Bacteriopurpurinimides: Highly Stable and Potent Photosensitizers for Photodynamic Therapy

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Abstract: The in situ conversion of the unstable bacteriochlorophyll *a* present in *Rhodobacter sphaeroides* produced highly stable bacteriopurpurin-18 which in a sequence of reactions was converted into a series of alkyl ether analogues of bacteriopurpurin-18-*N*-alkylimides with long wavelength absorption near 800 nm. The effective photosensitizers were found to localize in mitochondria but did not show any specific displacement of ^{3}H-PK11195, suggesting that the mitochondrial peripheral benzodiazepine receptor is not the cellular binding site for this class of compounds.

Introduction. The porphyrins and related tetrapyrrolic systems are among the most widely studied of all macrocyclic compounds.[1] In fact, in one capacity or another these versatile molecules have influenced nearly all disciplines in chemistry. Porphyrins are 18π-electron aromatic macrocycles that exhibit characteristic optical spectra with a strong $\pi-\pi^*$ transition around 400 nm (Soret band) and usually four Q bands in the visible region. In the porphyrin system, two of the peripheral double bonds in opposite pyrrolic rings are cross-conjugated and are not required to maintain aromaticity. Thus reduction of one or both of these cross-conjugated double bonds (to give chlorins and bacteriochlorins, respectively) maintains much of the aromaticity, but the change in symmetry results in bathochromically shifted Q bands with high extinction coefficients. Nature uses these optical properties of the reduced porphyrins to harvest solar energy for photosynthesis with chlorophylls and bacteriochlorophylls as both antenna and reaction-center pigments.[1] The long wavelength absorption of these natural chromophores led to explorations of their use as photosensitizers in photodynamic therapy (PDT).[2]

PDT is based on the interaction of a photosensitizer retained in tumors with photons of visible light, resulting in the formation of singlet oxygen ($^1O_2$), the putative lethal agent.[2] To achieve an effective destruction of tumor cells, a high quantum yield of singlet oxygen is required. Even in the absence of heavy atom substitution(s) and coordination of transition-metal ions, porphyrin systems generally satisfy these criteria, and that is why most of the sensitizers currently under clinical evaluation for PDT are porphyrins or porphyrin-based molecules.

It is well established that both absorption and scattering of light by tissue increases as the wavelength decreases and that most efficient sensitizers are those which have strong absorption bands from 700 to 800 nm. Light transmission by tissues drops rapidly below 550 nm; however, it doubles from 550 to 630 nm and doubles again from 630 to 700 nm. This is followed by an additional 10% increase in tissue penetration as the wavelength increases toward 800 nm.[2] Another reason to set the ideal wavelength for PDT at 700−800 nm is due to the availability of easy to use diode lasers. Although diode lasers are now available at 630 nm (where clinically approved Photofrin absorbs), photosensitizers with absorptions between 700 and 800 nm in conjunction with diode lasers are still desirable for treating deeply seated tumors.

Most of the naturally occurring bacteriochlorins have absorptions between 760 and 780 nm, and several have been studied by various investigators[3] for their use as photosensitizers for PDT. They were found to be extremely sensitive to oxidation, resulting in a rapid transformation into the chlorin state which generally has an absorption maxima at or below 660 nm. Furthermore, if a laser is used to excite the bacteriochlorin in vivo, oxidation may result in the formation of new chromophore absorbing outside the laser window, reducing the photodynamic efficacy.[3] Due to the desirable photophysical properties and promising in vitro/in vivo photosensitizing efficacy of bacteriochlorins, there has been increasing interest in the synthesis of stable bacteriochlorins either from bacteriochlorophyll *a* or from the other related tetrapyrrolic systems.

Results and Discussion. Chemistry: In our previous studies with various photosensitizers derived from pyropheo-phorbide *a*, chlorin-e$_6$, benzoporphyrin derivatives, and *N*-alkylpurpurinimides,[3] lipophilicity has proven to be an important molecular descriptor that often was found to be well-correlated with the bioactivity of drugs. In these analogues, the overall lipophilicity of the molecule was altered by introducing a series of the alkyl ether side chains with variable carbon units. In continuation of our studies to investigate the effect of such substituents in longer wavelength absorbing compounds related to bacteriochlorins, we developed a simple and efficient methodology for the preparation of stable bacteriochlorins.

