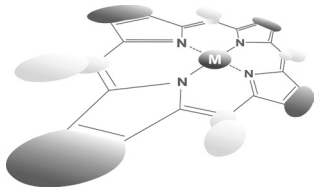
Journal of Porphyrins and Phthalocyanines
J. Porphyrins Phthalocyanines 2018; **22**: 1–14
DOI: 10.1142/S1088424618500347

Published at http://www.worldscinet.com/jpp/



Semi-synthesis and HPLC analysis of (bacterio)chlorophyllides possessing a propionic acid residue at the C17-position

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Received 3 February 2018
Accepted 20 February 2018

ABSTRACT: Various chlorophyll and bacteriochlorophyll derivatives possessing a magnesium or zinc atom at the central position and a free carboxylic acid group at the C17^2-position, also known as (bacterio)chlorophyllides, were synthesized through a combination of organic synthesis techniques and enzymatic steps. The semi-synthetic (bacterio)chlorophyllides were purified and analyzed using reversed-phase high-performance liquid chromatography with UV-vis spectroscopy and mass spectrometry. These free propionic acid-containing chlorophyllous pigments can be useful research materials for the study of (bacterio)chlorophyll metabolisms.

KEYWORDS: bacteriochlorophyll, chlorophyll, enzymatic reaction, high-performance liquid chromatography, hydrolysis, propionate residue.

INTRODUCTION

Chlorophylls (Chls) play essential roles in the initial stages of natural photosynthesis to capture sunlight, transfer the absorbed light energy, and carry out charge separation [1, 2]. The biologically important Chl pigments are biosynthesized through sequential enzymatic reactions. The following facts are known regarding the last steps for Chl *a* (the major Chl of oxygenic phototrophs) biosynthesis (Fig. 1) [3–5]. Magnesium is inserted into the central position of protoporphyrin IX which is a common biosynthetic precursor to Chls and hemes [6]. The C13^2-carboxylic acid group of magnesium protoporphyrin IX is methyl-esterified by *S*-adenosylmethionine (SAM) [7, 8]. This methyl ester plays a key role in forming the isocyclic ring (E-ring) that is characteristic of Chls [9, 10]. The resulting 3,8-divinyl-protochlorophyllide *a* (DV-PChlide *a*) is subsequently subjected to 8-vinyl hydrogenation [11] and C17=C18 reduction to give chlorophyllide *a* (Chlide *a*) [12–14]. Finally, Chl *a* is synthesized by esterification with a phytyl group at the free C17-propionate (C17-propionic acid) residue [15]. The biosynthesis of bacteriochlorophyll *a* (BChl *a*), which is the major photosynthetically active pigment in anoxygenic bacteria, requires three additional steps: formation of a bacteriochlorin ring through the C7=C8 reduction [16], introduction of the C3-acetyl group through hydration of the C3-vinyl group [17], and oxidation of the resulting secondary alcohol [18]. In the last step of BChl *a* biosynthesis, the C17^2-carboxylic acid group of bacteriochlorophyllide *a* (BChlide *a*) is esterified with a phytyl group to give BChl *a* [15].

In the above biosyntheses, (bacterio)chlorophyllides [(B)Chlides], magnesium complexes of chlorophyllous pigments possessing a free C17^2-carboxylic acid group, are enzymatically modified, and esterification occurs at the last step. To the contrary, hydrolysis at the C17-propionate residue proceeds in the early stages of Chl degradation in plants [19]. The esterified phytyl group is not π-conjugated with the (bacterio)chlorin systems and has little effect on their optical properties, but enhances the lipophilicity of the molecules. Most photosynthetically active (B)Chls have a long hydrocarbon chain [20] that is useful for anchoring into photosystems through hydrophobic interactions with proteins, lipids, and other esterified chains.

◊ SPP full member in good standing

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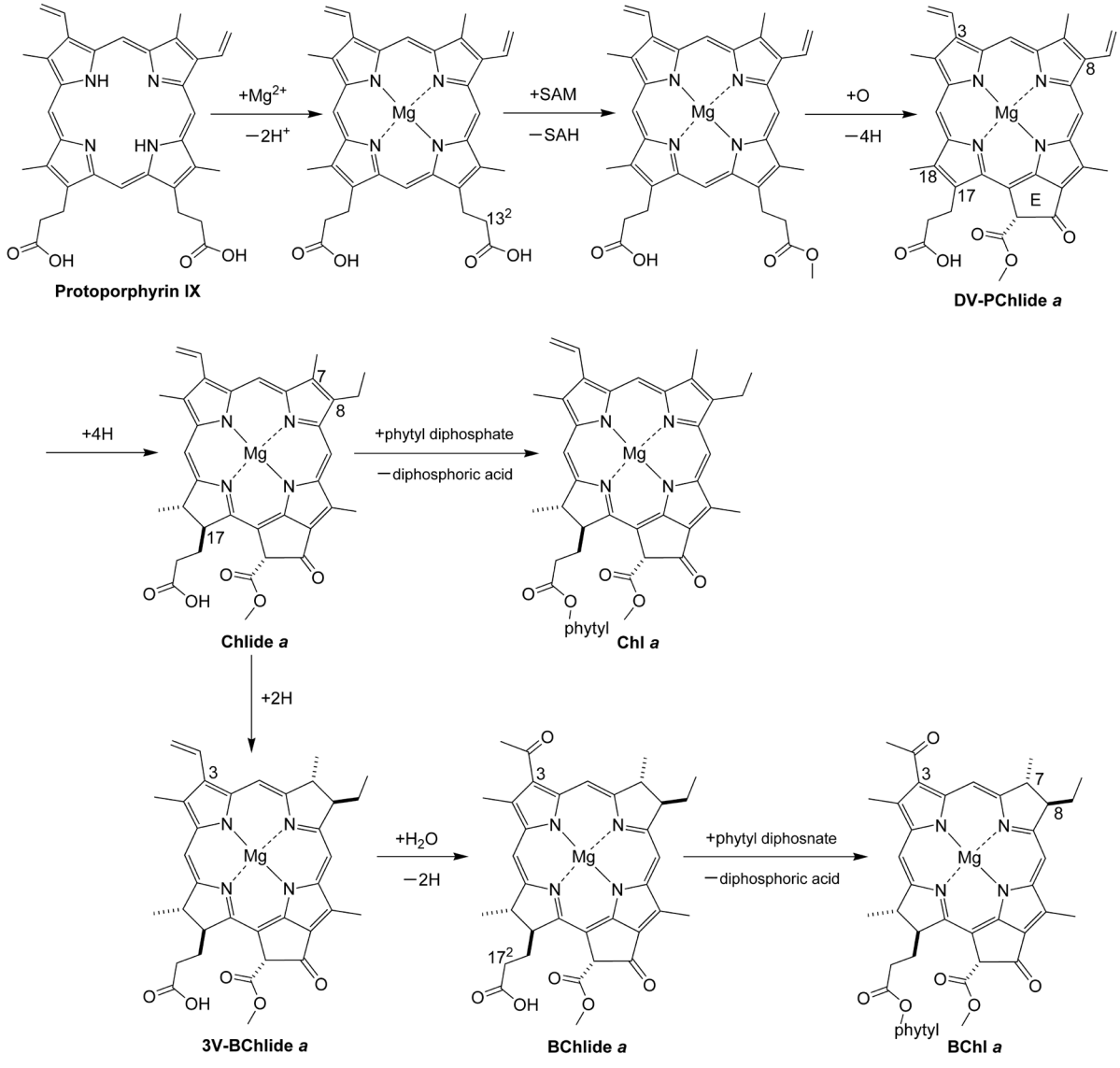

Fig. 1. Biosynthetic pathway from protoporphyrin IX to (B)Chl *a*

Although (B)Chlides are essential as research materials for studying (B)Chl metabolism and the effect of esterified chains, the availability of (B)Chlides has been limited. Pigments extracted from photosynthetic organisms are usually esterified at the C17 position, and a very small amount of (B)Chlides are available from natural materials. Mutant strains accumulating (B)Chlides can also be used as a resource, but the available quantities and varieties of (B)Chlides are still limited [10, 16, 21]. Another route for obtaining (B)Chlides is through semi-synthesis from natural (B)Chls: (1) organic synthetic methods [22] and (2) enzymatic modifications [23]. Using photosynthetic chlorophyllous pigments that are naturally abundant, a variety of (B)Chlides are obtainable in an adequate amount. In this study, we report the preparation of several (B)Chlides by combining the above two techniques. The semi-synthetic (B)Chlides were identified and purified by high-performance liquid chromatography(–mass spectrometry) (HPLC(–MS)) and their retention times were compared.

