Design, Synthesis, and Photophysical Characterization of Novel Pentacyclic Red Shifted Azine Dyes

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Received April 1, 1998

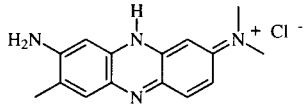
As part of our continuing efforts to develop second generation photodynamic therapeutic agents [1], we synthesized three pentacyclic azine dyes that were designed with the aid of MNDO calculations to absorb visible light having wavelengths longer than 600 nm. Photophysical measurements for the azine dyes 1,4,8,11-tetraethyl-1,2,3,4,8,9,10,11,13-nonahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazinium acetate, **13**, 1,4,8,11-tetraethyl-2,3,4,8,9,10,11,12,13-octahydro-13-methyldipyrazino[2,3-*b*:2',3'-*i*]phenazinium iodide, **14**, and 1,4,8,11,13-pentaethyl-2,3,4,8,9,10,11,12,13-octahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazinium iodide, **15**, are highlighted by a 35 nm red shift in their absorption spectra and a 5-7 fold increase in their singlet oxygen quantum yield relative to tricyclic model compounds 3,7-bis(diethylamino)phenazinium chloride, **20**, and 3,7-bis(diethylamino)-5-ethyl-phenazinium iodide, **21**, which were also synthesized for this study. Incorporation of rigid peripheral tetrahydropyrazino ring systems in the pentacyclic azines **13**, **14**, and **15** are responsible for the improved fluorescence and singlet oxygen quantum yields relative to the tricyclic azines **20** and **21**.

J. Heterocyclic Chem., **36**, 25 (1999).

Introduction.

Photodynamic therapy is a novel treatment for neoplasia which utilizes light and a heterocyclic photosensitizing dye [2] to destroy diseased tissue. In addition to staining cancerous tissue, photodynamic therapeutic agents must absorb light between 600-1300 nm, a spectral region defined as the "therapeutic window" [3], and upon photoirradiation, generate the potent cytotoxin [2f] singlet oxygen. Wavelengths of light shorter than 600 nm do not penetrate tissue efficiently because much of the light is scattered at the skin's surface or is absorbed by endogenous chromophores, *i.e.*, melanin and hemoglobin [3], while wavelengths greater than 1300 nm, in general, are not energetic enough to elicit a photodynamic response. The advantage of photodynamic therapy over traditional chemotherapy resides in its dual selectivity [4]; cell killing occurs only where photosensitizer and light are present simultaneously.

The encouraging results obtained using photodynamic therapy to treat a variety of tumor types coupled with the limitations of the first generation drugs [2a] has stimulated the search for improved tumor localizing photodynamic therapeutic agents. Our approach for developing second generation photodynamic therapeutic agents is based on the results of studies by Lewis [5] and Riley [6] which independently showed that selected cationic heterocyclic compounds, belonging primarily to the azine and thiazine family of dyes, had the uncommon properties of selectively staining and inhibiting tumor growth in mice in the dark. The most effective dye in Riley's study was the relatively non-toxic azine, Neutral Red, **1**. Endo [7] extended these observations with regard to tumor inhibition and lack of dark toxicity for Neutral Red to the 180 sarcoma and the 63 carcinoma solid tumors. Although Neutral Red has been shown to be an effective photodynamic therapeutic agent [8] against various cancer cell lines [9], bacteria [8b], and viruses [8c] *in vitro*, we are unaware of any photodynamic therapeutic investigations using Neutral Red to treat solid tumors. Presumably this is because Neutral Red (λ max 540 nm), like most azine dyes, does not have significant absorption within the therapeutic window. Herein we describe the design and synthesis of novel, water soluble azine dyes engineered to absorb in the therapeutic window, and report their absolute fluorescence and singlet oxygen quantum yields.



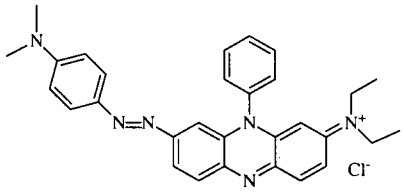
Neutral Red

λ max 540 nm

1

Results and Discussion.

