Water-Soluble, Core-Modified Porphyrins as Novel, Longer-Wavelength-Absorbing Sensitizers for Photodynamic Therapy

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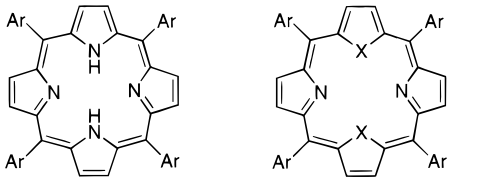
Water-soluble, core-modified 5,10,15,20-tetrakis(4-sulfonatophenyl)-21,23-dithiaporphyrin (**1**) and 5,10,15,20-tetrakis(4-sulfonatophenyl)-21,23-diselenaporphyrin (**2**) were prepared as the tetrasodium salts by the sulfonation of 5,10,15,20-tetraphenyl-21,23-dithiaporphyrin (**3**) and -21,23-diselenaporphyrin (**4**), respectively, with sulfuric acid. Compounds **3** and **4** were prepared by the condensation of pyrrole with either 2,5-bis(phenylhydroxymethyl)thiophene (**5**) or 2,5-bis(phenylhydroxymethyl)selenophene (**6**) in propionic acid. The addition of benzaldehyde to 2,5-dilithiothiophene or 2,5-dilithioselenophene gives **5** or **6**, respectively, as a nearly equimolar mixture of *meso*- and *d*,*l*-diastereomers. Careful crystallization of **5** gives a single diastereomer by removing the crystalline product from the equilibrating mixture of diastereomers in solution. Photodynamic therapy (PDT) with **1** has an LD_{50} of less than 25 μg/mL against Colo-26 cells in culture and exhibits a lethal dose for 90% or more at concentrations greater than 50 μg/mL. In contrast, PDT with 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin ($TPPS_4$) requires concentrations of greater than 100 μg/mL to achieve LD_{50}. Neither **1** nor $TPPS_4$ shows significant photoactivity against the murine T-cell line, MOLT-4, above the dark toxicity. Sensitizer **1** shows no toxicity or side effects in BALB/c mice observed for 30 days following a single intravenous injection of 10 mg (9.1 μmol)/kg. Distribution studies show that sensitizer **1** accumulates in the tumors of BALB/c mice bearing Colo-26 or EMT-6 tumors with sensitizer concentration roughly doubling as the dosage of **1** increased from 5 to 10 mg/kg. In vivo studies show that PDT with sensitizer **1** at both 3.25 and 10 mg/kg with 135 J cm^{-2} of 694-nm light is effective against Colo-26 tumors in BALB/c mice.

Photodynamic therapy (PDT) has been developed as a cancer therapy over the last 25 years and has regulatory approval in many countries for cancers of the lung, digestive tract, and genitourinary tract using Photofrin as a photosensitizer.[1−4] PDT with Photofrin is also being evaluated as a protocol for treating cancers of the head and neck region[5] and for treating pancreatic cancer.[6] The ideal sensitizer would absorb light strongly in the red region of the spectrum (600−900 nm), where light has greater penetration into tissue,[7] would have highly efficient photochemistry for killing tumor cells, would localize in or around the tumor and not in normal tissues, and would rapidly clear the system following treatment. Although Photofrin (a mixture of materials containing as one component dimeric porphyrin ethers) has received regulatory approval, Photofrin and many other porphyrin sensitizers have weak absorbance at the shorter end of the red region of the spectrum (maxima at approximately 630 nm) and induce long-lasting skin photosensitivity. These drawbacks have resulted in the preparation and evaluation of many other synthetic, porphyrin-related molecules.[8−10]

Chart 1



TPPS$_4$, Ar = $C_6H_4SO_3Na$

1, X = S; Ar = $C_6H_4SO_3Na$
2, X = Se; Ar = $C_6H_4SO_3Na$
3, X = S; Ar = C_6H_5
4, X = Se; Ar = C_6H_5

Part of the challenge to chemists trying to replace Photofrin with other porphyrin-like molecules is the synthesis of water-soluble porphyrins and related materials with suitable solubility and bioavailability for use in PDT and with minimal dark toxicity. Some approaches to increased water solubility and bioavailability include the incorporation of sugar residues around the porphyrin perimeter to enhance aqueous solubility[11] as well as the incorporation of ionic groups such as pyridinium, sulfonato, or carboxylato groups.[8,12]

5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrin ($TPPS_4$, Chart 1) and less highly sulfonated derivatives of tetraphenylporphyrin have been prepared and studied as photosensitizers.[13−15] Although the sulfonated derivatives are soluble in water and have shown mem-

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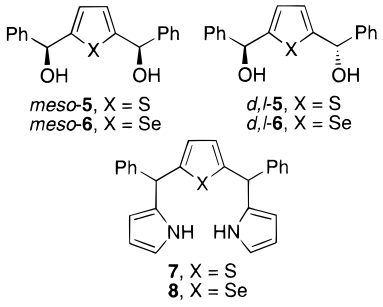
Chart 2



brane permeability, $TPPS_4$ and derivatives still absorb weakly near the 630-nm maxima. A greater concern with $TPPS_4$ is its reported neurotoxicity.[14] 21,23-Core-modified porphyrins containing the chalcogen atoms sulfur, selenium, and tellurium have been described[16,17] and are characterized by significantly longer wavelengths of absorption in band I. The 21,23-core-modified porphyrins do not bind divalent metals and the intramolecular 21,23-heteroatom contacts become significantly less than the sum of van der Waals' radii as the heteroatoms become larger (2.85 Å for Se···Se distances and 2.65 Å for Te···S distances). These features suggested that water-soluble derivatives might be interesting new sensitizer candidates for PDT with optical and photophysical properties and perhaps biochemical properties different than tetraarylporphyrin derivatives.