RESULTS AND DISCUSSION

Synthesis of Chlide *a* and its derivatives

Chl *a* was extracted from commercially available, dry cultured cells of the cyanobacterium *Spirulina geitleri* with a mixture of petroleum ether and methanol and purified according to previous procedures [24] (see Fig. 2). The purified Chl *a* was converted into Chlide *a* with one of the esterases, chlorophyllase derived from *Chenopodium album* [25] as follows. The chlorophyllase was overexpressed in *E. coli*, and its

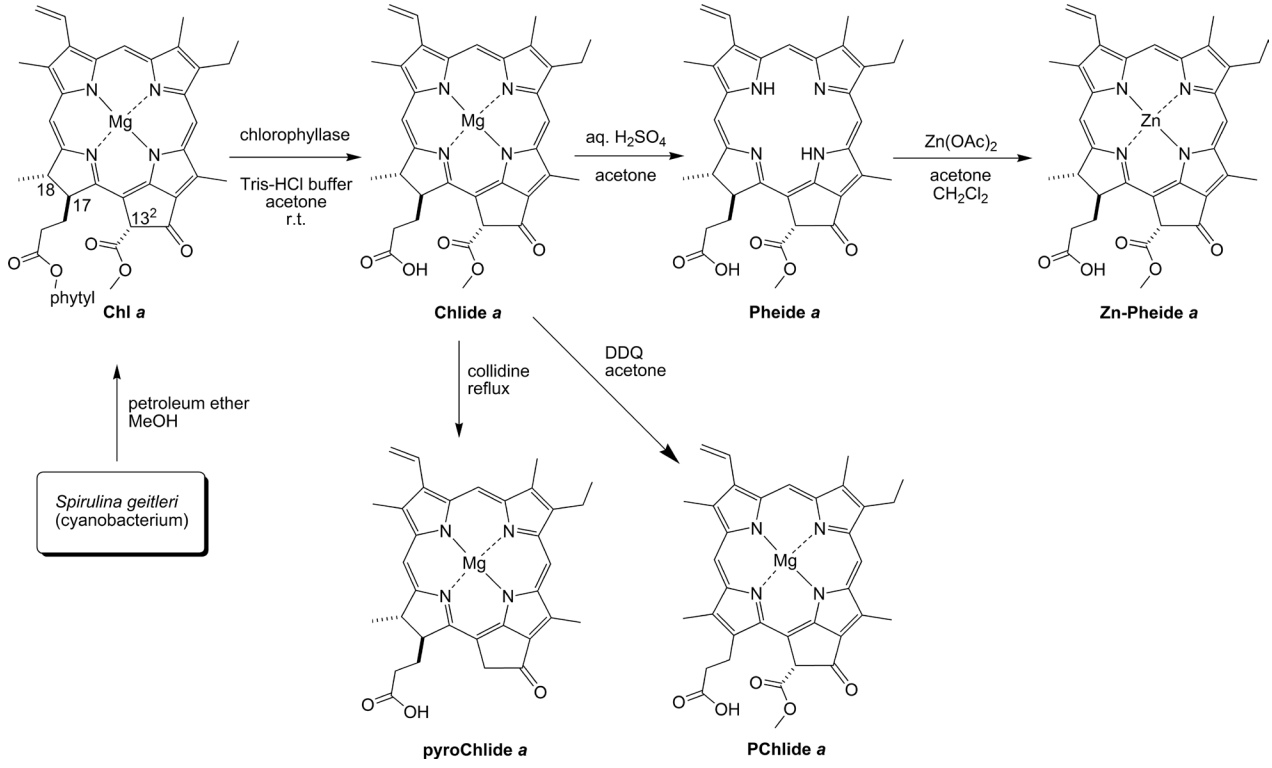

Fig. 2. Synthesis of Chlide *a* and its derivatives from a Chl-*a* producing cyanobacterium

cell lysate was used for the following hydrolysis. Chl *a* in acetone was dispersed in a mixture of aqueous Tris-HCl buffer (pH 7.8) with sodium ascorbate, and stirred at room temperature with an aliquot of the above chlorophyllase solution. After incubation for 1 h, the reaction was quenched by the addition of acetone, and the mixture was centrifuged. The resulting supernatant was washed with hexane, diluted with diethyl ether, washed with distilled water, and concentrated to dryness to give Chlide *a* [26]. The obtained Chlide *a* was used without further purification as the starting material in the following reactions. This enzymatic hydrolysis should be carried out before the removal of the C13^2-methoxycarbonyl group and the formation of the porphyrin π-skeleton by dehydrogenation of the C17H–C18H to the corresponding double bond because of the chlorophyllase substrate specificity. Chlide *a* was demethoxycarbonylated by refluxing in collidine to yield pyrochlorophyllide *a* (pyroChlide *a*) [27]. To obtain protochlorophyllide *a* (PChlide *a*), a dry acetone solution of Chlide *a* was treated with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), and the chlorin π-system was oxidized to the fully π-conjugated porphyrin moiety in PChlide *a* [28]. Chlide *a* in acetone was acidified with an aqueous 5% (v/v) H_2SO_4 solution, resulting in the smooth demetalation of Chlide *a* to pheophorbide *a* (Pheide *a*). Pheide *a* in dichloromethane was treated with zinc acetate dihydrate in acetone [29] to yield zinc pheophorbide *a* (Zn-Pheide *a*).

To analyze and purify the above Chlides, reversed phase (RP) HPLC was performed using a mixture of methanol and aqueous 50 mM ammonium acetate solution (pH 5.2) as a mobile phase. Chlide *a* and pyroChlide *a* were eluted at 4.50 and 7.46 min, respectively, under the same HPLC conditions: column, Cosmosil 2.5C_{18}-MS-II 3.0 φ × 75 mm octadecyl-silica gel (ODS); eluent, MeOH/50 mM $AcONH_4$ = 80/20; flow rate, 0.6 mL·min^{-1}. The slow elution of the 13^2-demethoxycarbonylation product was ascribed to a decrease of the molecular polarity, as supported by the estimated hydrophobicity, calculated values of logP (ClogP) [30, 31]: ClogP = 8.88 (Chlide *a*) < 9.20 (pyroChlide *a*). The dehydrogenation to the porphyrin π-skeleton as Chlide *a* → PChlide *a* slowed down the elution of the pigment: retention time (t_R) = 3.30 (Chlide *a*) and 3.93 min (PChlide *a*); eluent, MeOH/50 mM $AcONH_4$ = 85/15. The change was also consistent with the ClogP values: 8.88 (Chlide *a*) < 8.93 (PChlide *a*). Zn-Pheide *a* was eluted at 6.90 min later than Chlide *a* at 4.50 min, whereas the former ClogP value (8.46) was smaller than that of Chlide *a* (8.88). This inconsistency might come from a higher coordination ability of the central magnesium to the polar HPLC eluent than the zinc of Zn-Pheide *a* [22].

Synthesis of BChl *a* derivatives

BChl *a* was extracted from the cultured cells of the purple bacterium *Rhodobacter sphaeroides* with a mixture

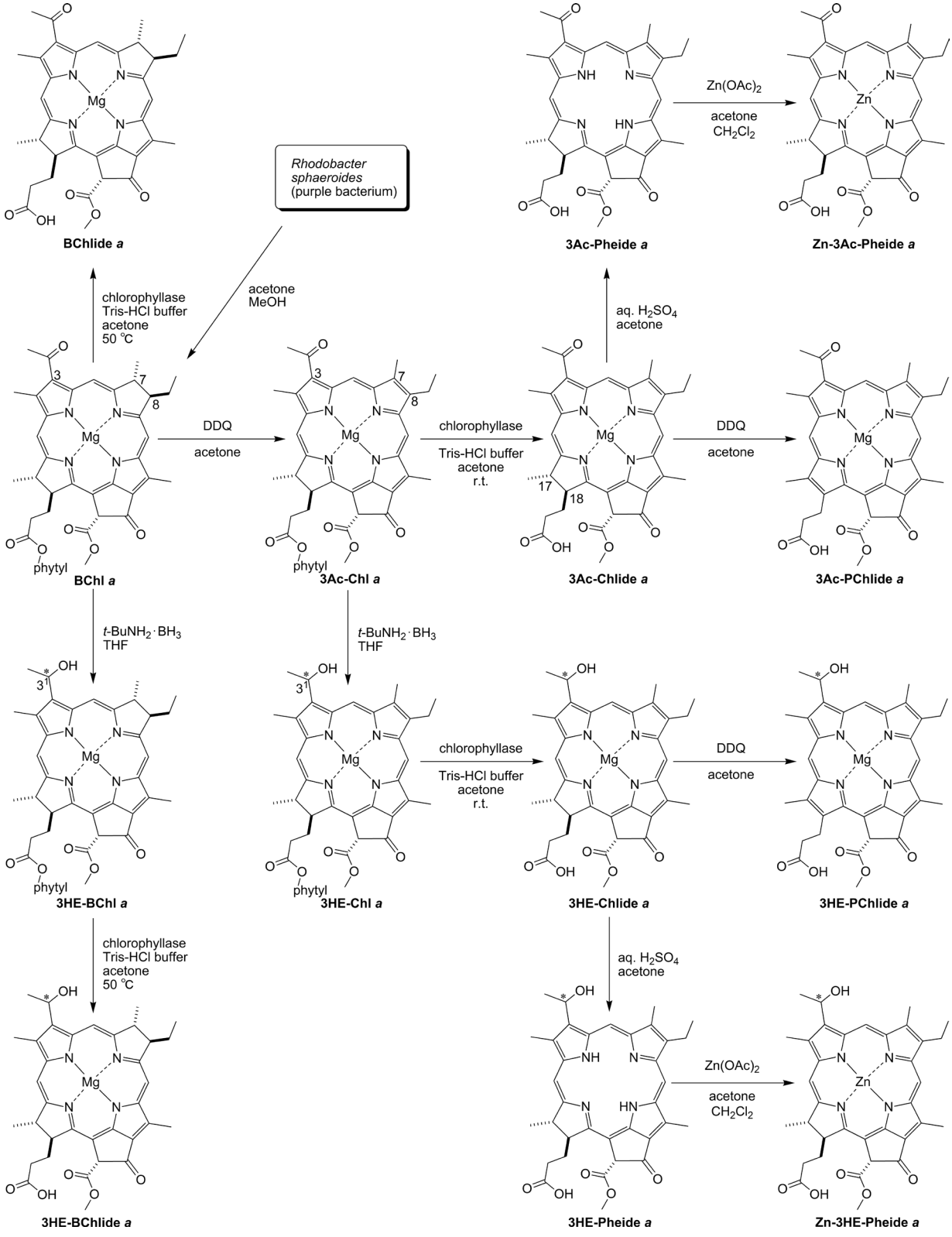

Fig. 3. Synthesis of BChlides, Chlides, and PChlides from a BChl-*a* producing purple bacterium

of acetone and methanol and purified by RP-HPLC [32] (see Fig. 3). BChl *a*, with a bacteriochlorin π-system, was oxidized to 3-acetyl-chlorophyll *a* (3Ac-Chl *a*), with a chlorin π-moiety, *via* dehydrogenation of C7H–C8H to C7=C8 using DDQ in a manner similar to the Chlide *a* to PChlide *a* transformation [33] (vide supra). The C3-acetyl groups of BChl *a* and 3Ac-Chl *a* were reduced using *tert*-butylamine borane in tetrahydrofuran (THF) to yield

