Synthesis and Photosensitizing Efficacy of Isomerically Pure Bacteriopurpurinimides

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Abstract: The isomerically pure 3-deacetyl-3-(1-heptyloxy)-ethylbacteriopurpurin-*N*-hexylimides exhibiting long-wavelength absorption near 800 nm were obtained from 3-acetyl-bacteriopurpurin-*N*-hexylimide in high stereospecificity by following Corey's synthetic approach. Both heptyl ether derivatives (*R*- and *S*-isomers) showed similar in vitro photosensitizing efficacy and limited skin phototoxicity and were found to localize in mitochondria. However, in preliminary in vivo screening, compared to the *S*-isomer, the corresponding *R*-isomer produced enhanced in vivo photodynamic therapy efficacy.

Photodynamic therapy (PDT) is now an established modality for the treatment of various types of tumors. Clinical studies have demonstrated that photodynamic therapy is a useful treatment for a wide variety of solid tumors. Regulatory approval in the U.S. was first obtained in 1995 for Photofrin (a porphyrin derivative), developed in our institute for the treatment of early lung cancer, and now includes treatment of late-stage lung and esophageal cancers and Barrett's esophagus. In addition to the U.S. approvals, numerous approvals for a wide range of PDT applications exist in Canada, Europe, and Japan.[1] However, Photofrin suffers from certain disadvantages: e.g., it is a chemically complex mixture, its longest absorption wavelength peak falls at 630 nm, where the tissue penetration of light is low, and it shows long-term (4−5 weeks) skin phototoxicity in patients if exposed to direct sunlight. Therefore, the objective of various laboratories has been to prepare tumor-avid photosensitizers with longer-wavelength absorption in the range 660−800 nm.[2−9]

One of our main goals has been to develop improved photosensitizer(s) compared with Photofrin. To develop effective photosensitizers with required photophysical characteristics, we used chlorophyll-a and bacteriochlorophyll-a as the substrates. On the basis of extensive SAR and QSAR studies on alkyl ether derivatives of pyrophephorbide-a (660 nm), purpurinimides (700 nm), and bacteriopurpurinimides (780 nm), three compounds (one from each series) were selected, and these are currently at various stages of human clinical and preclinical trials.[1,2]

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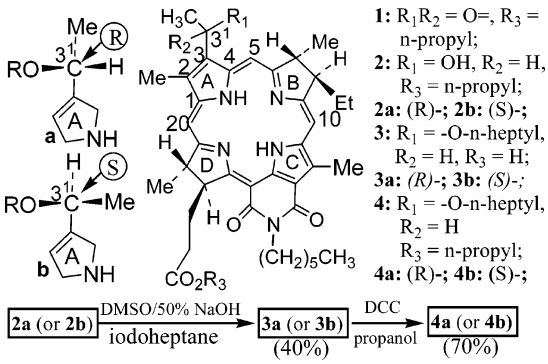
Scheme 1. Synthesis of *R*- and *S*-Isomers of 3-Deacetyl-3-(1′-heptyloxy)ethylbacteriopurpurin-*N*-hexylimides **4a** and **4b**, Respectively



In preliminary in vivo screening, among the alkyl ether analogues of bacteriopurpurin-18-*N*-hexylimides ($\lambda_{max} \approx 800$ nm) the corresponding 3-(1-heptyloxy)ethyl analogue **4** (Scheme 1) produced the best efficacy.[10] However, because of the presence of the chiral center at position 3, **4** was obtained as a mixture of two diastereomers of equal ratio confirmed by HPLC [retention times 37.4 and 39.8 (Figure 1)] and NMR analyses (Figure 2) [the resonances observed at 8.83 and 8.80 ppm, each integrated for 0.5 and corresponding to the meso proton at position 5 (close to the chiral center at position 3^1)].

To determine the impact of chirality in biological efficacy, we were interested in separating, identifying, and synthesizing diastereomerically pure isomers in large quantities so that the individual isomers could be investigated for in vitro and in vivo biological efficacy. In recent years one of the major goals of bioorganic chemists has been to develop stereoselective synthesis of the biologically active compounds (isomeric mixtures) and to compare their in vivo efficacy. There are examples where both stereoisomers were found to have similar therapeutic properties, for example, ibuprofen [2-(*p*-isobutylphenyl)propionic acid], a commonly used nonsteroidal anti-inflammatory drug that is effective in treating fever and pain. On the other hand, there are cases where one of the stereoisomers produces the desired effect, whereas the other caused serious side effects. The most tragic example of this situation is thalidomide (*N*-(2,6-dioxo-3-piperidinyl)phthalimide), a well-known drug with antinauseal activity for pregnant women. Between the two possible isomers, the *S*-enantiomer was found to produce the desired sedative efficacy whereas the *R*-enantiomer was teratogenic and caused severely underdeveloped limbs.