In this paper, we describe the synthesis of core-modified derivatives of $TPPS_4$, 21,23-dithia derivative **1**, and 21,23-diselena derivative **2** (Chart 1), with water solubility and with longer wavelength absorption maxima (λ_{max} 695 nm) than $TPPS_4$. Dithia derivative **1** was evaluated as the first member of a new class of photosensitizers and was found to display in vitro and in vivo phototoxicity. Importantly, no toxicity was observed in animals given a single dosage of 10 mg/kg of 5,10,15,20-tetrakis(4-sulfonatophenyl)-21,23-dithiaporphyrin (**1**).

Chemistry

Synthesis. 5,10,15,20-Tetrakis(4-sulfonatophenyl)-21,23-dithiaporphyrin (**1**) and 5,10,15,20-tetrakis(4-sulfonatophenyl)-21,23-diselenaporphyrin (**2**) were each prepared as the tetrasodium salts by sulfonation of the corresponding 5,10,15,20-tetraphenylporphyrin **3** or **4** (Chart 1) with sulfuric acid followed by treatment with NaOH, a procedure that has worked well for the sulfonation of other porphyrins.[13] The water solubility of **1** and **2** made purification somewhat difficult, but Soxhlet extraction followed by a final recrystallization gave **1** and **2** as the crystalline hexahydrates. Both **1** and **2** were isolated in 85% yield from the sulfonation, which suggests that the chalcogenophene rings survive the functionalization.

5,10,15,20-Tetraphenyl-21,23-dithiaporphyrin (**3**) was prepared in 5% overall yield by the condensation of pyrrole with 2,5-bis(phenylhydroxymethyl)thiophene (**5**), which in turn was prepared by the addition of benzaldehyde to 2,5-dilithiothiophene.[16] 5,10,15,20-Tetraphenyl-21,23-diselenaporphyrin (**4**) was prepared in 2% overall yield by the reaction of 2,5-dilithioselenophene with benzaldehyde to give 2,5-bis(phenylhydroxymethyl)selenophene (**6**, Chart 2), which was then condensed with pyrrole in propionic acid. The direct conversion of **6** to **4** has been described, although **6** was prepared by the addition of selenium reagents to 1,6-diphenylhexa-2,4-diyne-1,6-diol.[17] Both **3** and **4** have been prepared in trace amounts from the conversion of **5** and **6** to dipyrroles **7** and **8**, respectively, followed by treatment with trifluoroacetic acid and chloranil.[18]

Characterization and Spectral Properties. The structures of **1** and **2** followed directly from the ^1H and ^{13}C NMR spectra. In comparing **1** to **3** and **2** to **4**, the chemical shifts for the singlets corresponding to the four pyrrole and four chalcogenophene protons remain unchanged and integrate to four protons for each signal. These data suggest that sulfonation has not occurred on either heterocyclic ring. The aromatic signals for **1** and **2** have collapsed to slightly broadened 16-proton singlets ($\nu_{1/2} \approx 8$ Hz), which do not allow unequivocal assignment of substitution patterns. However, the ^{13}C NMR spectra are only consistent with para-substitution of the phenyl groups. In each case, nine lines were observed, corresponding to a 4-fold symmetric molecule with four aromatic signals from each of the identical para-substituted phenyl substituents, one signal from the four identical *meso*-carbons, and two signals each from the two pyrrole and thiophene rings that are each 2-fold symmetric.

The absorption spectra of compounds **1** and **2** in water as a solvent (Table 1) are nearly identical to the absorption spectra of compounds **3** and **4** in dichloromethane as solvent. Importantly, the absorption maxima of band I, the long-wavelength band, are little affected by the sulfonation or by the change in solvent. The absorption maxima are at 695 nm, which are significantly longer than the Photofrin maximum at 630 nm in water as well as the $TPPS_4$ maximum at 630 nm in water.

Diastereoselectivity in the Formation of Diols 5 and 6. One intriguing feature in the syntheses of diols **5**[16] and **6**[18] is that only a single diastereomer has been described for the addition of benzaldehyde to either 2,5-dilithiothiophene or 2,5-dilithioselenophene. The diols **5** and **6** can exist as either *meso*- or *d*,*l*-diastereomers and, energetically, both diastereomers should be comparable and, spectroscopically, would be expected to have similar but not necessarily identical spectral properties. As described in the literature examples,[16] recrystallization of the crude product mixture for the preparation of **5** in our hands gave a single, crystalline diastereomer in up to 89% isolated yield. The ^1H NMR spectrum of the crystalline product displayed two two-proton singlets corresponding to the 2,5-disubstituted thiophene ring and the α-protons on the hydroxymethyl substitutents in addition to the expected patterns for the phenyl substituents. The seven lines observed in the ^{13}C NMR spectrum (six aromatic, one alkyl) are also consistent with a single diastereomer.

However, examination of the crude product mixture (from a preparation of **5** prior to recrystallization) by ^1H and ^{13}C NMR spectroscopy indicated that both diastereomers were present in nearly equal amounts. The 500-MHz ^1H NMR spectrum of crude **5** showed superimposable resonances for the *meso*- and *d*,*l*-phenyl substituents but two distinct singlets for the thiophene protons (δ 6.67 and 6.64) and two distinct singlets for

Table 1. UV−Visible Band Maxima and Extinction Coefficients for $TPPS_4$, **1**, and **2** in Water

	λ_{max}, nm ($\epsilon \times 10^{-3}$ cm^{-1} mol^{-1} L) in water					
compd	Soret	Band IV	Band III	Band II	Band I	$\Phi(^1O_2)$[b]
$TPPS_4$[a]	411 (464)	513 (15.5)	549 (7.0)	577 (6.5)	630 (3.9)	0.71[c]
1	434 (190)	513 (19.3)	546 (5.5)	633 (2.0)	695 (4.0)	0.50 ± 0.01
2	434 (221)	513 (22.4)	546 (6.2)	631 (2.2)	695 (4.5)	0.17 ± 0.01

