Chalcogenapyrylium Dyes as Photochemotherapeutic Agents. 2. Tumor Uptake, Mitochondrial Targeting, and Singlet-Oxygen-Induced Inhibition of Cytochrome c Oxidase

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Cationic selena- and tellurapyrylium dyes 1d–g and 1i were found to inhibit cytochrome c oxidase upon irradiation of isolated mitochondrial suspensions treated with 10 μM solutions of dye. The amount of inhibition by these dyes was found to be related to oxygen concentration and inversely related to the concentration of added imidazole, a singlet-oxygen trap, suggesting that singlet oxygen is responsible, at least in part, for the inhibition of the enzyme. Dyes 1d–g and 1i, containing either selenium or tellurium, produce singlet oxygen with a quantum efficiency, $\Phi(^1O_2)$, between 0.005 and 0.09 in methanol. Dyes 1a–c, containing the lighter chalcogens oxygen and sulfur, have values of $\Phi(^1O_2)$ that are less than 0.0008 in methanol and do not inhibit cytochrome c oxidase in irradiated mitochondrial suspensions. Dyes 1c and 1d have nearly identical spectral and redox properties. Only the selenapyrylium dye 1d inhibits the enzyme, suggesting that neither ground-state nor excited-state electron transfer is important in inhibition of the enzyme. Electron micrographs of human U251 glioma cells, treated in vitro with 1i and light, showed pronounced morphology changes in the mitochondrial membranes relative to electron micrographs of untreated cells. Epifluorescence microscopy of the treated cells showed granular yellow-green fluorescence presumably from photooxidized dye in the mitochondria.

Photodynamic therapy (PDT) is a relatively recent development in cancer therapy that employs photochemistry as an integral part of the treatment. A tumor-specific photosensitizer is activated by light to produce a cytotoxic reagent or a cytotoxic reaction in the tumor cell. Ideally, the photosensitizer is localized in or around the tumor mass, is nontoxic to normal tissues, is activated by light that can penetrate deeply into both tumor and tumor-involved normal tissues, and is photochemically efficient at producing the cytotoxic agent.[1]

At the present time, one of the limiting factors on the successful application of PDT to the treatment of solid tumors has been the lack of a photosensitizer that absorbs light with a high degree of penetrance in tissue (wavelength range of 700–1200 nm for maximum penetration).[2] A second factor limiting the application of PDT is the low molar extinction coefficients of many sensitizers at longer wavelengths of absorption.[1] Near-infrared-absorbing tellurapyrylium dyes with molar extinction coefficients of greater than 10^5 in the near-infrared have been identified as potential photosensitizers for PDT from in vitro studies.[3] In an earlier communication, we described the efficient production of singlet oxygen by irradiating air-saturated solutions of tellurapyrylium dyes, suggesting that singlet oxygen might be the cytotoxic species produced by tellurapyrylium dyes in vitro.[4]

A variety of singlet-oxygen-producing photosensitizers have been examined for use in PDT including fluorescein, eosin, tetracycline, acridine orange, hematoporphyrin derivative (HpD) and related porphyrins, phthalocyanines, and metallophthalocyanines.[5-11] While these materials are all capable of producing singlet oxygen when irradiated in vivo, these materials are not selectively bound to tumor. Instead, as in the case of HpD and related derivatives, tumor binding of the photosensitizer appears to be at the extracellular level and depends more upon changes in vascular permeability, lack of adequate lymphatic drainage, and nonspecific binding of proteins to stromal elements in tumors.[12] Thus, light treatment of the tumor region after injection of the photosensitizer often results in nondiscriminant damage to normal as well as cancerous tissues.

Cancer cells (even those with increased glycolytic activity) derive a significant fraction of their total adenosine triphosphate (ATP) from mitochondrial oxidative phosphorylation. Some tumor cells, in vitro, utilize very little glucose and instead rely on fatty acids and/or amino acids such as glutamine as fuel sources.[13] Therefore, mitochondrial oxidative phosphorylation is a major source of

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(1) (a) Dougherty, T. J. *Photochem. Photobiol.* **1987**, *45*, 879. (b) Powers, S. K.; Pribil, S.; Gillespie, G. Y.; Watkins, P. J. *J. Neurosurg.* **1986**, *64*, 918. (c) Ben-Hur, E.; Rosenthal, I. *Photochem. Photobiol.* **1985**, *42*, 129. (d) Ben-Hur, E.; Carmichael, A.; Riesz, P.; Rosenthal, I. *Int. J. Radiat. Biol.* **1985**, *48*, 837. (e) Morgan, A. R.; Garbo, G. M.; Kreimer-Birnbaum, R.; Keck, R. W.; Chaudhuri, K.; Selman, S. H. *Cancer Res.* **1987**, *47*, 496. (f) Oseroff, A. R.; Ohuoha, D.; Ara, G.; McAuliffe, D.; Foley, J.; Cincotta, L. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9729.
(2) Wan, S.; Parrish, J. A.; Anderson, R. R.; Madden, M. *Photochem. Photobiol.* **1981**, *34*, 679.
(3) Powers, S. K.; Walstad, D. L.; Brown, J. T.; Detty, M. R.; Watkins, P. J. *J. Neuro-Oncology* **1989**, *72* (2), 179.
(4) Detty, M. R.; Merkel, P. B.; Powers, S. K. *J. Am. Chem. Soc.* **1988**, *110*, 5920.
(5) Figge, F. H. J.; Weiland, G. S.; Manganiello, L. O. J. *Proc. Soc. Exp. Biol. Med.* **1948**, *68*, 640.
(6) Kochevar, I. E. *J. Invest. Dermatol.* **1981**, *77*, 59.
(7) Moore, G. E. *Science (Washington, D.C.)* **1947**, *106*, 130.
(8) Rall, D. P.; Loo, T. L.; Lane, M. *J. Natl. Cancer Inst. (U.S.)* **1957**, *19*, 79.
(9) Rassmussen-Taxdal, D. S.; Ward, G. E.; Figge, F. H. J. *Cancer* **1955**, *8*, 78.
(10) (a) Dougherty, T. J.; Thoma, R. E.; Boyle, D. G.; Weishaupt, K. R. *Cancer Res.* **1981**, *41*, 401. (b) Weishaupt, K. R.; Gomer, C. J.; Dougherty, T. J. *Cancer Res.* **1976**, *36*, 2326. (c) Stenstrom, A. G. K.; Moan, J.; Brunborg, G.; Eklund, T. *Photochem. Photobiol.* **1980**, *32*, 349.
(11) Firey, P. A.; Ford, W. E.; Sounik, J. R.; Kenney, M. E.; Rogers, M. A. G. *J. Am. Chem. Soc.* **1988**, *110*, 7626.
(12) (a) Henderson, B. W.; Waldow, S. M.; Mang, T. S.; Potter, W. R.; Malone, P. B.; Dougherty, T. J. *Cancer Res.* **1985**, *45*, 572. (b) Gomer, C. J.; Jester, J. V.; Razum, N. J.; Szirth, B. C.; Murphree, A. L. *Cancer Res.* **1985**, *45*, 3718. (c) Boggan, J. E.; Walter, R.; Edwards, M. S. B.; Borcich, J. K.; Davis, R. L.; Koonce, M.; Berns, M. W. *J. Neurosurg.* **1984**, *61*, 1113.
(13) Walstad, D. L.; Brown, J. T.; Powers, S. K. *Photochem. Photobiol.* **1989**, *49*, 285.

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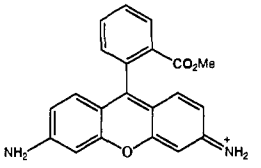
energy for cancer cells. Due to the structural and functional differences between normal and tumor mitochondria and the dependence of the tumor cell on the mitochondria for energy production, this cellular structure appears to be a suitable target for directing drug therapy against the cancer cell.

Tumor mitochondria differ from normal mitochondria in morphology, ultrastructure, and their ability to undergo configurational changes.[14] Tumor mitochondria also differ in their membrane and/or matrix compositions from mitochondria of nonneoplastic cells. Most tumors have a decreased number of mitochondria per cell which is generally less than 50% the number seen in nontransformed cells of the same cell type. In addition, tumor mitochondria more effeciently accumulate and retain Ca^{2+} and some other cations relative to normal mitochondria. There appears to be impairment of the normal release mechanism of Ca^{2+} in tumor mitochondria as well. The greater accumulation of cationic species in tumor mitochondria offers the potential for differentiation of tumor and normal cells by a chemical agent.

Studies with rhodamine 123 (Rh-123) have been of particular interest toward the design of a photosensitizer.

