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Bioconjugatable, PEGylated hydroporphyrins for photochemistry and photomedicine. Narrow-band, red-emitting chlorins†

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Chromophores that absorb and emit in the red spectral region (600–700 nm), are water soluble, and bear a bioconjugatable tether are relatively rare yet would fulfill many applications in photochemistry and photomedicine. Here, three molecular designs have been developed wherein stable synthetic chlorins – analogues of chlorophylls – have been tailored with PEG groups for use in aqueous solution. The designs differ with regard to order of the installation (pre/post-formation of the chlorin macrocycle) and position of the PEG groups. Six PEGylated synthetic chlorins (three free bases, three zinc chelates) have been prepared, of which four are equipped with a bioconjugatable (carboxylic acid) tether. The most effective design for aqueous solubilization entails facial encumbrance where PEG groups project above and below the plane of the hydrophobic disk-like chlorin macrocycle. The chlorins possess strong absorption at ~400 nm (B band) and in the red region (Q_y band); regardless of wavelength of excitation, emission occurs in the red region. Excitation in the ~400 nm region thus provides an effective Stokes shift of >200 nm. The four bioconjugatable water-soluble chlorins exhibit Q_y absorption/emission in water at 613/614, 636/638, 698/700 and 706/710 nm. The spectral properties are essentially unchanged in DMF and water for the facially encumbered chlorins, which also exhibit narrow Q_y absorption and emission bands (full-width-at-half maximum of each <25 nm). The water-solubility was assessed by absorption spectroscopy over the concentration range ~0.4 μM–0.4 mM. One chlorin was conjugated to a mouse polyclonal IgG antibody for use in flow cytometry with compensation beads for proof-of-principle. The conjugate displayed a sharp signal when excited by a violet laser (405 nm) with emission in the 620–660 nm range. Taken together, the designs described herein augur well for development of a set of spectrally distinct chlorins with relatively sharp bands in the red region.

Introduction

Photochemistry in the red (600–700 nm) and near infrared (NIR, 700–1400 nm) spectral regions has been less investigated than in the shorter wavelength visible or ultraviolet regions, yet holds powerful attractions. For solar photoconversion, nearly half of the solar photons are at wavelengths >600 nm.[1] For optical imaging in medicine,[2] the deepest penetration in soft tissue occurs in the NIR spectral region wherein light exhibits significantly less scattering than at shorter wavelengths and minimal absorption by natural pigments and the vibrational overtones of water.[3] For flow cytometry and related analytical methods in clinical diagnostics, an objective is to squeeze as many spectrally distinct fluorophores into the ultraviolet-visible-NIR region as possible thereby enabling multiplex analyses (*i.e.*, polychromatic flow cytometry[4–6]). Nonetheless, far fewer chromophores are available for photochemical studies in the red and NIR spectral regions than at wavelengths of 200–600 nm, and most NIR absorbers that are available have quite broad spectral features.[7,8]

Nature's chosen chromophores for the red and NIR spectral regions are chlorophylls and bacteriochlorophylls, respectively.[9] The fundamental chromophore of a chlorophyll or bacteriochlorophyll is a chlorin or bacteriochlorin, respectively, wherein

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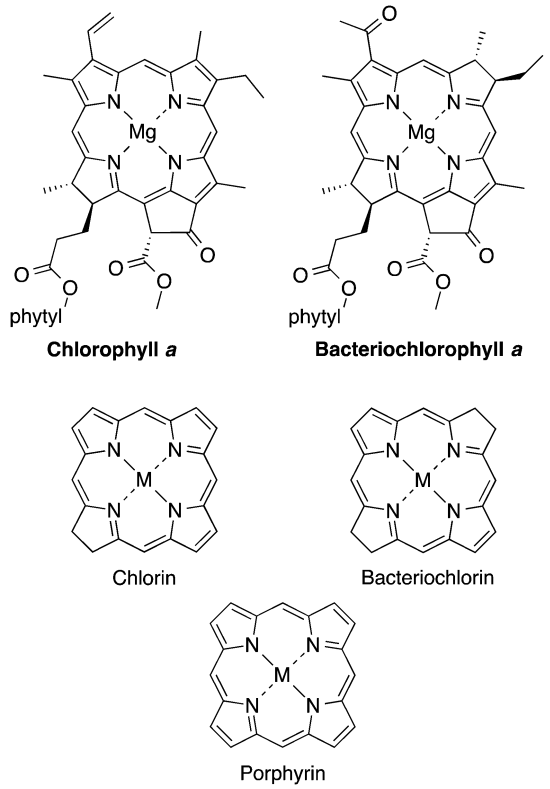


Chart 1 Natural pigments and core chromophores.

one or two β,β′-double bonds has been reduced relative to the π-system of a porphyrin (Chart 1). Synthetic methodology for preparing such tetrapyrrole macrocycles has advanced enormously over the years, with the ability now in hand to create reasonably diverse macrocycles and tailor the structure of the immediate environment.[10–21] Yet, suitable molecular designs for the aforementioned photochemical applications typically must satisfy multiple criteria. The criteria encompass wavelength tunability, features for bioconjugation, strong absorption, good fluorescence quantum yield (a proxy for a reasonably long-lived singlet excited state), and tailorable polarity for solubility in diverse media.[22] Meeting all such criteria simultaneously often poses design challenges and can stretch the limits of synthetic capabilities. Moreover, many of the applications of red- and NIR-functional chromophores require solubilization in water. In this regard, the development of general strategies for aqueous solubilization of tetrapyrrole macrocycles has remained a stubborn unsolved challenge.

A classic design for aqueous solubilization of porphyrins relies on appending polar groups at the perimeter of the macrocycle, exemplified by pyridyl and sulfonato porphyrins.[23,24] A more recent example entails similar appendages of oligoethyleneoxy (*i.e.*, PEG) groups, which have found extensive application and success across organic chemistry for aqueous solubilization.[25–27] The mere introduction of PEG groups is not necessarily a panacea, however, as illustrated by the *meso*-tetrakis[(*p*-PEG_{10})phenyl]-porphyrin, **T(*p*-PEG_{10})PP** (Chart 2), which exhibited significant aggregation in water at a concentration as low as 0.1 μM.[28]

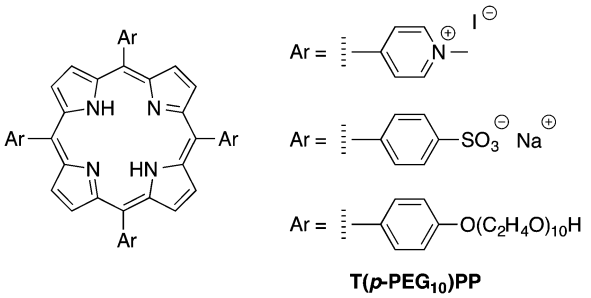


Chart 2 Synthetic porphyrins designed for aqueous solubility.

A variety of porphyrins bearing PEG groups similarly positioned in the plane of the macrocycle have been prepared.[29–32]

One strategy implemented to mitigate aggregation has been to place polar groups above and below the plane of the macrocycle, thereby shielding the intrinsically hydrophobic disk-like macrocycle by facial encumbrance. This approach has been employed with tetrapyrrole macrocycles as illustrated in Chart 3. The representative examples displayed include porphyrins bearing 3,5-disubstituted *meso*-aryl groups (**I**),[33] porphyrins bearing 2,6-disubstituted *meso*-aryl groups (**II**),[34–36] and the phthalocyanine **La Jolla Blue**.[37,38] In **La Jolla Blue** the apical bonding sites of the centrally coordinated silicon bear bulky polar groups, and the perimeter of the macrocycle is substituted with two carboxylic acid groups for bioconjugation. The chlorin **mTHPC**,[39] prepared by hydrogenation of the corresponding porphyrin, contains only one polar site on each *meso*-aryl group. The expected statistical distribution of conformers (due to rotation about the carbon–carbon single bond between the tetrapyrrole and each aryl unit) would afford positioning of groups on both (α,β) sides of the macrocycle for three conformers (αααβ,αβαβ,ααββ) but not the fourth conformer (αααα), likely limiting solubility (not shown).[40] Regardless, the chlorins **mTHPC** and PEGylated derivative **PEG-mTHPC**,[41] while widely used in photomedicine, lack a stabilizing structural motif in the pyrroline ring and are susceptible to aerobic dehydrogenation leading to the corresponding porphyrin.

Our own efforts to thwart aggregation in water have given rise to chlorins[42] that contain facially encumbering phosphonates (Chart 3, lower right), as well as a broader selection of ionic groups with porphyrins[43–46] and to some extent with bacteriochlorins.[47,48] Because the ionic groups impart solubility in water but not (typically) in non-aqueous solvents, the polar group typically must be installed or unveiled in the last synthetic step, whereupon purification often entails merely washing the product with solvents. Moreover, the preparation of an activated ester (for bioconjugation) in the presence of ionic groups such as phosphates, phosphonates, and carboxylates can result in deleterious cross-reaction.[47] The PEG group is an attractive alternative to these ionic species.[49] The PEG group is nonionic, is commercially available in various lengths[27] (monodisperse or polydisperse) with a single derivatizable handle,[25,27,50,51] and is soluble in water as well as a variety of organic solvents.[49] Accordingly, workup of PEGylated compounds can be achieved by partitioning crude reaction mixtures between an aqueous phase and dichloromethane.

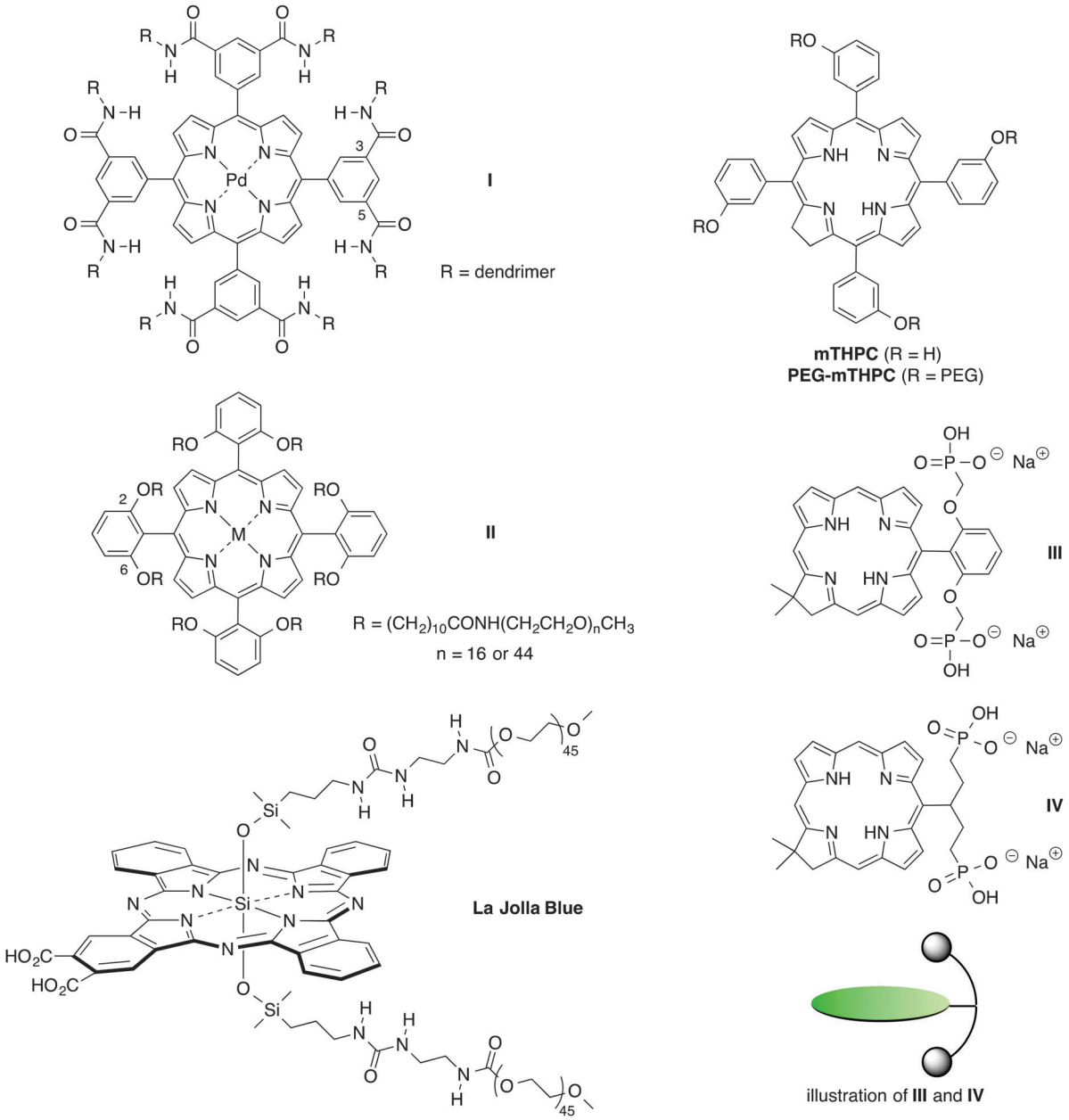


Chart 3 Tetrapyrroles with facially encumbering motifs.

In this paper we report the design and synthesis of a handful of chlorins bearing PEG groups and a bioconjugatable handle. A companion paper reports the development of corresponding bacteriochlorins.[52] Our specific motivations for preparing bioconjugatable, PEGylated (bacterio)chlorins stemmed from a desire to pursue two research areas. One area entails utilization of the PEGylated molecules as light-harvesting constituents in biohybrid antenna complexes *via* attachment at hydrophilic terminal regions of membrane-spanning photosynthetic peptides.[53] A second research area focuses on the utilization of the PEGylated (bacterio)chlorins as fluorophores in clinical diagnostics, particularly flow cytometry, where multiple antibodies are to be labeled. While diverse porphyrins have been used in bioconjugation studies,[24,54] few PEGylated synthetic chlorins have heretofore been reported,[41,55–58] and only one was equipped (but not tested) with a bioconjugatable tether.[58] The chlorins described herein have been characterized for solubility in dilute aqueous solution. The photophysical properties, including rate constants and yields for fluorescence emission, have been measured. One chlorin 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 red spectral region, particularly where sharp absorption and emission bands are advantageous.

Results and discussion

Molecular design and synthesis strategy

The *de novo* synthesis of chlorins developed in our laboratory provides the foundation for the creation of stable chlorin macrocycles that contain diverse substituents about the perimeter.[15] Each synthetic chlorin bears a geminal dimethyl group in the reduced, pyrroline ring to block adventitious dehydrogenation (Chart 4). Tuning the position of the long-wavelength (Q_y) absorption band can be accomplished chiefly by (1) introduction of auxochromes at positions along the *y*-axis (*i.e.*, the β-pyrrole positions 2, 3, 12 and 13 in rings A and C), and (2) metalation, and to lesser extent by (3) introduction of auxochromes at positions along the *x*-axis (*i.e.*, the β-pyrrole positions 7 and 8, and the β-pyrroline position 17) in rings B and D. Given the desire to exploit the β-pyrrole positions for wavelength tuning, a water-solubilization motif and a bioconjugatable tether are ideally installed at positions 5, 10, and/or 15. Chlorin nomenclature is described in detail in ref. 15.

