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Polarity-tunable and wavelength-tunable bacteriochlorins bearing a single carboxylic acid or NHS ester. Use in a protein bioconjugation model system†

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To broaden the scope of near-infrared (NIR)-active chromophores available for bioconjugation with proteins, 10 new bacteriochlorins have been synthesized: six are lipophilic and bear a carboxylic acid tether; four are hydrophilic and bear four carboxylic acids and one *N*-hydroxysuccinimido (NHS) ester tether. Each bacteriochlorin exhibits a sharp long-wavelength absorption (Q_y) band in the NIR region (727–823 nm). The lipophilic bacteriochlorins were examined in DMF (fluorescence quantum yield Φ_f = 0.037–0.19) whereas the hydrophilic bacteriochlorins were examined in DMF (Φ_f also = 0.037–0.19) or aqueous phosphate buffer (Φ_f = 0.0011–0.13). Two bacteriochlorins were conjugated to myoglobin (Mb), which contains ~14 accessible amino groups. Use of 2, 10, or 50 equivalents of a hydrophilic bacteriochlorin–NHS ester (**BC7**) gave average loadings of 0.62, 1.6, or 7.1 bacteriochlorins/Mb as determined by absorption spectral comparison with the strongly absorbing heme ligand. MALDI-MS analysis showed a distribution of 0–9 bound bacteriochlorins for the conjugate sample with average loading of 7.1. The **Mb–BC7** conjugates exhibited characteristic absorption and fluorescence spectra in aqueous buffer, yet the Φ_f value was markedly low (Φ_f ~ 0.02) regardless of loading *versus* that of the **BC7** monomer (Φ_f = 0.12), attributed in part to heme quenching. Removal of the heme revealed a loading-dependent Φ_f, which ranged from 0.091 (0.62 loading) to 0.023 (7.1 loading). The decrease in Φ_f with increased loading is attributed to self-quenching perhaps facilitated by excited-state energy transfer among the bacteriochlorins (Förster R_0 = 59 Å). Taken together, the results show facile access to a collection of useful bacteriochlorins for NIR spectroscopic studies, along with a pigment–protein system that serves the dual purposes of a convenient testbed for evaluating protein bioconjugation processes as well as a nanosized architecture for use in photochemical studies.

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Introduction

The conjugation of chromophores to biological molecules has a rich history both in methods and applications.[1–9] Yet, a comparatively unexplored topic in this domain concerns the use of NIR-active chromophores. The challenge to filling this lacuna entails synthetic tailoring of NIR-active chromophores to achieve the molecular design requirements, which typically include adjusting the polarity of the chromophore, tuning the wavelength of absorption/emission, and incorporating a single bioconjugatable tether. Examples of synthetic NIR chromophores range from long-chain cyanine dyes to quantum dots.[10–13] Nature's NIR-active chromophores are built around the bacteriochlorin π-system, which provides the basis for bacterial photosynthesis (Bchl *a*, *b* and *g*)[14] and unknown roles in other organisms (*e.g.*, tolyporphins)[15–21] (Chart 1).

Semisynthesis beginning with naturally occurring macrocycles has been a mainstay for preparing and tailoring bacteriochlorins,[22,23] but the presence of a number of substituents about the perimeter of such macrocycles limits synthetic manipulations particularly for wavelength tuning, polarity tuning, and installation of a single bioconjugatable tether. Methods to prepare synthetic bacteriochlorins are under active investigation[24–44] and have been recently reviewed.[45,46] Two approaches that define the range of such methods include (1) double addition to a porphyrin thereby converting two, opposite pyrrole rings to pyrroline rings, and (2) *de novo* synthesis wherein the pyrroline rings are incorporated as pre-made constituents upon macrocycle formation.[40–44,47–50] Bioconjugatable bacteriochlorins have

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† Electronic supplementary information (ESI) available: Sequence and structure of Mb; MALDI-MS and spectral data for bacteriochlorin–Mb conjugates. See DOI: 10.1039/c4nj01340a

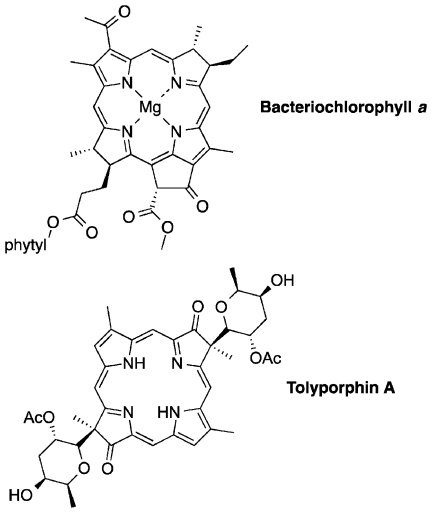


Chart 1 Representative natural bacteriochlorins.

been prepared by both approaches, as illustrated by the examples shown in Chart 2. Bacteriochlorins **I** and **II** were prepared by OsO_4 treatment of a porphyrin,[25,27] whereas the set of **III–V** were prepared by *de novo* synthesis.[51–55] Note the nature of the bioconjugatable groups [isothiocyanate (**I**), carboxylic acid (**II**), maleimide (**III**, **IV**), and NHS ester (**V**)] as well as the polarity: bacteriochlorin members of sets **III** and **V** are hydrophobic, whereas **IV** is hydrophilic.

The ability to tune the position of the long-wavelength absorption band (and hence the position of the fluorescence emission band) relies on introduction of auxochromes at the perimeter of the macrocycle.[56] The long-wavelength absorption (Q_y) band stems from a transition that is polarized along the long axis of the molecule, as shown in Fig. 1. Accordingly, the introduction of substituents at the β-pyrrole positions (2, 3, 12, 13) or adjacent meso-positions (5, 15) enables the band to be shifted from ~700 to nearly 900 nm. For the members of set **III**, the Q_y band ranges from 713 to 756 nm.[51,53] Such bacteriochlorins have been bioconjugated to analogues of the native membrane-spanning peptides of the light-harvesting complexes of photosynthetic bacteria. The resulting biohybrid light-harvesting architectures self-assemble in aqueous-detergent media. The appended synthetic bacteriochlorins – attached *via* a maleimide–cysteinyl linkage – absorb NIR light and funnel the resulting excited-state energy to lower-energy-absorbing chromophores as part of the light-harvesting process.[51,53]

We set out to develop a more broadly viable set of wavelength-tunable and polarity-tunable bacteriochlorins. Because one objective is to be able to conjugate multiple copies of a bacteriochlorin to a given peptide, we turned to the use of carboxylic acid or NHS esters (conjugatable with amines)[57] to avoid the problems anticipated if multiple cysteines were employed to accommodate bacteriochlorin–maleimides. To our knowledge, the only bacteriochlorin–NHS esters prepared by *de novo* synthesis are **Va** and **Vb** of Chart 2.

Two sets of target bacteriochlorins were identified (Chart 3). The members of the first set are lipophilic and bear a carboxylic acid for bioconjugation (**BC1–BC6**). The members of the second set are hydrophilic (each contains four carboxylic acid groups for aqueous solubilization) and bear a single NHS ester for bioconjugation (**BC7–BC10**). All of the bacteriochlorins are free base macrocycles, except for one zinc bacteriochlorin (**BC4**). **BC7** and **BC8** differ only in the *meta- versus para*-substitution of the tether. On the basis of the spectral properties of analogous bacteriochlorins (lacking a carboxylic acid tether), absorption in the NIR region (726–823 nm) is expected.

In this paper, we report the synthesis of the 10 bacteriochlorins along with their absorption and fluorescence properties in DMF

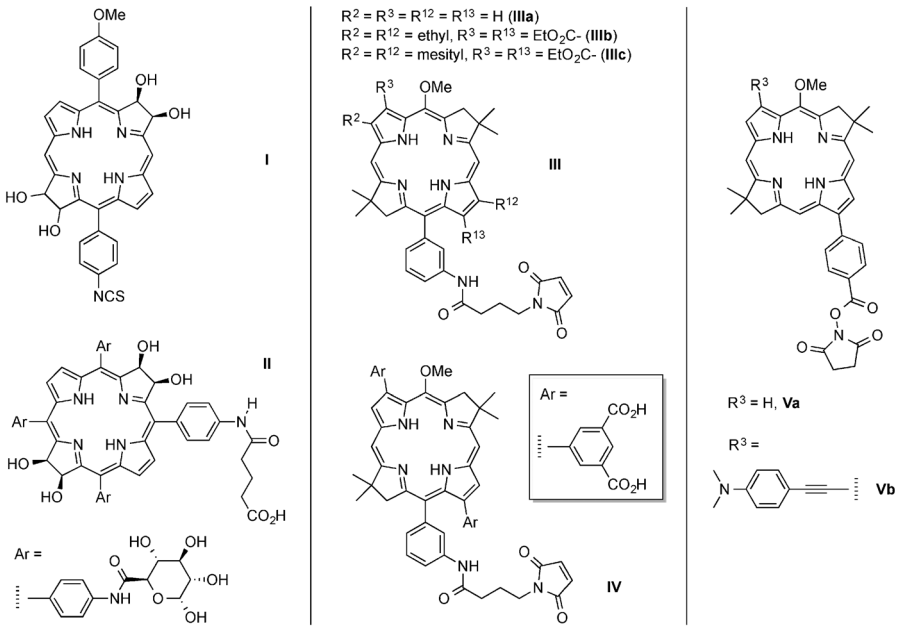


Chart 2 Representative bioconjugatable synthetic bacteriochlorins.

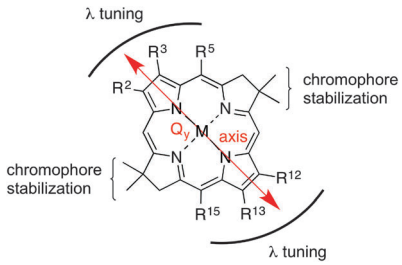


Fig. 1 Molecular design features of synthetic bacteriochlorins.

and/or aqueous solution. Many applications of the bacteriochlorins can be envisaged. We have employed myoglobin (Mb) as a globular protein for bioconjugation with selected hydrophilic bacteriochlorins, thereby affording a nanoscale analogue of pheophytin-coated polystyrene particles described by Cellarius and Mauzerall nearly a half-century ago.[58] The absorption and fluorescence properties of the resulting Mb–bacteriochlorin conjugates have been examined in aqueous solution in the presence or absence of the heme ligand. Taken together, the studies afford a new set of synthetic bacteriochlorins for use in cases where one seeks aqueous or membrane solubility, wavelength tunability (726 to 823 nm), and bioconjugation *via* one of the simplest joining reactions (amidation).

Results and discussion

Synthesis of lipophilic bacteriochlorins

The six monocarboxy-bacteriochlorins (**BC1–BC6**) were derivatized from three known bacteriochlorin building blocks (**BC11**,[51] **BC12**,[42] **BC13**[53]) bearing substituents at the 2, 3, 12, 13, 15 positions for wavelength tailoring and derivatization (Chart 4). Three distinct methods were employed to introduce the carboxylic acid group. (1) For bacteriochlorins with a 3-aminophenyl group, nucleophilic ring-opening of succinic anhydride gave the carboxylic acid group directly (**BC1**, **BC4** and **BC5**). (2) For 15-brominated bacteriochlorins, Suzuki coupling (with compounds bearing a protected carboxylic acid group) followed by deprotection with trifluoroacetic acid (TFA) unveiled the carboxylic acid group (**BC2** and **BC3**). (3) Pd-mediated carbonylation with a BOC-protected amine formed the bacteriochlorin–imide, which upon TFA deprotection unveiled the carboxylic acid group (**BC6**). All of these methods proceeded smoothly to afford the monocarboxy-bacteriochlorins in good to excellent yields.

Reaction of aminophenylbacteriochlorin **BC11** with succinic anhydride in $CHCl_3$ afforded **BC1** in 67% yield (Scheme 1). Metalation[44] of bacteriochlorin **BC11** with zinc triflate in the presence of sodium hydride afforded **BC14** in 52% yield.

