Published in final edited form as:

New J Chem. 2016 September 1; 40(9): 7750–7767. doi:10.1039/C6NJ01155A.

Bioconjugatable, PEGylated Hydroporphyrins for Photochemistry and Photomedicine. Narrow-Band, Near-Infrared-Emitting Bacteriochlorins

Nuonuo Zhang^a, Jianbing Jiang^a, Mengran Liu^a, Masahiko Taniguchi^a, Amit Kumar Mandal^b, Rosemary B. Evans-Storms^c, J. Bruce Pitner^c, David F. Bocian^d, Dewey Holten^b, and Jonathan S. Lindsey^a

^aDepartment of Chemistry, North Carolina State University, Raleigh, NC 27695-8204

^bDepartment of Chemistry, Washington University, St. Louis, MO 63130-4889

^cNIRvana Sciences, Research Triangle Park, NC 27709-3169

^dDepartment of Chemistry, University of California, Riverside, CA 92521-0403

Abstract

Synthetic bacteriochlorins absorb in the near-infrared (NIR) region and are versatile analogues of natural bacteriochlorophylls. The utilization of these chromophores in energy sciences and photomedicine requires the ability to tailor their physicochemical properties, including the incorporation of units to impart water solubility. Herein, we report the synthesis, from two common bacteriochlorin building blocks, of five wavelength-tunable, bioconjugatable and water-soluble bacteriochlorins along with two non-bioconjugatable benchmarks. Each bacteriochlorin bears short polyethylene glycol (PEG) units as the water-solubilizing motif. The PEG groups are located at the 3,5-positions of aryl groups at the pyrrolic β -positions to suppress aggregation in aqueous media. A handle containing a single carboxylic acid is incorporated to allow bioconjugation. The seven water-soluble bacteriochlorins in water display Q_y absorption into the NIR range (679–819 nm), sharp emission (21–36 nm full-width-at-half-maximum) and modest fluorescence quantum yield (0.017–0.13). Each bacteriochlorin is neutral (non-ionic) yet soluble in organic (e.g., CH_2Cl_2 , DMF) and aqueous solutions. Water solubility was assessed using absorption spectroscopy by changing the concentration ~ 1000 -fold (190–690 μM to 0.19–0.69 μM) with a reciprocal change in pathlength (0.1–10 cm). All bacteriochlorins showed excellent solubility in water, except for a bacteriochlorin–imide that gave slight aggregation at higher concentrations. One bacteriochlorin was conjugated to a mouse polyclonal IgG antibody for use in flow cytometry with compensation beads for proof-of-principle. The antibody conjugate of **B2-NHS** displayed a sharp signal upon ultraviolet laser excitation (355 nm) with NIR emission measured with a 730/45 nm bandpass filter. Overall, the study gives access to a set of water-soluble bacteriochlorins with desirable photophysical properties for use in multiple fields.

Correspondence to: J. Bruce Pitner; David F. Bocian; Dewey Holten; Jonathan S. Lindsey.

Electronic Supplementary Information (ESI) available: Studies and results concerning attempted syntheses of target bacteriochlorins; solvent-dependence of the fluorescence quantum yield for bacteriochlorin **B5**; and spectral data for new compounds

Conflict of Interest Statement: D.F.B., D.H. and J.S.L. are cofounders of NIRvana Sciences, which develops chlorins and bacteriochlorins for use in clinical diagnostics.

Introduction

Photochemistry in the NIR spectral region (700–1400 nm) has been far less explored than the visible or ultraviolet regions owing chiefly to availability of suitable chromophores. Yet, the NIR presents a number of distinct opportunities. The broadest spectral distribution of sunlight harvested for photosynthesis is achieved by anoxygenic bacteria, which deploy bacteriochlorophylls in light-harvesting assemblies that capture light with wavelengths in the 700–900 nm region, and in some cases to ~1000 nm.¹ An optical window for penetration of soft tissue by light occurs with midpoint near ~800 nm.² More generally, NIR light gives rise to excited states of relatively low energy (~30–40 kcal/mol), far less than that of typical chemical bonds, versus ultraviolet light which affords excited-state energies (up to ~140 kcal/mol) that exceed those of many chemical bonds.

Our work over the past decade has focused on developing synthetic bacteriochlorins as a class of compounds for use in diverse photochemical studies.³ A number of routes are available for gaining access to bacteriochlorins.^{4–13} The synthetic bacteriochlorins contain the core chromophore of natural bacteriochlorophylls¹⁴ yet also afford amenability toward molecular tailoring to meet diverse objectives (Chart 1). The presence of a geminal dimethyl group in each pyrroline ring stabilizes the macrocycle toward adventitious dehydrogenation. One objective has been to gain access to a palette of water-soluble, bioconjugatable, wavelength-tunable bacteriochlorins.¹⁵ In energy sciences, such bacteriochlorins can be employed in light-harvesting architectures and as constituents of energy-transfer cascades.^{16,17} In photomedicine, the bacteriochlorins are effective in photodynamic therapy^{18,19} and in optical imaging.^{20–25} In clinical diagnostics, the bacteriochlorins are candidates for fluorescent markers in flow cytometry.²⁶ For polychromatic flow cytometry,^{27–29} a palette of bacteriochlorins spanning the NIR spectral region would complement that of a corresponding set of chlorins in the red spectral region as described in the companion paper.³⁰

The construction of bacteriochlorins with the combination of features for water solubility, bioconjugation, and wavelength tunability has proven to be very challenging. The general structure of the synthetic bacteriochlorins is shown in Chart 1. Installing a single bioconjugatable tether has been achieved in a straightforward manner by regioselective 15-bromination of a 5-methoxybacteriochlorin followed by Pd-mediated coupling.¹¹ Tuning the position of the long-wavelength (Q_y) absorption band from ~700–900 nm has chiefly been achieved by appropriate choice of auxochromes located at the β -pyrrolic (2, 3, 12, 13) positions about the perimeter of the macrocycle.³ Indeed, a palette of lipophilic bacteriochlorins bearing diverse tethers was recently prepared for bio-orthogonal labeling.³¹ On the other hand, achieving water solubility has proved quite challenging alone and has been even more daunting to accomplish while maintaining the other desired features of bioconjugation and wavelength tunability.¹⁵

In the companion paper, a general strategy for water-solubilization of chlorins entailed use of PEG groups located at the 2,6-positions of a meso-aryl substituent, which caused the polar groups to project above and below the plane of the macrocycle.³⁰ The present synthetic

route to bacteriochlorins is not amenable to incorporation of such sterically hindered 2,6-substituted aryl groups at any macrocycle position. Accordingly, we recently incorporated 3,5-disubstituted aryl groups located at the β -pyrrole positions wherein the 3,5-substituents are quite polar.¹⁵ The substituents **A–F** are shown in Chart 2. The polar substituents are not thrust over the plane of the macrocycle as in the case for the 2,6-substitution pattern with chlorins, but still imparted a significant degree of aqueous solubility. The substituents include phosphonate (**A**), carboxylate (**B**), ammonium (**C**), choline-ester (**D**), phosphatidylcholine-ester (**E**), and oligoethyleneoxy (PEG, **F**) units. The common scaffold resulted in each bacteriochlorin exhibiting essentially the same spectral properties.¹⁵ Among these various motifs, the PEG group was most attractive for aqueous solubilization and bioconjugation. The nonionic nature of the PEG facilitates handling – the PEG derivatives are soluble in both water and organic solvents,^{32,33} but readily partition from water into dichloromethane thereby enabling partitioning of crude reaction mixtures. Moreover, the PEG group is compatible with bioconjugation strategies without cross-reactions as can occur with carboxylates, phosphonates, and amines.

In this paper, we report the design and synthesis of five bioconjugatable bacteriochlorins (**B1–B5**) that bear PEG groups at the 3,5-positions of appended aryl moieties (Chart 3). Two benchmark bacteriochlorins (**I**, **II**) lacking bioconjugatable groups also were prepared. The seven bacteriochlorins were prepared by elaboration of the bacteriochlorin building blocks **1** and **2**, which in turn were obtained by *de novo* synthesis.^{10–13} The bacteriochlorins afford distinct Q_y band positions owing to the presence of auxochromes at the macrocycle β -positions (2,12-, 3,13-, 7-sites) and meso-positions (15-site), and hence are of interest for use in light-harvesting and in flow cytometry. The bacteriochlorins have been characterized for solubility in dilute aqueous solution. The spectroscopic properties of bacteriochlorin **I** were reported previously;¹⁵ the synthesis is provided herein. The photophysical properties, including rate constants and yields for fluorescence emission, have been measured. One bacteriochlorin was attached to an antibody and examined via flow cytometry. Taken together, the advances reported herein broaden the scope of synthetically accessible chromophores for use in the NIR spectral region, particularly where sharp absorption and emission bands are advantageous.

Results and Discussion

Synthesis

Bacteriochlorin B1—An oxobacteriochlorin was synthesized to shift the Q_y band hypsochromically into the red spectral region. Treatment of bacteriochlorin **1** with MnO_2 in CH_2Cl_2 afforded oxobacteriochlorin **3** in 54% yield.³⁴ The steric hindrance caused by the methoxy group at the 5-position causes Pd-mediated coupling with the 3,13-dibromobacteriochlorin to proceed regioselectively at the 13-position with one equivalent of the ethyne.^{24,35,36} Therefore, the Sonogashira coupling reaction of **3** with methyl 4-ethynylbenzoate (**4**) regioselectively proceeded at the 13-position of the macrocycle to give ethynylbacteriochlorin **5** in 42% yield (Scheme 1). Further reaction of **5** with ethyne **6** in the presence of $Pd(PPh_3)_2Cl_2$ and Et_3N in DMF at 80 °C gave unsymmetrically substituted bacteriochlorin **7**.³⁵ The alkynes in **7** were reduced³⁷ to the fully saturated alkyl moieties by

catalytic hydrogenation to obtain dialkylbacteriochlorin **8** in 85% yield. Absorption spectroscopy was used to monitor the reduction, given that prolonged reaction afforded side products. The Boc groups were cleaved by treatment with 17% TFA in CH₂Cl₂ to release the free amines. The methyl group was removed under 4 M aqueous NaOH solution to afford the carboxylic acid. Without purification, PEGylation of deprotected bacteriochlorin with **PEG₈-NHS** was carried out directly in the presence of Cs₂CO₃ to give target oxobacteriochlorin **B1** in 63% yield.

Bacteriochlorins B2 and I—Suzuki coupling reaction of bacteriochlorin **1** with the coupling partner **9** previously gave the corresponding 3,13-diaryl bacteriochlorin **10** in 85% yield (Scheme 2).¹⁵ This procedure was carried out here at 6-fold larger scale in 90% yield. The four Boc groups were smoothly removed upon treatment with 40% TFA in CH₂Cl₂ to give bacteriochlorin **11** in 78% yield.¹⁵ The tetraaminobacteriochlorin **11** was subjected to PEGylation with **PEG₄-NHS** to give the water-soluble benchmark bacteriochlorin **I** in 80% yield.

Bromination of bacteriochlorin **10** with NBS afforded the 15-brominated bacteriochlorin¹¹ along with an almost equal amount of unreacted bacteriochlorin (on the assumption of equal desorption and ionization efficiencies upon MALDI mass spectrometry) (Scheme 2). The crude reaction mixture was not separable by column chromatography, and was used directly in the next step. The Suzuki coupling reaction with partner **12**³⁸ gave the corresponding 3,5-diaryl-15-(carboxyaryl)bacteriochlorin **13** in 31% yield from two steps. Upon treatment of 40% TFA in CH₂Cl₂, four Boc groups were removed smoothly, as well as the *tert*-butyl protecting group. The amine-containing bacteriochlorin **14** was subjected to PEGylation in the presence of Cs₂CO₃ to afford the water-soluble PEGylated bacteriochlorin **B2** in 83% yield. The aforementioned synthesis of **B2** was based on a small-scale synthesis (~10 mg); a larger scale synthesis afforded ~40 mg of **B2** starting from **10** (Supplementary Information).