In light of the demonstrated abilities of Neutral Red to both localize to tumor tissue and to photoinactivate cancer cells *in vitro*, our goal was to design azine photosensitizers with absorption bands in the therapeutic window that would still possess the beneficial properties reported for Neutral Red. Of the limited techniques available to obtain a red shifted absorption, a common method that has been successfully applied to azine dyes, as exemplified by Janus Green B (λ max 660 nm) **2** and Induline 6 B (λ max 595 nm) **3**, is to extend the length of the chromophore; unfortunately, the strategies used to achieve this extension



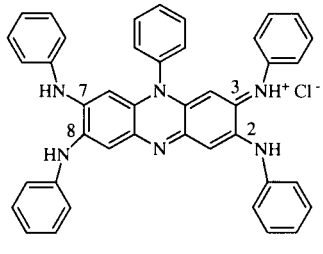
Janus Green B

λ max 660 nm

2

would likely diminish their ability to serve as effective photodynamic therapeutic agents. Thus, the use of an azo moiety in Janus Green B to increase the conjugation results in a twofold liability. First, Lazarow and Cooperstein [10] showed that in living cells the azo linkage is susceptible to enzymatic reduction which destroys the extended conjugation, thereby nullifying the red shift. Second, flexible azo linkages [11] alter the photophysical properties of dyes in a manner that is detrimental to the production of singlet oxygen by providing a pathway for the rapid deactivation of the excited state.

The absorption spectrum of Induline 6 B, **3**, was extended towards the red due to the increased conjugation of the π system brought about by the addition of phenyl groups on the nitrogen atoms at the 3 and 7 positions and the incorporation of anilino groups at the 2 and 8 positions. However, like Janus Green B, Induline 6 B also has a mechanism that promotes rapid relaxation from the excited state that diminishes the likelihood of singlet oxygen formation. While contribution from the 2 and 8 anilino groups to this rapid decay is unknown, it is well documented that phenyl rings on the 3 and 7 nitrogen atoms of this class of dyes can cause ultra fast deactivation of the excited state [12].



Induline 6 B

λ max 595 nm

3

A different approach to obtain a bathochromic shift, which usually does not introduce the rapid decay mentioned above, is to append auxochromes to the chromophore. In this method, the extension is caused by interactions between the lone pair of electrons on an auxochrome and the chromophore's delocalized π system. Inspection of the 2 and 8 positions of Induline 6 B indicates that the observed absorption shift may be influenced by both extension of the chromophore into the phenyl rings and by a separate contribution from the lone pair of electrons on the 2 and 8 nitrogen auxochromes. In order to determine the contribution solely from the lone pair of electrons on the 2 and 8 nitrogen auxochromes to the direction of the absorption shift, MNDO calculations were carried out on theoretical compounds **4** and **5**. To ensure a maximum effect from the amino auxochromes, the 2,3,7, and 8 nitrogens were constrained in rings forcing optimum overlap between their lone pairs and the π orbitals in the azine nucleus. *N*-Methyl substitution was chosen to avoid convergence to a local minimum, rather than a global minimum, which can occur when larger alkyl groups are modeled.

Computational Studies.

Restricted Hartree-Foch (RHF) theoretical calculations were carried out according to the semiempirical MNDO [13] SCF method using the PM3 [14] Hamiltonian as implemented in the MOPAC 93.00 [15] package of programs. All geometric degrees of freedom were optimized without any symmetry constraints using a 100 fold increase in the criteria normally required for convergence and termination. These calculations utilize BFGS geometry optimization [16] and do not take the energetics of solvation into account. Since Frank-Condon photoexcitation requires an initially formed excited state to have the same geometry as its precursor state, the vertical electronic transition energy for each chromophore was determined by calculating the difference in the magnitude of the SCF heats of formation for both the ground and the first excited singlet states using optimized ground state molecular geometries [17].

Indeed, the calculations indicated that target dipyrazino **5** was red shifted relative to model azine **4**. Transition energies for **4** and **5** were predicted to be 73.90 kcal/mol and 70.24 kcal/mol, respectively. Since the MNDO calculations employed did not consider solvation energy, the 3.66 kcal/mol relative energy difference calculated between **4** and **5** was more important than the absolute calculated absorption bands which were centered at 387 nm and 407 nm, respectively. When applied to the region in which azine dyes absorb, this energy difference would correspond to a red shift of approximately 40 nm. Since the primary difference between compounds **4** and **5** are the substituents at the 2 and 8 positions, it can be concluded that alkylamino groups at the those positions are responsible for the calculated red shift in dipyrazino **5**. Working within the MNDO theoretical framework, insight into the origin of this calculated energy difference can be gained by inspection of the Pz electron densities for the HOMO and the LUMO of aziniums **4** and **5** (Figure 1). In compound **5**, antibonding