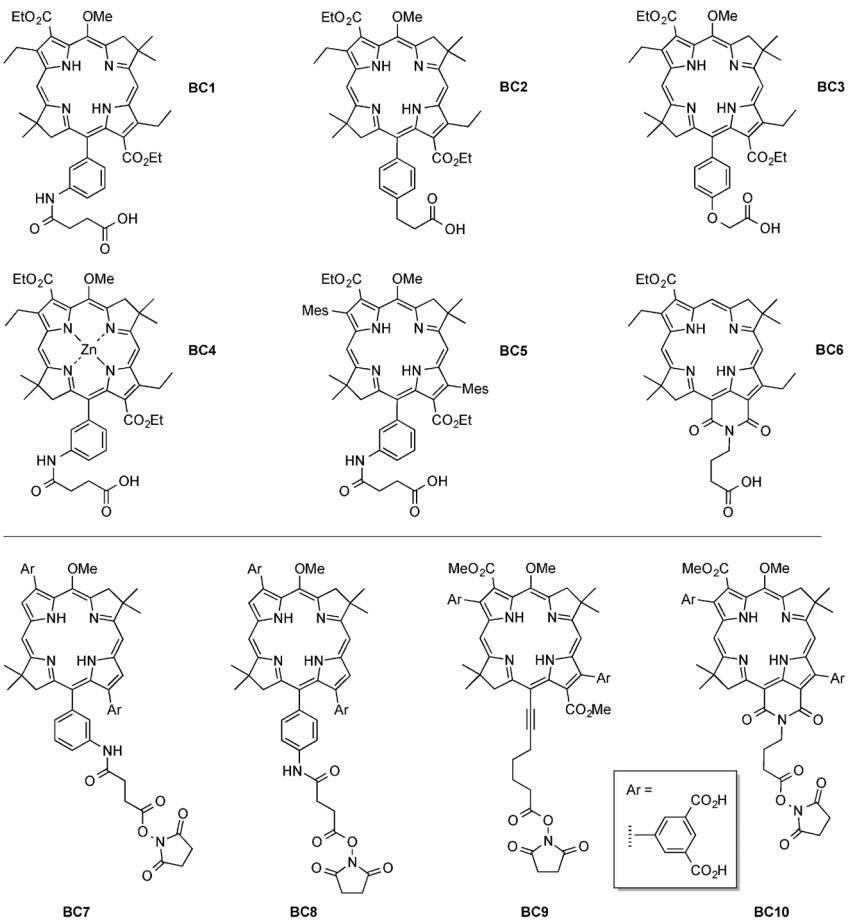


Chart 3 Structures of six lipophilic monocarboxy-bacteriochlorins (top) and four hydrophilic tetracarboxy-bacteriochlorins bearing an NHS ester (below).

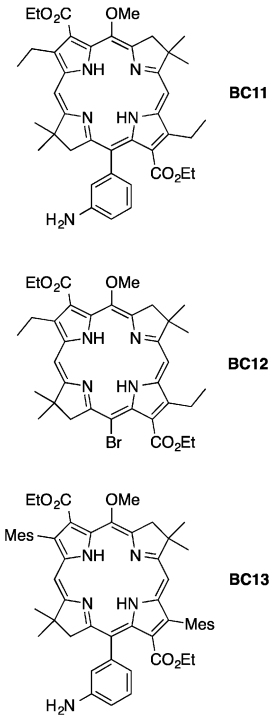
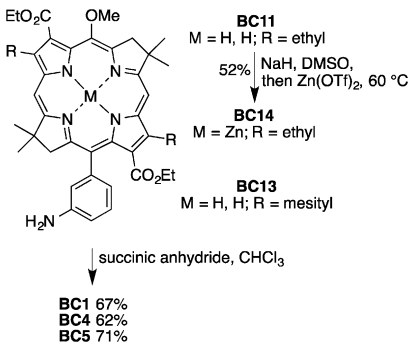


Chart 4 Three known bacteriochlorin building blocks.



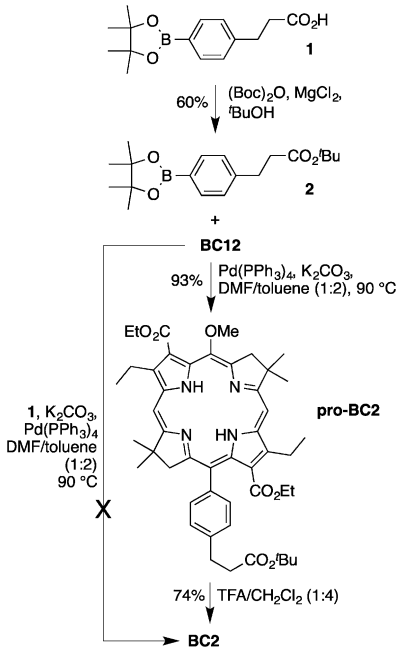
Scheme 1 Synthesis of monocarboxy-bacteriochlorins **BC1**, **BC4** and **BC5**.

Reaction of **BC14** with succinic anhydride gave the carboxy-bacteriochlorin **BC4** in 62% yield. In a similar manner to that of **BC1** and **BC4**, treatment of bacteriochlorin **BC13** with succinic anhydride afforded **BC5** in 71% yield.

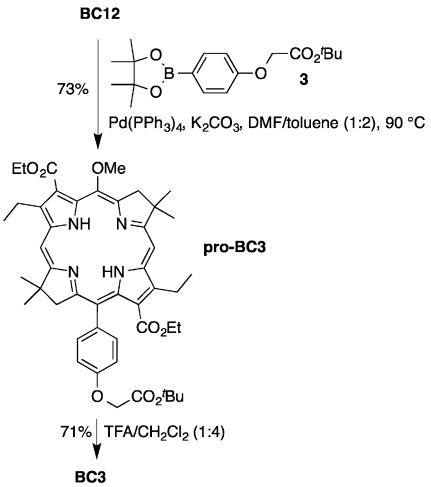
Suzuki coupling of bacteriochlorin **BC12** with compound **1** failed to give the desired carboxy-bacteriochlorin **BC2**, presumably because of the presence of the free carboxylic acid group of **1** (Scheme 2). Alternatively, protection of the free carboxylic acid group of **1** by treatment with di-*tert*-butyl dicarbonate [$(Boc)_2O$] in the presence of $MgCl_2$[59] afforded the *tert*-butyl ester **2** in 60% yield. Suzuki coupling of bacteriochlorin **BC12** with **2** gave **pro-BC2** in excellent yield (93%). Cleavage of the *tert*-butyl protecting group in 20% TFA gave **BC2** in 74% yield.[52]

BC3 was obtained in a similar manner as for **BC2**, using the known Suzuki coupling partner **3**[41] (Scheme 3).

Bacteriochlorin-13,15-dicarboximides with a methoxy group at the 5-position have been synthesized previously.[43]

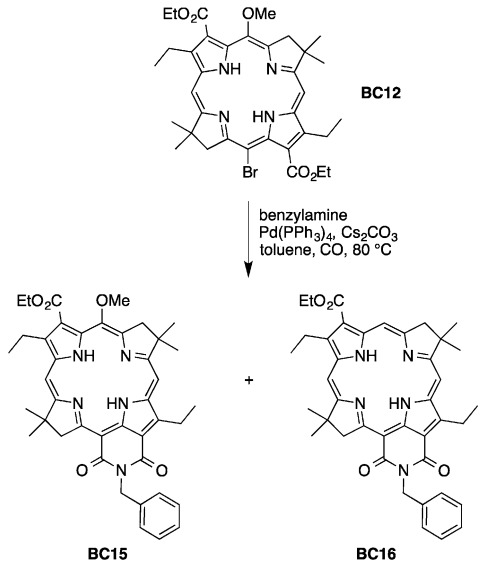


Scheme 2 Synthesis of monocarboxy-bacteriochlorin **BC2**.



Scheme 3 Synthesis of monocarboxy-bacteriochlorin **BC3**.

The imide-forming reaction entails treatment of a bacteriochlorin (bearing a 13-carboethoxy group and a 15-bromo group; *e.g.*, **BC12**) to Pd-mediated carbamoylation in the presence of an amine and CO. The reaction is carried out in the presence of a base, typically Cs_2CO_3. Thus, **BC12** was converted to **BC15** in 62% yield upon use of 3 equivalents of Cs_2CO_3.[43] Upon repeating this synthesis, **BC15** was obtained in 55% yield, and we noted the presence of a trace amount (<5%) of the corresponding bacteriochlorin–imide lacking the 5-methoxy group (**BC16**). When the reaction was repeated with 9 equivalents of Cs_2CO_3, the ratio reversed: the demethoxylated **BC16** was obtained in 84% yield whereas the 5-methoxybacteriochlorin **BC15** was obtained in <5% yield (Scheme 4). The reaction is readily monitored by absorption spectroscopy (as well as MALDI-MS), given that the long-wavelength absorption maximum is at 726 nm (**BC12**),



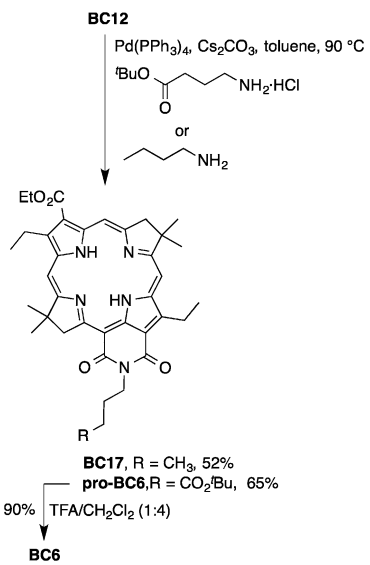
Scheme 4 Demethoxylation upon imide formation (see text for yields).

798 nm (**BC15**) and 820 nm (**BC16**). Removal of the 5-methoxy group thus provides a convenient means to impart a bathochromic shift of the long-wavelength absorption band of the bacteriochlorin.

Herein, 15 equivalents of Cs_2CO_3 were used to form the bacteriochlorin–imide as well as remove the 5-methoxy group. The synthesis was first carried out with *n*-butylamine, which gave the 5-demethoxylated bacteriochlorin–imide **BC17** in 52% yield (Scheme 5). Similar use of *tert*-butyl 4-aminobutyrate gave **pro-BC6** in 65% yield. Cleavage of the protecting group with TFA gave the monocarboxy-bacteriochlorin **BC6** in 90% yield.

Synthesis of hydrophilic bacteriochlorins

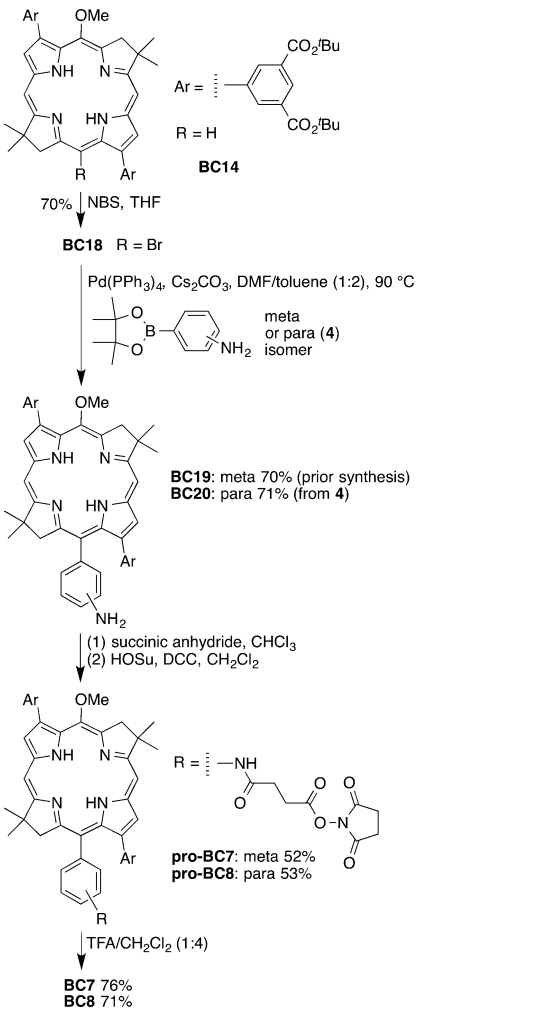
The generic route for the synthesis of the four tetracarboxy-bacteriochlorin–NHS esters (**BC7–BC10**, Chart 3) is as follows:



Scheme 5 Synthesis of monocarboxy-bacteriochlorin **BC6**.

(1) self-condensation of a bromodihydrodipyrrin–acetal to form the 3,13-dibromo-5-methoxybacteriochlorin macrocycle;[40,42] (2) Pd-mediated Suzuki coupling to install the 3,13-bis(3,5-di-*tert*-butoxycarbonylphenyl) groups;[52] (3) regioselective bromination at the 15-position;[41] (4) Pd-mediated Suzuki or Sonogashira coupling to install the unprotected carboxylic acid tether at the 15-position;[41] (5) reaction with *N*-hydroxysuccinimide (HOSu) in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to afford the penultimate, fully protected bacteriochlorin target bearing four *tert*-butyl esters and one NHS ester; and (6) selective cleavage of the *tert*-butyl groups to give the tetracarboxy-bacteriochlorin bearing a single NHS ester.