Bacteriochlorins B3, B4 and II—The Suzuki coupling reaction of the dibromobacteriochlorin bearing two ester groups (**2**) with **9** gave Boc-protected bacteriochlorin **15** in 80% yield (Scheme 3). Bacteriochlorin **15** was treated with 20% TFA in CH₂Cl₂ to unveil the four primary amines, which were used as handles for attaching four PEG₈ units to give benchmark bacteriochlorin **II** in 54% yield. Bromination of bacteriochlorin **15** with NBS gave the 15-brominated bacteriochlorin **16** in 50% yield. Sonogashira coupling of **16** initially with *tert*-butyl 6-heptynoate did not afford the desired product, while the unprotected 6-heptynoic acid (**17**) gave bacteriochlorin **18** in 15% yield. Deprotection of the four amines followed by PEGylation with **PEG₄-NHS** afforded the water-soluble bioconjugatable bacteriochlorin **B4** in 55% yield from two steps.

Target bacteriochlorin **ZnB3** with a predicted Q_y absorption band at ~760 nm was designed using the same strategy as for bacteriochlorin **B2** (Scheme 3). Suzuki coupling reaction of bacteriochlorin **16** with compound **12** smoothly afforded bacteriochlorin **19** in 56% yield. Cleavage of Boc and *tert*-butyl protecting groups using 20% TFA in CH₂Cl₂ followed by PEGylation of the four amino groups with **PEG₈-NHS** yielded **B3** in 66% yield. Bacteriochlorin **B3** was metalated with zinc (in DMF containing NaH)³⁹ to tune the

wavelength from 742 nm to 754 nm. However, the resulting zinc-metalated bacteriochlorin **ZnB3** demetalated to give the free-base bacteriochlorin **B3** on standing for 2 h in solution at ambient temperature or as a solid overnight at $-20\text{ }^{\circ}\text{C}$.

Bacteriochlorin B5—A further design entails the synthesis of a bacteriochlorin-imide. Carbamoylation³ of bacteriochlorin **16** with CO (ambient pressure) and *tert*-butyl 4-aminobutanoate hydrochloride **20** gave the corresponding bacteriochlorin-imide **21** in 25% yield (Scheme 4). Satisfactory separation of **21** from various byproducts by column chromatography required a very slow rate of elution. Each fraction was collected and preliminarily identified by absorption spectroscopy, and the desired product was further characterized by MALDI-MS, ESI-MS and ^1H NMR spectroscopy. The four Boc and one *tert*-butyl groups were easily removed upon treatment with 20% TFA in CH_2Cl_2 . The amine-containing bacteriochlorin was subjected to PEGylation with **PEG₈-NHS** in the presence of Cs_2CO_3 to afford the water-soluble PEGylated bacteriochlorin **B5** in 68% yield. A preceding model reaction of **21** (not in pure form, containing <10 mol % of **15**) was conducted for deprotection and PEGylation with **PEG₄-NHS**, and the PEGylated bacteriochlorin was found to afford broad absorption band and low fluorescence quantum yield (Φ_f) in water (0.0055). We assumed the poor water-solubility and low Φ_f was derived from the short PEG₄ chain, and switched to the longer PEG reagent **PEG₈-NHS** for the synthesis of **B5**.

Handling and synthesis robustness—The procedures for handling PEGylated chlorins are described in the companion paper³⁰ and generally apply to bacteriochlorins, although size exclusion chromatography was not employed. The routes for preparation of **B2–B5** are amenable to scale-up given the following attributes: (i) Each step for bacteriochlorin derivatization has a moderate to good yield (except 15% and 25% yields for the preparation of bacteriochlorin **18** and **21**, respectively). (ii) Each target compound or the corresponding intermediate is stable enough for purification and storage. (iii) The relatively expensive PEG reagents were used in the final step to make the purification facile (no column chromatography) and the synthesis more economical. The bacteriochlorin building block **1** or **2** could be prepared within three weeks in a scale of hundreds of mgs for each, and the diversification of these two precursors to the three target compounds could be achieved within three additional weeks in a 50–100 mg scale. The procedures for the four-step conversion of **1** to **B2** and of **2** to **B4** are described in the Supplementary Information.

Characterization—The PEGylated, bioconjugatable bacteriochlorins **B1–B5**, the benchmarks **I** and **II**, and the corresponding precursors typically were characterized by absorption and fluorescence spectroscopy (not for precursors), ^1H NMR spectroscopy, ^{13}C NMR spectroscopy (where quantity and solubility allowed), MALDI-MS and ESI-MS. The ^1H NMR spectra of the PEGylated bacteriochlorins typically showed the far upfield, aryl region, and far downfield peaks characteristic of bacteriochlorins, but were rather uninformative in the region of the PEG resonances; moreover, the spectrum was quite broadened for bacteriochlorin **B4**. Several other target molecules were also designed with expectation to tune the wavelength and were the subject of exploratory syntheses (see the Supplementary Information).

Photophysical properties

Absorption and fluorescence properties—The absorption and emission spectra of **B1**, **B2**, **B3**, and **B4** were collected in water at room temperature; the spectra of **B5** were collected in DMSO (Fig. 1). The absorption and fluorescence spectra of the same five bacteriochlorins collected in DMF and water are shown in Fig. 2, along with those for PEGylated benchmarks **I** and **II**, which lack a bioconjugatable tether. The absorption and fluorescence spectra for bacteriochlorin **8** (the non-PEGylated precursor of **B1**) were also obtained; these spectra were measured only in DMF (see Supplementary Information) because **8** is insoluble in water. Each absorption spectrum shown in Fig. 2 is normalized to the total intensity (300–1000 nm) obtained by integration when plotted in wavenumbers (cm^{-1}), which is convenient for comparing effects of substituents on relative band intensities.⁴⁰

The absorption spectra of the bacteriochlorins exhibit general features expected for this genre of macrocycle in three spectral domains. These regions are an intense NIR Q_y band (670–820 nm), weaker visible Q_x band (500–580 nm), and the strong near-UV (NUV) B_x and B_y features (360–400 nm), also known as the Soret bands. Typically a weaker (1,0) satellite feature can be resolved to higher energy than each (0,0), or origin, band. There is considerable overlap of the B_y and B_x origin and vibronic components in the NUV. The peak positions for the various absorption features are listed in Table 1.

Bacteriochlorins **B3**, **B4** and **II** possess ester groups at the 3,13-positions, which result in a bathochromic shift in the Q_y band of ~16 nm, ~28 nm and ~25 nm, respectively relative to that for **B2** (in DMF or water). **B3** and **B4** differ from **II** by the presence of a bioconjugatable tether via phenylation or ethynylation, respectively. **B5** differs from the other bacteriochlorins by the six-membered imide ring spanning the 13–15 positions, giving rise to a ~50 nm bathochromic shift of the Q_y absorption band (808 nm in DMF and 819 nm in water) compared to **B4** and **II** (750–754 nm).

The Q_y absorption bands of the bacteriochlorins have a full-width-at-half-maximum (FWHM) in the range 21–32 nm (25 nm average) in DMF and 21–36 nm (27 nm average) in water. The greater FWHM in water versus DMF is generally paralleled by a decrease in Q_y peak intensity (relative to the Soret maximum). The absorption profile for **B5** in water (Fig. 2B) shows an apparent increase in baseline starting at ~600 nm and increasing into the NUV region as may be expected for light scattering, perhaps due to formation of small aggregates. The extent of scattering is comparatively less for the same compound in DMF (Fig. 2A).

Each bacteriochlorin fluorescence spectrum in both DMF and water (Fig. 2) is dominated by the Q_y origin band, with evidence in some cases of the weaker (0,1) band to longer wavelength. The Q_y fluorescence maximum is (Stokes) shifted to longer wavelength than Q_y absorption peak by relatively small amount (5–7 nm) for all the cases, except **B3** and **II** in water (8–9 nm) and **B5** in DMF (11 nm) or water (15 nm).

Excited-state properties—The measured excited-state properties of the bacteriochlorins are the lifetime (τ_S) of the lowest singlet excited state (S_1), the $S_1 \rightarrow S_0$ fluorescence quantum yield (Φ_F) and the $S_1 \rightarrow T_1$ intersystem crossing yield (Φ_{isc}). The $S_1 \rightarrow S_0$

internal conversion yield is obtained by subtraction: $\Phi_{ic} = 1 - \Phi_f - \Phi_{isc}$. The fluorescence (k_f), intersystem crossing (k_{isc}), and internal conversion (k_{ic}) rate constants are obtained from these data via the expression $k_x = \Phi_x/\tau_s$, where $x = f, isc$ or ic . The latter three values are given as corresponding time constants ($1/k_x$) in units of nanoseconds in Table 2, which summarizes the excited-state photophysical properties of the bacteriochlorins in DMF and water.

The τ_s values of all the bacteriochlorins except **B3** (and **II**) in water are 7–18% lower in water (1.4–3.4 ns) than in DMF (1.7–3.7 ns). The lifetime for bacteriochlorin-imide **B5** in DMF or water (1.7 or 1.4 ns, respectively) is shorter than that of **B2**, **B4**, **II** and **B1** in DMF (3.0–3.7 ns) and water (2.7–3.4 ns). For all the bacteriochlorins, the Φ_f values are more substantially reduced (29–87%) in water (0.017–0.16) versus DMF (0.11–0.21). The reduction of Φ_f in water vs DMF follows the trend: **B5** (0.017 vs 0.13; 87%) > **B4** (0.035 vs 0.12; 71%) > **B1** (0.075 vs 0.19; 61%) > **B2** (0.098 vs 0.16; 39%) ~ **B3** (0.13 vs 0.21; 38%) > **II** (0.078 vs 0.11; 29%) > **I** (0.16 vs 0.20; 20%).

Collectively, the above comparisons make the following key points: (1) Comparison of the non-PEGylated **8** with PEGylated **B1** shows only a 10% drop in τ_s (3.3 to 3.0 ns) and Φ_f (0.20 to 0.19) in DMF. (Comparisons in water cannot be made because bacteriochlorin **8** is essentially insoluble in that medium.) (2) In DMF, **B2** gives comparable τ_s and Φ_f values to non-PEGylated bacteriochlorin analogues with similar Q_y wavelength (~ 731 nm).⁴¹ (3) The reduction in τ_s and Φ_f for PEGylated bacteriochlorin-imide **B5** is consistent with prior studies on non-PEGylated bacteriochlorin-imides, and primarily reflects the effect of the fused ring to give a lower S_1 energy (and longer Q_y wavelength), enhancing nonradiative decay.⁴¹ (4) The reduction in τ_s for **B5** relative to the other bacteriochlorins may reflect in part formation of small aggregates (even dimers) in the ground or excited state or both. Such a possibility is consistent with the above-noted light scattering in the absorption spectrum for the compound in water and to some degree in DMF (Fig. 2).

The average five-fold larger reduction of Φ_f (40%) than τ_s (15%) for the seven bacteriochlorins in water versus DMF reflects the fact that k_f is small compared to $k_{isc}+k_{ic}$; thus, the lifetime is only modestly dependent on k_f , via the expression $\tau_s = (k_f + k_{isc} + k_{ic})^{-1}$, whereas the fluorescence yield is directly proportional to k_f (and also modestly dependent through τ_s) via the expression $\Phi_f = k_f/\tau_s$. Thus, the (disparate) reductions in τ_s and Φ_f reflect in part a decrease in k_f in water versus DMF (in addition to an increase in k_{ic} as discussed below). Examination of Table 2 indicates that the values for k_f for all the bacteriochlorins (except **B5**) are reduced (k_f^{-1} is larger) by a factor of 1.1–3.3 (average 1.9) in DMF versus water. The larger, 6.5-fold difference in k_f between the two media for **B5** may stem from an effect of aggregation on the excited-state decay (*vide supra*), giving a τ_s that is smaller and thus, a k_f that is smaller than the value expected for the monomer. To further explore medium effects on the Φ_f value of **B5**, fluorescence studies were carried out in a variety of solvents in addition to DMF and water (Supplementary Information).

