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ORIGINAL PAPER

5,9-Diaminodibenzo[a,j]phenoxazinium Chloride: A Rediscovered Efficient Long Wavelength Fluorescent Dye

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Abstract We have evaluated the chemical, photophysical and photostability properties of 5,9-diaminodibenzo[a,j] phenoxazinium chloride, **3**, and its bis-5,9-ethylamino analogue, **4**, with the goal of determining if they have characteristics that are compatible with the requirements of a useful fluorescent probe. In order to gauge the potential utility of these fluorophores in biological and non-biological applications, these data were compared to those obtained for Oxazine 118, **1**, and Cresyl Violet, **2**, two well known fluorescent dyes that differ in molecular structure from the title dye **3** by having two or one fewer benzo moieties fused to a generic oxazine ring structure, respectively. The findings of this investigation show that **3**, as well as bis-ethylamino analogue, **4**, have fluorescent lifetimes, quantum yields and photostabilities that compare favorably with the lower order benchmark fluorophores **1** and **2**. Moreover, both dibenzo dyes have the highly desirable properties of absorbing and emitting further in the red and far red /near infrared spectral region, respectively, than do their less conjugated analogues. Taken together, these results suggest that **3** constitutes an archetype upon which a new class of long wavelength fluororescent reporters might be based.

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Introduction

Many of the seminal advances in modern physical chemistry, biophysics, and molecular biology have been made possible because of the availability of sophisticated extrinsic fluorescent reporter dyes that, with exquisite selectivity and sensitivity, provide the means to measure physical and photophysical phenomena and to observe the location and activity of biomolecules. Although the use of organic dyes as fluorescent probes is not new, it remains a dynamic and growing technology that continues to find new, more demanding applications such as in the fields of single molecule detection and spectroscopy. Currently there is considerable interest in developing efficient, photostable fluorophores that absorb and emit in the red and near infrared (NIR) spectral regions because light of these wavelengths, as compared to shorter ultraviolet, blue and green wavelengths: (1) penetrates tissues to greater depths, (2) minimizes undesirable biomolecular autofluorescent background signals, and (3) is more benign to live cells [1, 2]. Additional benefits associated with the use of red and NIR absorbing fluorophores include expanding the palette of multi-color imaging systems, such as those used in DNA sequencing and intracellular organelle labeling, and increasing the spectral range available for FRET experiments [3].

Members of the oxazinium family of dyes constitute an important group of fluorophors that emit above 600 nm. Oxazine 118, **1**, the archetype of the family, and its benzo analogue Cresyl Violet, **2**, possess chemical and photophysical characteristics that are typical of the class (Fig. 1).

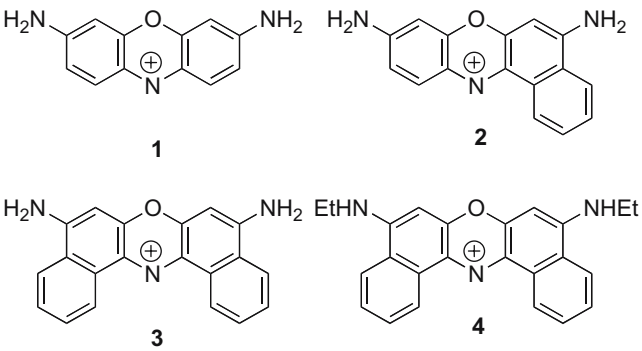

Fig. 1 Molecular structures of pertinent fluorescent dyes. All dyes have an accompanying chloride counter ion that is not shown