The synthesis of the bacteriochlorin–NHS esters **BC7** and **BC8** is shown in Scheme 6. **BC18** and **BC19** were reported in our previous paper,[52] and are presented here for comparison. The Suzuki coupling of bacteriochlorin **BC18** with *p*-anilinoboronic ester **4** afforded **BC20** in 71% yield. Treatment of **BC19** or **BC20** with succinic anhydride in $CHCl_3$ afforded the intermediate 15-carboxybacteriochlorin, which was partially purified by column

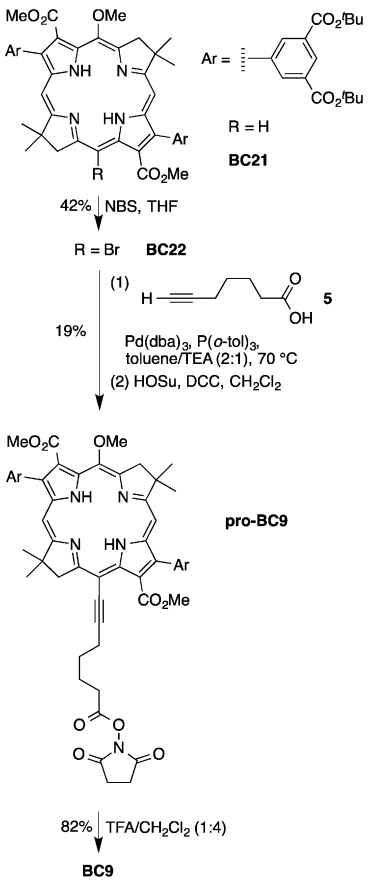


Scheme 6 Synthesis of tetracarboxy-bacteriochlorin–NHS esters **BC7** and **BC8**.

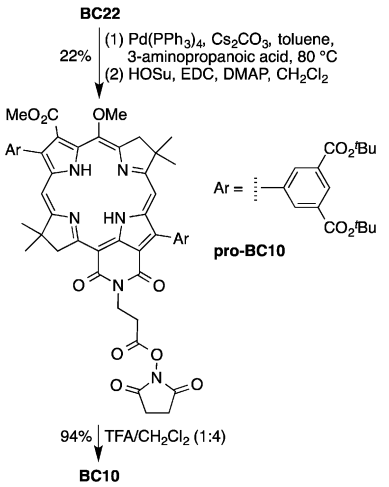
chromatography. Esterification of each crude bacteriochlorin with HOSu in the presence of DCC gave **pro-BC7** or **pro-BC8** in 52% or 53% yield (for two steps), respectively. Treatment of **pro-BC7** or **pro-BC8** with 20% TFA in CH_2Cl_2 unveiled the four carboxylic acid groups in 76% or 71% yield, respectively, while keeping the bacteriochlorin chromophore and NHS ester intact.

Treatment of bacteriochlorin **BC21**[52] with *N*-bromosuccinimide (NBS) in THF afforded the 15-brominated product **BC22** in 42% yield. The presence of the 3,13-aryldiester substituents on the bacteriochlorin ring caused a slightly adverse effect given that the yield was lower than that of bacteriochlorin **BC18** (70%).[52] The copper-free Sonogashira reaction[60] of **BC22** and 6-heptynoic acid (**5**) was carried out in toluene/triethylamine (TEA) containing $Pd_2(dba)_3$ and P(*o*-tol)$_3$ at 70 °C (Scheme 7). The resulting monocarboxy–bacteriochlorin was esterified with HOSu–DCC to afford the bacteriochlorin–NHS ester **pro-BC9** in 19% yield for two steps. The low yield could be attributed to two factors: (1) deprotonation of the free carboxylic acid of **5** under the basic reaction conditions, which would result in low solubility; and (2) purification of bacteriochlorin–NHS ester **pro-BC9** by preparative TLC (instead of column chromatography), from which recovery was poor. Finally, cleavage of the *tert*-butyl ester with 20% TFA in CH_2Cl_2 give the final bacteriochlorin **BC9** in 82% yield.

Pd-mediated carbonylation of 15-bromobacteriochlorin **BC22** with 3-aminopropanoic acid in toluene afforded the bacteriochlorin–imide, which was purified by column chromatography and used directly in the next step. Treatment with HOSu–EDC and 4-dimethylaminopyridine (DMAP) gave the bacteriochlorin–NHS ester **pro-BC10** in 22% yield for two steps (Scheme 8). Cleavage of the protecting group with TFA gave the free tetracarboxy-bacteriochlorin **BC10** in 94% yield.



Scheme 7 Synthesis of tetracarboxy-bacteriochlorin–NHS ester **BC9**.



Scheme 8 Synthesis of the tetracarboxy-bacteriochlorin–imide bearing an NHS ester.

The bacteriochlorins **BC1–BC10** and precursors typically were characterized by absorption and fluorescence spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy (where quantity and solubility allowed), MALDI mass spectrometry, and ESI mass spectrometry.

Photophysical properties

The parameters of interest include (1) the position of the Q_y absorption band, (2) the position of the companion fluorescence emission band, and (3) the sharpness of each band as measured by the full-width-at-half-maximum (fwhm). The absorption and emission spectra of the lipophilic bacteriochlorins were collected in *N*,*N*-dimethylformamide (DMF) (Fig. 2). All of these parameters, including the Φ_f values, are

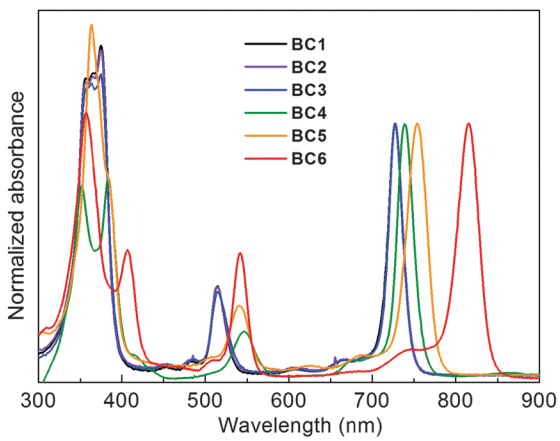


Fig. 2 Normalized absorption spectra in DMF at room temperature. Spectral parameters are given in Table 1.

Table 1 Absorption and fluorescence properties of lipophilic bacteriochlorins **BC1–BC6**[a]

Compounds	λ_{abs}/nm	fwhm nm (Abs)	λ_{em}/nm	fwhm nm (Flu)	Φ_f
BC1	727	21	733	24	0.18
BC2	728	20	734	24	0.18
BC3	727	19	733	24	0.19
BC4	737	24	745	39	0.14
BC5	754	28	764	27	0.18
BC6	816	30	822	27	0.037

[a] All spectra were recorded in DMF at room temperature.

listed in Table 1. Each bacteriochlorin gave characteristic absorption and fluorescence spectra,[61] indicating the absence of any adverse effect due to the presence of the bioconjugatable tether. The Q_y band of members of the set of six carboxybacteriochlorins is in the NIR region, ranging from 730–820 nm. As expected, an increase in the number of electron-withdrawing groups (*e.g.*, ester or imide moieties) along the *y*-axis of the bacteriochlorin caused a bathochromic shift in the absorption and emission spectra.

The absorption and emission spectra of the hydrophilic bacteriochlorins were collected in DMF and in aqueous potassium phosphate buffer (Fig. 3). The spectroscopic parameters, along with the fluorescence quantum yield (Φ_f) values, are listed in Table 2. The data for the parent bacteriochlorins **BC23** and **BC24** (Chart 5) also are included for comparison.

Each bacteriochlorin exhibited absorption and fluorescence in DMF characteristic of the bacteriochlorin chromophore: a strong B band (UV region), modest Q_x band (green-yellow region), and intense Q_y band (NIR region). **BC7–BC9** gave similar spectra in aqueous phosphate buffer, whereas that of **BC10** was significantly broadened characteristic of aggregation. Other than this lone exception, all bacteriochlorins displayed sharp absorption and emission bands with fwhm 22–35 nm. As with the lipophilic bacteriochlorins, introduction of the bioconjugatable tether in **BC7** and **BC8** caused little absorption or emission shift (by comparison with the parent compound **BC23**),

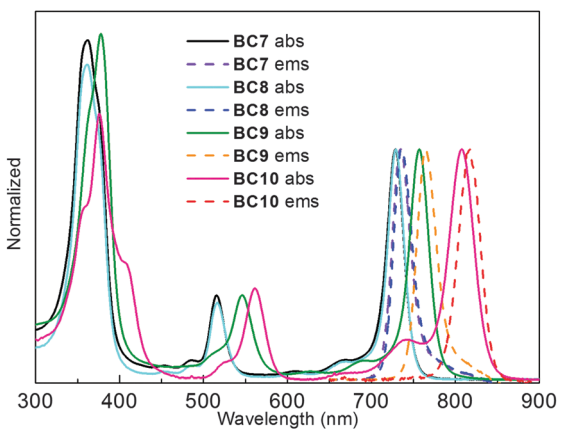


Fig. 3 Normalized absorption and fluorescent spectra in potassium phosphate buffer (0.5 M, pH 7, for **BC7–BC9**) and DMF (for **BC10**) at room temperature.

Table 2 Absorption and fluorescence properties of bacteriochlorins[a]

Compounds	solvent	λ_{abs}/nm	fwhm nm (Abs)	λ_{em}/nm	fwhm nm (Flu)	Φ_f
BC23[b]	DMF	729	22	735	23	0.19
BC23[b]	Buffer	730	26	736	26	0.078
BC24[b]	DMF	746	31	753	23	0.16
BC24[b]	Buffer	749	35	758	37	0.11
BC7	DMF	727	23	732	24	0.17
BC7	Buffer	729	24	735	26	0.12
BC8	DMF	727	21	733	24	0.19
BC8	Buffer	729	23	736	26	0.13
BC9	DMF	754	26	760	24	0.133
BC9	Buffer	757	27	765	27	0.13
BC10	DMF	808	35	818	33	0.037
BC10	Buffer	823	50	829	N/A[c]	0.0011

[a] Each sample contains 1% DMF to facilitate initial dissolution. The buffer is potassium phosphate (0.5 M, pH 7.0). [b] Data reported in ref. 52. [c] Low signal-to-noise ratio precluded the determination of the fwhm value.

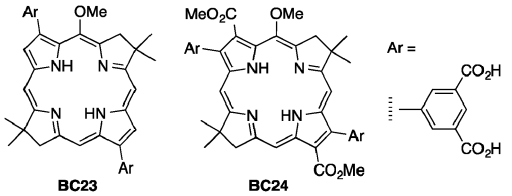


Chart 5 Parent bacteriochlorins lacking bioconjugatable tethers.

while the ethynyl group in **BC9** and the 13,15-imide moiety in **BC10** gave the expected bathochromic shift (in comparison with **BC24**). The Φ_f values ranged from 0.037–0.19, with exception for (aggregated) **BC10** in buffer, which gave 0.0011.

Bioconjugation study

Cellarius and Mauzerall employed polystyrene nanoparticles bearing surface-adsorbed pheophytins as prototypical photoreactors.[58] Their ingenious strategy "combines the structural features of an interface with the simplicity of studying photochemistry in solution" and in particular enabled studies of pigment loading, pigment–pigment interactions, excited-state energy transfer, and perhaps exciton trapping.[58] From the vantage of 50 years hence, the work was unavoidably limited by the polydispersity of the particles (24–260 nm diameter) and by the adsorption rather than covalent attachment of the tetrapyrrole chromophore. Accordingly, we felt that a modern analogue of the pheophytin-on-polystyrene particles could be constructed of bioconjugatable bacteriochlorins covalently attached to a globular protein, with the latter providing a 'particle' with known surface derivatization sites and uniform nanoscale size ($\sim$5 nm diameter).

We examined bioconjugation of selected hydrophilic bacteriochlorins with the protein Mb. The specific goals of this investigation include (1) quantitative analysis of the bacteriochlorin/Mb ratios, and (2) comparison of the spectral properties (absorption, fluorescence, Φ_f) of the bacteriochlorins bound to Mb with those for the bacteriochlorins free in solution. In addition to the more

exacting analogue of the pheophytin-on-particles system of Cellarius and Mauzerall,[58] we felt the Mb-conjugates could provide a testbed that is more simple and controlled than those in typical fluorophore–protein conjugation studies. The latter range from the widespread conjugation of fluorophores to antibodies[62–66] to our own use of biomimetic light-harvesting peptides.[51,53] For these experiments we chiefly examined bacteriochlorin **BC7** but also looked briefly at **BC8**.

