *with* ^1H–^1H *NOE correlations*. To determine the configurations at the 13^2-, 17- and 18-positions, first we use NOE correlations in NMR spectroscopy. Here, we noted that only the *relative* configurations and not the *absolute* configurations were determined because a pair of enantiomers (*e.g.* as shown in Fig. 1c) could not be distinguished in the NOE experiments.



Scheme 2. Chemical modification of BChl g_F to Phe a_M: (i) extraction with acetone/methanol; (ii) demetallation with diluted HCl; (iii) isomerization with HCl/acetone; (iv) transesterification with H_2SO_4/methanol.

Table 3 lists the observed ^{1}H–^{1}H NOE correlations around ring-D and -E in BPhe g_F and 8^1-OH-Phe a_F. The NOE correlation between 13^2-H and 17-CH_2 supported the location of 13^2-H and 17-CH_2CH_2 in the *syn*-orientation. The three NOE correlations between 18-H and 17^1-H, 18-H and 17^2-H and 18-CH_3 and 17-H supported the location of 17-CH_2CH_2 and 18-H (17-H and 18-CH_3) also in the *syn*-orientation. From these NOE correlations, the configurations around ring-D and -E in both BPhe g_F and 8^1-OH-Phe a_F were confirmed to be 13^2-(*R*)-, 17-(*S*)- and 18-(*S*)-configurations or 13^2-(*S*)-, 17-(*R*)- and 18-(*R*)-configurations shown in Fig. 1c. No apparent NOE correlations originating from the *anti* orientation of the relevant protons were observed except for weak correlations between 13^2-H and 17-H in both BPhe g_F and 8^1-OH-Phe a_F.

Configurations of the 13^2-, 17- and 18-positions in BPhe g_F *with chemical modifications*. To confirm the relative configuration in BPhe g_F discussed above, we used chemical modification to the structurally well-defined Chl derivative (Phe a_M) and HPLC analyses. The chemical modification of BChl g_F to ''Phe a_M'' was performed as shown in Scheme 2. Phe a_M was also prepared from the structurally well-defined Chl a_P as an authentic sample. It is noted that Chl a_P has the 13^2-(*R*)-, 17-(*S*)- and 18-(*S*)-configurations (20,23). First, major BChl g_F extracted from *Hbt. modesticaldum* was treated with aqueous 0.175% HCl to give the metal-free derivative BPhe g_F. No significant epimerization at the 13^2-position under the demetallation reaction was observed (see Fig. 1a,b). Second, ''Phe a_F'' was prepared from isomerization of BPhe g_F under acidic conditions (10). Figure 3a,b show the HPLC profiles before and after acidic isomerization of BPhe g_F, respectively. Under the present isomerization conditions, slight epimerization (a peak indicated by an asterisk in Fig. 3b) and some degradation were observed. Finally, ''Phe a_M'' was prepared from acidic transesterification of ''Phe a_F'' (8,15,16). HPLC analyses before and after treatment of purified ''Phe a_F'' with methanol containing sulfuric acid shown in Fig. 3c, d were also performed to check the epimeric purities. ''Phe a_M'' obtained from BChl g_F was identical with Phe a_M from Chl a_P because the cochromatographic analysis of ''Phe a_M'' and Phe a_M exhibits only a single peak (data not shown). Therefore, the configurations at the 13^2-, 17- and 18-positions in BChl g_F were determined to be (*R*)-, (*S*)- and (*S*)-configurations, respectively, which were usually seen in photosynthesis or its mirror image (*SRR*).

Absolute stereochemistries at the 13^2-, 17- and 18-positions in BPhe g_F *and 8^1-OH-Phe* a_F *with CD spectra*. To distinguish the pair of enantiomers confirmed above, we used their CD spectra in acetone. The solid lines of Fig. 4a show the visible (upper) and CD spectra (lower) of the structurally well-defined Phe a_P from *Spirulina geitleri*; the 13^2-(*S*)-epimerical isomer Phe a'_P gave the dotted spectrum. The CD spectra of the epimers, Phe a_P [13^2-(*R*)] and Phe a'_P [13^2-(*S*)] are considerably different, whereas their visible spectra are essentially the same (13). The comparison is limited to the three sets of electronic transitions in the visible region, the Q_y, Q_x and Soret absorption bands.