3-Acetyl-bacteriopurpurin-18-*N*-hexylimide **1** obtained from bacteriopurpurin, which in turn was isolated from *Rb. Sphaeroides* by following the methodology developed in our laboratory,[10] was reacted with sodium borohydride and converted into the corresponding hydroxyethyl derivative **2** and as expected was found to be an isomeric mixture by NMR spectroscopy (Figure 2). On the basis of 2D NMR, the singlets observed at δ 8.815 and 8.796 were assigned to the meso protons adjacent to the chiral center (position 5). Our initial efforts to separate the

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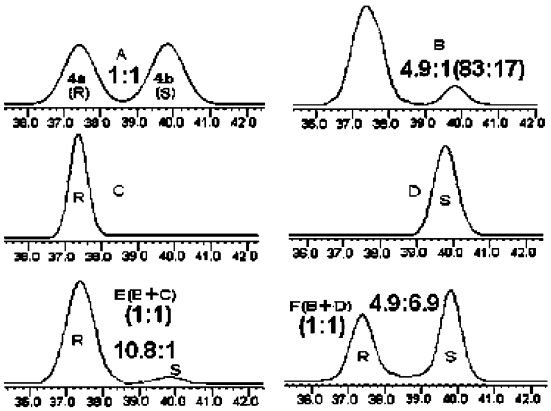


Figure 1. HPLC analysis of **4a** and **4b** (column, Symmetry analytical reverse-phase C18; solvent, methanol; flow rate, 1.0 mL/min; the wavelength set at 366 and 787 nm: (A) heptyl ether analogue **4** (ratio of *S* to *R*, 1:1); (B) heptyl ether analogue **4** (the ratio of *R* to *S* was 4.9 to 1) derived from the hydroxy analogue **2**, which was obtained by using S-CBS as the ligand (ratio of **2a** (*R*) to **2b** (*S*) was 4.9/1); (C and D) isomerically pure heptyl ether analogue **4a** and **4b** derived from the corresponding *R*- and *S*-hydroxy derivatives, respectively; (E) co-injection of (B) and (C) in the equivalent amount (the final ratio of *R* to *S* was 10.8/1); (F) co-injection of (B) and (D) in equal amount (the final ratio of *R* to *S* was 4.9/6.9).

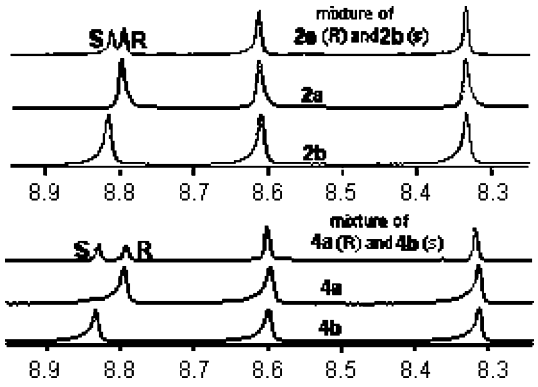


Figure 2. Partial NMR spectra (only the meso region is shown) of hydroxy analogue **2** (mixture of **2a** and **2b**), isomerically pure *R*- (**2a**) and *S*-isomers (**2b**), the heptyl ether analogue **4** (mixture of **4a** and **4b**), and isomerically pure *R*- (**4a**) and *S*-isomers (**4b**).

individual isomers in sufficient quantity by HPLC had limited success because of the subtleness of the differences between the two bulky bacteriochlorin diastereomers. It was a painstaking task to find a reliable and practical system to separate the two isomers. Several normal and reverse-phase HPLC columns and solvent systems were tried to separate the individual isomers with little success. However, the Develosil reverse-phase C30 column with a flow rate of 1.5 mL/min and 96% MeOH/4% H_2O as an eluting solvent produced the best separation.

