A Selenopyrylium Photosensitizer for Photodynamic Therapy Related in Structure to the Antitumor Agent AA1 with Potent in Vivo Activity and No Long-Term Skin Photosensitization

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Cationic chalcogenopyrylium dyes **5** were synthesized in six steps from *p*-aminophenylacetylene (**9**), have absorption maxima in methanol of 623, 654, and 680 nm for thio-, seleno-, and telluropyrylium dyes, respectively, and generate singlet oxygen with quantum yields [$\Phi(^1O_2)$] of 0.013, 0.029, and 0.030, respectively. Selenopyrylium dye **5-Se** was phototoxic to cultured murine Colo-26 and Molt-4 cells. Initial acute toxicity studies in vivo demonstrate that, at 29 mg (62 μmol)/kg, no toxicity was observed with **5-Se** in animals followed for 90 days under normal vivarium conditions. In animals given 10 mg/kg of **5-Se** via intravenous injection, 2−8 nmol of **5-Se**/g of tumor was found at 3, 6, and 24 h postinjection. Animals bearing R3230AC rat mammary adenocarcinomas were treated with 10 mg/kg of **5-Se** via tail-vein injection and with 720 J cm^{-2} of 570−750-nm light from a filtered tungsten lamp at 200 mW cm^{-2} (24 h postinjection of **5-Se**). Treated animals gave a tumor-doubling time of 9 ± 4 days, which is a 300% increase in tumor-doubling time relative to the 3 ± 2 days for untreated dark controls. Mechanistically, the mitochondria appear to be a target. In cultured R3230AC rat mammary adenocarcinoma cells treated with 0.1 and 1.0 μM **5-Se** and light, mitochondrial cytochrome *c* oxidase activity was inhibited relative to cytochrome *c* oxidase activity in untreated cells. Irradiation of isolated mitochondrial suspensions treated with 10 μM dye **5-Se** inhibited cytochrome *c* oxidase activity. The degree of enzyme inhibition was abated in a reduced oxygen environment. Superoxide dismutase, at a final concentration of 30 U, did not alter the photosensitized inhibition of mitochondrial cytochrome *c* oxidase by dye **5-Se**. The data suggest that singlet oxygen may play a major role in the photosensitized inhibition of mitochondrial cytochrome *c* oxidase.

Introduction

Various classes of cationic dyes have been explored as sensitizers for photodynamic therapy (PDT), which is a recently approved protocol for the treatment of cancer combining light and endogenous oxygen with a photosensitizer localized in or around the tumor.[1] While Photofrin has approval for clinical use in PDT against several tumor types and has shown clinical efficacy, Photofrin has several disadvantages including: (1) prolonged light sensitivity in treated patients, (2) weak absorption at the 630-nm maximum, and (3) difficulties in characterization as Photofrin is a mixture of products.[1] These disadvantages have driven the search for other porphyrin and porphyrin-like materials as sensitizers for PDT. Several second- and third-generation materials show considerable clinical promise.[1d] Of the non-porphyrin sensitizers, the cationic dyes are perhaps most important and include representative examples from the Victoria blue dyes,[2] methylene blue[1d,3] and related Nile blue dyes,[4] cyanine dyes,[5] rhodacyanine dyes,[6] telluropyrylium dyes,[7] and selenopyrylium dyes.[8] All of these dye classes typically have absorption maxima of greater than 600 nm and have molar extinction coefficients of $\geq 10^4$ M^{-1} cm^{-1}, which make them more effective at harvesting light during PDT than Photofrin and other porphyrin-derived sensitizers.[1] Many of the cationic dyes accumulate selectively in transformed cells relative to normal cells with the mitochondria as important cellular targets.[2,3,5−8]

The cationic dyes rhodamine-123 (Rh-123)[9] and 2,6-bis(4-aminophenyl)-4-(dimethylaminophenyl)thiopyrylium chloride (AA1)[10] accumulate selectively in tumors, presumably through localization in the mitochondria (Chart 1). Although these materials show selective toxicity toward carcinoma cells relative to normal epithelial cells, they are poor sensitizers for PDT. In addition to having absorption maxima of <600 nm, their poor performance in PDT perhaps correlates with their low quantum yields for the generation of singlet oxygen [$\Phi(^1O_2) < 0.01$], which is one cytotoxic species that can be produced by irradiation of the sensitizer.[1]

We have recently described chalcogenopyrylium dyes **1**−**4** (Chart 1) and related structures that are similar in structure to AA1 and have evaluated these dyes as

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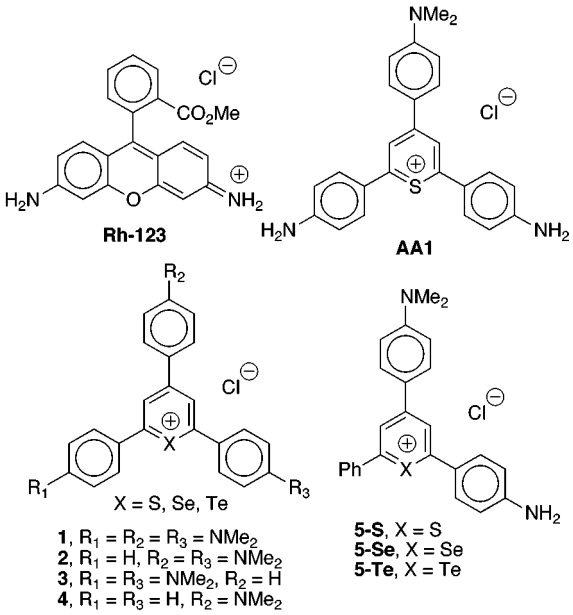
Chart 1

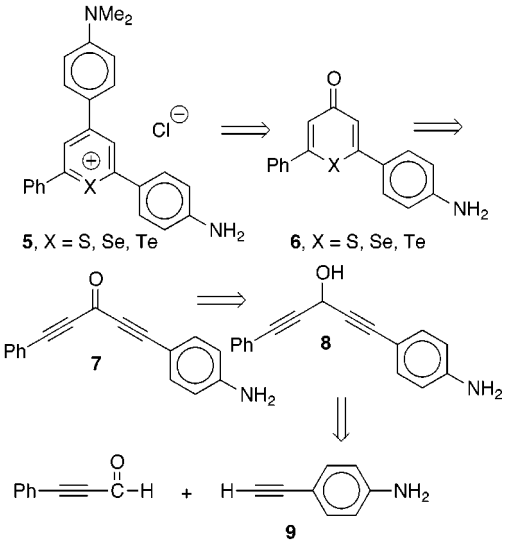


sensitizers in vitro.[8] Two trends have emerged that can be exploited in the design of better sensitizers. The heavier chalcogen atoms selenium and tellurium in these molecules increase the quantum yield for singlet oxygen generation relative to the thiopyrylium analogues and give effective photosensitizers in vitro. A second trend is that two aniline nitrogens appear to be sufficient to provide a photosensitizer based on in vitro evaluation. All three chalcogen analogues of dyes **1** bearing three dimethylanilino substituents were active as photosensitizers in vitro.[8a] Similarly, dyes **2** and **3** bearing two dimethylanilino substituents were also active as photosensitizers in vitro.[8b] In contrast, dyes **4** bearing one 4-dimethylanilino substituent displayed no phototoxicity in vitro.[8b]

Of the dye classes **1**−**4**, selenopyrylium dye **1-Se** is perhaps the most promising as a sensitizer candidate for PDT with strong absorption at 630 nm and $\Phi(^1O_2)$ of 0.064 (Table 1), which is the largest value of $\Phi(^1O_2)$ measured for dyes **1**-**4** and related structures. However, one concern with dye classes **1**−**3** is their relatively large values of the *n*-octanol/water partition coefficient (log $P \geq 2.2$), which might limit their ability to circulate freely in vivo and concentrate in tumor (as opposed to binding tightly to the first fatty deposits they encounter).

We describe here the synthesis of chalcogenopyrylium dyes **5** bearing an anilino substituent at the 2-position, a dimethylanilino substituent at the 4-position, and a phenyl substituent at the 6-position. Dyes **5** have higher values of $\Phi(^1O_2)$ than the corresponding dyes **2**, bearing two dimethylanilino substituents at the 2- and 4-positions, and significantly lower values of log *P*. Selenopyrylium dye **5-Se** was found to be an effective sensitizer for PDT against various carcinoma cell lines in culture and in vivo against R3230AC mammary adenocarcinomas in female Fischer rats, where a 300% increase in tumor-doubling time was observed in treated animals relative to untreated controls. Furthermore, no skin photosensitivity was observed in treated animals. In vitro experiments in whole cells and in isolated mitochondrial suspensions suggest that the mitochondria may be a target for PDT with **5-Se**.