Q_y transition region. An intense peak of Chl derivatives at the longest wavelength corresponds to the $Q_y(0,0)$ transition, and a smaller peak at the blue side is assigned to the $Q_y(0,1)$ band. In 13^2-(*S*)-Phe a'_P, a more intense negative CD band was observed at the $Q_y(0,0)$ region than in 13^2-(*R*)-Phe a_P (13).

Q_x transition region. $Q_x(0,0)$ absorption peaks appear at 535 nm for Phe a_P (a'_P) with a well-resolved $Q_x(0,1)$ satellite at 505 nm. The 13^2-(*R*)-Phe a_P exhibits almost no CD peak in this region, and

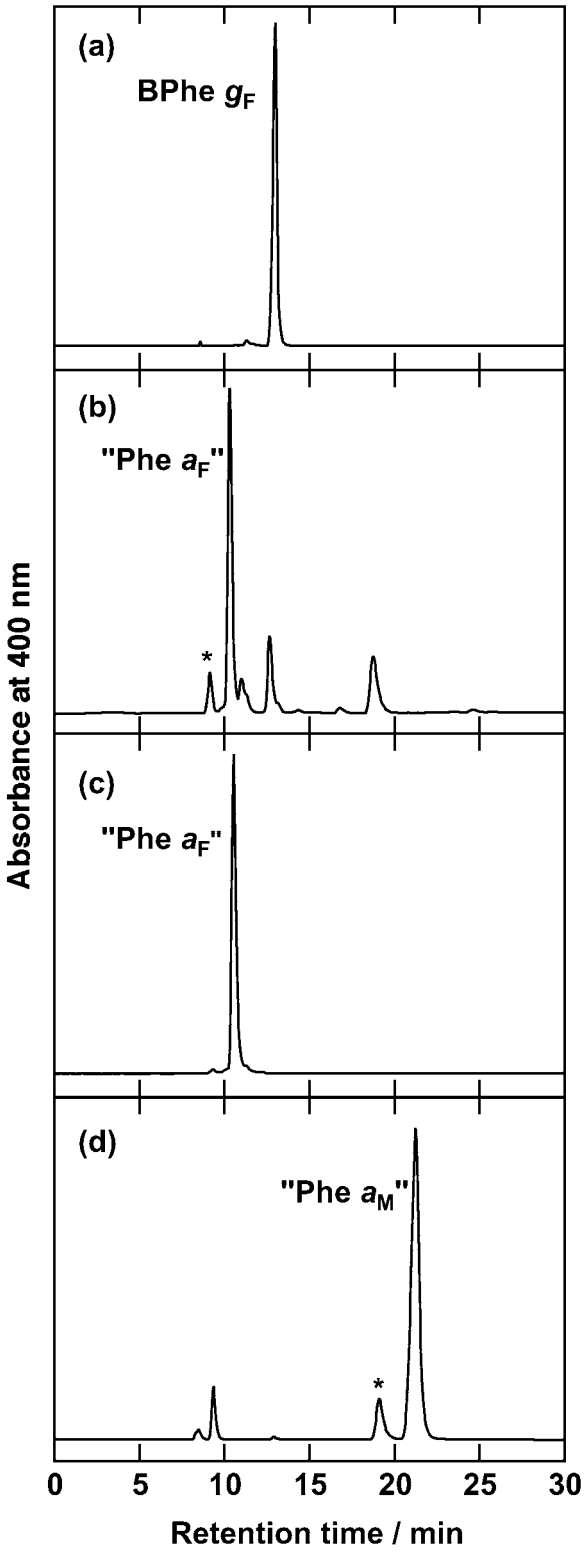


Figure 3. HPLC profiles before (**a**) and after acidic isomerization of BPhe g_F to ''Phe a_F'' (**b**) and transesterification of C17-farnesyl in pure ''Phe a_F'' (**c**) to methyl ester in ''Phe a_M'' (**d**). A small amount of the corresponding 13^2-(*S*)-isomer shown by asterisks was observed under the reaction.

