2,7-Diaminobenzopyrylium Dyes Are Live-Cell Mitochondrial Stains

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ABSTRACT: Small-molecule fluorescent stains enable the imaging of cellular structures without the need for genetic manipulation. Here, we introduce 2,7-diaminobenzopyrylium (DAB) dyes as live-cell mitochondrial stains excited with violet light. This amalgam of the coumarin and rhodamine fluorophore structures yields dyes with high photostability and tunable spectral properties.

KEYWORDS: fluorescent probes, cellular imaging, chemical biology, organic chemistry, mitochondria, coumarin, benzopyrylium

INTRODUCTION

Fluorescence microscopy is an essential tool to interrogate biological structure. A key element in any imaging experiment is the labeling strategy used to localize a fluorophore to the cellular component of interest. In addition to measuring the position and movement of specific biomolecules, cellular imaging experiments often involve the visualization of different organelles to quantify their dynamics or provide useful subcellular reference marks. Fusing a fluorescent protein to a targeting motif can allow labeling of cellular organelles but requires expression of an exogenous molecule. Fluorescent reagents with affinities for organelle-specific biomolecules can allow imaging without genetic manipulation but typically involve preparation of a small-molecule fluorophore conjugated to an antibody or drug. An alternative labeling strategy is the use of fluorescent stains that accumulate in specific organelles due to the different chemical environments found in these distinct subcellular regions. Examples include tertiary amine-containing dyes accumulating in acidic lysosomes or hydrophobic fluorophores partitioning into lipid droplets.

A widely used fluorescent staining strategy involves mitochondria, whose double membrane structure reflects their role as the powerhouse of the cell. The proteins that comprise the electron transport chain reside in the inner mitochondrial membrane that separates the matrix from the intermembrane space. Their activity drives protons across the inner membrane, resulting in a large voltage difference between these two compartments. This unique membrane potential drives the accumulation of lipophilic cations into the matrix or inner membrane. This was first observed with Rhodamine 123 (1), where esterification of the standard ortho-carboxyl group found in rhodamines endows the molecule with a fixed cationic charge. The strategy was expanded to tetramethylrhodamine methyl ester (TMRM, 2), yielding a red-shifted mitochondrial stain. This general idea was refined with the development of MitoTracker Orange (3), in which the carboxyl ester functionality found in rhodamines 1 and 2 is discarded entirely. Compound 3 also incorporates a chloromethyl moiety to allow formation of a glutathione adduct, thereby trapping the fluorophore inside the cell.

The majority of fluorescent mitochondrial stains are based on rhodamine (e.g., 1–3) and cyanine structures. These dyes exhibit relatively long absorption maxima (λ<sub>abs</sub>) and fall into the standard blue (488 nm), green-yellow (560 nm), and red (640 nm) excitation windows used in fluorescence imaging. Mitochondrial stains excited with violet light (405 nm) have received less attention since there is no general cationic dye scaffold with an excitation maximum in this wavelength range. We sought, and now report, a new class of mitochondrial stains based on 2,7-diaminobenzopyrylium (DAB) dyes.

RESULTS AND DISCUSSION

To create a violet-excited mitochondrial stain, we first considered coumarin dyes, which remain the most utilized

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fluorophores excited by ultraviolet (UV) and violet light. The simplest fluorescent coumarins are 7-hydroxy derivatives such as 4-methylumbelliferone (4, Figure 1b). We noted that the classic fluorophore fluorescein (5) is effectively the phenyl-ogous derivative of 4. Similarly, 7-aminocoumarins such as Coumarin 1 (6) and N,N-diethylrhodol (7); 2,7-diaminobenzopyrylium 8 and Rhodamine B (9). Inspired by the structural relationship between compounds 4/5 and 6/7, we considered 2,7-diaminobenzopyrylium (DAB) structures exemplified by the tetraethyl derivative 8; this is the “coumarin-sized” analog of Rhodamine B (9). Although iminocoumarins have received some attention as dyes15 and indicators,16 the cationic 2,7-diaminobenzopyrylium fluorophore scaffold is essentially unexplored, with a lone report in the Soviet chemistry literature from 1989.17 We began our investigation by synthesizing the known tetraethyl DAB dye 8 starting from Coumarin 1 (6, Scheme 1a).17 Treatment with Et3OBF4 generates a 2-ethoxychrome-nylium intermediate that reacts with diethylamine to give 8, which we obtained in 41% yield. Based on the success of this simple synthetic protocol, we prepared additional DAB dyes. We transformed the bright azetidinylcoumarin18 10 into the diazetidinyl dye 11 (Scheme 1b). We also explored dyes derived from Coumarin 102 (12); reaction with diethylamine, azetidine, or piperidine yielded compounds 13−15 (Scheme 1c).