[a] Reference 16. [b] In 0.01 M PBS at pH 7.4, 1.6% by weight NaCl at 25 °C. [c] Reference 19.

the α-protons of the hydroxymethyl substituents (δ 5.83 and 5.82). The two sets of signals are consistent with a mixture of both the *meso*- and *d,l*-diastereomers. The ^{13}C NMR spectrum of the crude product mixture displayed, in addition to the seven signals for the crystalline diastereomer of **5** (δ 149.37, 144.97, 128.09, 127.06, 125.99, 122.91, and 70.75), six new signals (δ 149.18, 144.44, 127.03, 126.06, 122.8, and 70.55) corresponding to the second diastereomer (with a 128.09-ppm signal common to both diastereomers). If the crystalline diastereomer is exposed to trifluoroacetic acid in dimethyl sulfoxide at ambient temperature, the mixture of diastereomers is reestablished.

The crystallization of a single diastereomer from the preparation of **5** is an excellent illustration of Le Châtelier's principle. As the single diastereomer crystallizes and is removed from the solution equilibrium of *meso*- and *d,l*-diastereomers, equilibration and further crystallization produce a single diastereomer. The equilibration of doubly benzylic alcohols is not surprising and could readily be catalyzed by trace amounts of acid.

The addition of benzaldehyde to 2,5-dilithioselenophene has been reported and the diol has been isolated.[18] In our hands, the reaction gave a nearly one-to-one mixture of both *meso*- and *d,l*-diastereomers in 63% yield as an oil. Crystallization/recrystallization of the diastereomeric mixture gave the same mixture of both diastereomers in 42% isolated yield. The 500-MHz ^1H NMR spectra in DMSO-d_6 for both crude and recrystallized **6** showed nearly superimposable resonances for the *meso*- and *d,l*-phenyl substituents ($\Delta\delta$ of approximately 0.005 ppm) but two distinct singlets for the selenophene protons (δ 6.81 and 6.77) and two distinct singlets for the α-protons of the hydroxymethyl substituents (δ 5.79 and 5.78). The two sets of signals are consistent with a mixture of both the *meso*- and *d,l*-diastereomers. The literature report of the ^1H NMR spectrum of **6** prepared in this manner describes only one singlet at δ 6.77 for the selenophene protons and another singlet at δ 5.85 for the phenylhydroxymethyl substituents (in $CDCl_3$), again illustrating that LeChâtelier's principle can give a single diastereomer under proper crystallization conditions.[18]

The ^{13}C NMR spectrum of either the crude or recrystallized product mixture displayed signals in seven different chemical shift regions. Five of these signals were doubled (δ 157.04/156.84, 145.30/145.24, 128.10/128.02, 127.06/127.02, 125.98/125.92) and two peaks were isolated singlets (δ 124.38, 72.40), which again is consistent with a diastereomeric mixture of diols **6**.

The crude diol mixtures of **5** and **6** gave **3** and **4** in 5% and 2% isolated yields, respectively, when treated with pyrrole as described above. The use of the crude diol mixtures gives somewhat higher overall yields for the preparation of **3** and **4**.

Quantum Yields for Singlet Oxygen Generation for 1 and 2. The quantum yield for singlet oxygen generation [$\Phi(^1O_2)$] by $TPPS_4$ has been determined to be 0.71.[19] Using rose bengal as a standard,[20] we confirmerd a value of 0.71 for $\Phi(^1O_2)$ for $TPPS_4$ and measured values of $\Phi(^1O_2)$ for **1** and **2** to be 0.50 ± 0.01 and 0.17 ± 0.01, respectively (Table 1). The lower value of $\Phi(^1O_2)$ for **2** relative to **1** was somewhat surprising since spin−orbit effects from the heavy atom would be more pronounced for **2** relative to **1**.[21] One possible explanation is that the close intramolecular Se···Se contacts[17] disrupt the planarity of the π-framework and lead to less efficient intersystem crossing relative to the various photophysical routes to return to the ground state.

Biology

In Vitro Studies. Photodynamic Therapy against Cells in Culture. Core-modified porphyrin **1** and $TPPS_4$ were evaluated in culture for dark- and light-induced toxicities toward Colo-26 cells, a murine colon carcinoma cell line. Cell cultures were incubated for 2 h in the dark with various concentrations of **1** or $TPPS_4$ and were then washed prior to treatment with red light at either 694 nm for **1** or 630 nm for $TPPS_4$ for a total light dose of 5 J cm^{-2}. Light-treated cells and dark controls were incubated for 24 h, and cell survival was determined. Results are shown in Figure 1. Compound **1** and $TPPS_4$ displayed comparable dark toxicity with dark toxicity becoming significant at concentrations >100 μg/mL. PDT with core-modified porphyrin **1** gave an LD_{50} of less than 25 μg/mL and exhibits lethality (LD_{90} or better) at doses greater than 50 μg/mL. In contrast, $TPPS_4$ requires concentrations of greater than 100 μg/mL to achieve LD_{50}.

Compound **1** and $TPPS_4$ were also evaluated in culture for dark and light toxicities toward MOLT-4, a murine T-cell line. As shown in Figure S1 (Supporting Information), neither **1** nor $TPPS_4$ showed significant photoactivity above the dark toxicity achieved at higher doses (>50 μg/mL).