Mb was selected for the bioconjugation for the following reasons: (1) Mb is a water-soluble globular protein (diameter ~50 Å) containing 19 lysine residues,[67] of which six are involved in stabilizing electrostatic interactions (Lys16–Asp122, Lys47–Asp44, Lys56–Glu52, Lys77–Glu18, Lys79–Glu4 and Lys133–Glu6).[68,69] The remaining 14 primary amines (13 Lys residues and 1 *N*-terminus amine) are considered accessible for the amine–NHS ester ligation. (2) The heme chromophore absorbs strongly at 408 nm (ε = 188 000 M^{-1} cm^{-1}).[70] The heme absorption is a better reference peak for calculation of intensely absorbing chromophore/protein ratios than the frequently used, weaker, broad (often non-descript) protein absorption at 280 nm (for apomyoglobin (apoMb), ε_{280nm} = 15 900 M^{-1} cm^{-1}),[71] a wavelength where solvent, impurities, and even the chromophore typically also absorb. A diarylbacteriochlorin[40] (*e.g.*, **BC7–BC10**), for example, exhibits ε_{280nm} = 52 900 M^{-1} cm^{-1}, which dwarfs that of Mb even for a 1 : 1 loading. (3) The heme ligand can be removed from the protein binding pocket as needed by organic extraction. (4) Mb can be purchased at low price in large quantity (hundreds of mgs) and with high purity (95–100%). We chose Mb from equine skeletal muscle for bioconjugation studies, although Mbs from different organisms have similar primary, secondary (helicity, 8 helical segments) and tertiary structures (see ESI,† Fig. S1 and S2).

The rationale for focus on Mb *versus* the more prevalent use of antibodies for fluorophore conjugation warrants emphasis: Mb is more compact (~17 kDa *versus* ~150 kDa); Mb is abundantly available as a pure compound; Mb and conjugates thereof readily afford MALDI-MS data; and the presence of the heme provides a convenient (removable) absorption spectrometric internal calibrant. The attachment of fluorophores to antibodies is an essential step for use in flow cytometry or cellular staining,[62,64,65] for example, yet for fundamental spectroscopic and photochemical studies, a small globular protein such as Mb (or apoMb) affords distinct advantages, as described below.

In one study, the bioconjugation of **BC7** was carried out at room temperature with 2, 10, or 50 equiv. of the bacteriochlorin–NHS ester *versus* Mb. The conjugation was performed in aqueous solution containing 10% DMSO. Purification by gel permeation chromatography (GPC) with potassium phosphate buffer (0.5 M, pH 7.0) caused elution of the conjugate as a clear dark green band, while the free bacteriochlorin (unreacted or hydrolyzed bacteriochlorin–NHS ester) remained on top of the column. The resultant conjugate solution was subjected to centrifugal filtration, and the absence of the bacteriochlorin absorption of the filtrates indicated the thorough removal of the free bacteriochlorin.

The absorption spectrum in potassium phosphate buffer of the Mb–bacteriochlorin conjugate **Mb–BC7** closely resembled

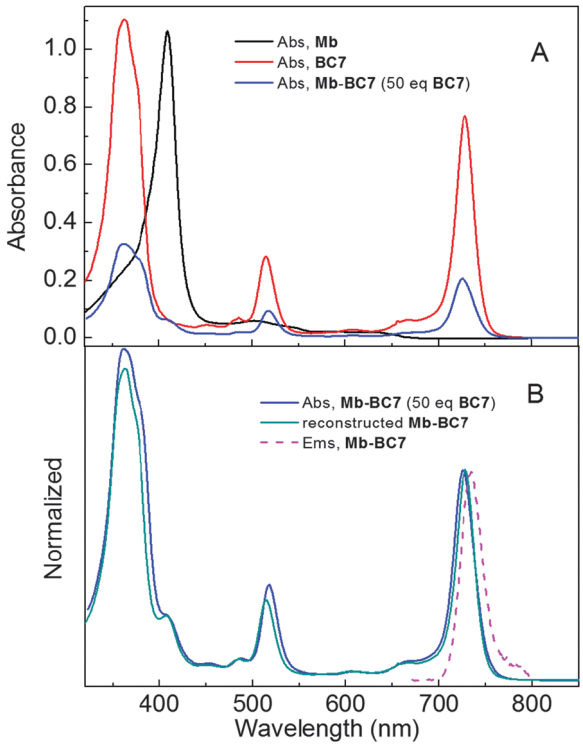


Fig. 4 (A). Absorption spectra of Mb, **BC7** and conjugate **Mb–BC7** in potassium phosphate buffer (0.5 M, pH 7.0). The concentration of each component is chosen arbitrarily. (B). The normalized experimental (blue), reconstructed (cyan) absorption, and emission (magenta, dashed) spectra of conjugate **Mb–BC7**. Spectral parameters are given in Table 3.

the sum of the component parts in each case (2, 10 or 50 equiv.) although a small amount of tailing (to long wavelength) of the bacteriochlorin Q_y band was observed. The spectrum of the conjugate prepared with 50 equiv. is shown in Fig. 4 (panel A) along with that of Mb and **BC7** (the spectra for 2 and 10 equiv. are shown in the ESI,† Fig. S3 and S4).

Multicomponent analysis (using the known absorption spectrum of **Mb** and of **BC7**) in each case was carried out using PhotochemCAD[72] to assess the bacteriochlorin/Mb ratio. The characteristic absorption peaks of Mb (408 nm) and bacteriochlorins (362, 516, 729 nm) were selected for the calculation. Reconstruction of the absorption of the conjugate *versus* the experimental absorption visually shows the accuracy of the absorption deconvolution for calculation of the bacteriochlorin/Mb ratio (Fig. 4, panel B). The results are listed in Table 3. The use of 2, 10, and 50 equiv. of **BC7** resulted in 0.62, 1.6, and 7.1 bacteriochlorins per Mb.

The same three conjugates were examined by MALDI-MS using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. The data are shown in Fig. 5. The increase in loading with number of equivalents of **BC7** was clearly seen, with a distribution of peaks separated by Δm = 920 Da, which corresponds to **BC7** minus the NHS moiety. The distribution shifts to higher mass with increasing number of equivalents. For the conjugate prepared with 50 equiv. of **BC7**, which gave an average loading of 7.1 (by absorption spectroscopy), individual peaks in the

Table 3 Absorption and fluorescence properties of conjugate **Mb–BC7** with different equivalents of **BC7** input[a]

Compound	Degree of loading[b]	λ_{abs}/nm	fwhm nm (Abs)	λ_{em}/nm	fwhm nm (Flu)	Φ_f
Mb–BC7 (2 equiv.)	0.62	729	27	735	26	0.019
apoMb–BC7 (2 equiv.)		726	23	730	23	0.091
Mb–BC7 (10 equiv.)	1.6	728	26	734	27	0.020
apoMb–BC7 (10 equiv.)		726	24	732	24	0.071
Mb–BC7 (50 equiv.)	7.1	726	27	735	27	0.018
apoMb–BC7 (50 equiv.)		721	30	731	27	0.023

[a] All data determined in potassium phosphate buffer (0.5 M, pH 7.0) at room temperature. [b] The ratio of **BC7** to Mb, determined by multicomponent absorption spectral analysis.

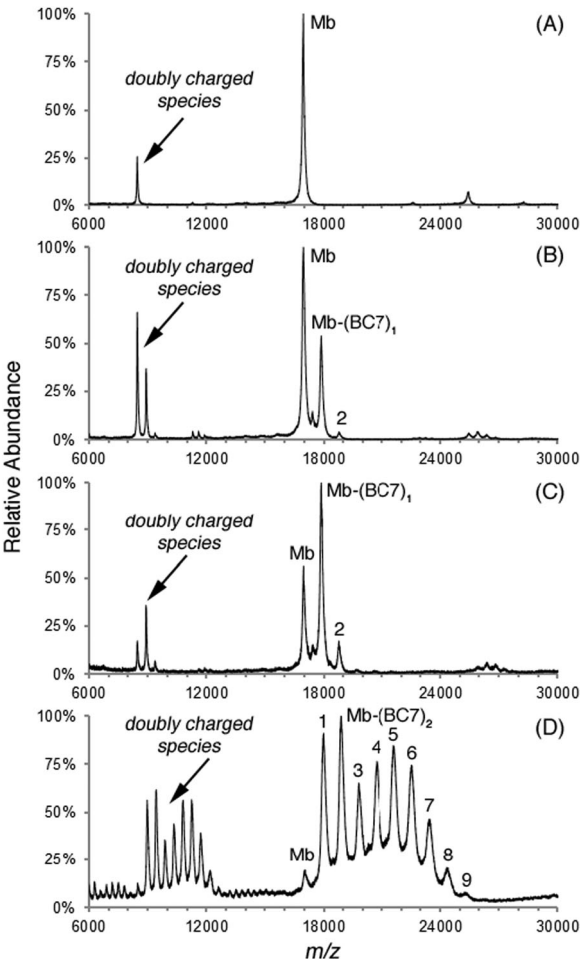


Fig. 5 The MALDI spectra of the conjugate samples of Mb to (A) 0, (B) 2, (C) 10, and (D) 50 equiv. of **BC7**. Peaks that match the mass of labeled Mb were marked by **Mb–(BC7)$_x$**, where *x* indicates the number of the bacteriochlorins attached.

progression of 0–9 were clearly observed. Since ionization efficiencies may vary with different amounts of chromophores attached, the MALDI-MS results, while insightful, are not reliable for calculations of bacteriochlorin/Mb ratios. The minimum conclusion is that the distribution is narrow for 10 equiv. (1.6 loading) yet quite broad for 50 equiv. (7.1 loading). In a separate experiment, **BC7** and **BC8** were conjugated at 60 equiv. relative to Mb, affording conjugates that also were quite soluble in aqueous solution. In both cases, the resulting loading was 9 and 12, respectively. The shift of the peaks in the distribution to higher mass was readily observed upon MALDI-MS analysis (see ESI,† Fig. S5).

The fluorescence properties of the **Mb–BC7** conjugates were examined. The spectrum for the conjugate derived from 50 equiv. of **BC7** is shown in Fig. 4 (panel B). The Φ_f value upon attachment to the protein was decreased to ~0.02, to be compared with the value of 0.12 for **BC7** in aqueous solution. The Φ_f value was essentially indifferent to the level of loading. To distinguish possible effects of heme as a quencher, the heme was removed by extraction with 2-butanone,[73] to afford the corresponding apoMb conjugates. In each case, the resulting **apoMb–BC7** conjugate (derived from 2, 10 or 50 equiv. of **BC7**) gave a characteristic bacteriochlorin absorption spectrum (see ESI,† Fig. S6–S8). Indeed, no trace of tailing of the long-wavelength, Q_y absorption band was observed. Unlike for **Mb–BC7**, however, the Φ_f values now were a function of loading (*i.e.*, **BC7**/Mb ratio). The results are illustrated in Fig. 6. The Φ_f value for the lowest-loading conjugate (2 equiv. of **BC7**, average 0.62 bacteriochlorins/Mb) was 0.091, only decreased by 25% from that of the parent **BC7** monomer. On the other hand, the decline with loading (to 0.023 for 50 equiv., average 7.1 bacteriochlorins/Mb) is attributed to self-quenching of the bacteriochlorins on the protein. Thus, a distinction between quenching due to the presence of heme *versus* quenching due to bacteriochlorin self-interactions is clearly obtained.

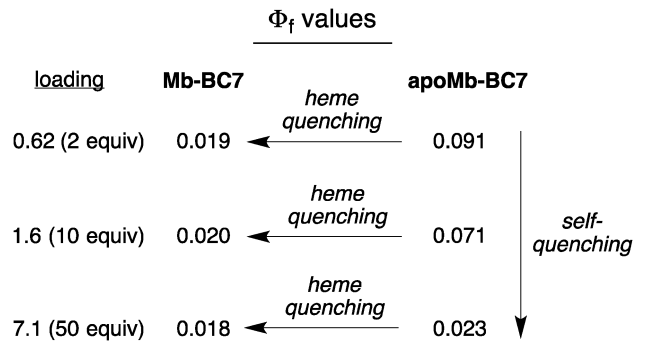


Fig. 6 Fluorescence quantum yield values as a function of loading and ±heme.

The origin of self-quenching is unclear. The absorption spectra and the emission spectra of the **apoMb–BC7** conjugates were essentially identical to those of the monomeric **BC7**. Calculations of the Förster through-space energy transfer (using PhotochemCad[72]) showed that the self-exchange process for bacteriochlorin–bacteriochlorin energy transfer exhibits R_0 = 59 Å (the distance at which energy transfer is 50% efficient). Given that the diameter of Mb is ~50 Å from most distant points, a considerable degree of energy transfer between bacteriochlorins attached to Mb is expected to be permissible. Hence, any excited-state trapping site(s) at/near the protein are likely to be encountered upon successive transfer steps.