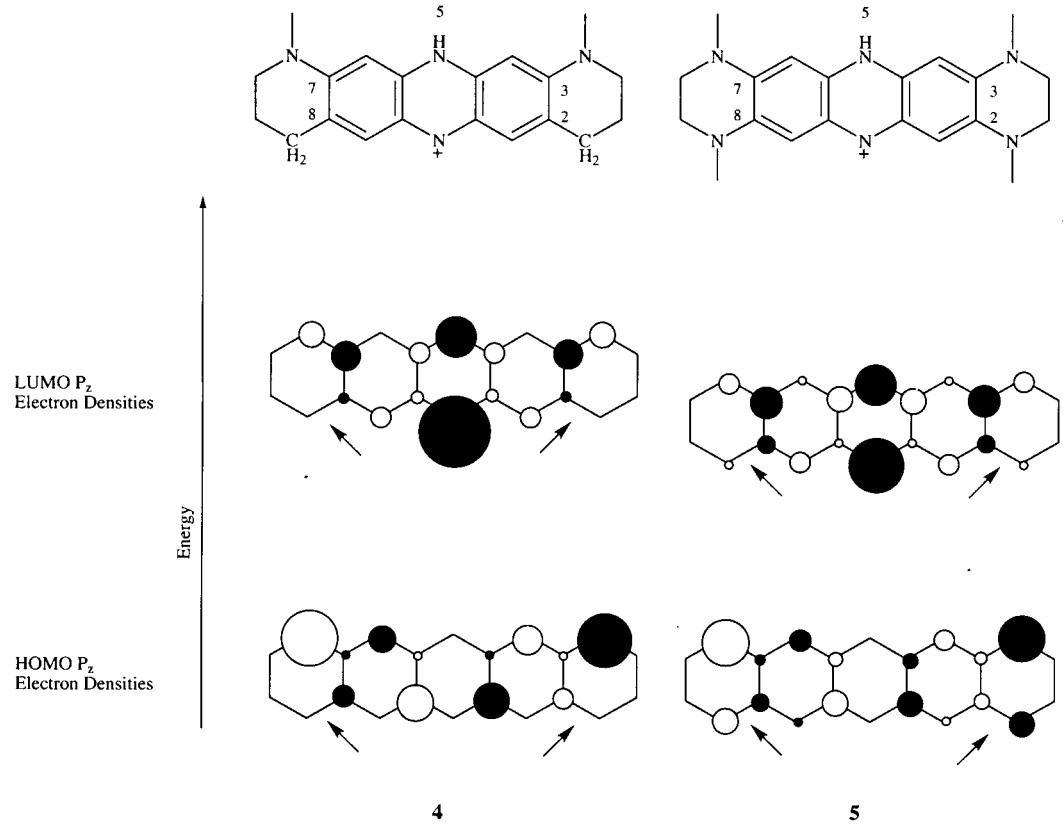


Figure 1. P_z Electron densities of the HOMO and the LUMO for model and red shifted azine dyes. The energies for the HOMOs of **4** and **5** were arbitrarily set equal to emphasize the difference in their calculated transition energy gaps. The numbering system applied is that which is used by Chemical Abstracts Service to identify the locations of appendages to a phenazinium core.