3-(1-hydroxyethyl)-bacteriochlorophyll *a* (3HE-BChl *a*) and 3-(1-hydroxyethyl)-chlorophyll *a* (3HE-Chl *a*) as a 1:1 epimeric mixture of 3^1*R*/*S*-epimers [34]. BChl *a*, 3HE-BChl *a*, 3Ac-Chl *a*, and 3HE-Chl *a* in acetone were hydrolyzed by chlorophyllase in aqueous Tris-HCl buffer to BChlide *a,* 3HE-BChlide *a*, 3Ac-Chlide *a*, and 3HE-Chlide *a*, respectively. The enzymatic hydrolysis reactions of the former two BChls were performed at 50 °C owing to their lower reactivities when compared with Chls. The hydrolysis was so mild that neither demetalation to (bacterio)pheophorbides nor oxidation of the bacteriochlorin to a chlorin ring was observed in the reaction mixtures. The substrate specificity of the present chlorophyllase was low enough that the substitution of the C3-vinyl group with the C3-acetyl or C3-(1-hydroxyethyl) group was tolerated. 3Ac-PChlide *a* and 3HE-PChlide *a* were synthesized from 3Ac-Chlide *a* and 3HE-Chlide *a* by selective oxidation of the chlorin moiety to a porphyrin moiety using DDQ [28]. It is noted that the DDQ-oxidation of bacteriochlorins gave chlorins, followed by the formation of porphyrins through the stepwise conversion of the first 7,8- and the second 17,18-dehydrogenations. Zn-3Ac-Pheide *a* and Zn-3HE-Pheide *a* were prepared from 3Ac-Chlide *a* and 3HE-Chlide *a* by demetalation followed by a Zn-metalation reaction (vide supra).

In the HPLC analysis, pigments possessing the same π-skeleton, but with different substituents at the C3-position, were compared with respect to their t_R-values. 3HE-BChlide *a* and BChlide *a* were eluted at 7.21/7.99 (3^1*R*/*S*-epimers) and 9.08 min, respectively, using MeOH/aqueous 50 mM $AcONH_4$ = 65/35 at 0.5 mL·min^{-1}. 3HE-Chlide *a* and 3Ac-Chlide *a* were eluted at 10.1/11.2 and 15.0 min, respectively, using aqueous 65% (v/v) MeOH at 0.5 mL·min^{-1}. 3Ac-Chlide *a* was eluted at 2.11 min, faster than Chlide *a* (t_R = 3.30 min), using aqueous 85% MeOH at 0.6 mL·min^{-1}. The same elution order was observed among the following porphyrin pigments: t_R = 1.77/1.98 (3^1*R*/*S*-epimers of 3HE-PChlide *a*) < 2.74 (3Ac-PChlide *a*) < 8.12 min (PChlide *a*) using aqueous 80% MeOH at 0.6 mL·min^{-1}. These data show that pigments possessing the same π-skeleton eluted in the order of 3-(1-hydroxyethyl) (3HE), 3-acetyl (3Ac), and 3-vinyl (3V). This order was consistent with the estimated hydrophobicity: ClogP = 7.32 (3HE-BChlide *a*) < 7.53 (BChlide *a*), 7.37 (3HE-Chlide *a*) < 7.59 (3Ac-Chlide *a*) < 8.88 (Chlide *a*), and 7.43 (3HE-PChlide *a*) < 7.65 (3Ac-PChlide *a*) < 8.93 (PChlide *a*). Zn-chelated 3HE/Ac-Pheides were eluted slower than the corresponding Mg-chelated pigments: t_R = 10.1/11.2 (3^1*R*/*S*-epimers of 3HE-Chlide *a*) < 16.5/18.0 min (3^1*R*/*S*-epimers of Zn-3HE-Pheide *a*) and 15.0 (3Ac-Chlide *a*) < 31.3 min (Zn-3Ac-Pheide *a*) using aqueous 65% MeOH at 0.5 mL·min^{-1}. These elution orders were opposite to the prediction based on estimated lipophilicity. This inconsistency may have resulted from the same reason as in the 3-vinylated pigments (vide supra): ClogP = 6.91 (Zn-3HE-Pheide *a*) < 7.37 (3HE-Chlide *a*) and 6.89 (3Ac-Chlide *a*) < 7.59 (Zn-3Ac-Pheide *a*).

Synthesis of BChl *c/d* derivatives

Homologous mixtures of BChl *c* and BChl *d* were extracted with a mixture of acetone and methanol from the cultured cells of green sulfur bacteria, *Chlorobaculum tepidum* and *Chlorobaculum parvum*, respectively (see Fig. 4). All the BChl *c/d* homologs were separated using RP-HPLC (see Experimental). *Chlorobaculum tepidum* gave almost exclusively the 3^1*R*-epimers of 8-ethyl-12-methyl-([E,M])BChl *c* and 8,12-diethyl-([E,E])BChl *c*, an approximate 9:1 mixture of the 3^1*R*- and 3^1*S*-epimers of 8-propyl-12-ethyl-([P,E])BChl *c*, and exclusively the 3^1*S*-epimer of 8-isopropyl-12-ethyl([I,E])BChl *c* [26]. *Chlorobaculum parvum* gave epimeric/homologous pigment compositions similar to *Chlorobaculum tepidum*, but also contained a small amount of 8-propyl-12-methyl-([P,M])BChl *d* [35]. [E,M]BChl *c* was converted into zinc 8-ethyl-12-methyl-bacteriopheophorbide *c* (Zn-[E,M]-BPheide *c*) using the following steps. [E,M]BChl *c* in THF was treated with aqueous H_2SO_4 to yield [E,M]-BPheide *c via* demetalation of magnesium and hydrolysis of the farnesyl ester [36]. [E,M]BPheide *c* was zinc-metalated to Zn-[E,M]BPheide *c* as mentioned above. The same procedures were used for the preparation of Zn-[E,M]BPheide *d*, Zn-[E,E]BPheides *c/d*, Zn-[P,E]-BPheides *c/d*, and Zn-[I,E]BPheides *c/d*.

To synthesize zinc 3-vinyl-8-ethyl-12-methyl-bacteriopheophorbide *c* (Zn-3V-[E,M]BPheide *c*), [E,M]BChl *c* in acetone was first treated with a diluted aqueous sulfuric acid solution to effect central magnesium demetalation. The resulting 8-ethyl-12-methyl-bacteriopheophytin *c* ([E,M]BPheo *c*) was refluxed in benzene containing *p*-toluenesulfonic acid (PTSA) monohydrate to yield 3V-[E,M]BPheo *c*. 3V-[E,M]BPheo *c* was hydrolyzed in THF with aqueous H_2SO_4 to 3V-[E,M]BPheide *c* and subsequently subjected to zinc-metalation (vide supra) to yield Zn-3V-[E,M]BPheide *c* [29]. Zn-3V-[E,M]BPheide *d*, Zn-3V-[E,E]BPheides *c/d*, Zn-3V-[P,E]BPheides *c/d*, and Zn-3V-[I,E]BPheides *c/d* were synthesized following similar procedures.

Zn-(3V-)[E,M]BPheide *d* was also synthesized from Chl *a* through an alternative pathway [28]. Chl *a* was first converted into methyl pyropheophorbide *a* through demetalation and *trans*-esterification with methanol under acidic conditions and a pyrolysis for the 13^2-demethoxycarbonylation product [37]. Zn-3V-[E,M]-BPheide *d* (= zinc pyropheophorbide *a*) was obtained from a subsequent hydrolysis at the propionate residue and zinc-metalation. To prepare Zn-[E,M]BPheide *d*, methyl pyropheophorbide *a* was hydrated at the C3-vinyl group and concomitantly hydrolyzed at the $C17^2$-ester with HBr in AcOH and with water, then zinc-metalated [29]. Because the hydration was not stereoselective, the

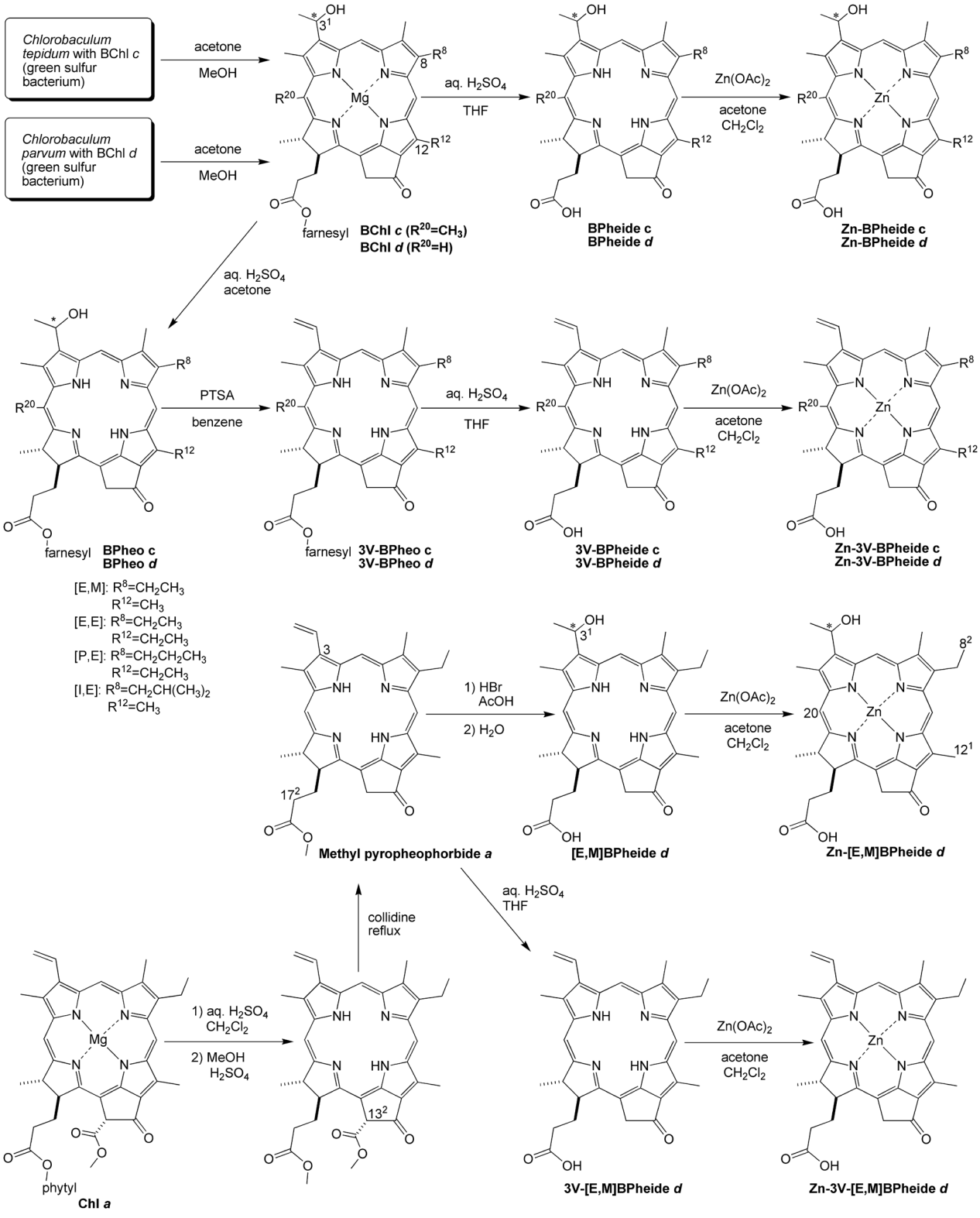

Fig. 4. Synthesis of Zn-(3V-)BChlide *c*/*d* homologs from BChl-*c*/*d* producing green bacteria and by modification of Chl *a*