Exploration of a number of designs has led to the synthesis of the target compounds shown in Chart 5. The synthesis relies on conversion of an aldehyde (compound **1** series) to a dipyrromethane (**2** series), a 1-acyldipyrromethane (**3** series), and a 1-acyl-9-bromodipyrromethane (**3-Br** series) to give the Eastern half, which is reacted with a tetrahydrodipyrrin Western half (**4** or **5**) to form the chlorin. The terminology for Eastern and Western halves dates to the work of Battersby some 40 years ago.[15] In the first design, the PEG groups are installed *via* click chemistry with a PEG-azide following construction of a trialkynyl-substituted chlorin macrocycle. The chlorin is prepared from a 2,4,6-tris-(propargyloxy)phenyl-substituted Eastern half and an unsubstituted Western half. The resulting zinc chlorin **ZnC1** and free base chlorin **FbC1** were examined for water solubility but lack a bioconjugatable tether. Click chemistry has found growing use in tetrapyrrole chemistry but only occasionally applied with chlorins.[59] In the second design, the PEG groups and a bioconjugatable tether were pre-installed in the Eastern half to give chlorins **ZnC2** and **FbC2**. The first two designs rely on a 2,6-disubstituted *meso*-aryl group to impart facial encumbrance and achieve water solubility. In the third design, a 3,13-diacetylchlorin bearing a bioconjugatable group was converted to the corresponding chalcone thereby affording chlorins **ZnC3** and **FbC3**. This third design lacks the facial encumbrance of the first two designs but, as in the first design, is attractive in the installation of the PEG groups in the final step of the synthesis. As part of this work, a number of other designs were investigated and abandoned owing to synthetic limitations (see the ESI†).

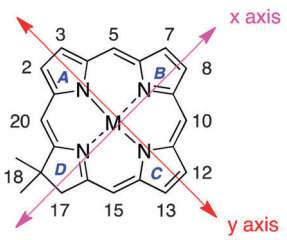


Chart 4 Chlorin nomenclature and spectroscopic axes.

Synthesis

Dipyrromethanes. The synthesis of an Eastern half begins with an aldehyde and pyrrole. Two aldehydes (**1a** and **1b**) required herein have been made from 2,4,6-trihydroxybenzaldehyde (phloroglucinol carboxaldehyde), whereas the other aldehyde **1c**[60] is a known compound. The reaction of 2,4,6-trihydroxybenzaldehyde and propargyl bromide in the presence of K_2CO_3 at 80 °C afforded 2,4,6-tris(propargyloxy)benzaldehyde **1a** in 87% yield without chromatographic purification (Scheme 1). The *O*-alkylation of 2,4,6-trihydroxybenzaldehyde with 1-bromo-3,6,9,12-tetraoxatridecane [$CH_3(OC_2H_4)_4Br$] in the presence of K_2CO_3 for 1.5 h[46] gave PEGylated benzaldehyde **1b** in a yield of 36%. Partially PEGylated product **1b′** was also isolated in 20% yield.

Dipyrromethanes were readily synthesized in a solventless process from the corresponding aldehyde in a solution of excess pyrrole containing $InCl_3$ or TFA as catalyst[60] (Scheme 2). Thus, reaction of aldehyde **1a** or **1b** with pyrrole in the presence of $InCl_3$ gave dipyrromethane **2a** or **2b** in yield of 52% or 74%, respectively. Alternatively, a streamlined route to dipyrromethane **2b** entailed the following: (1) the *O*-alkylation of 2,4,6-trihydroxybenzaldehyde was prolonged to 16 h; (2) the crude product **1b** (containing a trace amount of **1b′**) was directly used in condensation with pyrrole and ~1 equiv. of $InCl_3$; and (3) flash chromatography afforded dipyrromethane **2b** in a total yield of 54% (see Experimental section). Dipyrromethane **2c**[60] was synthesized as described in the literature.

Design I – chlorins without a bioconjugatable tether. Design I relies on a tris(propargyloxy)phenyl motif at the chlorin 10-position, whereupon click chemistry can be employed with the three alkynes and a PEG-azide. The synthesis of the Eastern half proceeded by Vilsmeier formylation[61] of **2a** with $POCl_3$/DMF, which afforded 1-formyldipyrromethane **3a** as the major product in 51% yield along with 1,9-diformylated byproduct **3a′** (not shown) in 20% yield (Scheme 3). Bromination of **3a** with 1 equiv. of NBS at −78 °C afforded **3a-Br**[9] in 68% yield. Because **3a-Br**[9] decomposed quickly at room temperature, the chlorin synthesis was initiated immediately. The chlorin-forming reaction[62] of **3a-Br**[9] and Western half **4**[63] entailed acid-catalyzed condensation (*p*-TsOH·H_2O in MeOH/CH_2Cl_2 under argon for 30 min) followed by cyclization with accompanying zinc chelation, oxidation, and HBr elimination [$Zn(OAc)_2$, 2,2,6,6-tetramethylpiperdine (TMPi), and AgOTf in CH_3CN at reflux exposed to air for 22 h]. In this manner, tris(propargyl) zinc chlorin **6** was obtained in 15% yield.

The click reaction of zinc chlorin **6** with PEG-azide **7** was examined under several conditions concerning the solvent and amount of copper (see Table S1, ESI†). Ultimately, treatment of **6** and **7** in DMSO with a stoichiometric amount of Cu(I) catalyst (freshly prepared from $CuSO_4·5H_2O$ and sodium ascorbate) in H_2O at 40 °C gave the zinc triazole-PEG-chlorin **ZnC1** in 58% yield. Demetalation with TFA gave the corresponding free base chlorin **FbC1** in 89% yield (Scheme 3). Performing the click reaction with the zinc rather than the free base chlorin was

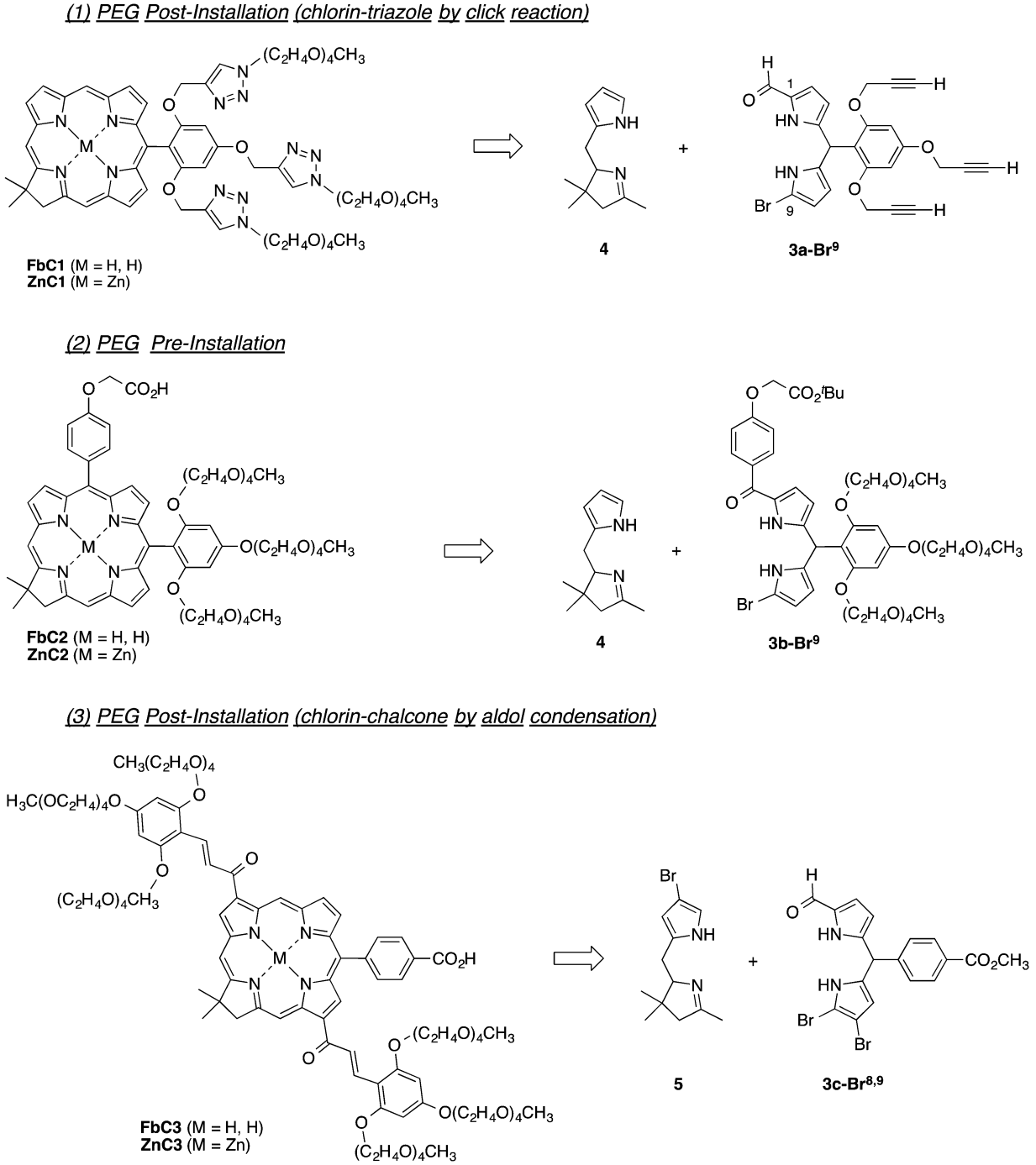
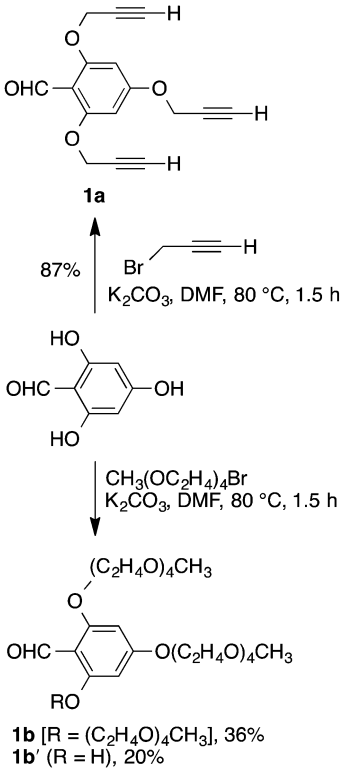


Chart 5 Molecular design and key precursors of synthetic chlorins.

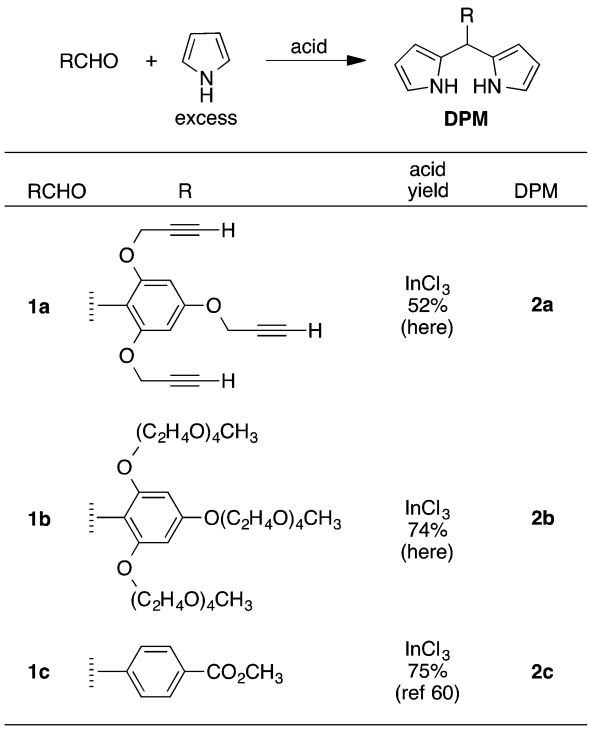
essential to avoid undesired copper insertion, given that (1) copper(II) chlorins are unsuitable for many photochemical applications due to the short excited-state lifetime,[64] and (2) demetalation of a copper chlorin requires strong acid and often proceeds in low yield.[65] Still, a zinc chlorin can potentially transmetalate with copper. Two tests proved the absence of any copper chlorin in the target chlorins: (1) upon demetalation of zinc chlorin **ZnC1** with TFA, the expected bathochromic shift of the Q_y band was observed for the neutralized sample; and (2) no copper chlorin peak was observed upon MALDI-MS analysis.

PEGylated chlorins **FbC1** and **ZnC1** were examined by UV-Vis spectroscopy in water and exhibited good water solubility (see the ESI,† Fig. S1). Successful installation of the PEG moiety by click reaction and resulting water solubility prompted examination of routes to analogous chlorins equipped with a bioconjugatable tether. Several synthetic problems were encountered, however, that limited the viability of this approach (see ESI†).

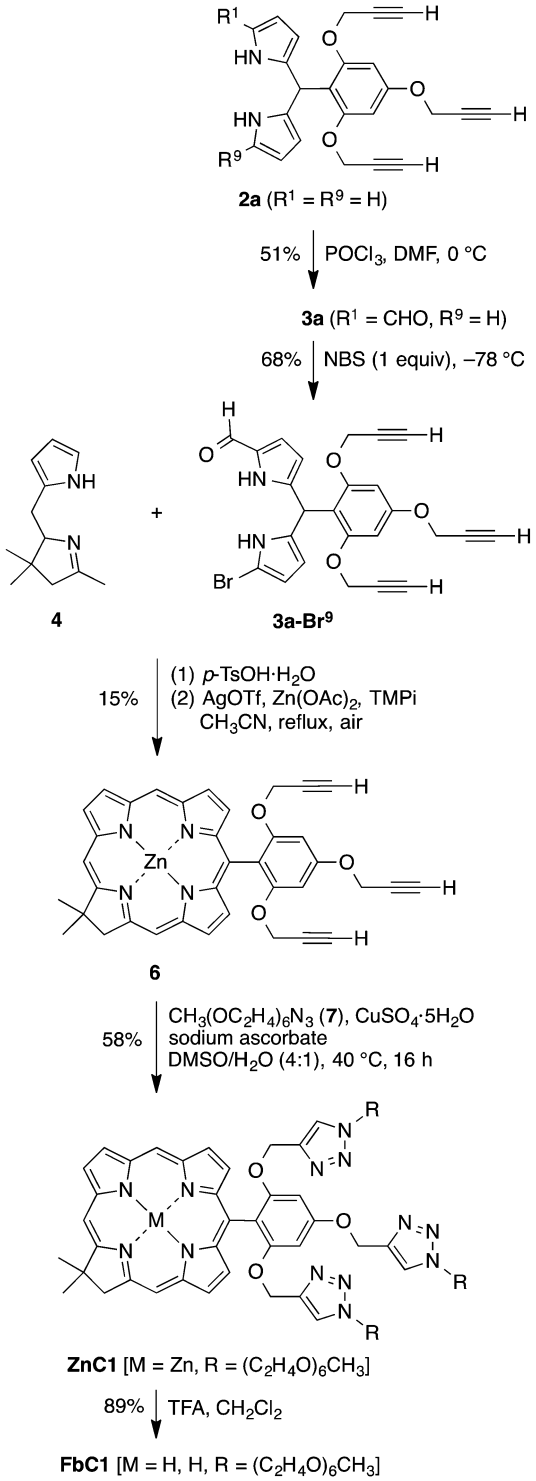
Design II – chlorins with a bioconjugatable tether at the 5-position. Incorporation of the bioconjugatable tether at the 5-position of chlorin can be achieved *via* acylation of the



Scheme 1 Synthesis of aldehydes.

