Progressive Cationic Functionalization of Chlorin Derivatives for Antimicrobial Photodynamic Inactivation and Related Vancomycin Conjugate

Short title: APDI with polycationic chlorins

Liyi Huang,[‡,§] Min Wang,[†] Ying-Ying Huang,[‡,§] Ahmed El-Hussein,[‡,§,♣] Lawrence Wolf,[†] Long Y. Chiang,*[,†] and Michael R. Hamblin*[,‡,§,¥]

[‡] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, Massachusetts 02114, United States

[§] Department of Dermatology, Harvard Medical School, Boston, Massachusetts 02115, United States

[†] Department of Chemistry, University of Massachusetts Lowell, Lowell, Massachusetts 01854, United States

[♣] The National Institute of Laser Enhanced Science (NILES), Cairo University, Egypt

[¥] Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts 02139, United States

- correspondence: Hamblin@helix.mgh.harvard.edu or Long_Chiang@uml.edu

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ABSTRACT: It is known that multiple cationic charges are required to produce broad-spectrum antimicrobial photosensitizers (PS) for photodynamic inactivation (aPDI) or photodynamic therapy of bacteria and fungi. In the present study we describe the synthesis and aPDI testing of a set of derivatives prepared from the parent pheophytin molecule with different numbers of attached sidearms (1–3) each consisting of five quaternized cationic groups (pentacationic), producing corresponding [Zn^{+2}]pheophorbide-*a*-N($C_2N^+C_1C_3$)$_5$ (Zn-Phe-N_5^+, 5 charges), [Zn^{+2}]chlorin e_6-[N($C_2N^+C_1C_3$)$_5$]$_2$ (Zn-Chl-N_{10}^+, 10 charges) and [Zn^{+2}]mesochlorin e_6-[N($C_2N^+C_1C_3$)$_5$]$_3$ (Zn-*m*Chl-N_{15}^+, 15 charges). Moreover, a conjugate between Zn-Phe-N_5^+ and the antibiotic vancomycin called Van-[Zn^{+2}]-*m*-pheophorbide-N($C_2N^+C_1C_3$)$_5$ (Van-Zn-*m*Phe-N_5^+) was also prepared. The aPDI activity of all compounds were based on Type-II photochemistry ($^1O_2$ generation). We tested these compounds against Gram-positive methicillin *Staphylococcus aureus* (MRSA), Gram-negative *Escherichia coli,* and fungal yeast *Candida albicans.* All three

compounds were highly active against MRSA giving eradication (≥ 6 logs of killing) with < 1.0 μM and 10 J/cm^2 of 415 nm light. The order of activity was Zn-Phe-N_5^+ > Zn-Chl-N_{10}^+ > Zn-*m*Chl-N_{15}^+. In the case of *E coli* the activity was much lower (eradication was only possible with 50 μM Zn-*m*Chl-N_{15}^+ and 20 J/cm^2). The order of activity was the reverse of that found with MRSA (Zn-*m*Chl-N_{15}^+ > Zn-Chl-N_{10}^+ > Zn-Phe-N_5^+). Activity against *C. albicans* was similar to *E. coli* with Zn-*m*Chl-N_{15}^+ giving eradication. The activity of Van-Zn-*m*Phe-N_5^+ was generally lower than Zn-Phe-N_5^+ (except for *E. coli*). Red (660 nm) light was also effective as might be expected from the absorption spectra. An initial finding that Van-Zn-*m*Phe-N_5^+ might have higher activity against vancomycin resistant *Enterococcus fecium* (VRE) strains (compared to vancomycin sensitive strains) was disproved when it was found that VRE strains were also more sensitive to aPDI with Zn-Phe-N_5^+. The minimum inhibitory concentrations of Van-Zn-*m*Phe-N_5^+ were higher than Van alone, showing antibiotic properties of the Van moiety were lessened in the conjugate. In conclusion Zn-Phe-N_5^+ is a highly active PS against Gram-positive species and deserves further testing. Increasing the number of cationic charges increased aPDI efficacy on *C. albicans* and Gram-negative *E. coli*.

KEYWORDS: Pentacationic pheophorbide; decacationic chlorin; pentadecacationic mesochlorin; photosensitizer; phototoxicity; antimicrobial photodynamic inactivation; bacterial cell-wall binding.

■ INTRODUCTION

Antimicrobial photodynamic inactivation (aPDI) is growing in popularity as an alternative route to killing pathogenic microbial cells,[1] and to treating localized infections.[2] The increasing interest in aPDI has been motivated by continuing concern about the seemingly unstoppable rise of multi-antibiotic resistance,[3] and emerging fears that formerly trivial infections will soon become untreatable by conventional antibiotics.[4]

Many different types of photosensitizer (PS) structure have been investigated over the years as antimicrobial PSs. A large number of these structures have been based on the tetrapyrrole backbone found in porphyrins, chlorophyll, and related molecules.[5] One of the attractions of using naturally occurring substances as starting materials for preparation of antimicrobial PS, is that the synthetic chemistry approach can be described as “green” in nature. Not only does the use of

natural products avoid wasting resources on multi-step syntheses,[6] but the financial costs are also lower.[7]

There are several important molecular properties that are required in the ideal antimicrobial PS.[8-10] These properties can be summarized as: water compatibility, a good absorbance peak in the red or near infrared region of the visible spectrum, satisfactory photostability (resistance to photobleaching), and lack of dark toxicity. Perhaps the single most important structural feature for preparing an antimicrobial PS, is the presence of one or more cationic charges, usually provided by introduction of at least one quaternary ammonium group into the molecular structure. Significantly

There are other desirable molecular features that should be incorporated into antimicrobial PSs, in addition to cationic charges[11-13]. An asymmetric structure with a high degree of amphiphilicity may allow the PS to better penetrate bacteria, than a more symmetric molecular structure.[14]

In the present study we describe the preparation of Zn^{+2}-pheophorbide, Zn^{+2}-chlorin and Zn^{+2}-mesochlorin derivatives that contain either, one, two, or three pentacationic chains, thus providing PSs with a total of five, ten, or fifteen cationic charges in each molecule. Owing to the negatively charged cell surface of many microorganisms, we hypothesized that a progressively increasing number of cations may allow us to investigate the variation of cell surface interactions to the influence of a-PDI activity. Moreover, one of these analogous derivatives having five cationic charges was further conjugated with the antibiotic, vancomycin with the goal of introducing a moiety that could target $_D$-Ala-$_D$-Ala residues present on the cell walls of Gram-positive bacteria.

■ RESULTS AND DISCUSSION

Synthesis and Characterization. Naturally occurring π-conjugated dye molecules exhibiting long-lived triplet excited states can be employed as a source of precursor synthons in the synthesis of new PSs for aPDI. Selection of these chromophore precursors should be based on compounds exhibiting a nearly quantitative efficiency for the process of intersystem crossing (*ISC*) from the excited singlet state of the π-conjugated photoreceptor moiety to the corresponding triplet excited state. Intersystem crossing will then be followed by subsequent intermolecular energy-transfer of the triplet energy to ground state triplet molecular oxygen ($^3O_2$) leading to the generation of excited singlet oxygen ($^1O_2$), a reactive oxygen species (ROS) that can also be converted to highly reactive free radicals in the presence of electron-rich substrates.[15, 16] One of these well-known natural dyes is pheophytin-*a* (Phe, Scheme 1) derived by demetalation of chlorophyll-*a*, which is the main acetone-soluble dye component of chloroplasts in spinach leaves.

Its unique photoreactivity arises from a ring system formed by four pyrroles linked *via* four methine bridges that contains a total of 18 π-conjugated electrons in the ring structure, responsible for the absorption of light to provide energy for photosynthesis. In order to covalently attach multicationic side-arms while preserving this central ring system, we designed a chemical functionalization strategy that was insensitive to ring olefinic carbons. Our main synthetic goal for the structural modification of pheophytin-*a* is to functionalize the porphyrin ring system with a tunable number of cationic charges to enhance the targeting ability of resulting PS toward Gram-negative bacteria.

