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Benzophenoxazine-based fluorescent dyes for labeling biomolecules

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1. Introduction

Meldola's Blue **1**, Nile Red **2**, and Nile Blue **3** have some desirable attributes as fluorescent probes.[3] Dyes **2** and **3** have reasonably high fluorescence quantum yields in apolar solvents and they fluoresce at reasonably long wavelengths. Nile Red, in particular, fluoresces far more strongly in apolar media than in polar ones, and the fluorescent emission shows a large bathochromic (i.e., red-) shift in polar media, hence it can be used as a probe for environment polarity;[4–6] these characteristics may be attributes for some applications, but limitations for others. None of these dyes are significantly soluble in aqueous media, and their quantum yields are dramatically reduced. One of the big challenges in the production of fluorescent dyes is to produce water-soluble probes that fluoresce strongly in aqueous media, particularly above 600 nm or at even longer wavelengths. Motivation for research in this area is drawn from needs for intracellular, tissue, and whole organism imaging where near-IR dyes are far more conspicuous than ones emitting at 550 nm or less.[7]

The phenoxazine skeleton may be extended by adding fused benzene rings to the *a–c* or *h–j* faces. Benzophenoxazines of this type are 'angular'[1] or 'linear' depending on the orientation of the ring fusion, as illustrated in Figure 1a.

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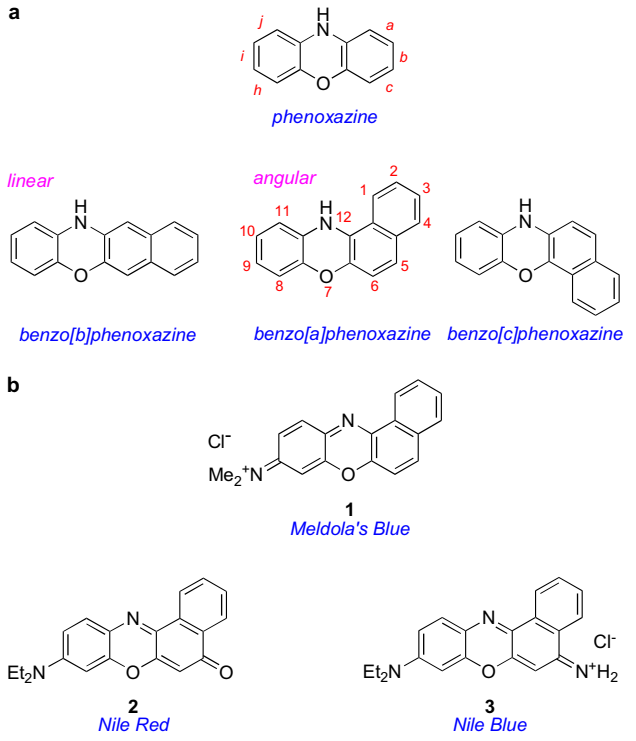


Figure 1. (a) Phenoxazines and benzophenoxazines; (b) structures of the three best known fluorescent dyes in this class.

Substituents that freely donate and/or accept electron density on benzophenoxazine cores can, in some orientations, give fluorescent compounds. The first notably fluorescent compound to be discovered in this class was Meldola's Blue **1**, but Nile Red **2**[2] and Nile Blue **3**[2] are far more frequently used in contemporary science.

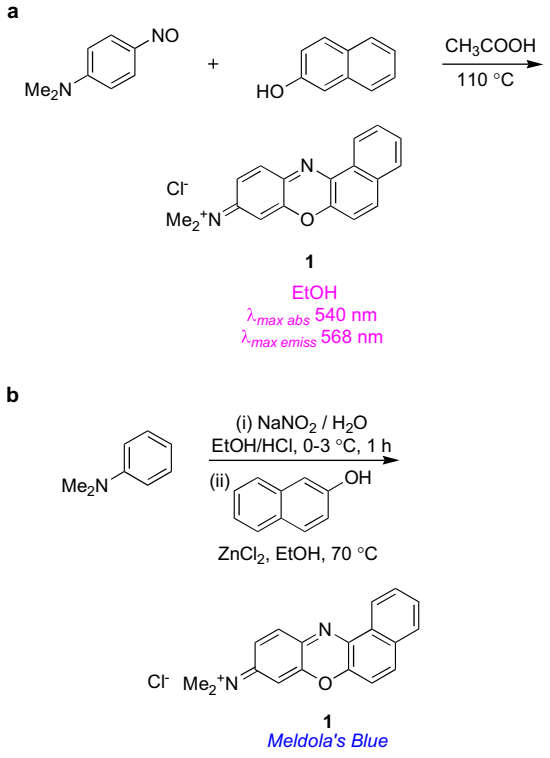
The objective of this review is to summarize the state of the art of benzophenoxazine dyes in such a way that all readers can understand quickly what has been done to develop useful probes in this category. Inspired readers should also be able to use this summary to design research approaches that are not yet explored but would lead to useful endpoints.