Multiple factors likely contribute to the differences in k_f for $S_1 \rightarrow S_0$ fluorescence (Table 2) and the parallel changes in radiative probability for $S_0 \rightarrow S_1$ absorption expected on the basis of the relationship of the Einstein coefficients. Variation in the $S_0 \rightarrow S_1$ absorption

strength is reflected in the Q_y/B absorption-intensity ratio (Table 1), which is one normalization method often used to gauge a change in the strength of absorption to the Q_y (S_1) excited state. A small difference in k_f values can be accounted for by the differences in refractive index (1.43 vs 1.33). Other potential contributions include a difference in solvent interactions with the macrocycle and peripheral substituents (e.g., H-bonding with oxo-moieties in ester or acid groups) that alter the relative energies of the bacteriochlorin frontier molecular orbitals.

Examination of Table 2 shows that the seven bacteriochlorins in DMF or water generally have an intersystem-crossing yield (Φ_{isc}) in the range 0.29–0.50 and a rate constant (k_{isc}) of $(6\text{--}10\text{ ns})^{-1}$. The exception is **B5** in water which has $\Phi_{isc} = 0.08$ and $k_{isc} = (18\text{ ns})^{-1}$, values likely compromised by aggregation effects. Except for **B5** in water, the values of the other cases are comparable but perhaps slightly smaller than those found for diverse bacteriochlorins having Q_y wavelength (and S_1 energies) in the same range.^{3,41}

The yield of $S_1 \rightarrow S_0$ internal conversion (Φ_{ic}) for the bacteriochlorins listed in Table 2 generally is in the range 0.31 to 0.58 for the compounds in DMF and 0.42 to 0.65 in water (**B5** in water is the exception with a value of 0.90). The average increase in Φ_{ic} in water versus DMF is 25%. Examination of Table 2 reveals that this effect arises from generally larger rate constant (k_{ic}) for internal conversion for the bacteriochlorins in water versus DMF. This difference and a general trend of increasing k_{ic} with increasing Q_y wavelength (decreasing S_1 energy) can be seen by collecting the bacteriochlorins in three groups considering uncertainties k_{ic} values): (1) Bacteriochlorins **B1–B3** and **I** have Q_y at (684–748 nm) and $k_{ic} = (9\text{--}11\text{ ns})^{-1}$ in DMF and (679–750 nm) and $k_{ic} = (6\text{--}8\text{ ns})^{-1}$ in water. (2) Bacteriochlorins **B4** and **II** have Q_y at (756–760 nm) and $k_{ic} = (5\text{--}6\text{ ns})^{-1}$ in DMF and (759–760 nm) and $k_{ic} = (5\text{ ns})^{-1}$ in water. (3) Bacteriochlorin-imide **B5** has Q_y at (819 nm) and $k_{ic} = (3\text{ ns})^{-1}$ in DMF and (834 nm) and $k_{ic} = (2\text{ ns})^{-1}$ in water. Such a trend of increasing k_{ic} with decreasing S_1 (Q_y) energy is consistent with results for a large number of bacteriochlorins^{3,41} and the energy-gap law for nonradiative decay.⁴²

Effect of concentration on spectral properties—Absorption versus concentration studies in neat deionized water were conducted to assess the aqueous solution properties of the bacteriochlorins over a concentration range of 1000-fold ($\sim 200\text{--}700\text{ }\mu\text{M}$ to $\sim 0.20\text{--}0.70\text{ }\mu\text{M}$). This type of study has been previously explained in detail,³³ and the same approach was adopted herein. The spectra for each bacteriochlorin are shown in Fig. 3 While **B5** exhibits obvious band broadening indicating some degree of aggregation, the other PEGylated bacteriochlorins exhibit almost unchanged spectroscopic properties in the NIR and visible regions and minimal changes in the NUV region over the 1000-fold concentration range. This observation indicates little or no aggregation of these molecules.

Flow cytometry

New fluorophores with spectrally distinct emission bands (i.e., distinct “colors”) and instrumentation that together enable polychromatic flow cytometry are essential for advancing the field of clinical diagnostics.^{27–29} Although ultraviolet lasers (UV, 355 nm) are less frequently found in flow cytometers used in the diagnostic sector than lasers that emit at

longer wavelengths, UV lasers are common in research instruments where they afford important analytic attributes. The attributes include the ability to perform cell cycle analyses with DNA intercalating dyes such as 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33258, and calcium tracking with Indo-1.⁴⁴ The 355-nm excitation wavelength is nearly ideal for bacteriochlorins, which typically exhibit strong absorption in this region, with resulting emission in the NIR region, providing an effective Stokes shift of >350 nm (Fig. 1, Fig. 2).

To examine the efficacy of bacteriochlorins in flow cytometry, bacteriochlorin-labeled antibodies were detected using antibody-capture compensation beads. Such beads are frequently used for building multicolor flow cytometry experiments to determine proper corrections due to spectral overlap between fluorophore-labeled reagents.⁴⁵ The bacteriochlorin **B2** was used to label mouse IgG antibody for detection using mouse IgG antibody-specific compensation beads. The resulting bacteriochlorin-antibody conjugate **B2-Ab** had a fluorophore/protein ratio of 2.5. Flow cytometry experiments used a 355-nm laser for excitation with 685 nm longpass and 730/45 nm bandpass filters for emission. Fig. 4 shows a histogram of the flow cytometry signals for the **B2-Ab**-bound compensation beads and for unbound beads (negative control).

A wide variety of fluorophores are available, albeit with spectra chiefly in the visible region.⁴⁶ Currently, the only two commercially available dye families that contain members that can be excited at 355 nm and emit in the NIR are conductive organic polymer-based Brilliant Ultraviolet (BUV) reagents and semiconductor based Quantum dots (QDs).^{47–49} Although these two fluorophore families offer large effective Stokes shifts for multiplexing, their broad emission spectra result in considerable spectral overlap as can be seen in Fig. 5. Thus, either two BUV fluorophores (BUV 737 and BUV 805) or two QDs (Qdot 705 and Qdot 800) could be used; however, in both cases the significant overlap between the members would require sensitivity-robbing compensation. In contrast, three or more bacteriochlorins could be used in the same window with much less spectral overlap and consequently less compensation.

The flow cytometry data presented herein indicate that bacteriochlorins afford valuable attributes as labels for polychromatic experiments, especially those requiring a large number of fluorophores excited with a UV laser. **B2** has far narrower emission (FWHM <20 nm) than any commercially available label (FWHM > 60 nm; Fig. 5) in this range. Accordingly, within the NIR spectral range, it should be possible to discriminate more biomolecules labeled with a palette of bacteriochlorins with less spectral overlap (“spillover”)⁴⁵ than with other fluorophore families that are currently available. Fig. 5 suggests that the overall advantage of the narrow-emitting bacteriochlorins would be further enhanced upon reduction of the filter bandpass (e.g., from 45 nm to 30 nm). Doing so would (1) fit more discrete emission channels in a given wavelength span, thereby enhancing multiplexing; (2) collect most of the emission from the bacteriochlorin but only a fraction from current fluorophores with broader emission; and (3) effectively collapse any apparent greater (integrated) brightness of another fluorophore compared to that of a bacteriochlorin.

Finally, a key issue for flow cytometry and many other photochemical applications concerns photostability. The PEGylated bacteriochlorins were stable for routine handling and measurements. For photophysical measurements the solutions are purged with argon to remove O₂. Such solutions during the course of spectroscopic studies typically showed less than 3% diminution in the overall spectral amplitude, generally without appearance of prominent new features. (Somewhat greater diminution was found for **B1** in water, and **B2** in DMF.) Such behavior has been observed previously for certain bacteriochlorins depending on substituents and medium (relative polarities) and attributed to decreased solubility in the excited state.^{15,50} Tetrapyrroles in the presence of O₂ and light do produce reactive oxygen species used for therapeutic (cell-killing) applications;^{18,19,51} however, the formation of photoproducts derived from reactions with the macrocycle appears to be a less prominent avenue of photoinstability than photoaggregation under conditions of routine spectroscopy (or higher light), at least for the bacteriochlorins assayed.⁵⁰ On the basis of such observations, it is anticipated that the PEGylated tetrapyrroles will show good photostability during flow-cytometry applications.

Conclusions

Bacteriochlorins are superb chromophores for use in the NIR spectral region, yet require extensive tailoring to fulfill many photochemical applications. Strategies for the installation of PEG groups at the 3,5-positions of aryl groups attached to the bacteriochlorin macrocycle have been developed. The resulting PEGylated bacteriochlorins are water-soluble but neutral and nonionic. The PEG moieties were incorporated in the final step to facilitate purification. A single carboxylic acid group was incorporated for bioconjugation purposes. All bacteriochlorins showed excellent solubility in water, except for a bacteriochlorin-imide that gave slight aggregation at higher concentrations. One bacteriochlorin-antibody conjugate displayed a sharp signal upon ultraviolet excitation (355 nm) with NIR emission (centered at 730 nm). The synthetic bacteriochlorins are distinguished from many other NIR chromophores by the relatively sharp long-wavelength absorption band and companion fluorescence emission band. Such sharp bands are particularly attractive for use in polychromatic flow cytometry, light-harvesting, and energy-cascade processes.

Experimental section

General methods

¹H NMR (300 MHz) and ¹³C NMR (100 MHz) were collected at room temperature in CDCl₃ unless noted otherwise. Matrix-assisted laser-desorption mass spectrometry (MALDI-MS) was performed with the matrix 1,4-bis(5-phenyl-2-oxazol-2-yl)benzene.⁵² 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. Sonication was carried out with a benchtop open-bath sonicator. Compounds **1**,¹² **2**,⁴³ **3**,³⁴ **9**,¹⁵ **11**¹⁵ and **12**³⁸ were prepared following reported procedures.

Photophysical measurements

Photophysical measurements were carried out as described in the companion paper.³⁰

Synthesis

3,13-Bis[3,5-bis(11-methoxy-3,6,9-trioxaundecylamidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (I)—A mixture of **11** (5.00 mg, 7.47 μmol) and **PEG₄-NHS** (100. mg, 299 μmol) in DMF (100 μL) was stirred under argon for 20 h. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous NaHCO_3 . The combined organic extract was dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (19:1) was added to the residue, and the suspension was sonicated (3 min) and centrifuged. The supernatant was discarded, leaving a green solid. This procedure (solvent addition–sonication–centrifuge) was conducted three more times to afford a green solid (9.2 mg, 80%): ^1H NMR δ -1.94 (s, 1H), -1.69 (s, 1H), 1.95 (s, 6H), 1.97 (s, 6H), 2.52–2.61 (m, 16H), 3.24 (s, 6H), 3.25 (s, 6H), 3.33–3.87 (m, 51H), 4.35 (s, 2H), 4.39 (s, 2H), 4.67 (d, J = 6.0 Hz, 4H), 4.72 (d, J = 6.0 Hz, 4H), 7.13–7.15 (m, 4H), 7.38 (s, 1H), 7.45 (s, 1H), 7.94 (s, 2H), 7.97 (s, 2H), 8.58–8.63 (m, 3H), 8.74–8.75 (m, 2H); MALDI-MS obsd 1541.8276; ESI-MS obsd 1563.8470, calcd 1563.8460 $[(\text{M} + \text{Na})^+]$, $\text{M} = \text{C}_{81}\text{H}_{120}\text{N}_8\text{O}_{21}$; λ_{abs} (water) 359, 506, 728 nm.