Recently, we reported a key well-defined synthon consisting of a water-soluble pentacationic *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine-penta(quaternary methyl-ammonium iodide) arm, $H_2N(C_2N^+C_1C_3)_5$, containing a fixed number of five constitutive positive charges.[17] The versatility in chemical reactivity of the primary amino end-group of its precursor $H_2N(C_2NC_3)_5$, in conjunction with five tertiary amine base units, toward many organic functional moieties makes it easy for us to attach the arm onto the pheophytin-*a* core. In addition, the multicationic nature of this type of the arm will provide sufficient hydrophilicity and high water solubility even when attached to hydrophobic molecules. The corresponding chemical reaction sequence is outlined in Scheme 1. Accordingly, the synthesis of [Zn^{+2}]pheophorbide-*a*-$N(C_2N^+C_1C_3)_5$ (Zn-Phe-N_5^+, 5 cationic charges) was performed by the trans-amination of pheophytin-*a* with $H_2N(C_2NC_3)_5$ in dichloromethane in the presence of trifluoroacetic acid at room temperature for a period of 10 h to afford the green solid of the precursor intermediate Phe-N_5 in a yield of 82%. It was followed by the metal insertion using $Zn(OAc)_2{\cdot}2H_2O$ as the reagent in DMF and quaternary methylation of all tertiary amines with CH_3I in an excessive amount at 45 ºC for 3 d to afford Zn-Phe-N_5^+ as a green solid. In the case of [Zn^{+2}]chlorin e_6-$[N(C_2N^+C_1C_3)_5]_2$ (Zn-Chl-N_{10}^+, 10 charges) synthesis, the intermediate Phe-N_5 was treated by $H_2N(C_2NC_3)_5$ in CH_2Cl_2 to give a green solid of the precursor intermediate Chl-N_{10} in a yield of 77%. It was followed by similar metal (Zn^{2+}) insertion and quaternary methylation reactions of all tertiary amines to afford Zn-Chl-N_{10}^+ as a green solid. To attach a third pentacationic $H_2N(C_2N^+C_1C_3)_5$ arm on Zn-Chl-N_{10}^+ for the synthesis of [Zn^{+2}]mesochlorin e_6-$[N(C_2N^+C_1C_3)_5]_3$ (Zn-*m*Chl-N_{15}^+, 15 charges), we carried out epoxidation of the olefin moiety located at the opposite side of the other two pentacationic arms on the chlorin e_6 core ring with *m*-chloroperoxybenzoic acid (MCPBA), followed by the reaction of the resulting epoxide intermediate with $H_2N(C_2NC_3)_5$ to afford the precursor product *m*Chl-N_{15} as a green solid in a yield of 67%. After similar metal (Zn^{2+}) insertion and quaternary methylation, the product of Zn-*m*Chl-N_{15}^+ was obtained also as a green solid. All product samples were purified using the corresponding neutral (non-ionic) tertiary amine precursor compounds using thin-layer

chromatographic (TLC) technique and subsequently checked by ^{1}H NMR spectroscopy to ensure they were free of impurity, prior to the metalation of pheophytin ring and the quaternization.

A slightly modified synthetic method was applied for the preparation of Van-Zn-*m*Phe-N_5^+, it was based on the structure of Phe-N_5 followed by epoxidation of the ethylene group by MCPBA followed by ring-opening of the resulting oxirane with vancomycin hydrochloride under catalytic conditions using di(*n*-butyl)tin(IV) dilaurate (T12) as a Lewis acid catalyst to afford green solids of Van-*m*Phe-N_5 intermediate. Similar subsequent metal (Zn^{2+}) insertion and quaternary methylation were carried out to give the desire product of Van-Zn-*m*Phe-N_5^+ as a green solid.

Various spectroscopic methods were applied for the characterization of all compounds with the corresponding data included in the experimental section. Since all methyl, ethyl side groups, and ring protons of Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, and Zn-*m*Chl-N_{15}^+ remained identical to those of the parent pheophytin core, therefore, all spectroscopic analyses were based on the comparison with the precursor compound to match with the consistent functional group changes. Accordingly, progressive changes of three infrared absorption bands (Figure S1) of these three compounds at 1668 [–**C**(=**O**)-NH–], 1618 (–**C**=**C**–), and 1463 [–C(=O)-(**N**–**H**)–] cm^{-1} were evaluated as the indicator of structural modification. Intensity increases of the former band going from pheophytin (Figure S1a) to Zn-Phe-N_5^+ (one amide arm, Figure S1b), Zn-Chl-N_{10}^+ (two amide arms, Figure S1c), and then Zn-*m*Chl-N_{15}^+ (three amide arms, Figure S1d) are apparently consistent with an increase of pentacationic arm(s). Similar phenomena were also detected on the latter band. In the case of ^{1}H NMR spectroscopic analyses, the number of *N,N',N,N,N*-hexapropyl-penta(aminoethyl)amide (N_5-amide) arms attached on either Phe or chlorin core can be accounted for easily by changes of the measured proton integration ratio in the spectrum. We selected the integration of H_δ located on C_δ or H_β on C_β of either the Phe or chlorin core at δ 8.61–8.64 or 9.58–9.60 (Figure S2), respectively, as the reference of one-proton count (1.0). Theoretically, integration of two types of protons of each N_5-amide arm with the chemical shift at δ 2.5–3.25 (–N-CH_2–, aminomethylene protons) and δ 0.65–1.08 (–CH_3, end-group methyl protons) should give proton counts of 32(H) and 18(H), respectively, in a ratio of 1.78. By taking the measured proton counts integrated over two chemical shift ranges at δ 2.5/2.75–3.25/4.0 (certain compound-related shifts occurred) and δ 0.65–1.08 as 47.0 and 23.7 in Figure S2b, 80.4 and 40.1 in Figure S2c, and 109.9 and 53.8, respectively, in Figure S2d as the base, we then deducted all other types of non-N_5-amide associated methyl and methylene protons appearing and overlapping in the same chemical shift regions. It resulted in adjusted values of 31.0 and 17.7 in a ratio of 1.75 for one N_5-amide-armed Phe-N_5, 62.4 and 34.1 in a ratio of 1.83 for two N_5-amide-armed Chl-N_{10}, and 91.9 and 47.8 in a

ratio of 1.92 for three N_5-amide-armed *m*Chl-N_{15}. These values corresponded well with a proportional ratio of –N-CH_2–/–CH_3 protons among Phe-N_5, Chl-N_{10}, and *m*Chl-N_{15} giving a roughly good agreement with that (1.78) of the N_5-amide arm. In addition, the total accumulative proton counts integrated also matched well with the number increase of N_5-amide arms from one, two, to three going from the structure of Phe-N_5, Chl-N_{10}, to *m*Chl-N_{15}. These results can be regarded as a good consistency with the structural assignment of these compounds after functional modification from the basic Phe or chlorin core moiety. Subsequent reactions of metal (Zn^{2+}) insertion and quaternary methylation to incorporate water-solubility characteristics should not alter the basic substituent structures on the core.

To verify the degree of quaternization, we selected the synthesis of *m*Chl-N_{15}^+ as an example. As a result, ^{1}H NMR spectra of neutral *m*Chl-N_{15} (in $CDCl_3$) and methyl quaternized multicationic *m*Chl-N_{15}^+ (in DMSO-d_6) showed nearly quantitative conversion using methyl iodide as the quaternization agent. The precursor *m*Chl-N_{15} compound displayed the chemical shift of methylene protons next to tertiary amines at δ 2.5–3.4. Upon quaternization, the chemical shift of these methylene protons down-shifted to δ 3.3–4.3 leaving the region of δ 2.5–3.0 with nearly no proton bands in the spectrum. This is indicative of approximately full chemical conversion to cationic states as *m*Chl-N_{15}^+. Meanwhile, a new sharp singlet peak of methyl protons next to the quaternary amine was detected at δ 3.10.

Furthermore, to increase the spectroscopic detection resolution, we used non-ionic precursor compounds, such as Phe-N_5, Chl-N_{10}, *m*Chl-N_{15}, and Van-*m*Phe-N_5, instead of multicationic PSs for the verification of their molecular mass after covalent conjugation reactions with different moieties. We detected the corresponding molecular mass ions using the matrix-assisted laser desorption ionization (MALDI-TOF) mass spectroscopy technique, where 3,5-dimethoxy-4-hydroxycinnamic acid was used as the matrix material. As a result, we were able to observe several molecular mass ions at *m/z* 1059–1061 (MH^+), 1545 (MH^+), 2045 (MH^+), and 2525 (M^+), respectively, for these four precursor compounds that indicated successful conjugation reactions of both pheophorbide and chlorin-e_6 ring moieties with either pentacationic N_5^+-arm(s) or the vancomycin unit. Apparently, the facile loss of vancomycin moiety occurred to give fragmented mass ions at *m/z* 1120 after bond cleavage shown in Figure S6 of supporting information. This substantiated the *m*Phe–Van conjugation. Low versatility of polycationic Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, Zn-*m*Chl-N_{15}^+, and Zn-Van-*m*Phe-N_5^+ prohibited their detection by MALDI-TOF mass spectroscopy.