resulting products were 1:1 3^1*R*/*S*-epimeric mixtures of (Zn-)[E,M]BPheide *d*. It is noted that other homologs, Zn-(3V-)[E,E]/[P,E]/[I,E]BPheides *c*/*d*, could not be synthesized from Chl *a* owing to synthetic difficulties in the methylation of the $C8^2$- and $C12^1$-positions [38].

HPLC analysis showed that the above homologs were eluted more slowly as the methylation level of the peripheral substituents increased. This observation paralleled the trend in hydrophobicity. The 20-methylation of Zn-(3V-)BPheide *d* homologs to Zn-(3V-)BPheide *c*

gave larger t_R-values compared with their corresponding homologs: *e.g*. Zn-3V-[E,M]BPheide *d* was eluted at 9.74 min, and Zn-3V-[E,M]BPheide *c* was eluted at 11.3 min (see Experimental). The stepwise methylation at the $C8^2$- and $C12^1$-positions also produced the same t_R effect, resulting in the elution order of [E,M] → [E,E] → [P,E] → [I,E]BPheides *c/d*: *e.g*. t_R = 11.3 (Zn-3V-[E,M]BPheide *c*) < 13.2 (Zn-3V-[E,E]BPheide *c*) < 17.1(Zn-3V-[P,E]BPheide *c*) < 20.6 min (Zn-3V-[I,E]-BPheide *c*). Isomerization at the C3-(1-hydroxyethyl) group partially occurred during acidic hydrolysis, but the 3^1-epimeric ratios of the semi-synthetic Zn-BPheide *c/d* homologs were comparable to that of each isolated BChl *c/d* homolog. Note also that the 3^1*R*-epimers of the BChlide *c/d* and Zn-BPheide *c/d* homologs were eluted faster than the corresponding 3^1*S*-epimers, which is consistent with the RP-HPLC elution orders of the other naturally occurring BChls *c/d/e/f* and their related secondary alcohols [39, 40].

Synthesis of (bacterio)chlorophyllides

BChlide *a* and 3HE-BChlide *a* could be synthesized from BChl *a* as mentioned above. However, 3V-BChlide *a* could not be prepared through the chemical modification of BChl *a* because the magnesium ion would be removed from the center of the tetrapyrrole ring during the acidic dehydration reaction. Therefore, to obtain 3V-BChlide *a*, we used a mutant of *Rhodobacter sphaeroides* that accumulated 3V-BChl *a*. A mixture of acetone and methanol was added to the cultured cells of this 3-vinyl hydratase gene *bchF* lacking mutant, and 3V-BChl *a* was extracted [33] (see Fig. 5, upper). The resulting residue was subjected to RP-HPLC purification and hydrolyzed with chlorophyllase to yield 3V-BChlide *a*. 3V-BChlide *a* should be carefully handled because it is significantly less stable than BChlide *a*. BChlide *a* is stabilized against oxidation and demetalation by the electron-withdrawing C3-acetyl group [41]. The RP-HPLC elution order was comparable to the dehydrogenation degree of the core tetrapyrrole skeletons of the 3-vinylated pigments: t_R = 2.74 (3V-BChlide *a*) < 3.30 (Chlide *a*) < 3.93 min (PChlide *a*) using aqueous 85% MeOH at 0.6 mL · min^{-1}. This matched the trend predicted using ClogP values: 8.82 (3V-BChlide *a*) < 8.88 (Chlide *a*) < 8.93 (PChlide *a*).

DV-Chl *a* was obtained from the cultured cells of a cyanobacterium mutant *Synechocystis* sp. PCC6803 that lacked the divinyl reductase gene *slr1923* [42] (see Fig. 5, middle). DV-Chlide *a* was synthesized with the aforementioned action of chlorophyllase, showing that chlorophyllase was active for the 8-vinylated substrate. DV-Chlide *a* was oxidized by DDQ (vide supra) to DV-PChlide *a*. DV-Chlide *a* was incubated with recombinant demethoxycarbonylase BciC from *Chlorobaculum tepidum* in an aqueous buffer to yield DV-pyroChlide *a* [33]. The aqueous acidic treatment of DV-Chlide *a* led to non-regioselective hydration of the C3- and/or C8-vinyl group(s) and simultaneously removed the central magnesium. Therefore, the synthesis of 3-(1-hydroxyethyl)-8-vinyl-chlorophyllide *a* (3HE-8V-Chlide *a*) was accomplished through a regio-selective hydration at the C3-vinyl group using recombinant hydratase BchF from *Chlorobaculum tepidum* in an aqueous buffer [26, 28, 43]. In the enzymatic hydration reaction, the 3^1*R*-epimer was produced as a major product owing to the stereoselectivity of BchF. In the HPLC analysis, DV-Chlide *a* was eluted at 4.89 min, slower than Chlide *a* (4.50 min), using aqueous 80% MeOH at 0.6 mL · min^{-1}. The same elution trend was observed for PChlide *a* (3.93 min) and DV-PChlide *a* (4.22 min) using aqueous 85% MeOH at 0.6 mL · min^{-1}.

Chl *b* and Chl *d* were extracted from commercially available spinach powder with a mixture of petroleum ether and methanol [44] and from *Acaryochloris marina* cells with acetone and methanol [45], respectively (see Fig. 5, lower). The pigment extracts were subjected to RP-HPLC to give pure Chl *b* and Chl *d*. Hydrolysis at the C17-propionate residues of phytylated Chl *b* and Chl *d* was performed using chlorophyllase to yield Chlide *b* and Chlide *d*, respectively. The chlorophyllase could readily catalyze the hydrolysis of pigments possessing a C7- or C3-formyl group. In comparison, between their t_R-values under RP-HPLC conditions using aqueous 80% MeOH at 0.6 ml · min^{-1}, the 7-formylated pigment (Chlide *b*) eluted at 1.96 min, which was slightly faster than the 3-formylated pigment (Chlide *d*) eluting at 2.05 min. Both pigments eluted faster than Chlide *a* (t_R = 4.50 min), and the order could be rationalized by their ClogP values: 8.09 (Chlide *b*) < 8.88 (Chlide *a*) and 7.50 (Chlide *d*) < 8.88 (Chlide *a*). However, between Chlide *b* and Chlide *d*, the elution order was inconsistent with the estimated hydrophobicity, and the coordination ability of these compounds to the eluent may have affected the elution order: the estimated axial coordination constant of Chl *b* is greater than that of Chl *d* [41].

EXPERIMENTAL

General

All the reactions on chlorophyllous pigments were carried out at room temperature in the dark under an air atmosphere unless otherwise stated.

Pigment purification and analysis by HPLC were performed using a Shimadzu Prominence liquid chromatography system consisting of a CBM-20A system controller, an SPD-M20A photodiode array (PDA) detector, LC-20AD pumps, a DGU-20A3 degasser and a CTO-20AC column oven (Japan). For all HPLC analysis, the column oven was set to 35 °C, and the PDA detector was set to 300–800 nm.