Outlook

The ability to synthesize hydrophilic/hydrophobic bacteriochlorins that exhibit some degree of wavelength tunability and bear a single carboxylic acid (or NHS-ester) opens the door to a wide variety of studies, particularly upon bioconjugation to proteins. The lipophilic bacteriochlorins can be used for attachment to the hydrophobic region of membrane-spanning light-harvesting peptides to give rise to self-assembled artificial photosynthetic architectures. The attachment of a hydrophilic bacteriochlorin to Mb affords a water-soluble NIR-active protein architecture that is likely a more exacting and versatile analogue of the pheophytin-coated polystyrene particles first reported by Cellarius and Mauzerall nearly 50 years ago. The bioconjugation of bacteriochlorins to Mb described herein also affords several attractive attributes as a testbed for attachment of chromophores to proteins: (1) the presence of heme provides a convenient absorption calibration standard for determining average loading of intensely absorbing chromophores, which often is difficult when relying solely on the weakly absorbing (and typically overlapped) 280 nm band of proteins; (2) mass spectrometry of the Mb–chromophore conjugate provides a more granular view of the loading distribution; and (3) heme can be readily removed following loading determination to assess spectral properties in the resulting apoMb, including absorption and fluorescence spectra as well as Φ_f values.

Experimental section

Protocols

General methods. ^{1}H NMR and ^{13}C NMR spectroscopies were performed at room temperature. MALDI-MS was performed with the matrix 1,4-bis(5-phenyl-2-oxaxol-2-yl)benzene for bacteriochlorins,[74] and α-cyano-4-hydroxycinnamic acid (CHCA) for Mb and conjugates. Electrospray ionization mass spectrometry (ESI-MS) data are reported for the molecular ion. Silica gel (40 μm average particle size) was used for column chromatography. All solvents were reagent grade and were used as received unless noted otherwise. THF was freshly distilled from sodium–benzophenone ketyl. $CHCl_3$ was stabilized with amylenes (≤1%). Compounds **1**, **2**, **4** and **5** were obtained from commercial sources. Known compound **3**[41] and bacteriochlorins **BC11**,[51] **BC12**,[42] **BC13**,[53] **BC14**,[52] **BC18**,[52] and **BC19**[52] were prepared following literature procedures. Equine Mb was obtained in 95–100% purity and used as received.

Preparation of Mb conjugate. The following procedure pertains to the use of 60 equiv. of bacteriochlorin–Mb. A sample of equine Mb (0.57 mg, 33 nmol) was dissolved in potassium phosphate buffer (0.1 M, pH 8.3, 0.17 mL). In a separate vial, bacteriochlorin **BC7** or **BC8** (2.0 mg, 2.0 μmol, 60 equiv.) was initially dissolved in DMSO (33 μL) and then 137 μL of the same phosphate buffer was added with stirring to make a homogeneous bacteriochlorin solution. The resulting bacteriochlorin solution was then transferred to the Mb solution, and incubated in the dark for 3 h at room temperature (~23 °C). The final concentration of Mb was 0.1 mM, in which DMSO accounts for 10% by volume.

The crude bacteriochlorin–Mb conjugate **Mb–BC7** or **Mb–BC8** was purified by passage (gravity-elution) over a PD-10 GPC column (Sephadex G-25 medium, bed dimension: 14.5 × 50 mm) with potassium phosphate buffer (0.5 M, pH 7.0) as eluent. The conjugate eluted as a clear dark green band, while free bacteriochlorin (unreacted or hydrolyzed bacteriochlorin–NHS ester) remained on top of the column. The resultant conjugate solution was subjected to centrifugal Amicon® Ultra-4 filtration (regenerated cellulose, molecular weight cutoff = 10 K) for 30 min. The resulting filtrate lacked bacteriochlorin absorption, consistent with the removal of any unconjugated bacteriochlorin. The solution that did not pass through the filter constituted the purified bacteriochlorin–Mb conjugate. The purification protocol is expected to remove all DMSO used in the bioconjugation reaction. MALDI-MS for **Mb–BC7**: m/z = 198 812, 20 695, 21 509, 22 484 (most intense), 23 401, and 24 319. MALDI-MS for **Mb–BC8**: m/z = 21 589, 22 509, 23 432 (most intense), 24 354 and 25 330. Further data are provided in the ESI† (Fig. S9 and S10).

The following protocol describes the use of 2, 10, or 50 equiv. of bacteriochlorin–Mb. A sample of Mb (0.52 mg, 30 nmol) was dissolved in potassium phosphate buffer (0.1 M, pH 8.3, 0.15 mL). In a separate vial, bacteriochlorin **BC7** (60 μg, 60 nmol, 2 equiv. or 0.30 mg, 0.30 μmol, 10 equiv., or 1.5 mg, 1.5 μmol, 50 equiv.) was initially dissolved in DMSO (30 μL) and then 120 μL of the same phosphate buffer was added with stirring to make a homogeneous bacteriochlorin solution. The resulting bacteriochlorin solution was then pipetted into the Mb solution, and incubated in the dark for 3 h at room temperature (~23 °C). The final concentration of Mb was 0.1 mM, and DMSO accounts for 10% by volume. The remainder of the protocol is identical for that above with 60 equiv. of bacteriochlorin–Mb. The characterization data are provided in the body of the paper.

Heme removal. Following a general procedure,[73] the **Mb–BC7** conjugate (2, 10, or 50 equiv, of **BC7**) in potassium phosphate buffer (100 mM, pH 8.3) was diluted with 2 N HCl to adjust to pH ~ 2 (pH paper). An equal volume of 2-butanone was added. The mixture was shaken gently and allowed to stand for 10 min. The organic layer was discarded. This procedure (2-butanone addition/phase separation/2-butanone removal) was repeated three times (total of four extractions). The aqueous

phase containing the resulting **apoMb–BC7** conjugate was titrated with 2 M NaOH to adjust to pH ~ 8 (pH paper) for subsequent spectroscopic studies. The characterization data are provided in the body of the paper.

Fluorescence quantum yield measurements. The Φ_f values were determined by excitation into the bacteriochlorin Q_x band (511–570 nm) with emission integrated from 650–850 nm. Samples were examined in a 1 cm pathlength cuvette at room temperature with absorption of the Q_x band of ~0.02–0.03. The absorption of the corresponding Q_y band was typically $\leq$0.1 thereby avoiding the inner filter effect. The yields were determined in the standard manner (with corrected spectra) by ratioing to 3,13-bis(3,5-dicarboxyphenyl)-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (Φ_f = 0.078) for studies in aqueous solution,[52] or to 2,12-di-*p*-tolyl-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (Φ_f = 0.18 in toluene) for studies in DMF.[40]

Förster energy-transfer calculations. The following parameters were utilized in the calculation:[72] refractive index n = 1.33; orientation factor κ^2 = 0.67; assumed ε = 120 000 M^{-1} cm^{-1} for **BC7** at 728 nm; Φ_f = 0.12 for **BC7**. The calculated R_0 was 59 Å for **BC7–BC7**.

Syntheses

2-[4-(2-(*tert*-Butoxycarbonyl)ethyl)phenyl]-3,3,4,4-tetramethyl-1,3,2-dioxaborolane (2). Following a general procedure,[59] a mixture of **1** (0.28 g, 1.0 mmol), di-*tert*-butyl dicarbonate (0.28 g, 1.3 mmol) and $MgCl_2$ (9.5 mg, 0.10 mmol) in *tert*-butyl alcohol (0.49 mL) and acetonitrile (0.15 mL) was stirred under argon for 16 h. The crude reaction mixture was diluted with water (10 mL) and extracted with ethyl acetate (3 × 10 mL). The combined extract was dried (Na_2SO_4), concentrated and chromatographed [silica, hexanes/ethyl acetate (9 : 1)] to afford a viscous colorless liquid (0.20 g, 60%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 12H), 1.41 (s, 9H), 2.53 (t, J = 7.5 Hz, 2H), 2.92 (t, J = 7.5 Hz, 2H), 7.21 (d, J = 7.8 Hz, 2H), 7.73 (d, J = 7.8 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.1, 28.3, 31.5, 37.1, 80.6, 83.9, 100.3, 128.0, 135.2, 144.4, 172.3; ESI-MS obsd 354.2083, calcd 354.2087 [$(M + Na)^+$, M = $C_{19}H_{29}BO_4$].

15-[3-(3-Carboxypropionylamino)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (BC1). Following a general procedure,[51] a solution of **BC11** (9.8 mg, 14 μmol) in $CHCl_3$ (0.60 mL) was treated with succinic anhydride (1.7 mg, 21 μmol) and stirred for 4 h at room temperature. 2 N HCl solution (~20 mL) was added to the reaction mixture, which then was extracted twice with CH_2Cl_2. The extract was dried (Na_2SO_4), concentrated and chromatographed (silica, ethyl acetate) to afford a greenish solid (7.5 mg, 67%): ^{1}H NMR (300 MHz, DMSO-d_6) δ −1.99 (s, 1H), −1.69 (s, 1H), 1.20 (t, J = 7.0 Hz, 3H), 1.19 (t, J = 7.0 Hz, 3H), 1.78 (d, J = 5.0 Hz, 6H), 1.82 (s, 6H), 1.94 (d, J = 5.0 Hz, 6H), 2.09 (d, J = 3.3 Hz, 6H), 2.21 (s, 3H), 2.49 (s, 3H), 2.87 (m, 4H), 3.65 (s, 3H), 3.68 (s 2H), 4.24 (d, J = 2.4 Hz, 2H), 4.32 (q, J = 7.2 Hz, 2H), 4.42 (q, J = 7.2 Hz, 2H), 6.56 (s, 1H), 6.64 (s, 1H), 7.08–7.15 (m, 4H), 7.34 (s, 1H), 7.39 (s, 1H), 7.47 (d, J = 7.2 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.79 (d, J = 8.4 Hz, 2H), 8.58 (br, 1H), 9.61 (s, 1H), 9.63 (s, 1H), 9.87 (s, 1H); MALDI-MS obsd 791.7302; ESI-MS obsd 792.3957, calcd 792.3967 [$(M + H)^+$, M = $C_{45}H_{53}N_5O_8$]; λ_{abs} (CH_2Cl_2) 356, 375, 515, 727 nm.

15-[4-(2-(*tert*-Butoxycarbonyl)ethyl)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (pro-BC2). Following a general procedure,[51] samples of **BC12** (34.0 mg, 50.0 μmol), **2** (49.8 mg, 150 μmol), $Pd(PPh_3)_4$ (17.3 mg, 15.0 μmol), and K_2CO_3 (83.0 mg, 600 μmol) were placed in a Schlenk flask which was then pump-purged three times with argon. DMF/toluene [5.0 mL, (1 : 2), degassed by bubbling with argon for 30 min] was added to the Schlenk flask, and the reaction mixture was stirred at 90 °C for 18 h. The reaction mixture was cooled to room temperature, concentrated to dryness, and diluted with CH_2Cl_2. The resulting solution was washed with aqueous $NaHCO_3$. The organic layer was separated, dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (49 : 1)] to obtain a greenish solid (37.6 mg, 93%): ^{1}H NMR (400 MHz, $CDCl_3$) δ −1.81 (brs, 1H), −1.51 (brs, 1H), 1.29 (t, J = 7.5 Hz, 3H), 1.55 (s, 9H), 1.63–1.70 (m, 6H), 1.77 (t, J = 7.5 Hz, 3H), 1.83 (s, 6H), 1.95 (s, 6H), 2.77 (t, J = 7.6 Hz, 2H), 3.16 (t, J = 7.6 Hz, 2H), 3.79 (q, J = 7.5 Hz, 2H), 3.83–3.89 (m, 6H), 4.27 (s, 3H), 4.38 (s, 2H), 4.80 (q, J = 7.5 Hz, 2H), 7.47 (d, J = 8.0 Hz, 2H), 7.73 (d, J = 8.0 Hz, 2H), 8.57 (s, 1H), 8.61 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 14.9, 17.8, 20.2, 20.3, 28.5, 31.2, 31.3, 31.4, 37.4, 45.2, 46.3, 47.5, 52.3, 61.4, 62.0, 64.4, 80.8, 94.5, 94.7, 112.9, 123.2, 126.2, 127.7, 127.8, 132.4, 132.6, 133.4, 135.0, 138.6, 139.7, 140.2, 154.7, 161.0, 168.1, 168.5, 169.2, 172.7; MALDI-MS obsd 804.6556; ESI-MS obsd 805.4520, calcd 805.4535 [$(M + H)^+$, M = $C_{48}H_{60}N_4O_7$]; λ_{abs} (CH_2Cl_2) 356, 365, 376, 515, 729 nm.