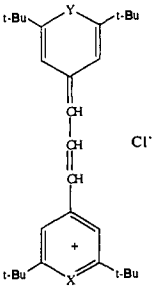


Rhodamine 123

Rh-123 is a cationic dye that is specifically taken up by the mitochondria of living cells.[15] It has been suggested that the electrochemical gradient driven by respiratory electron transport in the mitochondria and the pH gradient generated by the mitochondrial proton pump are responsible for the selective mitochondrial uptake of Rh-123.[16] In general, if cells maintain a plasma membrane potential of 59 mV and a mitochondrial membrane potential of 177 mV, then a given concentration of a cationic dye such as Rh-123 in culture medium may lead to a 10-fold increase in dye concentration in cytoplasm and a 10 000-fold increase in dye concentration relative to culture medium in the mitochondria based on the Nernst equation.[17]

It has been demonstrated that Rh-123 accumulates more in carcinoma cells and muscle cells relative to other body cell types[17] and that Rh-123 is selectively toxic to carcinoma cells in vitro.[18,19] Presumably, the greater metabolic activity of cancer cells (and muscle cells) relative to normal cells leads to an increased membrane potential in tumor cells relative to normal cells.[14] Although Rh-123 has been examined as a photosensitizer for PDT,[21,22] Rh-123 absorbs light of too short a wavelength for good tissue penetration and produces singlet oxygen only inefficiently upon irradiation.

Structurally, the rhodamine dyes are benzo-fused members of the pyrylium class of dyes. One would expect other pyrylium dyes, as well as the heavier chalcogenapyrylium dyes containing sulfur, selenium, and/or tellurium, to behave similarly to the rhodamines with respect to mitochondrial uptake. The longer wavelength absorbing tellurapyrylium dyes, with efficient quantum yields for singlet-oxygen production, would offer particular advantages if such dyes were mitochondrial specific.



1a; X = Y = O	1f; X = Y = Se
1b; X = S, Y = O	1g; X = Te, Y = O
1c; X = Y = S	1h; X = Te, Y = S
1d; X = Se, Y = O	1i; X = Te, Y = Se
1e; X = Se, Y = S	1j; X = Y = Te

The chalcogenapyrylium dyes that have exhibited significant potential for PDT are of general structure 1, where X and Y are some combination of oxygen, sulfur, selenium, or tellurium atoms. The chloride anion was chosen as the counterion for these studies for increased aqueous solubility while the *tert*-butyl substituents were chosen to impart greater kinetic stability toward the biological environment. Herein, we report the details of the preparation and purification of chalcogenapyrylium dyes 1 as well as preliminary studies on the entire class of chalcogenapyrylium dyes 1 as mitochondrial toxic agents from the production of singlet oxygen.

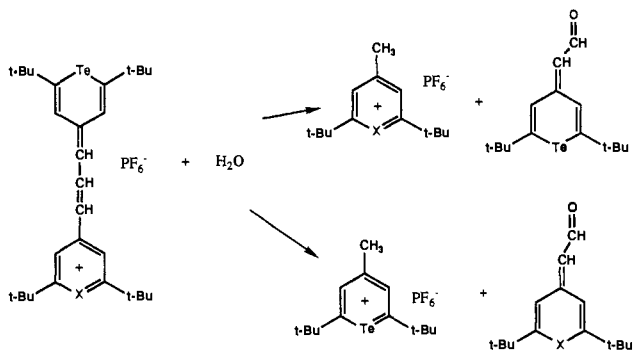
Results and Discussion

Reverse-Aldol Reactions and the Preparation of Pure Chalcogenapyrylium Dyes 1. Trimethine dyes such as 1 have been prepared by the condensation of a 4-methylchalcogenapyrylium nucleus with an appropriate (chalcogenapyranyl)acetaldehyde in acetic anhydride.[23,24] The preparations of symmetrical dyes **1a**, **1c**, **1f**, and **1j** are straightforward. From a practical standpoint, the chloride salts of the dyes 1 are most easily prepared by exchange of chloride ion for another anion such as tetrafluoroborate or hexafluorophosphate. In this manner, **1a**, **1c**, **1f**, and **1j** are prepared in high yield and in greater than 99% purity.

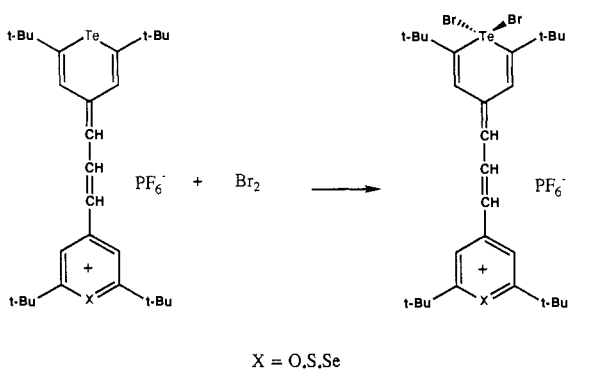
The unsymmetrical dyes **1b**,**d**,e and, in particular, **1g–i** are not as straightforward in their preparation. While **1b**,**d**,e can be prepared in greater than 98% purity, trace amounts of the symmetrical dyes **1a**, **1c**, and **1f** in appropriate combinations can be detected by ^1H NMR. In

(14) Pederson, P. L. *Prog. Exp. Tumor Res.* **1978**, *22*, 190.
(15) Johnson, L. V.; Walsh, M. L.; Chen, L. B. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 990.
(16) Mitchell, P. *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*; Glynn Research Ltd.: Bodmin, 1966; pp 1–205.
(17) Summerhayes, I. C.; Lampidis, T. J.; Bernal, S. D.; Chen, L. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5292.
(18) Lampidis, T. J.; Bernal, S. D.; Summerhayes, I. C.; Chen, L. B. *Ann. N. Y. Acad. Sci.* **1982**, *395*, 299.
(19) Lampidis, T. J.; Bernal, S. D.; Summerhayes, I. C.; Chen, L. B. *Cancer Res.* **1983**, *43*, 716.
(20) Bernal, S. D.; Lampidis, T. J.; Summerhayes, I. C.; Chen, L. B. *Science (Washington, D. C.)* **1982**, *218*, 1117.
(21) Powers, S. K.; Pribil, S.; Gillespie, G. Y.; Watkins, P. J. *J. Neurosurg.* **1986**, *64*, 918.
(22) Beckman, W. C., Jr.; Powers, S. K.; Brown, J. T.; Gillespie, G. Y.; Bigner, D. D.; Camps, J. L., Jr. *Cancer* **1987**, *59*, 266.
(23) (a) Detty, M. R.; Murray, B. J. *J. Org. Chem.* **1982**, *47*, 5235. (b) Detty, M. R.; McKelvey, J. M.; Luss, H. R. *Organometallics* **1988**, *7*, 1131.
(24) Detty, M. R.; Luss, H. R. *Organometallics* **1986**, *5*, 2250.

Scheme I



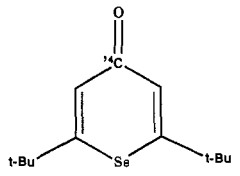
Scheme II



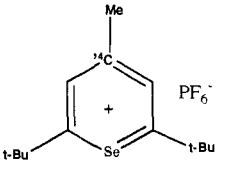
preparing the unsymmetrical tellurapyrylium dyes **1g–i**, the scrambling of heteroatoms can be extensive to the point that a statistical distribution of all combinations can be isolated. As an example, the preparation of **1i** from the appropriate 4-methylselenapyrylium chloride and the appropriate tellurapyranyl aldehyde in acetic anhydride gives a 1/2/1 mixture of **1f**, **1i**, and **1j**, respectively. Use of the hexafluorophosphate salt, of which the anion has less Lewis base character than chloride, gives less scrambling but still gives a 7/91/2 mixture of **1f**, **1i**, and **1j**, respectively, as the hexafluorophosphate salts. The preparation of **1g** and **1h** are similarly complicated.

The condensation reactions employed to prepare these dyes produce a molecule of water for every dye molecule formed. The water that is produced can lead to scrambling among the heteroatoms of these dyes by catalyzing a reverse-aldol process as shown in Scheme I. The reverse-aldol reaction can follow either of two routes. Depending upon the kinetics of the reverse-aldol reaction under the conditions employed, statistical scrambling of the heteroatoms is possible in the aldol reaction.

An alternative mechanism for the scrambling process would involve nucleophilic addition to the 2-position of the chalcogenapyrylium ring followed by ring-opening and scrambling of only the heteroatoms. A simple labeling experiment can distinguish between the reverse-aldol process and a heteroatom scrambling process. Ethyl formate labeled at the carbonyl carbon with carbon-14 (specific activity of 1.59 Ci/mol) was converted in several steps to 2,6-di-*tert*-butylselenapyran-4-one (i).[25] The



i



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selenapyranone i was converted to 4-methyl-2,6-di-*tert*-butylselenapyrylium hexafluorophosphate (ii) labeled with carbon-14 at position 4.[23] Condensation of this species with unlabeled tellurapyranyl aldehyde gave a 7/91/2 mixture of **1f**, **1i**, and **1j**, respectively.