2. Meldola's Blue

This dye is used in textiles, paper, and paints, mainly as a pigment. It is not a particularly useful fluorescent dye for labeling proteins because of its poor water solubility. Further, its fluorescence is weak in all common media (e.g., EtOH) and does not give a clear indication of the surrounding polarity. However, Meldola's Blue has been used as a component in redox sensors[16] for detection of materials such as NADH,[8] pyruvates,[9] hydrogen peroxide,[10] glucose,[11] and 3-hydroxybutyrate.[12] It has also been used in electrochemical experiments involving DNA wherein the dye mediates electron transport.[13]

2.1. Syntheses

The original (1879) synthesis of Meldola's Blue involved condensation of a nitroso compound with 2-naphthol at elevated temperatures (Scheme 1a).[14] Details of the reaction conditions and the yield were not given. Subsequently, Meldola's Blue has been made with a variety of counter ions via reactions involving different Lewis acids (Scheme 1b).[15] Counter ion modifications have been used to modulate the electronic properties of solid materials for applications not directly related to labeling biomolecules.



Scheme 1. (a) Original synthesis of Meldola's Blue; (b) a more recent approach.

2.2. Photophysical properties

Phenoxazines without strongly electron withdrawing or donating substituents have unexceptional absorbance characteristics, and they are not particularly fluorescent compounds. Fusion of a benzene ring onto the heterocycle does not alter this situation dramatically. Meldola's Blue has one dimethylamino substituent and the heterocyclic core is oxidized. These changes in the composition of the heterocycle shift its absorption and emission characteristics into a useful range: $\lambda_{max\ abs}$ 540 nm, $\lambda_{max\ emiss}$ 568 nm EtOH. This dye is not noted for its solvatochromic properties.[17]

3. Nile Red derivatives

3.1. Introduction

Nile Red has a neutral oxidized phenoxazine system, i.e., it is a phenoxazinone. The 9-diethylamino substituent is able to donate electron density into the carbonyl group across the ring; this electronic arrangement probably accounts for its highly fluorescent properties (Fig. 2).

The water-solubility of Nile Red is extremely poor, but in other solvents its fluorescence maxima and intensity are good indicators of the dye's environment-polarity. As

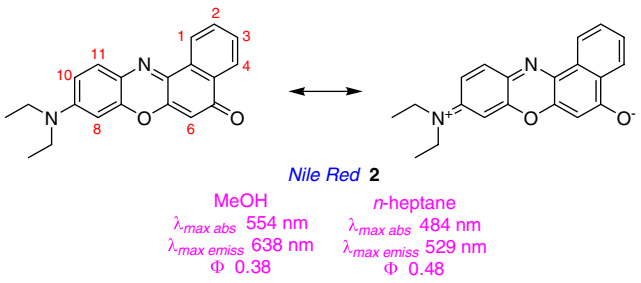
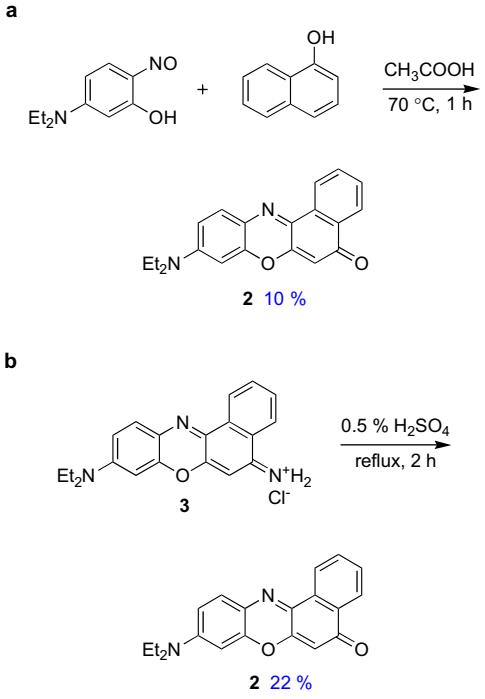


Figure 2. Electron delocalization in Nile Red.

mentioned above, this solvatochromic effect is such that polar media cause a red-shift but decreased fluorescence intensity. This decreased fluorescence intensity is probably due to self-quenching of the dye in face-to-face aggregates. Consequently, this dye is particularly useful for studying lipids and events that involve impregnation of the dye in apolar media. Surprisingly, very few water-soluble analogs of Nile Red have been reported, and only limited fluorescence data have been given for those.