Scheme 1



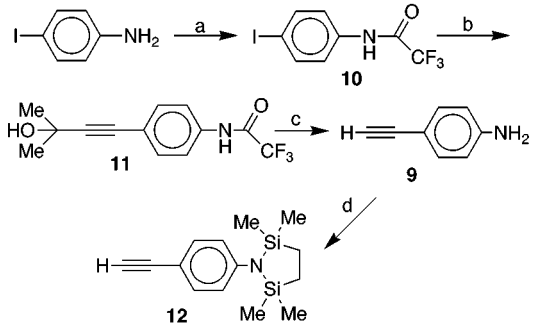
Chemical Results and Discussion

Retrosynthetic Analysis and Synthesis. The chalcogenopyranones **6** are the key intermediates for the synthesis of dyes **5** as shown in the retrosynthesis of Scheme 1. In analogy to the preparation of AA1, the addition of Grignard reagents to the pyranone carbonyl followed by acid-induced dehydration should lead to the chalcogenopyrylium dyes **5** even with an unprotected amino group on the aniline substituent.[10] However, existing synthetic methods to selenopyranone **6-Se** and telluropyranone **6-Te** must go through 1,4-pentadiyn-3-one **7** as an intermediate.[8] We were concerned that a free amino group in **7** might be problematic with respect to self-addition reactions to either the triple bonds or the carbonyl carbon. Compound **7** can be prepared via the oxidation of diynol **8**, which in turn is most readily prepared by the addition of an acetylide anion to phenylpropargylaldehyde. Although *p*-aminophenylacetylene (**9**) is known,[11] reactions of **9** with Grignard reagents and alkyllithium compounds are complicated by the free amino substituent.

The successful synthesis of chalcogenopyrylium dyes **5** required a suitable protecting group for **9** that would withstand the basic and nucleophilic conditions for the synthesis of chalcogenopyranones **6**. The bis-1,2-(dimethylsilyl)ethano (Stabase) protecting group[12] for the amine group appeared to be ideal for our purposes. It is stable to strong bases under aprotic conditions and comes off easily under either aqueous acidic or basic conditions.

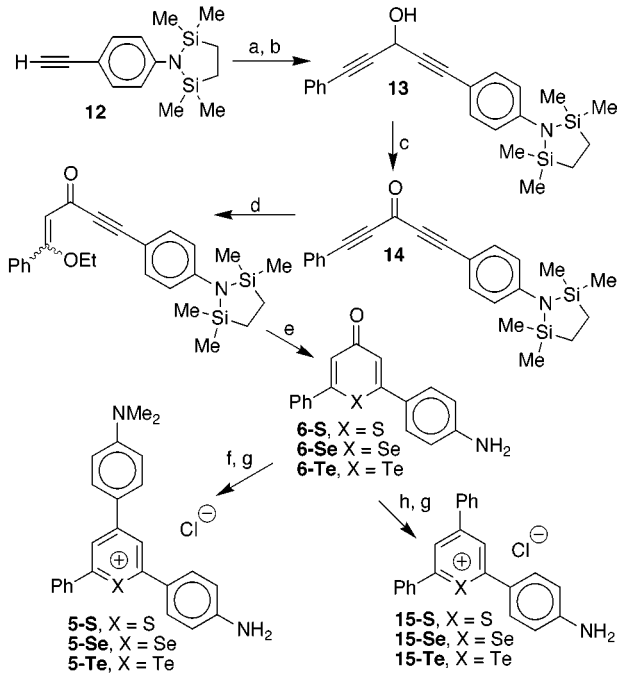
p-Aminophenylacetylene (**9**) was prepared according to Scheme 2.[11] *p*-Iodoaniline was protected as its trifluoroacetamide derivative **10** with trifluoroacetic anhydride in 98% yield, and **10** was coupled with 2-methyl-3-butyn-2-ol in the presence of Pd(II) to give acetylene **11** in 82% isolated yield. Both the amine and acetylene were deprotected with potassium hydroxide in 2-propanol to give **9** in 86% isolated yield. Acetylene **9** was treated with 1,2-bis(chlorodimethylsilyl)ethane in the

Scheme 2[a]



[a] Key: (a) $CF_3C(=O)OC(=O)CF_3$, THF (98%); (b) $(PPh_3)_2PdCl_2$, PPh_3, CuI_2, Et_3N, $HC{\equiv}CCMe_2OH$ (82%); (c) KOH/isopropanol (86%); (d) $ClSiMe_2CH_2CH_2SiMe_2Cl$, Et_3N, CH_2Cl_2 (75%)

Scheme 3[a]



[a] Key: (a) *n*-BuLi, THF, −78 °C; (b) PhC ≡ CCHO; (c) MnO_2, CH_2Cl_2 (86%); (d) 0.25 M NaOEt in EtOH; (e) S, Se, Te, $NaBH_4$ (2.5 equiv), 0.1 M NaOEt in EtOH, add Na_2 X to **14**; (f) (i) $Me_2NC_6H_4MgBr$, (ii) HPF_6; (g) Amberlite IRA-400 (Cl); (h) (i) PhMgBr, (ii) HPF_6

presence of triethylamine[12] to give protected acetylene **12** in 75% isolated yield (52% yield overall from *p*-iodoaniline).

Acetylene **12** was deprotonated with *n*-BuLi in THF at −78 °C, and the resulting acetylide was added to phenylpropargylaldehyde to give 1,4-pentadiyn-3-ol **13** with the Stabase group intact in 78% isolated yield (Scheme 3). Compound **13** was not stable to chromatography on either alumina or silica so the crude product was oxidized directly with MnO_2 to give 1,4-pentadiyn-3-one **14** in 59% overall yield from **12**.

The formal addition of hydrogen chalcogenides to **14** was best accomplished in two steps to minimize 5-exo-trig cyclization to give dihydrochalcogenophene products.[13] Diynone **14** was stirred with 0.25 M sodium ethoxide in ethanol for 2 h at ambient temperature to give addition of ethanol across one triple bond of **14** (Scheme 3). The addition of disodium chalcogenides to the enol ethers derived from **14** gave chalcogenopyranones **6** in 40−63% isolated yields.

Table 1. Physical Properties of Chalcogenopyrylium Dyes **2** and **5**

dye	λ_{max} (log ϵ)[a]	$\Phi(^1O_2)$[b]	log *P*
1-Se	634 (4.71)	0.064 ± 0.006[c]	2.2[c]
2-S	627 (4.54)	0.005 ± 0.001[d]	2.5
2-Se	657 (4.56)	0.015 ± 0.001[d]	2.4
2-Te	689 (4.54)	0.010 ± 0.001[d]	2.5
5-S	623 (4.49)	0.013 ± 0.003	0.0
5-Se	654 (4.47)	0.029 ± 0.005	0.8
5-Te	680 (4.50)	0.030 ± 0.004	0.5

[a] In *n*-octanol. [b] In methanol. [c] Ref 8a. [d] Ref 8b.

The chalcogenopyrylium dyes **5** were prepared by the addition of 4-dimethylaminophenylmagnesium bromide to pyranones **6** followed by dehydration with HPF_6 (Scheme 3). The hexafluorophosphate salts were converted to the chloride salts **5** using an ion-exchange resin.

As discussed below, dyes **15** bearing a 2-anilino substituent and phenyl groups at the 4- and 6-positions were prepared as control compounds. Dyes **4** isolate the contributions from the 4-dimethylanilino substituent, while dyes **15** isolate the contributions from the 2-anilino substituent. Dyes **15** were prepared via the addition of phenylmagnesium bromide to chalcogenopyranones **6** (Scheme 3). Selected properties of dyes **4** and **15** are compiled in Table 1.

Absorption Spectra of Dyes 2 and 5. As shown in Table 1, 2,4-bis(4-dimethylanilino) dyes **2** and 2-anilino-4-dimethylanilino dyes **5** have nearly identical absorption maxima and extinction coefficients for corresponding pairs of chalcogenopyrylium dyes in organic solvents such as *n*-octanol or dichloromethane. As with other series of chalcogenopyrylium dyes, replacing a lighter chalcogen atom with a heavier atom leads to a bathochromic shift in absorption maxima.

One interesting feature of the absorption spectra of anilino-substituted dyes **5** is the large hypsochromic shift observed when the dyes are dissolved in water. While absorption maxima are at 623, 654, and 680 nm for **5-S**, **5-Se**, and **5-Te**, respectively, in *n*-octanol, absorption maxima in water are shifted to shorter wavelengths by 40−50 nm at 573, 615, and 640 nm, respectively. The ability of the free amino group to act as both a hydrogen bond donor as well as a hydrogen bond acceptor is most likely responsible for the large solvent effect that is observed. Hydrogen bond donation in more polar solvents decreases the effective cationic charge on the chromophore and the corresponding cyanine character.