The dyes **1f**, **1i**, and **1j** were separated by analytical high-pressure liquid chromatography and the activity under each peak was determined by scintillation counting. Symmetrical selenapyrylium dye **1f** had twice the relative activity of unsymmetrical tellurapyrylium dye **1i**. Symmetrical tellurapyrylium dye **1j** showed no activity by scintillation counting. These data are consistent with a scrambling mechanism in which the entire chalcogenapyranyl ring is involved in scrambling. The reverse-aldol mechanism suggested in Scheme I is consistent with these facts.

The use of an Amberlite ion-exchange resin to replace perchlorate, tetrafluoroborate, or hexafluorophosphate anions with chloride anion also gave some heteroatom scrambling with dyes **1** when the resin was not dried prior to use. Apparently, water present in the resin catalyzed the reverse-aldol reaction. The ion-exchange resin was conveniently dried by washing with several portions of absolute methanol prior to use.

The ability of tellurapyrylium dyes to undergo oxidative addition of halogens to give isolable compounds offers a method of purification for unsymmetrical tellurapyrylium dyes.[24] As shown in Scheme II, the addition of bromine to dye mixtures containing tellurapyrylium dyes should give oxidative addition to give tellurium(IV) products. Oxygen, sulfur, and selenium incorporated into the dyes **1** do not react with bromine. Mixtures of tellurium(IV) compounds, such as a 91/2 mixture of the products of oxidative bromine addition to **1i** and **1j**, respectively, are more easily purified by recrystallization to give pure dihalide dyes than recrystallization of the mixture containing **1i** and **1j**.

The tellurium(IV) compounds give tellurapyrylium dyes **1** upon reduction and anion exchange. However, tellurapyrylium dyes are easily reduced as well. Since the dihalide dyes are reduced at potentials more positive than 0.0 V (vs SCE),[24] mild reducing agents might be employed that would leave the dye chromophore intact. Sodium bisulfite reduction gives pure tellurapyrylium dyes from the tellurium(IV) compounds in nearly quantitative yield. In this manner, pure tellurapyrylium dyes **1g–i** were prepared for in vitro and in vivo studies.

Lipophilicity of Chalcogenapyrylium Dyes and Comparisons to Rhodamine 123. Within the past decade, rhodamine 123 (Rh-123) has been shown to have selective affinity for certain types of tumors in vitro.[17–20] The selective accumulation of Rh-123 appears to depend on its cationic charge and consequent affinity for the electronegative environment of the mitochondrial membrane.[26] The preferential accumulation of Rh-123 by neoplastic cells is responsible for its ability to kill selectively tumorigenic cells. Although Rh-123 exists in an ionic form in aqueous solution, it remains lipophilic enough[27] to permeate the hydrophobic barrier of the plasma and mitochondrial membranes. Furthermore, Rh-123 passes through vascular

(25) Detty, M. R.; Hassett, J. W.; Murray, B. J.; Reynolds, G. A. *Tetrahedron* **1985**, *41*, 4853.

(26) Johnson, L. V.; Walsh, M. C.; Bockus, B. J.; Chen, L. B. *J. Cell. Biol.* **1981**, *88*, 526.

(27) As a comparison, the Rh-123 *n*-octanol/water partition coefficient is somewhat smaller with a log *P* of 1.5.

Table I. Inhibition of Mitochondrial Cytochrome c Oxidase by and Physical Properties of Chalcogenapyrylium Dyes 1

dye	log *P*	% inhibn/ J/cm^2 [a]	λ_{max}, [b] nm (log ϵ)	Ep_a(ox), V vs SCE[c]	$E^{0\prime}$(red), V vs SCE[c,d]	$\Phi(^1O_2)$[e]
1j	2.02		810 (5.18)	+0.86	−0.32	0.12 ± 0.01
1i	2.41	3.00	770 (5.10)	+0.77	−0.42	0.09 ± 0.01
1b	2.26		750 (5.04)	+0.73	−0.50	0.07 ± 0.007
1g	2.41	10.95	700 (5.27)	+0.78	−0.56	0.05 ± 0.005
1f	1.92	4.20	730 (5.48)	+0.75	−0.50	0.014 ± 0.002
1e	2.39	4.05	708 (5.40)	+0.82	−0.54	0.007 ± 0.001
1d		2.55	665 (5.38)	+0.71	−0.61	0.004 ± 0.0004
1c	1.97	0.045	685 (5.42)	+0.72	−0.62	0.0006 ± 0.0001
1b		0.030	640 (5.32)	+0.84	−0.68	0.0006 ± 0.0001
1a	2.51	0.000	593 (5.31)	+0.92	−0.75	0.0004 ± 0.0001

[a] The inhibition values were established on the basis of constant absorbance (optical density of 1.5 ± 0.2 in a 1-cm quartz cuvette at maximum absorption in water) from sample to sample. Total light energy over 375–800 nm was measured assuming that each dye would absorb the same fraction of the total energy if absorbance of the samples were the same. [b] In water as solvent. [c] 5×10^{-4} M solutions of 1 (as BF_4 salt) in CH_3CN with 0.2 M $Bu_4N^+BF_4^-$ as supporting electrolyte at a Pt disk electrode at a scan rate of 0.1 V s^{-1}. [d] Separation of Ep_c and Ep_a was 0.06 V at a scan rate of 0.05 V s^{-1}. [e] In methanol.

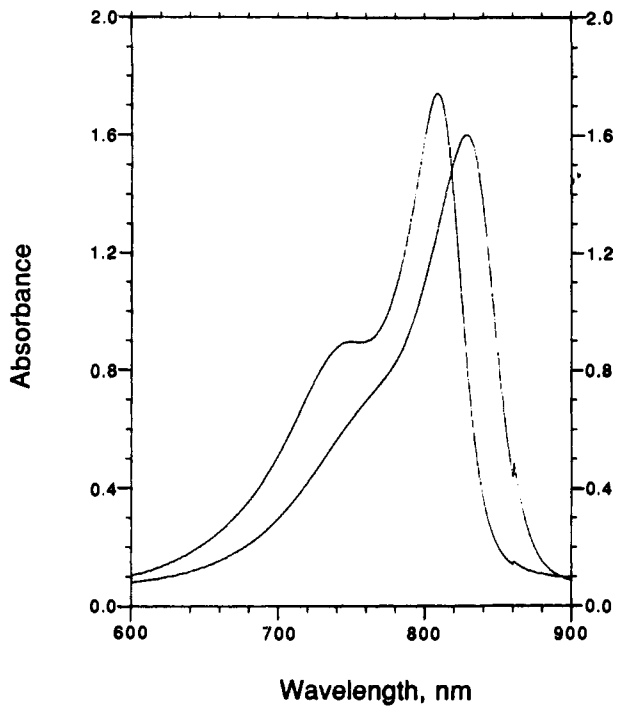


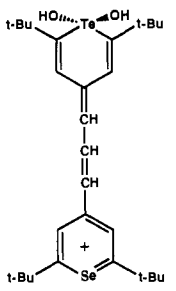
Figure 1. The effect of added bovine serum albumin (1%) on the absorption spectrum of **1j**. Note that the dye is red-shifted upon addition of the protein. This shift is expected if the dye molecules are in a less polar environment. These data also sugggest that the dyes 1 are quite lipophilic.

endothelial barriers such as the blood–brain barrier.

The chalcogenapyrylium dyes should behave similarly to Rh-123. Partition coefficients (*P*) between *n*-octanol and water for the chalcogenapyrylium dyes 1 have been measured. Values of log *P* (Table I) are between 1.92 and 2.51, which suggests that the dyes 1 are quite lipophilic.[27] As shown in Figure 1, the addition of bovine serum albumin to a phosphate-buffered saline solution of **1j** gives a red shift in the dye chromophore as the dye is surrounded by a less polar medium (protein). The dye appears to be bound completely to the serum protein which is a further indication of the lipophilicity of the dyes 1.

Effect of Chalcogenapyrylium Dyes on Mitochondria. As with other lipophilic cationic compounds, we assumed that the chalcogenapyrylium dyes are found on the mitochondrial membrane after treatment of cells with dye.[15-22] Of the 10 dyes 1, the dye **1i** was chosen as a representative example of the chalcogenapyrylium dyes 1 because of its favorable near-infrared chromophore and because of its quantum yield for singlet-oxygen generation (vide infra). Evidence for presence of the dye **1i** was found in electron micrographs of dye-treated cells. Electron-micrographs of U251MG cells after 15 min of treatment with **1i** show swelling of the mitochondria and disruption of the mitochondrial inner membranes. This mitochondrial membrane damage is even more extensive after exposing dye-laden cells to near-infrared light (Figure 2).

Exposure of the dye-treated cells to light leads to the photooxidation of intracellularly bound **1i** to give **2** via the



2

reaction of singlet oxygen and water with the tellurium atom of **1i**. Epifluorescence microscopy of light-exposed, dye-treated cells shows granular fluorescence from **2**, presumably localized in the mitochondria (Figure 3). The excitation and emission maxima of **2** in aqueous solution are 480 and 560 nm, respectively, consistent with the observed yellow-green fluorescence. This fluorescence is absent from cells that are not dye-treated.