The biological antimicrobial aPDI efficacy of Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, and Zn-*m*Chl-N_{15}^+ is critically dependent on the photophysical characteristics that enable the efficient generation of singlet oxygen ($^1O_2$) upon illumination in physiologic media, therefore, we carried out the evaluation of their UV-vis absorption and $^1O_2$ production with the results shown in Figures 1 and 3, respectively. Steady-state photophysical absorption of all compounds is originated mainly from a nearly identical 18 π-conjugated ring system of either pheophytin or chlorin core. A close resemblance of peak profiles among them is expected. This is the case showing the major Soret (excited $S_o \rightarrow S_2$ transition) absorption band centered at 412 nm along with two Q_x transition bands at 506 and 533–535 nm and two Q_y transition bands (excited $S_o \rightarrow S_1$ transitions) at 607–609 and 666–667 nm (Figures 1a–1c). The later bands were in an extinction coefficient around 30–33% lower than those of the former band at 412 nm. Both Soret and Q bands arise from π–π* transitions that can be correlated to their frontier HOMO and LUMO orbitals. A large number of reports on the study of lifetime and quantum yield of fluorescence and quantum yields of triplet state and singlet oxygen of pheophorbide and chlorin derivatives were reviewed recently with their correlation to photodynamic therapeutic (PDT) effects.[18] In the case of Van-Zn-*m*Phe-N_5^+ (Figure 1d), the extinction coefficients of both Soret and Q_x bands were reduced slightly with a small blue-shift of the former band to 406 nm. More significantly, intensity of the Q_y transition band was red-shifted to 672 nm with a large reduction of absorption intensity ($\varepsilon = 2.4 \times 10^3$ L·mol^{-1}·cm^{-1}). It is clearly indicative of partial disruption of long-range π–π* transitions of the mesopheophorbide ring system caused by the covalently bound bulky vancomycin moiety in the vicinity. This may result in the alteration of the long-range co-planarity thus reducing long wavelength absorptions.

To confirm the nature of the transitions in the absorption spectra, density functional theory (DFT) quantum-chemical calculations were performed on a simplified system (Figure 2). The highest occupied and lowest unoccupied orbitals (HOMO and LUMO) correspond with the $S_0 \rightarrow S_1$ transition ($\pi \rightarrow \pi$* type) with a predicted energy gap of 1.61 eV, in reasonable agreement with that observed in the absorption spectrum of 1.86 eV (667 nm). The predicted energy gap for the $S_0 \rightarrow S_2$ transition ($\pi \rightarrow \pi$* type) is 2.47 eV, which is somewhat lower than the observed $S_0 \rightarrow S_2$ gap of 3.01 eV (412 nm). Furthermore, the presence of the pendant vinyl moiety in Zn-Phe-N_5^+ and Zn-Chl-N_{10}^+ does not affect the absorbance measurably as compared with the saturated analogs and is predicted by DFT (see supporting information, Figure S9). Additionally, having an ammonium cation present is predicted to not alter the $S_0 \rightarrow S_1$ measurably as observed from the absorption spectra.

ROS Detection by Fluorescent Probes. Facile intersystem crossing efficiency from the singlet excited states ^{1}(Zn-Phe-N_5^+)*, ^{1}(Zn-Chl-N_{10}^+)*, ^{1}(Zn-*m*Chl-N_{15}^+)*, and ^{1}(Van-Zn-*m*Phe-N_5^+)* to their corresponding triplet excited states ^{3}(Zn-Phe-N_5^+)*, ^{3}(Zn-Chl-N_{10}^+)*, ^{3}(Zn-*m*Chl-N_{15}^+)*, and ^{3}(Van-Zn-*m*Phe-N_5^+)* accounted for the efficient production of $^1O_2$ in Type-II triplet energy-transfer processes and other types of ROS in biological media. We selected two reliable fluorescent (FL) probes for the detection of, specifically, $^1O_2$ and $O_2^{-}\cdot$ in different experiments in deionized water–DMSO at a concentration of 5.0×10^{-6} M of all compounds. For the detection of $^1O_2$, a highly fluorescent tetrasodium *α*,*α*'-(anthracene-9,10-diyl)bis(methylenemalonic acid salt) (ABMA) was used as the reactive probe.[14] The chemical trapping of $^1O_2$ by highly fluorecent ABMA leads to the formation of non-fluorescent 9,10-endoperoxide product ABMA-O_2. The conversion allows us to follow the loss of fluorescence emission intensity at 429 nm that can then be correlated to the proportional quantity of $^1O_2$ produced. Due to the high kinetic rate of the trapping reaction in aqueous media, we assume the internal decay of $^1O_2$ in the solvent is negligible and identical among different probe experimental runs.

Experimentally, an aqueous solution of the sample was irradiated by a white LED light for a pre-defined exposure period (fluence) first, followed by fluorescence measurement to collect the data, as shown in Figure 3A. They were calibrated by the probe solution without photosensitizers (blank, Figure 3Aa). Fluorescent emission (λ_{em} 428 nm) spectra of ABMA were recorded upon excitation at 350 nm (λ_{ex}). Over a white LED illumination period of 250 s, slight photodegradation of the ABMA probe in H_2O was observed. In the presence of Zn-Phe-N_5^+ (Figure 3Ab), Zn-Chl-N_{10}^+ (Figure 3Ac), Zn-*m*Chl-N_{15}^+ (Figure 3Ad), and Van-Zn-*m*Phe-N_5^+ (Figure 3Ae), all four samples photoinduced a rapid reduction in ABMA fluorescence in response to a cumulative increase in light fluence, in contrast to the blank sample in Figure 3Aa. In the short irradiation period (less than 25 s), all decay profiles were almost linear in respect to the cumulative fluence indicating the approximately linear production of $^1O_2$ without much photodegradation of PSs. At the later stage of irradiation, the decay rate became nonlinear and decreased in the order Zn-*m*Chl-N_{15}^+ > Zn-Chl-N_{10}^+ ≈ Van-Zn-*m*Phe-N_5^+ > Zn-Phe-N_5^+ indicating the same order of singlet oxygen production efficiency using white-light excitation, having the highest efficiency for Zn-*m*Chl-N_{15}^+. The nonlinearity may be caused by progressive decrease of the ABMA concentration in solution. In the cases of former three compounds, the fluorescence intensity reached nearly zero within 150 s revealing a high production rate of $^1O_2$. Apparently, higher water-solubility of this type of derivatives possessing hydrophilic moieties more than one pentacationic arm is able to enhance the molecular dispersion and minimize the compound aggregation in H_2O. In general, aggregation

phenomena of hydrophobic planar pheophorbide and chlorin rings in H_2O were regarded as the cause of "self-quenching" during photophysical processes that can significantly reduce the quantity of the effective triplet energy for exchange with molecular oxygen and diminish the production quantity of $^1O_2$. Accordingly, a slightly slower fluorescence reduction rate of ABMA in Figure 3Ab may reveal a higher tendency of Zn-Phe-N_5^+ (with only one pentacationic arm) to aggregate as compared with the other three analogous compounds.

We previously reported the possibility to achieve a Type-I photomechanism in the solution of water-soluble fullerene derivatives $C_{60}[>M(C_3N_6^+C_3)_2]$-$(I^-)_{10}$ *via* photoinduced electron-transfer from iodide anions to the excited fullerene cage moiety leading to, eventually, the formation of $O_2^{-}\cdot$.[19] It has also been reported that the iodide anion (I^-) was capable of initiating a reaction with $^1O_2$ to form several types of ROS, including $O_2^{-}\cdot$, H_2O_2, and HO·.[20] The reaction was associated with a plausible mechanism of electron-transfer event from I^- to $^1O_2$ that led to the possible formation of $O_2^{-}\cdot$. Therefore, we performed a similar experiment to monitor the production of $O_2^{-}\cdot$ by the same series of photosensitizers to address whether it is the case. A regioisomeric mixture of superoxide radical-reactive fluorescent probe, potassium bis(2,4-dinitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein-10'(or 11')-carboxylate isomers (DNBs-TFFC) were used for this purpose. High reaction selectivity of this probe toward $O_2^{-}\cdot$ was reported with a $O_2^{-}\cdot/^1O_2$ sensitivity ratio of 46.[21] However, possibility of DNBs-TFFC to undergo photodegradation is much higher than that of ABMA. Thus, we applied a dialysis film with the molecular weight cut-off (MWCO) of 100−500 Daltons to separate the solution of PSs (in the upper portion of the cuvette) from the aqueous $O_2^{-}\cdot$-probe solution (in the lower portion of the cuvette). We allowed only the upper solution portion of photosensitizers in the membrane sack to be subjected to white LED light exposure. Upon rapid stirring of the solution, $O_2^{-}\cdot$ produced in the sack was able to diffuse into the lower solution portion through the membrane to react with the probe molecules. The reaction initiated the desulfonylation of DNBs-TFFC and yield the corresponding bisphenol intermediate. Rearrangement of this bisphenol intermediate *via* ring-opening of the lactone moiety resulted in highly fluorescent potassium 2',4',5',7'-tetrafluorofluorescein-10'-carboxylate regioisomers (TFFC), as shown in Figure 2A. The compound TFFC emits fluorescence at 530 nm by photoexcitation at 480 nm. Since DNBs-TFFC is a non-fluorescent agent, the detected fluorescence intensity counts can be correlated directly to the quantity of TFFC produced. This, in terms, was used for the calculation of relative $O_2^{-}\cdot$ production among four photosensitizers studied.