Inhibition of Cytochrome c Oxidase in Isolated Mitochondrial Suspensions. $TPPS_4$ and other sulfonated photosensitizers accumulate in the lysosomes, where they are released to the cytoplasm upon irradiation.[15] The mitochondria are one possible subcellular target[23] for the sulfonated sensitizers following release. To evaluate the ability of compound **1** to target mitochondria during PDT, mitochondria, isolated from R3230AC rat mammary adenocarcinoma, were treated as suspensions with 10^{-5} M solutions of **1** for 5 min. The mixtures were centrifuged and the pellets were resuspended. The cytochrome c oxidase activity in sensitizer-treated mitochondrial suspensions was measured over time and compared to samples kept in the dark for comparable periods of time as shown in Figure

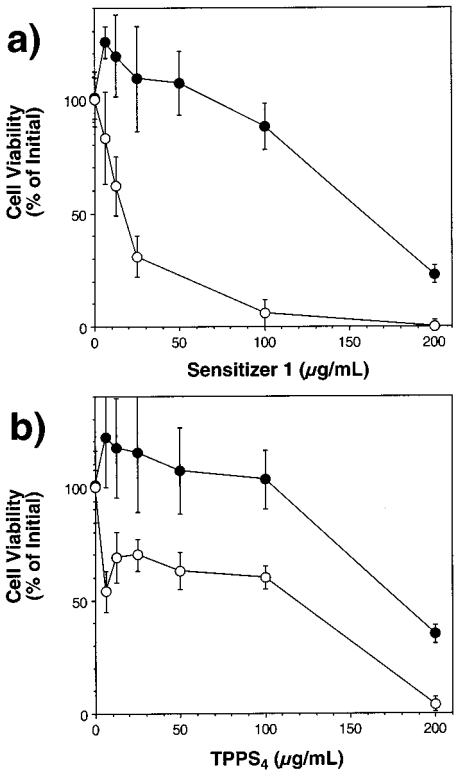


Figure 1. Effect of (a) sensitizer **1** and (b) $TPPS_4$ photosensitization on the cell viability of cultured Colo-26 tumor cells. Details of experimental conditions are described in the Experimental Section. Data are expressed as percent viable cells, compared to untreated cells, for cells treated with sensitizer in the dark (●) or for cells treated with sensitizer and 5.0 J cm^{-2} irradiation (○). Each datum point represents the mean percent viable cells calculated from at least three separate experiments performed in duplicate; bars are the SEM.

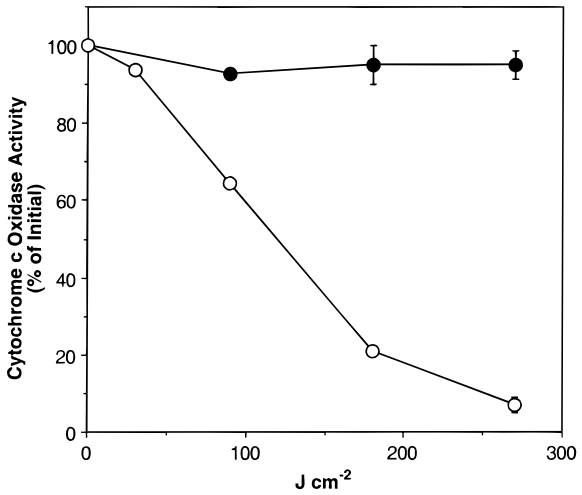


Figure 2. Effect of sensitizer **1** (10 μM) on mitochondrial cytochrome c oxidase activity in the dark (●) and with photosensitization (○). Details of experimental conditions are described in the Experimental Section. Data are expressed as the percent of initial preirradiation cytochrome c oxidase activity in mitochondrial suspensions. Each datum point represents the mean of two separate experiments performed in duplicate; bars are the individual values of the separate runs.

2. Cytochrome c oxidase is the last enzyme in the mitochondrial respiration chain and the loss of activity shown in Figure 2 is due to direct photodamage to this enzyme or to other sites preceding it in the respiration chain.[23]

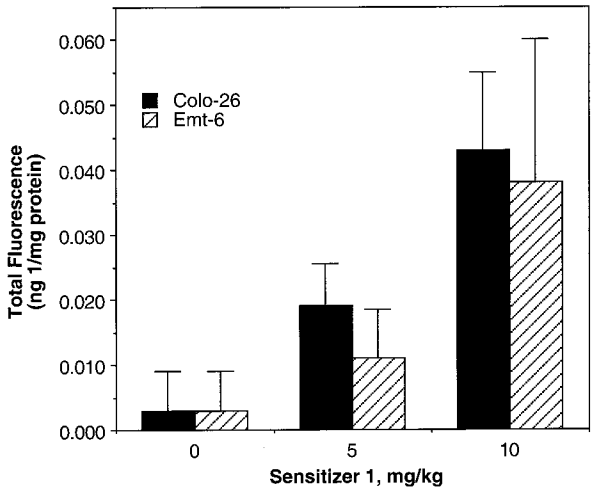


Figure 3. Accumulation of sensitizer **1** in Colo-26 and EMT-6 tumors in Balb/c mice determined by total fluorescence and expressed as nanograms of **1** per milligram of protein. Tumors were excised 24 h postinjection of 5% sodium bicarbonate solutions of **1** at either 5 mg/kg or 10 mg/kg. Details of experimental conditions are described in the Experimental Section; bars are the SEM.

In Vivo Studies. Toxicity and Distribution. In applications of PDT with porphyrin-related sensitizers, the therapeutic dose for rodents is typically on the order of 1−5 mg/kg.[1,9] Sensitizer **1** was evaluated for dark toxicity in BALB/c mice given a single tail-vein injection of 10 mg (9.1 μmol)/kg of **1** in saline. No toxicity, morbidity, or side effects were observed in a group of five animals followed for 30 days postinjection.