Quantum Yields for Singlet Oxygen Generation. In earlier studies with dyes **1**, we demonstrated that the inhibition of cytochrome *c* oxidase observed upon irradiation of dye-treated mitochondrial suspensions was oxygen-dependent and corresponded, at least in part, to the generation of singlet oxygen upon irradiation of the sensitizer.[8] While Aveline and Redmond have demonstrated that solution photophysics need not necessarily correspond to cellular phototoxicity,[14] trends within structurally related series of molecules should be more reliable predictors of relative phototoxicity. Dyes **1** and **2** have relatively low solution values of $\Phi(^1O_2)$, which may be due to rotational modes of relax-

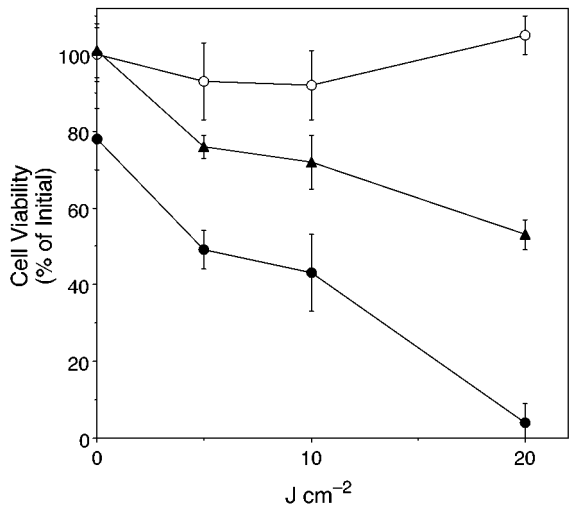


Figure 1. (a) Cell viability of cultured Colo-26 cells exposed to 0.8 μM (▲) or 1.6 μM (●) **5-Se** and light. Details of experimental conditions are described in the Experimental Section. Data are expressed as the fraction of viable cells compared to dye-free cells and light (○). Each datum point represents the mean percent viable cells calculated from at least three separate experiments; bars are the standard deviation.

ation of the excited state.[8] However, when dyes **1** and **2** are bound to biological membranes in vitro, they are effective sensitizers for PDT.[8] Values of $\Phi(^1O_2)$ for dyes **2** and **5** are compared in Table 1. Values of $\Phi(^1O_2)$ were higher for each dye **5** relative to the same heteroatom analogue of dye **2**. One might reasonably expect dyes **5** to be more effective as sensitizers for PDT than the corresponding dye **2** if singlet oxygen were primarily responsible for the observed phototoxicity.

***n*-Octanol/Water Partition Coefficients.** The *n*-octanol/water partition coefficients (log *P* values) for dyes **2** and **5** are compiled in Table 1. The free amino group on the aniline of dyes **5** greatly enhances the partitioning into water by approximately 2 orders of magnitude relative to the corresponding dyes **2**. Again, the ability of the free amino group to act as both a hydrogen bond donor and acceptor is most likely responsible for this observation. The greater partitioning into water also suggests that the sensitizer should circulate more freely in vivo.

Biological Results and Discussion

In Vitro Studies. Photodynamic Therapy Against Cells in Culture. Selenopyrylium dye **5-Se** was evaluated in culture for dark- and light-induced toxicities toward Colo-26 cells, a murine colon carcinoma cell line, and Molt-4 cells, a murine T-cell line. Cell cultures were incubated for 2 h in the dark with various concentrations of **5-Se** and were then washed prior to treatment with red light from a 630-nm laser for a total light dose of 5−20 J cm^{-2}. Light-treated cells and dark controls were incubated for 24 h, and cell survival was determined. Results are shown in Figure 1a for Colo-26 cells and Figure S1b (Supporting Information) for Molt-4 cells and indicate significant phototoxicity for **5-Se** against both cell lines.

Initial in Vivo Toxicity Studies. Cationic sensitizers for PDT are typically delivered as a single dosage in the 5−10 μmol/kg range.[2−7,15] In this study, neither morbidity nor mortality was observed in groups of five BALB/c mice given single dosages of 10 mg (21 μmol)/kg, 20 mg (43 μmol)/kg, or 29 mg (62 μmol)/kg of selenopyrylium dye **5-Se** in saline solutions. The animals were observed for 90 days under normal vivarium conditions (a 24-h cycle of 12 h of fluorescent light and 12 h of darkness). Higher concentrations were limited by the solubility of **5-Se** in saline solution. Animals sacrificed after 90 days showed no macroscopic signs of abnormalities in internal organs or tissues.

Similar results were obtained in female Fischer rats given dosages of 10 mg (21 μmol)/kg or 20 mg (43 μmol)/kg of selenopyrylium dye **5-Se** in saline solutions. Again no morbidity or mortality was observed in animals kept for 90 days under normal vivarium conditions. Based on the results with both BALB/c mice and female Fischer rats, a treatment dosage of 10 mg/kg was chosen for initial studies with a therapeutic index of at least 3.

Distribution of Dye 5-Se in Tumor-Bearing Animals. Although dye **1-Se** was phototoxic to cells in culture,[8] **1-Se** did not distribute to the tumor and other organs following injection into tumor-bearing animals. The relatively low value of log *P* (Table 1) for dye **5-Se** would be expected to impact favorably the time course of distribution of the sensitizer through a longer circulating lifetime. Saline solutions of **5-Se** were injected via the tail vein into 100-g female Fischer rats bearing R3230AC mammary tumors to give 10 mg (21 μmol)/kg of animal. The animals were sacrificed at 10 min and 3, 6, and 24 h postinjection (2 animals/time point). The tumors were excised and individually homogenized in an equal volume mixture of methanol and 1 N $HClO_4$. Following centrifugation, levels of dye **5-Se** were determined spectrophotometrically from the supernatant. No dye was detected in the tumors of treated animals 10 min following injection. However, dye **5-Se** was measured at 2−8 nmol of dye **5-Se**/g of tissue in tumors excised at 3, 6, and 24 h postinjection. At 24 h, no **5-Se** was detected in the liver, lungs, or kidneys of treated animals, which suggested 24 h postinjection as a starting point for PDT (Table S2, Supporting Information). Dye **5-Se** (5−12 nmol/g of tissue) was also found in the Colo-26 tumors of BALB/c mice given 29.3 mg (62 μmol) of **5-Se**/kg examined 3 and 24 h postinjection.

In both tumor-bearing Fischer rats and BALB/c mice sacrificed 72 h postinjection, no **5-Se** was detected in tumor, liver, lungs, spleen, or kidneys, which suggests that the drug has cleared the animals at this time. More quantitative assay of dye distribution in both models awaits the synthesis of appropriate radiolabeled materials.

Tumor Response to PDT with Selenopyrylium Dye 5-Se. The long-wavelength absorption band of **5-Se** is broad with apparent shoulders. As a consequence, we began our PDT investigation with a filtered, broadband light source to irradiate the entire absorption band as opposed to using a single-wavelength laser source for initial studies. Female Fischer rats bearing R3230AC mammary tumors were treated with **5-Se** and 570−750-nm light at 200 mW cm^{-2} for 1 h (720 J cm^{-2}) from a filtered tungsten lamp. Eight animals were irradiated 24 h after injection of saline solutions of **5-Se** [10 mg (21 μmol)/kg]. The data are compiled in Figure 2 as a

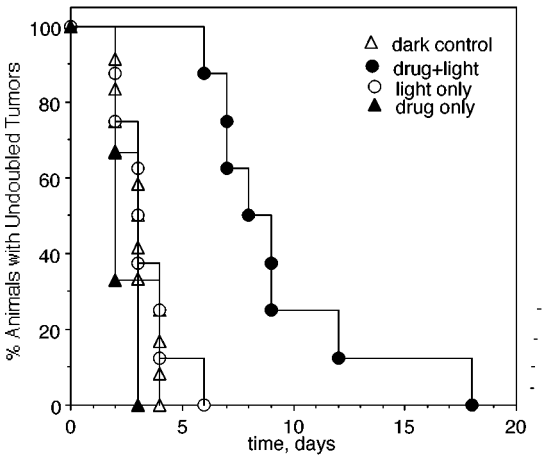


Figure 2. PDT with sensitizer **5-Se** at 10 mg/kg and 720 J cm^{-2} of 570−750-nm light delivered at 100 mW cm^{-2} for 60 min against implanted R3230AC rat mammary adenocarcinoma in female Fischer rats (●). These results can be compared with control groups that received neither light nor drug (○), light only (△), and drug only (▲). Times were measured posttreatment until tumor volumes had doubled.