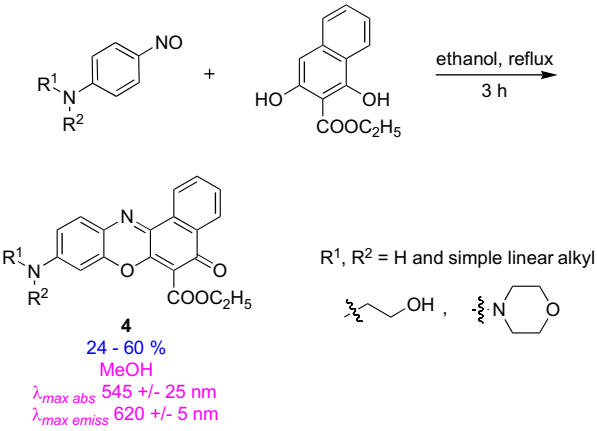
3.2. Syntheses

The first synthesis of Nile Red was a condensation reaction of a nitrosophenol (Scheme 2a). The patent literature indicates that other solvent systems can be used. It is also possible to prepare Nile Red via hydrolysis of Nile Blue as indicated in Scheme 2b. The product is usually isolated via chromatography on silica.



Scheme 2. (a) Original synthesis of Nile Red; (b) synthesis from Nile Blue.

Substituted or modified Nile Red derivatives may be prepared via variations of the syntheses above, i.e., *de novo* methods, or by functionalizing Nile Red itself. For instance, a *de novo* approach was used to prepare the 6-carboxyethyl derivatives **4** (reaction 1). These are potentially interesting since hydrolysis of the ester would give a carboxylic acid for attachment to biomolecules; however, this does not appear to have been attempted yet.[19] The patent literature also describes a synthesis of the 2-carboxy Nile Red derivative.[20]



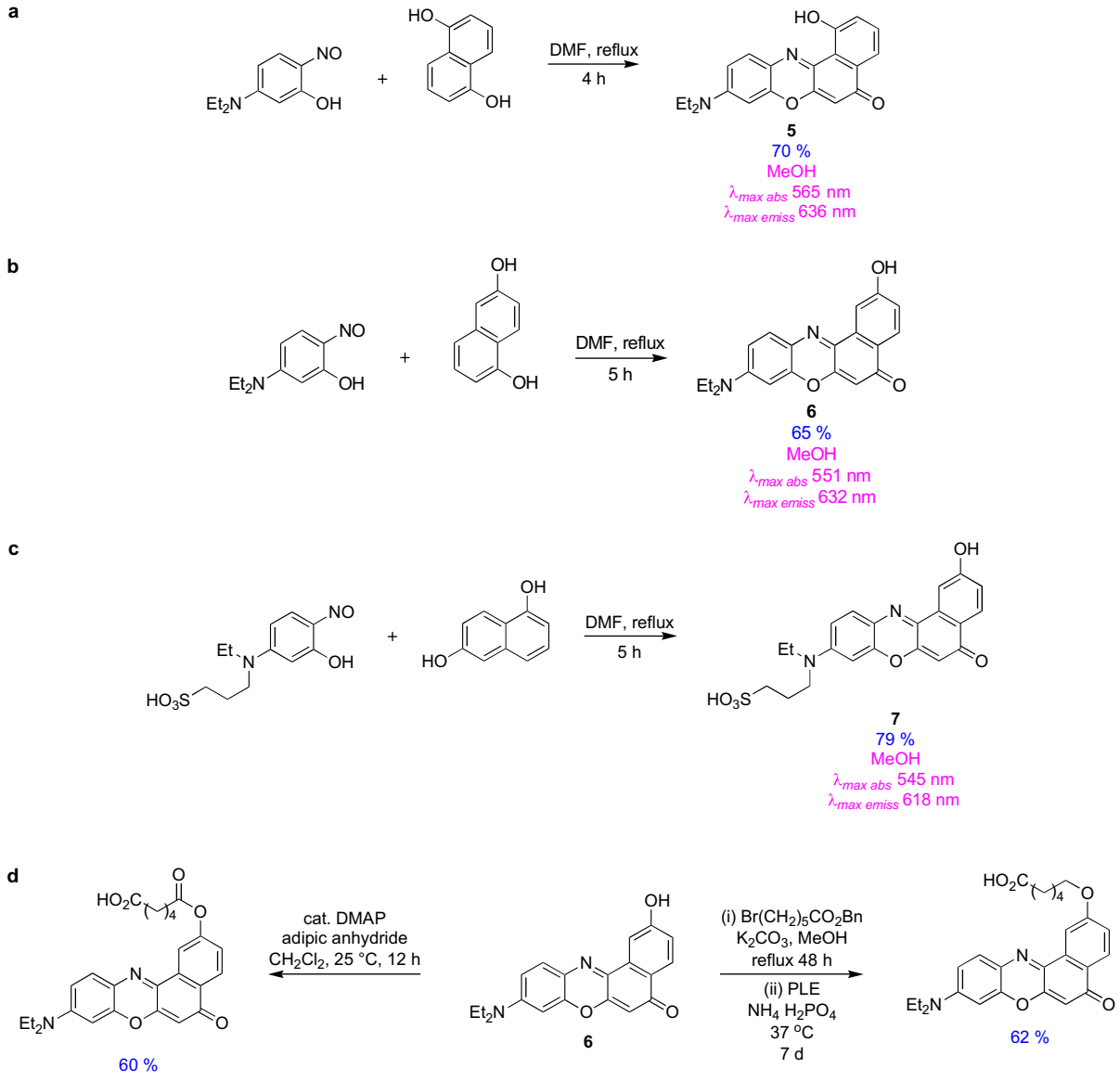
(1)