15-[4-(2-Carboxyethyl)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (BC2). Following a general procedure,[52] a sample of **pro-BC2** (9.0 mg, 11 μmol) in CH_2Cl_2 (2.0 mL) was stirred under argon for 2 min, followed by addition of TFA (0.40 mL). After 1 h, the reaction mixture was washed with saturated aqueous $NaHCO_3$, 2 N HCl, and water. The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with hexanes, sonicated in a benchtop sonication bath, centrifuged, and the supernatant was discarded, leaving a reddish solid (6.2 mg, 74%): ^{1}H NMR (400 MHz, DMSO-d_6) δ −1.97 (brs, 1H), −1.67 (brs, 1H), 1.17 (t, J = 7.2 Hz, 3H), 1.50–1.56 (m, 6H), 1.63 (t, J = 7.6 Hz, 3H), 1.77 (s, 6H), 1.90 (s, 6H), 2.73 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.2 Hz, 2H), 3.68–3.81 (m, 8H), 4.17 (s, 3H), 4.31 (s, 2H), 4.66 (q, J = 7.6 Hz, 2H), 7.45 (d, J = 7.6 Hz, 2H), 7.73 (d, J = 7.6 Hz, 2H), 8.67 (s, 1H), 8.72 (s, 1H), 12.30 (br, 1H); MALDI-MS obsd 747.9117; ESI-MS obsd 749.3912, calcd 749.3909 [$(M + H)^+$, M = $C_{44}H_{52}N_4O_7$]; λ_{abs} (CH_2Cl_2) 356, 365, 376, 515, 729 nm.

15-[4-(*tert*-Butoxycarbonylmethoxy)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (pro-BC3). Following a general procedure,[51] samples of **BC12** (34 mg, 50 μmol), **3** (50 mg, 0.15 mmol), $Pd(PPh_3)_4$ (17 mg, 15 μmol), and K_2CO_3 (83 mg, 0.60 μmol) were placed in a Schlenk flask which was then pump-purged three times with argon. DMF/toluene [5.0 mL, (1 : 2), degassed by bubbling with argon for 30 min] was added to the Schlenk flask, and the reaction mixture was stirred at 90 °C for 18 h. The reaction mixture was cooled to room temperature, concentrated to

dryness, diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$. The organic layer was separated, dried (Na_2SO_4) and concentrated. Column chromatography [silica, CH_2Cl_2/ethyl acetate (49 : 1)] provided a greenish solid (29 mg, 73%): ^{1}H NMR (400 MHz, $CDCl_3$) δ −1.83 (brs, 1H), −1.53 (brs, 1H), 1.30 (t, J = 7.5 Hz, 3H), 1.59 (s, 9H), 1.62–1.69 (m, 6H), 1.76 (t, J = 7.5 Hz, 3H), 1.82 (s, 6H), 1.94 (s, 6H), 3.76 (q, J = 7.5 Hz, 2H), 3.82–3.89 (m, 4H), 3.95 (q, J = 7.5 Hz, 2H), 4.26 (s, 3H), 4.36 (s, 2H), 4.72 (s, 2), 4.78 (q, J = 7.2 Hz, 2H), 7.14 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 8.56 (s, 1H), 8.60 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.5, 14.9, 17.9, 20.2, 20.3, 28.4, 31.2, 31.4, 45.2, 46.3, 47.6, 52.3, 61.6, 62.0, 64.4, 66.1, 82.8, 94.5, 94.7, 112.4, 113.9, 123.2, 126.4, 127.7, 132.4, 132.5, 132.8, 134.4, 135.0, 135.1, 135.2, 138.5, 154.7, 157.7, 161.3, 168.2, 168.4, 168.6, 169.3; MALDI-MS obsd 806.6729; ESI-MS obsd 807.4323, calcd 807.4327 [$(M + H)^+$, M = $C_{47}H_{58}N_4O_8$]; λ_{abs} (CH_2Cl_2) 356, 364, 376, 515, 729 nm.

15-[4-(Carboxymethoxy)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (BC3). Following a general procedure,[52] a sample of **pro-BC3** (14 mg, 17 μmol) in CH_2Cl_2 (3.1 mL) was stirred under argon for 2 min, followed by addition of TFA (0.62 mL). After 1 h, the reaction mixture was washed with saturated aqueous $NaHCO_3$, 2 N HCl, and water. The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with hexanes. The resulting suspension was sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a reddish solid (9.2 mg, 71%): ^{1}H NMR (400 MHz, DMSO-d_6) δ −1.97 (brs, 1H), −1.67 (brs, 1H), 1.22 (t, J = 7.5 Hz, 3H), 1.51–1.59 (m, 6H), 1.65 (t, J = 7.5 Hz, 3H), 1.80 (s, 6H), 1.92 (s, 6H), 3.70–3.88 (m, 8H), 4.19 (s, 3H), 4.32 (s, 2H), 4.68 (q, J = 8.0 Hz, 2H), 4.87 (s, 2H), 7.15 (d, J = 8.4 Hz, 2H), 7.64 (d, J = 8.4 Hz, 2H), 8.69 (s, 1H), 8.74 (s, 1H), 13.13 (br, 1H); MALDI-MS obsd 750.5661; ESI-MS obsd 751.3706, calcd 751.3701 [$(M + H)^+$, M = $C_{43}H_{50}N_4O_8$]; λ_{abs} (CH_2Cl_2) 357, 365, 375, 515, 728 nm.

Zn(II)-15-[3-(3-Carboxypropionylamino)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin (BC4). Following a general procedure,[51] a solution of **BC14** (14.3 mg, 18.9 μmol) in $CHCl_3$ (1.00 mL) was treated with succinic anhydride (2.50 mg, 25.0 μmol) and stirred at room temperature for 4 h. The resulting mixture was chromatographed (silica, ethyl acetate) to afford a reddish solid (10.0 mg, 62%): ^{1}H NMR (300 MHz, THF-d_8) δ 1.21 (t, J = 6.9 Hz, 3H), 1.51–1.69 (m, 9H), 1.82 (s, 3H), 1.83 (s, 3H), 1.95 (s, 6H), 2.61–2.63 (m, 4H), 3.61–3.84 (m, 8H), 3.97 (s, 1H), 4.14 (s, 3H), 4.38 (s, 2H), 4.61 (t, J = 7.2 Hz, 2H), 7.35–7.43 (m, 2H), 7.61 (s, 1H), 8.14 (d, J = 7.5 Hz, 1H), 8.46 (s, 1H), 8.450 (s, 1H), 9.28 (s, 1H); MALDI-MS obsd 853.40; ESI-MS obsd 854.3029, calcd 854.3107 [$(M + H)^+$, M = $C_{45}H_{51}N_5O_8Zn$]; λ_{abs} (CH_2Cl_2) 353, 384, 551, 735 nm.

15-[3-(3-Carboxypropionylamino)phenyl]-3,13-bis(ethoxycarbonyl)-2,12-dimesityl-8,8,18,18-tetramethylbacteriochlorin (BC5). Following a general procedure,[51] a solution of **BC13** (14.2 mg, 16.3 μmol) in $CHCl_3$ (652 μL) was treated with succinic anhydride (8.10 mg, 81.4 μmol) and stirred for 1 h at room temperature. The reaction mixture was dried and chromatographed [silica, CH_2Cl_2/ethyl acetate (9 : 1) to CH_2Cl_2/methanol (4 : 1)] to yield a greenish solid (11.2 mg, 71%): ^{1}H NMR (400 MHz, THF-d_8, the CO_2H proton was not observed) δ −0.80 (s, 1H), −0.47 (s, 1H), 0.97 (t, J = 7.2 Hz, 3H), 1.09 (t, J = 7.2 Hz, 3H), 1.77 (s, 3H), 1.81 (s, 3H), 1.83 (s, 3H), 1.87 (s, 3H), 1.93 (s, 6H), 2.07 (s, 3H), 2.09 (s, 3H), 2.24 (s, 3H), 2.47 (s, 3H), 2.59–2.67 (m, 4H), 3.63 (s, 3H), 3.75 (d, J = 2.4 Hz, 2H), 4.20–4.27 (m, 4H), 4.31 (t, J = 7.2 Hz, 2H), 6.45 (s, 1H), 6.72 (s, 1H), 6.97–7.04 (m, 2H), 7.10 (s, 2H), 7.54 (d, J = 8.0 Hz, 1H), 7.65 (s, 1H), 8.94 (s, 1H), 9.68 (s, 1H), 9.72 (s, 1H); ^{13}C NMR (100 MHz, THF-d_8) δ 16.9, 17.0, 24.2, 24.4, 24.7, 32.3, 34.0, 34.1, 34.2, 35.2, 48.9, 49.6, 50.7, 55.7, 63.59, 63.65, 65.8, 119.0, 121.1, 124.4, 124.8, 128.2, 129.1, 130.8, 131.09, 131.16, 131.20, 131.7, 136.1, 137.5, 138.1, 138.6, 138.8, 139.7, 139.9, 140.4, 140.5, 140.6, 141.2, 141.9, 144.1, 160.1, 166.3, 169.08, 169.18, 172.9, 173.8, 174.7, 177.2; MALDI-MS obsd 971.0664; ESI-MS obsd 972.4897, calcd 972.4906 [$(M + H)^+$, M = $C_{59}H_{65}N_5O_8$]; λ_{abs} (CH_2Cl_2) 364, 543, 756 nm.

15^2-[*N*-(3-*tert*-Butoxycarbonyl)propyl]-3-ethoxycarbonyl-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboximide (pro-BC6). Following a reported procedure,[43] a mixture of **BC12** (19.0 mg, 28.0 μmol), $Pd(PPh_3)_4$ (51.7 mg, 44.7 μmol), Cs_2CO_3 (137 mg, 419 μmol) and *tert*-butyl 4-aminobutyrate (22.0 mg, 112 μmol) was placed in a Schlenk flask, and deaerated under high vacuum for 40 min. The flask was then filled with CO and toluene (3.0 mL, deaerated by bubbling with argon for 30 min, and then with CO for 30 min). The reaction mixture was stirred at 90 °C for 14 h under a CO atmosphere at ambient pressure. The reaction mixture was cooled to room temperature, dried and washed (saturated aqueous $NaHCO_3$ solution). The combined organic layer was dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (22 : 3)]. The resulting solid was extracted with hexanes, sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a reddish solid (13.0 mg, 65%): ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.72 (s, 1H), −0.51 (s, 1H), 1.47 (s, 9H), 1.68–1.78 (m, 9H), 1.92 (s, 6H), 1.93 (s, 6H), 2.25–2.32 (m, 2H), 2.54 (t, J = 8.4 Hz, 2H), 4.08 (q, J = 7.2 Hz, 2H), 4.21 (q, J = 7.2 Hz, 2H), 4.33 (s, 2H), 4.50 (t, J = 7.2 Hz, 2H), 4.73 (s, 2H), 4.77 (q, J = 7.2 Hz, 2H), 8.57 (s, 1H), 8.70 (s, 1H), 9.55 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.8, 17.4, 17.6, 20.1, 20.9, 24.7, 28.4, 30.0, 31.2, 31.6, 33.8, 39.6, 45.7, 46.1, 52.1, 53.3, 61.5, 80.4, 94.7, 99.2, 99.5, 102.0, 115.0, 122.1, 133.4, 134.6, 136.2, 136.9, 140.3, 144.4, 162.8, 163.3, 165.9, 168.3, 168.5, 170.4, 172.9, 176.2; MALDI-MS obsd 709.4791; ESI-MS obsd 710.3921, calcd 710.3912 [$(M + H)^+$, M = $C_{41}H_{51}N_5O_6$]; λ_{abs} (CH_2Cl_2) 358, 408, 544, 819 nm.

15^2-[*N*-(3-Carboxypropyl)]-3-ethoxycarbonyl-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboximide (BC6). Following a general procedure,[52] a solution of **pro-BC6** (14.5 mg, 20.0 μmol) in CH_2Cl_2 (1.60 mL) was stirred under argon for 2 min, followed by addition of TFA (400 μL). After 30 min, the reaction mixture was diluted with ethyl acetate and then washed with saturated aqueous $NaHCO_3$. The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with hexanes, sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a reddish solid (12.0 mg, 90%): ^{1}H NMR (400 MHz, $CDCl_3$, the COOH proton was not observed) δ −0.70 (s, 1H), −0.50 (s, 1H),

1.64–1.75 (m, 9H), 1.90 (s, 12H), 2.23–2.27 (m, 2H), 2.54 (t, J = 7.2 Hz, 2H), 4.03 (q, J = 7.2 Hz, 2H), 4.17 (q, J = 7.2 Hz, 2H), 4.30 (s, 2H), 4.42 (t, J = 7.2 Hz, 2H), 4.68 (s, 2H), 4.77 (q, J = 7.2 Hz, 2H), 8.53 (s, 1H), 8.66 (s, 1H), 9.52 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.8, 17.4, 17.5, 20.1, 20.9, 24.3, 31.1, 31.5, 32.1, 39.3, 45.6, 46.2, 52.1, 53.3, 61.5, 94.7, 99.1, 99.6, 102.0, 114.6, 122.2, 133.3, 134.5, 136.3, 137.1, 140.2, 144.5, 162.8, 163.4, 165.8, 168.46, 168.49, 170.4, 176.5, 178.3; MALDI-MS obsd 654.1035; ESI-MS obsd 654.329, calcd 654.3286 [$(M + H)^+$, M $C_{37}H_{43}N_5O_6$]; λ_{abs} (CH_2Cl_2) 357, 408, 544, 819 nm.