Kaplan−Meyer plot indicating the time in days for the tumors to double their initial, pretreatment tumor volume for the groups studied in these experiments. A tumor-doubling time of 9 ± 4 days was observed in the treated animals compared to 3 ± 2 days for the untreated, dark controls ($P < 0.01$) with error limits expressed as the SEM. Animals treated with 570−750 nm-light at 200 mW cm^{-2} for 1 h and no sensitizer gave tumor-doubling times [(3 ± 2) days] equivalent to untreated dark controls as did animals treated with drug (10 mg/kg) and no light [(2.3 ± 0.5) days]. The temperature of the tumors during irradiation of either the control group or treatment group did not exceed 40 °C, which excludes hyperthermia during irradiation as a source of the tumor response and indicates that light and sensitizer are both necessary for response.

Skin Photosensitization. One side effect associated with Photofrin-mediated PDT is the long-term skin photosensitivity (up to 6 weeks) that patients may experience.[1] On the basis of the rapid clearance of **5-Se** from normal tissues in both Fischer rats and BALB/c mice, one would expect minimal long-term skin photosensitization from **5-Se**. Mouse footpad and ear-swelling response models have been developed to examine skin photosensitivity after PDT.[16] These tests were used to evaluate **5-Se**-mediated PDT for induction of skin photosensitivity. The right rear footpads of groups of five BALB/c mice receiving 10 mg (21 μmol)/kg of **5-Se** were irradiated using 630-nm laser light delivered at 75 mW cm^{-2} for a total fluence of 135 J cm^{-2} at 24 h postinjection. The response of irradiated right footpads was compared to the left footpads that were not irradiated. Footpads were scored 24 h after irradiation. There was no sign of swelling or redness in any of the treated footpads indicating that skin photosensitization was undetectable using this method after **5-Se**-mediated PDT.

Similar results were obtained irradiating the right earflaps of groups of three BALB/c mice treated with 10 mg/kg of **5-Se** and light (630-nm laser light, 75 mW cm^{-2}, 135 J cm^{-2}) at intervals of 24 h and 3 and 7 days. The thickness of the irradiated right earflap was measured at 5 min (control for light-induced swelling) and 24 h (acute skin photosensitization response) postirradiation with an engineer's micrometer and compared to the preirradiation thickness of the earflap. There was no sign of redness or swelling in any of the treated earflaps at any time point indicating that skin photosensitization was undetectable using this method after **5-Se**-mediated PDT.

Mechanistic Studies. Inhibition of Cytochrome *c* Oxidase in Cultured Cells. One might expect the tumor mitochondria to be a target during PDT with **5-Se** due to its structural similarity to AA1. To examine this hypothesis, whole cells in culture were treated with 0.01−1.0 μM solutions of **5-Se** and were then washed and irradiated for 5−10 min with fluorescent lights at 0.2 mW cm^{-2}, and the activity of cytochrome *c* oxidase in suspensions of treated cells was determined. The data are presented in Figure S3 (Supporting Information) and indicate that mitochondrial cytochrome *c* oxidase activity is greatly diminished upon treatment with **5-Se** and light. Cells treated with **5-Se** only and no light did not display any inhibition of cytochrome *c* oxidase relative to untreated controls. Cytochrome *c* oxidase is the last enzyme in the mitochondrial respiration chain, and the loss of activity shown in Figure S3 is due to direct photodamage to this enzyme or to other sites preceding it in the respiration chain.[17] The data are consistent with the dye entering the mitochondria of whole cells upon treatment and with the mitochondria being a target during PDT or at least with cytochrome *c* oxidase being a target.

Inhibition of Cytochrome *c* Oxidase in Isolated Mitochondrial Suspensions. The identity of the phototoxic species produced was probed with studies of isolated mitochondrial suspensions. Mitochondria, isolated from R3230AC rat mammary adenocarcinoma, were treated with 10^{-5} M solutions of **5-Se** for 5 min and centrifuged, and the pellet was resuspended. The cytochrome *c* oxidase activity in the dye-treated suspensions was determined at selected times during 1 h of irradiation with 570−750-nm light at 100 mW cm^{-2} and showed a decrease in activity with increasing light dose as shown in Figure S4 (Supporting Information). It should be noted that cytochrome *c* oxidase activity in dye-treated mitochondrial suspensions was unaltered during a 1-h incubation in the dark.

The photosensitized inhibition of mitochondrial cytochrome *c* oxidase by **5-Se** can be prevented by reducing the oxygen in the mitochondrial suspensions via nitrogen purging (Figure S4, Supporting Information). Reduction of the oxygen concentration to 0 ± 2% prior to and during irradiation of the dye-treated mitochondria prevented the inhibition of cytochrome *c* oxidase activity by **5-Se**, which strongly suggests that oxygen is involved in the inhibition process. The generation of singlet oxygen upon irradiation or the photoinduced transfer of an electron to generate superoxide is the most likely path available for oxygen involvement. Superoxide dismutase (SOD), added at a concentration of 30 enzyme units/mL, did not reduce the photosensitized inhibition of cytochrome *c* oxidase (Figure S4, Supporting Information), which suggests that singlet oxygen is responsible for the photoinhibition of cytochrome *c* oxidase activity that is observed.

Summary and Conclusions

Effective cationic sensitizers for PDT based on the thiopyrylium structure of AA1 can be prepared by replacing the sulfur atom with selenium in AA1-like molecules. The heavy atom increases the wavelength of absorption of the dye as well as the quantum yield for the production of singlet oxygen as illustrated for selenopyrylium dye **5-Se** (Table 1). Desirable photophysical properties are achieved in molecules with at least two anilino substituents (*N*-substituted or unsubstituted). However, more desirable biodistribution is observed with one anilino substituent and one dimethylanilino subtituent as in **5-Se**. Selenopyrylium dye **5-Se** is an effective photosensitizer against carcinoma cell lines in vitro (Figure 1) and gives prolonged tumor-doubling times in female Fischer rats bearing R3230AC rat mammary adenocarcinomas (Figure 2). Furthermore, sensitizer **5-Se** clears the skin of treated animals 24 h postinjection, which is a significant advantage over Photofrin.

The necessity of both anilino substituents in **5-Se** is inferred from the lack of photosensitization observed in vitro using either selenopyrylium dye **4-Se** with 2,6-diphenyl and 4-dimethylanilino substituents[8b] or selenopyrylium dye **15-Se** with 2,4-diphenyl and 6-anilino substituents in this study. Furthermore, neither **4-Se** nor **15-Se** produces singlet oxygen in significant amounts [$\Phi(^1O_2) < 0.001$] upon irradiation. Mechanistically, dye **5-Se** remains in the tumor longer relative to other tissues and phototoxicity appears to involve singlet oxygen-induced damage to the mitochondria as at least one mechanism of action.

Relatively few chalcogenopyrylium dyes have been examined as photosensitizers for PDT or as mitochondrial-specific agents.[2a,7,8,10] Although the synthesis of chalcogenopyrylium dyes is somewhat tedious requiring several steps, we have developed general synthetic methodology to prepare thiopyrylium, selenopyrylium, and telluropyrylium compounds bearing three different substituents in the 2-, 4-, and 6-positions. With two anilino substituents being sufficient to provide sensitizers for PDT and perhaps sufficient to provide mitochondrial-specific agents, the third site in the chalcogenopyrylium compounds can be used to optimize biological and physical properties.

We are currently optimizing PDT with selenopyrylium dye **5-Se** with respect to sensitizer dose, light fluence, total light energy, and the time between sensitizer injection and treatment. The results from our initial conditions in Figure 2, which show a 300% increase in tumor-doubling time, are encouraging even though they are not optimized. In addition, we are preparing structurally related analogues of **5-Se** in which one anilino substituent and one 4-dimethylanilino substituent remain while the third substituent is varied to optimize properties 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 on a Varian Gemini-300, Inova 400, or Inova 500 instrument with residual solvent signal as internal standard: $CDCl_3$ (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 SX-18 MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.). Both were equipped with a circulating constant-temperature bath for the sample chambers. Elemental analyses were conducted by Atlantic Microanalytical, Inc. Dyes were analyzed as the hexafluorophosphate salts since the chloride salts were hygroscopic.