It has been shown that certain chlorophyll *a* analogues containing a five-member isocyclic ring (λ_{max} 660 nm) can be converted into the related chlorins bearing either a fused six-member anhydride or imide ring systems and named as purpurin-18 and purpurinimide, respectively. The presence of such fused ring systems extended their long wavelength absorptions from 660 to 700 nm.[3] Despite their high stability in vitro, results obtained from in vivo reflectance spectroscopy indicated that compounds containing the six-member imide ring system were more stable in vivo than the photosensitizers with anhydride and isoimide ring systems.[3]

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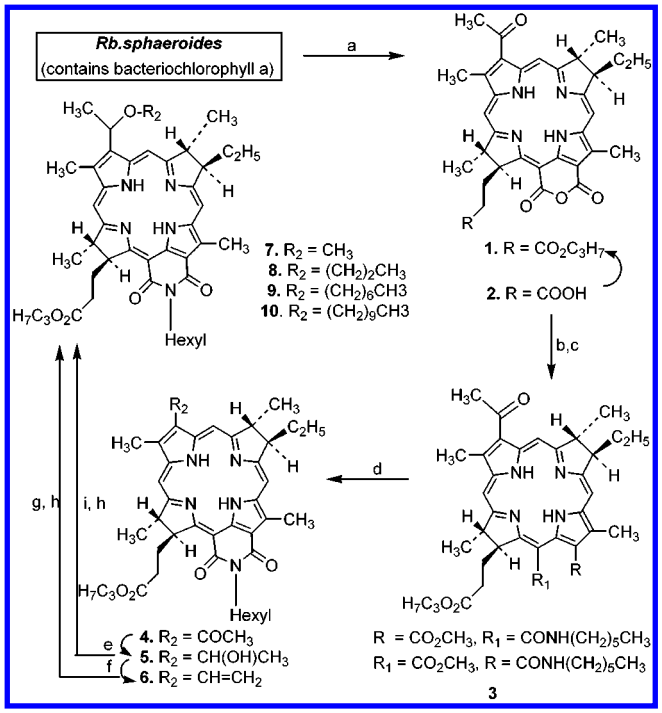
Scheme 1. Synthesis of Various Alkyl Ether Analogues of Bacteriopurpurin-18-*N*-hexylimide[a]



[a] Reagents: (a) KOH/1-propanol, H_2SO_4; (b) $H_2N(CH_2)_5CH_3$; (c) CH_2N_2; (d) KOH/MeOH; (e) $NaBH_4$; (f) *o*-dichlorobenzene (140 °C, 2 min); (g) HBr/AcOH; (h) R_2OH; (i) HBr gas.

Therefore, for synthesizing stable bacteriopurpurinimides, *Rhodobacter sphaeroides* enriched with bacteriochlorophyll *a* was reacted with KOH/1-propanol solution and the air was bubbled through the reaction mixture for 1.5 h. The solution was acidified to pH ~3 and extracted with CH_2Cl_2/THF, and after heating at 45 °C, bacteriopurpurin-18 **1** was isolated as a solid product. It was then converted into *N*-hexylpurpurinimide **4** by following the methodology depicted in Scheme 1. Depending on the nature of substituent present at position-3, two different approaches (HBr/AcOH or HBr gas method) were used for introducing the alkyl ether side chain at position-3 of the macrocycle (Scheme 1). Compared to the HBr/AcOH method, the HBr gas procedure was found to be more efficient for the synthesis of purpurinimides **7**−**10** with variable lipophilicity. These bacteriopurpurinimides exhibit long wavelength absorption at 780 nm (ϵ = 47 300), and if compared to Photofrin (622 nm, ϵ = 5000), HPPH (660 nm, ϵ = 45 500), and alkyl ether analogues of purpurin-*N*-alkylimide (700 nm, ϵ = 45 000), significant red shifts of 158, 120, and 80, respectively, were observed. The structures of the newly synthesized compounds were confirmed by ^{1}H NMR and mass spectrometry.

In Vitro and in Vivo Biological Studies: (a) Determination of Tumor Uptake by in Vivo Reflectance Spectroscopy. The bacteriopurpurinimides **4**−**10** had limited water solubility. Therefore, for biological studies, these compounds were formulated in a 1% Tween 80/5% dextrose solution, and the drug concentrations were determined on the basis of their extinction coefficient values.

The tumor and skin uptake of each compound was determined by in vivo reflectance spectroscopy. For measuring tumor uptake, the compounds were injected into mice at a dose of 5.0 μmol/kg. All photosensitizers showed a greater uptake in tumor than skin at 24 h postinjection, indicating some degree of tumor selectivity, the ratio varying less than 2.5 (compound **5**) to nearly 5 (compound **4**).