De novo syntheses of 1- and 2-hydroxy Nile Red, compounds **5** and **6**, respectively, have also been performed, and the products are easily modified via other reactions.[21,6] Thus, Briggs and co-workers at Amersham prepared the parent hydroxy compounds as shown in Scheme 3. Some of the reactions used to derivatize these materials are also shown. In some ways the hydroxyl group of the hydroxy Nile Reds **5** and **6** is an inconvenience because the spectroscopic properties of the dye under physiological conditions become highly pH dependent. However, this phenol is useful as a functional group to incorporate a handle for attachment to biomolecules (Scheme 3d).

A series of fluorinated phenoxazines (not shown) and benzophenoxazines (Scheme 4) have been prepared via sequential S_NAr substitutions reactions of fluorinated aromatics.[22] The 6-fluoro substituent in the compound shown below changes its spectroscopic properties slightly (see below) and, presumably its pH dependence.

The so-called 'FLAsH dyes' feature bisarsenic(3+)-based dye precursors that are non-fluorescent, presumably due to rapid quenching of the excited state via intramolecular energy transfer. However, the disposition of the arsenic dyes is such that they are thought to react with dicysteine units engineered into modified proteins that are expressed within cells. This type of reaction has at least two effects, it: (i) alters the oxidation potential of the arsenic centers and (ii) restricts rotations about the C–As bonds. For whatever reason, structural changes such as this render the dye-protein complex fluorescent. Only proteins within the cell that have the special arrangement of Cys-residues are likely to become labeled in this way, so the approach allows for highly selective visualization of the engineered protein (Fig. 3).

The original FLAsH dyes were fluorescein derivatives.[23] A range of bisarsenic derivatives on different dye skeletons



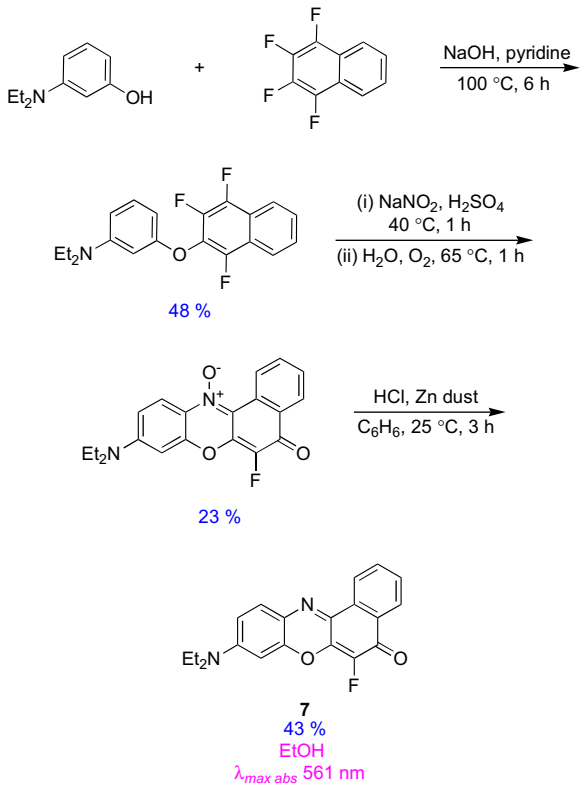
Scheme 3. (a) Synthesis of 1-hydroxy Nile Red; (b) synthesis of 2-hydroxy Nile Red; and (c) some derivatization reactions of 1-hydroxy Nile Red.