We then evaluated the spectral properties of the DAB dyes 8, 11, and 13−15 in phosphate-buffered saline (PBS; Table 1, Figure 2a−c) comparing them to the parent coumarin fluorophores 6, 10, and 12. In general, the transformation of the carbonyl group into an iminium moiety elicits a bathochromic shift of ~50 nm in λabs and a shift of 25−35 nm in fluorescence emission maxima (λem). Thus, all the DAB dyes exhibit λabs > 400 nm, absorbing in the violet-blue region of the visible spectrum. The reduced shift in λem results in smaller Stokes shifts for the DAB fluorophores relative to the coumarin starting materials. Despite this decrease, the Stokes shifts of the DAB dyes (65−100 nm) remain substantially larger than those of fluoresceins or rhodamines (~25 nm).

Finally, for each matched pair, the DAB congeners show substantially higher photobleaching time constants (tθ) and average number of photons emitted before photobleaching (Np) relative to the corresponding coumarin dye. Comparison of specific dye pairs reveal more nuanced differences. The transformation of Coumarin 1 (6) to DAB dye 8 yields a shift of 45 nm in λabs and 26 nm in λem resulting in a Stokes shift of 65 nm (Table 1, Figure 2a). Conversion of coumarin 10 into diazetidinyl DAB derivative 11 results in a bathochromic shift of 48 nm in λabs and a difference of 34 nm in λem (Table 1, Figure 2b). This larger shift in emission maximum combined with the properties of the parent coumarin 10 gives a Stokes shift of 99 nm for 11. For DAB
Dyes 13–15 derived from Coumarin 102 (12), the $\lambda_{\text{abs}}$ does not depend on the different secondary amine auxochromes (Table 1, Figure 2c). The fluorescence emission does vary with structure; the acyclic $\text{N}_2\text{N}$-diethylamino derivative 13 exhibits $\lambda_{\text{em}} = 513$ nm, which is 10 nm shorter than 14 and 15, resulting in a smaller Stokes shift of 68 nm. Across the series, we found that the absorbivity of the DAB dyes is substantially higher than the parent coumarin resulting in a smaller Stokes shift of 68 nm.

Figure 2. Spectra and photobleaching of representative DAB dyes. (a–c) Normalized absorption (abs) and fluorescence emission (f) of (a) 6 and 8, (b) 10 and 11, and (c) 12–15. (d–f) Plot of normalized fluorescence vs irradiation time of (d) 6 and 8, (e) 10 and 11, and (f) 12–15 with monexponential fit.

DAB compounds showing consistent $t_b$ values that are 5-fold higher than the parent coumarin 12 (Table 1, Figure 2d). Since photobleaching reactions stem from excited states, it is difficult to compare photobleaching time constants between dyes with different fluorescence quantum yields and lifetimes; this is reflected in the different bleaching rates observed across the various dye types (Table 1, Figure 2d–f). We therefore calculated the average total photons ($N_{\text{ph}}$) emitted by each dye. This parameter highlights the increased photostability of the DAB fluorophores and revealed remarkably consistent photostability for compounds 11 and 13–15 with an average of $\sim$8300 photons/dye (Table 1).