We assumed that, as a class, all of the chalcogenapyrylium dyes 1 would show similar uptake by mitochondria. All of the dyes 1 have the same charge, are structurally similar, and show similar partitioning between *n*-octanol and water.

Inhibition of Cytochrome c Oxidase in Isolated Mitochondrial Suspensions. The effect of photosensitizers of structure 1 on mitochondria was modeled with isolated mitochondrial suspensions for direct examination of the effect of the photosensitizer on cytochrome c oxidase. Mitochondria isolated from R3230AC rat mammary adenocarcinomas were treated with 10^{-5} M solutions of 1 and washed. The inhibition of cytochrome c oxidase as a function of light energy was monitored over the first 5 min of exposure and initial rates of inhibition of cytochrome c oxidase in dye-treated mitochondrial suspensions are compiled in Table I as well as quantum efficiencies of singlet-oxygen generation, $\Phi(^1O_2)$, and redox potentials for the chalcogenapyrylium dyes 1.

Cytochrome c oxidase is a multisubunit complex which contains four one-electron redox centers including two

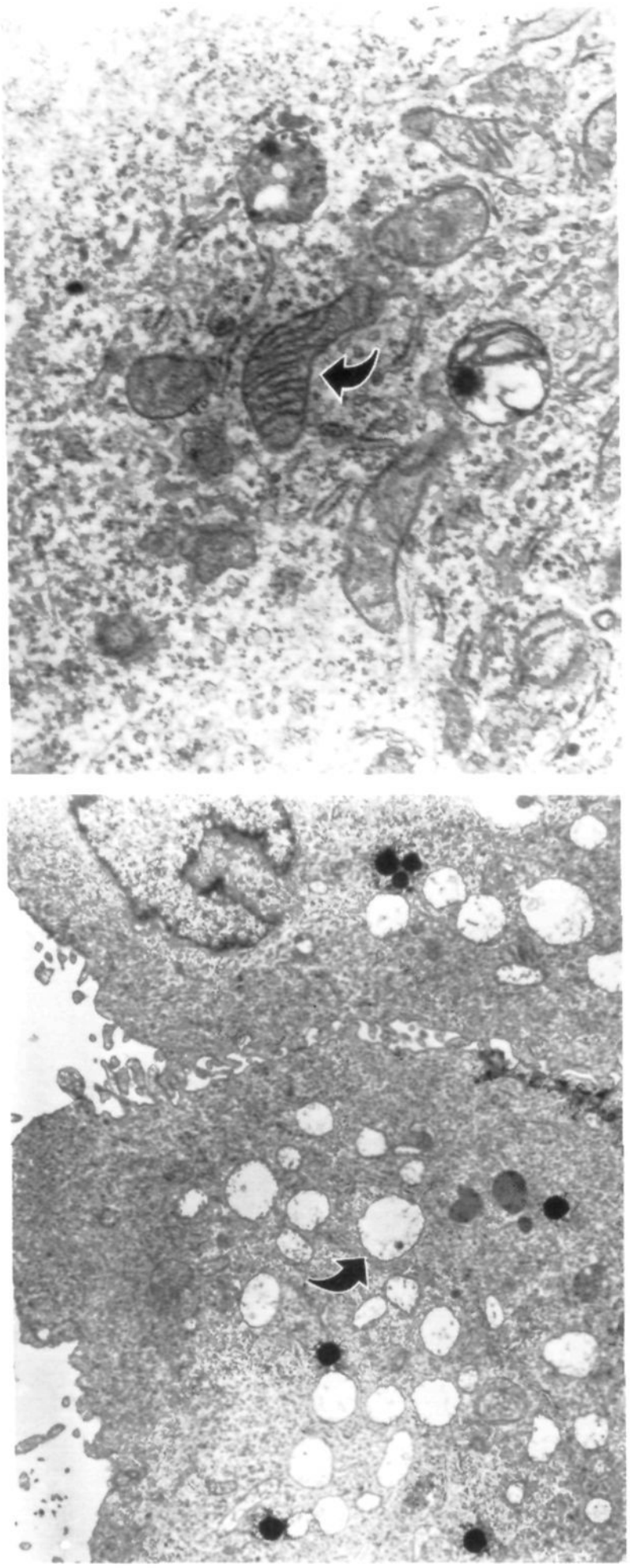


Figure 2. Electronmicrograph [40% reduction of original magnification (×12500)] of an untreated human U251Mg glioma cell showing normal mitochondria (top); Electronmicrograph [40% reduction of original magnification (×12500)] of glioma cell 1 h after incubation for 15 min in 10^{-6} M **1i** followed by 5 min of irradiation with 775 nm light (bottom). There is disruption of the cristae and internal mitochondrial membranes in addition to swelling of the mitochondria.

copper ions and two heme units.[28] The mechanism of inhibition of the enzyme could involve single-electron transfer (SET) during irradiation or in the ground state, or inhibition could be the result of singlet-oxygen-induced damage to the enzyme.

Only those dyes **1** containing at least one of the heavier chalcogen atoms, selenium or tellurium, inhibited the cytochrome c oxidase activity in the mitochondrial suspensions. As shown in Table I, the selenium- and tellurium-containing dyes produce singlet oxygen much more efficiently than those dyes containing only oxygen and sulfur. A comparison of dyes **1c** an **1d**, which have nearly identical spectral and redox properties, shows that inhibition of cytochrome c oxidase by **1d**, containing selenium, is nearly 2 orders of magnitude greater than thiapyrylium dye **1c**. Reactions involving SET would be expected to be similar for **1c** and **1d** in both the ground and excited states. However, the heavy-atom effect in **1d** should promote a higher triplet yield and greater production of singlet oxygen.

The inhibition of cytochrome c oxidase observed upon irradiation of mitochondria treated with chalcogenapyrylium dyes **1** can be reversed by controlling either oxygen concentration or the concentration of added singlet-oxygen acceptors. As shown in Figure 4 for selenium-containing dye **1e** and tellurium-containing dye **1i**, reducing the oxygen concentration in dye-treated mitochondrial suspensions during irradiation lowers the amount of inhibition observed which is consistent with the involvement of singlet oxygen. As shown in Figure 5 for selenium-containing dye **1e** and tellurium-containing dye **1i**, the addition of imidazole, an acceptor of singlet oxygen,[29] to dye-treated mitochondrial suspensions reduces the amount of inhibition observed, which is again consistent with singlet oxygen being the damaging agent.

The addition of catalase to scavenge hydrogen peroxide, superoxide dismutase to scavenge superoxide anion, or mannitol to scavenge hydroxy radicals had no effect on the inhibition of cytochrome c oxidase by the combination of dyes **1e** or **1i** and light. Again, these data are consistent with singlet oxygen being the species responsible for cytotoxic damage to the mitochondria.

Although other photosensitizers for PDT may have higher solution values for the quantum yield of singlet-oxygen production than chalcogenapyrylium dyes **1**, the effect of rigidization of dyes such as **1** (upon binding to membranes) on singlet-oxygen yield has not been determined. Rotational degrees of freedom in rigid, planar molecules, such as the porphyrins and related compounds, are little affected by binding to membranes. In dyes such as those of structure **1**, rotation and cis–trans isomerization can return the excited state to the ground state. Dye-binding to a membrane might eliminate these routes to return the excited state to the ground state, resulting in an increase in the excited state lifetime, which might improve triplet yield and, consequently, singlet-oxygen yield. As an example, chalcogenapyrylium dyes **1g** and **1i** are much more effective than Photofrin II, a commercially available hematoporphyrin dimer, at inhibiting cytochrome c oxidase in isolated mitochondrial suspensions (Figure 6) even though the quantum yields of singlet-oxygen production in solution of **1g** and **1i** are much less than that of Photofrin II. While part of this difference may be due to the higher extinction coefficients of the chalcogenapyrylium dyes, improved efficiency of singlet-oxygen generation in membrane-bound dyes cannot be excluded.

Summary and Conclusions

Within the specifications of dye design described here

(28) (a) Wikstrom, M.; Krab, K.; Saraste, M. In *Cytochrome Oxidase—A Synthesis*; Academic: New York, 1981. (b) Holm, L.; Saraste, M.; Wikstrom, M. *EMBO J.* **1987**, *6*, 2819.

(29) Kraljik, I.; El Mohani, F. *Photochem. Photobiol.* **1978**, *28*, 577.