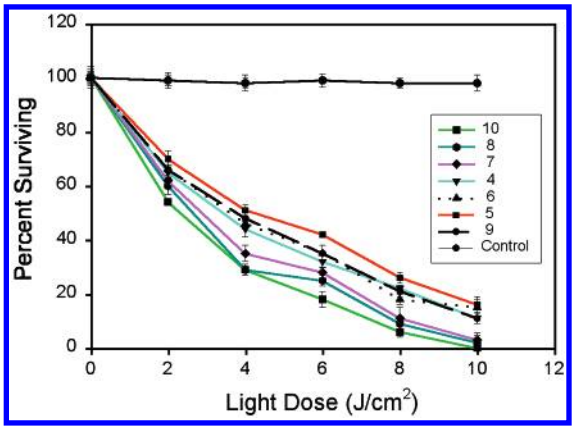


Figure 1. In vitro PDT efficacy of bacteriochlorin **4**−**10** in RIF tumor cells at a dose of 1.5 μM at 48 h MTT. The cells were treated with light (780 nm) after 4 h incubation (see the text). Control: cell exposed to light without any photosensitizer.

(b) In Vitro Photosensitizing Efficacy. RIF cells were grown in α-DMEM with 10% fetal calf serum, penicillin, and streptomycin.[2] Cells were maintained in 5% CO_2 and 95% air at 100% humidity. For phototoxicity studies, RIF cells were plated in 96-well plates at a density of 1 × 10^4 cells per well, in complete media. Twenty-four hours later compounds were added at a final concentration of 1.5 μM. After a 3 h incubation in the dark at 37 °C, the cells were irradiated with 780 nm laser light from an argon pumped dye laser using fluences of 0−10 J/cm^2 at a dose rate of 5.6 mW/cm^2. After PDT, the cells were washed once and placed in complete media and incubated for 48 h. Then 10 μL of 4 mg/mL solution of MTT was added to each well. After 4 h incubation at 37 °C, the MTT + media were removed and 100 μL of DMSO was added to solubilize the formazin crystals. The 96-well plate was read on a microtiter plate reader at an absorbance of 560 nm. The results were plotted as percent survival of the corresponding dark (drug no light) control for each compound tested. Each data point represents the mean from three separate experiments, and the error bars are the standard deviation. Each experiment was done with five replicate wells. The results are summarized in Figure 1, and it can be seen that all bacteriochlorins (**4**−**10**) produced a significant photosensitizing activity.

(c) In Vivo Photosensitizing Efficacy. C_3H mice (6 mice/group) were subcutaneously implanted with radiation induced fibrosarcoma (RIF) cells.[2] The tumors were allowed to grow for a period of 5 days to a size of about 4−5 mm. In our preliminary studies, the treatment consisted of a tail vein injection of photosensitizer **7** at a dose of 0.2 μmol/kg. These mice were exposed to light for 30 min (135 J/cm^2) at λ_{max} 785 nm (in vivo absorption) at 24 h postinjection, and the tumor response (reappearance of tumor) was recorded daily. The other bacteriochlorins **4**−**6** and **8**−**10** were treated under similar conditions. Compared to in vitro results that showed a similar PDT efficacy of all bacteriochlo-

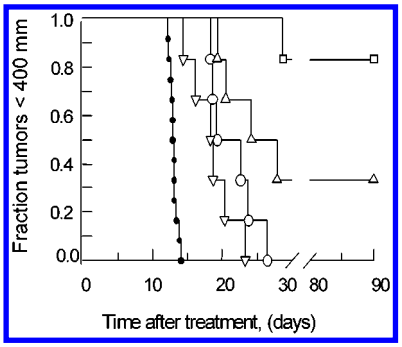


Figure 2. In vivo photosensitizing efficacy of bacteriochlorins **7** (−▽−), **8** (−○−), **9** (−□−), and **10** (−△−) in C3H mice (6 mice/group) bearing RIF tumors at a dose of 0.2 μmol/kg. The mice were treated with laser light (780 nm, 135 J/cm^2) at 24 h postinjection of the drug. Control (−●−): 12 mice were exposed to light without any photosensitizer.