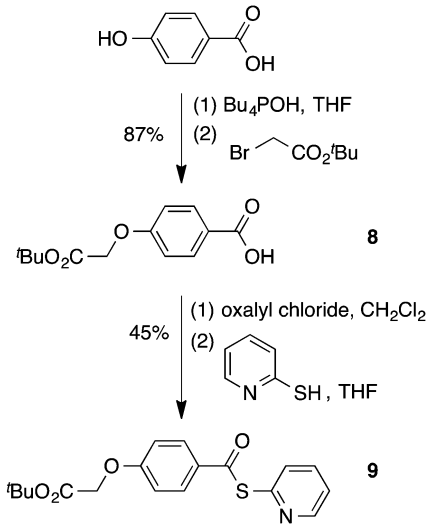


Scheme 2 Synthesis of dipyrromethanes from the corresponding aldehydes.



Scheme 3 Synthesis of PEGylated chlorins **FbC1** and **ZnC1** *via* click chemistry.

corresponding dipyrromethane.[62,66] The acylation reagent (an *S*-2-pyridyl benzothioate)[67] was prepared by selective alkylation of 4-hydroxybenzoic acid. Thus, 4-hydroxybenzoic acid was treated with *n*-Bu_4POH in THF at 0 °C under argon, followed by the addition of *tert*-butyl bromoacetate[68] to form the phenolic ether **8** in 87% yield (Scheme 4). Benzoic acid **8** was prepared previously *via* a 3-step procedure.[69] Reaction of **8** with oxalyl chloride under argon in anhydrous CH_2Cl_2 afforded the acid chloride, which was treated with 2-mercaptopyridine in anhydrous THF to give pyridyl

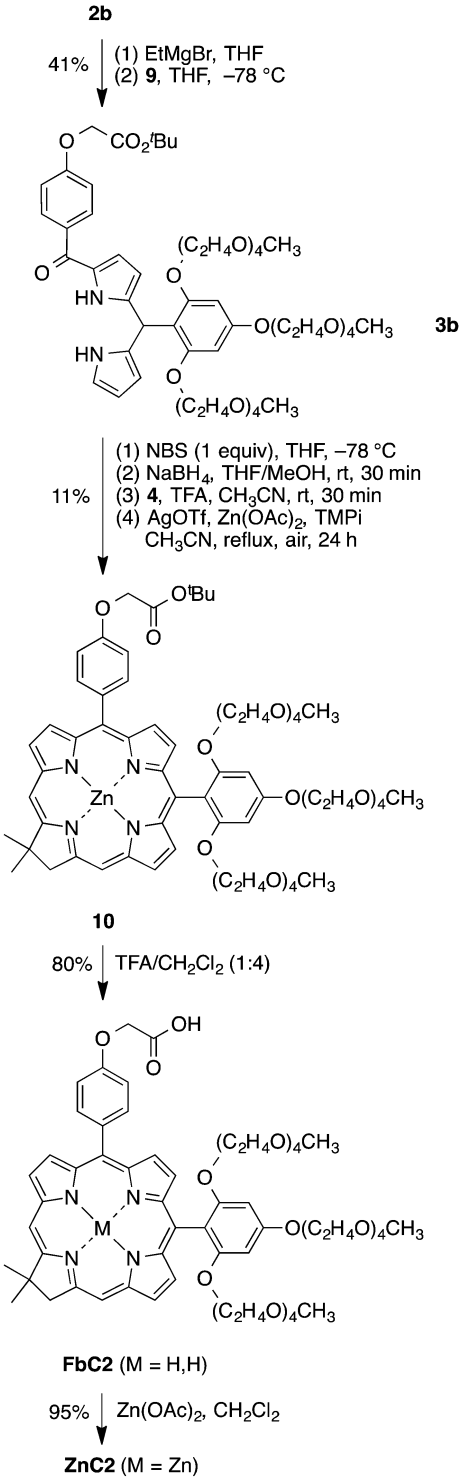


Scheme 4 Synthesis of Mukaiyama acylation reagent.

thioester **9** in 45% yield. Alternatively, streamlined procedures beginning with 4-hydroxybenzoic acid and use of recrystallization in lieu of chromatography afforded **9** in 32% yield.

Treatment of PEGylated dipyrromethane **2b** with **9** afforded acyldipyrromethane **3b** in 41% yield (Scheme 5). Bromination[70] of acyldipyrromethane **3b** with 1 equivalent of NBS at −78 °C followed by reduction with $NaBH_4$ in THF/MeOH at room temperature afforded the 9-bromodipyrromethane-1-carbinol. Condensation of the latter with Western half **4** in the presence of TFA followed by zinc-mediated oxidative cyclization gave zinc chlorin **10** in 11% yield. Treatment of **10** with 20% TFA in CH_2Cl_2 to hydrolyze the *tert*-butyl ester afforded PEGylated free base chlorin **FbC2**. Subsequently, **FbC2** was treated with $Zn(OAc)_2$ in CH_2Cl_2 to give zinc chlorin **ZnC2** in 95% yield. Chlorins **FbC2** and **ZnC2** constitute water-soluble, bioconjugatable, spectrally distinct chromophores with absorption and emission in the red spectral region. Examination of **10** by ^{1}H NMR spectroscopy validated the geometric design, showing the projection of the 2,6-substituted PEG groups in the vicinity of the π-electron cloud of the chlorin macrocycle, but the 4-substituted PEG group not in the ring current (see ESI,† Fig. S3). Attempts to extend this synthetic approach to a collection of chlorins encompassing a wider spectral region, which required incorporation of auxochromes in rings A and C, were not successful (see ESI†); hence, we turned to an alternative design.

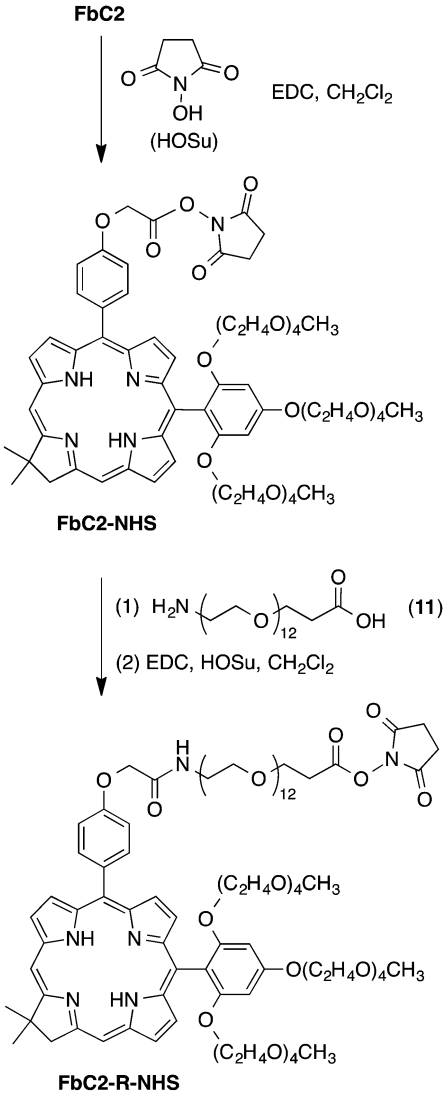
Chlorin **FbC2** was converted to the *N*-hydroxysuccinimide (NHS) active ester by reaction with *N*-hydroxysuccinimide (HOSu) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as shown in Scheme 6. The resulting active ester **FbC2-NHS** could be used for protein derivatization, but also was amidated with a longer PEG-azide (**11**) followed by NHS activation with EDC to give chlorin **FbC2-R-NHS**, which contains 24 PEG (*i.e.*, –OCH_2CH_2O–) units. The two chlorin NHS esters were prepared for use in flow cytometry studies (*vide infra*). As is customary in bioconjugate chemistry, the active esters were not purified to homogeneity given that the chlorin–protein conjugate can be readily freed of unbound



Scheme 5 Synthesis of a PEGylated chlorin.

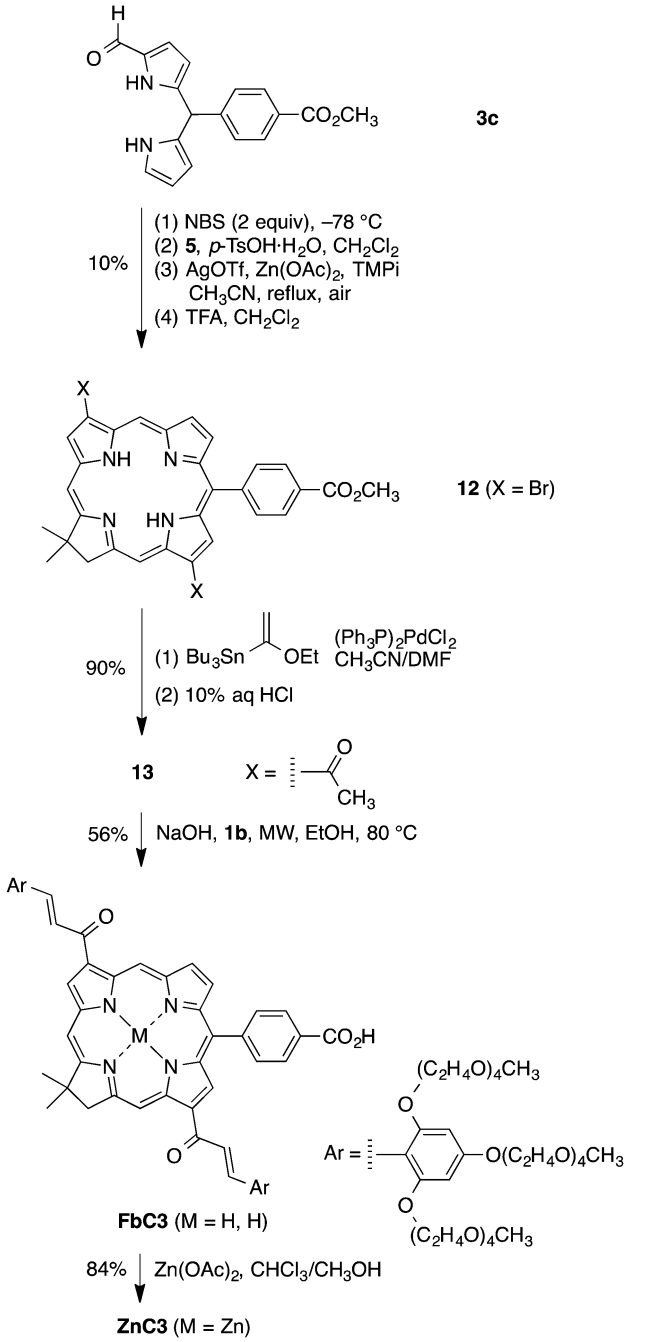
chlorin prior to use in biological applications. The chlorin NHS esters were characterized (absorption spectroscopy, ^{1}H NMR spectroscopy and MALDI mass spectrometry) and were estimated to be ~80% pure.

Design III – chlorins with a bioconjugatable tether at the 10-position. Chlorins with chalcones at the 3,13-positions are known to have a bathochromically shifted Q_y band, but this



Scheme 6 Synthesis of a PEGylated, bioconjugatable chlorin.

design had not previously been adapted for water solubility and bioconjugation. While the PEGylated porphyrin shown in Chart 2 had very poor water solubility, we sought to examine whether the presence of multiple PEG groups on each arene as well as the nonplanar configuration of the chlorin–chalcone architecture would impart aqueous solubility. Thus, vicinal dibromination of known 1-formyldipyrromethane **3c** gave Eastern half **3c-Br**[8,9] (Chart 5),[18] which was used directly without further purification (Scheme 7). Condensation of **3c-Br**[8,9] with bromo-Western half[71] **5** gave 3,13-dibromochlorin **12** in 10% yield from **3c**. Stille coupling[72] with tributyl(1-ethoxyvinyl)tin[73] and a catalytic amount of $(Ph_3P)_2PdCl_2$ in CH_3CN/DMF (3 : 2) followed by acidic hydrolysis gave 3,13-diacetylchlorin **13** in 90% yield. The aldol condensation[74] of **13** with excess aldehyde **1b** in ethanolic NaOH under microwave (MW) irradiation afforded chlorin–chalcone **FbC3**. Metalation of **FbC3** with $Zn(OAc)_2$ in $CHCl_3/CH_3OH$ gave **ZnC3** in 84% yield. This chlorin–chalcone strategy provides a concise and efficient route, particularly given the ease of purification afforded by installation of the PEG moieties in the last step of the process.



Scheme 7 Synthesis of PEGylated chlorins **ZnC3** and **FbC3**.

Handling of PEGylated compounds. The PEG groups are distinct from many other groups used to impart water solubility in two regards: (1) PEG groups are non-ionic, and (2) PEGylated compounds can be partitioned preferentially into organic solvents from an aqueous medium.[49] Thus, the PEGylated chlorins were quite soluble in CH_2Cl_2 or ethyl acetate, and could be extracted from aqueous solution to CH_2Cl_2 or ethyl acetate without significant loss. The purification of PEGylated compounds entailed gradient elution chromatography. For some of the PEGylated tetrapyrrole macrocycles, the improved purification involved a sequence of three chromatography procedures:[75] (1) removal of common impurities

by silica chromatography; (2) separation of PEG-containing impurities by gravity-flow size-exclusion chromatography (SEC); and (3) final silica chromatography to remove residual impurities (see the ESI†).

Characterization. The PEGylated, bioconjugatable chlorins and the corresponding precursors typically were characterized by absorption and fluorescence spectroscopy (not for precursors), ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy (where quantity and solubility allowed), MALDI-MS and ESI-MS. Several other target molecules were also designed with expectation to tune the wavelength and were the subject of exploratory syntheses (see the ESI†).

Photophysical characterization

Static spectral properties. Fig. 1A shows the absorption spectra of chlorins **ZnC2**, **FbC2**, **ZnC3**, and **FbC3** in DMF at room temperature normalized to the B band maximum (416–455 nm). Fig. 1B focuses on the NIR region with spectra normalized to the characteristic sharp Q_y band (610–687 nm). The corresponding fluorescence emission bands are in the range 613–692 nm.

The absorption and fluorescence spectra of the same four chlorins in DMF are shown in Fig. 2A along with those for **FbC1** and **ZnC1**. The spectra of all six chlorins in water are shown in Fig. 2B. The absorption spectra in Fig. 2 are normalized to the total (300–900 nm) absorption intensity obtained upon integration of the spectra plotted against wavenumbers (cm^{-1}). This approach is useful for comparing relative peak-intensity changes for related tetrapyrroles, and overcomes some of the drawbacks with other normalization methods or using (uncertain) extinction coefficients.[76] Each absorption spectrum contains three main features: the strong near-ultraviolet (NUV) Soret bands (B_x and B_y, 410–455 nm), a very weak green-orange Q_x band (520–560 nm) and a red or NIR Q_y band (609–706 nm). The Soret and Q_y band positions are listed in Table 1. Several general spectral characteristics are as follows: (1) the NUV B_y and B_x bands overlap completely with each other for the zinc chlorins but only partially overlap for the free base chlorins. (2) The weak Q_x bands of free base chlorins are further weakened in the case of zinc chlorins. (3) The Q_y bands for the two chlorin–chalcones (**ZnC3** and **FbC3**) are moderately strong whereas the Q_y bands for the other four chlorins (**ZnC1**, **FbC1**, **ZnC2** and **FbC2**) are relatively weak. (4) All the origin bands are typically accompanied by one weaker vibronic satellite band to higher energy by roughly 1000 cm^{-1}.