has been prepared for evaluation, but only fluorescein and Nile Red derivatives have emerged as useful. This is because several parameters must be controlled tightly if this type of experiment will work. For instance, the As-to-As distance must match the disposition of the target thiols, the ethanedithiol (EDT) concentration in the cell is critical, and the dye must permeate into the cell.

The FLAsH Nile Red derivative **8** was prepared as indicated in Scheme 5. This involves a standard condensation reaction to form the benzophenoxazine core, but without the *N*,*N*-diethyl substituents of Nile Red. These were omitted to allow more space for manipulations at the 6- and 8-positions. FLAsH dye **8** gives less fluorescent enhancement on binding than similar fluorescein dyes, but it emits at a longer wavelength (604 nm) and that, as mentioned before, is a more transparent region of the spectrum for intracellular imaging. Calcium-induced conformational changes of appropriately modified, intracellular calmodulin have been followed using FLAsH dye **8**.[24]

Scheme 6 describes syntheses of two more classical thiol-selective dyes, ones that rely on S_N2 displacement of iodide from iodoacetyl groups.[25] Thus probes **9** and **10** were prepared from a Nile Red derivative and from a 2-hydroxy Nile Red compound, respectively. Curiously, when these were complexed to a particular Cys-residue in maltose binding protein, dye **9** showed a three-fold enhancement of fluorescence, while the emission from **10** was *reduced* by a factor of five. The authors explain this by proposing that **10** is less constrained when bound to protein.

Two interesting questions arise from the work on Nile Red derivatives that is described above. First, would water-soluble derivatives of Nile Red have high quantum yields in aqueous media? As already stated, most Nile Red derivatives do not fluoresce strongly in polar media, but this could be due to aggregation effects that might be avoided if the dye has some intrinsic water solubility. Second, do water-soluble Nile Red derivatives also show the pronounced bathochromic shift in polar media, that is, observed for



Scheme 4. Preparation of 6-fluoro Nile Red.

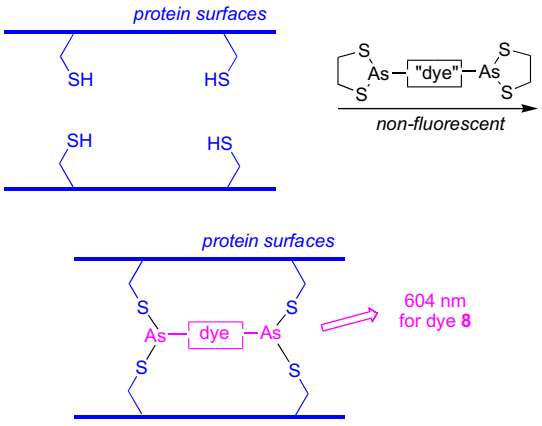
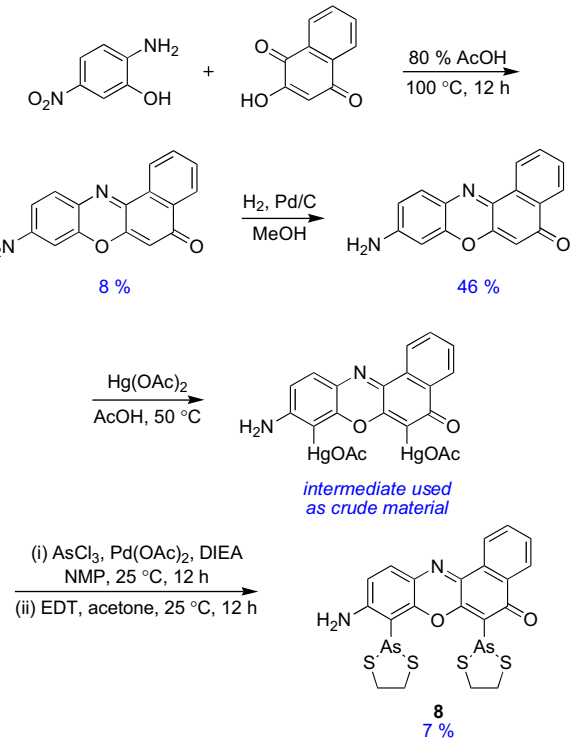


Figure 3. Conceptual basis of fluorescent arsenic dyes.

compounds in the series with little or no significant aqueous solubility?