rins, a remarkable difference was observed in in vivo activity. Under similar treatment conditions, bacteriopurpurinimides containing an acetyl-**4**, (1′-hydroxy)ethyl substituent **5**, and vinyl group **6** at position-3 did not show any PDT efficacy. Among the alkyl ether analogues, the 3-(1-methoxy)ethyl **7** (log P = 8.18) and 3-(1-propyloxy)ethyl **8** (log P = 9.16) produced limited activity. Bacteriochlorins **9** containing heptyl ether group **9** (log P = 11.19) showed excellent PDT efficacy (5/6 mice were tumor free at day 90), whereas the corresponding bacteriochlorin **10** with a decyl ether group (log P = 12.72) was found to be less effective (2/6 mice were tumor free on day 90). The results are summarized in Figure 2. The limited activity of photosensitizers **4**−**8**, suggest that for a compound to be biologically effective, besides the tumor uptake, the clearance of the drugs might play a significant role in its activity. These findings show that, to some extent, increasing the length of the alkyl ether carbon chain increases the photosensitizing capacity; however, it then diminishes by further increase of the overall lipophilicity. To establish a QSAR in this class of compounds, further detailed pharmacokinetic and pharmacodynamic studies with these and other related analogues at variable drug/light doses and time intervals are currently in progress.

(d) Determination of Intracellular Localization by Fluorescence. Determination of the critical site(s) of photosensitizer binding has long been a goal of the PDT research. It has been shown that effective photodynamic agents have very diverse patterns of localization, based on structure, lipophilicity, charge, and amphilicity. Two sites of localization are predominant in the lysosomes and the mitochondria.[4]

In our attempt to investigate the site(s) of localization of bacteriopurpurinimide analogues, photosensitizers were co-incubated in the RIF and FaDu cell lines with Rhodamine-123 (Molecular Probes, Eugene, OR) or Fluorspheres, 0.1 μm diameter latex beads labeled with fluorescein isothiocyanate 1/10000 dilution (Molecular Probes, Eugene, OR), which target mitochondria and lysosomes, respectively. Briefly, 1 × 10^5 cells were plated on poly-L-lysine coated coverslips and grown for 2 days. Cells were incubated with 1 μM of the individual bacteriochlorins **7**−**10** for 4 or 24 h at 37 °C, 5% CO_2 in the dark, then washed with fresh growth medium for 1 h to remove loosely bound drug, and gently rinsed with PBS immediately prior to microscopy (Zeiss Axiovert 35, Carl Zeiss, Inc., Germany). Localization was similar in both cell lines and at both time points. For the co-incubation studies, fluorospheres were added 1/10000 dilution of the stock for 24 h, or R-123 at 1.0 μM, for the final 30 min before washing with PBS and examining by microscopy. Fluorescence was imaged with a CCD camera and intensifier as previously described.[5] The images were captured and processed by Metamorph, version 4.0 (Universal Imaging Corp., Westchester, PA). The cells were illuminated by a mercury arc lamp filtered through a filter cube containing a 530−585 nm excitation filter, a 600 nm dichroic filter, and a 615 nm long-pass emission filter for detection of photosensitizers. R-123 and Fluorspheres were detected using a 450−490 nm excitation filter, a 510 nm dichroic, and a 520−560 nm long pass emission filter. Images of photosensitizer and Rhodamine-123 or Fluorspheres localization were taken in rapid succession. The imaging data clearly indicate that bacteriopurpurinimides **8**−**10** localize to the same subcellular regions as Rhodamine-123, suggesting that these compounds selectively localize in

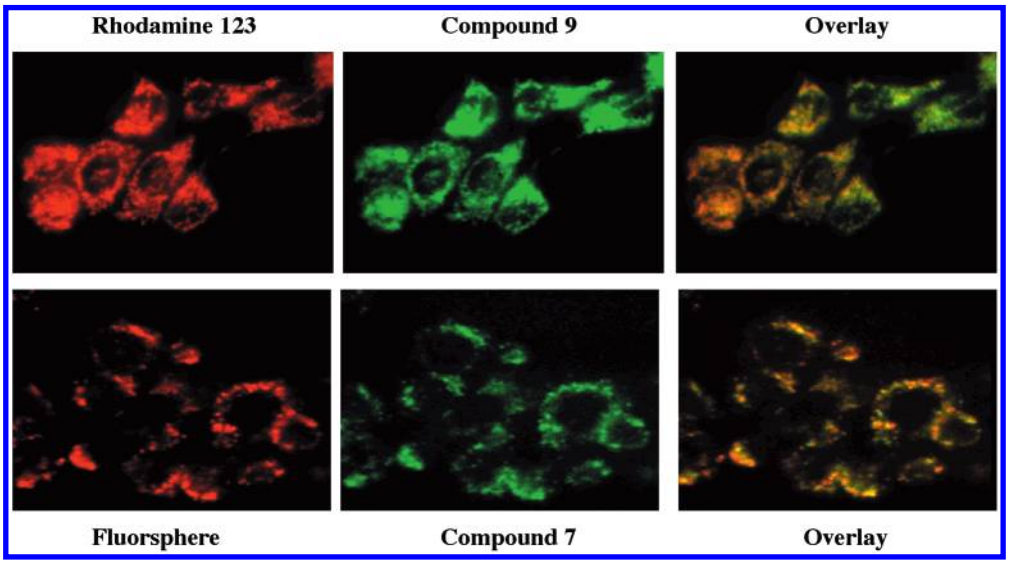


Figure 3. Comparative intracellular localization of bacteriopurpurinimides **7** and **9** with Fluorspheres and Rhodamine 123, respectively.