***p*-Iodoaniline Trifluoroacetamide (10).**[11] Trifluoroacetic anhydride (64.5 mL, 457 mmol) was added dropwise to *p*-iodoaniline (50.0 g, 228 mmol) in THF (200 mL) at 0 °C. The reaction mixture was warmed to ambient temperature and stirred for 1 h, concentrated and the residue was dissolved in ethyl acetate (250 mL). The ethyl acetate solution was washed with an aqueous solution of saturated sodium bicarbonate (3 × 100 mL), dried over $MgSO_4$, and concentrated. The product was collected as a white solid in 70.4 g (98%) isolated yield: mp 136−138 °C; ^{1}H NMR (DMSO-d_6) δ 11.51 (s, 1 H), 7.93 (AA′BB′, 2 H, J(doub) = 8.7 Hz), 7.69 (AA′BB′, 2 H, J(doub) = 8.7 Hz); ^{13}C NMR (DMSO-d_6) δ 155.6 (q, J = 5.0 Hz), 138.3, 136.9, 123.7, 116.1 (q, J = 30 Hz); EIMS m/z 315 ($C_8H_5NOIF_3$).

Preparation of 4-(4-Aminophenyl)-2-methyl-3-butyn-2-ol Trifluoroacetamide (11).[11] Bis(triphenylphosphine)-palladium(II) chloride (0.382 g, 0.544 mmol) was added to a solution of *p*-iodoaniline trifluoroacetamide (**10**; 71.2 g, 227.0 mmol), triphenylphosphine (1.43 g, 5.45 mmol), copper(II) iodide (0.375 g, 1.97 mmol), and 2-methyl-3-butyn-2-ol (55.8 mL, 577 mmol) in triethylamine (675 mL). The reaction was stirred for 0.5 h at 50 °C and then at reflux for 1 h. The reaction was then cooled to ambient temperature and ether (330 mL) was added. Triethylammonium iodide precipitated and was filtered from solution and the filtrate was concentrated. The product was recrystallized from toluene (315 mL). The white, crystalline product was collected by filtration in 50.4 g (82%) isolated yield: mp 120−122 °C; ^{1}H NMR (DMSO-d_6) δ 11.36 (br s, 1 H, NH), 7.70 (AA′BB′, 2 H, J(doub) = 5.4 Hz), 7.42 (AA′BB′, 2 H, J(doub) = 5.1 Hz), 5.48 (s, 1 H, OH), 1.46 (s, 6 H); IR (KBr) 3277, 2985, 1710 cm^{-1}; EIMS m/z 271 ($C_{13}H_{12}NO_2F_3$).

Preparation of 4-Ethynylaniline (9).[11] 4-(4-Aminophenyl)-2-methyl-3-butyn-2-ol trifluoroacetamide (**11**; 50.0 g, 184 mmol) was added to a refluxing solution of potassium hydroxide (31.0 g, 554 mmol) in 2-propanol (625 mL). The resulting mixture was heated at reflux for 3.0 h. The reaction was concentrated and diluted with cold H_2O (400 mL), which dissolved the water-soluble salts. The remaining solid was collected by filtration and washed with cold H_2O. The product was then dissolved in CH_2Cl_2 (250 mL), dried over Na_2SO_4, and concentrated. The product was collected as a yellow solid in 18.5 g (86%) isolated yield: mp 88−90 °C; ^{1}H NMR (DMSO-d_6) δ 7.32 (AA′BB′, 2 H, J(doub) = 8.4 Hz), 6.71 (AA′BB′, 2 H, J(doub) = 8.1 Hz), 5.67 (s, 2 H, NH_2), 3.93 (s, 1 H); EIMS m/z 117 (C_8H_7N); IR (KBr) 3485, 3388, 3260, 2097, 1618 cm^{-1}. Anal. (C_8H_7N) C, H, N.

Preparation of 4-Ethynyl-*N*,*N*-[1,2-bis(dimethylsilyl)-ethano]aniline (12). *p*-Ethynylaniline (**9**; 18.67 g, 159.6 mmol) and triethylamine (44.4 mL, 319 mmol) were dissolved in methylene chloride (70 mL) at ambient temperature. 1,2-Bis(chlorodimethylsilyl)ethane (34.31 g, 159.6 mmol) was added dropwise and the resulting mixture was stirred for 3 h. Upon completion, hexanes (280 mL) were added to the reaction mixture precipitating triethylammonium chloride, which was removed by filtration. The resulting orange oil was purified by column chromatography on basic alumina, eluted with hexane/CH_2Cl_2 (5/1) (R_f = 0.72) to give the aniline in 31.0 g (75%) isolated yield: ^{1}H NMR (CD_2Cl_2) δ 7.33 (AA′BB′, 2 H, J(doub) = 8.7 Hz), 6.86 (AA′BB′, 2 H, J(doub) = 8.7 Hz), 3.05 (s, 1 H), 0.89 (s, 4 H), 0.27 (s, 12 H); ^{13}C NMR ($CDCl_3$) δ 149.0, 133.0, 122.1, 112.7, 84.2, 75.6, 8.3, 0.2; IR (CCl_4) 3309, 2965, 2107 cm^{-1}; FAB(+)MS m/z 259 ($C_{14}H_{21}Si_2N$). Anal. ($C_{14}H_{21}NSi_2$) C, H, N.

Preparation of 1-[4-(*N,N*-(Dimethylsilylethano)amino)-phenyl]-5-phenyl-1,4-pentadiyn-3-ol (13). 4-Ethynyl-*N,N*-[1,2-bis(dimethylsilyl)ethano]aniline (**12**; 2.94 g, 11.3 mmol) and THF (80 mL) were added to a flame dried flask and cooled to −78 °C. *n*-Butyllithium (1.6 M in hexanes, 7.80 mL, 12.4 mmol) was added dropwise to the cooled solution and the resulting solution was stirred for 20 min at −78 °C. The reaction mixture was then added via cannula to a solution of phenylpropargylaldehyde (1.38 mL, 11.3 mmol) in THF at −78 °C. The solution was then warmed to ambient temperature over a period of 1 h and a few drops of methanol were added to protonate the alkoxide. The reaction mixture was concentrated and then redissolved in CH_2Cl_2 (75 mL). The CH_2Cl_2 solution was then washed with dilute aqueous sodium bicarbonate (3 × 50 mL), dried over $MgSO_4$, and concentrated. The product was collected as a red oil in 3.30 g (78%) isolated yield and was used without further purification: ^{1}H NMR ($CDCl_3$) δ 7.47 (AA′BB′, 2 H, J(doub) = 3.6 Hz), 7.31 (m, 5 H), 6.79 (AA′BB′, 2 H, J(doub) = 6.3 Hz), 5.55 (s, 1 H), 2.42 (br s, 1 H, OH), 0.83 (s, 4 H), 0.22 (s, 12 H); FAB(+)MS m/z 389 ($C_{23}H_{27}NOSi_2$).

Preparation of 1-[4-(*N,N*-(Dimethylsilylethano)amino)-phenyl]-5-phenyl-1,4-pentadiyn-3-one (14). 1-[4-(*N,N*-(Dimethylsilylethano)amino)phenyl]-5-phenyl-1,4-pentadiyn-3-ol (**13**; 4.40 g, 11.3 mmol) was dissolved in CH_2Cl_2 (200 mL) and stirred with manganese dioxide (4.92 g, 56.5 mmol) for 14 h. The reaction mixture was filtered through Celite and the filtrate was concentrated in vacuo. The product was collected as a yellow-red oil in 3.28 g (75%) isolated yield: ^{1}H NMR ($CDCl_3$) δ 7.66 (AA′BB′, 2H, J(doub) = 7.2 Hz), 7.46 (m, 5H), 6.90 (AA′BB′, 2H, J(doub) = 8.7 Hz), 0.89 (s, 4H), 0.31 (s, 12H); FAB(+)MS m/z 387 ($C_{23}H_{25}NOSi_2$). Anal. ($C_{23}H_{25}NOSi_2$) C, H, N.

Preparation of Δ-4*H*-2-(4-Aminophenyl)-6-phenylthiopyran-4-one (6-S). Sulfur (0.048 g, 1.50 mmol) and sodium borohydride (0.124 g, 3.30 mmol) were heated at reflux in 0.25 M sodium ethoxide in ethanol (30 mL) for 3 h. 1-(4-*N,N*-[1,2-Bis(dimethylsilyl)ethano]aminophenyl)-5-phenyl-1,4-pentadiyn-3-one (**14**; 0.500 g, 1.30 mmol) was stirred in 0.25 M sodium ethoxide in ethanol (30 mL) at ambient temperature for 2 h and was then added to the solution of Na_2S at reflux. The reaction mixture was stirred for 2 h, was concentrated, and was diluted with water (75 mL). The product was extracted with CH_2Cl_2 (3 × 25 mL) and the combined organic extracts were dried over Na_2SO_4. The crude product was concentrated and purified via chromatography on SiO_2 eluted with EtOAc (R_f = 0.45). The product was collected as an orange solid in 0.181 g (50%) isolated yield: mp 138−140 °C; ^{1}H NMR (CD_2Cl_2) δ 7.89 (m, 2 H), 7.74 (m, 5 H), 7.31 (AA′BB′, 2 H, J(doub) = 7.5 Hz), 6.98 (AA′BB′, 2 H, J(doub) = 8.4 Hz), 4.30 (br s, 2 H, NH_2); IR (KBr) 3155, 2927, 1580 cm^{-1}; FAB(+)MS m/z 279 (M + 1, $C_{17}H_{14}NOS$). Anal. ($C_{17}H_{13}N_2OS$) C, H, N.