They have: (1) high fluorescent quantum yields, (2) low triplet yields, (3) good photostabilities, (4) rigid ring structures that preclude undesirable light-mediated cis–trans conformational changes and (5) amino auxochrome moieties that can be fitted with alkyl groups that concomitantly cause a significant red shift in excitation and emission bands and afford a method for attaching bioconjugation functional groups to the chromophore [4]. Of particular importance to the present investigation is the observation that the added π conjugation accompanying benzo group fusion to **1** to give benzo[a]phenoxazinium dye 2 results in a 25 nm red shift in the wavelength of maximum emission (λ_{max}F). [4] This finding suggests that the addition of an additional benzo moiety to **2** to give 5,9-diaminodibenzo[a,j]phenoxazinium, **3** (Fig. 1), should increase the fluorescence waveband to even longer wavelengths. Surprisingly, in stark contrast to the more than 2,000 literature citations devoted to cataloging the photophysical properties or uses of **1** and **2**, a perusal of the literature found only a single, 100 year old reference to **3**: [5] moreover, a SciFinder® substructure search failed to find any derivatives of this intriguing compound.

The demonstrated advantages of using oxazinium dyes as fluorescent reporters, coupled with the expectation that a dibenzo derivative would extend emission to the far-red spectral region provided the incentive for this investigation. The main purpose of this article, therefore, is to present the results of a study designed to evaluate the chemical and photophysical characteristics of **3**, paying special attention to any deficiencies in these properties that might account for its absence from contemporary usage. We relate these findings to pertinent chemical and photophysical properties of its lower order benzo-analogues, **1** and **2**.

Experimental

Materials and methods 1-Amino-2-naphthol hydrochloride was obtained from Lancaster Synthesis Ltd. Oxazine 118 was synthesized according to the method of Calzaferri et al. [6] Silica gel (32–63 μm particle size) was purchased from Scientific Absorbents, Inc. All other chemicals were purchased from Sigma-Aldrich. Solvents were reagent grade and used as received.

Spectroscopic methods Absorption spectra were recorded using an HP 8453 spectrophotometer; samples were analyzed in capped 1 cm cells. Corrected fluorescence spectra were recorded using a SPEX Fluorolog-2 spectrophotometer; fluorescence quantum yields were measured relative to Cresyl Violet standard [7] at 25 °C and are the average of three measurements. Fluorescence lifetime data was generated using a PicoQuant Fluo Time 100 time-resolved spectrophotometer; a dilute aqueous solution of LUDOX was used to measure the instrument response function. ^{1}H NMR spectra were obtained using a Varian 400 MHz spectrometer using TMS as internal standard; chemical shifts are reported as δ, coupling constants as J (cps) using standard peak splitting terminology. Mass spectral measurements were performed using a Waters Quattro Ultima coupled HPLC/mass spectrometer.

Synthetic and photostability methods

Synthesis of 5,9-diaminodibenzo[a,j]phenoxazinium chloride (**3**). This compound was synthesized using the reaction sequence depicted in Fig. 2 according to the method of Nietzki and Becker [5]. Because this report appeared more than a century ago and was based on the structure of the starting material and combustion analysis data, we verified the structure of **3** using mass spectroscopy and high field NMR analysis. ^{1}H NMR (δppm, DMSO-d_6): 6.95 (s, 2H), 7.86 (ddd, J=8.4, 8.0, 1.2, 2H), 8.01 (dd, J=8.0, 8.0, 2H), 8.49 (d, J=8.0, 2H), 9.12 (dd, J=8.4, 1.2, 2H) and 9.2 (broad, NH_2, 4H). MS (ESI) m/z $C_{20}H_{14}N_3O$ (M+) calc 312; found 312.