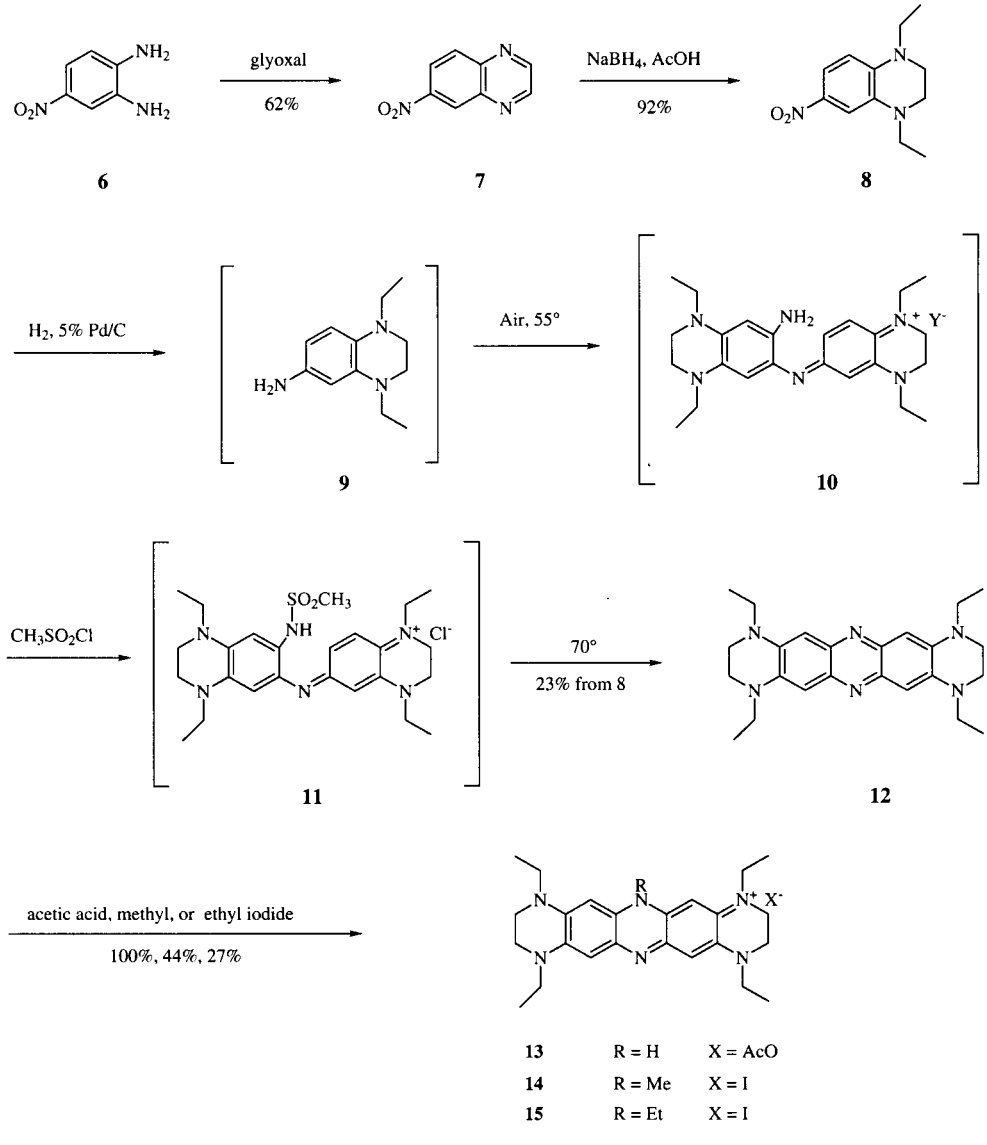
interactions are calculated to exist in both the HOMO and the LUMO between the carbon atoms at the 2 and 8 positions with their adjoining nitrogen auxochromes. This considerably greater interaction in the HOMO, relative to the analogous interaction in the LUMO, for dipyrazino **5** (highlighted by the arrows in Figure 1) contributes to the decrease in the transition energy gap which results in a calculated red shift. A comparison of the highlighted regions of **4** and **5** clearly show that the corresponding interactions are absent in compound **4**.

Synthesis.

The syntheses of red shifted 2,3,7,8 alkylamino substituted and the model 3,7-alkylamino substituted azines are outlined in Schemes 1 and 2, respectively. While the safranines, azines that are substituted in the 5 position with an aromatic group, are a well known family of dyes and have long been a staple of the dyestuff industry, the literature contains surprisingly few examples of azine dyes with alkylamino groups at the 3 and 7 without an aromatic group at the 5 position. Thus, model azines **20** and **21** were synthesized to serve as reference compounds so that the effects of the added alkylamino groups at the 2 and 8 positions in the target azine dyes upon wavelength of maximum absorption could be determined and to increase the scope of knowledge of a relatively unstudied subset of azine dyes.

6-Nitroquinoxaline, **7**, was obtained by employing a method developed by Weisman *et al.* for the macrocyclization of tetraamines [18]. Reductive alkylation of **7** with sodium borohydride and acetic acid [19] gave *N,N*-diethyl-1,2,3,4-tetrahydro-6-nitroquinoxaline, **8**. In a one pot reaction sequence to give **12**, the transient intermediate triamine **9**, obtained by catalytic hydrogenation of **8**, spontaneously self condensed to presumably give intermediate indamine **10**. Since the treatment of **8** with a variety of oxidizing agents to give **12** directly proved impractical, a more favorable pathway was found that involved the addition of methanesulfonyl chloride presumably to give sulfonamide **11** that cyclized upon heating to give **12**. Following purification by column chromatography and crystallization, **12** was acidified with acetic acid to provide **13** or alkylated using methyl or ethyl iodide to give derivatives **14** and **15**, respectively.

Scheme 1



N,N-Diethyl-1,3-benzenediamine, **17**, was obtained by reduction of *N,N*-diethyl-3-nitroaniline, **16**, with hydrogen over palladium on carbon and then converted to sulfonamide [20] **18**. Addition of the hydrochloride salt of *p*-phenylenediamine **19** to an aqueous solution of **18** and potassium dichromate gave model azine **20** which was isolated and purified as its hydrochloride salt. Alkylation of **20** using ethyl iodide gave 3,7-bis(diethylamino)-5-ethylphenazinium iodide, **21**.