Preparation of Δ-4*H*-2-(4-Aminophenyl)-6-phenylselenopyran-4-one (6-Se). Selenium (0.244 g, 3.10 mmol) and sodium borohydride (0.246 g, 6.50 mmol) were heated at reflux in 0.25 M sodium ethoxide in ethanol (60 mL) for 3 h. 1-(4-*N,N*-[1,2-Bis(dimethylsilyl)ethano]aminophenyl)-5-phenyl-1,4-pentadiynol-3-one (**14**; 1.00 g, 2.60 mmol) was stirred in 0.25 M sodium ethoxide in ethanol (60 mL) at ambient temperature for 2 h and was then added to the Na_2Se solution at reflux. The reaction mixture was stirred for 2 h, was concentrated, and was then diluted with water (150 mL). The product was extracted with CH_2Cl_2 (3 × 50 mL) and the combined organic extracts were dried over Na_2SO_4. The crude product was concentrated and purified via chromatography on SiO_2 eluted with 3:1 CH_2Cl_2/EtOAc (R_f = 0.35). The product was collected as an orange solid in 0.535 g (63%) isolated yield: mp 181−183 °C; ^{1}H NMR (CD_2Cl_2) δ 7.63 (m, 2 H), 7.49 (m, 5 H), 7.19 (AA′BB′, 2 H, J(doub) = 9.3 Hz), 6.76 (AA′BB′, 2 H, J(doub) = 8.4 Hz), 5.33 (br s, 2 H, NH_2); IR (KBr) 3155, 2931, 1581 cm^{-1}; FAB(+)MS m/z 328 (M + 1, $C_{17}H_{14}NO^{80}Se$). Anal. ($C_{17}H_{13}N_2OSe$) C, H, N.

Preparation of Δ-4*H*-2-(4-Aminophenyl)-6-phenyltelluropyran-4-one (6-Te). Tellurium (0.154 g, 1.20 mmol) and sodium borohydride (0.950 g, 2.50 mmol) were heated at reflux in 0.25 M sodium ethoxide in ethanol (17 mL) for 3 h. 1-(4-*N,N*-[1,2-Bis(dimethylsilyl)ethano]aminophenyl)-5-phenyl-1,4-pentadiynol-3-one (**14**; 0.390 g, 1.00 mmol) was stirred in 0.25 M sodium ethoxide in ethanol (17 mL) at ambient temperature for 2 h and was then added to the solution of Na_2Te at reflux. The reaction mixture was concentrated and was then diluted with water (100 mL). The product was extracted with CH_2Cl_2 (3 × 30 mL) and the combined organic extracts were dried over Na_2SO_4. The crude product was concentrated and purified via chromatography on SiO_2 eluted with 3:1 CH_2Cl_2/EtOAc (R_f = 0.2). The product was collected as an orange solid in 0.375 g (40%) isolated yield: mp 198−200 °C; ^{1}H NMR (CD_2Cl_2) δ 7.49 (m, 5 H), 7.32 (m, 4 H), 6.72 (AA′BB′, 2 H, J(doub) = 8.1 Hz), 3.98 (br s, 2 H); IR (KBr) 3155, 2984, 1587 cm^{-1}; FAB(+)MS m/z 378 (M + 1, $C_{17}H_{14}NO^{130}Te$). Anal. ($C_{17}H_{13}N_2OTe$) C, H, N.

Preparation of 2-(4-Aminophenyl)-4-(4-*N,N*-dimethylanilino)-6-phenylthiopyrylium Chloride (5-S). *p*-Bromo-*N,N*-dimethylaniline (0.376 g, 1.90 mmol) and magnesium (0.076 g, 3.20 mmol) were stirred at reflux in anhydrous THF (6.3 mL) for 0.5 h then cooled to ambient temperature. Δ-4*H*-2-(4-Aminophenyl)-6-phenylthiopyran-4-one (**6-S**; 0.175 g, 0.630 mmol) in anhydrous THF (4 mL) was added to the to the grignard reagent and the resulting mixture was stirred an additional 0.5 h. The reaction mixture was poured into 10% aqueous HPF_6 (60 mL). The resulting precipitate was collected by filtration and then washed with H_2O (20 mL) and ether (20 mL). The product was recrystallized from acetonitrile and ether to give the hexafluorophosphate salt in 0.295 g (89%) isolated yield: mp 240−242 °C. The hexafluorophosphate salt (0.150 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.102 g (80%) of **5-S**: mp 208−211; ^{1}H NMR (CD_3OD) δ 8.59 (AA′BB′, 2 H, J(doub) = 9 Hz), 8.36 (AA′BB′, 2 H, J(doub) = 9 Hz), 8.08 (s, 1 H), 8.06 (s, 1 H), 8.01 (AA′BB′, 2 H, J(doub) = 9 Hz), 7.68 (m, 3H), 6.90 (AA′BB′, 2H, J(doub) = 9 Hz), 6.79 (AA′BB′, 2H, J(doub) = 9 Hz), 3.18 (s, 6H); FAB(+)MS m/z 383 ($C_{25}H_{23}N_2S$); λ_{max} (CH_2Cl_2) 623 nm (ϵ = 31 000 M^{-1} cm^{-1}. Anal. ($C_{25}H_{23}N_2S\cdot PF_6$) C, H, N.

Preparation of 2-(4-Aminophenyl)-4-(4-*N,N*-dimethylanilino)-6-phenylselenopyrylium Chloride (5-Se). *p*-Bromo-*N,N*-dimethylaniline (0.600 g, 3.00 mmol) and magnesium turnings (0.120 g, 5.00 mmol) were stirred at reflux in anhydrous THF (10 mL) for 0.5. The reaction mixture was cooled to ambient temperature and Δ-4*H*-2-(4-aminophenyl)-6-phenylselenopyran-4-one (**6-Se**; 0.326 g, 1.00 mmol) dissolved in anhydrous THF (7 mL) was then added. The reaction mixture was stirred for 0.5 h was then poured into a 10% aqueous solution of HPF_6 (100 mL). The resulting precipitate was collected by filtration and washed with H_2O (20 mL) and ether (20 mL). The product was recrystallized from acetonitrile and ether to give the hexafluorophosphate salt of the dye in 0.51 g (89%) isolated yield: mp 223−226 °C. The hexafluorophosphate salt (0.150 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.113 g (83%) of **5-Se**: mp 168−170; ^{1}H NMR (CD_3OD) δ 8.09 (AA′BB′, 2 H, J(doub) = 9 Hz), 7.82 (s, 1 H), 7.81 (s, 1 H), 7.76 (AA′BB′, 2 H, J(doub) = 9 Hz), 7.61 (m, 5 H), 6.89 (AA′BB′, 2 H, J(doub) = 9 Hz), 6.77 (AA′BB′, 2 H, J(doub) = 9 Hz), 3.33 (s, 6 H); FAB(+)MS m/z 431 ($C_{25}H_{23}N_2{}^{80}Se$); λ_{max} (CH_2Cl_2) 654 nm (ϵ = 31 500 M^{-1} s^{-1}). Anal. ($C_{25}H_{23}N_2Se\cdot PF_6$) C, H, N.

Preparation of 2-(4-Aminophenyl)-4-(4-*N,N*-dimethylanilino)-6-phenyltelluropyrylium Chloride (5-Te). *p*-Bromo-*N,N*-dimethylaniline (0.30 g, 2.0 mmol) and magnesium turnings (0.082 g, 3.4 mmol) were stirred at reflux in anhy-

drous THF (3 mL) for 0.5 h. The reaction mixture was cooled to ambient temperature and Δ-4*H*-2-(4-aminophenyl)-6-phenyltelluropyran-4-one (**6-Te**; 0.25 g, 0.66 mmol) dissolved in anhydrous THF (2 mL) was then added. The reaction mixture was stirred for 2 h and was then poured into a 10% aqueous solution of HPF_6 (25 mL). The resulting precipitate was collected by filtration and washed with H_2O (4 mL) and ether (4 mL). The product was recrystallized from acetonitrile and ether to give the hexafluorophosphate salt of the dye in 0.173 g (42%) isolated yield: mp 256−258 °C. The hexafluorophosphate salt (0.150 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.122 g (85%) of **5-Te**: mp 163−165 °C; ^{1}H NMR (CD_2Cl_2) δ 8.31 (AA′BB′, 2 H, *J*(doub) = 9 Hz), 7.99 (AA′BB′, 2 H, *J*(doub) = 9 Hz), 7.68 (m, 7 H), 7.06 (AA′BB′, 2 H, *J*(doub) = 9 Hz), 6.92 (AA′BB′, 2 H, *J*(doub) = 9 Hz), 6.49 (br s, 2 H), 3.18 (s, 6 H); FAB(+)MS *m*/*z* 481 ($C_{25}H_{23}N_2{}^{130}Te$); λ_{max} (CH_2Cl_2) 680 nm (ϵ = 29 500 M^{-1} s^{-1}). Anal. ($C_{25}H_{23}N_2Te{\cdot}PF_6$) C, H, N.