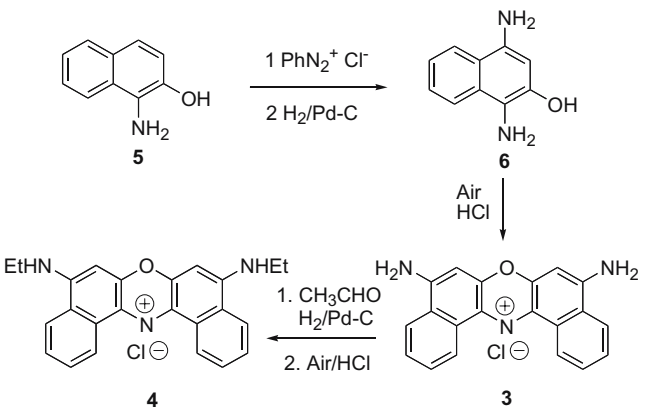

Fig. 2 Synthetic methodology used to prepare **3** and **4**

Synthesis of 5,9-bis(ethylamino)dibenzo[a,j]phenoxazinium chloride (**4**). A mixture of a 100 mg (0.3 mmol) of **3**, 25 mg (0.6 mmol) of acetaldehyde in 50 mL of anhydrous ethanol was hydrogenated using 200 mg of 10% Pd/charcoal catalyst (50 psi, RT). The reaction mixture was filtered through celite to give a light blue solution that rapidly turned a deep blue color upon air oxidation. Tlc analysis showed three blue reaction products that were tentatively identified by their wavelengths of maximum absorption and their order of appearance during the course of the reaction via tlc analysis to be the mono- di- and triethylated derivatives of **3**. In order to ensure that the reaction products were chloride salts, the mixture was treated with two drops of concentrated hydrochloric acid. After removing the solvent and excess hydrochloric acid in vacuo, a portion of the reaction mixture was separated using column chromatography with methanol/methylene chloride (1:10, v/v) as eluent. The predominant component of the mixture was identified by mass spectroscopy and NMR analysis to be title compound **4**. Purity of the sample was verified by tlc and NMR spectroscopy. ^{1}H NMR ($\widetilde{\delta}$ppm, MeOH-d_4): 1.42 (t, J=7.2, CH_3, 6H), 3.45 (q, J=7.2, CH_2, 4H), 6.39 (s, 2H), 7.60 (d, J=8.0, 2H), 7.71 (dd, J=8.0, 7.6, 2H), 7.98 (d, J=8.0, 2H), 8.52 (d, J=8.0, 2H) and 4.89 (s, NH_2 plus MeOH). MS (ESI) m/z $C_{24}H_{22}N_3O$ (M+) calc 368; found 368.

Photostability measurements. In order to determine the relative stabilities of dyes **1**, **2** and **3**, each was dissolved in a solvent mixture consisting of one volume of aqueous porcine gelatin (3% by weight) and one volume of methanol; the inclusion of gelatin was to partially emulate a biomolecular environment while the role of the methanol was to minimize chromophore dimerization which could possibly skew the results. The solution was placed in a capped 1 cm standard cuvette (optical density (OD) ≈ 1.5 at wavelength of maximum absorption (λ_{max}A) for each dye) and placed in a cell holder. One face of the cuvette was subjected to the unfiltered, focused beam emanating from a slide projector (Polaroid 610 having an irradiance of ca. 20 mW per 100 nm wide band). The OD at the λ_{max}A of each solution was measured before illumination and every 30 min thereafter until the OD decreased by a minimum of 10% (typically 1.5 h).

Apparent pKa (pKa′) Measurements. pKa′ values were determined using a titration method wherein the OD of the dye was observed in the pH range of 4–12 using dilute potassium hydroxide as titrant. Because of concerns that the deprotonated, neutral form of the dye would be insoluble in pure water, which would invalidate the experiment, the measurements were performed in a water/ethanol (equal volumes) mixture using an electrode that had been stabilized in a solution of pH=7.0 buffer/ethanol (equal volumes). Results are the average of two determinations.

Results and discussion

In 1896 Kehrmann and Hertz reported that colorless aqueous solution of 1,4-diamino-2-naphthol hydrochloride rapidly turned violet in the presence of air [8]. Ten years later, Nietzki and Becker isolated this oxidation product as a blue crystalline solid that fluoresces red in solution which together account for the apparent violet color of the dye in solution; moreover, such an observation is suggestive of *intense* far-red emission. On the basis of color, the molecular structure of the dye's precursor and elemental analysis data, Nietzki and Becker deduced the compound to be 5,9-diaminodibenzo[a,j]phenoxazinium chloride, **3** [5]. To the best of our knowledge this dye has never again been mentioned in searchable scientific or patent literature. In light of renewed interest in developing efficient fluorophores that absorb and emit red and near infrared photons, we have resurrected this promising chromophore and compared its chemical and physical properties with those of benchmark oxazine analogs **1** and **2** with the goal of determining if it has the potential to emulates these dyes in serving as a generic template upon which new fluorophores might be derived.