In BALB/c mice bearing Colo-26 tumors or EMT-6 tumors, a murine mammary tumor, the presence of sensitizer **1** in the tumors was followed by fluorescence spectroscopy. Twenty-four hours following intravenous injection of a saline solution of **1**, tissues were excised, minced, and homogenized. The homogenate was taken up in Solvable and the emission from sensitizer **1** was quantified in terms of nanograms of **1**/milligrams of protein in tissue. Fluorescence from **1** that was above background levels was found only in the tumor and the fluorescence level was roughly linear with respect to dosage, as shown in Figure 3.

PDT in Vivo. BALB/c mice bearing Colo-26 tumors were given 3.25 or 10 mg/kg of sensitizer **1** as an aqueous solution in 5% sodium bicarbonate via tail-vein injection. Four hours postinjection, the animals were treated with 135 J cm^{-2} of laser light at 694 nm (75 mW cm^{-2} for 30 min). The times to 400 mm^3 tumor volume were noted, and the results are presented as a Kaplan−Meyer plot in Figure 4. Animals treated with sensitizer **1** and light gave a significant response at both 3.25 mg/kg (198 ± 19 h, $P < 0.01$) and 10 mg/kg (276 ± 133 h, $P < 0.014$) relative to control animals (104 ± 25 h) receiving neither drug nor light.

Summary and Conclusions

Core-modified 5,10,15,20-tetraaryl-21,23-dichalcogenaporphyrins can be prepared and sulfonated without destruction of the chalcogenophene rings. The sulfonated dithia derivative **1** and diselena derivative **2**

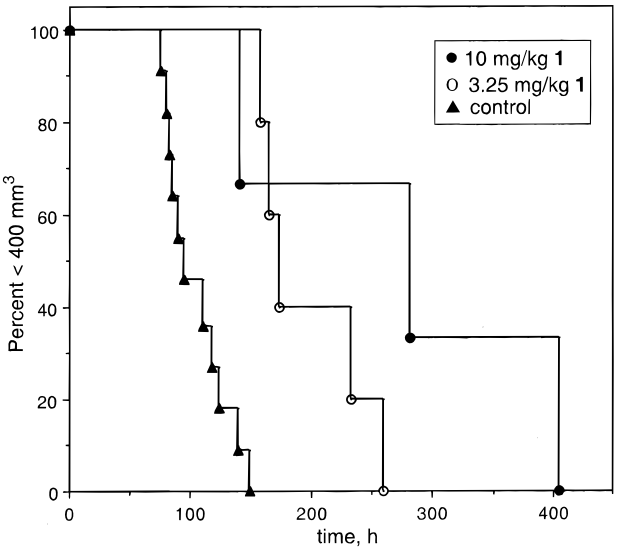


Figure 4. PDT with sensitizer **1** at 3.25 mg/kg (○, $n = 5$ animals, mean = 198 ± 19 h) and 10 mg/kg (●, $n = 3$ animals, mean = 276 ± 133 h) against implanted Colo-26 tumors in BALB/c mice. These results can be compared with a control group (▲, $n = 11$ animals, mean = 104 ± 25 h) that received neither light nor drug. Animals received 135 J cm^{-2} of 694-nm light delivered at 75 mW cm^{-2} for 30 min. Times were measured posttreatment till tumor volumes reached 400 mm^3.

have absorption maxima at 695 nm in water, which is 65 nm longer than that of $TPPS_4$. The dithia derivative **1** is an effective photosensitizer in vitro against Colo-26 cells and is more effective than $TPPS_4$ at comparable concentrations and light dose. In vivo studies indicated that **1** is not toxic at an intravenous dosage of 10 mg (9.1 μmol)/kg with no signs of toxicity, morbidity, or side effects in animals followed for 30 days postinjection. In animals bearing Colo-26 or EMT-6 tumors, sensitizer **1** is found in the tumor 24 h postinjection as determined by fluorescence spectroscopy with levels corresponding to dosage. In animals bearing Colo-26 tumors, PDT with 694-nm light was effective at both 3.25 and 10 mg/kg of sensitizer **1** relative to untreated controls.

Although $TPPS_4$ is one of the more selective porphyrins for accumulation in tumors, its development as a sensitizer has been hindered by its reported neurotoxicity.[14] Sensitizer **1** appears to be as effective as $TPPS_4$ as a sensitizer for PDT and also appears to localize in the tumor. Hopefully, the changes to the core in **1** and **2** and related molecules will avoid the neurotoxicity associated with $TPPS_4$. We are currently optimizing appropriate in vivo protocols for PDT with **1** and **2** with the Colo-26 tumor line. Encouraged by this preliminary survey of biological results, we are also preparing other core-modified porphyrin derivatives for functionalization with water-solubilizing groups and evaluation as sensitizers for PDT.

Experimental Section

General Methods. Solvents and reagents were used as received from Sigma−Aldrich Chemical Co (St. Louis, MO) unless otherwise noted. Cell culture media was purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Concentration in vacuo was performed on a Büchi rotary evaporator. NMR spectra were recorded at 30.0 °C on a Varian Gemini-300, Inova 400, or Inova 500 instruments with the residual solvent signal of $CDCl_3$ as internal standard (δ 7.26 for proton, δ 77.0 for carbon). Infrared spectra were recorded on a Perkin-Elmer FT-IR instrument. UV−visible−near-IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer or on a Sequential DX17 MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK). Both were equipped with a circulating constant-temperature bath for the sample chambers. Elemental analyses were conducted by Atlantic Microanalytical, Inc.