As shown in Figure 3Ba, the $O_2^{-}\cdot$-probe itself displayed only a slight increase of fluorescence emission over a white LED light irradiation period of 60 min in the absence of PSs, indicating a

low degree of photoinduced desulfonylation (or photohydrolysis) of DNBs-TFFC in aqueous solution. By using this curve profile as the reference for comparison, all compounds of Zn-Phe-N_5^+ (Figure 3Bb), Zn-Chl-N_{10}^+ (Figure 3Bc), Zn-*m*Chl-N_{15}^+ (Figure 3Bd), and Van-Zn-*m*Phe-N_5^+ (Figure 3Be) produced roughly similar intensity of fluorescence over the same irradiation period of 60 min with the fluctuation of only ±1% from that of Figure 3Ba. Therefore, we concluded that no significant production of $O_2^{-}\cdot$ was observed regardless the variation of PSs. It also revealed a negligible electron-transfer reaction of iodide ions with $^1O_2$ in forming $O_2^{-}\cdot$.

Relative Efficacy of Zn-Phe-N_5^+, Zn-Chl-N_{10}^+ and Zn-*m*Chl-N_{15}^+ as Antimicrobial PSs. Gram-positive MRSA was very susceptible to light mediated killing, particularly, with Zn-Phe-N_5^+ with 5 cationic charges. A fluence of 20 J/cm^2 of 415-nm light gave complete eradication using a concentration as low as 100 nM, and only 10 J/cm^2 fluence was required in conjunction with 500 nM concentration (Figure 4A). The second compound Zn-Chl-N_{10}^+ with 10 cationic charges was less effective; a concentration of 500 nM was needed to be excited by a light fluence of 20 J/cm^2 to produce the same eradication effect (Figure 4B). The third compound Zn-*m*Chl-N_{15}^+ having 15 cationic charges was the least effective one giving 5-logs of killing (Figure 4C), without full eradication being obtained. Therefore, the order of effectiveness can be summarized as Zn-Phe-N_5^+ > Zn-Chl-N_{10}^+ > Zn-*m*Chl-N_{15}^+.

Gram-negative *E. coli* was much less susceptible overall as compared to those of MRSA. The least effective compound Zn-Phe-N_5^+ gave very little killing efficacy (only around 1.0-log) even at a concentration as high as 100 μM (Figure 5A). The compound Zn-Chl-N_{10}^+ with 10 cationic charges was somewhat better, with more than 4.0-logs of killing efficacy being achieved using a combination of 100 μM and 20 J/cm^2 (Figure 5B). The most effective compound was Zn-mChl-N_{15}^+ having 15 cationic charges, which was able to give complete eradication with a combination of only 50 μM and 20 J/cm^2 (Figure 5C). The order of effectiveness in this experiment was summarized as Zn-*m*Chl-N_{15}^+ > Zn-Chl-N_{10}^+ > Zn-Phe-N_5^+; this order is the opposite of that found in the case of MRSA.

In the case of fungal yeast *C. albicans*, it was more sensitive than *E. coli*, but still much more resistant than MRSA. The compound Zn-Phe-N_5^+ with 5 cationic charges was just able to achieve eradication with a combination of 100 μM and 20 J/cm^2 (Figure 6A). By contrast, Zn-Chl-N_{10}^+ was much more effective giving eradication under conditions of 10 μM and 20 J/cm^2 (Figure 6B). Zn-*m*Chl-N_{15}^+ was considered to be the most effective achieving eradication with 50 μM and a fluence of 10 J/cm^2 (Figure 6C). Thus, the order of effectiveness was similar to that found with *E. coli* as Zn-*m*Chl-N_{15}^+ > Zn-Chl-N_{10}^+ > Zn-Phe-N_5^+ and different from the order found with MRSA.

We carried out the studies with a blue LED array (415±15 nm) that matched with the absorption Soret band wavelength of all Phe and chlorin derivatives investigated (Figure 1). We expected that high wavelength coherence and overlap between the light emission for excitation and PS absorption for $^1O_2$ production should facilitate efficient killing. Meanwhile, for the similar reason to match with the absorption energy of Q_y bands in the spectrum of Phe/chlorins, we also performed replicated studies using a red light (660±15 nm) for photoexcitation. We considered that if the compounds were to be used for the PDT treatment of localized infections, it is likely that the better penetration of red light into tissue would be a beneficial property. The data is summarized and shown in Figures S8(A–I). As we mentioned above, extinction coefficient of the long Q_y band in red region is roughly 33% in intensity of that of the Soret band in the blue region. This can be the explanation why the killing of red light is less than that found with blue light. We observed less killing with red light as compared to that found using blue light (Figures 4–6). Even though, the killing curves still assumed the same overall relative shapes as those obtained with the blue light.

Investigations with Van-Zn-*m*Phe-N_5^+ in Vitro. We first asked whether the antibiotic activity of vancomycin was retained in the Van-Zn-Phe-N_5^+ conjugate. To do this, we compared inhibitory activity against MRSA, two different strains of vancomycin-sensitive *E. fecium*, and two different vancomycin-sensitive strains of *E. fecium*. We used a standard broth-microdilution technique to determine minimum inhibitory concentration (MIC). Table 1 shows that while both VRE strains were indeed vancomycin resistant, VRE1 was significantly more resistant than VRE2. However, the Van-Zn-Phe-N_5^+ conjugate was almost completely inactive as a traditional antibiotic. There was hardly any difference between the MIC values of Van-Zn-Phe-N_5^+ and Zn-Phe-N_5^+.

We next asked whether there was any evidence of the Van-Zn-*m*Phe-N_5^+ conjugate being able to target Gram-positive bacteria to deliver aPDI, and how this compound compared with the non-targeted Zn-Phe-N_5^+. In Figure 6A, it can be seen that both vancomycin-resistant VRE strains were significantly more easily killed by the Van-Zn-*m*Phe-N_5^+ conjugate excited by blue light than the VSE-sensitive strains. However, when we conducted the control experiment (Figure 7B) with non-targeted Zn-Phe-N_5^+, it became clear that the VRE strains we had chosen were both intrinsically more susceptible to aPDI than the VSE strains and this difference was statistically significant.

Discussion of Biological Data. It is known that cationic groups are required to produce highly active antimicrobial PSs. Moreover, cationic groups also provide water solubility for hydrophobic tetrapyrrole backbones such as the present chlorin derivatives. Green chemistry

approaches, which take advantage of the rich variety of naturally occurring molecular frameworks, such as chlorophyll, have risen in popularity in recent years.[22-24] In the present study, our development of synthetically feasible methods for attachment of either 1, 2, or 3 of pentacationic quaternary ammonium arms to the [Zn^{+2}]pheophorbide and [Zn^{+2}]chlorin nucleus formed a panel of multicationic PSs with either 5, 10, or 15 discrete cationic charges in Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, or Zn-*m*Chl-N_{15}^+, respectively. We excited these PSs both with blue light (415±15 nm) or red light (660±15 nm). As might be expected from the absorption spectra, there was more bacterial killing with blue light compared to that found with red light (compare Figures 4, 5, and 6 with Figure S1). If these compounds are to find any practical application in the PDT treatment of actual localized infections, it is likely that the better penetration of red light into tissue would be a beneficial property.

The Gram-positive MRSA was more easily killed by all the compounds, compared to the Gram-negative *E. coli* and the fungal yeast *C. albicans.* MRSA was eradicated by a very low concentration (100 nM) of Zn-Phe-N_5^+, and the order of activity was Zn-Phe-N_5^+ > Zn-Chl-N_{10}^+ > Zn-*m*Chl-N_{15}^+. It has been previously reported that highly active antimicrobial PS can kill Gram-positive bacteria at comparably concentrations as low as 5–10 nM.[25] Amphiphilicity, as well as an overall cationic charge, is a highly beneficial attribute in PS, in order to provide killing of Gram-positive bacteria. The mono-substituted cationic bacteriochlorins (BCs) described in Huang *et al*[26] had much higher activity against MRSA (100–200 nM), than broadly comparable symmetrically disubstituted BCs that required concentrations of about 1.0 μM.[27]

In sharp contrast to MRSA, the Gram-negative *E.coli* was much less susceptible to aPDI. Only Zn-*m*Chl-N_{15}^+ with 15 cationic charges was able to produce total eradication and that needed a relatively high concentration of 50 μM and the higher light dose. The compounds Zn-Phe-N_5^+ and Zn-Chl-N_{10}^+ were much less active, with Zn-Phe-N_5^+ giving only about 2 logs of killing even at 100 μM. The order of activity was opposite of that found with MRSA, with the 15-charge compound being best against *E. coli* and worst against MRSA*;* Zn-*m*Chl-N_{15}^+ > Zn-Chl-N_{10}^+ > Zn-Phe-N_5^+. Therefore, the relationship between increasing numbers of cationic charges and increasing activity was as we expected with *E. coli.* It should be noted that the differences between Gram-positive and Gram-negative bacterial cells are more complicated than just the structure of the outer-membrane alone. Gram-positive cell walls have a thick multi-layered peptidoglycan structure with a low lipid content and a high proportion of murein. By contrast, Gram-negative cells have a thinner peptidoglycan layer with more lipid and less murein.