In order for a fluorophor to be of practical value, it should be easily synthesized and purified. Such is the case for dibenzophenoxazinium **3**, which we prepared using the sequence of reactions outlined in Fig. 2; the dye is formed cleanly and can readily be purified by crystallization from ethanol. We also demonstrated that it is a relatively simple matter to append alkyl groups to the pendant amino-auxochrome N atoms by synthesizing the symmetrically substitutes diethyl derivative **4**, which also serves as a model for the future attachment of one or two bioconjugating moieties to the chromophore if warranted by the results of the present study (Fig. 2).

A comparison of the molecular structure of **3** to that of **1** and **2** shows that it is distinguished from the latter two dyes by the presence of one or two benzo moieties fused to the [a] and/or [j] positions of the generic oxazine ring system of **1** (Fig. 1). As expected [9], the increased π conjugation that accompanies this sequential addition is mirrored by a significant bathochromic shift in both the absorption and emission spectra for **3** relative to the two lower order members of the series and thus constitutes an improvement in this desirable characteristic (Fig. 3). Furthermore, because the position of the long wavelength band of donor-acceptor dyes depends on the ionization potential of the amino auxochrome group, [10] we predicted, and indeed found, that the substitution of the amino moieties of **3** by ethylamino groups to give **4** augments the shift in the absorption and emission bands even further to the far red and NIR spectral regions, respectively (Fig. 3).

Physical and photophysical data for the oxazine derivatives of the present study are summarized in Table 1.

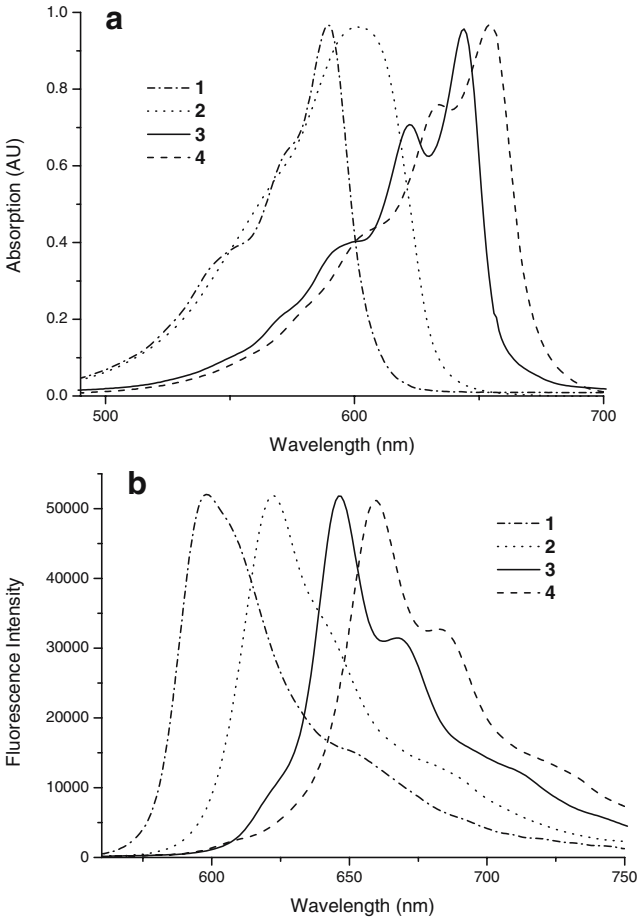

Fig. 3 Normalized absorption (**a**) and emission (**b**) spectra of oxazinium analogues in ethanol