3,13-Bis[3,5-bis(*tert*-butoxycarbonyl)phenyl]-5-methoxy-15-[3-(4-(*N*-succinimidooxy)-1,4-dioxobutylamino)phenyl]-8,8,18,18-tetramethylbacteriochlorin (pro-BC7). Following a general procedure,[51] a solution of **BC16** (14.7 mg, 14.0 μmol) in $CHCl_3$ (560 μL) was treated with succinic anhydride (2.80 mg, 27.8 μmol) and stirred for 2 h at room temperature. The crude reaction mixture was chromatographed [silica, CH_2Cl_2/ethyl acetate (23 : 2)] to afford a greenish solid, which was used directly in the next step. The greenish solid was dissolved in CH_2Cl_2 (1.23 mL) followed by addition of DCC (38.1 mg, 0.185 mmol). The mixture was stirred under argon for 3 min. Then HOSu (21.3 mg, 0.185 mmol) was added. The resulting mixture was stirred for 40 min and then filtered to remove insoluble material. The filtrate was concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (9 : 1)] to yield a greenish solid (9.0 mg, 40%): ^{1}H NMR (300 MHz, $CDCl_3$) δ −1.59 (s, 1H), −1.21 (s, 1H), 1.64 (s, 18H), 1.69 (s, 18H), 1.90 (s, 6H), 2.00 (s, 6H), 2.74–2.81 (m, 6H), 3.06 (t, J = 7.5 Hz, 2H), 3.70 (s, 3H), 3.91–4.18 (m, 2H), 4.38 (s, 2H), 7.14 (t, J = 7.8 Hz, 1H), 7.38 (d, J = 5.4 Hz, 1H), 7.41–7.43 (m, 3H), 7.58 (s, 1H), 7.96 (s, 1H), 8.18 (s, 1H), 8.38 (s, 1H), 8.66 (t, J = 9.9 Hz, 2H), 8.76 (s, 1H), 8.91 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.4, 22.9, 25.2, 25.78, 25.81, 26.9, 28.48, 28.54, 31.4, 34.2, 45.4, 46.0, 47.8, 49.4, 52.1, 63.6, 81.9, 97.3, 97.8, 113.2, 119.6, 123.4, 125.5, 126.6, 128.16, 128.27, 128.31, 129.2, 129.6, 132.0, 132.6, 133.71, 133.75, 134.3, 134.9, 135.2, 136.0, 136.3, 137.1, 138.6, 139.0, 141.6, 155.4, 157.0, 160.8, 165.8, 168.20, 168.37, 169.1, 169.2, 169.7; MALDI-MS obsd 1242.5563; ESI-MS obsd 1241.5811, calcd 1241.5811 [$(M + H)^+$, M = $C_{61}H_{71}BrN_4O_{13}$]; λ_{abs} (CH_2Cl_2) 366, 517, 730 nm.

3,13-Bis(3,5-dicarboxyphenyl)-5-methoxy-15-[3-(4-(*N*-succinimidooxy)-1,4-dioxobutylamino)phenyl]-8,8,18,18-tetramethylbacteriochlorin (BC7). Following a general procedure,[52] a solution of **pro-BC7** (32.2 mg, 26.0 μmol) in CH_2Cl_2 (4.00 mL) was stirred under argon for 2 min, followed by addition of TFA (1.00 mL). After 2 h, the reaction mixture was diluted with ethyl acetate and then washed with brine until the aqueous phase was neutral (pH paper). The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with CH_2Cl_2, sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a green solid (20.0 mg, 76%): ^{1}H NMR (300 MHz, DMSO-d_6) δ −1.67 (s, 1H), −1.32 (s, 1H), 1.74 (s, 3H), 1.86 (s, 3H), 1.92 (s, 3H), 1.94 (s, 3H), 2.67 (t, J = 5.1 Hz, 2H), 2.77 (s, 4H), 2.94 (t, J = 5.1 Hz, 2H), 3.58 (s, 3H), 3.80 (d, J = 13.2 Hz, 1H), 4.06 (d, J = 13.2 Hz, 1H), 4.34 (s, 2H), 7.05 (t, J = 5.4 Hz, 1H), 7.26 (t, J = 8.1 Hz, 2H), 7.77 (s, 1H), 7.87 (s, 1H), 8.10 (s, 1H), 8.27 (d, J = 1.2 Hz, 1H), 8.69 (d, J = 1.2 Hz, 1H), 8.85–8.97 (m, 6H), 9.88 (s, 1H), 13.4–13.6 (br, 4H); MALDI-MS obsd 1015.4610; ESI-MS obsd 1017.3289, calcd 1017.3301 [$(M + H)^+$, M = $C_{55}H_{48}N_6O_{14}$]; λ_{abs} (0.5 M potassium phosphate buffer, pH 7.0) 362, 516, 729 nm.

3,13-Bis[3,5-bis(*tert*-butoxycarbonyl)phenyl]-5-methoxy-15-[4-(4-(*N*-succinimidooxy)-1,4-dioxobutylamino)phenyl]-8,8,18,18-tetramethylbacteriochlorin (pro-BC8). Following a general procedure,[51] a solution of **BC20** (18 mg, 17 μmol) in $CHCl_3$ (0.63 mL) was treated with succinic anhydride (6.2 mg, 62 μmol) and stirred for 1 h at room temperature. The crude reaction mixture was chromatographed (silica, ethyl acetate) to afford a greenish solid (14 mg), which was used directly in the next step. The resulting greenish solid (8.0 mg) was dissolved in CH_2Cl_2 (0.70 mL) followed by the addition of DCC (14 mg, 70 μmol), and the mixture was stirred under argon for 3 min. HOSu (8.1 mg, 70 μmol) was then added. The resulting mixture was stirred for 40 min and then filtered to remove insoluble material. The filtrate was concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (4 : 1)]. The resulting solid was treated with hexanes/CH_2Cl_2 (9 : 1), sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a green solid (6.5 mg, 53%): ^{1}H NMR (300 MHz, $CDCl_3$, the NH proton peaks were not observed) δ −1.58 (s, 1H), −1.20 (s, 1H), 1.63 (s, 18H), 1.69 (s, 18H), 1.84 (s, 6H), 1.98 (s, 6H), 2.85–2.90 (m, 6H), 3.15 (t, J = 6.6 Hz, 2H), 3.68 (s, 3H), 3.95 (s, 2H), 4.38 (s, 2H), 7.25–7.27 (m, 1H), 7.41–7.46 (m, 3H), 8.01 (d, J = 1.8 Hz, 2H), 8.40 (s, 1H), 8.66–8.69 (m, 4H), 8.76 (t, J = 1.8 Hz, 1H), 8.91 (d, J = 1.8 Hz, 2H); MALDI-MS 1240.4706; ESI-MS obsd 1241.5795, calcd 1241.5805 [$(M + H)^+$, M = $C_{71}H_{80}N_6O_{14}$]; λ_{abs} (CH_2Cl_2) 366, 518, 730 nm.

3,13-Bis(3,5-dicarboxyphenyl)-5-methoxy-15-[4-(4-(*N*-succinimidooxy)-1,4-dioxobutylamino)phenyl]-8,8,18,18-tetramethylbacteriochlorin (BC8). Following a general procedure,[52] a solution of **pro-BC8** (6.5 mg, 5.2 μmol) in CH_2Cl_2 (0.42 mL) was stirred under argon for 2 min, followed by addition of TFA (0.11 mL). After 1.5 h, the reaction mixture was diluted with ethyl acetate and then washed with brine until the aqueous phase was neutral (pH paper). The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with CH_2Cl_2, sonicated in a benchtop sonication bath, and centrifuged. The supernatant was discarded, leaving a green solid (3.8 mg, 71%): ^{1}H NMR (300 MHz, CD_3OD–$CDCl_3$, the COOH and NH proton peaks were not observed) δ 1.87 (s, 6H), 2.01 (s, 6H), 2.88–2.92 (m, 6H), 3.14 (t, J = 7.2 Hz, 2H), 3.68 (s, 3H), 3.97 (s, 2H), 4.40 (s, 2H), 7.37–7.45 (m, 4H), 8.15 (d, J = 1.5 Hz, 2H), 8.52 (s, 1H), 8.73–8.76 (m, 4H), 8.91 (s, 1H), 9.02 (d, J = 1.5 Hz, 2H); MALDI-MS obsd 1016.2656; ESI-MS obsd 1017.3289, calcd 1017.3301 [$(M + H)^+$, M = $C_{55}H_{48}N_6O_{14}$]; λ_{abs} (0.5 M potassium phosphate buffer, pH 7.0) 362, 516, 729 nm.

2,12-Bis[3,5-bis(*tert*-butoxycarbonyl)phenyl]-3,13-bis(methoxycarbonyl)-5-methoxy-15-[7-(*N*-succinimidooxy)-7-oxohept-1-ynyl]-8,8,18,18-tetramethylbacteriochlorin (pro-BC9). Following a general procedure[41] for copper-free Sonogashira reactions,[60] samples of **BC22** (26 mg, 23 μmol), 6-heptynoic acid (5, 15 μL, 0.12 mmol), $Pd_2(dba)_3$ (6.2 mg, 6.7 μmol), and P(*o*-tol)$_3$ (11 mg, 35 μmol) were placed in a Schlenk flask and dried under high

vacuum for 30 min. Toluene/TEA [2.4 mL, (2 : 1), deaerated by bubbling with argon for 30 min] was added to the Schlenk flask under argon and deaerated by three freeze–pump–thaw cycles. The reaction mixture was stirred at 70 °C for 18 h. The reaction mixture was cooled to room temperature, concentrated to dryness, diluted with CH_2Cl_2 and washed (saturated aqueous $NaHCO_3$ solution). The organic layer was separated, dried (Na_2SO_4) and concentrated. Column chromatography [silica, CH_2Cl_2/EtOAc (19 : 1) to CH_2Cl_2/CH_3OH (19 : 1)] provided a reddish solid (8.0 mg, total yield is given below): MALDI-MS obsd 1192.7728; ESI-MS obsd 1193.5701, calcd 1193.5693 [$(M + H)^+$, M = $C_{68}H_{80}N_4O_{15}$]; λ_{abs} (CH_2Cl_2) 382, 547, 756 nm. Half of the product (4.0 mg), DCC (6.9 mg, 34 μmol) and HOSu (3.9 mg, 34 μmol) were stirred in CH_2Cl_2 (0.34 mL) under argon at room temperature for 40 min. The resulting mixture was filtered to remove insoluble material. The filtrate was concentrated and separated by preparative TLC [silica, CH_2Cl_2/methanol (99 : 1)] to yield a reddish solid (2.8 mg, 19%): ^{1}H NMR (300 MHz, $CDCl_3$) δ −1.22 (s, 1H), −0.97 (s, 1H), 1.54–1.58 (m, 2H), 1.66 (s, 36H), 1.82 (s, 6H), 1.83 (s, 6H), 2.22–2.28 (m, 2H), 2.78–2.91 (m, 8H), 4.12 (s, 3H), 4.17 (s, 3H), 4.26 (s, 3H), 4.32 (s, 2H), 4.42 (s, 2H), 8.45 (s, 1H), 8.49 (s, 1H), 8.82 (s, 1H), 8.83 (s, 1H), 8.86–8.87 (m, 4H); MALDI-MS 1289.4308; ESI-MS obsd 1290.5865, calcd 1290.5857 [$(M + H)^+$, M = $C_{72}H_{83}N_5O_{17}$]; λ_{abs} (CH_2Cl_2) 381, 547, 756 nm.