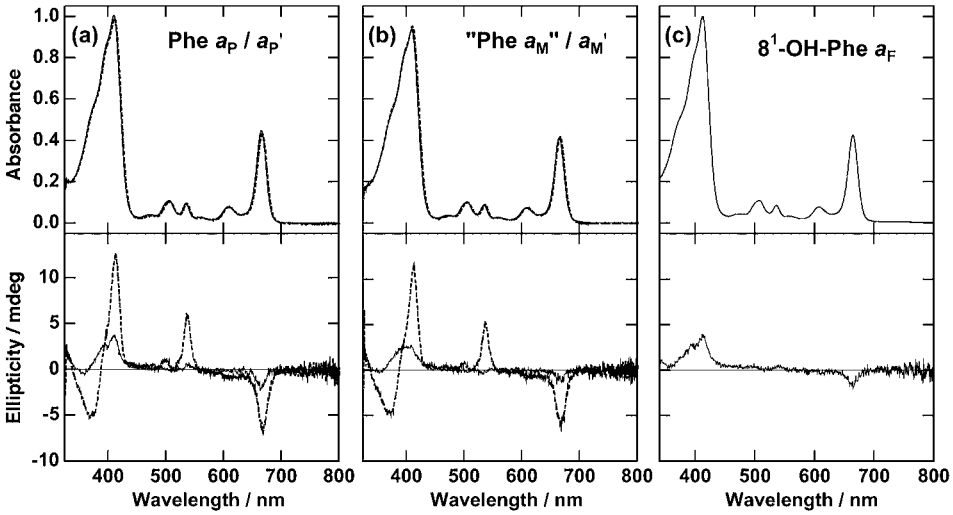


Figure 4. Visible (**upper**) and CD spectra (**lower**) Chl derivatives in acetone; Phe a_P/a'_P (**a**), "Phe a_M"/a'_M (**b**) and 8^1-OH-Phe a_F (**c**). Nonprimed-type Chl derivatives gave the solid lines and the primed-type the dotted lines. All the samples were adjusted to absorbance = 1.0/cm at the Soret band.

13^2-(*S*)-Phe a'_P shows a fairly intense CD whose sign is opposite that of the Q_y transitions described above (13).

Soret band region. 13^2-(*S*)-Phe a'_P gives strong S-shape CD signals at around 400 nm, the Soret region, whereas the CD spectrum of 13^2-(*R*)-Phe a_P gives weak positive CD signals (13).

On the basis of the above findings, the absolute configurations at the 13^2-position of metal-free Chl derivatives were confirmed from the CD spectra (13).

Figure 4 also indicates the visible and CD spectra of "Phe a_M" prepared from BPhe g_F (panel b) and 8^1-OH-Phe a_F (panel c). Compared with the CD spectrum of Phe a_P (solid line of Fig. 4a, bottom), "Phe a_M" exhibits a similar spectral pattern in the above three transitions (solid line of Fig. 4b, bottom). Similarly, 8^1-OH-Phe a_F shown at the bottom of Fig. 4c gives a weak negative CD at $Q_y(0,0)$, almost no CD peak at $Q_x(0,0)$ and weak positive CD signals at the Soret band. Thus 8^1-OH-Phe a_F also has the 13^2-(*R*)-configuration. From the combination of the above CD spectral findings with the observed NOE correlations, the stereochemistry at ring-D and -E in both BPhe g_F and 8^1-OH-Phe a_F was unambiguously determined to be 13^2-(*R*), 17-(*S*) and 18-(*S*). The complete molecular structures of BChl g_F and 8^1-OH-Chl a_F that have been determined in this study are shown in Scheme 3a.