3,13-Dimethoxycarbonyl-2,12-bis[3,5-bis(3,6,9,12,15,18,21,24-octaoxahexacosanyl-26-amidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (II)—A solution of **15** (4.6 mg, 4.2 μmol) in CH_2Cl_2 (0.80 mL) was stirred under argon for 2 min followed by addition of TFA (0.20 mL). After 1 h, the reaction mixture was dried under an argon flow. Tributylamine (50 μL) was added to the solid residue, and the resulting mixture was sonicated for 3 min. A mixture of THF/hexanes (1:1) was then added, and the resulting suspension was sonicated (5 min) and centrifuged. The supernatant was discarded to afford a dark red solid (3.3 mg), which was partially characterized as below: MALDI-MS obsd 784.05, calcd 785.41 $[(\text{M} + \text{H})^+]$, $\text{M} = \text{C}_{45}\text{H}_{52}\text{N}_8\text{O}_5$; λ_{abs} (CH_3OH) 368, 525, 745 nm. A mixture of the resulting bacteriochlorin (3.3 mg, 4.2 μmol), Cs_2CO_3 (23 mg, 85 μmol) and **PEG₈-NHS** (50 mg, 98 μmol) in DMF (85 μL) was stirred under argon for 2.5 h. The reaction mixture was diluted with saturated aqueous NaHCO_3 (1.0 mL) and stirred for 2 h. The reaction mixture was extracted with CH_2Cl_2 . The combined organic extract was dried (Na_2SO_4) and concentrated. A solution of hexanes/ CH_2Cl_2 (2:1) was added to the residue, and the resulting suspension was sonicated (3 min) and centrifuged. The supernatant was discarded to afford a dark red solid. This procedure (solvent addition–sonication–centrifuge) was conducted three more times to afford a dark red semi-solid (5.0 mg, 54%): ^1H NMR (the four amide protons were not observed) δ -1.57 (s, 1H), -1.30 (s, 1H), 1.82 (s, 6H), 1.86 (s, 6H), 2.65 (m, 10H), 3.34–3.65 (m, 122H), 3.77–3.82 (m, 8H), 4.04 (s, 3H), 4.14 (s, 3H), 4.23 (s, 3H), 4.34 (s, 2H), 4.39 (s, 2H), 4.70 (s, 4H), 4.72 (s, 4H), 7.59 (s, 2H), 7.72 (s, 2H), 7.86 (s, 2H), 8.44 (s, 1H), 8.56 (s, 1H), 9.51 (s, 1H); MALDI-MS obsd 2363.15, calcd 2362.2950 $[(\text{M} + \text{H})^+]$, $\text{M} = \text{C}_{117}\text{H}_{188}\text{N}_8\text{O}_{41}$; ESI-MS obsd 1203.6304, calcd 1203.6336 $[(\text{M} + 2\text{Na})^{2+}]$, $\text{M} = \text{C}_{117}\text{H}_{188}\text{N}_8\text{O}_{41}$; λ_{abs} (H_2O) 369, 525, 751 nm.

13-[(4-Carboxyphenyl)ethyl]-5-methoxy-8,8,18,18-tetramethyl-3-(26-oxo-2,5,8,11,14,17,20,23-octaoxa-27-azatriacontan-30-yl)-7-oxobacteriochlorin (B1)—A solution of **8** (6.0 mg, 8.2 μmol) in CH_2Cl_2 (1.1 mL) was stirred under argon for 2 min followed by addition of TFA (219 μL). After 1 h, the mixture was dried under an argon flow. The residue was dissolved in THF (4 mL) and methanol (2 mL). Aqueous NaOH (4 M, 2 mL) was added, and the mixture was stirred at room temperature for 16 h. Aqueous HCl solution (3 M, 8 mL) was added to stop the reaction. The mixture was extracted with CH_2Cl_2 . The organic phase was washed with brine and dried over Na_2SO_4 . Solvent was removed in vacuum. A mixture of the resulting crude, Cs_2CO_3 (53.4 mg, 0.164 mmol), and **PEG8-NHS** (167 mg, 0.328 mmol) in DMF (200 μL) was stirred under argon for 2.5 h. Saturated aqueous NaHCO_3 (1 mL) was added. After 2 h, the organic phase was extracted by CH_2Cl_2 , dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (10:1) was added to the residue, the suspension was sonicated and centrifuged. This procedure (solvent addition-sonication-centrifuge) was conducted three more times to afford a green solid (5.0 mg, 63%): ^1H NMR δ -1.21 (s, 1H), -1.16 (s, 1H), 1.87 (s, 6H), 1.90 (s, 6H), 2.40–2.48 (m, 2H), 2.52–2.60 (m, 2H), 3.34 (s, 3H), 3.41–3.45 (m, 4H), 3.45–3.50 (m, 8H), 3.50–3.54 (m, 6H), 3.55–3.59 (m, 8H), 3.60–3.64 (m, 8H), 3.76–3.80 (m, 2H), 3.90–4.00 (m, 3H), 4.28 (s, 2H), 4.45 (s, 3H), 4.68 (s, 1H), 7.38 (d, J = 8.0 Hz, 2H), 7.99 (d, J = 8.0 Hz, 2H), 8.27 (s, 1H), 8.40 (m, 2H), 8.45 (s, 1H), 8.47 (s, 1H); MALDI-MS obsd 1015.2222; ESI-MS obsd 1014.5407, calcd 1014.5434 [(M+H) $^+$, M = $\text{C}_{55}\text{H}_{75}\text{N}_5\text{O}_{13}$]; λ_{abs} (H_2O) 409, 676 nm.

15-[4-(3-Carboxyethyl)phenyl]-3,13-bis[3,5-bis(11-methoxy-3,6,9-trioxaundecylamidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (B2)—A mixture of **14** (10.0 mg, 12.2 μmol), Cs_2CO_3 (80 mg, 250 μmol) and **PEG4-NHS** (164 mg, 490 μmol) in DMF (150 μL) was stirred under argon for 2.5 h. The reaction mixture was diluted with saturated aqueous NaHCO_3 (2.5 mL) and stirred for 4 h. The reaction mixture was acidified with 2N HCl solution and extracted with CH_2Cl_2 . The combined organic extract was dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (19:1) was added to the residue, and the resulting suspension was sonicated (3 min) and centrifuged. The supernatant was discarded to afford a green solid. This procedure (solvent addition-sonication-centrifuge) was conducted three more times to afford a green solid (17.2 mg, 83%): ^1H NMR (the carboxylic acid proton was not observed) δ -1.62 (s, 1H), -1.23 (s, 1H), 1.83 (s, 6H), 1.97 (s, 6H), 2.59–2.68 (m, 16H), 2.76 (t, J = 7.8 Hz, 2H), 3.01 (t, J = 7.8 Hz, 2H), 3.25 (s, 6H), 3.26 (s, 6H), 3.34–3.89 (m, 57H), 4.35 (s, 2H), 4.44 (br, 4H), 4.68 (s, 4H), 6.98 (s, 1H), 7.10–7.14 (m, 4H), 7.39–7.46 (m, 3H), 7.97 (s, 2H), 8.54 (s, 1H), 8.61 (s, 3H); MALDI-MS obsd 1691.1589; ESI-MS obsd 845.4628, calcd 845.4619 [(M + 2H) $^{2+}$, M = $\text{C}_{90}\text{H}_{128}\text{N}_8\text{O}_{23}$]; λ_{abs} (water) 363, 513, 726 nm. A larger-scale synthesis afforded 40.0 mg of the title compound (77% yield, see the Supplementary Information).

3,13-Bis[3,5-bis(4,7,10,13-tetraoxaamido)phenyl]-5-methoxy-15-[(3-(4-succinimidooxycarboxyethyl)phenyl)]-8,8,18,18-tetramethylbacteriochlorin (B2-NHS)—A mixture of **B2** (8.0 mg, 4.6 μmol), EDC (5.9 mg, 31 μmol), and HOSu (7.0 mg, 61 μmol) in DMF (0.10 mL) was stirred overnight under argon at room temperature. The reaction mixture was diluted with CH_2Cl_2 and washed with acidic saturated aqueous

brine (0.05 N HCl). The combined organic extract was dried (Na_2SO_4) and concentrated. A mixed solvent of hexanes/ CH_2Cl_2 (19:1) was added to the residue, and the suspension was sonicated for 3 min on a benchtop sonication bath and centrifuged. The supernatant was discarded, leaving a green solid (6.6 mg, 60%): ^1H NMR δ -1.64 (s, 1H), -1.27 (s, 1H), 1.84 (s, 6H), 1.97 (s, 6H), 2.52 (t, J = 5.7 Hz, 2H), 2.61 (t, J = 5.7 Hz, 2H), 2.81–2.94 (m, 20H), 3.31–3.84 (m, 63H), 3.92 (s, 2H), 4.35 (s, 2H), 4.39 (d, J = 5.7 Hz, 4H), 4.68 (d, J = 5.7 Hz, 4H), 6.16–6.18 (m, 4H), 6.98 (s, 1H), 7.09–7.14 (m, 4H), 7.39–7.44 (m, 3H), 7.97 (s, 2H), 8.55 (d, J = 1.8 Hz, 1H), 8.62 (s, 3H); MALDI-MS obsd 1787.0947; ESI-MS obsd 1808.9091, calcd 1808.9148 $[(\text{M} + \text{Na})^+]$, $\text{M} = \text{C}_{94}\text{H}_{131}\text{N}_9\text{O}_{25}$; λ_{abs} (CH_2Cl_2) 366, 517, 729 nm; λ_{abs} (water) 363, 513, 726 nm.

15-[4-(3-Carboxyethyl)phenyl]-3,13-dimethoxycarbonyl-2,12-bis[3,5-bis(3,6,9,12,15,18,21,24-octaohexacosanyl-26-amidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (B3)—A solution of **19** (10. mg, 7.2 μmol) in CH_2Cl_2 (0.63 mL) was stirred under argon for 2 min followed by addition of TFA (0.16 mL). After 1 h, the reaction mixture was dried under an argon flow. Tributylamine (50 μL) was added to the solid residue, and the resulting mixture was sonicated for 3 min. A mixture of THF/hexanes (1:1) was then added, and the resulting suspension was sonicated (5 min) and centrifuged. The supernatant was discarded to afford a greenish solid (6.7 mg), which was partially characterized as below: MALDI-MS obsd 933.50, calcd 933.47 $[(\text{M} + \text{H})^+]$, $\text{M} = \text{C}_{54}\text{H}_{60}\text{N}_8\text{O}_7$; λ_{abs} (CH_3OH) 370, 521, 736 nm. The resulting crude solid was used for the next reaction. A mixture of this solid (6.7 mg, 7.2 μmol), Cs_2CO_3 (67 mg, 0.21 mmol) and **PEG8-NHS** (0.13 g, 0.27 mmol) in DMF (0.27 mL) was stirred under argon for 2.5 h. The reaction mixture was diluted with saturated aqueous NaHCO_3 (1 mL) for 2 h. The reaction mixture was extracted with CH_2Cl_2 . The combined organic extract was dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (5:1) was added to the residue, and the resulting suspension was sonicated (3 min) and centrifuged. The supernatant was discarded to afford a dark red solid. This procedure (solvent addition-sonication-centrifuge) was conducted three more times to afford a dark red semi-solid (12 mg, 66%): ^1H NMR (the carboxylic acid proton was not observed) δ -1.53 (s, 1H), -1.23 (s, 1H), 1.75 (s, 6H), 1.85 (s, 6H), 2.58 (q, J = 5.7 Hz, 8H), 2.86 (t, J = 6.9 Hz, 2H), 3.19 (t, J = 6.9 Hz, 2H), 3.32–3.65 (m, 123H), 3.78 (q, J = 5.7 Hz, 12H), 3.86 (s, 2H), 4.14 (s, 3H), 4.28 (s, 3H), 4.36 (s, 2H), 4.65 (d, J = 5.7 Hz, 4H), 4.70 (d, J = 5.7 Hz, 4H), 7.15–7.21 (m, 4H), 7.43–7.49 (m, 4H), 7.67 (s, 1H), 7.70 (s, 1H), 7.74 (s, 2H), 7.89 (s, 2H), 8.56 (s, 1H), 8.59 (s, 1H); MALDI-MS obsd 2508.94; ESI-MS obsd 1255.6828, calcd 1255.6771 $[(\text{M} + 2\text{H})^{2+}]$, $\text{M} = \text{C}_{126}\text{H}_{196}\text{N}_8\text{O}_{43}$; λ_{abs} (water) 374, 522, 742 nm.