Preparation of Tetrasodium 5,10,15,20-Tetrakis(4-sulfonatophenyl)-21,23-dithiaporphyrin (1). 5,10,15,20-Tetraphenyl-21,23-dithiaporphyrin (**3**, 0.18 g, 0.28 mmol) was dissolved in 6 mL of sulfuric acid and the resulting solution was heated at 100 °C for 18 h. The solution was cooled to ambient temperature and was added slowly to 50 mL of ice water. A 30% sodium hydroxide solution was added dropwise to raise the pH of the solution to 8. Ice was continually added to keep the solution cold. After the pH adjustment, the reaction mixture was concentrated in vacuo to give crude product, which was collected by filtration. The crude crystals were washed with several small portions of methanol (5 × 30 mL), the filtrate was combined with the aqueous filtrate, and more product precipitated that was again collected by filtration and washed with several small portions of methanol. The crude product was purified via Soxhlet extraction with 90% ethanol to give 0.25 g (85%) of **1** as a purple crystalline solid, mp > 300 °C: ^{1}H NMR (CD_3OD) δ 9.77 (s, 4 H, thiophene H's), 8.69 (s, 4 H, pyrrole H's), 8.34 (s, 16 H, Ar); ^{13}C NMR (CD_3OD) δ 157.5, 148.9, 146.3, 143.9, 136.7, 135.4, 134.9, 134.5, 126.2; IR (KBr) 3413 (br), 1635, 1185, 1130 cm^{-1}. Anal. ($C_{44}H_{24}N_2Na_4O_{12}S_6 \cdot 6H_2O$) C, H, N.

Preparation of Tetrasodium 5,10,15,20-Tetrakis(4-sulfonatophenyl)-21,23-diselenaporphyrin (2). 5,10,15,20-Tetraphenyl-21,23-diselenaporphyrin (**4**, 0.030 g, 0.040 mmol) was dissolved in 2 mL of sulfuric acid and the resulting solution was heated at 100 °C for 18 h. The solution was cooled to ambient temperature and was added slowly to 25 mL of ice water. A 30% sodium hydroxide solution was added dropwise to raise the pH of the solution to 8. Ice was continually added to keep the solution cold. After the pH adjustment, the reaction mixture was concentrated in vacuo to give crude product, which was collected by filtration. The crude crystals were washed with several small portions of methanol (5 × 5 mL) to remove the soluble porphyrin from the less soluble sodium sulfate. The crude product was purified via Soxhlet extraction with 90% ethanol to give 0.040 g (85%) of **2** as a purple crystalline solid, mp > 300 °C: ^{1}H NMR (CD_3OD) δ 9.77 (s, 4 H, selenophene H's), 8.69 (s, 4 H, pyrrole H's), 8.34 (s, 16 H, Ar); ^{13}C NMR (CD_3OD) δ 170.67, 150.27, 145.23, 144.86, 138.79, 138.13, 136.92, 135.64, 127.71; IR (KBr) 3448 (br), 2955, 1558, 1418, 1247 cm^{-1}. Anal. ($C_{44}H_{24}N_2Na_4O_{12}Se_2S_4 \cdot 6H_2O$) C, H, N.

Preparation of 5,10,15,20-Tetraphenyl-21,23-dithiaporphyrin (3). 2,5-Bis(phenylhydroxymethyl)thiophene (**5**, 8.50 g, 0.0297 mol) and acetic anhydride (30 mL) were dissolved in 1.5 L of propionic acid. The pyrrole (2.0 mL, 0.029 mol) was then added and the resulting solution was stirred for 1 h at reflux. The reaction mixture was cooled to ambient temperature and was then added slowly to a mixture of 400 mL of a NH_4OH solution and 800 mL of ice water. The products were extracted with CH_2Cl_2 (3 × 300 mL) and the combined extracts were washed with more of the NH_4OH−ice water solution. The organic extracts were dried over $MgSO_4$ and concentrated. The crude product was purified via chromatography on SiO_2 eluted with 1:1 $CHCl_3$/toluene. The initial red band was collected and concentrated to give **3**, which was recrystallized from CH_2Cl_2/MeOH to give 0.56 g (6.0%) of metallic purple crystal, mp > 300 °C:[16] ^{1}H NMR ($CDCl_3$) δ 9.69 (s, 4 H, thiophene H's), 8.69 (s, 4 H, pyrrole H's), 8.25 (d, 8 H, $J = 7$ Hz, Ph), 7.81 (m, 12 H, Ph); IR (KBr) 3454, 2915, 2850, 1595, 1458, 1358 cm^{-1}; FAB(+)MS, m/z 649 ($C_{44}H_{28}N_2S_2$ + H, M^+ + 1). Anal. ($C_{44}H_{28}N_2S_2$) C, H, N.

Preparation of 5,10,15,20-Tetraphenyl-21,23-diselenaporphyrin (4). 2,5-Bis(phenylhydroxymethyl)selenophene (**6**, 2.32 g, 6.76 mmol) and acetic anhydride (10 mL) were dissolved in 500 mL of propionic acid. The pyrrole (0.50 mL, 0.029 mol) was then added and the resulting solution was stirred for 1 h at reflux. The reaction mixture was cooled to ambient temperature and was then added slowly to a mixture of 200 mL of a NH_4OH solution and 400 mL of ice water. The products were extracted with CH_2Cl_2 (3 × 300 mL) and the combined extracts were washed with more of the NH_4OH−ice water solution. The organic extracts were dried over $MgSO_4$ and concentrated. The crude product was purified via chromatography on SiO_2 eluted with 1:1 $CHCl_3$/toluene. The initial red band was collected and concentrated to give **4**, which was recrystallized from CH_2Cl_2/MeOH to give 0.056 g (2.2%) of metallic purple crystal, mp > 300 °C:[17] ^{1}H NMR ($CDCl_3$) δ 9.725 (s, 4 H, selenophene H's), 8.72 (s, 4 H, pyrrole H's), 8.29 (m, 8 H, Ph), 7.85 (m, 12 H, Ph); IR (KBr) 3454, 2915, 2850, 1595, 1458, 1358 cm^{-1}; FAB(+)MS, *m/z* 745 ($C_{44}H_{28}N_2{}^{80}Se_2$ + H, M^+ + 1). Anal. ($C_{44}H_{28}N_2Se_2$) C, H, N.