However, it cannot be denied the overall level of activity against *E. coli* was rather disappointing. If we look at the relative ability of the BCs described in the previous studies[26, 27] to kill MRSA and *E. coli*, we can see remarkable differences. In the case of the mono-substituted BCs, the concentrations needed for MRSA and *E. coli* were 100–200 nM and 1.0–5.0 μM, respectively (approximately 1:10).[26] While in the case of the symmetrically di-substituted BCs the concentrations needed for MRSA and *E. coli* were 1.0 μM and 1.0–10 μM, respectively (again approximately 1:10 or even lower).[27] Here in the present study, the ratio between the concentrations needed for MRSA and *E. coli* were 100 nM and 40–100 μM respectively (approximately 1:400 or even higher). How can we explain these large differences in the ratio? Perhaps the answer lies in the molecular weights of the compounds. In the previous study[27] with di-substituted BCs there was a big increase in activity against *E. coli* with increasing numbers of cationic charges (6 >> 4 > 2 > 0). However due to differences in the molecular structure and how the quaternary nitrogen groups were attached to the tetrapyrrole nucleus, the molecular weights were: 1762 (6 charges), 1364 (4 charges), 882 (2 charges) and 604 (0 charges). In the study[26] with mono-substituted BCs (that were more active than the disubstituted compounds) the molecular weights were: 569 (1 charge), 853 (2 charges), and 653 (1 charge). In the present case the molecular weights were 1198, 1758, 2334, and 2665 for Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, Zn-*m*Chl-N_{15}^+, and Van-Zn-*m*Phe-N_5^+, respectively. If it is indeed the case that the molecular weight makes much less difference for Gram-positive bacteria with their porous outer cell wall structure, than it does for Gram-negative bacteria with their highly impermeable outer membrane structure, then we may have the answer. Even if the compounds possess a large number of cationic charges (for example as many as 15), then the very high molecular weight may still present a major barrier to diffusion through the cell wall. A report described the "molecular-sieving" function of the Gram-negative Salmonella cell wall with a cut-off estimated at 650 Da.[28] Younes *et al* showed that the antibacterial activity of a range of chitosan derivatives against Gram-negative species inversely depended on the molecular weight.[29] Furthermore, in our cases, the relatively low aPDI activity was associated only with the sample of Van-Zn-*m*Phe-N_5^+. In the chemical consideration, a close covalent-bond linkage between the Phe core and vancomycin moieties in structure, the large macrocyclic ring of vancomycin unit can induce significant steric hindrance on the conjugation ring of the Phe moiety that reduces co-planarity of π-conjugation and, thus, optical absorption extinction coefficient of the Phe ring. This may serve as the primary reason to result in the alternation and photoactivity reduction.

With regard to the activity against *C. albicans,* the relative activity increased as the number of cationic charges increased. This was somewhat surprising, as our studies with cationic BCs had shown that compounds with fewer cationic charges were better at killing fungal cells.[27] We were also somewhat surprised and disappointed by the lack of activity of our conjugate between Zn-Phe-N_5^+ and vancomycin. If out theory is correct that higher molecular weights correlate with lower antibacterial photodynamic activity, then we could explain the lower aPDI activity of Van-Zn-*m*Phe-N_5^+ (M.W. 3209) compared to Zn-Phe-N_5^+ (M.W. 1760).

There have been several studies that have reported conjugates between various PS and vancomycin for the purposes of targeting Gram-positive bacteria. Xing *et al* prepared conjugates between a porphyrin and either one or two vancomycin molecules.[30] They found that the conjugate with two vancomycin molecules (and to a lesser extent the conjugate with one vancomycin) mediated more aPDI killing of both VRE and VSE compared to porphyrin alone. Gao *et al* conjugated fluorescein to vancomycin and showed that the product could fluorescently label and photo-inactivate Gram-positive *Bacillus subtilis,* but not *E. coli* or VRE.[31] Choi and co-workers attached vancomycin and a platinum porphyrin to iron oxide nanoparticles to prepare a targeted magnetic antimicrobial PS.[32] Feng *et al*[33] conjugated an "aggregation-induced emission fluorogen[34] based on a perylene derivative, to vancomycin to produce a compound that could fluorescently label, and also mediate aPDI against *B. subtilis*, VRE and VSE.

In our studies it must be the case that conjugation of the relatively large Zn-*m*Phe-N_5^+ destroyed the ability of the vancomycin moiety to recognize the $_D$-Ala-$_D$-Ala motif to which the antibiotic binds on the Gram-positive bacterial cell wall. This loss of binding activity is consistent with the lack of antibiotic activity found in the MIC assay. An initial finding that somehow the Van-Zn-*m*Phe-N_5^+ might indeed have some selective activity against VRE compared to VSE, was disproved, when it was found that our two VRE strains were intrinsically more sensitive compared to the VSE strains, to aPDI with the non-vancomycin containing Zn-Phe-N_5^+.

Furthermore, our functionalization of the chlorin ring did not alter the basic π-conjugation of central ring system, as shown by similar optical absorptions to those of other reported chlorin derivatives.[35-38] In principle, the ROS production efficiency should be similar as well to those corresponding known compounds. The difference of observed in vitro aPDI efficacy in our experiments led us to conclude that some important structure-function relationships, in the design of antimicrobial PSs, in our present studies should be highlighted. The overall molecular weight and interaction forces between the PS and the cell wall surface are important factors in addition to the number of cationic charges, and also a degree of molecular asymmetry.

■ EXPERIMENTAL SECTION

Materials. Reagents of trifluoroacetic acid, $Zn(OAc)_2 \cdot 2H_2O$, iodomethane (CH_3I), *m*-chloroperoxybenzoic acid (MCPBA), sodium borohydride, pentaethylene hexamine, *n*-propionaldehyde, and potassium carbonate were purchased from Aldrich Chemicals and used without further purification. Chlorella powder was purchased from Cellusyn Labs, LLC. Vancomycin hydrochloride was purchased from Gold Biotechnology. Solvents were routinely distilled prior to the use. The reagent *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine was prepared by a routine protection-deprotection reaction [39] of one primary amine group of pentaethylene hexamine. It was followed by the conversion of 1° or 2° alkylamines to tertiary amines using *n*-propionaldehyde, as the capping agent, and sodium borohydride as the reducing agent.[40]

Characterization. Infrared spectra were recorded as KBr pellets on a Thermo Nicolet Avatar 370 FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Bruker Avance Spectrospin–500 spectrometer. UV-vis spectra were recorded on a Perkin Elmer Lambda 750 UV-vis-NIR Spectrometer. Mass spectroscopic measurements were performed by the use of positive ion matrix-assisted laser desorption ionization (MALDI–TOF) technique on a micromass M@LDI-LR mass spectrometer. The sample blended or dissolved in the matrix material was irradiated by nitrogen UV laser at 337 nm with 10 Hz pulses under high vacuum. Mass ion peaks were identified for the spectrum using the MassLynx v4.0 software.

Preparation of Pheophytin-a. Chlorella powder (broken cell wall, 120 g) was washed with water (500 mL) and 50% ethanol in water (300 mL) to remove polar materials. The residue was extracted twice with a mixture of dichloromethane–ethanol (1:2, v/v, 500 mL) to give a chlorophyll rich fraction. After evaporating the solvents, raw chlorophyll materials were collected as green semi-solids. They were then stirred in a mixture of HCl (1.0 N)–ethanol (1:1, v/v) for 3.0 h at r.t. to afford pheophytin mixtures in the form of precipitates. They were dissolved in dichloromethane, washed with distilled water, dried over Na_2SO_4, rotary-evaporated to dryness, and purified by column chromatography (neutral alumina) using a gradient eluent in a composition from hexane–dichloromethane (7:3, v/v) to 100% dichloromethane. A major green band was collected and evaporated to dryness to afford green viscous semi-solids (5.8 g) of pheophytin-*a*. Spectroscopic data: FT-IR (KBr) ν_{max} 3437.1 (s), 3396.5 (s), 2951.7 (s), 2924.0 (s), 2865.7 (m), 1736.2 (s), 1700.7 (s), 1618.4 (m), 1579.8 (w), 1551.5 (m), 1535.2 (w), 1499.1 (m), 1460.0 (m), 1451.1 (m), 1439.9 (m), 1400.6 (w), 1377.6 (m), 1365.6 (m), 1346.5 (m), 1296.6 (w), 1221.9 (m), 1159.9 (m), 1122.0

(w), 1198.1 (w), 1059.9 (w), 1033.9 (m), 984.9 (m), 965.9 (m), 909.5 (w), 895.0 (w), 842.4 (w), 813.5 (w), 784.2 (w), 770.0 (w), 750.8 (w), 730.8 (m), 717.4 (w), 669.6 (m), and 604.6 (m) cm^{-1}; UV–vis (dichloromethane, 1.0×10^{-5} M) λ_{max} (ε) 405 nm (2.8×10^4), 504 nm (4.5×10^3), 539 nm (2.7×10^3), 610 nm (2.4×10^3), 668 nm (1.3×10^4), and 697 nm (3.9×10^3 $L \cdot mol^{-1} \cdot cm^{-1}$); ^1H NMR (500 MHz, $CDCl_3$, ppm) δ 9.58 (s, 1H), 9.45 (s, 1H), 8.61 (s, 1H), 8.03 (1H), 6.35 (1H), 6.30 (s, 1H), 6.22 (1H), 5.17 (1H), 4.52 (3H), 4.25 (1H), 3.91 (s, 3H), 3.73 (5H), 3.44 (3H), 3.28 (3H), 2.65 (1H), 2.52 (1H), 2.37 (1H), 2.25 (1H), 1.90 (2H), 1.83 (3H),1.69 (3H), 1.57 (3H), 0.94–1.45 (19H), and 0.76–0.94 (m, 12H).