We then evaluated the stability of the iminium linkage in aqueous solution under “dark” conditions and under illumination with violet (405 nm) light. Monitoring with tandem liquid chromatography–mass spectrometry (LC–MS) revealed that all the DAB dyes (8, 11, and 13–15) show excellent stability with minimal iminium hydrolysis after 48 h at pH 7.4 in the absence of light (Figure 3a,b, Figure S1). We also evaluated the stability of dye 15 in different buffer conditions, observing modestly higher rates of hydrolysis at elevated pH or in cell culture media containing serum (Figure S2). We then undertook a comprehensive photochemistry study of 15 using 405 nm illumination and analysis by LC–MS (Figure 3c,d, Figure S3) The photochemical reactions of 15

Figure 3. Stability of DAB dye 15. (a) Spontaneous hydrolysis of 15 to form coumarin 12. (b) LC–MS chromatograms of 15 at $t = 0$ (top), 15 at $t = 48$ h (middle), and standard 12 (bottom) in PBS, pH 7.4. (c) Photochemistry of 15 to form oxidized adducts 16–18. (d) LC–MS chromatograms in PBS, pH 7.4, of 15 in the absence of light (top), 15 after 5 min illumination with 405 nm light (middle), and 15 after 20 min illumination with 405 nm light (bottom).
are similar to those observed with parent coumarin 12 where oxidation appears to be centered on the julolidine ring system,\textsuperscript{20,21} we did not detect any photochemically driven oxidation on the piperidine ring. Overall, these data show that the DAB dyes exhibit reasonable chemical stability and the iminium motif is not susceptible to photochemical degradation.

Given the propensity of cationic rhodamines to accumulate in the mitochondria (Figure 1a), we investigated the similarly charged DAB dyes as live-cell mitochondrial stains. We incubated live U2OS cells with dyes 8, 11, and 13–15 at 200 nM and co-stained the mitochondria either using MitoTracker Deep Red (Figure 4) or by transient transfection of HaloTag–TOMM20 and staining with the far-red fluorogenic label Janelia Fluor 635-HaloTag ligand\textsuperscript{22} (Figure S4). In both experiments, we observed similar staining patterns between the DAB dyes and the established far-red stain or genetically encoded label, confirming our hypothesis that this positively charged fluorophore scaffold would accumulate in mitochondria. Imaging using the same settings revealed that the julolidine-containing derivatives 13–15 showed brighter staining, perhaps due to increased lipophilicity of the compact cationic structure.

In these cellular imaging experiments, we noted that the cellular intensity of even the best DAB mitochondrial stain 15 rapidly decreased upon media exchange. To further improve this reagent, we prepared a dimer derivative of this molecule by reacting Coumarin 102 (12) with dipiperidine 19 to yield “diDAB” 20 (Figure 5a). This design is predicated on two concepts. First, the relatively long Stokes shift of the parent dye 15 (Table 1, Figure 2c) should minimize FRET between the two fluorophore units and preserve fluorescence quantum yield. Second, the presence of two cationic moieties per molecule of 20 should improve mitochondrial retention. Examination of the chemical properties of 20 revealed a slightly higher rate of hydrolysis relative to 15 in different pH conditions even when considering the presence of two iminium groups (Figure S5).

We then measured the spectral properties of the dye dimer. Compound 20 exhibited similar absorption (\(\lambda_{\text{abs}} = 447 \text{ nm}\)) and fluorescence emission (\(\lambda_{\text{em}} = 524 \text{ nm}\)) spectra compared to DAB monomer 15 (Figure 5b); both dyes showed a linear relationship between absorption and concentration \(\leq 20 \mu\text{M}\) (Figure S6). In aqueous solution, the diDAB 20 did not show the expected 2-fold increase in absorptivity but exhibited \(\epsilon = 45 \text{ 200 M}^{-1} \text{ cm}^{-1}\) along with a modestly lower \(\Phi_f = 0.45\) (Table S1). To reconcile this observation and mimic the

![Figure 4. DAB dyes stain mitochondria. Chemical structures of DAB dyes 8, 11, and 13–15 and corresponding fluorescence images of U2OS cells co-stained with the DAB dye (200 nM) and MitoTracker Deep Red (100 nM). Scale bar, 10 \(\mu\text{m}\).](https://doi.org/10.1021/acsbiomedchemau.1c00068)