Absorption maxima and fluorescence maxima values were recorded in ethanol rather than water in order to preclude dimer or possibly higher order aggregate formation that could affect these data. The small Stoke's shifts and large overlap of the absorption and fluorescence spectra observed for the symmetrical dyes of the series, **1**, **3** and **4**, are consistent with negligible differences in molecular structure between the ground and first excited singlet state species. All of the dyes are efficient absorbers of light with extinction coefficients greater than 80,000 L/mol−cm. Especially pertinent to this work was the finding that dibenzo-derivatives **3** and **4** have high fluorescence quantum yields that rival those of **1** and **2**, with the modest decrease observed for the former pair attributable to the decreased energy gap of longer wavelength absorbers (energy gap law [11]).

Drexhage et al. have found that the rates of internal conversion of oxazinium dyes substituted with primary, secondary but not tertiary amino-donor groups can significantly be diminished if fluorescence measurements are conducted in ethanol-OD rather than ethanol-OH as evidenced by greatly increased fluorescent quantum yields [7]. Not surprisingly, we observed a large increase in the fluorescent lifetimes of **1**, **2** and **3** when we used the same technique to exchange the hydrogen atoms of the amino-auxochrome groups of the series with deuterium (Table 1); thus, provided deuterium substitution affects only the nonradiative rate constant, the fluorescent quantum yield for **3** is calculated to increase from 0.53 to 0.84 in the deuterated solvent. This finding provides further support for the suggestion that N–H stretching modes are the predominant nonradiative mechanism operative in this class of dyes [7] which we now extend to include dibenzo-derivatives.

Fluorescent lifetime measurements performed in water generally gave multi-exponential fits for the data for all four dyes of this study if we used solutions that were too concentrated whereas single exponential behavior was routinely observed for the series when organic solvents were used (only data obtained in ethanol shown). This discrepancy suggests that the flat planar structures of even the smallest of the oxazine dyes are prone to dimer or high order aggregation formation in aqueous media. Nonetheless, when a single exponential did fit the data, the lifetime of **3** (2.1 ns) (Table 1), which most likely is associated with a monomeric species, compares favorably to lifetimes of a series of commercially available red emitters recently measured in water by the Sauer group (representative listing: Bodipy 630/650—3.9 ns; DY650—1.9 ns; ATTO 665—0.6 ns; Alexa 647—1.0 ns; Cy5—0.9 ns) [1]. As a consequence, at least in the relatively benign environment afforded by water, we expect that a bioconjugated version

Table 1 Physical and photophysical properties of pertinent dyes

Dye	**1**	**2**	**3**	**4**
λ_{abs} (nm)[a]	589	601	644	654
log ε	4.92[f]	4.92[f]	4.99	4.91
λ_{fl} (nm)[b]	598	622	646	660
Φ_{fl}^{c}	0.61	0.56	0.53	0.55
τ_{EtOH} (ns)[d]	3.1	3.2	3.1	3.4
τ_{EtOD} (ns)	4.9	5.0	5.0	ND[g]
τ_{HOH} (ns)	2.4	2.1	2.2	ND
pKa′[e]	9.8	9.1	9.8	ND

[a] Absorption maximum in ethanol containing 0.1% acetic acid. [b] Fluorescence maximum in ethanol containing 0.1% acetic acid. [c] Absolute fluorescence quantum yield relative to **2** [7]. [d] Fluorescence lifetime in denoted solvent. [e] Apparent pKa in ethanol/water (1:1, v/v). [f] Literature value in methanol [6, 13]. [g] Not determined.

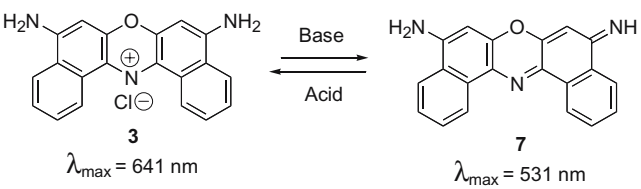

Fig. 4 Change in molecular structure and wavelength of maximum absorption accompanying protonation/deprotonation reactions of **3**

of **3**, wherein dimer formation is precluded, has the potential to have a fluorescence efficiency that is as good or better than most of these commercial fluorophores.