Synthesis of 15b-methyl-17c-[N,N',N,N,N,N-hexapropyl-penta(aminoethyl) amide]-[Zn+2]pheophorbide a (Zn-Phe-N_5^+). The compound was first synthesized through the trans-amination of pheophytin-*a* with *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine, followed by the insertion of Zn^{2+} and quaternization with CH_3I. Briefly, to the solution of pheophytin-*a* (0.14 g, 0.16 mM) in dichloromethane (20 mL), was added *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine (0.082 g, 0.17 mmol) and stirred under N_2. Trifluoroacetic acid (0.14 mL, 1.8 mmol) was slowly added and the mixture was stirred at r.t. for 10 h. After evaporating the solvent, the product obtained was neutralized by saturated K_2CO_3 in DMF, and then purified by column chromatography (neutral alumina) using the eluent of dichloromethane–acetone (1:2), to afford green solids of the precursor intermediate Phe-N_5 in a yield of 82% (0.14 g). It was then treated with $Zn(OAc)_2 \cdot 2H_2O$ (0.035 g, 0.16 mmol) in DMF overnight, followed by reacting with a large excess amount of CH_3I at 45 °C for 3 d to form the product Zn-Phe-N_5^+. After evaporating the excessive amount of CH_3I using the rotavapor, it was precipitated by the addition of ethyl acetate, washed with methanol to remove the small amount of unreacted $Zn(OAc)_2$, and dried in vacuum to obtain Zn-Phe-N_5^+ as green solids in a yield of 88% (calculated from the amount of Phe-N_5 used). Spectroscopic data of Zn-Phe-N_5^+: FT-IR (KBr) ν_{max} 2972.6 (m), 2941.3 (w), 2880.7 (w), 2853.6 (w), 1732.2 (s), 1675.9 (s), 1618.4 (m), 1579.8 (w), 1551.5 (m), 1535.2 (w), 1499.1 (m), 1464.9 (m), 1451.1 (w), 1431.6 (m), 1400.5 (w), 1372.6 (m), 1368.6 (m), 1350.5 (m), 1231.9 (m), 1165.9 (m), 1198.1 (w), 1061.9 (w), 1035.9 (m), 986.9 (m), 966.9 (m), 895.0 (w), 845.4 (w), 817.5 (w), 784.0 (w), 765.0 (w), 758.8 (w), 738.8 (m), 669.6 (m), and 604.6 (m) cm^{-1}; UV–vis (DMF, 1.0×10^{-5} M) λ_{max} (ε) 412 nm (4.3×10^4), 506 nm (4.7×10^3), 535 nm (3.8×10^3), 560 (1.9×10^3), 607 nm (3.0×10^3), and 667 nm (1.5×10^4 $L \cdot mol^{-1} \cdot cm^{-1}$); ^1H NMR (500 MHz, DMSO-d_6, ppm) δ 9.54 (s, 1H), 9.29 (s, 1H), 8.32 (s, 1H), 7.98 (1H), 6.01–6.24 (m, 3H), 2.51–4.46 (m, br, 61H), 2.52 (2H), 2.34 (2H), 2.06 (2H), 1.58–2.04 (m, br, 18H), and 0.98–1.08 (m, 18H). ^1H NMR spectrum of Phe-N_5 (500 MHz, $CDCl_3$, ppm) δ 9.50 (s, 1H), 9.21 (s, 1H), 8.62 (s, 1H), 8.00 (1H),

6.00–6.22 (m, 3H), 4.52 (2H), 3.90 (s, 3H), 2.29–3.73 (m, br, 43H), 2.47 (2H), 2.06 (2H), 1.38–1.86 (m, br, 18H), and 0.93 (m, 18H).

Synthesis of 15b-methyl-13a,17c-di[N,N',N,N,N,N-hexapropyl-penta(aminoethyl) amide]-[Zn^{+2}]chlorin e_6 (Zn-Chl-N_{10}^{+}). It was found earlier that the exocyclic ring moiety of the pheophytin-*a* can be easily opened by primary amines.[41, 42] According to this reaction mechanism, the compound was prepared by treating the intermediate Phe-N_5 with *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine in dichloromethane at r.t., followed by the insert of Zn^{2+} and quaternization with CH_3I. Briefly, to the solution of Phe-N_5 (0.15 g, 0.14 mmol) in dichloromethane (20 mL) was added *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine (0.10 g, 0.21 mmol) and stirred at r.t. overnight. The resulting raw products were purified by column chromatography (neutral alumina) using the eluent of dichloromethane–acetone (1:1) to afford green solids of the precursor intermediate Chl-N_{10} in a yield of 77% (0.17 g).

A similar procedure as that described for the preparation of Zn-Phe-N_5^+ from Phe-N_5 (above) was applied to obtain Zn-Chl-N_{10}^+ as green solids in a yield of 92% (calculated from the amount of Chl-N_{10} used). Spectroscopic data of Zn-Chl-N_{10}^+: FT-IR (KBr) ν_{max} 2997.7 (w), 2966.6 (m), 2928.3 (w), 2874.7 (w), 2849.6 (w), 1732.2 (s), 1663.9 (s), 1632.0 (m), 1622.4 (m), 1586.8 (w), 1549.5 (m), 1504.1 (m), 1467.9 (s), 1431.6 (m), 1389.6 (m), 1375.6 (m), 1357.5 (m), 1231.9 (m), 1176.9 (m), 1210.1 (w), 1062.9 (w), 1035.9 (m), 988.9 (m), 966.9 (m), 895.2 (w), 845.9 (w), 819.5 (w), 784.5 (w), 762.0 (w), 760.8 (w), 740.0 (m), 670.1 (m), and 602.2 (m) cm^{-1}; UV–vis (DMF, 1.0×10^{-5} M) λ_{max} (ε) 412 nm (4.2×10^4), 506 nm (5.2×10^3), 534 nm (4.5×10^3), 560 (2.7×10^3), 607 nm (3.4×10^3), and 666 nm (1.4×10^4 $L \cdot mol^{-1} \cdot cm^{-1}$); ^1H NMR (500 MHz, DMSO-d_6, ppm) δ 9.54 (s, 1H), 9.29 (s, 1H), 8.32 (s, 1H), 7.98 (1H), 6.19 (1H), 6.01 (1H), 3.10–4.46 (m, br, 110H), 2.52 (2H), 2.45 (2H), 2.34 (2H), 1.58–1.90 (m, br, 30H), and 1.08 (m, 36H). ^1H NMR spectrum of Chl-N_{10} (500 MHz, $CDCl_3$, ppm) δ 9.60 (s, 1H), 9.48 (s, 1H), 8.63 (s, 1H), 8.04 (1H), 6.31 (1H), 6.20 (1H), 4.48 (2H), 2.29–4.00 (m, br, 80H), 2.50 (2H), 2.06 (2H), 1.73 (m, br, 30H), and 0.94 (m, 36H).

Synthesis of 3a-hydroxy-15b-methyl-3b,13a,17c-tri[N,N',N,N,N,N-hexapropyl-penta(aminoethyl)amide]-[Zn^{+2}]mesochlorin e_6 (Zn-mChl-N_{15}^{+}). To the solution of the intermediate Chl-N_{10} (0.1 g, 0.065 mmol) in THF was added *meta*-chloroperoxybenzoic acid (MCPBA, 0.013 g, 0.075 mmol) to perform the epoxidation of the olefin bond located at the opposite side to the amide moieties of the chlorin-e_6 core structure. *N,N',N,N,N,N*-hexapropyl-penta(aminoethyl)amine (0.035 g, 0.073 mmol) was then added to react with the resulting epoxide with the catalyst of di(*n*-butyl)tin(IV) dilaurate (T-12, 70 μL) and triethylamine (70 μL, 0.5 mmol).

After collected by evaporating the solvents and purified by column chromatography (neutral alumina) using the eluent of dichloromethane–methanol (1:1), the precursor intermediate *m*Chl-N_{15} was obtained as green solids in a yield of 67% (0.085 g).