![Figure 5. Dimerization of DAB dye 15 improves performance as a mitochondrial stain. (a) Synthesis of diDAB 20. (b) Normalized absorption (abs) and fluorescence emission (fl) of 15 and 20. (c) Plot of cellular fluorescence vs time of cells incubated with 15, 20, or MitoView 405 during photobleaching; error bars indicate SEM. (d) Live U2OS cells incubated with DAB 15 (200 nM), diDAB 20 (200 nM), or MitoView 405 (100 nM) after 0, 1, or 2 dye-free media exchange washes. Scale bar, 10 \(\mu\text{m}\).](https://doi.org/10.1021/acsbiomedchemau.1c00068)
nonpolar environment of the mitochondrial inner membrane, we measured the spectral properties of compounds 15 and 20 in sodium dodecyl sulfate (SDS) micelles and dioxane/water mixtures (Table S1). We observed the expected higher $\varepsilon = 78,900 \text{ M}^{-1} \text{ cm}^{-1}$ in PBS containing 0.1% w/v SDS along with a larger $\Phi_f = 0.83$; compound 20 is also brighter in aqueous dioxane. Although dye 15 also showed higher absorptivity and fluorescence quantum yield in these nonpolar conditions relative to PBS, the effect was less pronounced. We postulate that the relatively low $\varepsilon$ and $\Phi_f$ observed for compound 20 in aqueous solution is due, in part, to intramolecular interactions between the two chromophore units. These interactions could be reduced in the more hydrophobic environment of SDS micelles or dioxane/water mixtures, resulting in higher absorptivity and fluorescence quantum yield.

We then compared the parent DAB 15, diDAB 20, and the commercial violet-excited (and structurally mysterious) MitoView 405 in live-cell experiments. Although MitoView 405 was modestly brighter than DAB 15 or diDAB 20 upon initial application, the dye bleached rapidly in live cells in our hands, preventing acquisition of a full confocal microscopy stack. The DAB compounds exhibited substantially higher resistance to photobleaching with the diDAB 20 showing the best overall photostability (Figure 5c). As expected, all the dyes showed excellent mitochondrial staining upon initial application (Figure 5d). Upon media exchange, however, the DAB 15 signal rapidly decreased whereas the diDAB 20 and MitoView 405 were retained after this cell washing protocol.

**CONCLUSION**

In summary, we demonstrate that the 2,7-diaminobenzopyrylium (DAB) framework is a modular scaffold for the synthesis of mitochondrial stains excited with violet light. These atom-efficient imaging reagents can be prepared from the broad palette of 7-aminocoumarin dyes with different N-substitution patterns (Scheme 1). Although the spectral properties can be tuned by choosing different coumarin starting materials, the structure of the secondary amine reactant has only minor effects on the properties of the resulting DAB dyes: use of azetidine or piperidine gave fluorophores with similar quantum yields and photostability (Table 1). This is in contrast to rhodamine dyes, where azetidine or piperidine auxochromes can elicit 10-fold changes in $\Phi_f$.16 The DAB dyes exhibit higher absorptivity and photostability than their coumarin parent dyes (Table 1, Figure 2), show reasonable chemical stability (Figure 3), and are effective mitochondrial stains (Figure 4); dimerization of the DAB dye affords a stain with better cellular retention (Figure 5). Looking forward, the utility of these bright, photostable, and biocompatible “mini-rhodamines” (Figure 1) could be expanded beyond mitochondrial stains. The stability of the iminium moiety could be improved through intramolecular cyclization to develop new conjugatable fluorescent labels or photolabile groups. The slow rate of hydrolysis could also be tuned and exploited to release coumarin-based drugs.13 Overall, the DAB dyes represent an underutilized chemical scaffold worthy of further attention.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.1c00068.

Chemical stability of dyes in PBS, stability of 15 under different conditions, photochemistry of 15, mitochondrial staining with DAB dyes, comparison of stability of 15 and 20, Beer–Lambert–Bouguer analysis of 15 and 20, spectral properties of 15 and 20 in different solvents, spectroscopy and imaging experimental details, synthetic organic chemistry details, and characterization of novel compounds (PDF).

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**Notes**

The authors declare no competing financial interest.

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