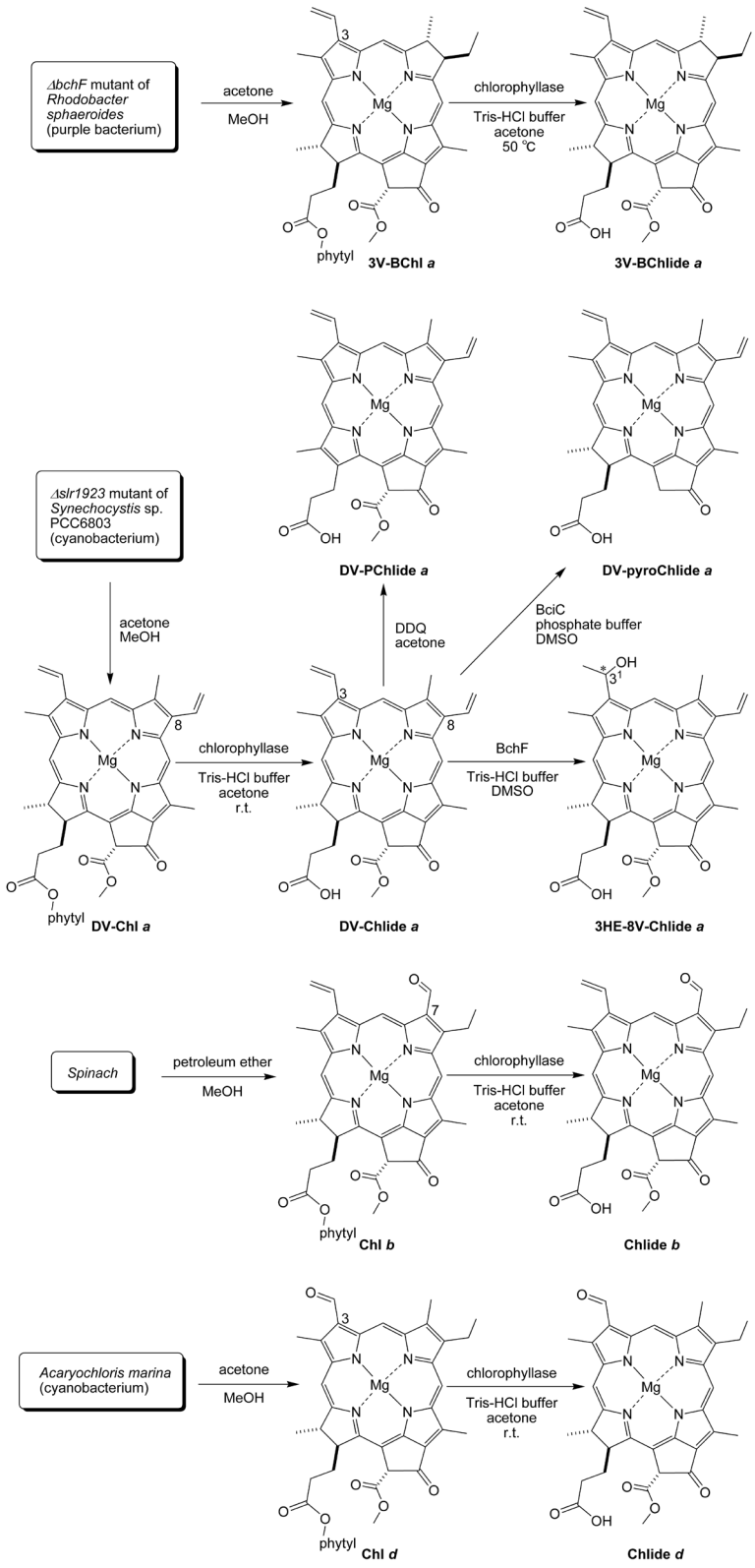

Fig. 5. Synthesis of 3V-BChlide *a* from the 3V-BChl-*a* producing Δ*bchF* mutant of a purple bacterium (upper), 8-vinylated (P)Chlides *a* from the DV-Chl-*a* producing Δ*slr1923* mutant of a cyanobacterium (middle), and Chlides *b*/*d* from Chl-*b* producing spinach and Chl-*d* producing cyanobacterium (lower)

Liquid chromatography–mass spectrometry (LCMS) was performed using a Shimadzu LCMS-2010EV system consisting of a quadrupole mass spectrometer equipped with electrospray ionization. All the solvents used for HPLC were of HPLC grade and purchased from Nacalai Tesque or Wako Pure Chemical Ind.

Synthetic procedures

Pigment extraction. Chl *a* was extracted from a commercially available spirulina powder (cyanobacterium *Spirulina geitleri*) with a mixture of petroleum ether and methanol (1:1, v/v) and purified according to the method described previously [24].

DV-Chl *a* was extracted from the Δ*slr1923* mutant of cyanobacterium *Synechocystis* sp. PCC6803 by stirring its cultured cells in a mixture of acetone and methanol (4:1, v/v) [42]. After the solvents were concentrated, the residue was purified by RP-HPLC: column, Cosmosil $5C_{18}$-AR-II (4.6 φ × 150 mm); eluent, MeCN/H_2O = 95/5; flow rate, 5 mL · min^{-1}.

Chl *b* was extracted from commercially available spinach powder with a mixture of petroleum ether and methanol (1:1, v/v) [44]. After centrifugation, the resulting supernatant was partitioned between diethyl ether and distilled water. The ether phase was washed several times with distilled water and concentrated, and Chl *b* was purified by RP-HPLC: column, Cosmosil $5C_{18}$-AR-II (4.6 φ × 150 mm); eluent, MeOH/H_2O = 97/3; flow rate, 2.5 mL · min^{-1}.

Chl *d* was extracted from cyanobacterium *Acaryochloris marina* cells using acetone and methanol (4:1, v/v) [45] in a manner similar to the procedure used for Chl *b*, and Chl *d* was purified by RP-HPLC: column, Cosmosil $2.5C_{18}$-MS-II (3.0 φ × 75 mm); eluent, MeCN/H_2O = 95/5; flow rate, 2.0 mL · min^{-1}.

BChl *a* was extracted from the cultured cells of purple bacterium *Rhodobacter sphaeroides* [32] in a manner similar to the procedure used for Chl *d*. BChl *a* was purified by normal–phase (NP) HPLC: column, Cosmosil 5SL-II (6.0 φ × 250 mm); eluent, hexane/acetone = 75/25 (v/v); flow rate, 6.0 mL · min^{-1}.

3V-BChl *a* was extracted from the cultured Δ*bchF* mutant cells of *Rhodobacter sphaeroides* [33] in a manner similar to the procedure used for BChl *a*. 3V-BChl *a* was purified by RP-HPLC: column, Cosmosil 2.5C18-MS-II (3.0 φ × 75 mm); eluent, MeOH/H_2O = 85/15 (0–15 min), then MeOH (15 min–end); flow rate, 0.6 mL · min^{-1}. The fraction containing 3V-BChl *a* was dialyzed with 50 mM phosphate buffer (pH 7.8) for the next chlorophyllase reaction.

BChl *c* was extracted from green sulfur bacterium *Chlorobaculum tepidum* [28] in a manner similar to the procedure used for Chl *d*. Each BChl *c* homolog was isolated from the mixture by RP-HPLC, and pure [E,M], [E,E], [P,E], and [I,E]BChls *c* were obtained: column, Cosmosil $5C_{18}$-AR-II (10 φ × 250 mm); eluent, MeOH; flow rate, 2.0 mL · min^{-1}.

BChl *d* was extracted from green sulfur bacterium *Chlorobaculum parvum* [43] in a manner similar to the above preparation of BChl *c* homologs. The following RP-HPLC purification yielded [E,M], [P,E], and [I,E] BChls *d* as well as a mixture of [E,E] and [P,M]BChls *d*: column, Cosmosil 5C18-AR-II (6.0 φ × 250 mm); eluent, MeCN/acetone/H_2O = 70/15/15; flow rate, 2.5 mL · min^{-1}. The mixture of [E,E] and [P,M]BChls *d* was separated by a further NP-HPLC process: column, Cosmosil 5C18-SL (6.0 φ × 250 mm); eluent, hexane/THF = 84/16; flow rate, 5 mL · min^{-1}.

Hydrolysis using chlorophyllase. Recombinant chlorophyllase derived from *Chenopodium album* was generously gifted by Dr. Yusuke Tsukatani of Japan Agency for Marine-Earth Science and Technology. The cell lysate of *E. coli* overexpressing chlorophyllase was used for the following hydrolysis [23]. An acetone solution (200 μL) of each substrate (Chl *a*, 3Ac-Chl *a*, 3HE-Chl *a*, DV-Chl *a*, Chl *b*, or Chl *d*) was dispersed in 25 mM Tris-HCl buffer (pH 7.8, 8 mL) containing 0.5 M aqueous sodium *L*-ascorbate (50 μL). To this was added the chlorophyllase solution (5.7 mg · mL^{-1}, 500 μL), and the mixture was stirred under nitrogen for 1 h. Similar reactions were performed at 50 °C for BChl *a*, 3HE-BChl *a*, and 3V-BChl *a*. To stop the reaction, acetone (30 mL) was added, and the mixture was centrifuged. The resulting supernatant was washed with hexane to remove unreacted (B)Chls, diluted with diethyl ether, and washed with distilled water to give the corresponding carboxylic acids, Chlide *a*, 3Ac-Chlide *a*, 3HE-Chlide *a*, DV-Chlide *a*, Chlide *b*, Chlide *d*, BChlide *a*, 3HE-BChlide *a*, and 3V-BChlide *a*. For HPLC purification of these products, see HPLC conditions mentioned in Spectral data section.

Acidic hydrolysis. Concentrated H_2SO_4 was added dropwise to BChl *c*/*d* and 3V-BPheo *c*/*d* homologs in THF and H_2O (4/1, v/v) in an ice bath. The reaction was monitored using NP-thin-layer chromatography with diethyl ether and dichloromethane (1:9, v/v) as the eluent. After the substrates were consumed, $CHCl_3$ was added to the reaction mixture, and the organic layer was washed with distilled water. The organic layer was concentrated to yield the corresponding BPheide *c*/*d* and 3V-BPheide *c*/*d* homologs.

DDQ oxidation. The bacteriochlorin of BChl *a* was oxidized to the chlorin (3Ac-Chl *a*), and the chlorins of Chlide *a*, 3Ac-Chlide *a*, 3HE-Chlide *a*, and DV-Chlide *a* were oxidized to the corresponding porphyrins ((un) substituted PChlide *a*) using DDQ [28, 42]. DDQ, dissolved in dry acetone, was added to a dry acetone solution of each substrate. The oxidation was monitored with UV-vis spectroscopy until the absorption peak changed as follows: Q_y absorption maxima = 770 (BChl *a*) to 677 nm (3Ac-Chl *a*), 663 (Chlide *a*) to 623 nm (PChlide *a*), 677 (3Ac-Chlide *a*) to 632 nm (3Ac-PChlide *a*), 654 (3HE-Chlide *a*) to 616 nm (3HE-PChlide *a*), and 663 (DV-Chlide *a*) to 625 nm (DV-PChlide *a*). The reaction mixture of BChl *a* was poured into water and extracted with dichloromethane. The organic layer was washed with aqueous 4% $NaHCO_3$ and water, and concentrated to give 3Ac-Chl *a*. The other reaction mixtures were suspended in 2-propanol and subjected to RP-HPLC: for PChlide *a*, Inertsil ODS-P (4.6 φ × 150 mm, GL Science Inc.) column and MeOH/50 mM

$AcONH_4$ = 85/15 at 2.0 mL·min^{-1}; for 3Ac-PChlide *a* and for 3HE-PChlide *a*, Cosmosil 2.5C_{18}-MS-II (3.0 φ × 75 mm) column and MeOH/50 mM $AcONH_4$ = 80/20 at 0.6 mL·min^{-1}; for DV-PChlide *a*, Cosmosil 5C_{18}-AR-II (4.6 φ × 150 mm) column and MeOH/50 mM $AcONH_4$ = 85/15 at 1.0 mL·min^{-1}.