Photophysical Properties.

Wavelengths of maximum absorption, molar absorptivities, absolute fluorescence quantum yields, and absolute singlet oxygen quantum yields are listed in Table 1. As predicted by MNDO calculations, absorption bands for the pentacyclic azines **13**, **14**, and **15** are red shifted relative to the tricyclic model azine dyes **20** and **21** (Figure 2); the observed bathochromic shift was 36-37 nm.

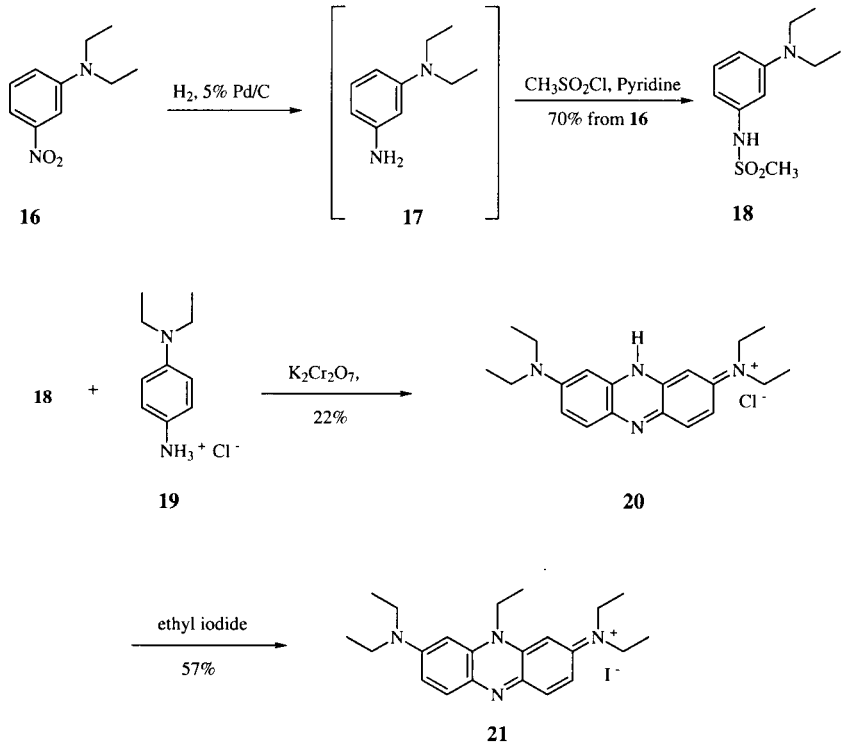
Since these red shifted azine dyes were designed to be photodynamic therapeutic agents, we were interested in

Table 1
Photophysical Properties of Azine Dyes

Compound	λ max [a]	ε	Φ_F [b]	Φ $^1O_2$ [c]
13	600	50,300	38.0	23.0
14	607	75,300	37.0	34.0
15	606	58,400	40.0	36.0
20	564	51,200	10.0	7.0
21	569	77,800	6.0	4.0

[a] Measured in ethanol. [b] Fluorescence quantum yields obtained by comparison with Cresyl Violet [21]. [c] Obtained by a diphenylisobenzofuran bleach method [22].

Scheme 2



evaluating their ability to produce singlet oxygen, the species generally regarded to be the active cytotoxin in photodynamic therapy [2f]. Absorption of a single photon initiates the stepwise process which ultimately results in the generation of singlet oxygen. After promotion to the first excited state the dye can undergo intersystem crossing into the triplet manifold or decay to the ground state. The ability of a dye to intersystem cross is a necessary characteristic of a photosensitizing dye because, with few exceptions, singlet oxygen can only be generated by the interaction between dyes in their excited triplet state with ground state oxygen.

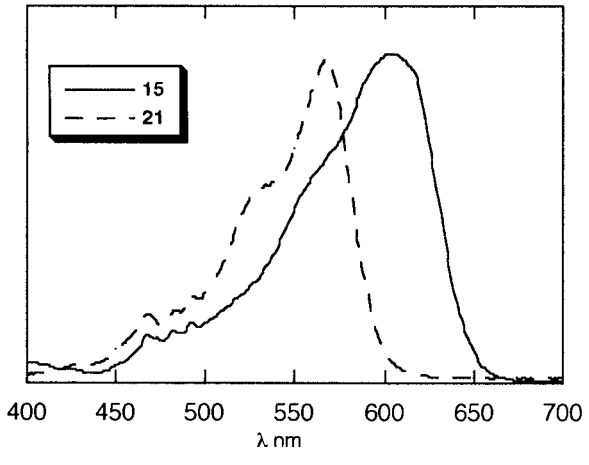


Figure 2. Normalized absorption curve overlay of red shifted and model azines 15 and 21, respectivelyy. Absolute extinctin coefficients can be found in Table 1.