Preparation of 2,5-Bis(phenylhydroxymethyl)thiophene (5). Thiophene (20.3 mL, 21.0 g, 0.250 mol) was added slowly via syringe to a solution of *n*-BuLi (390 mL of a 1.6 M solution in hexanes, 0.62 mol) and tetramethylethylenediamine (75 mL) in 1 L of dry hexanes under an argon atmosphere. The resulting solution was warmed to reflux for 1 h, cooled to ambient temperature, and transferred via cannula to a pressure-equalizing addition funnel. The dilithiothiophene solution was added slowly to a solution of benzaldehyde (61 g, 0.58 mol) in 1 L of dry THF (distilled from sodium benzophenone ketyl) cooled to 0 °C in an ice bath. The ice bath was removed after addition was complete and the reaction was warmed to ambient temperature. A cold, saturated NH_4Cl solution was added (2 L) and the organic phase was separated. The aqueous phase was extracted with ether (3 × 500 mL). The combined organic phases were washed with water (3 × 1 L) and brine (1 L), dried over Na_2SO_4, and concentrated. The crude product was recrystallized from chloroform to give 65.9 g (89%) of crystalline **5** as one diastereomer (*meso*- or *d*,*l*-), mp 137−138 °C (lit.[19] mp 137−138 °C): ^{1}H NMR (DMSO-d_6) δ 7.38 (d, 4 H, J = 7 Hz, Ph), 7.31 (t, 4 H, J = 7 Hz, Ph), 7.22 (t, 2 H, J = 7 Hz, Ph), 6.67 (s, 2 H, thiophene H's), 6.11 (br s, 2 H, −CHO*H*), 5.83 (s, 2 H, −C*H*-OH); ^{13}C NMR (DMSO-d_6) δ 149.37, 144.97, 128.09, 127.06, 125.99, 122.91, 70.75; IR (KBr) 3403 (br), 3075, 2874, 1453 cm^{-1}; FAB(+)MS *m/z* 297 ($C_{18}H_{16}O_2S$ + H, M + 1). Anal. ($C_{18}H_{16}O_2S$) C, H.

The crude diol prior to crystallization displayed approximately a 1:1 mixture of both diastereomers. For the second diastereomer of **5**: ^{1}H NMR (DMSO-d_6) δ 7.38 (d, 4 H, J = 7 Hz, Ph), 7.31 (t, 4 H, J = 7 Hz, Ph), 7.22 (t, 2 H, J = 7 Hz, Ph), 6.64 (s, 2 H, thiophene H's), 6.11 (br s, 2 H, −CH−O*H*), 5.82 (s, 2 H, −C*H*OH); ^{13}C NMR (DMSO-d_6) δ 149.18, 144.44, 128.09, 127.03, 126.06, 122.80, 70.65.

Preparation of 2,5-Bis(phenylhydroxymethyl)selenophene (6). Selenophene (10.0 g, 0.0760 mol) was added slowly via syringe to a solution of *n*-BuLi (100 mL of a 1.6 M solution in hexanes, 0.16 mol) and tetramethylethylenediamine (25 mL) in 350 mL of dry hexanes under an argon atmosphere. The resulting solution was warmed to reflux for 1 h, cooled to ambient temperature, and transferred via cannula to a pressure-equalizing addition funnel. The dilithiothiophene solution was added slowly to a solution of benzaldehyde (17.0 g, 0.160 mol) in 350 mL of dry THF (distilled from sodium benzophenone ketyl) cooled to 0 °C in an ice bath. The ice bath was removed after addition was complete and the reaction was warmed to ambient temperature. A cold, saturated NH_4Cl solution was added (1 L) and the organic phase was separated. The aqueous phase was extracted with ether (3 × 200 mL). The combined organic phases were washed with water (3 × 300 mL) and brine (300 mL), dried over Na_2SO_4, and concentrated. The crude product was recrystallized from chloroform to give 11.1 g (42%) of crystalline **6** as one-to-one mixture of diastereomers (*meso*- and *d*,*l*-), mp 92−93 °C for roughly half of the crystals and 140−144 (dec) °C for the other half (lit.[18] mp 143−144 °C): ^{1}H NMR (CD_3OD) δ 7.49 (d, 4 H, J = 7 Hz, Ph), 7.36 (t, 4 H, J = 7 Hz, Ph), 7.26 (t, 2 H, J = 7 Hz, Ph), 6.81/6.77 (s, 2 H, selenophene H's), 5.79/5.78 (s, 2 H, −C*H*OH), 2.88 (br s, 2 H, −CHO*H*); ^{13}C NMR (CD_3OD) δ 157.04/156.84, 145.30/145.24, 128.10/128.02, 127.06/127.02, 125.98/125.92, 124.38, 72.40; IR (KBr) 3437 (br), 3062, 3029, 2867, 1493 cm^{-1}; FAB(+)MS *m/z* 345 ($C_{18}H_{16}O_2{}^{80}Se$ + H, M + 1). Anal. ($C_{18}H_{16}O_2Se$) C, H.

Quantum Yield Determinations. Quantum yields for singlet oxygen were measured in 0.01 M phosphate-buffered saline (PBS) at pH 7.4 with 1.6% NaCl at 25 °C using methods we have previously described.[20,21]

Cells and Culture Conditions. Colo-26, a murine colon carcinoma cell line, was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics (all components purchased from GIBCO Laboratories, Grand Island, NY) at 37 °C, 5% CO_2. EMT-6, a murine mammary carcinoma cell line, was maintained in MEM supplemented with 15% fetal calf serum and antibiotics. MOLT-4, a murine T cell leukemia cell line, was maintained in RPMI 1640, 5% FCS and antibiotics at 37 °C, 5% CO_2.