For chlorins, **ZnC1**, **FbC1**, **ZnC2** and **FbC2**, the Q_y absorption bands have a full-width-at-half-maximum (FWHM) in the range 12–18 nm (14 nm average) in DMF and 14–19 nm (17 nm average) in water (Table 1). The greater FWHM in water *versus* DMF is paralleled by a decrease in Q_y-band peak intensity (relative to the Soret maximum) in water *versus* DMF. The compensating effects of bandwidth with peak height indicate that the integrated intensity (oscillator strength) of the Q_y band generally does not change appreciably with solvent. For the two chlorin–chalcones (**ZnC3** and **FbC3**), the peak intensity of the Q_y band (relative to the Soret maximum) is much higher than that of the other four chlorins, and the FWHM again increases in water *versus* DMF.

The spectra in Fig. 2 and Q_y band data in Table 1 reveal that the addition of a bioconjugatable tether at the 5-position in chlorins **ZnC2** and **FbC2** gives a small (~2 nm) bathochromic shift in the Q_y bands with respect to **ZnC1** and **FbC1** in DMF. A small diminution of intensity of the Q_y band is also observed in the case of **FbC1** with respect to that of **FbC2** in water for incorporation of the bioconjugatable tether at the 5-position (Table 1). For chlorin–chalcone **FbC3**, the Q_y band shows a ~15 nm bathochromic shift with respect to the corresponding zinc chlorin **ZnC3** in DMF.

The fluorescence spectra of all PEGylated chlorins are dominated by the $Q_y(0,0)$ emission band, with a weaker (0,1) band to longer wavelength (Fig. 2A and B). For chlorins **ZnC1**, **FbC1**, **ZnC2** and **FbC2**, the fluorescence maximum lies within 3 nm of the $Q_y(0,0)$ absorption maximum in both DMF and water, indicating a very small Stokes shift. On the other hand, in DMF the Stokes shift for chlorin–chalcones **ZnC3** and **FbC3** are 10 nm and 5 nm, respectively. In water, the shift is smaller, 2 nm for **ZnC3** or 4 nm for **FbC3**. For chlorins **ZnC1**, **FbC1** and **FbC2**, the solvent effect causes a hypsochromic shift of 1–2 nm in the Q_y fluorescence maximum in water *versus* DMF. Contrarily, the Q_y fluorescence maximum shifts bathochromically for **ZnC2** (1 nm), **ZnC3** (18 nm) and **FbC3** (8 nm).

Excited-state properties. The measured photophysical properties of the chlorins are the lifetime (τ_S) of the lowest singlet excited state (S_1), the fluorescence quantum yield (Φ_f), *i.e.* the yield of S_1 to S_0, and the triplet yield (Φ_{isc}), *i.e.*, the yield of S_1 to T_1 intersystem crossing. The yield of S_1 to S_0 internal conversion is obtained by the

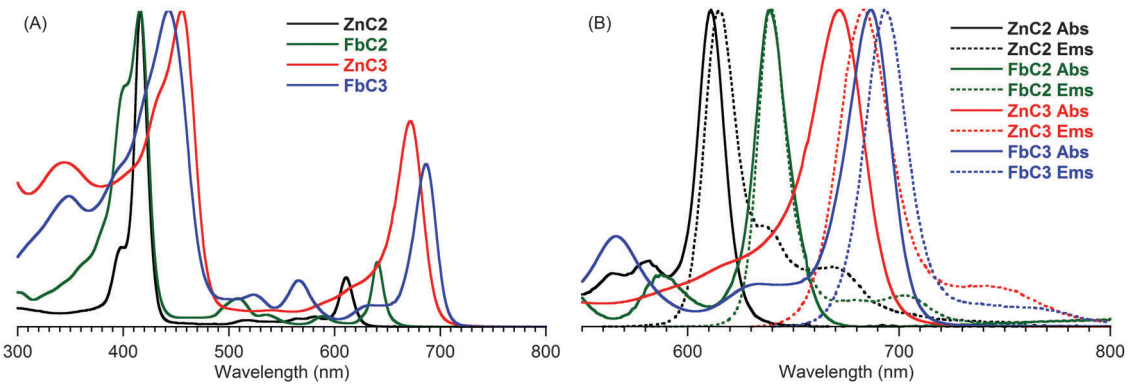


Fig. 1 (A) Absorption spectra of PEGylated chlorins in DMF; (B) normalized Q_y absorption and emission spectra of chlorins in DMF.

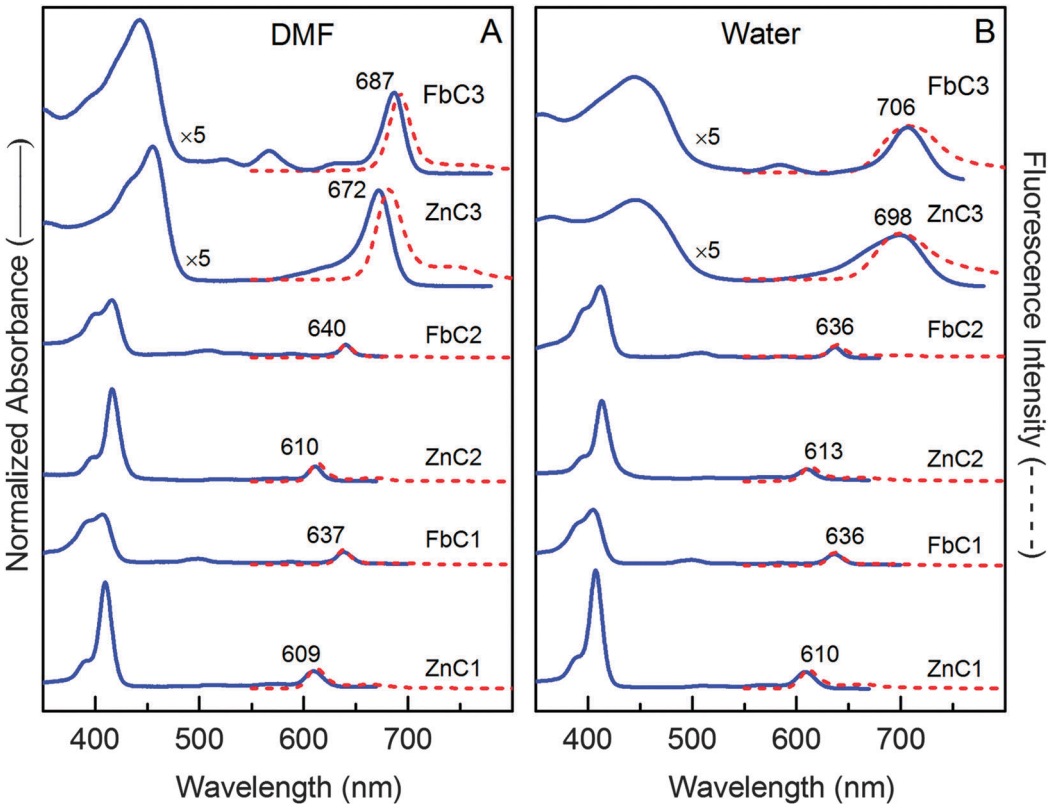


Fig. 2 Absorption (blue solid) and fluorescence (red dashed) spectra of PEGylated chlorins in (A) DMF and (B) water. The emission intensities are normalized to the Q_y absorption for ease of presentation.

Table 1 Spectral properties of PEGylated chlorins in DMF and in water

Chlorin	Solvent[a]	B_{max} abs (nm)	Q_y abs FWHM (nm)	Q_y abs (nm)	I_{Q_y}/I_{B_y}	$\sum Q_y/\sum B_y$	Q_y em (nm)	Q_y em FWHM (nm)
ZnC1	DMF	410	609	18	0.16	0.20	612	20
	Water	408	610	19	0.14	0.15	611	22
FbC1	DMF	406	637	15	0.25	0.12	639	16
	Water	404	636	17	0.20	0.12	637	18
ZnC2	DMF	416	610	14	0.15	0.16	613	17
	Water	414	613	16	0.15	0.15	614	24
FbC2	DMF	416	640	12	0.25	0.15	640	15
	Water	412	636	14	0.14	0.06	638	17
ZnC3	DMF	455	672	28	0.68	0.38	682	30
	Water	445	698	71	0.57	0.38	700	60
FbC3	DMF	443	687	25	0.52	0.19	692	26
	Water	445	706	42	0.52	0.22	710	58

[a] The samples in water contained 5% DMF as a cosolvent.

Table 2 Photophysical properties of PEGylated chlorins in DMF and water[a]

Chlorin	Solvent[b]	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)
ZnC1	DMF	612	2.0	0.066	0.92	0.01	30	2	140
	Water	611	1.8	0.030	0.88	0.09	61	2	20
FbC1	DMF	639	8.4	0.20	0.73	0.07	42	11	120
	Water	637	8.2	0.14	0.76	0.10	58	11	82
ZnC2	DMF	613	2.1	0.080	0.91	0.01	26	2	210
	Water	614	1.8	0.013	0.93	0.06	138	2	31
FbC2	DMF	640	10.2	0.25	0.63	0.12	41	16	85
	Water	638	9.7	0.17	0.68	0.15	57	14	65
ZnC3	DMF	682	5.1	0.34	0.48	0.18	15	11	28
	Water	700	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
FbC3	DMF	692	6.0	0.33	0.43	0.24	18	14	25
	Water	710	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]

[a] The typical errors (percent of value) of the photophysical properties are as follows: τ_S (±7%), Φ_f (±5%), Φ_{isc} (±15%), Φ_{ic} (±20%), k_f (±10%), k_{isc} (±20%), k_{ic} (±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] The samples in water contained 5% DMF as a cosolvent. [c] Not examined due to low solubility.

difference: $\Phi_{ic} = 1 - \Phi_f - \Phi_{isc}$. The fluorescence ($k_f$), internal conversion ($k_{ic}$), and intersystem crossing (k_{isc}) decay rate constants of the lowest singlet excited state are related *via* the expressions $\tau_S = (k_f + k_{ic} + k_{isc})^{-1}$ and $\Phi_x = k_x \cdot \tau_S$, where x = f, isc or ic. Thus, all three excited-state rate constants can be calculated from the lifetime and yields (Table 2).

The Φ_f values range from 0.013 to 0.080 and the τ_S values range from 1.8 ns to 2.1 ns for the zinc chlorins **ZnC1** and **ZnC2** (Table 2) in both DMF and water. For the corresponding free base analogues, the Φ_f values are higher (0.14–0.25) and the τ_S values are much longer (8.2–10.2 ns). For the chlorin–chalcones, **ZnC3** and **FbC3**, the Φ_f values are relatively higher (0.34 and 0.33, respectively) than for the other chlorins in DMF (Table 2).

Some important characteristic features gleaned from Table 2 are as follows: (1) the Φ_f values of the free base chlorins (**FbC1**, **FbC2**) are greater than the zinc chlorins (**ZnC1**, **ZnC2**), and are similar for the two chlorin–chalcones (**FbC3**, **ZnC3**). (2) The τ_s values of the zinc chlorins are much shorter than the free

base chlorins. These changes can be traced largely to greater k_{isc} values for the zinc chelates relative to the free base forms, as expected due to more facile intersystem crossing associated with the heavy-atom effect on spin–orbit coupling in the metallochlorins (Table 2). Metalation only increases k_{isc} modestly for chlorin–chalcone **ZnC3** relative to **FbC3**. The k_{ic}^{-1} values decrease (k_{ic} increases) for chlorins **ZnC1**, **FbC1**, **ZnC2** and **FbC2** in water *versus* DMF. As noted above, the low solubility of the two chlorin–chalcones (**ZnC3** and **FbC3**) precludes the photophysical studies in water. In each case, the calculated radiative rate constant (k_f) in water is reduced considerably from the values in DMF. Similarly, the intensity of the Q_y band relative to the Soret is either reduced or similar in water *versus* DMF (Table 1). The effects are in the same direction and in good agreement considering experimental error. The connection between these two observables is the direct proportionality of the Einstein coefficients for absorption and spontaneous emission. This connection explains the slight diminution in relative Q_y absorption intensity (Table 1) and the moderate decrease of Φ_f (Table 2) in water compared to DMF. Some small change in radiative probability for the compounds in the two media is expected due to the difference in refractive index values. However, other effects may also come into play, including the influence of the media on the relative energies of the frontier molecular orbitals of the complexes. The latter issue has not been explored in detail for tetrapyrroles.

Effect of concentration on spectral properties. Absorption *versus* concentration studies were conducted to assess the aqueous solution properties of the PEGylated chlorins over a 1000-fold range of concentration (~450 μM to ~0.45 μM). The methodology for this type of study has been previously reported in detail;[77] the same approach was utilized herein. The spectral properties of the PEGylated chlorins **ZnC2** and **FbC2** in neat deionized water are shown in Fig. 3; data for **ZnC1** and **FbC1** are shown in ESI† (Fig. S1). **ZnC2** exhibits almost unchanged spectral properties over a concentration range of 1000-fold, indicating exceptionally high solubility of this chlorin in water. On the other hand, **FbC2** exhibits changes in the shape of the B bands at higher concentrations (between 4.8 and 48 μM) indicating some degree of aggregation. The absorption spectra of chlorin–chalcones, **ZnC3** and **FbC3** exhibit clear broadening in neat water (see ESI,† Fig. S2) at ~5 μM, which indicates limited water solubility of these compounds.

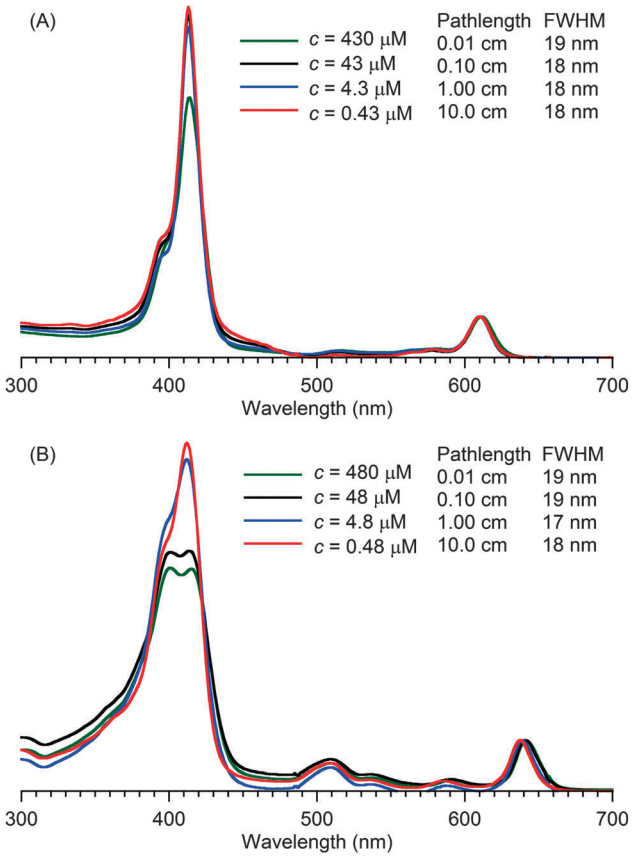


Fig. 3 Absorption *versus* concentration of **ZnC2** (top) and **FbC2** (bottom) over a 1000-fold range. The spectra are normalized at the Q_y band; the path length of the sample cell and FWHM of the Q_y band are also listed in the inset. The concentrations are based on the absorbance of the ~5 μM solution measured at the B band (assuming[64] ε = 160 000 M^{-1} cm^{-1}).