We designed our red shifted azine dyes to ensure that the first excited state would be long lived in order to maximize the likelihood of intersystem crossing. To this end the nitrogen atoms at the 2,3,7, and 8 positions of the azine core were restricted from rotation by their incorporation into tetrahydro-pyrazino rings, thus eliminating the formation of a twisted intramolecular charge transfer complex. In general, this change in geometry of the excited molecule lowers the energy to a point where the excited dye can mix with highly energetic ground states resulting in internal conversion [12] on the picosecond time scale.

By employing a diphenylisobenzofuran bleach method [21], it was determined that upon photoirradiation, dyes **13**, **14**, and **15** generated 23%, 34%, and 36% singlet oxygen, respectively, whereas model azines **20** and **21** only generated 7% and 4% singlet oxygen, respectively. The absolute fluorescence quantum yields, using Cresyl Violet as a standard [22], for rigidized pentacyclic dyes **13**, **14**, and **15** are 38%, 37%, and 40%, respectively. These values are approximately 4 times greater than those for models **20** and **21** which are 6% and 10%, respectively. The increase in fluorescence and singlet oxygen yield are a consequence of designing rigid rings into the pentacyclic azine dyes. Thus, it can be concluded that elimination of

twisted intramolecular charge transfer complex mediated internal conversion in compounds **13**, **14**, and **15** by rigidizing the exocyclic amino groups in tetrahydropyrazino rings is responsible for the remarkable increase in fluorescence and singlet oxygen quantum yields.

Conclusion.

Incorporation of rigid dipyrazino rings into the azine dyes **13**, **14**, and **15** is responsible for the absorption shift from the green to the red, as well as, the markedly enhanced absolute fluorescence and absolute singlet oxygen quantum yields, relative to tricyclic azines **20** and **21**.

EXPERIMENTAL

All reagents were used as received from Aldrich Chemical Co., Fisher Scientific Inc., or Pfaltz and Bauer Inc. All solvents were reagent grade and were used without further purification. Thin layer chromatography was carried out with silica gel (K5F Silica Gel 150 Å) tlc plates. Medium pressure column chromatography was performed at 80 psi using silica gel (32-63 micron). Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. The uv data was collected on a Perkin-Elmer Lambda 5 spectrometer and reported in nanometers. Nuclear magnetic resonance spectra (^{1}H nmr) were recorded on a JEOL 400 MHz spectrometer in deuteriochloroform using tetramethylsilane as an internal standard. Fluorescence quantum yields were determined using a SPEX Fluorolog 1680 spectrofluorometer. Elemental analyses were performed at the University of New Hampshire Instrumentation center.

6-Nitroquinoxaline (**7**).

The following adaptations to a procedure reported for the macrocyclization of tetraamines [18] was employed to obtain 6-nitroquinoxaline in good yield. 4-Nitro-1,2-phenylenediamine (12.0 g, 78.4 mmoles) and glyoxal (40%, 25 ml, 207 mmoles) in acetonitrile (250 ml) were stirred at 50° for 12 hours. The reaction mixture was cooled to room temperature and then diluted with water (100 ml) to precipitate a brown solid to yield 8.75 g (62%) of 6-nitroquinoxaline, mp 175-176° (lit [23] 177-179°).

N,N-Diethyl-1,2,3,4-tetrahydro-6-nitroquinoxaline (**8**).

A procedure reported for the reductive alkylation of quinoxaline [19] (method C) gave a solid whose components were separated by medium pressure chromatography eluting with a gradient of dichloromethane:methanol (1-4% of methanol by volume) to give 3.10 g (92%) of *N,N*-diethyl-1,2,3,4-tetrahydro-6-nitroquinoxaline, mp 59-60°; ^{1}H nmr: δ 1.20 (t, 7.1 Hz, 3H, CH_3), 1.21 (t, 7.1 Hz, 3H, CH_3), 3.28-3.30 (m, 2H), 3.39 (q, 7.1 Hz, 2H, CH_2), 3.44 (q, 7.1 Hz, 2H, CH_2), 3.50-3.55 (m, 2H), 6.43 (d, 9.2 Hz, 1H, phenyl protons), 7.38 (d, 2.2 Hz, 1H, phenyl protons), 7.67 (dd, 9.2, 2.2 Hz, 1H, phenyl protons).

Anal. Calcd. for $C_{12}H_{17}N_3O_2$: C, 61.24; H, 7.28; N, 17.86. Found: C, 61.31; H, 7.44; N, 17.91.