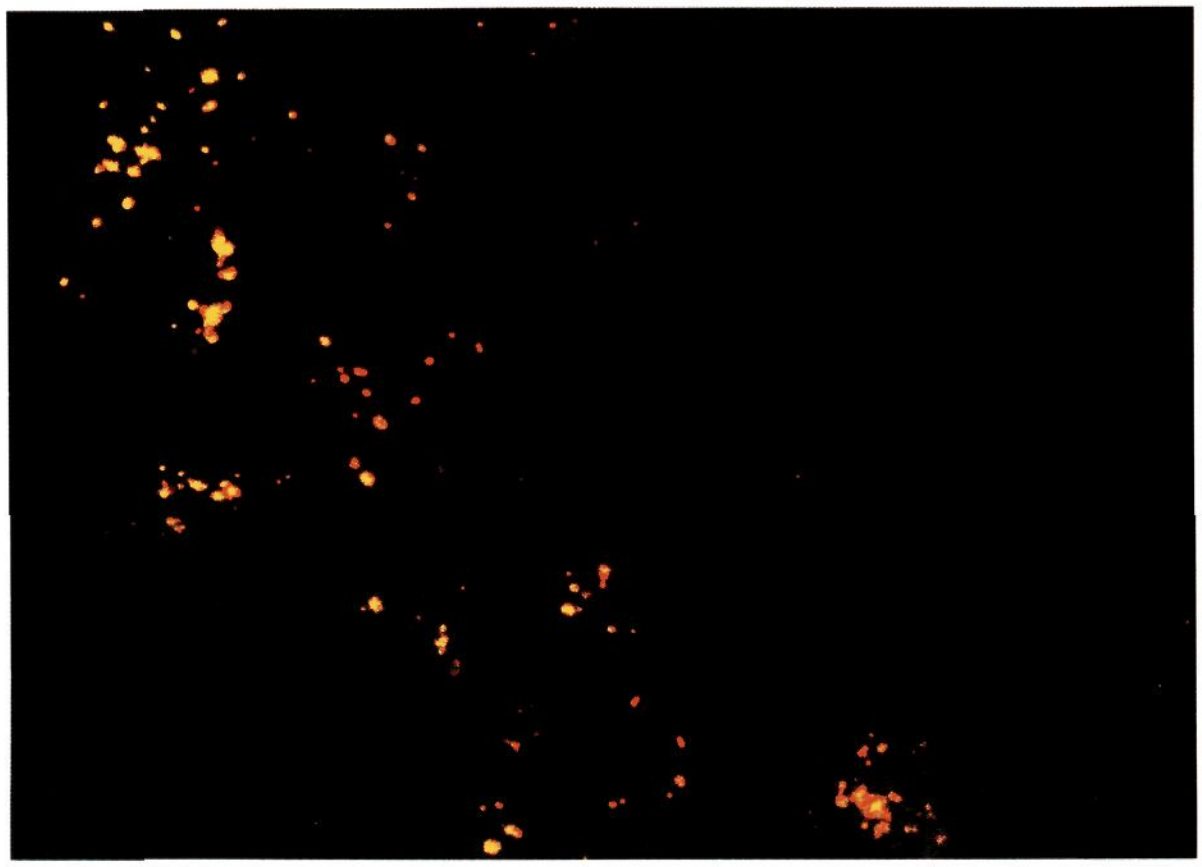


Figure 3. Epifluorescence microscopy of a U251MG cell treated with 10^{-7} M **1i** and light. The granular fluorescence is presumably from the mitochondria and the emitting species is the photooxidation product of **1i**, compound **2**.

for photosensitizers for photodynamic therapy, the chalcogenapyrylium dyes **1** containing selenium and/or tellurium offer significant potential. The chalcogenapyrylium dyes appear to be cytotoxic via the generation of singlet oxygen in those systems where a heavy atom would be expected to increase triplet yield. Furthermore, in vitro studies with these dyes suggest that these materials are targeted to mitochondria and that the activity of mitochondrial cytochrome c oxidase is inhibited upon exposure of the dye-treated cells to light. The fact that these dyes absorb light in the near-infrared would allow maximum penetration of light into tissue in vivo and would allow deeper treatment of neoplastic tissue. Furthermore, the large molar extinction coefficients in dyes of structure **1** should permit lower concentrations of sensitizer to be effective in treatment.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are corrected. ^{1}H NMR spectra were recorded on a General Electric QE 300 instrument. Microanalyses were obtained with a Perkin-Elmer C, H, and N analyzer. Solvents (Kodak Laboratory Chemicals) were dried over 3A molecular sieves prior to use. UV–visible–near-IR spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer. The preparation of tetrafluoroborate, hexafluorophosphate, and/or perchlorate salts of the chalcogenapyrylium dyes **1** has been described.[23,24]

General Procedures for the Preparation of Chloride Salts via Ion-Exchange Resins. Preparation of the Chloride Salt of Dye 1i. The perchlorate salt of dye **1i** (0.19 g, 0.27 mmol) and 1.5 g of Amberlite IRA-400 (Cl) ion-exchange resin (washed three times with 15 mL of absolute methanol prior to use) were stirred in 75 mL of methanol for 4 h. The ion-exchange resin was removed by filtration and the filter cake was washed with 10 mL of methanol. The combined filtrates were stirred for an additional 2 h with 1.5 g of the ion-exchange resin. The ion-exchange resin was removed by filtration and the filter cake was washed with 10 mL of methanol. The combined filtrates were concentrated in vacuo. The residue was dissolved in 5 mL of acetonitrile, which was then diluted with ether to 50 mL. Chilling precipitated the dye as yellow-green crystals which were collected by filtration, washed with ether, and dried to give 0.14 g (82%) of the dye: mp 213.5–215 °C; λ_{max} (CH_2Cl_2) 786 nm (ϵ 304 000); ^{1}H NMR (CD_3OD) δ 8.87 (t, 1 H, J = 13.3 Hz), 7.78 (br s, 4 H), 6.76 (d, 1 H, J = 13.3 Hz), 6.71 (d, 1 H, J = 13.3 Hz), 1.47 (s, 27 H), 1.45 (s, 9 H). Anal. Calcd for $C_{29}H_{43}SeTeCl$: C, 55.0; H, 6.8; Cl, 5.6. Found: C, 55.2; H, 6.8; Cl, 5.5.

Preparation of the Chloride Salt of Dye 1f. The hexafluorophosphate salt of **1f** (0.11 g, 0.20 mmol) was dissolved in 10 mL of methanol. Two grams of Amberlite IRA-400 (Cl) ion-exchange resin (washed three times with 15 mL of absolute methanol prior to use) was added and the resulting mixture was stirred for 1 h at ambient temperature. The resin was removed by filtration and the filter cake was washed with 5 mL of methanol. The combined filtrates were concentrated. The residue was recrystallized from 1 mL of acetonitrile and 20 mL of ether to give 0.053 g (50%) of **1f**: mp 209–209.5 °C; λ_{max} (H_2O) 730 nm (ϵ 300 000); ^{1}H NMR (CD_3OD) δ 8.78 (t, 1 H, J = 13 Hz), 7.75 (br s, 4 H), 6.67 (d, 2 H, J = 13 Hz), 1.46 (s, 36 H). Anal. Calcd for $C_{29}H_{43}Se_2$: C, 59.5; H, 7.4; Cl, 6.1. Found: C, 59.6; H, 7.5; Cl, 7.1.

Preparation of the Chloride Salt of Dye 1h. The hexafluorophosphate salt of **1h** (0.070 g, 0.10 mmol) was dissolved in 20 mL of methanol. One gram of the Amberlite IRA-400 (Cl) ion-exchange resin (washed three times with 15 mL of absolute methanol prior to use) was added. The resulting mixture was stirred at ambient temperature for 4.0 h. The ion-exchange resin was removed by filtration and the filter cake was washed with 10 mL of methanol. The combined filtrates were concentrated. The residue was recrystallized from 1 mL of acetonitrile and 20 mL of ether. Chilling precipitated copper-bronze needles of the dye which were collected by filtration, washed with ether, and dried to give 0.048 g (81%) of **1h**: mp 200.5–203.5 °C; λ_{max} (H_2O) 745 nm (ϵ 110 000); ^{1}H NMR (CD_3OD) δ 8.77 (t, 1 H, J = 13 Hz), 7.93 (br s, 2 H), 7.7 (br s, 2 H), 6.69 (d, 2 H, J = 13 Hz), 1.48 (s, 18 H), 1.42 (s, 18 H). Anal. Calcd for $C_{29}H_{43}STeCl$: C, 59.4; H, 7.4; Cl, 6.0. Found: C, 59.3; H, 7.4; Cl, 5.8.

The other chloride salts of chalcogenapyrylium dyes **1** were prepared in a similar fashion.

For **1a**: mp 199–202 °C; λ_{max} (H_2O, log ϵ) 593 nm (5.31). Anal. Calcd for $C_{29}H_{43}O_2Cl$: C, 75.9; H, 9.4; Cl, 7.7. Found: C, 75.7; H, 9.4; Cl, 7.4.