Reduction of 3Ac to 3HE group. BChl *a* and 3Ac-Chl *a* were reduced using *tert*-butylamine borane (140 mg) in THF (10 mL) under nitrogen [34]. During the reduction, their Q_y absorption maxima were blue-shifted: from 771 (BChl *a*) to 722 nm (3HE-BChl *a*) and from 680 (3Ac-Chl *a*) to 653 nm (3HE-Chl *a*). The reaction was quenched by the addition of acetone and stirred for a further 30 min. Extraction with diethyl ether and washing with distilled water gave 3HE-BChl *a* and 3HE-Chl *a*.

Dehydration of 3HE to 3V group. Homologously pure BPheo *c*/*d* were refluxed in benzene containing PTSA for 15 min [46]. After cooling down to room temperature, $CHCl_3$ was added to the reaction mixture, and the organic layer was washed with distilled water. The organic layer was concentrated to yield the corresponding 3V-BPheo *c*/*d* homologs.

Demetalation. Removal of the magnesium ion coordinated to Chlide *a*, 3Ac-Chlide *a*, 3HE-Chide *a*, and BChls *c*/*d* was achieved under acidic conditions. Diluted aqueous H_2SO_4 was added to an acetone solution of each magnesium-chelated pigment and the reaction mixtures were stirred. The progress of the demetalation reaction was monitored with UV-vis spectroscopy until blue-shifts of the Soret maxima and red-shifts of the Q_y absorption maxima were observed. The reaction mixtures were extracted using CH_2Cl_2, washed with distilled water, and concentrated to yield the desired metal-free pigments.

Zn metalation. An acetone solution containing zinc acetate dihydrate was added to Pheide *a*, 3HE-Pheide *a*, 3Ac-Pheide *a*, BPheide *c*/*d*, or 3V-BPheide *c*/*d* in CH_2Cl_2 and stirred. After red-shifts of the Soret absorption maxima and blue-shifts of the Q_y absorption maxima were observed, $CHCl_3$ was added to the reaction mixture and the organic layer was washed with distilled water and concentrated to yield Zn-Pheide *a*, Zn-3HE-Pheide *a*, Zn-3Ac-Pheide *a*, Zn-BPheide *c*/*d* homologs, or Zn-3V-BPheide *c*/*d* homologs. For HPLC purification of these products, see HPLC conditions mentioned in Spectral data section.

Pyrolysis for* 13^2*-demethoxycarbonylation. Chlide *a* was refluxed in collidine to yield pyroChlide *a* [27]. For its HPLC purification, see HPLC conditions mentioned in Spectral data section.

Hydration of 3V group using BchF. BchF hydratase derived from *Chlorobaculum tepidum* was generously gifted by Dr. Jiro Harada of Kurume University School of Medicine. The cell lysate of *E. coli* overexpressing BchF was used for the following hydration. DV-Chlide *a* in dimethyl sulfoxide (DMSO) was dispersed in 25 mM Tris-HCl (pH 7.8) buffer containing BchF. The reaction mixture was incubated at 35 °C for 30 min and acetone was added to stop the enzymatic reaction [43]. The solution was concentrated and applied to RP-HPLC for purification of 3HE-8V-Chlide *a*: column, Cosmosil 2.5C_{18}-MS-II (3.0 φ × 75 mm); eluent, MeOH/aqueous 50 mM $AcONH_4$ = 70/30; flow rate, 0.6 mL·min^{-1}.

13^2*-Demethoxycarbonylation by BciC.* BciC demethoxycarbonylase derived from *Chlorobaculum tepidum* was generously gifted by Dr. Jiro Harada of Kurume University School of Medicine. The cell lysate of *E. coli* overexpressing BciC was used for the following enzymatic reaction. DV-Chlide *a* in DMSO was dispersed in 25 mM NaH_2PO_4 (pH 7.0) buffer containing 100 mM NaCl. After the addition of BciC protein, the reaction mixture was incubated at 45 °C for 30 min and acetone was added to stop the enzymatic reaction [33]. The solution was concentrated and applied to RP-HPLC for purification of DV-pyroChlide *a*: column, Cosmosil 2.5C_{18}-MS-II (3.0 φ × 75 mm); eluent, MeOH/aqueous 50 mM $AcONH_4$ = 85/15; flow rate, 0.6 mL·min^{-1}.

Spectral data

***Chlorophyllide a* (*Chlide a*).** t_R = 4.50 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S1); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 666 (relative intensity, 1.00), 619 (0.24), 431 nm (0.94); MS (ESI) found: *m*/*z* 615.5, calcd for $C_{35}H_{35}N_4O_5Mg$: MH^+, 615.2 [23, 33, 47].

***Chlorophyllide b* (*Chlide b*).** t_R = 1.96 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S2); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 652 (relative intensity, 0.36), 604 (0.12), 469 nm (1.00); MS (ESI) found: *m*/*z* 629.5, calcd for $C_{35}H_{33}N_4O_6Mg$: MH^+, 629.2 [47, 48].

***Chlorophyllide d* (*Chlide d*).** t_R = 2.05 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S3); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 702 (relative intensity, 1.00), 650 (0.21), 456 (0.72), 402 nm (0.72) [49]; MS (ESI) found: *m*/*z* 617.6, calcd for $C_{34}H_{33}N_4O_6Mg$: MH^+, 617.2.

3-*Acetyl*-chlorophyllide *a* (3*Ac*-Chlide *a*). t_R = 15.0 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 65/35, 0.5 mL·min^{-1}, Fig. S4); VIS (MeOH/aq. 50 mM $AcONH_4$ = 65/35) λ_{max} = 688 (relative intensity, 1.00), 634 (0.28), 442 (0.93), 397 nm (0.94); MS (ESI) found: *m*/*z* 631.4, calcd for $C_{35}H_{35}N_4O_6Mg$: MH^+, 631.2 [18].

3-(1-*Hydroxyethyl*)-chlorophyllide *a* (3*HE*-Chlide *a*). t_R = 10.1/11.2 min for 3^1*R*/*S*-epimers (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 65/35, 0.5 mL·min^{-1}, Fig. S5); VIS (MeOH/aq. 50 mM $AcONH_4$ = 65/35) λ_{max} = 659 (relative intensity, 1.00), 614 (0.25), 426 (0.93), 414 nm (0.96); MS (ESI) found: *m*/*z* 633.4, calcd for $C_{35}H_{37}N_4O_6Mg$: MH^+, 633.3 [18, 26].

3,8-*Divinyl-chlorophyllide a* (*DV-Chlide a*). t_R = 4.89 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S6); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 668 (relative intensity, 0.82), 624 (0.20), 442 nm (1.00); MS (ESI) found: *m/z* 613.5, calcd for $C_{35}H_{33}N_4O_5Mg$: MH^+, 613.2 [33, 43, 50].

3-(1-*Hydroxyethyl*)-8-*vinyl-chlorophyllide a* (*3HE-8V-Chlide a*). t_R = 4.36/4.75 min for 3^1*R*/*S*-epimers (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 70/30, 0.6 mL·min^{-1}, Fig. S7); VIS (MeOH/aq. 50 mM $AcONH_4$ = 70/30) λ_{max} = 660 (relative intensity, 0.88), 617 (0.19), 438 nm (1.00); MS (ESI) found: *m/z* 631.6, calcd for $C_{35}H_{35}N_4O_6Mg$: MH^+, 631.2 [43].

***Pyrochlorophyllide a* (*pyroChlide a*).** t_R = 7.46 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S8); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 666 (relative intensity, 1.00), 621 (0.22), 433 nm (0.97); MS (ESI) found: *m/z* 557.6, calcd for $C_{33}H_{33}N_4O_3Mg$: MH^+, 557.2 [26, 33].

3,8-*Divinyl-pyrochlorophyllide a* (*DV-pyroChlide a*). t_R = 7.41 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 0.6 mL·min^{-1}, Fig. S9); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 666 (relative intensity, 0.88), 622 (0.20), 441 nm (1.00); MS (ESI) found: *m/z* 555.4, calcd for $C_{33}H_{31}N_4O_3Mg$: MH^+, 555.2 [33].

3-*Vinyl-bacteriochlorophyllide a* (*3V-BChlide a*). t_R = 2.74 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 0.6 mL·min^{-1}, Fig. S10); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 725 (relative intensity, 0.77), 670 (0.34), 587 (0.29), 356 nm (1.00); MS (ESI) found: *m/z* 617.6, calcd for $C_{35}H_{37}N_4O_5Mg$: MH^+, 617.3 [16, 33, 43].

***Bacteriochlorophyllide a* (*BChlide a*).** t_R = 1.12 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 0.6 mL·min^{-1}, Fig. S11); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 773 (relative intensity, 0.88), 698 (0.26), 607 (0.27), 366 nm (1.00); MS (ESI) found: *m/z* 633.1, calcd for $C_{35}H_{37}N_4O_6Mg$: MH^+, 633.3 [47, 51].

3-(1-*Hydroxyethyl*)-*bacteriochlorophyllide a* (*3HE-BChlide a*). t_R = 7.21/7.99 min for 3^1*R*/*S*-epimers (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 65/35, 0.5 mL·min^{-1}, Fig. S12); VIS (MeOH/aq. 50 mM $AcONH_4$ = 65/35) λ_{max} = 709 (relative intensity, 0.62), 667 (0.33), 576 (0.27), 346 nm (1.00); MS (ESI) found: *m/z* 635.4, calcd for $C_{35}H_{39}N_4O_6Mg$: MH^+, 635.3 [52].

***Protochlorophyllide a* (*PChlide a*).** t_R = 3.93 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 0.6 mL·min^{-1}, Fig. S13); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 630 (relative intensity, 0.17), 577 (0.05), 532 (0.03), 434 nm (1.00); MS (ESI) found: *m/z* 613.3, calcd for $C_{35}H_{33}N_4O_5Mg$: MH^+, 613.2 [18, 28, 33, 43, 52].