After identification of the HPLC parameters for separating the mixture into individual isomers, our next step was to confirm their stereochemistry. We initially investigated the utility of Corey's reagent[11] (*R*- or *S*-CBS-2-methyloxazaborolidine **6**) (Figure 3) as the ligand for stereoselective reduction of 3-acetylbacteriopurpurinimide **1**. When *R*-CBS-2-methyloxazaborolidine **6** was used with the borane−methyl sulfide complex (BMS) as the reducing agent, the resulting product was isolated as a mixture of mainly two components

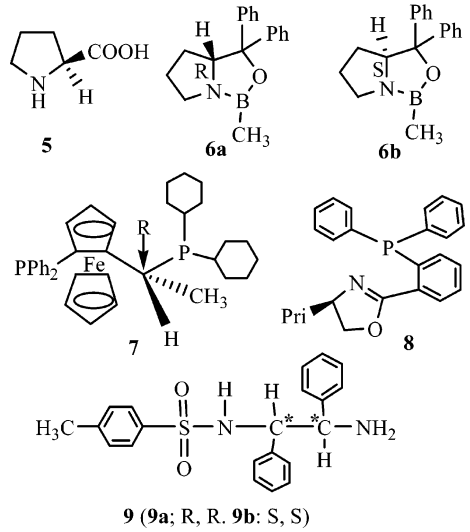


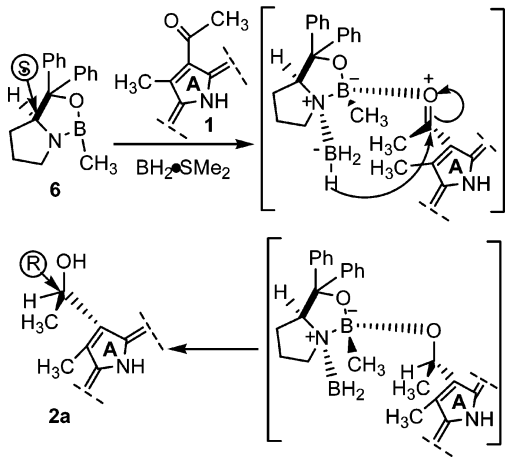
Figure 3. Structures of various chiral ligands.

with retention times of 35.1 and 38.8 min in a ratio of 3 to 1 and were assigned *S*- and *R*-isomers respectively. The stereochemistry of the individual isomers was further confirmed by the HPLC analysis of bacteriochlorin **2**, produced by using the reducing agent (BMS) and *S*-CBS-2-methyloxazaborolidine as the ligand. The resulting products with retention times of 35.1 and 38.1 min were isolated in a ratio of 1 to 4. A comparative NMR analysis of the mixture **2** and the related isomers **2a** and **2b** also confirmed the predicted assignments. The ^{1}H NMR spectrum of the isomeric mixture **2** showed two resonances with equal intensity at 8.815 and 8.796 ppm (attributed to position 5 meso proton) (Figure 2) and was thus assigned to isomers with *S*- and *R*-configuration, respectively. These NMR results were consistent with those reported by Smith et al.[12] for optically pure methyl bacteriopheophorbides *c* and *d* in that *S*-configuration showed downfield shift compared to the *R*-isomer (see Supporting Information for detailed NMR data).

A possible mechanism for the enantioselective reduction of 3-acetylbacteriopurpurinimide **1** by using borane as the reducing agent and chiral CBS-2-methyloxazaborolidine as a ligand to the corresponding hydroxy analogue **2a** is shown in Scheme 2. The ligand is commercially available with both *R*- and *S*-configurations and produces the corresponding hydroxy analogues with opposite configuration. As can be seen from Scheme 2, both BH_3 and acetylbacteriopurpurinimide react with the ligand from the less obstructive side.

We followed a number of methodologies that have been used successfully in a variety of systems for converting the isomerically pure hydroxyethylbacteriochlorins (**2a** or **2b**) into the corresponding heptyl ether derivative, which resulted in limited success. However, treating these individual isomers **2a** or **2b** with aqueous 50% NaOH for a short period (−1 min) and then subsequently reacting it with *n*-iodohepatane (0.5 h) at room temperature produced the corresponding isomerically pure alkyl ether analogue as a carboxylic acid **3** in moderate yield (40%). Although **3** was converted into **4** in almost quantitative yield by reacting with oxalyl chloride/*n*-propanol, it produced a racemic mixture of 3^1-*R* and 3^1-*S* isomers in equal ratio. Reaction of **3** with *n*-propanol/DCC at room temperature produced the

Scheme 2. Possible Mechanism for the Stereoselective Reduction of 3-Acetylbacteriopurpurinimide **1** with CBS Reagent (*S*) To Produce the Corresponding Hydroxy Analogue Containing the Opposite Configuration (*R*) (both BH_3 and Compound **1** React with CBS-2-methyloxazaborolidine from the Less-Obstructive Side)



desired propyl ester analogue **4** in 70% yield. The HPLC conditions followed for separating the isomerically pure hydroxy analogues **2a** and **2b** (Develosil reverse-phase C30 column) were found to have limited application in separating the related heptyl ether analogues **4a** and **4b**. However, a Symmetry reverse-phase C18 column was quite useful, and the two isomers with retention times of 37.4 and 39.8 min were separated by eluting with pure methanol with a flow rate of 1.0 mL/min. The structural assignments to individual isomers **4**, **4a**, and **4b** were further confirmed by extensive NMR studies. Interestingly, similar to the NMR spectra of the individual isomers related to 2-(hydroxy)ethylbacteriopurpurinimide **2**, the position-5 proton of **4b** with the *S*-configuration (8.831 ppm) showed a downfield shift compared to the *R*-isomer (8.795 ppm).