For **1b**: mp 222.5–225 °C; λ_{max} (H_2O, log ϵ) 640 nm (5.32); ^{1}H NMR (MeOH-d_4) δ 8.44 (t, 1 H, J = 13.3 Hz), 6.42 (d, 1 H, J = 13.3 Hz), 6.17 (d, 1 H, J = 13.3 Hz), 1.45 (s, 18 H), 1.33 (s, 18 H). Anal. Calcd for $C_{29}H_{43}OSCl$: C, 73.3; H, 9.1; Cl, 7.5. Found: C, 73.0; H, 9.0; Cl, 7.2.

For **1c**: mp 225–227 °C; λ_{max} (H_2O, log ϵ) 685 nm (5.42). Anal. Calcd for $C_{29}H_{43}S_2Cl$: C, 70.9; H, 8.8; Cl, 7.2. Found: C, 70.8; H, 8.8; Cl, 7.1.

For **1d**: mp 193–197 °C; λ_{max} (H_2O, log ϵ) 665 nm (5.38); ^{1}H NMR (MeOH-d_4) δ 8.41 (t, 1 H, J = 12.5 Hz), 6.52 (s, 1 H, J =

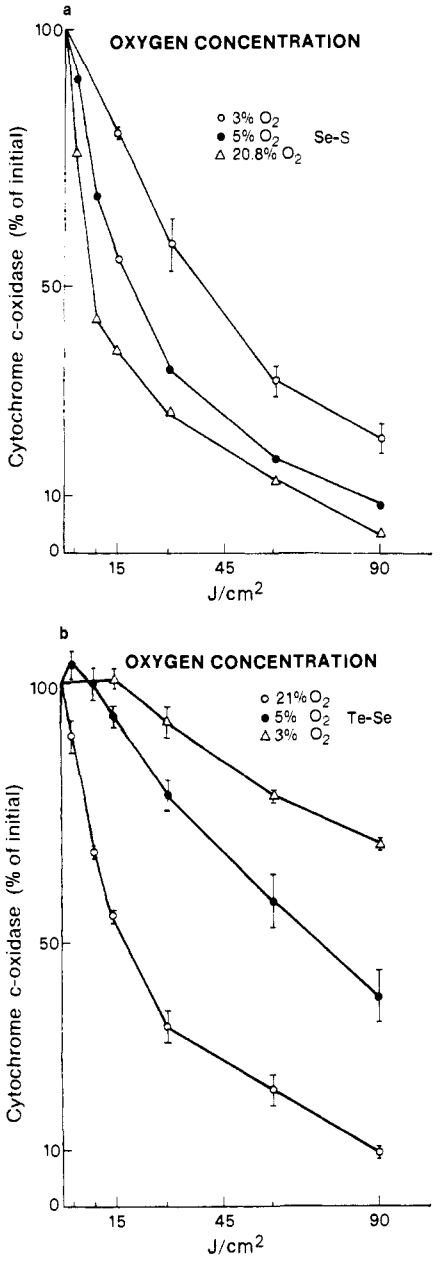


Figure 4. The effect of oxygen concentration on the inhibition of cytochrome c oxidase in isolated mitochondria treated (a) with 1e and (b) with 1i.

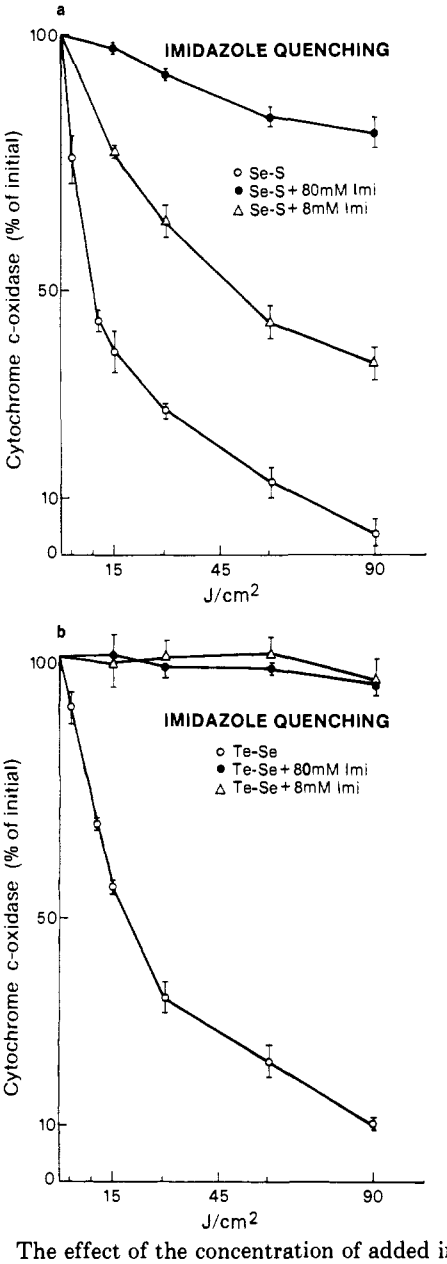


Figure 5. The effect of the concentration of added imidazole on the inhibition of cytochrome c oxidase in isolated mitochondria treated (a) with 1e and (b) with 1i.

12.5 Hz), 6.30 (d, 1 H, J = 12.5 Hz), 8.0–6.3 (br, 4 H), 1.47 (s, 18 H), 1.39 (s, 18 H). Anal. Calcd for $C_{29}H_{43}OSeCl$: C, 66.7; H, 8.3; Cl, 6.8. Found: C, 66.7; H, 8.4; Cl, 6.5.

For 1e: mp 222.5–225 °C; λ_{max} (H_2O, log ϵ) 708 nm (5.40). Anal. Calcd for $C_{29}H_{43}SSeCl$: C, 64.7; H, 8.1; Cl, 6.6. Found: C, 64.6; H, 7.9; Cl, 6.3.

For 1g: mp 197–199 °C; λ_{max} (H_2O, log ϵ) 700 nm (5.27); ^1H NMR (MeOH-d_4) δ 8.29 (t, 1 H, J = 12.5 Hz), 7.8 (br s, 2 H), 7.2 (br s, 2 H), 6.70 (d, 1 H, J = 12.5 Hz), 6.45 (d, 1 H, J = 12.5 Hz), 1.45 (s, 18 H), 1.38 (s, 18 H). Anal. Calcd for $C_{29}H_{43}OTeCl$: C, 61.0; H, 7.6; Cl, 6.2. Found: C, 61.0; H, 7.6; Cl, 6.2.

For 1j: mp 218–220 °C; λ_{max} (H_2O, log ϵ) 810 nm (5.18); ^1H NMR ($CDCl_3$) δ 8.75 (t, 1 H, J = 13.5 Hz), 7.71 (s, 4 H), 6.86 (d, 2 H, J = 13.5 Hz). Anal. Calcd for $C_{29}H_{43}Te_2Cl\cdot H_2O$: C, 49.7; H, 6.5; Cl, 5.1. Found: C, 49.8; H, 6.3; Cl, 4.8.

Attempted Preparation of the Chloride Salt of Dye 1i with Condensation Technology. 2,6-Di-*tert*-butyl-4-methylselenapyrylium chloride (6.00 g, 18.8 mmol) and (2,6-di-*tert*-butyltellurapyran-4-ylidine)acetaldehyde (6.72 g, 19.4 mmol) in 20 mL of acetic anhydride were heated on a steam bath for 11 min. The reaction mixture was cooled to ambient temperature and 15 mL of acetonitrile was added. The resulting solution was filtered through a pad of glass wool. The filtrate was diluted with 250 mL of ether and the resulting solution was chilled. The dye precipitated as copper-bronze crystals which were collected by filtration, washed with ether, and dried to give 10.51 g (88%) of the dye. ^1H NMR and absorption spectroscopies showed the product to be a 1/2/1 mixture of 1f/1i/1j. This mixture is expected from a statistical distribution of the heteroatoms if random scrambling of the heteroatoms were to occur during reaction.

General Procedures for Purification of Unsymmetrical Tellurapyrylium Dyes. Purification of 1i as Its Hexafluorphosphate Salt. A mixture containing 91% 1i, 7% 1f, and 2% 1j (0.37 g, 0.50 mmol) was dissolved in 10 mL of dichloromethane. Bromine (0.09 g, 0.55 mmol) in 1 mL of dichloromethane was added via a syringe to give a magenta solution. After addition was complete, the reaction mixture was slowly diluted with 10 mL of ether. The resulting mixture was chilled, precipitating dark red crystals of the dibromide salt of 1i which were collected by filtration and dried to give 0.32 g (71%) of material: mp 188–190 °C; ^1H NMR (CD_3CN) δ 8.65 (d × d, 1 H, J = 12, 15 Hz), 8.52 (s, 2 H), 7.22 (d, 1 H, J = 15 Hz), 7.07 (s, 1 H), 7.01 (d, 1 H, J = 12 Hz), 6.56 (s, 1 H), 1.66 (s, 27 H), 1.57 (s, 9 H); λ_{max} (CH_2Cl_2, log ϵ) 546 nm (4.76).