1,4,8,11-Tetraethyl-1,2,3,4,8,9,10,11-octahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazine (**12**).

N,N-Diethyl-1,2,3,4-tetrahydro-6-nitroquinoxaline (**8**) (8.0 g, 34.0 mmoles) and palladium on carbon (catalytic, 5%) in ethanol (300 ml) in a heavy walled Parr bottle were shaken under an atmosphere of hydrogen (50 psi) until the red color of the reaction mixture disappeared. The catalyst was removed by filtration through Celite. The filtrate was then warmed to 50° and maintained at that temperature for two hours while a continuous stream of air was passed through the warmed filtrate. The solution was then cooled to 0° and then treated with methanesulfonyl chloride (2.64 ml, 34.0 mmoles). The solution was stirred at 0° until thin layer chromatographic analysis, eluting with dichloromethane:-methanol, (20:1, v/v) showed one spot. The solution was heated at reflux temperature for 1 hour, cooled to room temperature, treated with water (300 ml) and then made basic with 1 *M* sodium hydroxide. The mixture was extracted with ether (5 x 40 ml) and the combined organic layers were dried over anhydrous sodium sulfate. The components of the reaction mixture were separated by medium pressure chromatography eluting with a gradient of dichloromethane:methanol, (1-4% methanol by volume) and then further purified by recrystallization from a mixture of dichloromethane and ethyl acetate (1:1, v/v) to give 1.57 g (23%) of 1,4,8,11-tetraethyl-1,2,3,4,8,9,10,11-octahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazine, mp >300°; uv: (ethanol:acetic acid, (250:1, v/v)) λ max 600 nm (ε 50,119); ^{1}H nmr: δ 1.28 (t, 7.1 Hz, 12 H, CH_3), 3.47 (s, 8H, CH_2), 3.53 (q, 7.1 Hz, 8H, CH_2), 6.88 (s, 4H, phenyl protons).

Anal. Calcd. for $C_{24}H_{32}N_6$: C, 71.25; H, 7.97, N, 20.77. Found: C, 71.33; H, 8.11; N, 20.78.

1,4,8,11-Tetraethyl-2,3,4,8,9,10,11,12,13-octahydro-13-methyldipyrazino[2,3-*b*:2',3'-*i*]phenazinium Iodide (**14**).

1,4,8,11-Tetraethyl-1,2,3,4,8,9,10,11-octahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazine (**12**) (0.36 g, 0.89 mmole) and methyl iodide (0.84 ml, 8.90 mmoles) were heated in a stainless steel bomb to 130° for 1/2 hour. The bomb was cooled to room temperature and its contents were separated by medium pressure chromatography eluting with a gradient of dichloromethane:methanol (1-4% methanol by volume) and then further purified by recrystallization from a mixture of dichloromethane and ethyl acetate (1:1, v/v) to give 0.24 g (44%) of 1,4,8,11-tetraethyl-2,3,4,8,9,10,11,12,13-octahydro-13-methyldipyrazino[2,3-*b*:2',3'-*i*]phenazinium iodide, mp 254-245°; uv: (ethanol) ν max 607 nm (ε 75,300); ^{1}H nmr: δ 1.28 (t, 7.1 Hz, 6H, CH_3), 1.40 (t, 7.1 Hz, 6H, CH_3), 3.46-3.48 (m, 4H), 3.53 (q, 7.1 Hz, 4H, CH_2), 3.67-3.70 (m, 4H), 3.85 (q, 7.1 Hz, 4H, CH_2), 4.56 (s, 3H, CH_3), 6.84 (s, 2H, phenyl protons), 6.89 (s, 2H, phenyl protons).

Anal. Calcd. for $C_{25}H_{35}IN_6$: C, 54.95; H, 6.46; N, 15.38. Found: C, 55.14; H, 6.52; N, 15.32.

1,4,8,11,13-Pentaethyl-2,3,4,8,9,10,11,12,13-octahydrodipyrazino[2,3-*b*:2',3'-*i*]phenazinium Iodide (**15**).