In Vitro Phototoxicity Measurements. Cells were plated at 5 × 10^3 cells/well of a 96-well tissue culture plate the evening before the assay. The day of the assay, the cells were washed twice with PBS, and 100 μL of HBSS containing various concentrations of either **1** or $TPPS_4$ was added to each well. The sensitizer and cells were incubated for 2 h at 37 °C followed by a wash with PBS and the addition of 100 μL of PBS. The plates were irradiated with red light at either 694 or 630 nm for a total light dose of 5 J cm^{-2}. Following irradiation 100 μL of growth media was added, and the plates were incubated for 24 h at 37 °C, 5% CO_2. Cell survival was monitored using the MTT assay as described in Mosmann.[24]

Animals. All animals were cared for under the guidelines of the Roswell Park Cancer Institute Committee on Animal Resources or the University Committee on Animal Resources at the University of Rochester.

Preparation of Mitochondrial Suspensions. The R3230AC mammary adenocarcinoma was transplanted subcutaneously in the axillary region of 80−100g female Fischer 344 rats using the sterile trochar method.[25] Two to three weeks after transplantation, when tumors had grown to 2−3 cm in diameter, the animals were sacrificed. The tumors were excised and placed in 0.9% sodium chloride on ice. The tissue was finely minced with scissors and homogenized on ice at a ratio of 1 g of tumor tissue to 5 mL of buffer containing 0.33 M sucrose, 1 mM dithiothreitol, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraaetic acid, 0.03% bovine serum albumin, and 0.1 M potassiom chloride (pH 7.4), using 30-s bursts with a Polytron PCU-2110 homogenizer at a setting of 6 (Brinkmann Ind., Westbury, NY). Preparation of isolated mitochondria from the homogenized tumor tissue followed a method described earlier.[23] Mitochondrial suspensions were divided into 0.5-mL aliquots (6−10 mg of mitochondrial protein/mL) and stored at −86 °C until used for in vitro experiments.

Exposure of Tumor Mitochondria to Sensitizer 1 and $TPPS_4$ in Vitro. Mitochondrial suspensions were removed from storage and thawed on ice. Solutions of sensitizer **1** and $TPPS_4$ were prepared by dissolving 2.5 mg of dye in 5.0 mL of mitochondrial preparation buffer, which approximated a 1.0 mM solution for each of the three dyes studied. Final concentrations of the sensitizers were determined using their absorbance. Ten microliters of the sensitizer/buffer solution was transferred to 1.0 mL mitochondrial preparation buffer and the absorbance determined using a diode array spectrophotometer (HP8452A, Hewlett-Packard, Palo Alto, CA). The sensitizers in mitochondrial preparation buffer, at a final concentration that gave an OD of 0.2, were then added to mitochondrial suspensions (1.0 mL) and allowed to incubate in the dark on ice for 15 min. The sensitizer/mitochondrial suspension was then centrifuged at 8000*g* for 3 min using an Eppendorf microcentrifuge (Model 3200, Brinkmann Ind.,

Westbuty, NY), the supernatant was aspirated with a Pasteur pipet, and the pellet was resuspended in 1.0 mL of mitochondrial preparation buffer. The suspension was then transferred to a 3.0-mL quartz cuvette which was positioned in a focused, 1.0-cm diameter, filtered (530 to 750 nm) light beam emitted from a 750-W tungsten source. The intensity of the beam was uniform over the wavelength band used and adjusted to a fluence rate of 100 mW cm^{-2} using neutral density filters. Beam intensity was measured using a radiometer (Model 210, Coherent Inc., Palo Alto, CA). The light was cooled by passing it through a water filter, eliminating thermal effects as the sample temperature did not rise above 25 °C. The mitochondrial suspensions were magnetically stirred continuously during the 1.0-h irradiation period. Aliquots (10 μL) were removed at various times during irradiation for determination of cytochrome c oxidase activity. A portion of the mitochondria/dye suspension was maintained in the dark, and determinations of cytochrome c oxidase activity were performed on aliquots from these samples as dark controls. Measurement of cytochrome c oxidase activity was performed according to a method described earlier.[23] Initial enzyme activity was adjusted to obtain a decrease in the reduced cytochrome c oxidase absorbance at 550 nm of 0.4−0.6 OD units/min. Data are expressed as the % of initial, preirradiation cytochrome c oxidase activity.

Photosensitizer Administration. For animal experimentation, the sensitizers were dissolved in 5% sodium bicarbonate solutions. Injection was intravenous via the tail vein.

Fluorescence Measurements (Solvable Assay.) Tissue samples and tumors were harvested at various times post-treatment and flash frozen at −70 °C. Samples were thawed on ice and 1 mL of Solvable (Packard Instrument Company, Meriden, CT) was added prior to incubation at 55 °C for 18−24 h. Samples were allowed to cool to ambient temperature, and the fluorescence per sample was determined and compared to a standard curve generated by dilution of pure **1**. Results are presented as total dye concentration per milligram of total protein. Total protein is measured using the Bio-Rad protein assay (Bio-Rad Laboratories).

PDT with 1. Colo-26 tumors were implanted in BALB/c mice via the sterile trochar method. Sensitizer at 3.25 or 10 mg/kg was administered 96 h following tumoring of the animals. Four hours following administration of sensitizer **1**, the tumors were irradiated with red light at 694 nm at 75 mW cm^{-2} for 30 min for a total light dose of 135 J cm^{-2}. Treated animals were followed until a tumor volume of 400 mm^3 was reached. An untreated control group received neither light nor drug.

Statistical Analyses. All statistical analyses were performed using the Student's *t*-test for pairwise comparisons. A *P* value of <0.05 was considered significant.

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Supporting Information Available: Figure S1 showing the dark and phototoxicity of $TPPS_4$ and **1** against the murine T-cell line MOLT-4. This information is available free of charge via the Internet at http://pubs.acs.org.

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