No significantly water-soluble Nile Red derivatives have been prepared to answer the questions posed above until recent work[18] from our laboratories. Classical condensation routes were used to prepare the three Nile Red derivatives **11**–**13** (Scheme 7). These were designed to be water soluble, but, surprisingly, the diol/phenol **11** was not, even in aqueous base. The other two compounds do have very good water solubilities. Their spectroscopic properties are discussed in the next sub-section; but the data shown in Table 3 show that the answers to both these questions were affirmative for compounds **12** and **13**.

Some researchers may be interested in access to more sophisticated analogs of Nile Red. One obvious approach



Scheme 5. Preparation of the FLAsH system **8**.

would be to prepare iodo-, bromo-, or chloro-substituted compounds then elaborate them via organometallic couplings. There have been reports of attempted halogenation of Nile Red, but the regioselectivity and extent of the halogenations proved hard to control and mixtures were produced.[26–28]

3.3. Spectroscopic properties

Table 1 summarizes spectroscopic data for Nile Red in different solvents, all taken from the same source.[4] These data show decreased fluorescence intensity of Nile Red correlates much more with hydrogen bonding than with solvent polarity, and the magnitude of this difference is best appreciated from the graphical presentation of only the emission wavelengths and intensities that is given in Figure 4. However, the bathochromic shift seems to be a function of solvent polarity, consistent with stabilization of relatively polar excited states.

A similar study featuring emission and absorption maxima, quantum yields, and solvent polarities was performed for

Table 1. Solvent dependency of emission intensities and wavelengths for Nile Red **2**

Solvent	$\lambda_{max\ abs}$ (nm)	$\lambda_{max\ emiss}$ (nm)	Relative fluorescence intensity
Water	591	657	18
EtOH	559	629	355
Acetone	536	608	687
$CHCl_3$	543	595	748
iso-Amyl acetate	517	584	690
Xylene	523	565	685
n-Dodecane	492	531	739
n-Heptane	484	529	585

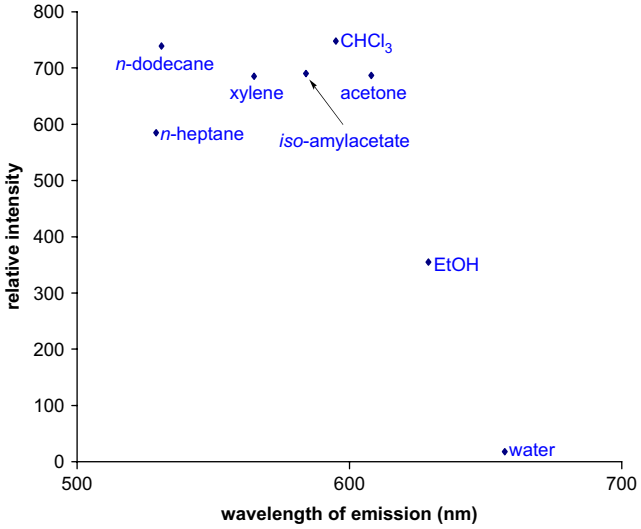


Figure 4. Solvent dependency of emission intensities and wavelengths for Nile Red **2**.

Table 2. Solvent dependency of quantum yield and wavelengths for 2-hydroxy Nile Red **6**

Solvent	$\lambda_{max\ abs}$ (nm)	$\lambda_{max\ emiss}$ (nm)	Quantum yield (Φ)
Cyclohexane	514	528	0.51
Dibutyl ether	514	555	0.66
Toluene	525	568	0.76
Acetone	528	608	0.78
EtOH	544	634	0.58
1,2-Ethanediol	584	655	0.40
CF_3CH_2OH	587	653	0.24
$(CF_3)_2CHOH$	612	670	0.09

2-hydroxy Nile Red **6** (Table 2). Selected data from this study are shown in Figure 5. Just as with Nile Red, polar hydrogen-bonding solvents correlate with reduced quantum yields and significant bathochromic shifts.