Flow cytometry

The development of fluorophores with spectrally distinct emission bands (*i.e.*, distinct "colors") as well as supporting technology that enables polychromatic flow cytometry is critically important for achieving increased accuracy and efficacy in clinical diagnostics.[4–6] In this regard, the 405 nm (violet) diode laser is becoming one of the most commonly used excitation sources in flow cytometers.[5] This excitation wavelength is ideal for chlorins because these molecules typically absorb strongly near 405 nm; the resulting emission in the red region affords an effective Stokes shift of >200 nm (Fig. 1). To demonstrate the use of chlorins in flow cytometry, chlorin-labeled antibodies were detected using antibody-capture compensation beads, a tool frequently used for setting up multicolor flow cytometry experiments to determine proper corrections due to spectral overlap between fluorophore-labeled reagents.[78]

The chlorin **FbC2-R-NHS** was used to label mouse IgG antibody for detection using mouse IgG antibody-specific compensation beads. The antibody was labeled at room temperature for 3 h with a 35-fold molar excess of **FbC2-R-NHS**. The conjugate was dialyzed against a 20 kD molecular weight cutoff membrane to remove byproducts, and the labeled antibody was further purified by affinity binding to Protein A agarose beads. The resulting chlorin–antibody conjugate **FbC2–Ab** had a fluorophore/protein ratio of 2.2 on the basis of absorption spectroscopy. This ratio is relatively low but it ensures minimal dye–dye quenching on the antibody. Flow cytometry experiments used a 405 nm laser for excitation with 600 nm longpass and 620/60 nm bandpass filters for emission. Fig. 4 shows a histogram of the flow cytometry signals for the **FbC2–Ab**-bound compensation beads, for unbound beads (negative population), and for beads bound to a monoclonal antibody labeled with Brilliant Violet 650 (**BV650–Ab**, the positive control). Brilliant Violet 650 is from a recently introduced dye family (based on conjugated polymers) that are among the brightest fluorophores available for flow cytometry.[79] The **FbC2–Ab** exhibits

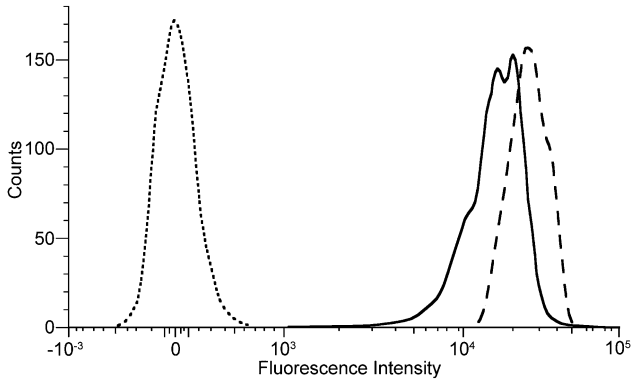


Fig. 4 Histogram from a flow cytometry experiment using compensation beads stained with either 0.5 μg of chlorin–antibody **FbC2–Ab** (solid line) or 0.12 μg of Brilliant Violet 650–antibody **BV650–Ab** (dashed line), plus unstained beads (dotted line). The coefficient of variation (CV) was 36 (**FbC2–Ab**), 30 (**BV650–Ab**), and 155 (unstained beads). The analysis used the 405 nm violet laser with 600 nm longpass and 620/60 nm bandpass filters.

roughly the same fluorescence intensity as a four-fold dilution of the **BV650–Ab** for the same relative protein concentrations. Fig. 5 shows the relative emission spectral overlap of both dyes with the bandpass filter. The **BV650–Ab** is a commercial product with an undisclosed fluorophore/protein ratio, so a more precise comparison of relative dye spectral properties was not possible.

The flow cytometry data suggest that chlorins should prove valuable as labels for polychromatic experiments, especially ones requiring a large number of fluorophores excited from a violet laser. Although not as bright as Brilliant Violet 650, **FbC2-R-NHS** has far narrower emission (FWHM <20 nm) than the commercial label (FWHM ~ 50 nm; Fig. 5). Thus, within the red spectral range, it should be possible to discriminate more chlorin-labeled biomolecules with less spectral overlap ("spillover")[78] than with other fluorophore families currently available for flow cytometry. Fig. 5 suggests that the overall advantage of the narrow-emitting chlorins would be further enhanced if the filter bandpass were reduced (*e.g.*, from 60 nm to 30 nm). This would (1) fit more discrete emission channels in a given wavelength span to enhance multiplexing; (2) collect most of the emission from the chlorin but only a fraction from current dyes with broader emission; and (3) effectively collapse any apparent greater (integrated) brightness of another dye compared to a chlorin.

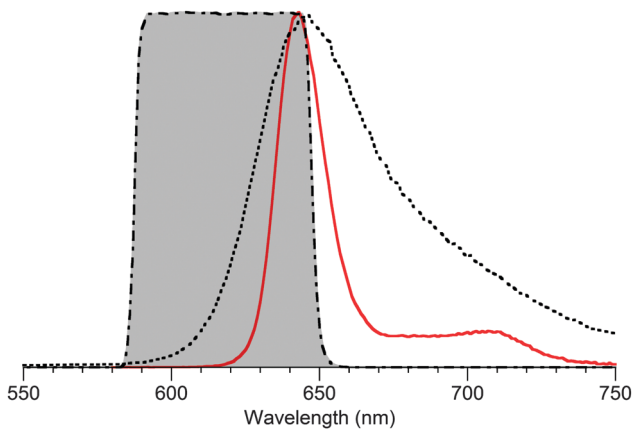


Fig. 5 Fluorescence spectra of chlorin **FbC2-R-NHS** (red line) and Brilliant Violet 650 (dotted line) overlaid with the Chroma bandpass filter ET620/60 (shadowed gray).

Finally, a key issue for flow cytometry and many other photochemical applications concerns photostability. The PEGylated chlorins were stable for routine handling and spectroscopic measurements. For photophysical measurements the solutions were purged with argon to remove O_2. Such solutions typically showed less than 3% change in the spectrum during the course of spectroscopic studies carried out here, as evidenced by the diminution in the overall spectral amplitude generally without appearance of prominent new features.

Conclusions

The installation of PEG groups in a facially encumbering arrangement enables aqueous solubilization of the hydrophobic, disk-like chlorin macrocycle. The resulting PEGylated chlorins are water-soluble but neutral and nonionic. A single PEG-substituted 2,4,6-trialkoxy arene (for **ZnC1**, **FbC1**, **ZnC2**, **FbC2**) suffices to impart aqueous solubility, whereas PEG groups at the terminus of chalcones (for **FbC3**, **ZnC3**) were only partially effective in this regard. Chlorins **ZnC1**, **FbC1** or **ZnC2**, **FbC2** are substituted with fewer PEG units (12 units or 18 units in total, respectively) than **T(*p*-PEG$_{10}$)PP** (40 units in total; Chart 2), yet the former designs impart significant solubility of the macrocycle in water. A distinct feature of the synthetic chlorins is the relatively sharp long-wavelength absorption and companion fluorescence emission band. Such sharp bands are very attractive for use in polychromatic flow cytometry, light-harvesting, and energy-cascade processes.

Experimental section

General methods

^{1}H NMR (400 MHz) spectra and ^{13}C NMR spectra (100 MHz) were collected at room temperature in $CDCl_3$ unless noted otherwise. Silica gel (40 μm average particle size) was used for adsorption column chromatography. Size-exclusion chromatography (SEC) was carried out at the preparative level using Bio Beads S-X3 (200–400 mesh) and elution with toluene (HPLC grade). All solvents were reagent grade and were used as received unless noted otherwise. THF was freshly distilled from sodium/benzophenone ketyl. Electrospray ionization mass spectrometry (ESI-MS) data are reported for the molecular ion or cationized molecular ion. Matrix-assisted laser-desorption mass spectrometry (MALDI-MS) was performed with the matrix 1,4-bis(5-phenyl-2-oxaxol-2-yl)benzene.[80] Sonication was carried out using a benchtop sonication bath. Compounds **2c**,[60] **3c**,[18] **4**,[63] and **5**[71] were prepared as described in the literature.

Synthesis

2,4,6-Tris(propargyloxy)benzaldehyde (1a). Following an alkylation procedure,[46] a solution of 2,4,6-trihydroxybenzaldehyde (5.05 g, 32.7 mmol) in anhydrous DMF (150 mL) was treated with

K_2CO_3 (27.6 g, 200 mmol), and the resulting suspension was heated to 60 °C. After 30 min, propargyl bromide (80% in toluene, 11.5 mL, 105 mmol) was added, and the temperature was increased to 80 °C. After stirring for 1.5 h, the reaction mixture was allowed to cool to room temperature, and then diluted with ethyl acetate (500 mL). The organic fraction was washed [water (3 × 200 mL) and brine (2 × 200 mL)], dried (Na_2SO_4), and concentrated to obtain a brown solid (7.65 g, 87%): mp 137–139 °C; ^{1}H NMR (300 MHz) δ 2.56 (t, J = 2.4 Hz, 2H), 2.60 (t, J = 2.4 Hz, 1H), 4.76 (d, J = 2.4 Hz, 2H), 4.78 (d, J = 2.4 Hz, 4H), 6.39 (s, 2H), 10.38 (s, 1H); ^{13}C NMR δ 32.9, 56.0, 56.6, 76.5, 77.4, 93.8, 95.0, 110.3, 161.5, 163.2, 187.2; ESI-MS obsd 269.08107, calcd 269.08084 [$(M + H)^+$, M = $C_{16}H_{12}O_4$].

2,4,6-Tris(3,6,9,12-tetraoxatridecyloxy)benzaldehyde (1b). Following an alkylation procedure,[46] a solution of 2,4,6-trihydroxybenzaldehyde (0.45 g, 2.9 mmol) in anhydrous DMF (5.0 mL) was treated with K_2CO_3 (1.5 g, 12 mmol), and the resulting suspension was heated to 60 °C. After 30 min, 1-bromo-3,6,9,12-tetraoxatridecane (3.0 g, 11 mmol) was added. The mixture was heated at 80 °C and stirred for 1.5 h. The reaction mixture was allowed to cool to room temperature, and then diluted with CH_2Cl_2. The mixture was washed with brine. The organic layer was dried (Na_2SO_4) and concentrated. Chromatography (silica, CH_2Cl_2 with 5% MeOH) afforded the title compound (0.77 g, 36%) and 2,4-bis(3,6,9,12-tetraoxatridecyloxy)-6-hydroxybenzaldehyde (**1b′**, 0.30 g, 20%), each as a pale yellow liquid. Data for **1b**: ^{1}H NMR δ 3.38 (s, 9H), 3.53–3.56 (m, 6H), 3.63–3.72 (m, 30H), 3.85–3.91 (m, 6H), 4.14–4.17 (m, 6H), 6.11 (s, 2H), 10.36 (s, 1H); ^{13}C NMR δ 59.0, 69.6, 68.7, 69.3, 70.6, 70.8, 71.0, 71.9, 92.2, 109.4 163.0, 165.0, 187.4; ESI-MS obsd 725.3942, calcd 725.3954 [$(M + H)^+$, M = $C_{34}H_{60}O_{16}$]. Data for **1b′**: ^{1}H NMR δ 3.38 (s, 6H), 3.54–3.56 (m, 4H), 3.63–3.73 (m, 20H), 3.84–3.89 (m, 4H), 4.13–4.16 (m, 4H), 5.97 (dd, J = 21.7 Hz, J = 2.01 Hz, 2H), 10.12 (s, 1H), 12.48 (s, 1H); ^{13}C NMR δ 59.0, 67.8, 68.1, 69.2, 70.6, 70.8, 70.9, 71.9, 91.84, 91.90, 93.5, 93.6, 106.1, 162.6, 166.2 167.1, 192.0; ESI-MS obsd 535.2725, calcd 535.2749 [$(M + H)^+$, M = $C_{25}H_{42}O_{12}$].

5-[2,4,6-Tris(propargyloxy)phenyl]dipyrromethane (2a). Following a reported procedure,[60] a solution of aldehyde **1a** (2.68 g, 10.0 mmol) in pyrrole (69.3 mL, 1.00 mol) was degassed with a stream of argon for 10 min. $InCl_3$ (221 mg, 1.00 mmol) was added, and the mixture was stirred for 1.5 h under argon flow. Powdered NaOH (1.2 g) was added, and the mixture was stirred for 45 min. The mixture was filtered. The filtrate was concentrated under high vacuum (to remove excess pyrrole) and then diluted with ethyl acetate (100 mL). The mixture was washed with water, dried (Na_2SO_4), concentrated and chromatographed [silica, hexanes/ethyl acetate (4 : 1)] to give a viscous oil (2.03 g, 52%): ^{1}H NMR (300 MHz) δ 2.50–2.57 (m, 3H), 4.52 (b, 4H), 4.66 (d, J = 2.7 Hz, 2H), 5.90–5.94 (m, 2H), 6.07–6.12 (m, 3H), 6.44 (s, 2H), 6.62–6.66 (m, 2H), 7.56–7.64 (b, 2H); ^{13}C NMR δ 14.0, 32.8, 56.2, 57.6, 76.1, 76.2, 78.4, 78.6, 96.2, 106.3, 108.0, 115.2, 116.5, 116.8, 132.8, 157.2, 157.8; ESI-MS obsd 383.13838, calcd 383.13902 [$(M–H)^+$, M = $C_{24}H_{20}N_2O_3$; this likely is the protonated dipyrrin, which is far more easily protonated than the dipyrromethane and can be detected in minute quantities].

5-[2,4,6-Tris(3,6,9,12-tetraoxatridecyloxy)phenyl]dipyrromethane (2b). Following a standard procedure,[60] a solution of pyrrole (7.4 mL, 0.11 mol) and **1b** (0.77 g, 1.1 mmol) was degassed with a stream of argon for 10 min. $InCl_3$ (2.4 mg, 11 μmol) was added, and the mixture was stirred under argon at room temperature for 1.5 h. The mixture turned yellow during the course of the reaction. Powdered NaOH (0.13 g, 3.3 mmol) was added to quench the reaction. After stirring for 45 min, the mixture was filtered by gravity flow. The filtrate was concentrated and chromatographed (silica, CH_2Cl_2 with 2% MeOH) to afford a pale yellow liquid (0.66 g, 74%): ^{1}H NMR δ 3.36 (s, 6H), 3.37 (s, 3H), 3.50–3.57 (m, 15H), 3.62–3.70 (m, 24H), 3.80–3.82 (m, 3H), 4.00–4.07 (m, 6H), 5.86 (s, 2H), 5.85–5.87 (m, 2H), 6.10 (s, 3H), 6.60–6.61 (m, 2H), 9.20 (s, 2H); ^{13}C NMR δ 32.6, 59.0, 67.5, 69.6, 70.4, 70.6, 70.75, 70.79, 71.9, 93.2, 105.5, 107.2, 113.5, 115.9; ESI-MS obsd 841.4682, calcd 841.4698 [$(M + H)^+$, M = $C_{42}H_{68}N_2O_{15}$].

Streamlined synthesis of 2b. A solution of 2,4,6-trihydroxybenzaldehyde (0.82 g, 5.3 mmol) in anhydrous DMF (10.6 mL) was treated with K_2CO_3 (2.4 g, 18 mmol), and the resulting suspension was heated to 60 °C. After 30 min, 1-bromo-3,6,9,12-tetraoxatridecane (5.0 g, 18 mmol) was added, and the temperature was raised to 80 °C. After 16 h, the reaction mixture was concentrated under vacuum (40 mmHg) at 60 °C. The resulting liquid was diluted with CH_2Cl_2, dried (Na_2SO_4) and filtered. The filtrate was concentrated in a 100 mL flask to afford a pale yellow liquid. Pyrrole (37 mL, 0.54 mol) was added, and the mixture was degassed with a stream of argon for 10 min. $InCl_3$ (0.60 g, 3.0 mmol) was added, and the mixture was stirred under argon at room temperature. After 1 h, ^{1}H NMR analysis showed the reaction was not complete. Additional $InCl_3$ (0.60 g, 3.0 mmol) was added, and the mixture was stirred for 2.5 h. NaOH (0.60 g, 0.020 mol) was added to quench the reaction. After stirring for 45 min, the mixture was filtered by gravity flow. The filtrate was concentrated and chromatographed (silica, CH_2Cl_2 with 2% MeOH) to afford a pale yellow liquid (2.4 g, 54%). The characterization data (^{1}H NMR) were consistent with those reported above.