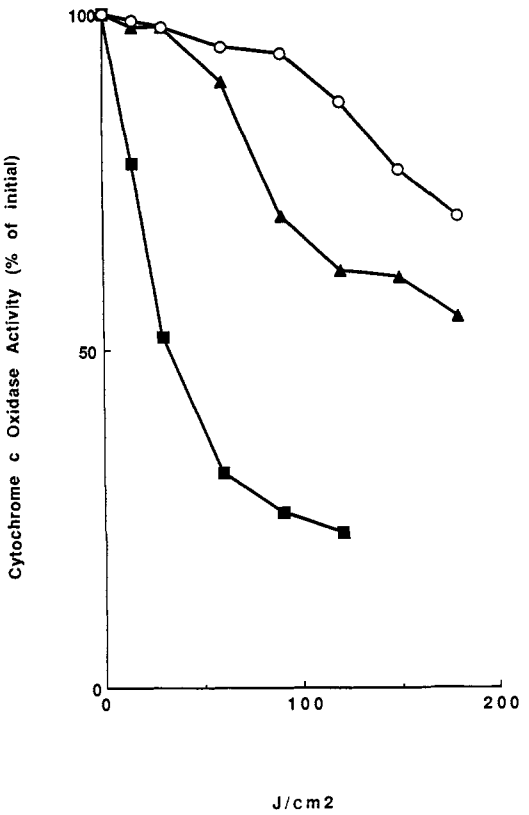


Figure 6. A comparison of Photofrin II (open circles) with chalcogenapyrylium dyes **1g** (filled squares) and **1i** (filled triangles) with respect to inhibition of cytochrome c oxidase in isolated liver mitochondria. Sensitizer concentration was 10^{-5} M with irradiation from 570 to 800 nm at 50 mW/cm^2.

The dibromide salt of **1i** (0.180 g, 0.20 mmol) was dissolved in 5 mL of dichloromethane and 5 mL of methanol. Sodium bisulfite (0.050 g, 0.50 mmol) in 5 mL of water was added. The resulting mixture was stirred for 5 min at ambient temperature. The reaction mixture was poured into 20 mL of water. The product was extracted with dichloromethane (2 × 25 mL). The combined organic extracts were dried over sodium sulfate and concentrated. The residue was recrystallized from 1 mL of acetonitrile and 20 mL of ether to give 0.142 g (96%) of **1i** as its hexafluorophosphate salt containing less than 1% of either **1f** or **1j** on the basis of ^{1}H NMR spectroscopy.

Purification of the Hexafluorophosphate Salt of 1h. A mixture containing 88% **1h** and 12% **1c** (0.70 g, 1.0 mmol) was dissolved in 20 mL of dichloromethane. Bromine (0.20 g, 1.2 mmol) in 1 mL of dichloromethane was added dropwise, giving a red solution. After addition was complete, the reaction mixture was stirred for 5 min at ambient temperature and was then diluted with 25 mL of ether. Chilling the resulting solution precipitated bright orange crystals of the dibromide salt of **1h** which were collected by filtration and dried to give 0.59 g (78%) of material: mp 190.5–194.0 °C; ^{1}H NMR (CD_2Cl_2) δ 8.48 (s, 2 H), 8.43 (d × d, 1 H, J = 12, 15 Hz), 7.28 (d, 1 H, J = 15 Hz), 6.95 (s, 1 H), 6.93 (d, 1 H, J = 12 Hz), 6.39 (s, 1 H), 1.67 (s, 18 H), 1.65 (s, 9 H), 1.60 (s, 1 H); λ_{max} (CH_2Cl_2, log ϵ) 524 nm (4.85).

The dibromide salt of **1h** (0.214 g, 0.25 mmol) was dissolved in 5 mL of dichloromethane and 5 mL of methanol. Sodium bisulfite (0.052 g, 0.50 mmol) in 5 mL of water was added and the resulting mixture was stirred for 5 min at ambient temperature. The reaction mixture was poured into 5 mL of water, and the products were extracted with dichloromethane (2 × 25 mL). The combined organic extracts were dried over sodium sulfate and concentrated. The residue was recrystallized from 1 mL of acetonitrile and 25 mL of ether to give yellow-green crystals of **1h** (0.141 g, 81%) as its hexafluorophosphate salt, mp 220–221 °C, containing less than 1% **1c** by ^{1}H NMR.

Purification of the Hexafluorophosphate Salt of 1g. A mixture containing 85% **1g** and 15% **1a** (0.034 g, 0.050 mmol) was dissolved in 1 mL of dichloromethane. Bromine (0.016 g, 0.10 mmol) in 1 mL of dichloromethane was added and the resulting solution was stirred for 5 min at ambient temperature. The reaction mixture was diluted with 5 mL of ether, precipitating an orange solid. The orange crystals of the dibromide salt of **1g** were collected by filtration and dried to give 0.035 g (92% based on **1g**) of material: mp 178–180 °C; ^{1}H NMR (CD_2Cl_2) δ 8.40 (d × d, 1 H, J = 12, 15 Hz), 7.73 (s, 2 H), 7.05 (d, 1 H, J = 15 Hz), 6.90 (s, 1 H), 6.89 (d, 1 H, J = 12 Hz), 6.38 (s, 1 H), 1.65 (s, 9 H), 1.60 (s, 9 H), 1.55 (s, 18 H); λ_{max} (CH_2Cl_2, log ϵ) 510 nm (4.60).

The dibromide salt of **1g** (0.021 g, 0.025 mmol) was dissolved in 2 mL of dichloromethane and 2 mL of methanol. Sodium bisulfite (0.010 g, 0.10 mmol) in 2 mL of water was added. The resulting mixture was stirred at ambient temperature for 10 min. The reaction mixture was poured into 20 mL of water and the products were extracted with dichloromethane (2 × 20 mL). The combined organic extracts were dried over sodium sulfate and concentrated. The residue was crystallized by the addition of 20 mL of ether. Green crystals of **1g** (0.014 g, 82%) were collected by filtration. The hexafluorophosphate salt of dye **1g**, mp 198–200 °C, contained less than 1% **1a** by ^{1}H NMR.

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. Two to three weeks after transplantation, when tumors had grown to 2–3 cm in diameter, the animals were sacrificed, and the tumors were excised and placed in 0.9% sodium chloride on ice. The tissue was finely minced with scissors and homogenized on ice with 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'*-tetraacetic acid, 0.03% bovine serum albumin, and 100 mM potassium chloride (pH 7.4), with 30-s bursts with a Polytron PCU-2110 homogenizer at a setting of six (Brinkmann Ind., Westbury, NY). Preparation of isolated mitochondria from the homogenized tumor tissue followed a method described earlier.[30] Mitochondrial suspensions were divided into 0.5-mL aliquots (6–10 mg of mitochondrial protein/mL) and stored at −70 °C until used for in vitro experiments.

Exposure of Tumor Mitochondria to Chalcogenapyrylium Dyes in Vitro. The chalcogenapyrylium dyes were prepared for in vitro testing by dissolving 1.0–1.5 mg of each (according to molecular weight) in 250 μL of 95% ethanol followed by dilution to 2.5 mL with distilled water (1 mM in dye). Ten microliters of each of the dye preparations was added to 1 mL of homogenizing buffer (10 μM in dye) and the absorption spectrum was obtained (500–850 nm) with a Cary 219 scanning spectrophotometer (Varian Instruments, Palo Alto, CA). Stock dye solutions were adjusted by dilution with distilled water to a concentration in the absorbance range of 1.3–1.6 units for each dye at its wavelength maximum. Absorbance spectra of each dye were obtained immediately before and after each experiment in vitro to determine whether the dye absorbance remained within the range stated above. Aliquots of mitochondrial suspensions were allowed to thaw at ambient temperature and diluted with homogenizing buffer to a concentration that would yield a 0.45–0.60 change in the absorbance of cytochrome c/min at 550 nm as previously described.[30] Ten microliters of each dye stock solution was added to 1 mL of the diluted mitochondrial suspension; the suspension was allowed to stand at room temperature in the dark for 5 min, followed by centrifugation at 800g with an Eppendorf microcentrifuge (Model 3200, Brinkmann Ind., Westbury, NY). The supernatant was carefully removed and saved for estimating dye uptake and the mitochondrial pellet was resuspended with vigorous vortexing in 1 mL of the buffer.