Zn(II)-15-[4-(3-Carboxyethyl)phenyl]-3,13-dimethoxycarbonyl-2,12-bis[3,5-bis(3,6,9,12,15,18,21,24-octaohexacosanyl-26-amidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (ZnB3)—Following a standard procedure,³⁹ a mixture of **B3** (1.0 mg, 0.4 μmol) and NaH (5.6 mg, 0.24 mmol, 600 equiv) in DMF (0.1 mL) was stirred under argon for 1 min, followed by addition of $\text{Zn}(\text{OAc})_2$ (22 mg, 0.12 mmol, 300 equiv) at 80 °C for 16 h. The reaction mixture washed with water three times and extracted with CH_2Cl_2 . The organic extract was dried (Na_2SO_4) and concentrated to give a dark red semisolid: MALDI-MS obsd 2572.9736, calcd 2572.2609 $[(\text{M} + \text{H})^+]$, $\text{M} =$

$C_{126}H_{194}N_8O_{43}Zn$]; λ_{abs} (H_2O) 358, 384, 563, 754 nm. The title compound was not stable in solution (toluene, DMF, or H_2O) or as solid at $-20\text{ }^{\circ}C$, and was not characterized further.

2,12-Bis[3,5-bis(11-methoxy-3,6,9-trioxaundecylamidomethyl)phenyl]-15-(7-carboxy-1-heptyl)-3,13-dimethoxycarbonyl-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (B4)—A solution of **18** (8.0 mg, 6.1 μ mol) in CH_2Cl_2 (1.6 mL) was stirred under argon for 2 min followed by addition of TFA (0.40 mL). After 1 h, the reaction mixture was dried by a stream of argon to thoroughly remove the volatile components (CH_2Cl_2 and TFA). The residue was dried under high vacuum. Tributylamine (10 μ L) was added to the solid residue, and the resulting mixture was sonicated for 3 min. (The thorough removal of TFA is critical to avoid decomposition of the bacteriochlorin.) A mixture of THF/hexanes (1:2) was then added, and the resulting suspension was sonicated (5 min) and centrifuged. The supernatant was discarded to afford a greenish solid (5.5 mg), which was partially characterized as follows: MALDI-MS obsd 908.3242, calcd 909.4663 [(M + H)⁺, M = $C_{52}H_{60}N_8O_7$]; λ_{abs} (CH_3OH) 375, 543, 750 nm. A mixture of the resulting bacteriochlorin (5.5 mg, 6.1 μ mol), Cs_2CO_3 (34 mg, 0.11 mmol, 17 equiv) and **PEG₄-NHS** (72 mg, 0.22 mmol, 34 equiv) in DMF (2.0 mL) was stirred under argon for 2 h. The reaction mixture was diluted with 0.6 M aqueous $NaHCO_3$ (3.0 mL) for 2 h. The reaction mixture was extracted with CH_2Cl_2 . The combined organic extract was dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (10:1) was added to the residue, and the resulting suspension was sonicated (3 min) and centrifuged. The supernatant was discarded to afford a dark red solid. This procedure (solvent addition-sonication-centrifuge) was conducted three times to afford a red solid (6.0 mg, 55%): 1H NMR (the carboxylic acid proton was not observed) δ -1.38 (s, 1H), -1.16 (s, 1H), 1.82 (s, 12H), 2.00 (br, 4H), 2.51 (br, 2H), 2.59 (s, 12H), 2.83 (br, 2H), 3.23–3.65 (m, 64H), 3.77 (s, 8H), 4.05 (s, 3H), 4.11 (s, 3H), 4.23 (s, 3H), 4.29 (s, 2H), 4.40 (s, 2H), 4.68 (br, 4H), 7.45 (s, 2H), 7.84 (s, 4H), 8.49 (s, 1H), 8.52 (s, 1H); MALDI-MS obsd 1780.9208, calcd 1781.9280 [(M + H)⁺, M = $C_{92}H_{132}N_8O_{27}$]; ESI-MS obsd 913.4495, calcd 913.4493 [(M + 2Na)²⁺, M = $C_{92}H_{132}N_8O_{27}$]; λ_{abs} (H_2O) 378, 543, 754 nm. A larger-scale synthesis afforded 20.0 mg of the title compound (86% yield, see the Supplementary Information).

2,12-Bis[3,5-bis(3,6,9,12,15,18,21,24-octaohexacosanyl-26-amidomethyl)phenyl]-15²-N-(3-carboxypropyl)-3-methoxycarbonyl-5-methoxy-8,8,18,18-tetramethylbacteriochlorin-13,15-dicarboimide (B5)—A solution of **21** (4.00 mg, 2.99 μ mol) in CH_2Cl_2 (400 μ L) was stirred under argon for 2 min followed by addition of TFA (120 μ L). After 1 h, the reaction mixture was dried under an argon flow. Tributylamine (48.0 μ L) was added to the solid residue, and the resulting mixture was sonicated for 3 min. A mixture of THF/hexanes (1:1) was then added, and the resulting suspension was sonicated (5 min) and centrifuged. The supernatant was discarded to afford a dark red solid (3.00 mg, 3.40 μ mol), which was used for the next reaction. A mixture of this solid (3.00 mg, 3.40 μ mol), Cs_2CO_3 (22.8 mg, 70.0 μ mol) and **PEG₈-NHS** (69.8 mg, 137 μ mol) in DMF (80.0 μ L) was stirred under argon for 2.5 h. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ (1.00 mL) for 2 h. The reaction mixture was extracted with CH_2Cl_2 . The combined organic extract was dried (Na_2SO_4) and concentrated. A mixture of hexanes/ CH_2Cl_2 (2:1) was added to the residue, and the resulting

suspension was sonicated (3 min) and centrifuged. The supernatant was discarded, leaving a dark red solid. This procedure (solvent addition-sonication-centrifuge) was conducted three more times to afford a dark red semi-solid (5.0 mg, 68%): ^1H NMR (the carboxylic acid proton and one of pyrrolic protons was overlaid with the solvent peak) δ -0.52 (s, 1H), 1.81 (s, 12H), 2.31 (br, 2H), 2.60 (m, 10H), 3.35 – 3.62 (m, 132H), 3.75 – 3.81 (m, 10H), 4.12 (s, 3H), 4.24 (s, 3H), 4.49 (br, 4H), 4.68 (s, 2H), 4.70 (s, 2H), 7.51 (s, 2H), 7.81 (s, 2H), 7.91 (s, 2H), 8.41 (s, 1H), 8.56 (s, 1H); MALDI-MS obsd 2457.98, calcd 2459.3114 [(M + H)⁺, M = C₁₂₁H₁₉₁N₉O₄₃]; ESI-MS obsd 1252.1376, calcd 1252.1416 [(M + 2Na)²⁺, M = C₁₂₁H₁₉₁N₉O₄₃]; λ_{abs} (water) 376, 566, 818 nm, λ_{abs} (CH₂Cl₂) 376, 563, 810 nm.

3-Bromo-5-methoxy-8,8,18,18-tetramethyl-13-[(4-methoxycarbonylphenyl)ethynyl]-7-oxobacteriochlorin (5)—Following a reported procedure,³⁵ a mixture of **3** (33 mg, 58 μmol), methyl 4-ethynylbenzoate (**4**, 10 mg, 64 μmol), K₂CO₃ (80 mg, 0.58 mmol), and Pd(PPh₃)₄ (6.7 mg, 5.8 μmol) in DMF (5.8 mL) was deaerated by three freeze-pump-thaw cycles, and then was stirred at 80 °C under argon. After 16 h, the mixture was diluted with ethyl acetate, washed with brine and dried over Na₂SO₄. The residue was chromatographed [silica, hexanes/CH₂Cl₂ (1:5)] to afford a brown solid (16 mg, 42%): ^1H NMR (400 MHz) δ -1.09 (s, 2H), 1.90 (s, 6H), 1.93 (s, 6H), 3.99 (s, 3H), 4.40 (s, 2H), 4.49 (s, 3H), 7.91 (d, J = 8.4 Hz, 2H), 8.17 (d, J = 8.4 Hz, 2H), 8.45 (s, 1H), 8.61 (s, 1H), 8.64 (d, J = 2.8 Hz, 1H), 8.82 (s, 1H), 8.92 (d, J = 1.6 Hz, 1H); MALDI-MS obsd 650.1274; ESI-MS obsd 651.1599, calcd 651.1601 [(M + H)⁺, M = C₃₅H₃₁BrN₄O₄]; λ_{abs} (toluene) 411, 512, 539, 649, 712 nm.

5-Methoxy-8,8,18,18-tetramethyl-13-[(4-methoxycarbonylphenyl)ethynyl]-3-[3-(*tert*-butoxycarbonylamino)propynyl]-7-oxobacteriochlorin (7)—Following a reported procedure,³⁵ a mixture of **5** (16 mg, 24 μmol), **6** (7.6 mg, 49 μmol), and Pd(PPh₃)₂Cl₂ (1.7 mg, 2.5 μmol) in DMF (5 mL) and Et₃N (2.5 mL) was deaerated by three freeze-pump-thaw cycles, and then was stirred at 80 °C under argon. After 3 h, the mixture was diluted with ethyl acetate, washed with brine, and dried over Na₂SO₄. The residue was chromatographed [silica, CH₂Cl₂/ethyl acetate (5:1)] to afford a yellow brown solid (10 mg, 57%): ^1H NMR δ -1.07 (s, 1H), -1.04 (s, 1H), 1.55 (s, 9H), 1.90 (s, 6H), 1.93 (s, 6H), 4.00 (s, 3H), 4.39 (s, 2H), 4.55 (s, 5H), 5.16 (br s, 1H), 7.91 (d, J = 8.1 Hz, 2H), 8.17 (d, J = 8.1 Hz, 2H), 8.45 (s, 1H), 8.59 (s, 1H), 8.61 (d, J = 2.1 Hz, 1H), 8.79 (s, 1H), 8.90 (d, J = 1.5 Hz, 1H); MALDI-MS obsd 724.6149; ESI-MS obsd 725.3202, calcd 725.3208 [(M + H)⁺, M = C₄₃H₄₃N₅O₆]; λ_{abs} (toluene) 416, 515, 543, 655, 720 nm.

5-Methoxy-8,8,18,18-tetramethyl-13-[(4-methoxycarbonylphenyl)ethyl]-3-[3-(*tert*-butoxycarbonylamino)propyl]-7-oxobacteriochlorin (8)—Following a reported procedure,³⁷ a mixture of **7** (10.0 mg, 0.0138 mmol) and Pd/C (4.4 mg, 0.0041 mmol, 10% Pd on carbon) under an inert atmosphere was treated with ethyl acetate (1 mL) and ethanol (1 mL). The mixture was stirred at room temperature under a H₂ balloon for 4 h. The mixture was filtered through a pad of Celite and rinsed with ethyl acetate and ethanol. The filtrate was concentrated and chromatographed [silica, CH₂Cl₂/ethyl acetate (9:1)] to obtain a green solid (8.6 mg, 85%): ^1H NMR (400 MHz) δ -1.18 (s, 1H), -1.16 (s, 1H), 1.49 (s, 9H), 1.87 (s, 6H), 1.90 (s, 6H), 2.38 – 2.43 (m, 2H), 3.45 – 3.49 (m, 2H), 3.59 (t, J =

7.6 Hz, 2H), 3.90 (s, 3H), 3.95 (t, $J = 7.6$ Hz, 2H), 4.06 (t, $J = 7.6$ Hz, 2H), 4.28 (s, 2H), 4.45 (s, 3H), 4.86 (br s, 1H), 7.41 (d, $J = 8.0$ Hz, 2H), 7.99 (d, $J = 8.0$ Hz, 2H), 8.33 (s, 1H), 8.37 (s, 1H), 8.44 (s, 1H), 8.46 (s, 1H), 8.47 (s, 1H); MALDI-MS obsd 732.4455; ESI-MS obsd 733.3825, calcd 733.3834 [(M + H)⁺, M = C₄₃H₅₁N₅O₆]; λ_{abs} (CH₂Cl₂) 398, 681 nm.