1-Formyl-5-[2,4,6-tris(propargyloxy)phenyl]dipyrromethane (3a). Following a standard procedure,[61] the Vilsmeier reagent was prepared by treatment of dry DMF (2 mL) with $POCl_3$ (341 μL, 3.65 mmol) at 0 °C and stirring of the resulting mixture for 10 min under argon. In a separate flask, a solution of **2a** (1.30 g, 3.38 mmol) in DMF (15 mL) was treated with the freshly prepared Vilsmeier reagent at 0 °C. The resulting mixture was stirred at 0 °C for 1.5 h. The reaction mixture was treated with saturated aqueous NaOAc (∼15 mL) for 2 h. CH_2Cl_2 was added, then the organic phase was washed (water, brine), dried (Na_2SO_4), concentrated, and chromatographed [silica, hexanes/ethyl acetate (3 : 2)] to afford an orange oil (713 mg, 51%): ^{1}H NMR δ 2.53 (t, J = 2.4 Hz, 2H), 2.56 (t, J = 2.4 Hz, 1H), 4.56 (d, J = 2.4 Hz, 4H), 4.66 (d, J = 2.4 Hz, 2H), 5.92–5.94 (m, 1H), 6.06 (s, 1H), 6.11–6.12 (m, 2H), 6.40 (s, 2H), 6.68–6.70 (m, 1H), 6.83–6.84 (m, 1H), 8.90 (s, 1H), 9.26 (s, 1H), 9.33 (s, 1H); ^{13}C NMR δ 32.7, 32.8, 56.8, 76.1, 76.2, 76.4, 77.9, 78.07, 95.44, 95.49, 107.7, 107.9, 109.4, 112.7, 117.6, 122.2, 129.8, 131.5, 144.1, 156.7, 158.2, 177.9; ESI-MS obsd 413.14984, calcd 413.14958 [$(M + H)^+$, M = $C_{25}H_{20}N_2O_4$].

1-{4-[2-(*tert*-Butoxy)-2-oxoethoxy]benzoyl}-5-[2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl]dipyrromethane (3b). Following a standard procedure[62] with slight modification of reaction time and reagent equivalents, a solution of **2b** (840. mg, 1.00 mmol) in THF (2.0 mL) was treated with EtMgBr (2.8 mL, 0.9 M, 2.5 mmol) at room temperature for 30 min. The solution was cooled to −78 °C, whereupon a solution of **9** (202 mg, 1.20 mmol) in THF (2.4 mL) was added. After 1 h, the reaction mixture was quenched by addition of saturated aqueous NH_4Cl solution (10 mL). The mixture was extracted with CH_2Cl_2 (3 × 20 mL). The organic layer was dried (Na_2SO_4), concentrated, and chromatographed (silica, CH_2Cl_2 with 2–5% MeOH) to afford a pale yellow liquid (439 mg, 41%): ^{1}H NMR δ 1.50 (s, 9H), 3.36 (s, 6H), 3.37 (s, 3H), 3.51–3.55 (m, 6H), 3.60–3.73 (m, 33H), 3.82–3.84 (m, 3H), 3.97–4.16 (m, 6H), 4.58 (s, 2H), 5.90–5.92 (m, 1H), 6.05–6.07 (m, 1H), 6.08–6.10 (m, 1H), 6.11–6.15 (m, 3H), 6.71–6.74 (m, 2H), 6.91–6.96 (m, 2H), 7.81–7.85 (m, 2H), 9.41 (s, 1H), 9.90 (s, 1H); ^{13}C NMR δ 28.0, 32.9, 59.0, 65.5, 67.5, 69.53, 70.46, 70.71, 70.77, 71.83, 82.6, 93.1, 107.0–107.3 (m), 109.0, 111.3, 114.0, 117.8, 119.7, 129.2, 130.5, 130.7, 132.2, 143.5, 157.2, 159.3, 160.5, 167.5, 182.3; ESI-MS obsd 1075.5566, calcd 1075.5585 [$(M + H)^+$, M = $C_{55}H_{82}N_2O_{19}$].

Zn(II)-10-[2,4,6-tris(propargyloxy)phenyl]-18,18-dimethylchlorin (6). Following a standard procedure,[70] a solution of **3a** (540 mg, 1.31 mmol) in anhydrous THF (13 mL) was treated with NBS (233 mg, 1.31 mmol) under argon at −78 °C. The reaction mixture was stirred for 1 h at −78 °C, after which the cooling bath was removed. Upon reaching 0 °C, hexanes and water were added. The mixture was extracted with ethyl acetate. The organic layer was dried (Na_2SO_4), concentrated, and chromatographed [silica, hexanes/ethyl acetate (2 : 1)] to afford 9-bromo-1-formyl-5-[2,4,6-tris(propargyloxy)phenyl]dipyrromethane (**3a-Br**[9]) as an orange oil (440 mg, 68%): ^{1}H NMR δ 2.56 (t, J = 2.4 Hz, 2H), 2.57 (t, J = 2.4 Hz, 1H), 4.62 (d, J = 2.4 Hz, 4H), 4.70 (d, J = 2.4 Hz, 2H), 5.96–5.97 (m, 1H), 6.00 (s, 1H), 6.04–6.05 (m, 2H), 6.42 (s, 2H), 6.85–6.87 (m, 1H), 8.76 (s, 1H), 9.05 (s, 1H), 9.20 (s, 1H). Bromodipyrromethane **3a-Br**[9] in $CDCl_3$ solution darkened and decomposed, and hence was used directly in the chlorin-forming process.

Following a general procedure,[62] a solution of **4** (170 mg, 0.896 mmol) and **3a-Br**[9] (440 mg, 0.896 mmol) in CH_2Cl_2 (24 mL) was treated with a solution of *p*-TsOH·H_2O (852 mg, 4.48 mmol) in methanol (6 mL) under argon. The reaction mixture immediately turned red. The mixture was stirred for 30 min under argon, then treated with 2,2,6,6-tetramethylpiperidine (1.10 mL, 6.72 mmol) and concentrated to dryness. The resulting brown solid was suspended in acetonitrile (90 mL) followed by the successive addition of 2,2,6,6-tetramethylpiperidine (3.00 mL, 17.9 mmol), $Zn(OAc)_2$ (2.46 g, 13.4 mmol), and AgOTf (691 mg, 2.69 mmol). The resulting suspension was refluxed for 22 h exposed to air. The crude mixture was filtered through a silica pad with CH_2Cl_2. The filtrate was chromatographed [silica, hexanes/CH_2Cl_2 (1 : 1)] to afford a green solid (86 mg, 15%): ^{1}H NMR (THF-d_8) δ 2.06 (s, 6H), 2.65–2.67 (m, 3H), 4.38 (d, J = 2.8 Hz, 4H), 4.55 (s, 2H), 5.03 (d, J = 2.8 Hz, 2H), 6.93 (s, 2H), 8.46 (d, J = 4.4 Hz, 1H), 8.54 (d, J = 4.4 Hz, 1H), 8.60–8.66 (m, 3H), 8.70 (d, J = 4.4 Hz, 1H), 8.74 (d, J = 4.4 Hz, 1H), 9.04 (d, J = 4.4 Hz, 1H), 9.55 (s, 1H); ^{13}C NMR (THF-d_8) δ 29.6, 30.8, 45.1 50.9, 57.4, 75.9, 79.0, 93.6, 94.7, 96.0, 108.6, 114.2, 116.0, 126.4, 126.5, 127.7, 127.8, 131.8, 132.2, 145.7, 146.1, 147.2, 148.3, 153.0, 153.4, 158.1, 159.2, 159.6, 168.4; MALDI-MS obsd 641.4263, calcd 641.1526 [$(M + H)^+$, M = $C_{37}H_{28}N_4O_3Zn$]; ESI-MS obsd 640.14376, calcd 640.14474 (M^+, M = $C_{37}H_{28}N_4O_3Zn$); λ_{abs} (toluene) 407, 609 nm.

4-[2-(*tert*-Butoxy)-2-oxoethoxy]benzoic acid (8). Following a procedure for phenol alkylation,[68] a solution of 4-hydroxybenzoic acid (3.00 g, 21.7 mmol) in THF (43.4 mL) at 0 °C under argon was treated with 40% aqueous *n*-Bu_4POH (30.3 mL, 43.4 mmol), whereupon *tert*-butyl bromoacetate (3.20 mL, 21.7 mmol) was added. The resulting reaction mixture was allowed to warm to room temperature for 1 h. The organic solvent was removed under vacuum, and the aqueous residue was acidified with 2 N aqueous HCl. No product precipitated after acidification, so ethyl acetate and brine were added. The organic phase was washed (water), dried (Na_2SO_4), and concentrated to give a colorless oil. The resulting oil was filtered through a silica pad with ethyl acetate. The filtrate was chromatographed [silica, hexanes/ethyl acetate (3 : 1)] to afford a white solid (4.76 g, 87%): mp 114–116 °C; ^{1}H NMR δ 1.46 (s, 9H), 4.57 (s, 2H), 6.91 (d, J = 9.0 Hz, 2H), 8.04 (d, J = 9.0 Hz, 2H), 11.23 (br s, 1H); ^{13}C NMR δ 28.0, 65.5, 82.9, 114.3, 122.6, 132.4, 162.3, 167.5, 171.8; ESI-MS obsd 275.08850, calcd 275.08899 [$(M + Na)^+$, M = $C_{13}H_{16}O_5$].

***S*-2-Pyridyl 4-[2-(*tert*-butoxy)-2-oxoethoxy]benzothioate (9).** Following a reported procedure,[67] a sample of **8** (4.76 g, 18.8 mmol) was dissolved in anhydrous CH_2Cl_2 (188 mL) under argon and several drops of DMF were added. The mixture was cooled to 0 °C and treated dropwise with oxalyl chloride (2.40 mL, 28.3 mmol). The resulting reaction mixture was allowed to warm to room temperature for 40 min, and then the solvent was removed under vacuum to afford a colorless oil. The resulting oil was dissolved in THF (37.6 mL) and treated with 2-mercaptopyridine (2.10 g, 18.8 mmol) under argon. After stirring for 30 min at room temperature, the reaction mixture was treated with diethyl ether/aqueous $NaHCO_3$. The organic phase was washed (water), dried (Na_2SO_4), concentrated, and chromatographed [silica, hexanes/ethyl acetate (3 : 1)] to afford a white solid (2.8 g, 45%): mp 105–106 °C; ^{1}H NMR δ 1.49 (s, 9H), 4.60 (s, 2H), 6.96 (d, J = 9.0 Hz, 2H), 7.31–7.34 (m, 1H), 7.71–7.73 (m, 1H), 7.76–7.80 (m, 1H), 8.00 (d, J = 9.0 Hz, 2H), 8.66–8.68 (m, 1H); ^{13}C NMR δ 28.2, 65.7, 83.0, 114.7, 123.6, 129.9, 130.1, 131.1, 137.2, 150.56, 150.64, 162.5, 167.3; ESI-MS obsd 346.11081, calcd 346.11076 [$(M + H)^+$, M = $C_{18}H_{19}NO_4S$].

Streamlined synthesis of 9. A solution of 4-hydroxybenzoic acid (6.0 g, 43 mmol) in THF (86 mL) at 0 °C under argon was treated with 40% aqueous *n*-Bu_4POH (60. mL, 87 mmol) followed by *tert*-butyl bromoacetate (6.4 mL, 43 mmol). The resulting reaction mixture was allowed to warm to room temperature for 1 h. Ethyl acetate (300 mL) and aqueous HCl solution (300 mL, pH = 2 by pH test paper) were added to the reaction mixture. The organic phase was separated and washed with aqueous HCl solution (150 mL × 3, pH = 2 by pH test paper), dried (Na_2SO_4), and then concentrated to give a white solid. Recrystallization (hot water) afforded **9** (8.2 g, 30 mmol, 69%), which was dissolved in its entirety in anhydrous CH_2Cl_2 (380 mL)

containing 1 mL of DMF under argon. The mixture was cooled to 0 °C and treated dropwise with oxalyl chloride (4.8 mL, 56 mmol). The resulting reaction mixture was allowed to warm to room temperature for 40 min, and then the solvent was removed under vacuum to afford a colorless oil. The oil was dissolved in THF (75 mL) and treated with 2-mercaptopyridine (4.7 g, 45 mmol) under argon. After stirring for 30 min at room temperature, the reaction mixture was treated with diethyl ether/aqueous $NaHCO_3$. The organic phase was washed (water), dried (Na_2SO_4), and concentrated. The resulting solid was recrystallized (hot ethanol) to afford a yellow solid (4.7 g, 32% from 4-hydroxybenzoic acid). The characterization data (^{1}H NMR) were consistent with those reported above.

Zn(II)-18,18-dimethyl-5-[4-(2-(*tert*-butoxy)-2-oxoethoxy)phenyl]-10-[2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl]chlorin (10). Following a standard procedure,[62] a solution of **3b** (482 mg, 449 μmol) in THF (12 mL) was treated with NBS (83.0 mg, 446 μmol) at −78 °C for 1 h. A mixture of hexanes (26 mL) and water (26 mL) was added, and the reaction mixture was allowed to warm to room temperature for 20 min. The organic phase was separated. The aqueous phase was extracted with CH_2Cl_2 (26 mL). The combined organic extract was dried (Na_2SO_4) and concentrated, whereupon THF (7.3 mL) and anhydrous methanol (1.8 mL) were added. The solution was treated with $NaBH_4$ (300. mg, 7.93 mmol) at room temperature for 45 min. The reaction mixture was quenched by the addition of saturated aqueous NH_4Cl (26 mL), and then extracted with CH_2Cl_2 (52 mL). The organic extract was dried (Na_2SO_4) and concentrated under reduced pressure without heating to ∼4 mL, whereupon anhydrous CH_3CN (10 mL) was added, and again the solution was concentrated by blowing with argon to ∼4 mL. The resulting solution (which contained the Eastern half **3b-Br**[9]) was treated with anhydrous CH_3CN (9 mL, total volume of ∼13 mL of CH_3CN), Western half **4** (86.6 mg, 456 μmol) and TFA (34 μL, 447 μmol). The reaction mixture was stirred at room temperature for 30 min and then diluted with CH_3CN (42 mL). Samples of triethylamine (1.8 mL, 14 mmol), $Zn(OAc)_2$ (1.2 g, 12 mmol), and AgOTf (0.34 mg, 1.3 mmol) were added. The resulting mixture was refluxed in the presence of air for 24 h. The reaction mixture was quenched by the addition of water, and extracted with CH_2Cl_2 (50 mL × 4). The organic extract was dried (Na_2SO_4) and concentrated. The mixture was purified by (*vide supra*): (1) flash chromatography (silica, CH_2Cl_2 with 5% MeOH), (2) preparative SEC, and (3) flash chromatography (silica, CH_2Cl_2 with 2–5% MeOH). The purification procedure afforded a blue solid (62 mg, 11%): ^{1}H NMR δ 1.60 (s, 9H), 1.77–1.80 (m, 4H), 1.89–2.18 (m, 12H), 2.02 (s, 6H), 2.23–2.41 (m, 8H), 2.46 (s, 6H), 2.99–3.02 (m, 4H), 3.40 (s, 3H), 3.57–3.60 (m, 2H), 3.68–3.80 (m, 8H), 3.84–3.87 (m, 2H), 3.89–3.92 (m, 4H), 4.01–4.04 (m, 2H), 4.35–4.37 (m, 2H), 4.49 (s, 2H), 4.77 (s, 2H), 6.54 (s, 2H), 7.19 (d, J = 8.8 Hz, 2H), 7.69 (d, J = 8.8 Hz, 2H), 8.30, 8.33 (AB, J = 4.4 Hz, 2H), 8.47 (s, 1H), 8.51 (d, J = 4.4 Hz, 1H), 8.53 (s, 1H), 8.58 (d, J = 4.4 Hz, 1H), 8.610 (d, J = 4.4 Hz, 1H), 8.614 (d, J = 4.4 Hz, 1H); ^{13}C NMR δ 28.2, 31.1, 45.0, 50.5, 58.2, 59.1, 66.0, 67.7, 68.64, 68.71, 68.89, 68.95, 69.14, 69.17, 69.5, 69.9, 70.7, 70.9, 71.9, 82.5, 93.4, 93.9, 95.8, 112.6, 115.1, 115.7, 123.2, 126.1, 126.9, 127.5, 128.6, 132.4, 132.6, 134.6, 136.4, 145.4, 146.8, 147.3, 148.6, 153.1, 153.2, 157.3, 158.7, 159.9, 160.4, 168.3, 169.7; ESI-MS obsd 1303.5576, calcd 1303.5614 [$(M + H)^+$, M = $C_{67}H_{90}N_4O_{18}Zn$].