3-*Acetyl-protochlorophyllide a* (*3Ac-PChlide a*). t_R = 2.74 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S14) [53]; VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 643 (relative intensity, 0.17), 588 (0.04), 543 (0.03), 436 nm (1.00); MS (ESI) found: *m/z* 629.4, calcd for $C_{35}H_{33}N_4O_6Mg$: MH^+, 629.2.

3-(1-*Hydroxyethyl*)-*protochlorophyllide a* (*3HE-PChlide a*). t_R = 1.77/1.98 min for 3^1*R*/*S*-epimers (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S15); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 626 (relative intensity, 0.16), 574 (0.06), 533 (0.04), 433 nm (1.00); MS (ESI) found: *m/z* 631.3, calcd for $C_{35}H_{35}N_4O_6Mg$: MH^+, 631.2.

3,8-*Divinyl-protochlorophyllide a* (*DV-PChlide a*). t_R = 4.22 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 0.6 mL·min^{-1}, Fig. S16); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 630 (relative intensity, 0.15), 578 (0.06), 542 (0.03), 439 nm (1.00); MS (ESI) found: *m/z* 611.5, calcd for $C_{35}H_{31}N_4O_5Mg$: MH^+, 611.2 [33].

***Zinc pheophorbide a* (*Zn-Pheide a*).** t_R = 6.90 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 80/20, 0.6 mL·min^{-1}, Fig. S17); VIS (MeOH/aq. 50 mM $AcONH_4$ = 80/20) λ_{max} = 661 (relative intensity, 0.89), 614 (0.21), 572 (0.12), 528 (0.05), 429 nm (1.00); MS (ESI) found: *m/z* 655.3, calcd for $C_{35}H_{35}N_4O_5Zn$: MH^+, 655.2 [54].

***Zinc* 3-*acetyl-pheophorbide a* (*Zn-3Ac-Pheide a*).** t_R = 31.3 min (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 65/35, 0.5 mL·min^{-1}, Fig. S18); VIS (MeOH/aq. 50 mM $AcONH_4$ = 65/35) λ_{max} = 679 (relative intensity, 0.96), 630 (0.26), 437 (1.00), 388 nm (0.84); MS (ESI) found: *m/z* 671.2, calcd for $C_{35}H_{35}N_4O_6Zn$: MH^+, 671.2 [18].

***Zinc*-3-(1-*hydroxyethyl*)-*pheophorbide a* (*Zn-3HE-Pheide a*).** t_R = 16.5/18.0 min for 3^1*R*/*S*-epimers (Cosmosil 2.5C_{18}-MS-II, 3.0 φ × 75 mm, MeOH/aq. 50 mM $AcONH_4$ = 65/35, 0.5 mL·min^{-1}, Fig. S19); VIS (MeOH/aq. 50 mM $AcONH_4$ = 65/35) λ_{max} = 655 (relative intensity, 1.00), 608 (0.26), 570 (0.17), 425 (0.98), 412 nm (0.95); MS (ESI) found: *m/z* 673.4, calcd for $C_{35}H_{37}N_4O_6Zn$: MH^+, 673.2 [18].

***Zinc* 8-*ethyl*-12-*methyl-bacteriopheophorbide c* (*Zn-[E,M]BPheide c*).** t_R = 4.09/4.66 min for 3^1*R*/*S*-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S20); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 665 (relative intensity, 0.78), 623 (0.18), 434 nm (1.00); MS (ESI) found: *m/z* 629.4, calcd for $C_{34}H_{37}N_4O_4Zn$: MH^+, 629.2 [28].

***Zinc* 8-*ethyl*-12-*ethyl-bacteriopheophorbide c* (*Zn-[E,E]BPheide c*).** t_R = 4.84/5.61 min for 3^{1}R/S-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S21); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 665

(relative intensity, 0.84), 624 (0.20), 433 nm (1.00); MS (ESI) found: m/z 643.3, calcd for $C_{35}H_{39}N_4O_4Zn$: MH^+, 643.2 [28].

***Zinc* 8-*propyl*-12-*ethyl*-bacteriopheophorbide *c* (*Zn*-[*P,E*]*BPheide c*).** t_R = 5.69/6.70 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S22); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 665 (relative intensity, 0.80), 626 (0.18), 434 nm (1.00); MS (ESI) found: *m*/*z* 657.2, calcd for $C_{36}H_{41}N_4O_4Zn$: MH^+, 657.2 [28].

***Zinc* 8-*isobutyl*-12-*ethyl*-bacteriopheophorbide *c* (*Zn*-[*I,E*]*BPheide c*).** t_R = 6.09/7.29 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S23); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 664 (relative intensity, 0.75), 628 (0.18), 434 nm (1.00); MS (ESI) found: *m*/*z* 671.2, calcd for $C_{37}H_{43}N_4O_4Zn$: MH^+, 671.3 [28].

***Zinc* 8-*ethyl*-12-*methyl*-bacteriopheophorbide *d* (*Zn*-[*E,M*]*BPheide d*).** t_R = 8.51/9.74 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 75/25, 1.0 mL·min^{-1}, Fig. S24); VIS (MeOH/aq. 50 mM $AcONH_4$ = 75/25) λ_{max} = 652 (relative intensity, 0.78), 610 (0.22), 425 nm (1.00); MS (ESI) found: *m*/*z* 615.0, calcd for $C_{33}H_{35}N_4O_4Zn$: MH^+, 615.2 [26, 43].

***Zinc* 8-*ethyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-[*E,E*]*BPheide d*).** t_R = 3.82/4.09 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S25); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 652 (relative intensity, 0.97), 607 (0.19), 425 nm (1.00); MS (ESI) found: *m*/*z* 629.4, calcd for $C_{34}H_{37}N_4O_4Zn$: MH^+, 629.2 [43].

***Zinc* 8-*propyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-[*P,E*]*BPheide d*).** t_R = 4.36/4.76 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S26); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 652 (relative intensity, 0.96), 607 (0.19), 426 nm (1.00); MS (ESI) found: *m*/*z* 643.3, calcd for $C_{35}H_{39}N_4O_4Zn$: MH^+, 643.2 [43].

***Zinc* 8-*isobutyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-[*I,E*]*BPheide d*).** t_R = 5.10/5.59 min for $3^1R/S$-epimers (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S27); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 653 (relative intensity, 0.95), 606 (0.20), 426 nm (1.00); MS (ESI) found: *m*/*z* 657.4, calcd for $C_{36}H_{41}N_4O_4Zn$: MH^+, 657.2 [43].

***Zinc* 3-*vinyl*-8-*ethyl*-12-*methyl*-bacteriopheophorbide *c* (*Zn*-3*V*-[*E,M*]*BPheide c*).** t_R = 11.3 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S28); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 668 (relative intensity, 0.77), 629 (0.19), 436 nm (1.00); MS (ESI) found: *m*/*z* 611.4, calcd for $C_{34}H_{35}N_4O_3Zn$: MH^+, 611.2 [28].

***Zinc* 3-*vinyl*-8-*ethyl*-12-*ethyl*-bacteriopheophorbide *c* (*Zn*-3*V*-[*E,E*]*BPheide c*).** t_R = 13.2 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S29); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 668 (relative intensity, 0.78), 630 (0.18), 435 nm (1.00); MS (ESI) found: *m*/*z* 625.2, calcd for $C_{35}H_{37}N_4O_3Zn$: MH^+, 625.2 [28].

***Zinc* 3-*vinyl*-8-*propyl*-12-*ethyl*-bacteriopheophorbide *c* (*Zn*-3*V*-[*P,E*]*BPheide c*).** t_R = 17.1 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S30); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 668 (relative intensity, 0.77), 629 (0.18), 436 nm (1.00); MS (ESI) found: *m*/*z* 639.3, calcd for $C_{36}H_{39}N_4O_3Zn$: MH^+, 639.2 [28].

***Zinc* 3-*vinyl*-8-*isobutyl*-12-*ethyl*-bacteriopheophorbide *c* (*Zn*-3*V*-[*I,E*]*BPheide c*).** t_R = 20.6 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S31); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 668 (relative intensity, 0.75), 630 (0.18), 436 nm (1.00); MS (ESI) found: *m*/*z* 653.3, calcd for $C_{37}H_{41}N_4O_3Zn$: MH^+, 653.3 [28].

***Zinc* 3-*vinyl*-8-*ethyl*-12-*methyl*-bacteriopheophorbide *d* (*Zn*-3*V*-[*E,M*]*BPheide d*).** t_R = 9.74 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S32); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 660 (relative intensity, 0.88), 614 (0.19), 430 nm (1.00); MS (ESI) found: *m*/*z* 597.2, calcd for $C_{33}H_{33}N_4O_3Zn$: MH^+, 597.2 [26, 43].

***Zinc* 3-*vinyl*-8-*ethyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-3*V*-[*E,E*]*BPheide d*).** t_R = 12.2 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S33); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 660 (relative intensity, 0.87), 613 (0.19), 429 nm (1.00); MS (ESI) found: *m*/*z* 611.4, calcd for $C_{34}H_{35}N_4O_3Zn$: MH^+, 611.2 [43].

***Zinc* 3-*vinyl*-8-*propyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-3*V*-[*P,E*]*BPheide d*).** t_R = 15.9 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S34); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 660 (relative intensity, 0.90), 612 (0.19), 430 nm (1.00); MS (ESI) found: m/z 625.5, calcd for $C_{35}H_{37}N_4O_3Zn$: MH^+, 625.2 [43].

***Zinc* 3-*vinyl*-8-*isobutyl*-12-*ethyl*-bacteriopheophorbide *d* (*Zn*-3*V*-[*I,E*]*BPheide d*).** t_R = 19.7 min (Cosmosil 5C18-AR-II, 4.6 φ × 150 mm, MeOH/aq. 50 mM $AcONH_4$ = 85/15, 1.0 mL·min^{-1}, Fig. S35); VIS (MeOH/aq. 50 mM $AcONH_4$ = 85/15) λ_{max} = 660 (relative intensity, 0.89), 612 (0.18), 430 nm (1.00); MS (ESI) found: *m*/*z* 639.6, calcd for $C_{36}H_{39}N_4O_3Zn$: MH^+, 639.2 [43].