In our attempt to increase the stereospecificity of the hydroxybacteriochlorin **2**, we further investigated the potential use of other known chiral ligands. A comprehensive literature survey revealed that for the stereoselective hydrogenation of the keto groups a variety of asymmetric reagents such as amino acids,[13] ferrocene complex,[14] phosphinooxazoline,[15] and diamine[16] have been used. However, for our system, the hydrogenation approach was not suitable because besides the reduction of the 3^1-keto group it could also reduce the functionalities present at the six-member fused ring system. However, inspired with the success of using CBS-2-methyloxazaborolidine as a chiral ligand, we investigated the utility of several known ligands as the chiral catalysts [(**5**, (L)-(*s*)-proline; **7**, (*R*)-(−)1-(*s*)-2-(diphennylphosphino)ferrocenyl)ethyldicyclohexyphosphine; **8**, (*R*)-(+)-2-[2-(diphenylphosphino)phenyl]-4-(1-methylethyl)-4,5-dihydrooxazole; **9** [**9a** (*R*), **9b** (*S*)], *N*-*p*-tosyl-1,2-diphenyl-ethylenediamine] and BMS as a reducing agent (Figure 3).

These results are summarized in Scheme 3. In fact, these ligands represent almost all the asymmetric catalysts reported so far for the stereospecific conversion of the keto group to the corresponding hydroxy derivative. In bacteriopurpurinimide system, compared to CBS-2-methyloxazaborolidine **6**, most of these ligands were found to be less selective.

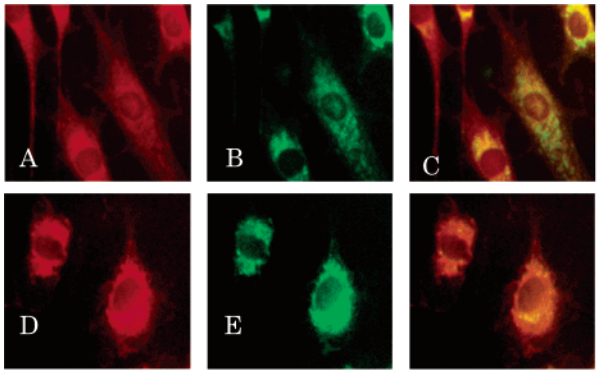
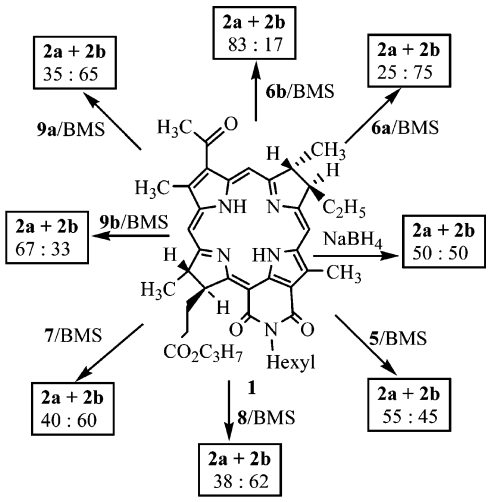


Figure 4. Comparative intracellular localization of **4a** and **4b** with MitoTracker Green: (A) **4a**; (B) MitoTracker Green; (C) overlap of **4a** with MitoTracker Green; (D) **4b**; (E) MitoTracker Green; (F) overlap of **4b** with MitoTracker Green.

Scheme 3. Stereospecificity of Borane−Methyl Sulfide Complex (BMS) for the Reduction of the Acetyl Group in the Presence of Various Chiral Ligands



A comparative intracellular localization characteristic of the isomerically pure bacteriopurpurinimides **4a** and **4b** (1 μM at 24 h postincubation) with Mito Tracker Green (a mitochondrial probe) in RIF tumor cells was determined by fluorescence light microscopy. The results summarized in Figure 4 indicate that both isomers localize in mitochondria.