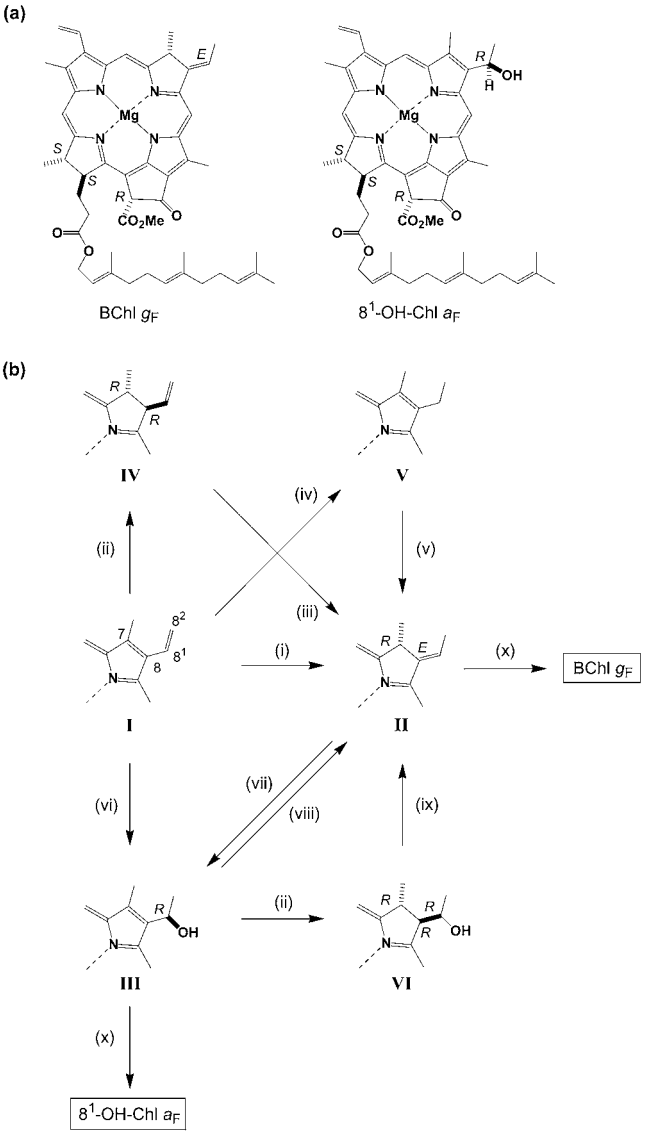
Possible biosynthetic pathways for BChl g_F and 8^1-OH-Chl a_F in *Hbt. modesticaldum*

Biosynthesis of chlorophyllous pigments has been widely investigated by various molecular genetic techniques to identify, clone, characterize and express the genes encoding their biosynthetic enzymes. Xiong *et al.* (24) reported that most of the genes for biosynthesis of (B)Chl from protoporphyrin IX (*bchI, bchD, bchH, bchM, bchE, bchJ, bchL, bchN, bchB* and *bchG*) are present in a gene cluster in *Heliobacillus* (*Hba.*) *mobilis*. Recently, Raymond *et al.* (25) also reported that *Hba. mobilis* possessed enzymes encoding *bchX* to reduce ring-B. No unique genes have been identified yet that have obvious roles in introducing an ethylidene group at the 8-position.

Scheme 3b depicts possible routes for the formation of BChl g_F and 8^1-OH-Chl a_F on the basis of the present stereochemical findings in combination with the reported enzymatic pathways for (B)Chl *a* (1,26,27). To clarify the routes, other parts except ring-B were omitted in Scheme 3b. Here, we assume that 8-vinyl-chlorophyllide *a* (**I**) is the precursor for the formation of both BChl g_F and 8^1-OH-Chl a_F because **I** would be a precursor found in (B)Chl biosyntheses and biosynthetically available in heliobacteria on the basis of the genetic evidence obtained so far mentioned above. This assumption is also consistent with the identical configurations at chiral 13^2-, 17- and 18-positions of both BChl g_F and 8^1-OH-Chl a_F to those of (B)Chl *a*. We propose several alternative routes from **I** to bacteriochlorophyllide *g* (**II**) and 8^1-hydroxy-chlorophyllide *a* (**III**) as follows. Both BChl g_F and 8^1-OH-Chl a_F would be prepared by esterification of **II** and **III**, respectively, with BchG as in step x of Scheme 3(b).