Fluorescence lifetimes contribute to two important physical parameters of fluorescent dyes. Dyes with long fluorescent lifetimes can emit strongly because non-radiative processes are relatively slow, and because intersystem crossing to triplet states is less competitive.

Fluorescence lifetimes for Nile Red in different solvents have been measured (Table 3). These data show that the fluorescence lifetime of Nile Red is 3.65 ns (EtOH)[29] in comparison to fluorescein (4.25 ns, EtOH)[30] and tetramethylrhodamine (i.e., rhodamine 6-G, 3.99 ns, EtOH).[30] The fluorescent lifetime of Nile Red does not seem to vary much with solvent polarity, but it is extremely sensitive to H-bonding. In hydrogen-bonding solvents the fluorescence lifetime of Nile Red decreases dramatically.

As stated above, the bias of this review is to highlight potential applications in biotechnology, especially for intracellular imaging. There are two common approaches to long wavelength dyes for imaging in tissues. The first, and most obvious, is to prepare analogs of known dyes with extended conjugated systems. Secondly, two-photon absorption can be used,[31] in which two long wavelength photons absorbed by a molecule promote it to an excited state that then emits

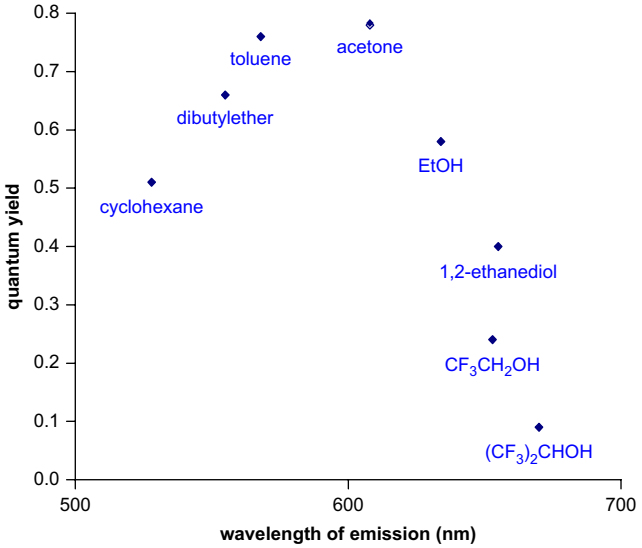


Figure 5. Solvent dependency of quantum yield and wavelengths for 2-hydroxy Nile Red **6**.

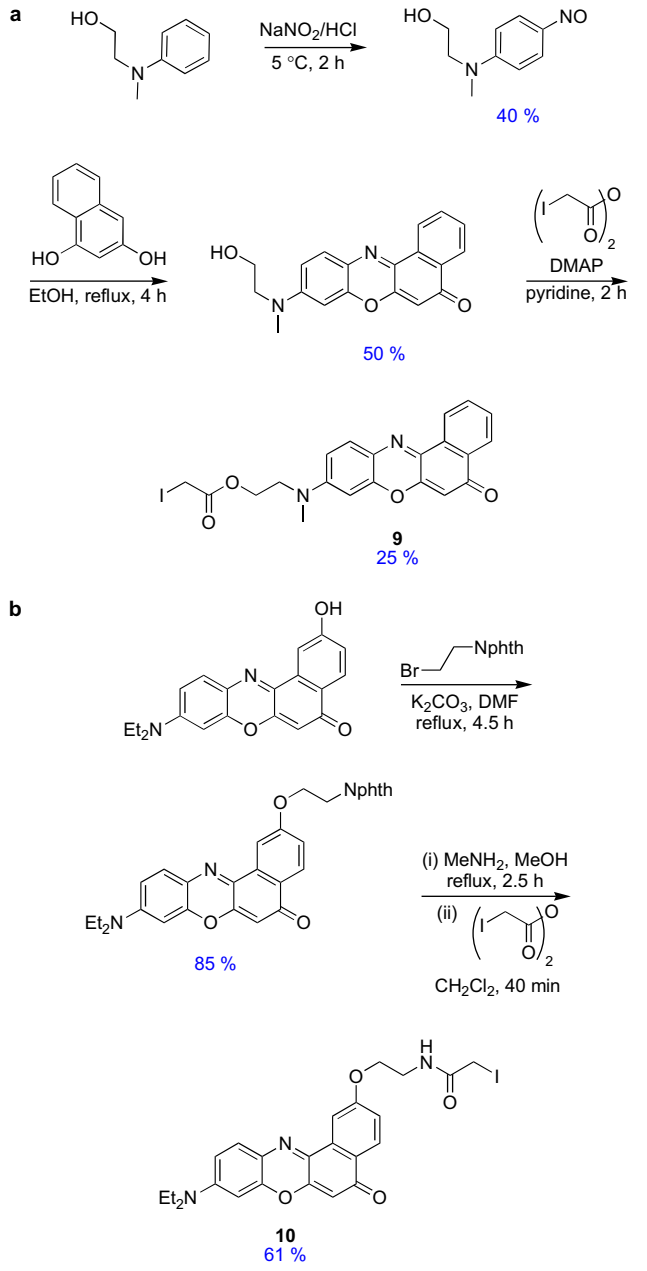
Table 3. Spectroscopic properties of water-soluble Nile Red derivatives **11–13** in different solvents