Inspection of the oxazinum dyes studied herein shows they possess a delocalized positive charge that can be neutralized by the removal of a proton from one of the pendant amino auxochrome groups; typical pKa values for this reaction fall in the 9.5–11 range,[12] depending on the exact structure of the dye and upon the solvent system used for the measurement. A consequence of this chemistry is that, as shown in Fig. 4, the resulting imino dye, **7**, has an absorption band that is shifted approximately 100 nm to the blue of its oxazinium precursor and therefore no longer functions as a red absorber. To ensure that dibenzo-oxaziniums are not susceptible to a deprotonation reaction at unusually low pH values (i.e. pKa′s in the 4–7 range) that would render them of little use in physiologically relevant environments, we studied the behavior of dyes **1**, **2** and **3** as a function of pH in aqueous ethanol. The results of these experiments were unremarkable and showed that the pKa' values found for **3** fall in the same range as those measured for **1** and **2**, and that therefore **3** is expected to primarily remain in its protonated, red-absorbing form in commonly encountered biological environments. We note that diethyl-analogue **4** exhibited spectral features suggestive of aggregate formation in the solvent system used for these experiments and was therefore excluded from this study; anecdotally, a crude pKa′ measurement of **4** placed it in the same range as the other dyes of the study.

Oxazinium fluorophores are reputed to be highly resistant to photodegradation processes when used as the active medium in dye lasers [4]. While this might portend well for sundry non-biological uses of the dibenzo dyes, it is not necessarily relevant to biological applications because the complex components that make up such systems tend to be more reactive than are the pure solvent systems usually employed in non-biological experiments. The best test for determining the suitability of any type of fluorophore for biological applications is to study its photostability while conjugated to a variety biomolecules. Since such an enterprise was beyond the scope of the present study, we initially attempted to evaluate the relative photostabilities of dyes **1**, **2** and **3** in aqueous gelatin with the goal of emulating the more stringent conditions of a biological environment. However, aggregation of the dyes in this medium forced us to compromise on solvent selection for this study and, we instead, used a solvent composed of equal volumes of aqueous gelatin and methanol; **4** showed signs of aggregate formation in this co-solvent and was therefore excluded from this aspect of the investigation. Photostability measurements were performed by measuring the bleaching rates of air saturated solutions of the dyes exposed to an unfiltered, focused beam emanating from a slide projector. Since the spirit of this experiment was to identify unusual instability of **3** relative to **1** and **2**, no attempt was made to ensure that each dye absorbed precisely the same number of photons from the broad band light source and therefore the results of this comparative study are qualitative rather than quantitative. The findings of this experiment indicated that all three dyes had similar photostability profiles, each requiring approximately 1.5 h exposure to the intense source to cause a 10% decrease in its OD. Thus, constraints in utility imposed by photostability should be no more limiting for dibenzooxazines than those generally recognized for its better known lower-order oxazine analogues.

Conclusions

We have shown that 5,9-diaminodibenzo[a,j]phenoxazinum chloride, a fluorescent dye devoid of reference in scientific and patent literature for over a century, has chemical, photophysical and photostability characteristics that are similar to those of its better known and valued, lower-order benzo-analogues Oxazine 118 and Cresyl Violet. Most important, this dye has the additional, highly desirable property of efficiently absorbing in the red and fluorescing in the far-red/NIR spectral region. Moreover, by converting the amino moieties of **3** to ethylamino groups, we have demonstrated the feasiblity of attaching one or two reactive bioconjugating groups to the chromophore while concomitantly extending the wavelengths of absorption and emission even further toward the red and NIR spectral region, respectively. The results of this preliminary investigation suggest that this long ignored chromophore deserves to be developed for its own right and for its potential to become a template upon which future red/NIR reporter fluorophores might be based.

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