A similar procedure as that described for the preparation of Zn-Phe-N_5^+ from Phe-N_5 (above) was applied to obtain Zn-*m*Chl-N_{15}^+ as green solids in a yield of 92% (calculated from the amount of *m*Chl-N_{15} used). Spectroscopic data of Zn-*m*Chl-N_{15}^+: FT-IR (KBr) ν_{max} 3002.7 (m), 2971.6 (m), 2928.6 (w), 2876.7 (w), 2849.8 (w), 1735.2 (m), 1668.0 (s), 1635.2 (m), 1582.8 (w), 1555.5 (m), 1500.1 (m), 1463.3 (s), 1436.3 (m), 1390.6 (m), 1375.6 (w), 1365.5 (m), 1234.9 (w), 1174.6 (m), 1213.2 (w), 1063.9 (w), 1035.5 (m), 988.9 (m), 966.4 (w), 891.2 (w), 842.9 (w), 820.6 (w), 779.5 (w), 760.1 (w), 742.0 (m), 670.4 (m), and 602.2 (m) cm^{-1}; UV–vis (DMF, 1.0×10^{-5} M) λ_{max} (ε) 412 nm (3.8×10^4), 506 nm (4.3×10^3), 533 nm (2.9×10^3), 560 (1.6×10^3), 609 nm (2.4×10^3), and 666 nm (1.3×10^4 $L \cdot mol^{-1} \cdot cm^{-1}$); ^1H NMR (500 MHz, DMSO-d_6, ppm) δ 9.54 (s, 1H), 9.29 (s, 1H), 8.32 (s, 1H), 3.10–4.46 (m, br, 164H), 2.45 (2H), 2.34 (2H), 1.58–2.00 (m, br, 42H), and 1.04 (m, 54H). ^1H NMR spectrum of *m*Chl-N_{15} (500 MHz, $CDCl_3$, ppm) δ 9.59 (s, 1H), 9.40 (s, 1H), 8.64 (s, 1H), 4.46 (2H), 2.15–3.78 (m, br, 117H), 2.49 (2H), 2.06 (2H), 1.32–1.89 (m, br, 42H), and 0.89 (m, 54H).

Synthesis of 3a-hydroxy-3b-vancomycin-15b-methyl-17c-[N,N',N,N,N,N-hexapropyl-penta(aminoethyl) amide]-[Zn^{+2}]mesopheophorbide (Van-Zn-mPhe-N_5^+). To the solution of Phe-N_5 (0.20 g, 0.19 mmol) in THF–H_2O (3.0:0.7, v/v) was added *m*-chloroperoxybenzoic acid (MCPBA, 0.035 g, 0.20 mmol) to perform the epoxidation of the olefin bond located at the opposite side to the amide moieties of the pheophytin core structure. Vancomycin hydrochloride (0.29 g, 0.20 mmol) was then added to react with the resulting epoxide with the catalyst of di(*n*-butyl)tin(IV) dilaurate (T12, 150 μL) and triethylamine (150 μL, 1.1 mmol) at 60 ºC for 3.0 h. After collected by evaporating the solvents, the resulting raw products were dissolved in dil. HCl to remove the insoluble 4-chlorobenzoic acid generated during the reaction. The precursor intermediate Van-*m*Phe-N_5 was obtained as green solids after neutralization using a saturated solution of K_2CO_3 in DMF, followed by extracting the product using methanol to remove excessive vancomycin and K_2CO_3. It was then treated with $Zn(OAc)_2 \cdot 2H_2O$ (0.044 g, 0.20 mmol) in DMF overnight, followed by reacting with a large excessive amount of CH_3I at 45 ºC for 3 d to form the product Van-Zn-*m*Phe-N_5^+. After evaporating the excess CH_3I using the rotavapor, it was precipitated by the addition of ethyl acetate, washed with methanol to remove the small amount of unreacted $Zn(OAc)_2$, and dried in vacuum to afford Van-Zn-*m*Phe-N_5^+ as green solids in a yield of 92% (calculated from the amount of Van-*m*Phe-N_5 used). Spectroscopic data: FT-IR (KBr) ν_{max}

2972.8 (m), 2960.0 (w), 2940.3 (w), 2924.6 (w), 2884.7 (w), 2849.6 (w), 1735.2 (m), 1675.9 (s), 1655.0 (s), 1618.4 (m), 1586.9 (m), 1553.5 (m), 1500.2 (m), 1464.9 (m), 1421.1 (m), 1400.5 (w), 1398.1 (m), 1375.6 (m), 1370.6 (m), 1340.5 (m), 1310.4 (w), 1233.2 (m), 1176.9 (w), 1153.2 (w), 1126.7 (w), 1061.9 (m), 1025.9 (m), 990.9 (m), 966.9 (m), 892.0 (w), 848.4 (w), 819.5 (w), 784.0 (w), 763.0 (w), 738.8 (m), 709.6 (m), and 610.6 (m) cm^{-1}; UV-vis (DMF, 1.0×10^{-5} M) λ_{max} (ε) 282 nm (2.5×10^4), 406 nm (2.1×10^4), 512 nm (3.0×10^3), 554 nm (2.4×10^3), 580 nm (2.0×10^3), and 672 nm (2.4×10^3 $L \cdot mol^{-1} \cdot cm^{-1}$).

Computational Methods. All geometries were fully optimized using the M06L functional [15] and the 6-31+G(d,p) basis set for all atoms. The energies were refined by performing single-point calculations employing the def2-TZVPP basis set [43-45] using the same functional. Stationary points were characterized by evaluating the harmonic vibrational frequencies at the optimized geometries. All calculations were performed with Gaussian09 program package.[46]

ROS Measurements. Fluorometric traces were collected using a PTI QuantaMaster™ 40 Fluorescence Spectrofluorometer. The light sources used was an ultrahigh power white-light LED lamp (Prizmatix, operated at the emission peak maxima centered at 451 and 530 nm with the collimated optical power output of >2000 mW in a diameter of 5.2 cm) for the generation of emission spectra at visible light range.

A superoxide radical reactive fluorescent probe DNBs-TFFC was used to detect $O_2^{-}\cdot$ generated in solution. A typical probe solution was prepared by diluting a stock probe solution of DNBs-TFFC in DMSO (5.0 mM) by 300 times with D.I. water. A dialysis film with a molecular weight cut-off (MWCO) of 100−500 Daltons was used to separate the solution of photosensitizers (Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, Zn-*m*Chl-N_{15}^+, and Van-Zn-*m*Phe-N_5^+) in H_2O−DMSO (19:1, 5.0 μM) from the probe solution kept in a cuvette with stirring during the fluorescent measurement. To minimize photodegradation side-reaction of DNBs-TFFC and TFFC, only the photosensitizer solution in the membrane sack was allowed to white LED light exposure (excitation wavelength of 420–650 nm with $\lambda_{em,max}$ at 451 and 530 nm). The quantity of $O_2^{-}\cdot$ generated was correlated and counted proportionately by the measured fluorescence emission intensity of TFFC at 520–530 nm upon excitation at 480 nm. TFFC is a reaction product of DNBs-TFFC probe with $O_2^{-}\cdot$ in solution.

A similar experimental method was applied to detect the generation of singlet oxygen ($^1O_2$) using highly water-soluble ABMA as a fluorescent probe by monitoring the loss of its emission maximum at 429 nm under excitation at 350 nm. The quantity of $^1O_2$ generated was correlated and counted by the relative fluorescence intensity decrease of ABMA at 429 nm.

Cells and Culture Conditions. The following microbial strains were used: Gram-positive bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA) US300; four strains of *Enterococcus fecium* (kind gifts from Prof Eleftherios Mylonakis, Brown University, Providence, RI), vancomycin-resistant *E. fecium* WC176 and WC312, and vancomycin-sensitive *E. fecium* D24 and D25; Gram-negative bacterium: *Escherichia coli* K-12 (ATCC 33780); fungal yeast luciferase-expressing strain of *Candida albicans (CEC 749)*. For bacteria, a single colony was suspended in 5.0 mL of brain heart infusion (BHI) broth and grown overnight in a shaker incubator (New Brunswick Scientific, Edison, NJ) at 120 rpm under aerobic conditions at 37 °C. An aliquot of 1.0 mL from an overnight bacterial suspension was refreshed in fresh BHI broth at 37 °C to mid-log phase. Cell concentration was estimated by measuring the optical density (OD) at 600 nm (OD of 0.6 = 10^8 CFU cells/mL). For fungal yeast, a colony was suspended in 20 mL of yeast extract-peptone-dextrose (YPD) broth and grown overnight in a shaker incubator (New Brunswick Scientific, Edison, NJ) at 120 rpm under aerobic conditions at 30 °C. The fungal yeast cell number was assessed with a hemocytometer.

aPDI Studies. Cells were grown overnight at 37 °C (30 °C for Candida) and bacteria were refreshed in fresh BHI broth for 2.0–3.0 hours before being collected through centrifugation and suspended in phosphate-buffered saline (PBS). A cell suspension consisting of 10^8 cells/mL for bacteria (10^7 cells/mL for Candida) was incubated with various concentrations of the different PS compounds for 30 min at room temperature in the dark, then we used one of two different light sources to illuminate the stirred suspension. For red light, 1.0-mL aliquots were transferred to a 24-well plate and illuminated at room temperature with a red light source (660 nm band-pass filter; Lumacare, Newport Beach, California) to deliver 10 J/cm^2 at an irradiance of 100 mW/cm^2 as measured with a power meter (Coherent, Santa Clara, California); for blue light, 200-uL aliquots were transferred to a 96-well plate and illuminated at room temperature with a blue light source (Omnilux clear-UTM light emitting diode (LED) array (Photo Therapeutics, Inc., Carlsbad, CA) with a central wavelength of 415-nm) to deliver 10 J/cm^2 at an irradiance of 50 mW/cm^2 as measured with a power meter. Cells treated with PS in the dark were incubated covered with aluminum foil for the same time as the PDT groups (30 min).