CONCLUSIONS

Various (B)Chlides were successfully semi-synthesized from natural (B)Chls extracted from photosynthetic

organisms, using a combination of organic synthetic techniques and enzymatic modifications. RP-HPLC on an ODS column with an eluent mixture of methanol and aqueous 50 mM ammonium acetate was useful for purification, identification, and analysis of the semi-synthetic (B)Chlides. The t_R-values of these (B)Chlides were dependent on the molecular lipophilicity and the coordination ability of the central metal to the HPLC eluent: (1) t_R(bacteriochlorin) < t_R(chlorin) < t_R(porphyrin) for π-conjugation of the tetrapyrrole skeleton, (2) t_R(3HE) < t_R(3Ac) < t_R(3V) for the 3-substituents, (3) t_R(Mg) < t_R(Zn) for the central metal, and (4) t_R(20-H) < t_R(20-Me) and t_R([E,M]) < t_R([E,E]) < t_R([P,E]) < t_R([I,E]) for 8^2-, 12^1-, and 20-methylation.

Acknowledgments

We thank Dr. Yusuke Tsukatani for his generous gift of chlorophyllase as well as Dr. Jiro Harada for BchF hydratase and BciC demethoxycarbonylase. We also thank Prof. Tadashi Mizoguchi of Ritsumeikan University for his assistance in HPLC analyses. This work was partially supported by JSPS KAKENHI Grant Number JP17H06436 in the Scientific Research on Innovative Areas "Innovation for Light-Energy Conversion (I^4LEC)" (to HT) as well as the Sasakawa Scientific Research Grant from The Japan Science Society and JSPS KAKENHI Grant Number JP17J08860 in the JSPS Research Fellow program (to MT).

Supporting information

Figures S1–S35 are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

REFERENCES

1. Fajer J. *Photosynth. Res*. 2004; **80**: 165–172.
2. Blankenship RE. Molecular mechanism of photosynthesis, 2nd ed., Wiley Blackwell: Oxford, 2014; pp. 1–9.
3. Tamiaki H, Teramura M and Tsukatani Y. *Bull. Chem. Soc. Jpn*. 2016; **89**: 161–173.
4. Senge MO, Ryan AA, Letchford KA, MacGowan SA and Milke T. *Symmetry* 2014; **6**: 781–843.
5. Chen M. *Annu. Rev. Biochem.* 2014; **83**: 317–340.
6. Walker JC and Willows DR. *Biochem. J*. 1997; **327**: 321–333.
7. Bollivar DW, Jiang ZY, Bauer CE and Beale SI. *J. Bacteriol.* 1994; **176**: 5290–5296.
8. Gibson LC and Hunter CN. *FEBS Lett.* 1994; **352**: 127–130.
9. Ouchane S, Steunou AS, Picaud M and Astier C. *J. Biol. Chem.* 2004: **279**: 6385–6394.
10. Minamizaki K, Mizoguchi T, Goto T, Tamiaki H and Fujita Y. *J. Biol. Chem*. 2008; **283**: 2684–2692.
11. Wang P, Gao J, Wan C, Zhang F, Xu Z, Huang X, Sun X and Deng X. *Plant. Physiol*. 2010; **153**: 994–1003.
12. Heyes DJ, Ruban AV, Wilks HM and Hunter CN. *Proc. Natl. Acad. Sci. USA* 2002; **99**: 11145–11150.
13. Raymond J, Siefert JL, Staples CR and Blankenship RE. *Mol. Biol. Evol*. 2004; **21**: 541–554.
14. Muraki N, Nomata J, Ebata K, Mizoguchi T, Shiba T, Tamiaki H, Kurisu G and Fujita, Y. *Nature* 2010; **465**: 110–114.
15. Oster U, Bauer CE and Rüdiger W. *J. Biol. Chem.* 1997; **272**: 9671–9676.
16. Nomata J, Mizoguchi T, Tamiaki H and Fujita Y. *J. Biol. Chem.* 2006; **281**: 15021–15028.
17. Bollivar DW, Suzuki JY, Beatty JT, Dobrowolski JM and Bauer CE. *J. Mol. Biol.* 1994; **237**: 622–640.
18. Lange C, Kiesel S, Peters S, Virus S, Scheer H, Jahn D and Moser J. *J. Biol. Chem*. 2015; **290**: 19697–19709.
19. Kräutler B. *Angew. Chem. Int. Ed*. 2016; **55**: 4882–4907.
20. Tamiaki H, Shibata R and Mizoguchi T. *Photochem. Photobiol*. 2007; **83**: 152–162.
21. Liu Z and Bryant DA. *J. Biol. Chem.* 2011; **286**: 22393–22402.
22. Tamiaki H and Kunieda M. Handbook of Porphyrin Science, Vol. 11, World Scientific, 2011; pp. 223–290.
23. Tsukatani Y, Yamamoto H, Harada J, Yoshitomi T, Nomata J, Kasahara M, Mizoguchi T, Fujita Y and Tamiaki H. *Sci. Rep.* 2013; **3**: 1217, doi: 10.1038/srep01217.
24. Iriyama K, Ogura N and Takamiya A. *J. Biochem.* 1974; **76**: 901–904.
25. Tsuchiya T, Ohta H, Okawa K, Iwamatsu A, Shimada H, Masuda T and Takamiya K. *Proc. Natl. Acad. Sci. USA* 1999; **96**: 15362–15367.
26. Harada J, Teramura M, Mizoguchi T, Tsukatani Y, Yamamoto K and Tamiaki H. *Mol. Microbial*. 2015; **98**: 1184–1198.
27. Pennington FC, Strain HH, Svec WA and Katz JJ. *J. Am. Chem. Soc*. 1964; **86**: 1418–1426.
28. Teramura M, Harada J and Tamiaki H. *Photosynth. Res*. 2016; **130**: 33–45.
29. Tamiaki H, Takeuchi S, Tsudzuki S, Miyatake T and Tanikaga R. *Tetrahedron* 1998; **54**: 6699–6718.
30. Soares AR, Thanaiah Y, Taniguchi M and Lindsey JS. *New. J. Chem.* 2013; **37**: 1087–1097.
31. Cui Y, Ogasawara S and Tamiaki H. *J. Porphyr. Phthalocy.* 2017; **21**: 692–699.
32. Tamiaki H, Kouraba M, Takeda K, Kondo S and Tanikaga R. *Tetrahedron Asymm.* 1998; **9**: 2101–2111.
33. Teramura M, Harada J, Mizoguchi T, Yamamoto K and Tamiaki H. *Plant Cell Physiol.* 2016; **57**: 1048–1057.
34. Kunieda M, Mizoguchi T and Tamiaki H. *Photochem. Photobiol*. 2004; **79**: 55–61.

35. Mizoguchi T, Saga Y and Tamiaki H. *Photochem. Photobiol Sci.* 2002; **1**: 780–787.
36. Miyatake T, Tamiaki H, Holzwarth AR and Schaffner K. *Photochem. Photobiol*. 1999; **69**: 448–456.
37. Tamiaki H, Machida S and Mizutani K. *J. Org. Chem.* 2012; **77**: 4751–4758.
38. Tamiaki H, Tomida T and Miyatake T. *Bioorg. Med. Chem. Lett.* 1997; **7**: 1415–1418.
39. Tamiaki H, Komada J, Kunieda M, Fukai K, Yoshitomi T, Harada J and Mizoguchi T. *Photosynth. Res.* 2011; **107**: 133–138.
40. Harada J, Shibata Y, Teramura M, Mizoguchi T, Kinoshita Y, Yamamoto K and Tamiaki H. *ChemPhotoChem* 2018; **2**, in press, doi: 10.1002/cptc.201700164.
41. Tamiaki H, Yagai S and Miyatake T. *Bioorg. Med. Chem.* 1998; **6**: 2171–2178.
42. Mizoguchi T, Nagai C, Kunieda M, Kimura Y, Okamura A and Tamiaki H. *Org. Biomol. Chem.* 2009; **7**: 2120–2126.
43. Teramura M, Harada J and Tamiaki H. *Photosynth. Res.* 2018; **135**: 319–328.
44. Oba T, Masada Y and Tamiaki H. *Bull. Chem. Soc. Jpn.* 1997; **70**: 1905–1909.
45. Mizoguchi T, Shoji A, Kunieda M, Miyashita H, Tsuchiya T, Mimuro M and Tamiaki H. *Photochem. Photobiol. Sci*. 2006; **5**: 291–299.
46. Tamiaki H, Ariki N, Sugiyama H, Taira Y, Kinoshita Y and Miyatake T. *Tetrahedron* 2013; **69**: 8412–8421.
47. Chou YL, Lee YL, Yen CC, Chen LFO, Lee LC and Shaw JF. *Biotechnol. Appl. Biochem.* 2016; **63**: 371–377.
48. Chen K, Ríos JJ, Roca M and Pérez-Gálvez A. *J. Chromatogr. A* 2015; **1412**: 90–99.
49. Fukusumi T, Matsuda K, Mizoguchi T, Miyatake T, Ito S, Ikeda T, Tamiaki H and Oba T. *FEBS Lett*. 2012; **586**: 2338–2341.
50. Harada J, Mizoguchi T, Tsukatani Y, Yokono M, Tanaka A and Tamiaki H. *J. Biol. Chem.* 2014; **289**: 12716–12726.
51. Kiesel S, Wätzlich D, Lange C, Reijerse E, Bröcker MJ, Rüdiger W, Lubitz W, Scheer H, Moser J and Jahn D. *J. Biol. Chem.* 2015; **290**: 1141–1154.
52. Guijarro-Díez M, Nozal L, Marina ML and Crego AL. *Anal. Bioanal. Chem.* 2015; **407**: 7197–7213.
53. Scholz B and Ballschmiter K. *Angew. Chem. Int. Ed.* 1981; **20**: 956–958.
54. Helfrich M and Rüdiger W. *Z. Naturforsch., C: J. Biosci.* 1992; **47**: 231–238.