Photoradiation Conditions. Mitochondrial suspensions (1 mL) containing chalcogenapyrylium dyes were transferred to 3-mL quartz cuvettes and photoirradiated by focusing a 1-cm-diameter, filtered beam (375–800 nm) emitted from a quartz–halogen source on the suspension. The mitochondrial suspensions were continuously stirred magnetically and the temperature of the suspensions was monitored via a YSI Model 41TD telethermometer connected to a needle type temperature probe (Yellow Springs Instruments, Yellow Springs, OH). The temperature of the suspensions did

(30) Gibson, S. L.; Hilf, R. *Cancer Res.* **1983**, *43*, 4191.

not rise above ambient (25 °C) under the irradiation conditions employed (50 mW/cm^2, 30 min). The intensity of the irradiation beam was measured with an Rk 5200 power radiometer connected to an RKP 545 radiometer probe (Laser Precision Corp., Utica, NY). An action spectrum of the beam emitted from the filtered quartz–halogen source was analyzed with a Model H10 precision monochrometer (Instruments SA, Londonderry, New Hampshire) in combination with the power radiometer. The intensity of the emitted beam remained fairly constant over the wavelength range (595–770 nm) at which the different dyes tested possessed their maximum light absorption. At selected times during photoirradiation, 10-μL aliquots were removed and analyzed for the activity of the cytochrome c oxidase as previously described.[30]

Uptake of Dye by the Mitochondria in Vitro. Dye uptake into the mitochondria was assessed by measuring the absorbance of the dye in homogenizing buffer versus the absorbance of the dye remaining in the supernatant following centrifugation of the mitochondrial suspensions incubated with the dyes in the dark. The diference was divided by the value obtained for the dye alone in buffer and the data are expressed as percentage uptake of each dye into mitochondria. Mitochondrial protein was determined by the Lowry method and ranged from 3.5 to 5 mg of mitochondrial protein/mL for all of the preparations employed in these experiments.

Exposure of Mitochondria to Chalcogenapyrylium Dyes and Light in the Presence of Imidazole. Imidazole, a singlet-oxygen acceptor employed for the detection of singlet oxygen in the RNO assay,[29] was added at 8 or 80 mM final concentration to mitochondrial suspensions containing either 1e or 1i to determine whether its presence would alter the photoradiation-induced inhibition of mitochondrial c oxidase. Photoradiation conditions and measurement of cytochrome c oxidase activity were as described above. The presence of imidazole in mitochondrial suspensions had no effect on cytochrome c oxidase activity if maintained in the dark or exposed to light in the absence of dye.

Exposure of Mitochondria to Chalcogenapyrylium Dyes and Light at Reduced Oxygen Concentrations. Mitochondrial suspensions containing 1e or 1i were maintained at 20.8% (ambient, 0.24 mM), 5% (0.06 mmol), or 3% (0.035 mmol) oxygen concentration during exposure to photoradiation. The concentration of oxygen in the suspensions was controlled with an Oxyreducer (Reming Bioinstruments, Redfield, NY) as described previously.[31] The oxygen concentration in solution was monitored using on OM-1 biological oxygen meter connected to a microprobe (Microelectrodes, Inc., Londonderry, NH). The suspensions were equilibrated at selected oxygen concentrations prior to photoradiation and maintained at selected levels (±0.4%) throughout each experiment. Photoradiation of mitochondria at reduced oxygen concentrations in the absence of dye or mitochondria maintained in the dark displayed no alteration in the activity of cytochrome c oxidase as compared to unperturbed controls.

Quantum Yields for Singlet-Oxygen Generation in Methanol. Photolyses were carried out using light from a 500-W tungsten-filament lamp filtered through appropriate narrow-band (10 nm) interference filters and cutoff filters. Irradiation intensity was monitored with an EG&G 450-1 radiometer calibrated vs a standard thermopile. Reinecke's salt actinometry[32] provided a calibration check. The singlet-oxygen acceptor 1,3-diphenylisobenzofuran (DPBF, purchased from Aldrich Chemical Co.) was recrystallized under yellow lights from an acetone/methanol mixture. Spectrograde methanol and certified rose bengal and methylene blue were used as received from Eastman Laboratory Chemicals.

Quantum yields for singlet-oxygen generation in air-saturated methanol were determined by monitoring the chalcogenapyrylium-sensitized photooxidation of DPBF. DPBF is a convenient acceptor since it absorbs in a region of chalcogenapyrylium dye transparency and rapidly scavenges singlet oxygen to give colorless products. This reaction occurs with little or no physical quenching.[33] Our measurements of both rose bengal and methylene blue sensitized photooxidation of DPBF in methanol indicate that one molecule of this acceptor is destroyed per molecule of singlet oxygen scavenged. According to the procedure of Foote and co-workers,[34] the β-value for an acceptor and the quantum yield of singlet-oxygen generation of a sensitizer may be determined from the slope and intercept of a plot of the inverse of the photooxidation quantum yield (Φ_{ox}) vs the inverse of the acceptor concentration. In this manner a β-value of 7.8×10^{-5} M was obtained for DPBF and a singlet-oxygen quantum yield [$\Phi(^1O_2)$] of 0.77 ± 0.05 was obtained for rose bengal in methanol. The value of $\Phi(^1O_2)$ is very close to the literature value[35] of 0.76, which supports a one-to-one reaction between DPBF and singlet oxygen.

With a similar procedure with methylene blue as the sensitizer, a singlet-oxygen quantum yield of 0.50 ± 0.05 and a β-value of 8.0×10^{-5} M were obtained. The $\Phi(^1O_2)$ value is reasonable based on the reported[36] quantum yield for triplet formation of 0.52 in both ethanol and water and on the observation that oxygen quenching of methylene blue triplets in methanol produces singlet oxygen with nearly unit efficiency.[37]

Singlet-oxygen quantum yields for the chalcogenapyrylium dyes were measured at low dye concentrations to minimize the possibility of singlet-oxygen quenching by the dyes. Irradiations were carried out in 2-cm cells containing a dye concentration (typically 2×10^{-6} M) sufficient to produce an optical density of approximately 0.3–0.5 at the irradiation wavelength. A DPBF concentration of 2×10^{-5} M was utilized.

The methylene blue sensitized bleach of DPBF (2×10^{-5} M) was measured with 2×10^{-6} M 1j or 1l added. The observed photobleaching quantum yields were the same with and without added dye within an experimental error of about 7%, indicating that rate constants for quenching of singlet oxygen (lifetime ≈ 10 μs) by these dyes are $\leq 4 \times 10^9$ M^{-1} s^{-1} in methanol.

To simplify kinetic analysis, DPBF was photolyzed to low conversions (≤10%) such that its concentration may be assumed to be fixed at the initial value. Photolyses required between 5 and 60 min, depending upon the efficiency of the chalcogenapyrylium photosensitizer. No thermal recovery of DPBF (from a possible decomposition of endoperoxide product) was observed under the conditions of these experiments. The quantum yield of singlet-oxygen generation by a chalcogenapyrylium dye may be calculated by comparing the quantum yield for photooxidation of DBPF sensitized by the dye of interest to the quantum yield of methylene blue (MB) sensitized DPBF photooxidation at the same DPBF concentration:

$$\Phi(^1O_2)(\text{dye}) = [\Phi_{ox,DPBF}(\text{dye}) \times \Phi(^1O_2)(\text{MB})]/\Phi_{ox,DPBF}(\text{MB})$$

Alternatively, $\Phi(^1O_2)$ values for the chalcogenapyrylium dyes may be calculated from the following expression, using the measured β-value for DPBF:

$$\Phi(^1O_2)(\text{dye}) = \{\Phi_{ox,DPBF}(\text{dye}) \times (\beta + [\text{DPBF}])\}/[\text{DPBF}]$$

Values obtained by the two procedures were equivalent.

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Registry No. 1a, 124620-00-4; 1b, 124620-01-5; 1c, 124620-02-6; 1d, 124620-03-7; 1e, 124620-04-8; 1f, 124620-05-9; 1g, 124620-06-0; 1g·PF_6^-, 124620-16-2; 1h, 124620-07-1; 1h·PF_6^-, 124620-14-0; 1i, 124620-08-2; 1i·PF_6^-, 124620-12-8; 1j, 124620-09-3; O_2, 7782-44-7; cytochrome *c* oxidase, 9001-16-5; 2,6-di-*tert*-butyl-4-methylselenapyrylium chloride, 124620-10-6; (2,6-di-*tert*-butyltellurapyran-4-ylidine)acetaldehyde, 103817-95-4.

(31) Gibson, S. L.; Hilf, R. *Photochem. Photobiol.* **1985**, *42*, 367.
(32) Wegner, E. E.; Adamson, A. W. *J. Am. Chem. Soc.* **1966**, *88*, 394.
(33) Merkel, P. B.; Kearns, D. R. *J. Am. Chem. Soc.* **1975**, *97*, 462.
(34) Foote, C. S. In *Singlet Oxygen*; Academic: New York, 1979; p 139.
(35) Gollnick, K.; Schenck, G. O. *Pure Appl. Chem.* **1964**, *9*, 507.
(36) Nemoto, M.; Kokubun, H.; Koizumi, M. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 1223, 2464.
(37) Merkel, P. B.; Kearns, D. R. *J. Am. Chem. Soc.* **1972**, *94*, 7244.