3,13-Bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (10)—Following a general procedure,¹¹ a mixture of **1** (270 mg, 484 μmol), **9** (492 mg, 1.06 μmol), Pd(PPh₃)₄ (336 mg, 290 μmol), Cs₂CO₃ (944 mg, 2.90 μmol) and toluene/DMF [48.4 mL (2:1), deaerated by bubbling with argon for 45 min] was added to a Schlenk flask and deaerated by three freeze-pump-thaw cycles. The remaining synthesis protocol, purification procedure and characterization data (ESI-MS data were not obtained) were essentially identical with those reported previously,¹⁵ whereupon the title bacteriochlorin was obtained in 90% yield (465 mg) versus 85% (73 mg) previously.

15-[4-(3-*tert*-Butoxycarbonyl)ethyl]phenyl]-3,13-bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (13)—Following a general procedure,¹¹ a solution of **10** (88.0 mg, 82.3 μmol) in THF (165 mL) was treated with NBS (17.6 mg, 98.8 μmol) in THF (988 μL) at room temperature for 1.5 h. The reaction mixture was diluted with CH₂Cl₂ and washed with saturated aqueous NaHCO₃. The organic layer was dried (Na₂SO₄), concentrated and chromatographed [silica, CH₂Cl₂/hexanes (4:1)] to afford a red solid, which was transferred to a Schlenk flask. The resulting bacteriochlorin, **12** (67.8 mg, 0.204 mmol), Pd(PPh₃)₄ (19.2 mg, 16.6 μmol), and Cs₂CO₃ (82.2 mg, 0.252 mmol) were placed in the Schlenk flask and dried under high vacuum for 30 min. Toluene/DMF [4.2 mL (2:1), deaerated by bubbling with argon for 30 min] was added to the Schlenk flask under argon, and the mixture was deaerated by three freeze-pump-thaw cycles. The reaction mixture was stirred at 90 °C for 19 h. The reaction mixture was allowed to cool to room temperature and then concentrated to dryness. The residue was dissolved in ethyl acetate and washed with saturated aqueous NaHCO₃. The organic layer was separated, dried (Na₂SO₄), concentrated and chromatographed [silica, hexanes/ethyl acetate (8:2 to 7:3)] to provide a greenish solid (33.0 mg, 31%): ¹H NMR (400 MHz) δ -1.61 (s, 1H), -1.24 (s, 1H), 1.48 (s, 18H), 1.50 (s, 18H), 1.52 (s, 9H), 1.83 (s, 6H), 1.97 (s, 6H), 2.59 (t, $J = 7.6$ Hz, 2H), 2.93 (t, $J = 7.6$ Hz, 2H), 3.66 (s, 3H), 3.89 (s, 2H), 4.27 (d, $J = 5.6$ Hz, 4H), 4.37 (s, 2H), 4.56 (d, $J = 5.6$ Hz, 4H), 4.90 (br, 2H), 5.07 (br, 2H), 6.98 (s, 1H), 7.04 (d, $J = 7.6$ Hz, 2H), 7.11 (s, 2H), 7.39–7.42 (m, 3H), 7.97 (s, 2H), 8.57 (d, $J = 2.4$ Hz, 1H), 8.62–8.64 (m, 3H); ¹³C NMR δ 28.4, 28.7, 30.0, 30.9, 31.3, 36.9, 44.7, 45.1, 45.2, 45.9, 47.8, 52.3, 63.5, 79.7, 80.8, 97.0, 97.5, 113.8, 123.1, 124.0, 125.2, 126.5, 127.2, 128.2, 129.2, 129.5, 133.6, 133.8, 134.0, 134.2, 136.2, 136.9, 137.7, 138.8, 138.9, 139.2, 139.3, 154.9, 156.1, 156.2, 160.9, 168.9, 172.7; MALDI-MS obsd 1274.9357; ESI-MS obsd 648.3581, calcd 648.3582 [(M + H + Na)²⁺, M = C₇₄H₉₆N₈O₁₁]; λ_{abs} (CH₂Cl₂) 367, 517, 729 nm. A larger-scale synthesis afforded 60.0 mg of the title compound (25% yield, see the Supplementary Information).

15-[4-(3-Carboxyethyl)phenyl]-3,13-bis[3,5-bis(aminomethyl)phenyl]-5-methoxy-8,8,18,18-tetramethylbacteriochlorin (14)—A solution of **13** (15.5 mg, 12.2 μmol) in CH₂Cl₂ (730 μL) was stirred under argon for 2 min, followed by addition of TFA

(470 μL). After 1 h, the reaction mixture was diluted with CHCl_3 and dried under an argon flow. Tributylamine (200 μL , 840 μmol) was added to the solid residue, and the mixture was sonicated for 3 min. A mixture of THF/hexanes (1:1) was then added, and the resulting suspension was sonicated (5 min) and centrifuged. The supernatant was discarded, leaving a green solid (9.90 mg, 99%): ^1H NMR (CD_3OD , the eight amine protons, two pyrrolic protons and one carboxylic acid proton were not observed) δ 1.82 (s, 6H), 1.98 (s, 6H), 2.57 (br, 2H), 2.89 (br, 2H), 3.69 (s, 3H), 3.88 (s, 2H), 4.06 (s, 4H), 4.28 (s, 4H), 4.37 (s, 2H), 7.12 (d, J = 8.1 Hz, 2H), 7.28 (s, 1H), 7.37 (s, 2H), 7.44 (d, J = 8.1 Hz, 2H), 7.64 (s, 1H), 8.20 (s, 2H), 8.67 (s, 1H), 8.74 (s, 2H), 8.77 (s, 1H); MALDI-MS obsd 816.0103; ESI-MS obsd 409.2308, calcd 409.2311 $[(\text{M} + 2\text{H})^{2+}]$, $\text{M} = \text{C}_{50}\text{H}_{56}\text{N}_8\text{O}_3$; λ_{abs} (CH_3OH) 363, 515, 726 nm. A larger-scale synthesis afforded 31.0 mg of the title compound (97% yield, see the Supplementary Information).

3,13-Dimethoxycarbonyl-2,12-bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl-5-methoxy-8,8,18,18-

tetramethylbacteriochlorin (15)—Samples of **2** (46.0 mg, 68.2 μmol), **9** (69.4 mg, 150 μmol), $\text{Pd}(\text{PPh}_3)_4$ (47.3 mg, 41.0 μmol), and Cs_2CO_3 (66.7 mg, 205 μmol) were placed in a Schlenk flask and dried under high vacuum for 30 min. Toluene/DMF [6.80 mL (2:1), deaerated by bubbling with argon for 30 min] was added to the Schlenk flask under argon and the resulting mixture was deaerated by three freeze-pump-thaw cycles. The reaction mixture was stirred at 90 $^\circ\text{C}$ for 22 h. The reaction mixture was allowed to cool to room temperature, concentrated to dryness, diluted with ethyl acetate 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 (8:2 to 7:3)]. A mixture of ethyl hexanes/acetate (19:1) was added to the product, and the resulting suspension was sonicated and centrifuged. The supernatant was discarded, leaving a red solid (65.0 mg, 80%): ^1H NMR (THF- d_8) δ -1.45 (s, 1H), -1.15 (s, 1H), 1.57 (s, 18H), 1.58 (s, 18H), 1.98 (s, 6H), 2.03 (s, 6H), 4.09 (s, 3H), 4.23 (s, 3H), 4.35 (s, 3H), 4.51 (s, 2H), 4.56 (s, 2H), 4.63 (br, 8H), 6.91 (br, 4H), 7.62 (s, 2H), 7.90 (s, 2H), 8.06 (s, 2H), 8.72 (s, 1H), 8.83 (s, 1H), 9.73 (s, 1H); MALDI-MS obsd 1184.7352; ESI-MS obsd 1185.6251, calcd 1185.6231 $[(\text{M} + \text{H})^+]$, $\text{M} = \text{C}_{65}\text{H}_{84}\text{N}_8\text{O}_{13}$; λ_{abs} (THF) 372, 526, 748 nm. A larger-scale synthesis afforded 326 mg of the title compound (62% yield, see the Supplementary Information).

15-Bromo-3,13-dimethoxycarbonyl-2,12-bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl-5-methoxy-8,8,18,18-

tetramethylbacteriochlorin (16)—Following a general procedure,^{11,43} a solution of **15** (28 mg, 24 μmol , 2.0 mM) in dry THF (12 mL) was treated dropwise (10 min) with a solution of NBS (4.2 mg, 24 μmol) in dry THF (0.24 mL) and stirred at room temperature under argon for 1 h. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous NaHCO_3 . The organic layer was separated, dried (Na_2SO_4) and concentrated. Column chromatography [silica, CH_2Cl_2 /ethyl acetate/ CH_3OH (100:2:1)] afforded a dark red solid (15 mg, 50%): ^1H NMR δ -1.61 (s, 1H), -1.36 (s, 1H), 1.48 (m, 36H), 1.83 (s, 6H), 1.85 (s, 6H), 4.11 (s, 3H), 4.15 (s, 3H), 4.26 (s, 3H), 4.35 (s, 2H), 4.20 (s, 2H), 4.58 (s, 8H), 5.05 (s, 4H), 7.47 (s, 2H), 7.84 (s, 2H), 7.88 (s, 2H), 8.53 (s, 2H); MALDI-MS obsd 1263.39; ESI-MS obsd 1263.5347, calcd 1263.5341 $[(\text{M} + \text{H})^+]$, $\text{M} =$

$C_{65}H_{83}BrN_8O_{13}$; λ_{abs} (CH_2Cl_2) 375, 530, 739 nm. A larger-scale synthesis afforded 250 mg of the title compound (72% yield, see the Supplementary Information).

15-(6-Carboxyhex-1-ynyl)-3,13-dimethoxycarbonyl-2,12-bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl]-5-methoxy-8,8,18,18-

tetramethylbacteriochlorin (18)—Following a reported procedure,³⁸ a mixture of **16** (50. mg, 40. μ mol), $Pd_2(dba)_3$ (15 mg, 18 μ mol, 0.45 equiv), and $P(o\text{-tol})_3$ (30. mg, 95 μ mol, 2.4 equiv) was dried under high vacuum in a Schlenk flask for 1 h. Toluene (2.6 mL) and TEA (1.3 mL) (both were bubbled with argon for 1 h) were added and the resulting mixture was degassed by three freeze-pump-thaw cycles. 6-Heptynoic acid (**17**, 50 μ L, 0.40 mmol, 10 equiv) was added. The reaction mixture was stirred at 70 °C for 16 h. The reaction mixture was allowed to cool to room temperature and washed with brine and 0.20 N HCl. The organic layer was separated, dried (Na_2SO_4), concentrated to dryness and chromatographed [silica, CH_2Cl_2 /ethyl acetate (2:1) to CH_2Cl_2/CH_3OH (50:1)] to afford a red solid, which was washed with hexanes to afford a red solid (8.0 mg, 15%): 1H NMR (the carboxylic acid proton was not observed) δ -1.29 (s, 1H), -1.06 (s, 1H), 1.47 (s, 36H), 1.82 (s, 6H), 1.83 (s, 6H), 1.95 (br, 4H), 2.53 (br, 2H), 2.84 (t, J = 7.5 Hz, 2H), 4.07 (br, 3H), 4.14 (s, 3H), 4.25 (s, 3H), 4.30 (s, 2H), 4.02 (s, 2H), 5.56 (s, 4H), 5.58 (s, 4H), 5.07 (br, 4H), 7.45 (s, 2H), 7.87 (s, 4H), 8.48 (s, 1H), 8.51 (s, 1H); MALDI-MS obsd 1308.6234; ESI-MS obsd 1309.6734, calcd 1309.6760 [(M + H)⁺, M = $C_{72}H_{92}N_8O_{15}$]; λ_{abs} (CH_2Cl_2) 381, 546, 755 nm. A larger-scale synthesis afforded 45.0 mg of the title compound (17% yield, see the Supplementary Information).