At the completion of illumination (or dark incubation), aliquots (100 μL) were taken from each well to determine colony-forming units (CFUs). The aliquots were serially diluted 10-fold in PBS to give dilutions of 10^{-1} to 10^{-5} times in addition to the original concentration, and 10-μL aliquots of each of the dilutions were streaked horizontally on square BHI (bacteria) or YPD

(*Candida*) plates by the method of Jett and colleagues.[47] Plates were streaked in triplicate and incubated for 12–36 h at 30 °C (*Candida*) or 37 °C (bacteria) in the dark to allow colony formation.

A control group of cells treated with light alone (no PS added) showed the same number of CFU as absolute control (data not shown). Survival fractions were routinely expressed as ratios of CFU of microbial cells treated with light and PS (or PS in the absence of light) to CFUs of microbes treated with neither.

MIC Determination. The antibacterial activities of vancomycin, Van-Zn-Phe-N_5^+ and Zn-Phe-N_5^+ were determined by the minimum inhibitory concentration (MIC) broth microdilution method[48] using a traditional turbidity endpoint measurement using MRSA, *E. fecium* D24, D25 and WC176, WC312, using sterile 96-well microliter plates to generate a checker-board of 2-fold serial dilutions from column 1 (128 mg/mL) to column 11 (0.125 mg/mL) (column 12 = zero) for these compounds in 50% BHI broth. A bacterial suspension (10 μL containing 10^4 cells) was added to each well, and the plate was incubated at 37 °C for 15 h with vigorous shaking. The bacterial turbidity was monitored with a plate reader (SpectraMax M5 plate reader, Molecular Devices, Sunnyvale, CA). The experiments were repeated three times.

Statistics. Experiments were repeated three times and biological killing curves are expressed as means and standard deviation.

■ ASSOCIATED CONTENT

Supporting Information

Additional details of spectroscopic data, including infrared spectra comparison among Phe, Phe-N_5, Chl-N_{10}, *m*Chl-N_{15}, and their multicationic derivatives (Figure S1), ^{1}H NMR spectra of Phe, Phe-N_5, Chl-N_{10}, and *m*Chl-N_{15} (Figure S2), ^{1}H NMR spectra of Phe-N_5, vancomycin, and Van-Zn-*m*Phe-N_5 (Figure S3), ^{1}H NMR spectra of neutral *m*Chl-N_{15} and methyl quaternized *m*Chl-N_{15}^+ (Figure S4), MALDI-TOF mass spectra of Phe-N_5, Chl-N_{10}, *m*Chl-N_{15}, and Van-*m*Phe-N_5 (Figure S5), proposed MALDI-TOF mass fragmentation of Van-*m*Phe-N_5 (Figure S6), photostability evaluation of Zn-*m*Chl-N_{15}^+ and the experimental setup (Figure S7), and orbital energy diagrams of chlorin core moiety (Figure S9). For the structural characterization of all compounds given in Scheme 1. Figure S8 included aPDI data of three microbial species using the compounds described in the main paper, but excited by red light (660±15 nm) instead of blue light. A, B, C, Gram-positive MRSA; D, E, F, Gram-negative *E. coli*; G, H, I, fungal yeast *C. albicans.* A, D, G, Zn-Phe-N_5^+; B, E, H, Zn-Chl-N_{10}^+; C, F, I, Zn-*m*Chl-N_{15}^+.

■ AUTHOR INFORMATION

Corresponding Authors

*For M.R.H.: phone, +1 617-726-6182, e-mail, hamblin@helix.mgh.harvard.edu. For L.Y.C.: phone, +1 978-934-3663; e-mail, Long_Chiang@uml.edu.

Notes

The authors declare no competing financial interest.

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Legends to the figures

Scheme 1. Synthetic Pathways for the Preparation of Antimicrobial PSs Zn-Phe-N_5^+, Zn-Chl-N_{10}^+, Zn-*m*Chl-N_{15}^+, and Van-Zn-*m*Phe-N_5^+.

Figure 1. UV-vis spectra (solid lines) of new multicationic photosensitizers. (a) Zn-Phe-N_5^+, (b) Zn-Chl-N_{10}^+, (c) Zn-*m*Chl-N_{15}^+, and (d) Van-Zn-*m*Phe-N_5^+ in DMF at a concentration of 1.0×10^{-5} M. Emission bands of blue ($\lambda_{em,max}$ 415 nm), white LED ($\lambda_{em,max}$ 451 and 530 nm), and red ($\lambda_{em,max}$ 660 nm) light used for photoexcitation. These curves are colored in blue, beige, and pink shades, respectively.

Figure 2. Molecular orbitals. (a) Model system investigated. (b) Molecular orbital energy diagram for the frontier orbitals determined at the M06L/def2-TZVPP//M06L/6-31+G(d,p) level of DFT. (c) The computed frontier orbitals (HOMO and LUMO) corresponding to the $\pi \rightarrow \pi^*$ transitions.

Figure 3. Fluorescent emission spectra. (A) Singlet oxygen production efficiency of (a) blank, (b) Zn-Phe-N_5^+, (c) Zn-Chl-N_{10}^+, (d) Zn-*m*Chl-N_{15}^+, and (e) Van-Zn-*m*Phe-N_5^+ in deionized water–DMSO (19:1) at a concentration of 5.0×10^{-6} M using ABMA as the $^1O_2$ trapping agent at λ_{ex} 350 nm and λ_{em} 428 nm for detection with the irradiation source of a white LED light operated with $\lambda_{em,max}$ at 451 and 530 nm. (B) Superoxide radical production efficiency of the same series of aPDI agents (b)–(e) using DNBs-TFFC as the $O_2^{-}\cdot$ acceptor agent with λ_{ex} 480 nm and λ_{em} 530 nm for the detection under the same conditions as those of (A).

Figure 4. aPDI of Gram-positive MRSA. Survival fractions of MRSA (10^8 cells/mL) incubated for 30 min with increasing concentrations of (A) Zn-Phe-N_5^+, (B) Zn-Chl-N_{10}^+, and (C) Zn-*m*Chl-N_{15}^+ by illumination with either 10 J/cm^2 or 20 J/cm^2 of a 415-nm LED light. Points are means from three experiments and error bars are SD. * signifies eradication (zero CFU)

Figure 5. aPDI of Gram-negative *E. coli.* Survival fractions of *E. coli* (10^8 cells/mL) incubated for 30 min with increasing concentrations of (A) Zn-Phe-N_5^+, (B) Zn-Chl-N_{10}^+, and (C) Zn-*m*Chl-N_{15}^+ by illumination with either 10 J/cm^2 or 20 J/cm^2 of a 415-nm LED light. Points are means from three experiments and error bars are SD. * signifies eradication (zero CFU)

Figure 6. aPDI of fungal yeast *C. albicans.* Survival fractions of *C. albicans* (10^8 cells/mL) incubated for 30 min with increasing concentrations of (A) Zn-Phe-N_5^+, (B) Zn-Chl-N_{10}^+, and (C) Zn-*m*Chl-N_{15}^+ by illumination with either 10 J/cm^2 or 20 J/cm^2 of a 415-nm LED light. Points are means from three experiments and error bars are SD. * signifies eradication (zero CFU)

Figure 7. aPDI of vancomycin-resistant and sensitive strains of *E. fecium*. Survival fractions of *E. fecium* (10^8 cells/mL) incubated for 30 min with 50 nM concentration of (A) Van-Zn-*m*Phe-N_5^+ and (B) Zn-Phe-N_5^+ by illumination with increasing fluences of a 415-nm LED. Points are means from three experiments and error bars are SD. * signifies eradication (zero CFU). † means significantly different ($P < 0.05$) from VSE1 and VSE2. ‡ means significantly different ($P < 0.05$) from VSE1

Table 1. MIC values for vancomycin, Van-Zn-*m*Phe-N_5^+, and Zn-Phe-N_5^+.

Compound	vancomycin	Van-Zn-*m*Phe-N_5^+	Zn-Phe-N_5^+
Bacterial species	MIC (μg/mL)	MIC (μg/mL)	MIC (μg/mL)
MRSA	2	64	64
VRE1 (WC176)	64	128	>128
VRE2 (WB312)	8	128	>128
VSE1 (D24)	1	128	>128
VSE1 (D25)	2	128	128