Preparation of 2-(4-Aminophenyl)-2,6-diphenylthiopyrylium Chloride (15-S). Phenylmagnesium bromide (0.60 mL of a 3.0 M solution, 1.8 mmol) was added dropwise to a solution of Δ-4*H*-2-(4-aminophenyl)-6-phenylthiopyran-4-one (**6-S**; 0.100 g, 0.357 mmol) in anhydrous THF (10 mL). After 1 h, the reaction mixture was poured into 10% aqueous HPF_6 (50 mL). The resulting precipitate was collected by filtration and then washed with H_2O (20 mL) and ether (20 mL). The product was recrystallized from acetonitrile and ether to give 0.120 g (69%) of the hexafluorophosphate salt: mp 160−161 °C. The hexafluorophosphate salt (0.120 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.099 g (85%) of the chloride salt **15-S**: mp 163−165 °C; ^{1}H NMR ($CDCl_3$) δ 8.49 (s, 1 H), 8.32 (s, 1 H), 7.91−7.82 (m, 6 H), 7.71−7.68 (m, 6 H), 6.92 (d, 2 H), 5.00 (br s, 2 H); FAB(+)MS *m*/*z* 341 ($C_{23}H_{18}NS$); λ_{max} (H_2O) 551 nm (ϵ = 16 600 M^{-1} s^{-1}). Anal. ($C_{23}H_{18}NS{\cdot}PF_6$) C, H, N.

Preparation of 2-(4-Aminophenyl)-2,6-diphenylselenopyrylium Chloride (15-Se). Phenylmagnesium bromide (1.2 mL of a 3.0 M solution, 3.6 mmol) was added dropwise to a solution of Δ-4*H*-2-(4-aminophenyl)-6-phenylselenopyran-4-one (**6-Se**; 0.200 g, 0.614 mmol) in anhydrous THF (10 mL). After 1 h, the reaction mixture was poured into 10% aqueous HPF_6 (50 mL). The resulting precipitate was collected by filtration and then washed with H_2O (20 mL) and ether (20 mL). The product was recrystallized from acetonitrile and ether to give 0.203 g (62%) of the hexafluorophosphate salt: mp 142−143 °C. The hexafluorophosphate salt (0.120 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.099 g (85%) of the chloride salt **15-Se**: mp 163−165 °C; ^{1}H NMR ($CDCl_3$) δ 8.41 (s, 1 H), 8.32 (s, 1 H), 7.91−7.82 (m, 6 H), 7.76−7.66 (m, 6 H), 6.93 (d, 2 H), 4.50 (br s, 2 H); FAB(+)MS *m*/*z* 388 ($C_{23}H_{18}N^{80}Se$); λ_{max} (H_2O) 578 nm (ϵ = 24 500 M^{-1} s^{-1}). Anal. ($C_{23}H_{18}NSe{\cdot}PF_6$) C, H, N.

Preparation of 2-(4-Aminophenyl)-2,6-diphenyltelluropyrylium Chloride (15-Te). Phenylmagnesium bromide (0.60 mL of a 3.0 M solution, 1.8 mmol) was added dropwise to a solution of Δ-4*H*-2-(4-aminophenyl)-6-phenyltelluropyran-4-one (**6-Te**; 0.100 g, 0.267 mmol) in anhydrous THF (10 mL). After 1 h, the reaction mixture was poured into 10% aqueous HPF_6 (50 mL). The resulting precipitate was collected by filtration and then washed with H_2O (20 mL) and ether (20 mL). The product was recrystallized from acetonitrile and ether to give 0.102 g (66%) of the hexafluorophosphate salt: mp 138−140 °C. The hexafluorophosphate salt (0.100 g) was dissolved in 10 mL of methanol and 0.3 g of Amberlite IRA-400 Cl ion-exchange resin was added. The resulting mixture was stirred 0.5 h and the exchange resin was removed via filtration. The process was repeated with two additional 0.3-g aliquots of the ion-exchange resin. The product was recrystallized from CH_3CN to give 0.082 g (85%) of the chloride salt **15-Te**: mp 143−145 °C; ^{1}H NMR ($CDCl_3$) δ 8.40 (m, 2 H), 7.84−7.36 (m, 12 H), 7.06 (m, 2 H), 3.83 (br s, 2 H); FAB(+)-MS *m*/*z* 438 ($C_{23}H_{18}N^{130}Te$); λ_{max} (H_2O) 609 nm (ϵ = 14 600 M^{-1} s^{-1}). Anal. ($C_{23}H_{18}NTe{\cdot}PF_6$) C, H, N.

Quantum Yields for Singlet Oxygen Generation. The singlet oxygen acceptor 1,3-diphenylisobenzofuran (DPBF), HPLC-grade methanol, and certified rose bengal and methylene blue were used as received from Aldrich Chemical Co. Quantum yields for singlet oxygen generation in air-saturated methanol were determined by monitoring the dye-sensitized photooxidation of DPBF in a stopped-flow spectrophotometer (Applied Photophysics, Ltd.; SX-18MV) using techniques that we have previously described.[8]

Determination of Partition Coefficients. The octanol/water partition coefficients were all measured at pH 7.4 in phosphate-buffered saline (PBS) using UV−visible spectrophotometry. The measurements were done using a "shake flask" direct measurement.[18] 3−5 min of mixing was followed by 1 h of settling time. Equilibration and measurements were made at 23 °C using a Perkin-Elmer Lambda 12 spectrophotometer. HPLC grade 1-octanol was obtained from Sigma-Aldrich.

Photobleaching of 5-Se. Solutions of selenopyrylium dye **5-Se** in 0.05 M 0.9% PBS were prepared with an absorbance of 0.5 at 600 nm (λ_{max} in water). The solutions were irradiated with 630-nm laser light (75 mW) with the tip of the fiber optic cable positioned 2 cm from the top of a 1-cm quartz cuvette. The rate of loss of absorbance at 600 nm over 900 s was determined by time-drive spectroscopy. The procedure was repeated in the presence of 0.5 mg/mL of bovine serum albumin (BSA) in the PBS. The procedure was also repeated for a methanol solution of **5-Se**. Little if any loss of chromophore was observed during irradiation in any of the experiments with the first-order rate constant for bleaching $k \leq 2.5 \times 10^{-5}$ s^{-1} under these conditions.

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. Molt-4, a murine T-cell leukemia cell line, was maintained in RPMI 1640, 5% FCS and antibiotics at 37 °C, 5% CO_2. R3230AC, a rat mammary adenocarcinoma cell line, was maintained in minimum essential media supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA), 50 units/mL of penicillin G, 50 mg/mL of streptomycin, and 1.0 mg/mL of fungizone (MEM).

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 **5-Se** 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 630 nm for a total light dose of 5−20 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.[19]

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.

Administration of Dyes 5-Se. For studies with Fischer rats, stock solutions of dye **5-Se** at 5 mg of dye/mL in 1% ethanol were prepared by first dissolving the dye in ethanol and diluting to the appropriate volume with 0.9% NaCl. The

solutions were filtered, and final concentration was confirmed spectrophotometrically.

For studies with BALB/c mice, a stock solution of 5 mg of **5-Se**/mL in 1% Tween 80/0.9% NaCl was prepared by first dissolving the dye in Tween 80 and diluting to the appropriate volume with saline. The solutions were filtered and final concentration was confirmed spectrophotometrically. Animals were give 50−100-μL aliquots of the stock solutions via tail-vein injection.