15-[4-(3-*tert*-Butoxycarbonyl)phenyl]-3,13-dimethoxycarbonyl-2,12-bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl]-5-methoxy-8,8,18,18-

tetramethylbacteriochlorin (19)—Samples of **16** (7.0 mg, 5.5 μ mol), $Pd(PPh_3)_4$ (3.8 mg, 3.3 μ mol), **12** (7.2 mg, 22 μ mol) and Cs_2CO_3 (8.0 mg, 23 μ mol) were placed in a Schlenk flask and dried under high vacuum for 1 h. Toluene/DMF [1.5 mL (2:1), deaerated by bubbling with argon for 1 h] was added to the Schlenk flask under argon, and the resulting mixture was deaerated by three freeze-pump-thaw cycles. The reaction mixture was stirred at 90 °C for 16 h. The reaction mixture was allowed to cool to room temperature, concentrated to dryness, diluted with ethyl acetate 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 (2:1)] to provide a greenish solid (4.3 mg, 56%): 1H NMR δ -1.50 (s, 1H), -1.19 (s, 1H), 1.46 (s, 18H), 1.47 (s, 18H), 1.52 (s, 9H), 1.74 (s, 6H), 1.84 (s, 6H), 2.75 (t, J = 7.7 Hz, 2H), 3.14 (t, J = 7.7 Hz, 2H), 3.33 (s, 3H), 3.84 (s, 2H), 4.16 (s, 3H), 4.28 (s, 3H), 4.36 (s, 2H), 4.52 (d, J = 6.0 Hz, 4H), 4.57 (d, J = 6.0 Hz, 4H), 4.98 (br, 4H), 7.40–7.45 (m, 4H), 7.69 (s, 1H), 7.72 (s, 1H), 7.74 (s, 2H), 7.90 (s, 2H), 8.52 (s, 1H), 8.58 (s, 1H); MALDI-MS obsd 1391.0250, ESI-MS obsd 1389.7320, calcd 1389.7386 [(M + H)⁺, M = $C_{78}H_{100}N_8O_{15}$]; λ_{abs} (CH_2Cl_2) 375, 525, 741 nm.

2,12-Bis[3,5-bis(*tert*-butoxycarbonamidomethyl)phenyl]-3-methoxycarbonyl-5-methoxy-15²-N-(3-*tert*-butoxycarbonyl)propyl-8,8,18,18-

tetramethylbacteriochlorin-13,15-dicarbodiimide (21)—Following a reported procedure,³ a mixture of **16** (15.0 mg, 11.9 μ mol), $Pd(PPh_3)_4$ (13.6 mg, 11.9 μ mol), and

Cs_2CO_3 (15.6 mg, 47.6 μmol) and **20** (9.20 mg, 47.6 μmol) was dried under high vacuum in a Schlenk flask for 1 h. The flask was then filled with CO, and toluene (1.20 mL) (bubbled with argon and CO, each for 30 min) was added. The reaction mixture was then stirred at 80 °C for 16 h under a CO atmosphere. The reaction mixture was cooled to room temperature and washed with saturated aqueous NaHCO_3 and water. The organic layer was dried (Na_2SO_4), concentrated to dryness and chromatographed [silica, CH_2Cl_2 /ethyl acetate/ CH_3OH (200:12:1)]. The resulting solid was washed with diethyl ether to afford a red solid (3.8 mg, 25%): ^1H NMR δ -0.55 (s, 1H), -0.14 (s, 1H), 1.43 (s, 9H), 1.48 (s, 36H), 1.81 (s, 12H), 2.21 (m, 2H), 2.49 (t, J = 7.5 Hz, 2H), 4.14 (s, 3H), 4.25 (s, 3H), 4.44 (t, J = 7.5 Hz, 2H), 4.57 (s, 2H), 4.59 (s, 6H), 4.60 (s, 2H), 4.70 (s, 2H), 5.05 (s, 2H), 5.11 (s, 2H), 7.48 (s, 1H), 7.51 (s, 1H), 7.84 (s, 2H), 7.87 (s, 2H), 8.41 (s, 1H), 8.52 (s, 1H); MALDI-MS obsd 1337.92; ESI-MS obsd 1338.6928, calcd 1338.7026 [(M + H) $^+$, M = $\text{C}_{73}\text{H}_{95}\text{N}_9\text{O}_{15}$]; λ_{abs} (CH_2Cl_2) 376, 562, 810 nm.

Flow cytometry measurements

Instrumentation and software—Samples were analyzed at the University of North Carolina Core Flow Cytometry Facility on a 19-parameter LSR-II SORP flow cytometer (BD Biosciences, San Jose, CA) equipped with seven lasers (355, 405, 488, 532, 561, 594, and 633 nm) using FACSDiva 8.0 acquisition software. Data were collected using the 100 mW 355 nm laser. Post-experimental analysis was performed with FlowJo software (version 10.0.8, FlowJo, LLC, Ashland, OR).

Materials—Simply CellularTM anti-mouse for violet laser compensation standard beads (Bangs Laboratories, Fishers, IN) were used for all flow cytometry experiments. Antibody used was Protein A-purified mouse IgG (MU-003-C, ImmunoReagents, Raleigh, NC).

Bioconjugation to form B2-Ab—Prior to bioconjugation, dialysis was used to exchange antibodies into 50 mM borate buffer (pH 8.5). Mouse IgG (0.5 μg) was added directly to 0.1 mg of the bacteriochlorin **B2-NHS** to achieve an 18-fold ratio of fluorophore to protein in a final reaction volume of 62 μL . This reaction solution was gently rotated in a microcentrifuge tube protected from light for 5 h at ambient temperature and then dialyzed against phosphate buffered saline (PBS) with a 20 kD MWCO dialysis membrane for 2 h at room temperature and then overnight at 4 °C to remove unreacted materials. The bacteriochlorin–antibody bioconjugate was further purified by affinity purification using Pierce Protein A Agarose beads (Thermo Scientific, Rockford, IL) and the manufacturer's protocol for immunoprecipitation. The resulting bacteriochlorin–antibody bioconjugate, **B2-Ab**, exhibited a fluorophore/protein ratio of 2.5 as determined by absorption spectroscopy using a molar absorption coefficient for the bacteriochlorin ($\epsilon_{726\text{ nm}} = 120,000\text{ M}^{-1}\text{cm}^{-1}$; $\epsilon_{280\text{ nm}} = 16,100\text{ M}^{-1}\text{cm}^{-1}$) and for the antibody⁵³ ($\epsilon_{280\text{ nm}} = 210,000\text{ M}^{-1}\text{cm}^{-1}$). The value for the bacteriochlorin was drawn from that for a similarly substituted bacteriochlorin analogue.¹⁰

Staining of compensation beads—All preparations used PBS with 0.5% bovine serum albumin (BSA) as the buffer for all steps. For blanks (negative controls), 1 drop of the anti-mouse compensation bead solution was added to buffer and adjusted to a final volume of

250 μ L. Blanks were treated by the following sequence of washes and centrifugation but without antibody addition, then resuspended in 1.0 mL buffer. For labeled antibodies, 1.0 mL volumes of bead solution were prepared from 4 drops of beads, then 50 μ L was aliquoted per sample. For the experiment shown in Fig. 4, 0.8 μ g of **B2–Ab** was added to each aliquot, and each sample was mixed gently for 30 min at ambient temperature in the dark. Solutions were washed twice by addition of buffer (1 mL) to each tube, centrifugation at 3000 μ g for 3 min, and removal of supernatant. Samples were resuspended in 1.0 mL of buffer for analysis.

Experiment and data analysis—The **B2–Ab** was excited with the 355 nm laser and read in channel A with a 685 nm longpass filter and a 730/45 nm bandpass filter in place (both provided with the instrument). Compensation beads were identified on the basis of forward and side light scatter. Gating was applied to exclude events with both lower and higher scatter than single beads and all further data were analyzed using this gating. For all experiments, 5000 gated events were collected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Synthesis and photophysical characterization were supported by the Photosynthetic Antenna Research Center (PARC), an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Award No. DE-SC0001035. Flow cytometry studies were supported by a grant to NIRvana Sciences (1R41 AI112302) from the National Institutes of Health Small Business Technology Transfer Program. Cytometry was performed at the UNC Flow Cytometry Core Facility, which is supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center. Mass spectra were obtained at the Mass Spectrometry Laboratory for Biotechnology at North Carolina State University. Partial funding for the facility was obtained from the North Carolina Biotechnology Center and the National Science Foundation. Mr. Nuonuo Zhang was supported by the China Scholarship Council (CSC, 201306250076) as a visiting Ph.D. student from Tianjin University for joint research at North Carolina State University.

References

1. Robert, B., Cogdell, R.J., van Grondelle, R. Advances in Photosynthesis and Respiration. In: Green, B.R., Parsons, W.W., editors. Light-Harvesting Antennas in Photosynthesis. Vol. 13. Kluwer Academic Publishers: Dordrecht, The Netherlands; 2003. p. 169-194.
2. Dawson JB, Barker DJ, Ellis DJ, Grassam E, Cotterill JA, Fisher GW, Feather JW. Phys. Med. Biol. 1980; 25:695–709. [PubMed: 7454759]
3. Vairaprakash P, Yang E, Sahin T, Taniguchi M, Krayner M, Diers JR, Wang A, Niedzwiedzki DM, Kirmaier C, Lindsey JS, Bocian DF, Holten D. J. Phys. Chem. B. 2015; 119:4382–4395. [PubMed: 25768657]
4. Minehan TG, Kishi Y. Angew. Chem. Int. Ed. 1999; 38:923–925.
5. Wang W, Kishi Y. Org. Lett. 1999; 1:1129–1132. [PubMed: 10825966]
6. Chen Y, Li G, Pandey RK. Curr. Org. Chem. 2004; 8:1105–1134.
7. Galewski M, Gryko DT. Curr. Org. Chem. 2007; 11:1310–1338.
8. Grin MA, Mironov AF, Shtil AA. Anti-Cancer Agents Med. Chem. 2008; 8:683–697.
9. Brückner, C., Samankumara, L., Ogikubo, J. Handbook of Porphyrin Science. Kadish, KM, Smith, KM., Guillard, R., editors. Vol. 17. Singapore: World Scientific; 2012. p. 1-112.
10. Kim H-J, Lindsey JS. J. Org. Chem. 2005; 70:5475–5486. [PubMed: 15989329]
11. Fan D, Taniguchi M, Lindsey JS. J. Org. Chem. 2007; 72:5350–5357. [PubMed: 17567079]