3,13-Dibromo-10-[4-(methoxycarbonyl)phenyl]-18,18-dimethylchlorin (12). Following a reported procedure,[18] a solution of **3c** (1.70 g, 5.52 mmol) in anhydrous THF (55 mL) was treated with NBS (1.96 g, 11.0 mmol) under argon at −78 °C. The reaction mixture was stirred for 1 h at −78 °C, after which the cooling bath was removed. Upon reaching −20 °C, hexanes and water were added. The mixture was extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and concentrated to afford 8,9-dibromo-5-[(4-methoxycarbonyl)phenyl]dipyrromethane (**3c-Br**[8,9])[18] as a yellow oil, which was used in the next step without further purification.

Following a general procedure,[62] a solution of **5** (5.5 mmol) and **3c-Br**[8,9] (1.48 g, 5.50 mmol) in CH_2Cl_2 (140 mL) was treated with a solution of *p*-TsOH·H_2O (5.20 g, 27.5 mmol) in methanol (37 mL) under argon. The reaction mixture immediately turned red. The mixture was stirred for 30 min under argon, then treated with 2,2,6,6-tetramethylpiperidine (7.00 mL, 41.3 mmol) and concentrated to dryness. The resulting brown solid was suspended in acetonitrile (550 mL) followed by the successive addition of 2,2,6,6-tetramethylpiperidine (23.60 mL, 137.5 mmol), $Zn(OAc)_2$ (25.00 g, 137.5 mmol), and AgOTf (4.20 g, 16.5 mmol). The resulting suspension was refluxed for 22 h exposed to air. The crude mixture was filtered through a silica pad with CH_2Cl_2. The filtrate was concentrated. The resulting crude product was dissolved in anhydrous CH_2Cl_2 (100 mL). TFA (500 μL) was added dropwise to the resulting mixture. After 10 min, saturated aqueous $NaHCO_3$ was added slowly. The organic layer was dried (Na_2SO_4), concentrated and chromatographed [silica, hexanes/CH_2Cl_2 (2 : 1)] to afford a green solid (332 mg, 10%): ^{1}H NMR δ −2.19 (br s, 1H), −1.79 (br s, 1H), 2.00 (s, 6H), 4.10 (s, 3H), 4.60 (s, 2H), 8.15 (d, J = 8.0 Hz, 2H), 8.40 (d, J = 8.0 Hz, 2H), 8.47 (d, J = 4.4 Hz, 1H), 8.73 (s, 1H), 8.75 (s, 1H), 8.91 (d, J = 4.4 Hz, 1H), 8.93 (s, 1H), 9.11 (s, 1H), 9.82 (s, 1H); ^{13}C NMR δ 31.3, 46.6, 52.1, 52.7, 94.9, 95.7, 106.0, 113.9, 118.8, 120.3, 124.7, 128.4, 128.8, 129.9, 132.3, 132.5, 133.4, 134.0, 134.2, 137.3, 140.2, 145.9, 151.3, 152.5, 159.6, 163.8, 167.5, 176.1; MALDI-MS obsd 631.0874; ESI-MS obsd 631.03316, calcd 631.03388 [$(M + H)^+$, M = $C_{30}H_{24}Br_2N_4O_2$]; λ_{abs} (toluene) 411, 652 nm.

3,13-Diacetyl-10-[4-(methoxycarbonyl)phenyl]-18,18-dimethylchlorin (13). Following a procedure for Stille coupling of chlorins,[72] a mixture of **12** (100 mg, 0.158 mmol), tributyl(1-ethoxyvinyl)tin (267 μL, 0.790 mmol), and $(Ph_3P)_2PdCl_2$ (22.2 mg, 0.0316 mmol) was stirred in CH_3CN/DMF [8 μL (3 : 2)] under argon for 4 h at 83 °C in a Schlenk line. The reaction mixture was treated with 10% aqueous HCl (5 mL) at room temperature for 20 min. CH_2Cl_2 was added. The organic layer was separated, washed (saturated aqueous $NaHCO_3$, water, and brine), dried (Na_2SO_4), concentrated, and chromatographed [silica, CH_2Cl_2/CH_3OH (99 : 1)]. The resulting solid product was washed [hexanes/ethyl ether (20 : 1)] five times (to remove impurities derived from the tin reagent) to afford a brown solid (80 mg, 90%): ^{1}H NMR δ −1.78 (br s, 1H), −1.58 (br s, 1H), 1.99 (s, 6H), 3.04 (s, 3H), 3.12 (s, 3H), 4.14 (s, 3H), 4.57 (s, 2H), 8.06 (d, J = 8.0 Hz, 2H), 8.38–8.40 (m, 3H), 8.77 (s, 1H), 8.84 (d, J = 4.0 Hz, 1H), 8.94 (s, 1H), 9.17 (s, 1H), 10.14 (s, 1H), 10.53 (s, 1H); ^{13}C NMR δ 29.8, 29.9, 31.1, 46.4, 52.3, 52.7, 66.1, 96.6, 99.3, 109.0, 122.1, 127.8, 128.3, 130.07, 130.12,

131.3, 132.3, 133.0, 133.2, 134.0, 134.1, 135.0, 137.4, 138.1, 145.8, 153.6, 154.7, 165.9, 167.4, 176.8, 196.7, 196.9; MALDI-MS obsd 559.2522; ESI-MS obsd 559.23347, calcd 559.23398 [$(M + H)^+$, M = $C_{34}H_{30}N_4O_4$]; λ_{abs} (toluene) 433, 686 nm.

Zn(II)-10-[2,4,6-tris(2,5,8,11,14,17-hexaoxanonadecyl-1*H*-1,2,3-triazol-4-ylmethoxy)phenyl]-18,18-dimethylchlorin (ZnC1). The Cu(I) catalyst was prepared by treatment of $CuSO_4{\cdot}5H_2O$ (15.7 mg, 0.0630 mmol) and sodium ascorbate (25.0 mg, 0.126 mmol) with 600 μL deionized H_2O under argon. The reaction mixture turned brown immediately and was stirred until homogeneous. In a separate vial, a solution of chlorin **6** (20.0 mg, 0.0315 mmol) and PEG-azide **7** (150 mg, 0.468 mmol) in DMSO (1200 μL) was treated with freshly prepared Cu(I) catalyst (300 μL) under argon. The reaction mixture was stirred at 40 °C for 16 h. H_2O was added, and the organic phase was extracted with CH_2Cl_2. The extract was washed (brine), dried (Na_2SO_4), and concentrated. The resulting mixture was purified as follows: (1) chromatography [silica, CH_2Cl_2/CH_3OH (93 : 7 → 19 : 1)], (2) preparative SEC, and (3) chromatography (silica, CH_2Cl_2 with 8% CH_3OH). The purification procedure afforded a blue oil (29 mg, 58%): ^{1}H NMR (THF-d_8) δ 1.98 (s, 6H), 2.78 (s, 6H), 2.92–2.94 (m, 4H), 3.04–3.06 (m, 4H), 3.12–3.17 (m, 6H), 3.22–3.25 (m, 4H), 3.27 (s, 3H), 3.30–3.36 (m, 12H), 3.38–3.41 (m, 4H), 3.44–3.45 (m, 2H), 3.53–3.68 (m, 28H), 3.80 (t, J = 5.2 Hz, 4H), 3.97 (t, J = 5.2 Hz, 2H), 4.52 (s, 2H), 4.62 (t, J = 5.2 Hz, 2H), 4.96 (s, 4H), 5.44 (s, 2H), 6.47 (s, 2H), 6.97 (s, 2H), 8.24 (s, 1H), 8.42 (d, J = 4.4 Hz, 1H), 8.52 (d, J = 4.0 Hz, 1H), 8.58–8.60 (m, 3H), 8.69 (d, J = 4.0 Hz, 1H), 8.76 (d, J = 4.4 Hz, 1H), 9.01 (d, J = 4.0 Hz, 1H), 9.54 (s, 1H); ^{13}C NMR (THF-d_8) δ 32.4, 47.2, 51.2, 51.9, 52.4, 60.0, 64.2, 65.0, 70.8, 71.6, 71.8, 72.12, 72.13, 72.18, 72.22, 72.31, 72.35, 72.40, 72.44, 72.5, 72.6, 72.7, 73.9, 74.0, 95.1, 96.0, 97.6, 110.5, 116.8, 117.4, 125.0, 126.4, 128.3, 129.6, 130.0, 133.7, 134.4, 145.4, 147.5, 147.8, 149.2, 150.4, 154.8, 155.4, 160.0, 161.8, 162.6, 171.5; MALDI-MS obsd 1604.8876, calcd 1604.7225 [$(M + H)^+$, M = $C_{76}H_{109}N_{13}O_{21}Zn$]; ESI-MS obsd 1625.70380, calcd 1625.69664 [$(M + Na)^+$, M = $C_{76}H_{109}N_{13}O_{21}Zn$]; λ_{abs} (toluene) 409, 609 nm.

10-[2,4,6-Tris(2,5,8,11,14,17-hexaoxanonadecyl-1*H*-1,2,3-triazol-4-ylmethoxy)phenyl]-18,18-dimethylchlorin (FbC1). A solution of **ZnC1** (8.0 mg, 0.0050 mmol) in CH_2Cl_2 (16 mL) was treated dropwise with TFA (40 μL). After 5 min, saturated aqueous $NaHCO_3$ was added slowly. The organic layer was separated, dried ($NaSO_4$), and concentrated. The crude product was chromatographed [silica, CH_2Cl_2/CH_3OH (19 : 1)] to afford a green solid (7.0 mg, 89%): ^{1}H NMR (THF-d_8) δ −2.22 (s, 1H), −1.94 (s, 1H), 2.06 (s, 6H), 2.55 (s, 6H), 2.92–2.94 (m, 4H), 3.04–3.06 (m, 4H), 3.12–3.17 (m, 6H), 3.22–3.25 (m, 4H), 3.27 (s, 3H), 3.30–3.36 (m, 12H), 3.38–3.41 (m, 4H), 3.44–3.45 (m, 2H), 3.53–3.68 (m, 28H), 3.80 (t, J = 5.2 Hz, 4H), 3.97 (t, J = 5.2 Hz, 2H), 4.52 (s, 2H), 4.63 (t, J = 5.2 Hz, 2H), 5.02 (s, 4H), 5.47 (s, 2H), 6.26 (s, 2H), 7.10 (s, 2H), 8.27 (s, 1H), 8.54 (d, J = 4.4 Hz, 1H), 8.75 (d, J = 4.4 Hz, 1H), 8.82 (d, J = 4.4 Hz, 1H), 8.86 (d, J = 4.4 Hz, 1H), 8.98–9.04 (m, 3H), 9.28 (d, J = 4.4 Hz, 1H), 9.87 (s, 1H); ^{13}C NMR (THF-d_8) δ 30.5, 30.6, 46.2, 49.3, 50.1, 52.0, 58.1, 58.2, 62.4, 63.2, 68.85, 68.95, 69.6, 69.7, 69.8, 69.94, 69.95, 70.01, 70.06, 70.09, 70.23, 70.38, 70.40, 70.44, 70.51, 70.56, 70.58, 70.74, 70.76, 70.80, 72.0, 72.1, 94.0, 94.2, 96.3, 107.0, 113.1, 114.3, 122.5, 122.8, 123.0, 123.6, 124.6, 127.5, 128.3, 131.9, 132.4, 132.6, 133.8, 136.6, 139.7, 140.5, 143.5, 150.8, 154.8, 159.9, 161.2, 162.5, 174.2; MALDI-MS obsd 1566.0934, calcd 1564.7910 [$(M + Na)^+$, M = $C_{76}H_{111}N_{13}O_{21}$]; ESI-MS obsd 771.90605, calcd 771.90815 [$(M + 2H)^{2+}$, M = $C_{76}H_{111}N_{13}O_{21}$]; λ_{abs} (toluene) 408, 640 nm.

5-[4-(Carboxymethoxy)phenyl]-18,18-dimethyl-10-[2,4,6-tris-(3,6,9,12-tetraoxatridecyloxy)phenyl]chlorin (FbC2). Following a standard procedure,[77] a solution of **10** (15 mg, 12 μmol) in CH_2Cl_2 (4.2 mL) was treated with TFA (1.1 mL). The reaction mixture was stirred at room temperature for 3 h. Water (∼6 mL) was added. The organic phase was separated, washed with water (6 mL × 3), dried (Na_2SO_4) and concentrated. Hexane (HPLC-grade) was added to the residue, and the suspension was sonicated for 3 min followed by centrifugation. The supernatant was discarded to afford a brown solid (11 mg, 80%): ^{1}H NMR (the CO_2H proton peaks were not observed) δ −1.91 (br s, 2H), 2.06 (s, 6H), 2.14–2.28 (m, 6H), 2.61–2.63 (m, 4H), 2.91–2.93 (m, 6H), 3.02–3.08 (m, 2H), 3.18–3.21 (m, 4H), 3.25 (s, 6H), 3.28–3.31 (m, 4H), 3.41 (s, 3H), 3.59–3.61 (m, 2H), 3.69–4.05 (m, 18H), 4.37–4.40 (m, 2H), 4.60 (s, 2H), 4.88 (s, 2H), 6.55 (s, 2H), 7.24 (d, J = 8.2 Hz, 2H), 8.02 (d, J = 8.2 Hz, 2H), 8.35, 8.50 (AB, J = 4.2 Hz, 2H), 8.72–8.75 (m, 3H), 8.80 (d, J = 4.8 Hz, 1H), 8.83 (s, 1H), 8.90 (s, 1H); ^{13}C NMR δ 31.2, 46.3, 51.8, 58.70, 58.79, 59.0, 65.2, 65.8, 67.7, 68.5, 69.1, 69.3, 69.41, 69.53, 69.62, 69.77, 69.83, 70.53, 70.65, 70.70, 70.9, 71.5, 71.9, 92.72, 92.80, 94.6, 96.1, 112.80, 112.93, 113.2, 114.1, 121.2, 122.6, 123.2, 128.0, 128.4, 131.2, 131.7, 134.4, 134.9, 135.6, 135.92, 140.04, 140.26, 151.4, 153.5, 157.4, 159.9, 160.8, 163.2, 170.8, 174.2; MALDI-MS obsd 1186.7898; ESI-MS obsd 1185.5815, calcd 1185.5853 [$(M + H)^+$, M = $C_{63}H_{84}N_4O_{18}$].