Distribution Studies with 5-Se. The R3230AC mammary adenocarcinoma was transplanted subcutaneously in the axillary region of 80−100-g female Fischer 344 rats using the sterile trochar method.[19] When the R3230AC tumors reached 2−3 cm in diameter, usually 2−3 weeks after tumor implantation, the animals were given 200 μL of the dye solution (10 mg of dye/kg) via tail-vein injection. Animals were sacrificed at the indicated time points and the tissues were finely minced with scissors and homogenized in one-to-one 1 M $HClO_4$ and MeOH using 30-s bursts with a Polytron PCU-2110 homogenizer at a setting of 6 (Brinkmann Ind., Westbury, NY). The suspensions were then centrifuged at 10000*g* for 20 min using an Eppendorf microcentrifuge (model 3200, Brinkmann Ind., Westbury, NY). The supernatant was transferred using a Pasteur pipet into a quartz cuvette and the absorption for dye **5-Se** was determined for each tissue.

Colo-26 tumors were implanted in BALB/c mice via the sterile trochar method. Animals were injected at 29.3 mg (62 μmol)/kg of **5-Se** in 1% Tween 80/saline and tissues were examined at 3 and 24 h postinjection as described.

PDT with Dye 5-Se. The R3230AC mammary adenocarcinoma was transplanted subcutaneously in the axillary region of 80−100-g female Fischer 344 rats using the sterile trochar method.[20] When the R3230AC tumors reached 0.5−0.6 cm in diameter (0.1−0.17 cm^3), usually 7−12 days after after tumor implantation, the animals were given 200 μL of the dye solution (10 mg of dye/kg) via tail-vein injection. A group of 8 animals was irradiated 24 h after injection of **5-Se** with 570−750-nm light at 200 mW cm^{-2} for 1 h focused to a 1-cm diameter field at the site. A total of 720 J cm^{-2} of light was delivered. The data compiled in Figure 1 show the tumor-doubling times, i.e., the time in days for the tumors to double their initial, pretreatment tumor volume for the groups studied in these experiments. A group of 12 animals provided untreated controls. Another group received light only and another group received drug only. Tumor temperature did not rise above 40 °C during irradiation either the control group or the group group receiving drug + light (monitored with an Omega HYP-O microthermocouple), which excludes hyperthermia during irradiation as a source of the tumor response.

Mouse Foot Response of Selenopyrylium Dye 5-Se. The back feet of 5 BALB/c mice were delapitated with Nare. The mice were injected 72 h later with 10 mg/kg of selenopyrylium dye **5-Se** as a 1% Tween 80/saline solution. The right foot was irradiated with laser light (630 nm light, 135 J cm^{-2} at 75 mW) 24 h postinjection. The feet were examined and rated 24 h postinjection.[16]

Ear-Swelling Response of Selenopyrylium Dye 5-Se. The time course of acute cutaneous photosensitivity following administration of **5-Se** was followed using the murine ear-swelling response as an end point.[16] Groups of BALB/c mice were given dosages of 10.0 mg/kg of **5-Se**. Right ears were irradiated with 630-nm laser light (75 mW, 135 J cm^{-2}) at selected time points postinjection. Ear thickness was measured with an engineer's micrometer at 5 min (control for light-associated swelling) and 24 h postirradiation (acute skin photosensitization response) and compared to the preirradiation thickness (0.25−0.30 mm) of the ears. No measurable swelling was observed with irradiation at 24 h, 3 days, or 7 days. A typical ear-swelling response from Photofrin administered at 5.0 mg/kg and irradiated 24 h later with 89 J cm^{-2} of 630-nm laser light is on the order of 0.25-mm swelling above normal ear thickness 24 h postirradiation.[16]

Cytochrome *c* Oxidase Measurements in Cultured R3230AC Tumor Cells. Cells cultured from the rodent mammary adenocarcinoma (R3230AC) were used for these studies. The R3230AC tumors were maintained by transplantation in the abdominal region of 100−120-g Fischer female rats, using the sterile trochar technique.[20] R3230AC cells were cultured from tumor homogenates using the method described earlier.[21] Cells were maintained in passage culture on 100-mm diameter polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ) in 10 mL of α-MEM supplemented with 10% FBS, 50 units/mL penicillin G, 50 μg/mL streptomycin and 1.0 mg/mL Fungizone. Only cells from passages 1−10 were used for experiments. A stock of cells, passages 1−4, were maintained at −86 °C to initiate the experimental cultures. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere (Forma Scientific, Marietta, OH). Passage was accomplished by removing the culture medium, then adding a 1-mL solution containing 0.25% trypsin, incubating at 37 °C for 2−5 min to remove them from the surface followed by seeding new culture dishes with an appropriate number of cells in 10 mL of α-MEM. Cell counts were performed using a particle counter (model ZM, Coulter Electronics, Hialeah, FL).

To determine whether **5-Se** photosensitized mitochondrial cytochrome *c* oxidase activity, R3230AC cells were plated on 12-well culture plates using an initial cell seeding of 3.0×10^5 cells/well in 1.0 mL α-MEM. Cells were incubated for 24−48 h at 37 °C in a 5% CO_2 humidified atmosphere until they reached $4-7 \times 10^5$ cells/well, a number where the cells were still in log phase growth. The α-MEM was removed and fresh α-MEM without FBS or phenol with **5-Se** at 1×10^{-8} to 1×10^{-6} final concentration or without **5-Se** for control cells. Cells were incubated for 3 h in the dark as above, the medium was removed, the cells were washed once with α-MEM minus FBS and phenol (no **5-Se** in the wash) and 1.0 mL of fresh α-MEM minus FBS and phenol was added. Monolayers were then exposed to fluorescent light (0.2 mW cm^{-2}) positioned 6 cm above the surface. At the end of the irradiation period (5−15 min), the medium was removed, 0.2 mL of trypsin was added to each well and cells were incubated at 37 °C for 3−5 min until all the cells detached from the surface. Cell suspensions were transferred to 1.0-mL microcentrifuge tubes and centrifuged at 8000*g* for 3 min. The supernatant was aspirated and cell pellets were immediately frozen and stored at −86 °C. Cytochrome *c* oxidase activity was determined on cells that had been thawed and sonicated for 10 s using a Bronson sonicator (model 185, Brinkmann Ind.) at a setting of 4. A sonicated cell suspension, representing $1-1.5 \times 10^6$ cells was used for measurements of cytochrome *c* oxidase according to the method described earlier.[19] Data are expressed as percent of control cytochrome *c* oxidase activity, mol of cytochrome *c* oxidized/min/cell, which was determined from cells not exposed to **5-Se** or light. Cytochrome *c* oxidase activity was also determined in cells exposed to **5-Se** alone or light alone, drug and light controls, respectively.

Preparation of Mitochondrial Suspensions. The R3230AC mammary adenocarcinoma was transplanted subcutaneously in the axillary region of 80−100-g female Fischer 344 rats using the sterile trochar method.[20] 2−3 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% BSA, 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.[17] 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 Dye 5-Se in Vitro. Mitochondrial suspensions were removed from storage and thawed on ice. Dye **5-Se** was prepared by dissolving 2.5 mg of dye in 5.0 mL of 95% ethanol, which approximated a 1.0 mM

solution. Final concentrations of the dye were determined using absorbance. Ten microliters of the dye/ethanol solution was transferred to 1.0 mL of mitochondrial preparation buffer and the absorbance determined using a diode array spectrophotometer (HP8452A, Hewlett-Packard, Palo Alto, CA). The dye in mitochondrial preparation buffer, at a final concentration that gave an OD of 0.2, was then added to mitochondrial suspensions (1.0 mL) and allowed to incubate in the dark on ice for 15 min. The dye/mitochondrial suspension was then centrifuged at 8000*g* for 3 min, 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 (570−750 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 irradiation period, 1.0 h. 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.[21] 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 percent (%) of initial, preirradiation cytochrome *c* oxidase activity.

Exposure of Mitochondria to Dye 5-Se and Oxygen Radical Quenchers or a Reduced Oxygen Environment. To determine whether singlet oxygen was the major factor involved in the inhibition of cytochrome *c* oxidase when tumor mitochondria were exposed to dye **5-Se** and light, we performed experiments using superoxide dismutase to remove superoxide. Superoxide dismutase was added to mitochondria/dye suspensions at 30 enzyme units/mL. An oxygen-free environment was established by nitrogen purging of the sample in a specifically designed chamber described earlier.[22] Control samples containing dye but not exposed to light were maintained in the nitrogen purged atmosphere in the dark for 1 h and no inhibition of mitochondrial cytochrome *c* oxidase activity was observed.

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 S1b showing the dark and phototoxicity of **5-Se** against the murine T-cell line Molt-4; Figure S3 showing the effect of **5-Se** photosensitization on mitochondrial cytochrome *c* oxidase activity in cultured whole R3230AC tumor cells; Figure S4 showing the effect of **5-Se** photosensitization on isolated mitochondrial suspensions; and Table S2 showing the distribution of **1-Se** and **5-Se** in female Fischer rats bearing R3230AC adenocarcinomas. This information is available free of charge via the Internet at http://pubs.acs.org.

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