First, we discuss the biotransformation of **I** to **II**. One is the direct route without any intermediates shown in step i, and the others are the indirect routes *via* intermediates (**IV** or **V**) shown in steps ii–iii and iv–v. Similar steps giving a unique ethylidene group to step i have been reported in phycobilin biosynthesis: phycocyano(phycoerythro)bilin possessing an ethylidene group at the 3-position was synthesized from (15,16-dihydro)biliverdin IXα possessing the 3-vinyl group by PcyA(PebB) (28). Such enzymes and the corresponding genes have not yet been found in heliobacteria. Both steps ii and iv were seen in (B)Chl *a* biosyntheses: step ii is the *trans*-hydrogenation of the 7,8–double bond catalyzed by BchX,Y,Z and step iv is the reduction of the 8-vinyl group catalyzed by BchJ (1,26,27). Both bchX and bchJ have been identified in *Hba. mobilis* as described above. Although no enzymatic activities have yet been reported to be present for steps iii and v, stereospecific hydrogen shifts in **IV** and **V** could produce isomerized **II**.

Next, we could propose two biotransformations of **I** to **III**: one is the direct route without any intermediates as shown in step vi, and the other is the indirect route *via* the intermediate **II** shown in steps i–vii. Similar hydration of a vinyl to 1-hydroxyethyl group at the 3-position to step vi can be seen in (B)Chl *a* biosyntheses: the enzyme encoded by *bchF* catalyzes the reaction. Recently, it has been postulated that BchF stereospecifically would hydrate to give the 3^1-*R* configuration (29), which is consistent with **I** → **III** at the 8-position. Unfortunately, the *bchF* gene has not yet been identified in heliobacteria. As in transformation of **II** to **III**, stereospecific oxidation of an allyl group occurs: 7C*H–8C=CH → 7C=8C–C*HOH. An oxygen species attacks the 8^1-position of **II** from the *re*-face, which is the *anti*-direction to the 7-methyl group and sterically less hindered. The resulting secondary alcohol does possess the *R*-



Scheme 3. (**a**) Determined molecular structures of BChl g_F and 8^1-OH-Chl a_F from *Hbt. modesticaldum*. (**b**) Possible biosynthetic routes from 8-vinyl-chlorophyllide *a* (**I**) to BChl g_F and 8^1-OH-Chl a_F: (i) $7,8^2$-hydrogenation/7-*si*-face; (ii) 7,8-*anti*-hydrogenation/7-*si*-face, 8-*si*-face; (iii) $8 \rightarrow 8^2$ H-shift isomerization; (iv) $8^1,8^2$-hydrogenation; (v) $8^1 \rightarrow$ 7H-shift isomerization/7-*si*-face; (vi) Markovnikov-type hydration of 8-vinyl group/8^1-*re*-face; (vii) 7-dehydrogenation and 8^1-hydroxygenation/8^1-*re*-face; (viii) 7-hydrogenation/7-*si*-face and 8^1-dehydroxygenation; (ix) $8,8^1$-*anti*-dehydration; (x) esterification of 17^2-COOH with farnesyl group.

configuration at the 8^1-position. The stereochemistry produced is ascribable to the *R*-configuration at the 7-position and the *E*-configuration of the 8-ethylidene group in **II**. *In vitro* isomerization of the 8-ethylidene group to C7=C8 is easily produced (4,10), and the route of **II** to **III** would be chemically favorable.

As with the preparation of **II**, we additionally consider the biosynthetic routes from **III**. One is the direct route without any intermediates shown in step viii, and the other is the indirect route *via* the intermediate **VI** shown in steps ii–ix. Step viii corresponds to the reverse reaction of step vii described above. The 7,8-double bond in **III** would be *trans*-hydrogenated by BchX,Y,Z (*vide supra*), and the resulting **VI** is *anti*-dehydrated at the $8,8^1$-positions and chemically expected to produce **II**.

In view of the stereochemistry around ring-B, we could propose several (bio)chemically acceptable routes for transformation of BChl g_F and 8^1-OH-Chl a_F from 8-vinyl-chlorophyllide *a* in heliobacteria. Although some enzymes catalyzing siteselective or stereoselective chlorophyllous biosynthesis (or both) proposed in Scheme 3b are completely unknown at present, these will be clarified by genetic studies in the near future. It is noteworthy that the biotransformation of the substituents on ring-B must be the same in BChl *b* and *g* because of their identical stereochemistry, although the full biosynthetic route for BChl *b* remains unknown.

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