Zn(II)-5-[4-(carboxymethoxy)phenyl]-18,18-dimethyl-10-[2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl]chlorin (ZnC2). A solution of **FbC2** (11 mg, 9.3 μmol) in CH_2Cl_2 (0.5 mL) was treated with a solution of $Zn(OAc)_2{\cdot}2H_2O$ (30.0 mg, 136 μmol) in methanol (0.5 mL). The reaction mixture was stirred at room temperature for 16 h, and then treated with water (∼4 mL). The organic phase was separated, washed with water (4 mL × 3), dried (Na_2SO_4) and concentrated. HPLC-grade hexanes was added to the residue, and the suspension was sonicated for 3 min followed by centrifugation. The supernatant was discarded to afford a blue solid (11 mg, 95%): ^{1}H NMR ($CDCl_3$, 300 MHz, the CO_2H proton peak was not observed) δ 2.02 (s, 6H), 2.08–2.69 (m, 24H), 2.71 (s, 6H), 2.93–3.05 (m, 4H), 3.40 (s, 3H), 3.57–3.60 (m, 4H), 3.68–3.94 (m, 10H), 4.01–4.04 (m, 4H), 4.35–4.38 (m, 2H), 4.48 (s, 2H), 4.77 (br s, 2H), 6.53 (s, 2H), 7.17 (d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.3 Hz, 2H), 8.26, 8.34 (AB, J = 4.3 Hz, 2H), 8.47 (s, 1H), 8.50 (d, J = 4.4 Hz, 1H), 8.52 (s, 1H), 8.57–8.61 (m, 3H); ^{13}C NMR δ 31.0, 45.0, 58.2, 59.0, 68.6, 68.8, 69.0, 69.2, 69.8, 70.50, 70.62, 71.9, 114.9, 125.3, 128.2, 129.0, 137.8, 159.8, 169.8; MALDI-MS obsd 1247.6318; ESI-MS obsd 1247.4956, calcd 1247.4988 [$(M + H)^+$, M = $C_{63}H_{82}N_4O_{18}Zn$].

18,18-Dimethyl-5-[4-(succinimidooxycarbonylmethoxy)phenyl]-10-[2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl]chlorin (FbC2-NHS). Following a standard procedure,[52] a mixture of **FbC2** (4.0 mg, 3.4 μmol), *N*-hydroxysuccinimide (0.47 mg, 4.1 μmol) and

EDC (0.78 mg, 4.1 μmol) in CH_2Cl_2 (5.0 mL) was stirred in the dark at room temperature. After 6 h, the reaction mixture was diluted with CH_2Cl_2 (3 mL) and washed with water. The organic layer was separated, dried (Na_2SO_4) and concentrated to a red-brown solid (4.6 mg, ~80% purity estimated by ^{1}H NMR spectroscopy and MALDI-MS): MALDI-MS obsd 1283.73, calcd 1282.60 [$(M + H)^+$, M = $C_{67}H_{87}N_5O_{20}$]; λ_{abs} (CH_2Cl_2) 414, 508, 641 nm.

18,18-Dimethyl-5-(4-((42-(succinimidooxy)-2,42-dioxo-6,9,12,15,-18,21,24,27,30,33,36,39-dodecaoxa-3-azadotetracontyl)oxy)phenyl)-10-(2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl)chlorin (FbC2-R-NHS). A mixture of **FbC2-NHS** (5.5 mg, 80% purity, 3.4 μmol), PEG-amine **11** (2.5 mg, 4.0 μmol) in dry CH_2Cl_2 (1.7 mL) was stirred in the dark at room temperature. After 6 h, the reaction mixture was diluted with CH_2Cl_2 (3 mL) and washed with water. The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting material was treated with *N*-hydroxysuccinimide (0.29 mg, 2.5 μmol) and EDC (0.48 mg, 2.5 μmol) in CH_2Cl_2 (2.1 mL) in the dark at room temperature. After 20 h, the reaction mixture was diluted with CH_2Cl_2 (3 mL) and washed with aqueous HCl solution (pH = 5). The organic layer was separated, dried (Na_2SO_4) and concentrated. The resulting material was treated with hexanes/ethyl acetate (1 : 2), sonicated, and centrifuged. The supernatant was discarded, leaving a red-brown solid (3.8 mg, ~80% purity estimated by ^{1}H NMR spectroscopy and MALDI-MS): MALDI-MS obsd 1882.75, calcd 1881.95 [$(M + H)^+$, M = $C_{94}H_{140}N_6O_{33}$]; λ_{abs} (CH_2Cl_2) 414, 508, 641 nm.

5-(4-Carboxyphenyl)-18,18-dimethyl-3,13-bis{(*E*)-3-[2,4,6-tris-(3,6,9,12-tetraoxatridecyloxy)phenyl]prop-2-en-1-onyl}chlorin (FbC3). A mixture of **13** (50.0 mg, 0.0895 mmol), benzaldehyde **1b** (649 mg, 0.895 mmol), and NaOH (143 mg, 3.58 mmol) in absolute ethanol (45 mL) was refluxed in the open air upon microwave irradiation at 60 W. The microwave irradiation protocol was as follows: (1) heat from room temperature to 100 °C (irradiate for 2 min), (2) hold at 100 °C (irradiate for 18 min), (3) allow to cool to room temperature. The reaction mixture was concentrated. The resulting crude product was dissolved in CH_2Cl_2 and washed with saturated aqueous NH_4Cl. The organic layer was separated, dried (Na_2SO_4), and concentrated. The mixture was purified as follows: (1) flash chromatography (silica, CH_2Cl_2 with 5% MeOH), (2) preparative SEC (toluene), and (3) flash chromatography (silica, CH_2Cl_2 with 10% MeOH). The purification procedure afforded a blue solid (98 mg, 56%): ^{1}H NMR δ −1.52 (br s, 1H), −1.36 (br s, 1H), 2.07 (s, 6H), 3.24 (s, 6H), 3.31 (s, 6H), 3.36–3.37 (m, 4H), 3.39 (s, 3H), 3.40 (s, 3H), 3.44–3.50 (m, 8H), 3.51–3.52 (m, 16H), 3.55–3.58 (m, 8H), 3.61–3.63 (m, 4H), 3.65–3.76 (m, 28H), 3.79–3.82 (m, 4H), 3.86–3.90 (m, 2H), 3.90–3.95 (m, 6H), 4.04–4.09 (m, 4H), 4.15–4.20 (m, 2H), 4.20–4.22 (m, 2H), 4.25–4.29 (m, 4H), 4.30–4.34 (m, 4H), 4.67 (s, 2H), 6.26 (s, 2H), 6.27 (s, 2H), 8.29 (d, J = 8.0 Hz, 2H), 8.46 (d, J = 16.0 Hz, 1H), 8.52–8.54 (m, 3H), 8.62 (d, J = 16.0 Hz, 1H), 8.67 (d, J = 15.6 Hz, 1H), 8.72 (d, J = 15.6 Hz, 1H), 8.93 (s, 1H), 8.00 (d, J = 4.4 Hz, 1H), 9.22 (s, 1H), 9.49 (s, 1H), 10.34 (s, 1H), 10.81 (s, 1H), the –CO_2H proton was not detected; ^{13}C NMR δ 31.2, 46.4, 52.4, 59.0, 59.1, 67.7, 68.2, 68.3, 69.4, 69.6, 69.7, 70.3, 70.40, 70.44, 70.52, 70.56, 70.61, 70.67, 70.70, 70.9, 71.8, 72.0, 92.6, 92.8, 96.2, 99.2, 107.4, 109.1, 121.7, 124.6, 125.5, 126.5, 128.6, 129.9, 132.4, 132.5, 132.9, 133.9, 134.2, 134.7, 135.5, 136.2, 137.1, 138.4, 138.6, 146.4, 153.3, 154.2, 161.1, 161.2, 162.38, 162.48, 165.4, 168.8, 176.5, 189.6, 190.2; MALDI-MS obsd 1980.6716, calcd 1979.9554 [$(M + Na)^+$, M = $C_{101}H_{144}N_4O_{34}$]; ESI-MS obsd 979.49094, calcd 979.49094 [$(M + 2H)^{2+}$, M = $C_{101}H_{144}N_4O_{34}$]; λ_{abs} (toluene) 442, 686 nm.

Zn(II)-5-(4-carboxyphenyl)-18,18-dimethyl-3,13-bis{(*E*)-3-[2,4,6-tris(3,6,9,12-tetraoxatridecyloxy)phenyl]prop-2-en-1-onyl}chlorin (ZnC3). A solution of **FbC3** (15 mg, 7.7 μmol) in $CHCl_3$ (0.5 mL) was treated with a solution of $Zn(OAc)_2$ (27.0 mg, 150 μmol) in methanol (0.5 mL). The reaction mixture was stirred at room temperature for 16 h, and then treated with water (~4 mL). The organic phase was separated, washed with water (4 mL × 3), dried (Na_2SO_4) and concentrated. HPLC-grade hexanes was added to the residue, and the suspension was sonicated followed by centrifugation. The supernatant was discarded to afford a green solid (13 mg, 84%): ^{1}H NMR (THF-d_8) δ 2.10 (s, 6H), 3.24 (s, 6H), 3.31 (s, 6H), 3.36–3.37 (m, 4H), 3.39 (s, 3H), 3.40 (s, 3H), 3.44–3.50 (m, 8H), 3.51–3.52 (m, 16H), 3.55–3.58 (m, 8H), 3.61–3.63 (m, 4H), 3.65–3.76 (m, 28H), 3.79–3.82 (m, 4H), 3.86–3.90 (m, 2H), 3.90–3.95 (m, 6H), 4.04–4.09 (m, 4H), 4.15–4.20 (m, 2H), 4.20–4.22 (m, 2H), 4.25–4.29 (m, 4H), 4.30–4.34 (m, 4H), 4.60 (s, 2H), 6.35 (s, 2H), 6.41 (s, 2H), 8.24 (d, J = 8.0 Hz, 2H), 8.41–8.45 (m, 3H), 8.47–8.48 (m, 3H), 8.61 (d, J = 15.6 Hz, 1H), 8.70 (s, 1H), 8.80 (d, J = 15.6 Hz, 1H), 8.91 (d, J = 4.4 Hz, 1H), 9.18 (s, 1H), 9.47 (s, 1H), 10.06 (s, 1H), the –CO_2H proton was not detected; ESI-MS obsd 1032.42903, calcd 1032.42907 [$(M + 2Na)^{2+}$, M = $C_{101}H_{142}N_4O_{34}Zn$]; λ_{abs} (toluene) 447, 666 nm.

Photophysical measurements

All studies were performed at room temperature for compounds in DMF, or in HPLC-grade water (Sigma Aldrich) with 5% DMF. The small amount of DMF was employed to pre-solubilize any solid compound with limited solubility in pure water, and this method was used in all cases for consistency. To simplify the presentation, the solvent consisting of water plus 5% DMF is referred to as "water". Dilute (μM) argon-purged samples were used for static emission spectral, fluorescence quantum yield (Φ_f) and singlet excited-state lifetime (τ_S) studies. Static emission spectra were acquired using 2–4 nm excitation and detection bandwidths and corrected for instrument spectral response. The Φ_f values were determined for samples having $A \leq 0.1$ at λ_{exc} (typically in the Soret region) using replicate measurements with an integrating sphere (Horiba, Quanti-Phi).

Singlet excited-state lifetimes (τ_S) are the average result of two measurements. The first method used a stroboscopic fluorescence decay apparatus with an ~1 ns Gaussian instrument response function (Laser Strobe TM-3; Photon Technology International) and samples excited in the blue to green spectral regions by a dye laser pumped by a nitrogen laser. The second method utilized transient absorption spectroscopy employing ~100 fs excitation flashes (typically in the Q_x region) from an ultrafast laser system (Spectra Physics) and acquisition of difference spectra (360–900 nm) using a white-light pulsed laser (~1 ns rise time) in 100 ps time bins with variable pump–probe spacing up to 0.5 ms (Ultrafast Systems, EOS). The latter

apparatus was also used to determine the yield of $S_1 \rightarrow T_1$ intersystem crossing (Φ_{isc}) by comparing the extent of bleaching of the ground-state B_x, Q_x or Q_y absorption bands (relative to the featureless excited-state absorption) for the T_1 state at long times compared to that due to S_1 right after the flash. The contribution of stimulated emission (to S_1 spectra) was taken into account for studies in the Q_y region.

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. The longpass filter in the "A" detector channel of the 355 nm laser was replaced with a 600 nm longpass filter provided with the instrument and the standard bandpass filter in this channel was replaced with a 620/60 nm bandpass filter (ET620/60, Chroma Technology Corp., Bellows Falls, VT). Data were collected using the 100 mW 405 nm laser. Post-experimental analysis was performed with FlowJo software (version 10.0.8, FlowJo, LLC, Ashland, OR).

Materials

Simply Cellular™ anti-Mouse for Violet Laser compensation standard beads (Bangs Laboratories, Fishers, IN) were used for all flow cytometry experiments. Antibodies used included BD Horizon Brilliant Violet 650-labeled CD8 clone RPA-T8 (**BV650–Ab**, positive control) and Protein A-purified mouse IgG (MU-003-C, Immuno-Reagents, Raleigh, NC).

Bioconjugation to form FbC2–Ab

Prior to bioconjugation, dialysis was used to exchange antibodies into 50 mM borate buffer (pH 8.5). The chlorin **FbC2-R-NHS** (0.1 mg) was dissolved in 20 μL of the borate buffer and added to 0.25 mg of mouse IgG solution to achieve a 35-fold ratio of fluorophore to protein in a final reaction volume of 86 μL. This reaction solution was gently rotated in a microcentrifuge tube protected from light for 3 h at ambient temperature and then dialyzed against phosphate buffered saline (PBS) with a 20 kD MWCO dialysis membrane for at least 4 h at ambient temperature to remove unreacted materials. The chlorin–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 chlorin–antibody bioconjugate, **FbC2–Ab**, exhibited a fluorophore/protein ratio of 2.2 as determined by absorption spectroscopy using a molar absorption coefficient for the chlorin (ε_{414nm} = 160 000 M^{-1} cm^{-1}; ε_{280nm} = 22 000 M^{-1} cm^{-1}) and for the antibody[81] (ε_{280nm} = 210 000 M^{-1} cm^{-1}). The value for the chlorin was drawn from that of the chlorin analogue lacking any *meso*-substituents.[64]

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 μL. Blanks were treated by the following sequence of washes and centrifugation but without antibody addition, then resuspended in 1.0 mL of buffer. For labeled antibodies, 1.0 mL aliquots of bead solution were prepared from 4 drops of beads, then 50 μL was aliquoted per sample. For the experiment shown in Fig. 4, 0.5 μg of **FbC2–Ab**, and 0.12 μg of the positive control Brilliant Violet 650-labeled antibody (**BV650–Ab**) were 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 × *g* for 3 min, and removal of supernatant. Samples were resuspended in 1.0 mL of buffer for analysis.

Experiment and data analysis

The **FbC2–Ab** and the positive control **BV650–Ab** were excited with the 405 nm laser and read in channel A with a 600 nm longpass filter and a 620/60 nm bandpass filter in place. 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.

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.

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