12. Kraye M, Ptasek M, Kim H-J, Meneely KR, Fan D, Secor K, Lindsey JS. *J. Org. Chem.* 2010; 75:1016–1039. [PubMed: 20088604]
13. Mass O, Lindsey JS. *J. Org. Chem.* 2011; 76:9478–9487. [PubMed: 22035424]
14. Scheer, H. *Advances in Photosynthesis and Respiration*. In: Grimm, B.Porra, R.J.Rudiger, W., Scheer, H., editors. *Chlorophylls and Bacteriochlorophylls. Biochemistry, Biophysics, Functions and Applications*. Vol. 25. Springer: Dordrecht, The Netherlands; 2006. p. 1-26.
15. Jiang J, Yang E, Reddy KR, Niedzwiedzki DM, Kirmaier C, Bocian DF, Holten D, Lindsey JS. *New J. Chem.* 2015; 39:5694–5714.
16. Harris MA, Jiang J, Niedzwiedzki DM, Jiao J, Taniguchi M, Kirmaier C, Loach PA, Bocian DF, Lindsey JS, Holten D, Parkes-Loach PS. *Photosynth. Res.* 2014; 121:35–48. [PubMed: 24604033]
17. Jiang J, Reddy KR, Pavan MP, Lubian E, Harris MA, Jiao J, Niedzwiedzki DM, Kirmaier C, Parkes-Loach PS, Loach PA, Bocian DF, Holten D, Lindsey JS. *Photosynth. Res.* 2014; 122:187–202. [PubMed: 24997120]
18. Huang Y-Y, Luo D, Hamblin MR. *Curr. Org. Chem.* 2015; 19:948–957.
19. Yin R, Hamblin MR. *Curr. Med. Chem.* 2015; 22:2159–2185. [PubMed: 25787964]
20. Kee HL, Nothdurft R, Muthiah C, Diers JR, Fan D, Ptasek M, Bocian DF, Lindsey JS, Culver JP, Holten D. *Photochem. Photobiol.* 2008; 84:1061–1072. [PubMed: 18673324]
21. Kee HL, Diers JR, Ptasek M, Muthiah C, Fan D, Lindsey JS, Bocian DF, Holten D. *Photochem. Photobiol.* 2009; 85:909–920. [PubMed: 19222800]
22. Ptasek M, Kee HL, Muthiah C, Nothdurft R, Akers W, Achilefu S, Culver JP, Holten D. *SPIE Proc.* 2010; 7576:75760E.
23. Alexander VM, Sano K, Yu Z, Nakajima T, Choyke P, Ptasek M, Kobayashi H. *Bioconjugate Chem.* 2012; 23:1671–1679.
24. Yu Z, Ptasek M. *J. Org. Chem.* 2013; 78:10678–10691. [PubMed: 24079536]
25. Harada T, Sano K, Sato K, Watanabe R, Yu Z, Hanaoka H, Nakajima T, Choyke PL, Ptasek M, Kobayashi H. *Bioconjugate Chem.* 2014; 25:362–369.
26. Borbas KE, Mroz P, Hamblin MR, Lindsey JS. *Bioconjugate Chem.* 2006; 17:638–653.
27. Chattopadhyay PK, Hogerkorp C-M, Roederer M. *Immunology.* 2008; 125:441–449. [PubMed: 19137647]
28. Chattopadhyay PK, Roederer M. *Methods.* 2012; 57:251–258. [PubMed: 22391486]
29. Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. *Trends Immunol.* 2012; 33:323–332. [PubMed: 22476049]
30. Liu M, Chen C-Y, Mandal AK, Chandrashaker V, Evans-Storms RB, Pitner JB, Bocian DF, Holten D, Lindsey JS. *New J. Chem.* 2016; 40 NJ-ART-04-2016-001154, submitted as preceding companion paper.
31. Jiang J, Taniguchi M, Lindsey JS. *J. New J. Chem.* 2015; 39:4534–4550.
32. Harris, JM. *Poly(ethylene glycol) Chemistry – Biotechnical and Biomedical Applications*. Harris, JM., editor. Plenum Press: New York; 1992. p. 1-14.
33. Zalipsky, S., Harris, JM. *Poly(ethylene glycol) Chemistry and Biological Applications*. Harris, JM., Zalipsky, S., editors. Washington, DC: American Chemical Society; 1997. p. 1-13.
34. Liu, M. Ph.D. Thesis. North Carolina State University; 2016. submitted
35. Yu Z, Ptasek M. *Org. Lett.* 2012; 14:3708–3711. [PubMed: 22783931]
36. Yu Z, Pancholi C, Bhagavathy GV, Kang HS, Nguyen JK, Ptasek M. *J. Org. Chem.* 2014; 79:7910–7925. [PubMed: 25061710]
37. Ruzic C, Kraye M, Balasubramanian T, Lindsey JS. *J. Org. Chem.* 2008; 73:5806–5820. [PubMed: 18576691]
38. Jiang J, Chen C-Y, Zhang N, Vairaprakash P, Lindsey JS. *New J. Chem.* 2015; 39:403–419.
39. Chen C-Y, Sun E, Fan D, Taniguchi M, McDowell BE, Yang E, Diers JR, Bocian DF, Holten D, Lindsey JS. *Inorg. Chem.* 2012; 51:9443–9464. [PubMed: 22916839]
40. Yuen JM, Harris MA, Liu M, Diers JR, Kirmaier C, Bocian DF, Lindsey JS, Holten D. *Photochem. Photobiol.* 2015; 91:331–342. [PubMed: 25488432]

41. Yang E, Kirmaier C, Krayner M, Taniguchi M, Kim H-J, Diers JR, Bocian DF, Lindsey JS, Holten D. J. Phys. Chem. B. 2011; 115:10801–10816. [PubMed: 21875047]
42. Birks, JB. Photophysics of Aromatic Molecules. London: Wiley-Interscience; 1970. p. 142-192.
43. Jiang J, Vairaprakash P, Reddy KR, Sahin T, Pavan MP, Lubian E, Lindsey JS. Org. Biol. Chem. 2014; 12:86–103.
44. Telford, WG. Recent Advances in Cytometry, Part A Instrumentation, Methods. In: Darzynkiewicz, Z., Holden, E., Orfao, A., Telford, WG., Wlodkowic, D., editors. Methods Cell Biol. 5th. Vol. 102. Atlanta, GA: Elsevier Inc; 2011. p. 375-409.
45. Nguyen R, Perfetto S, Mahnke YD, Chattopadhyay P, Roederer M. Cytometry Part A. 2013; 83A: 306–315.
46. Ptaszek, M. Progress in Molecular Biology and Translational Science. Morris, MC., editor. Vol. 113. Burlington: Academic Press; 2013. p. 59-108.
47. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Dose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Science. 2005; 307:538–544. [PubMed: 15681376]
48. Chattopadhyay PK, Gaylord B, Palmer A, Jiang N, Raven MA, Lewis G, Reuter MA, Rahman AKMN, Price DA, Betts MR, Roederer M. Cytometry Part A. 2012; 81A:456–466.
49. Telford WG. Cytometry Part A. 2015; 87A:1127–1137.
50. Yang E, Diers JR, Huang Y-Y, Hamblin MR, Lindsey JS, Bocian DF, Holten D. Photochem. Photobiol. 2013; 89:605–618. [PubMed: 23163632]
51. Huang Y-Y, Balasubramanian T, Yang E, Diers JR, Bocian DF, Lindsey JS, Holten D, Hamblin MR. ChemMedChem. 2012; 7:2155–2167. [PubMed: 23065820]
52. Srinivasan N, Haney CA, Lindsey JS, Zhang W, Chait BT. J. Porphyrins Phthalocyanines. 1999; 3:283–291.
53. Tech Tip #6. Thermo Fisher Scientific, Inc; 2008.

Fig. 1.
(A) Absorption spectra of PEGylated bacteriochlorins normalized at the Soret maximum.
(B). Normalized Q_y-region absorption (solid lines) and fluorescent (dotted lines) spectra of bacteriochlorins. **B1**, **B2**, **B3**, and **B4** are in water, whereas **B5** is in DMSO.

Fig. 2. Absorption (blue solid) and fluorescence (red dashed) spectra of bacteriochlorins in (A) DMF or (B) water with 5% DMF as a co-solvent. Emission spectra were collected using excitation at the Soret maximum and are normalized to the Q_y absorption intensity for ease of presentation.

Fig. 3.

Absorption versus concentration of **I**, **II**, and **B1-B5**, each over a range of 1000-fold. All spectra were recorded in water and normalized at the Q_y band. The concentration of was calculated on the basis of absorption in the 1-cm cuvette assuming $\epsilon(Q_y) = 120,000 \text{ M}^{-1}\text{cm}^{-1}$.¹⁰

Fig. 4. Histogram from a flow cytometry experiment using compensation beads stained with 0.8 μg of bacteriochlorin-antibody **B2-Ab** (solid line) plus unstained beads (dotted line). The coefficient of variation (CV) was 31 for **B2-Ab** and 406 for the unstained beads. The analysis used the 355-nm UV laser with 685 nm longpass and 730/45 nm bandpass filters.

Fig. 5. Fluorescence spectra of bacteriochlorin **B2** (red line), Qdot 705 (dashed line) and BUV-737 (dotted line) overlaid with a 730/45 nm bandpass filter (shadowed gray).

Scheme 1.
 Synthesis of a PEGylated oxobacteriochlorin.

Scheme 2.
Synthesis of PEGylated bacteriochlorins.

Scheme 3.
Synthesis of PEGylated bacteriochlorin-diester.

Scheme 4.
 Synthesis of a PEGylated bacteriochlorin-imide.

Bacteriochlorophyll *a*

synthetic bacteriochlorin

Chart 1.
Natural and synthetic bacteriochlorins

$$\vdots - \text{CO}_2\text{H}$$

Chart 3.
Target PEGylated bacteriochlorins and precursor building blocks.

Table 1

Spectral properties of bacteriochlorins^a

Compound	Solvent	B _{max} abs (nm)	Q _y abs (nm)	Q _y abs fwhm	I _{Q_y} /I _B	Q _y /B	Q _y em (nm)	Q _y em fwhm
8	DMF	397	679	23	0.41	0.23	686	26
B1	DMF	398	679	22	0.40	0.23	684	26
	Water	411	672	25	0.29	0.20	679	32
B2	DMF	366	726	21	0.75	0.48	731	25
	Water	362	726	21	0.69	0.40	731	25
I^b	DMF	363	729	22	0.86	0.53	735	24
	PBS ^c	358	727	22	0.77	0.49	733	23
B3	DMF	375	741	23	0.74	0.52	748	27
	Water	374	742	24	0.81	0.57	750	28
II	DMF	373	750	24	0.79	0.42	756	27
	Water	369	751	26	0.75	0.40	760	31
B4	DMF	377	754	30	0.63	0.55	760	31
	Water	368	753	32	0.62	0.52	759	33
B5	DMF	375	808	32	0.78	0.50	819	35
	Water	376	819	36	0.50	0.38	834	44

^a All data acquired at room temperature in DMF or water with 5% DMF as a cosolvent.^b From reference 15.^c PBS is standard aqueous phosphate buffered saline solution.

Table 2

Photophysical properties of bacteriochlorins.^a

Compound	Solvent	Q_y em (nm)	τ_S (ns)	Φ_f	Φ_{isc}	Φ_{ic}	k_f^{-1} (ns)	k_{isc}^{-1} (ns)	k_{ic}^{-1} (ns)
8	DMF	686	3.28	0.20	0.48	0.32	16	7	10
B1	DMF	684	3.03	0.19	0.50	0.31	16	6	10
	water	679	2.76	0.075	0.47	0.45	37	6	6
B2	DMF	731	3.74	0.16	0.43	0.41	23	9	9
	water	731	3.29	0.098	0.35	0.55	34	9	6
I^b	DMF	735	3.20	0.20	0.42	0.38	16	8	9
	PBS ^c	733	2.70	0.16	0.40	0.44	17	7	6
B3	DMF	748	3.27	0.21	0.47	0.32	15	7	10
	water	750	3.39	0.13	0.45	0.42	27	8	8
II	DMF	756	3.02	0.11	0.32	0.57	27	9	5
	water	760	2.96	0.078	0.31	0.61	38	10	5
B4	DMF	760	3.46	0.12	0.33	0.55	28	10	6
	water	759	3.21	0.035	0.31	0.65	92	10	5
B5	DMF	819	1.70	0.13	0.29	0.58	13	6	3
	water	834	1.40	0.017	0.08	0.90	84	18	2

^a All data were acquired at room temperature in DMF or in water with 5% DMF as cosolvent (unless indicated otherwise). The typical errors (percent of value) of the photophysical properties are as follows: τ_S ($\pm 7\%$), Φ_f ($\pm 5\%$), Φ_{isc} ($\pm 15\%$), Φ_{ic} ($\pm 20\%$), k_f ($\pm 10\%$), k_{isc} ($\pm 20\%$), k_{ic} ($\pm 25\%$). The error bars for τ_S , Φ_f , and Φ_{isc} were determined from select repeat measurements, and those for the Φ_{ic} , k_f , k_{isc} and k_{ic} were obtained from propagation of errors.

^b From reference 15.

^c PBS is standard aqueous phosphate buffered saline solution.