2,12-Bis(3,5-dicarboxyphenyl)-3,13-bis(methoxycarbonyl)-5-methoxy-15-[7-(*N*-succinimidooxy)-7-oxohept-1-ynyl]-8,8,18,18-tetramethylbacteriochlorin (BC9). Following a general procedure,[52] a solution of **pro-BC9** (3.4 mg, 2.6 μmol) in CH_2Cl_2 (0.22 mL) was stirred under argon for 2 min, followed by addition of TFA (44 μL). After 1.5 h, the reaction mixture was diluted with ethyl acetate and then washed with brine until the aqueous phase was neutral (checked by pH paper). The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with CH_2Cl_2, sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a reddish solid (2.3 mg, 82%): ^{1}H NMR (300 MHz, CD_3OD–$CDCl_3$, the COOH and NH proton peaks were not observed) δ 1.26–1.30 (m, 2H), 1.85 (s, 12H), 2.02–2.22 (m, 2H), 2.78–2.93 (m, 8H), 4.14 (s, 3H), 4.18 (s, 3H), 4.29 (s, 3H), 4.42 (s, 2H), 4.55 (s, 2H), 8.50 (s, 1H), 8.53 (s, 1H), 8.95 (s, 1H), 8.96 (s, 1H), 8.99–9.01 (m, 4H); MALDI-MS obsd 1068.5653; ESI-MS obsd 1066.3371, calcd 1066.3353 [$(M + H)^+$, M = $C_{56}H_{51}N_5O_{17}$]; λ_{abs} (0.5 M potassium phosphate buffer, pH 7.0) 378, 546, 757 nm.

15^2-*N*-(3-(*N*-succinimidooxy)propyl)-3-methoxycarbonyl-2,12-bis-[3,5-bis(*tert*-butoxycarbonyl)phenyl]-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboximide (pro-BC10). Following a reported procedure,[43] a mixture of **BC22** (12 mg, 10 μmol), $Pd(PPh_3)_4$ (12 mg, 10 μmol), Cs_2CO_3 (10 mg, 30 μmol) and 3-aminopropanoic acid (4.0 mg, 40 μmol) was placed in a Schlenk flask, and deaerated under high vacuum for 40 min. The flask was then filled with CO and toluene (1.0 mL, deaerated by bubbling with argon for 30 min, and then with CO for 30 min). The reaction mixture was stirred at 80 °C for 18 h under a CO atmosphere at ambient pressure. The reaction mixture was cooled to room temperature, dried and washed (saturated aqueous $NaHCO_3$ solution). The combined organic layer was dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/methanol (4 : 1)]. The resulting solid was mixed with EDC (9.6 mg 50 μmol), DMAP (0.20 mg, 2.0 μmol) and HOSu (5.7 mg, 50 μmol) in CH_2Cl_2 (0.20 mL) and stirred under argon for 3 h. The reaction residue was chromatographed [silica, CH_2Cl_2/ethyl acetate (19 : 1 to 4 : 1)] to afford a reddish solid (2.7 mg, 22%): ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.43 (s, 1H), 0.09 (s, 1H), 1.66 (s, 18H), 1.67 (s, 18H), 1.79 (s, 6H), 1.82 (s, 6H), 2.83 (s, 4H), 3.31 (t, J = 6.6 Hz, 2H), 4.18 (s, 3H), 4.25 (s, 2H), 4.27 (s, 3H), 4.70 (s, 2H), 4.83 (t, J = 6.6 Hz, 2H), 8.38 (s, 1H), 8.45 (s, 1H), 8.83 (d, J = 2.1 Hz, 2H), 8.84 (d, J = 2.1 Hz, 2H), 8.89 (t, J = 1.5 Hz, 2H); MALDI-MS obsd 1248.9579; ESI-MS obsd 1249.5371, calcd 1249.5340 [$(M + H)^+$, M = $C_{68}H_{76}N_6O_{17}$]; λ_{abs} (CH_2Cl_2) 377, 564, 811 nm.

15^2-*N*-(3-(*N*-succinimidooxy)propyl)-3-methoxycarbonyl-2,12-bis(3,5-dicarbonylphenyl)-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboximide (BC10). Following a general procedure,[52] a solution of **pro-BC10** (2.6 mg, 2.1 μmol) in CH_2Cl_2 (0.12 mL) was stirred under argon for 2 min, followed by addition of TFA (92 μL). After 1 h, the reaction mixture was diluted with ethyl acetate and then washed with brine until the aqueous phase was neutral (checked by pH paper). The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting solid was treated with CH_2Cl_2, sonicated in a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a reddish solid (2.0 mg, 94%): ^{1}H NMR (300 MHz, THF-d_8, four CO_2H protons were not observed) δ −0.24 (s, 1H), 0.29 (s, 1H), 1.99 (s, 6H), 2.01 (s, 6H), 2.93 (s, 4H), 3.43 (t, J = 7.2 Hz, 2H), 4.28 (s, 3H), 4.46 (s, 3H), 4.50 (s, 2H), 4.90–4.93 (m, 4H), 8.70 (s, 1H), 8.74 (s, 1H), 9.12–9.15 (m, 6H); MALDI-MS obsd 1025.4487; ESI-MS obsd 1025.2875, calcd 1025.2836 [$(M + H)^+$, M = $C_{52}H_{44}N_6O_{17}$]; λ_{abs} (0.5 M potassium phosphate buffer, pH 7.0) 377, 570, 824 nm.

Zn(II)-15-(3-Aminophenyl)-3,13-bis(ethoxycarbonyl)-2,12-diethyl-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (BC14). Following a general procedure,[44] a mixture of **BC11** (16.0 mg, 23.1 μmol) and NaH (16.6 mg, 1.20 mmol, 30.0 equiv.) was added to DMSO (2.30 mL) under argon. The mixture was stirred for 5 min. $Zn(OTf)_2$ (252 mg, 694 μmol, 30.0 equiv.) was then added, and the suspension was stirred for 16 h in an oil bath at 80 °C. The crude mixture was washed with water and extracted with ethyl acetate. The combined extract was dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (9 : 1)] to afford a reddish solid (9.1 mg, 52%): ^{1}H NMR (300 MHz, THF-d_8) δ 1.25 (t, J = 7.2 Hz, 3H), 1.51–1.69 (m, 9H), 1.81 (s, 3H), 1.83 (s, 3H), 1.94 (s, 6H), 3.61–3.76 (m, 4H), 3.89–4.02 (m, 4H), 4.13 (s, 3H), 4.37 (s, 2H), 4.55 (s, 2H), 4.61 (t, J = 7.5 Hz, 2H), 6.71–6.74 (m, 1H), 6.92–6.94 (m, 2H), 7.17 (t, J = 7.8 Hz, 1H), 8.44 (s, 1H), 8.48 (s, 1H); MALDI-MS obsd 753.39; ESI-MS obsd 754.2917, calcd 754.2947 [$(M + H)^+$, M = $C_{41}H_{47}N_5O_5Zn$]; λ_{abs} (CH_2Cl_2) 355, 385, 553, 738 nm.

15^2-*N*-Butyl-3-ethoxycarbonyl-2,12-diethyl-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboximide (BC17). Following a reported procedure,[43] a mixture of **BC12** (25.0 mg, 36.8 μmol), $Pd(PPh_3)_4$ (68.0 mg, 58.9 μmol), Cs_2CO_3 (180 mg, 552 μmol) and *n*-butylamine (18.0 μL, 184 μmol) was placed in a Schlenk flask, and deaerated under high vacuum for 40 min. The flask was then filled with

CO and toluene (4.0 mL, deaerated by bubbling with argon for 30 min, and then with CO for 30 min). The reaction mixture was stirred at 90 °C for 18 h under a CO atmosphere at ambient pressure. The reaction mixture was cooled to room temperature, dried and washed with saturated aqueous $NaHCO_3$ solution. The combined organic layer was dried (Na_2SO_4), concentrated and chromatographed [silica, hexanes/CH_2Cl_2 (5 : 5 to 3 : 7)] to afford a reddish solid (11.9 mg, 52%): ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.74 (s, 1H), −0.54 (s, 1H), 1.10 (t, J = 7.5 Hz, 3H), 1.62–1.79 (m, 13H), 1.92 (s, 6H), 1.93 (s, 6H), 4.08 (q, J = 7.2 Hz, 2H), 4.22 (q, J = 7.2 Hz, 2H), 4.33 (s, 2H), 4.44 (t, J = 7.5 Hz, 2H), 4.74 (s, 2H), 4.78 (q, J = 7.2 Hz, 2H), 8.58 (s, 1H), 8.71 (s, 1H), 9.56 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.3, 14.8, 17.4, 17.6, 20.1, 20.9, 21.0, 30.0, 31.2, 31.3, 31.6, 40.4, 45.7, 46.1, 52.1, 53.36, 53.45, 61.5, 94.7, 99.4, 99.5, 101.9, 115.2, 122.0, 133.5, 134.6, 136.2, 136.8, 140.3, 144.3, 162.6, 163.4, 165.9, 168.2, 168.5, 170.5, 176.0; MALDI-MS obsd 622.9292; ESI-MS obsd 624.3532, calcd 624.3544 [$(M + H)^+$, M = $C_{37}H_{45}N_5O_4$]; λ_{abs} (CH_2Cl_2) 357, 408, 543, 818 nm.

15-(4-Aminophenyl)-3,13-bis[3,5-bis(*tert*-butoxycarbonyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (BC20). Following a general procedure,[51] samples of bacteriochlorin **BC18** (53.0 mg, 51.4 μmol), **4** (56.3 mg, 0.257 mmol), $Pd(PPh_3)_4$ (23.7 mg, 20.6 μmol), and Cs_2CO_3 (101 mg, 0.308 mmol) were placed in a Schlenk flask and dried under high vacuum for 30 min. Toluene/DMF [5.1 mL, (2 : 1), deaerated by bubbling with argon for 30 min] was added to the Schlenk flask under argon and deaerated by three freeze–pump–thaw cycles. The reaction mixture was stirred at 90 °C for 18 h. The reaction mixture was cooled to room temperature, concentrated to dryness, diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic layer was separated, dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (23 : 2)] to provide a greenish solid (38.0 mg, 71%): ^{1}H NMR (300 MHz, $CDCl_3$) δ −1.52 (s, 1H), −1.18 (s, 1H), 1.65 (s, 18H), 1.69 (s, 18H), 1.85 (s, 6H), 1.98 (s, 6H), 3.60 (s, 2H), 3.68 (s, 3H), 4.00 (s, 2H), 4.38 (s, 2H), 6.41 (d, J = 8.4 Hz, 2H), 7.22 (d, J = 8.1 Hz, 1H), 7.26 (s, 1H), 7.62 (d, J = 8.1 Hz, 1H), 8.48 (t, J = 1.5 Hz, 1H), 8.64–8.67 (m, 4H), 8.76 (t, J = 1.5 Hz, 1H), 8.91 (d, J = 1.5 Hz, 1H) (two anilino NH protons were not observed); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.1, 28.5, 31.27, 31.38, 45.1, 46.0, 47.7, 52.4, 63.5, 81.4, 81.8, 83.5, 97.31, 97.37, 114.1, 114.3, 122.9, 127.0, 127.5, 128.0, 129.1, 131.07, 131.12, 131.9, 132.1, 133.9, 134.1, 134.2, 134.8, 136.0, 136.2, 136.6, 138.7, 138.9, 145.5, 154.8, 161.9, 165.6, 165.8, 169.06, 169.22; MALDI-MS 1043.6068; ESI-MS obsd 1044.5475, calcd 1044.5481 [$(M + H)^+$, M = $C_{63}H_{73}N_5O_9$]; λ_{abs} (CH_2Cl_2) 366, 520, 729 nm.

15-Bromo-2,12-bis[3,5-bis(*tert*-butoxycarbonyl)phenyl]-3,13-dimethoxycarbonyl-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (BC22). Following a general procedure,[42] a solution of bacteriochlorin **BC21** (44 mg, 41 μmol) in THF (8.3 mL) was treated with NBS (7.3 mg, 41 μmol) in THF (0.41 mL) at room temperature for 1.5 h. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and chromatographed [silica, CH_2Cl_2/ethyl acetate (19 : 1)] to afford a reddish solid (20 mg, 42%): ^{1}H NMR (300 MHz, $CDCl_3$) δ −1.52 (s, 1H), −1.25 (s, 1H), 1.66 (s, 36H), 1.83 (s, 6H), 1.86 (s, 6H), 4.16 (s, 3H), 4.20 (s, 3H), 4.28 (s, 3H), 4.37 (s, 2H), 4.44 (s, 2H), 8.50 (s, 2H), 8.83–8.85 (m, 2H), 8.87–8.88 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.4, 28.5, 29.9, 31.0, 31.3, 46.0, 47.9, 53.27, 53.39, 54.7, 64.6, 82.04, 82.10, 94.6, 96.7, 97.1, 98.2, 125.2, 126.0, 129.2, 130.0, 130.4, 131.5, 132.8, 133.1, 133.4, 133.7, 133.9, 134.4, 134.5, 136.3, 136.6, 158.2, 160.8, 165.08, 165.14, 168.6, 168.8, 169.2, 173.4; ESI-MS obsd 1147.4245, calcd 1147.4274 [$(M + H)^+$, M = $C_{61}H_{71}BrN_4O_{13}$]; λ_{abs} (CH_2Cl_2) 375, 531, 740 nm.

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