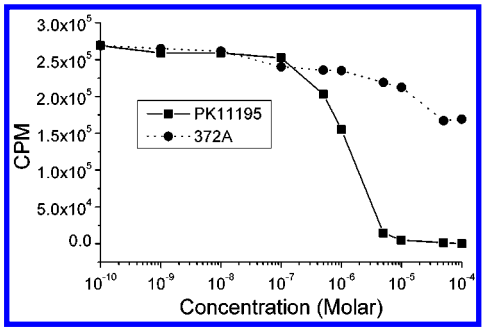


Figure 4. Displacement of ^{3}H-PK11195 by PK11195 (−■−) and with bacteriochlorin **9** (− ●−).

mitochondria (see Figure 3, a representative example). In other experiments, the results obtained by using Fluorospheres as a counterstain indicated that in contrast to bacteriopurpurinimides **8**−**10**, 3-(1-methoxy)ethyl purpurinimide **7** that had shown limited in vivo PDT efficacy was found to localize in lysosomes which is generally thought of as an inefficient site for producing photodamage.[4]

In previous studies it has been implied by us and others that certain photosensitizers that show mitochondrial localization exhibit peripheral benzodiazepine receptor (PBR) binding which may be an important target for PDT.[4] Therefore, using the peripherally active benzodiazepine ligand PK11195 as a tool for measuring binding and displacement at the PBR, we looked for evidence to support this hypothesis with the bacteriopurpurinimides. We determined the capacity of biologically effective bacteriopurpurinimides to displace PK11195 from its specific cellular binding site, the IQ site on PBR. Specific radioactive binding assays using ^{3}H-labeled ligand were used to determine the dissociation constant (K_d) of PK 11195 in whole cells. Initially, saturation binding of PK11195 was determined on FaDu and RIF cells in the presence and absence of excess unlabeled ligands. Bound and free concentrations of ligand were calculated, and binding affinities were determined by Scatchard analysis. Nonspecific binding of PK11195 was 10% or less. The K_d of PK11195 was 23 nM in FaDu and 44 nM in RIF, which agrees with values quoted in the literature in the low nanomolar range of 3−50 nM.[5a] The concentration of ^{3}H-PK11195 which causes 50% of the maximum binding was used for subsequent displacement studies. Preliminary experiments with increasing concentration of mitochondrially localized bacteriopurpurinimide did not indicate any specific displacement of ^{3}H-PK11195. These results are in contrast to those obtained from HPPH and benzoporphyrin derivatives (BPD)[5b] and suggest that the PBR is not the target for the highly effective bacteriopurpurinimide analogues. The results obtained from the most effective photosensitizer are depicted in Figure 4.

Conclusion. In summary, we present an efficient approach to in situ conversion of extremely unstable bacteriochlorophyll *a* present in bacteria *Rb. sphaeroides* into into a series of stable *N*-hexylimide analogues exhibiting long wavelength absorption near 780−815 nm. Preliminary in vitro and in vivo results of some of the related 3-(1′-alkoxy)ethyl- analogues demonstrate their potential as promising photosensitizers for photodynamic therapy. Among the bacteriopurpurinimides studied so far, the methyl ether analogue **7** that localizes in lysosomes produced limited in vivo PDT efficacy, whereas two of the more effective higher alkyl ether derivatives (**9**, **10**) were found to localize in mitochondria. The displacement studies with PK11195 suggest that, unlike the hexyl ether derivative of pyropheophorbide *a* (HPPH) and benzoporphyrin derivatives (BPD), the effective alkyl ether derivatives of bacteriopurpurin-*N*-hexylimides that localize in mitochondria did not have significant affinity toward the peripheral benzodiazepine receptor. Among the compounds tested so far, the bacteriopurpurinimide **9** (log P = 11.19) was found to be most effective at a dose of 0.2 μmol/kg. To select the best candidate, further in vitro and in vivo studies with this and other related bacteriochlorins are currently in progress.

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Supporting Information Available: ^{1}H NMR, mass spectrometry data, electronic absorption spectra, and tumor vs skin uptake results of new compounds are available free of charge via the Internet at http://pubs.acs.org.

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