The in vitro photosensitizing efficacy of the isomerically pure isomers **4a** and **4b** was investigated in RIF tumor cells by MTT assay and produced similar efficacy. For example, cells incubated for 3 h at a drug concentration of 1.5 μM and exposed to laser light (780 nm, 16 J/cm^2) produced 100% cell kill (data not shown). MTT assay is a long-term assay, which roughly parallels clonogenic assay.[17]

The in vivo photosensitizing efficacy of both isomers was also evaluated in C3H mice (10 mice/group) bearing RIF tumors. The results are summarized in Figure 5. The efficacy of the heptyl ether analogue with *R*-configuration **4a** (6 out of 10 mice were tumor-free on day 90) was greater than the corresponding *S*-isomer **4b** (2 out of 10 mice were tumor-free on day 90). The difference in photosensitizing efficacy in two isomers may be due to the nature of interactions between mitochondrial proteins and the photosensitizers of different chirality. Mitochondria are dynamic intracellular

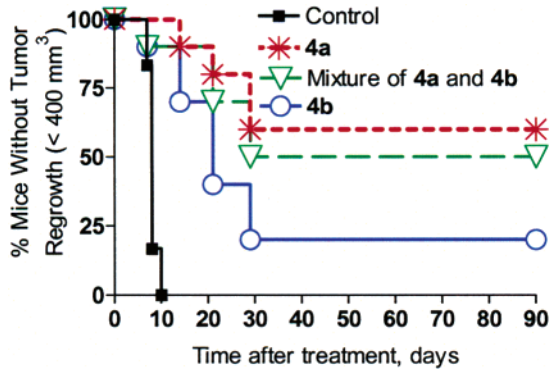


Figure 5. In vivo photosensitizing efficacy of isomerically pure isomers **4a** and **4b** and the mixture **4** at 0.4 μmol/kg in mice implanted with RIF tumors. The tumors were exposed to light (780 nm, 135 J/cm^2) at 24 h postinjection. As the control, mice implanted with tumors were exposed to light without injecting the photosensitizer.

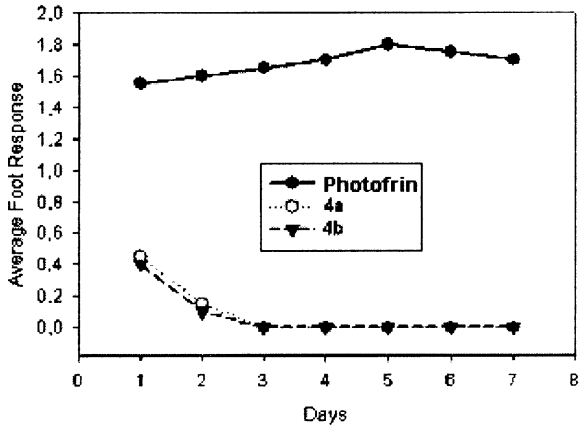


Figure 6. Comparative skin phototoxicity of bacteriochlorins **4a** and **4b** (*R*- and *S*-isomers) with Photofrin at the therapeutic doses (Photofrin, 8.5 μM; **4a**, **4b**, 0.4 μM. Photosensitizers were individually injected to mice (four Swiss mice/group), then one of the hind feet of each mouse was exposed to light (similar to PDT conditions) after 1−7 day postinjection, while another unexposed foot was used as the control.

organelles that play a central role in oxidative metabolism and apoptosis, and a detailed mechanistic study with isomerically pure isomers is currently in progress.

At therapeutic doses, compared to Photofrin, both photosensitizers produced significantly reduced skin phototoxicity (for detailed explanation, see Supporting Information). As can be seen from Figure 6, no skin phototoxicity was observed at 72 h postinjection. Foot response was judged using a 0−3 scale. Response greater than 2.0 indicates unacceptable severe normal tissue reaction.

In summary, the present work describes a first report on the synthesis, separation, and identification of the isomerically pure 3-(1-heptyloxyethyl)-3-deacetylbacteriopurpurin-18-*N*-hexylimides *(R*- and *S*-isomers) and the impact of chirality in photosensitizing efficacy, which seems to make a significant difference. However, these results could be limited to only *N*-alkylbacteriopurpurinimide series. Therefore, to further establish the structural requirements, the synthesis and biological evaluation of other isomerically pure isomers present in certain alkyl ether analogues of pyropheophorbide-a and purpurinimides are currently in progress.

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Supporting Information Available: Data for compounds **1**−**4** including detailed NMR and detailed foot response scale description. This material is available free of charge via the Internet at http://pubs.acs.org.

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