1,4,8,11-Tetraethyl-1,2,3,4,8,9,10,11-octahydrodipyrazino-[2,3-*b*:2',3'-*i*]phenazine (**12**) (0.50 g, 1.24 mmoles) and ethyl iodide (1.01 ml, 12.4 mmoles) were heated in a stainless steel bomb to 130° for 1 hour and then cooled to room temperature. The contents were separated by medium pressure chromatography eluting with a gradient of dichloromethane:methanol (1-4% methanol by volume) and then further purified by recrystallization from a mixture of dichloromethane and ethyl acetate (1:1, v/v) to give 0.19 g (27%) of 1,4,8,11,13-pentaethyl-2,3,4,8,9,10,11,12,13-octahydrodipyrazino[2,3-*b*:

2',3'-*i*]phenazinium iodide, mp 233-235° dec; uv: (ethanol) λ max 606 nm (ε 58,400); ^{1}H nmr δ 1.28 (t, 7.1 Hz, 6H, CH_3), 1.39 (t, 7.1 Hz, 6H, CH_3), 1.67 (t, 7.3 Hz, 3H, CH_3), 3.47-3.50 (m, 4H), 3.53 (q, 7.1 Hz, 4H, CH_2), 3.68-3.71 (m, 4H), 3.85 (q, 7.1 Hz, 4H, CH_2), 5.19 (q, 7.3 Hz, 2H, CH_2), 6.78 (s, 2H, phenyl protons), 6.90 (s, 2H, phenyl protons).

Anal. Calcd. for $C_{26}H_{37}IN_6$: C, 55.71; H, 6.65; N, 14.99. Found: C, 55.61; H, 7.04; N, 14.86.

3,7-Bis(diethylamino)phenazinium Chloride (**20**).

An aqueous solution (50 ml) of *p*-phenylenediamine (0.5 g, 2.89 mmoles) was added dropwise to an ice cold methanolic solution (30 ml) of sulfonamide **18** [20] (0.7 g, 2.89 mmoles) and a saturated solution of potassium dichromate in water (1 ml). The mixture was then heated at reflux temperature for 15 minutes and then cooled to 0°, and then diluted with water (100 ml). The aqueous mixture was acidified with 1 *M* hydrochloric acid and then extracted with dichloromethane (3 x 30 ml). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated to give a solid that was purified by recrystallization from a mixture of dichloromethane and ethyl acetate (1:1, v/v) to give 0.24 g (22%) of 3,7-bis(diethylamino)phenazinium chloride, mp 142-143°; uv: (ethanol) λ max 564 nm (ε 51,200); ^{1}H nmr: δ 1.34 (t, 7.1 Hz, 12H, CH_3), 1.83 (bs, 1H, NH), 3.61 (q, 7.1 Hz, 8H, CH_2), 7.23 (dd, 9.8, 2.6 Hz, 2H, phenyl protons), 7.38 (d, 2.6 Hz, 2H, phenyl protons), 7.83 (d, 9.8 Hz, 2H, phenyl protons).

Anal. Calcd. for $C_{20}H_{27}ClN_4 \cdot H_2O$: C, 63.73; H, 7.75; N, 14.86. Found: C, 64.03; H, 8.00; N, 14.51.

3,7-Bis(diethylamino)-5-ethylphenazinium Iodide (**21**).

A mixture of 3,7-bis(diethylamino)phenazinium chloride (0.20 g, 0.558 mmoles), sodium acetate (1.83 g, 22.34 mmoles), and ethyl iodide (3.12 ml, 39.1 mmoles) were heated in a stainless steel bomb to 130° for 1 hour. The reaction mixture was then cooled to room temperature and the components were separated by medium pressure chromatography eluting with a gradient of dichloromethane:methanol (1-4% methanol by volume) and then further purified by recrystallization from a mixture of dichloromethane and ethyl acetate (1:1, v/v) to give 0.15 g (57%) of 3,7-bis(diethylamino)-5-ethylphenazinium iodide, mp 289-291°; uv: (ethanol) λ max 569 nm (ε 77,800); ^{1}H nmr: δ 1.39 (t, 7.1 Hz, 12H, CH_3), 1.66 (t, 7.3 Hz, 3H, CH_3), 3.78 (q, 7.1 Hz, 8H, CH_2), 5.19 (q, 7.3 Hz, 2H, CH_2), 6.81 (d, 2.2 Hz, 2H, phenyl protons), 7.27 (dd, 10.0, 2.2 Hz, 2H, phenyl protons), 7.91 (d, 10.0 Hz, 2H, phenyl protons).

Anal. Calcd. for $C_{22}H_{31}IN_4$: C, 55.23; H, 6.53; N, 11.71. Found: C, 54.88; H, 6.32; N, 11.63.

Computational Methods.

The MNDO semiempirical calculations were carried out using the MOPAC 93.00 [15] program package which were run on a Power Macintosh 7500/100 computer.

Acknowledgments.

We thank The Rowland Research Institute for Science for funding this work. We also thank Donald Rogers and Robert Newton for help with the lasers, Dr. Peter J. Connolly for help with the nmr spectrometer, and Nancy D. Kackley and Joseph Fitzgerald for their expert assistance.

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