Dye	$\lambda_{max\ abs}$ (nm)	$\lambda_{max\ emiss}$ (nm)	Quantum yield (Φ)	Solvent
11	542	631	0.56	EtOH
12	520	632	0.43	EtOH
12	560	648	0.33	Phos[a] 7.4
12	556	647	0.07	Bor[b] 9.0
13	548	632	0.42	EtOH
13	558	652	0.37	Phos[a] 7.4
13	556	650	0.18	Bor[b] 9.0

[a] pH 7.4 Phosphate buffer.
[b] pH 9.0 Borate buffer.

a single photon of higher energy. This is a way to excite intracellular or tissue samples at a wavelength that is more transparent to these media. The dependence of two-photon absorption on the intensity of laser beam allows for high spatial selectivity by focusing the laser beam on the target cell and thus preventing any damage to adjacent cells. Relatively few dyes are suitable for practical experiments using two-photon excitation because most do not absorb two long wavelength photons efficiently, i.e., they have poor two-photon cross-sections. Unfortunately, Nile Red has a relatively poor two-photon cross-section.[31]

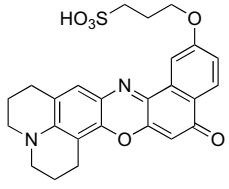
One issue with two-photon excitation experiments is that the emitted light is of a short wavelength compared to the excitation source, and this might not be in a convenient region to permeate out of cells of other tissues, and for detection. One strategy to circumvent this problem is to arrange a fluorescence energy transfer system (FRET) featuring a donor with a large two-photon cross-section and an acceptor with a more convenient, longer wavelength, and emission maxima. 2-Hydroxy Nile Red derivatives are being used in such systems. Thus a series of compounds (of which **14** and **15** are two of the simplest; Fig. 6) have been prepared toward this end; and donor-to-acceptor energy transfer efficiencies of more than 70% were observed (Fig. 5).[32,33] These compounds presumably do not have the solubility and size characteristics that render them suitable for a range of



Scheme 6. Two thiol-selective dye electrophiles: (a) a Nile Red derivative; (b) a 2-hydroxy Nile Red derivative.

applications in biotechnology, so further developments in this area would be opportune.

Finally, there is the possibility that the fluorescence of Nile Red is somewhat attenuated if the excited state of the benzophenoxazinone core is reduced via electron transfer from the 9-amino substituent. We mention this because the possibility has been explored via AM1 calculations.[34] These gauge the degree of charge transfer in a planar state relative to a twisted one in which the amine lone pair is not disposed to electron donation to the heterocycle. The degree of electron transfer from the 9-amino substituent will be dependent on the conformation *and* on the reduction potential of the heterocycle in its excited state. If intramolecular charge transfer was a major pathway for quenching the fluorescence of Nile Red derivatives, it would be expected that compound **16** would be less fluorescent than Nile Red itself; this dye itself has not been prepared.



16
more or less fluorescent than Nile Red?

4. Nile Blue

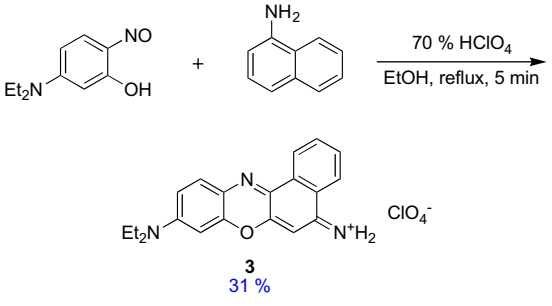
4.1. Introduction

Nile Blue has a positively charged, oxidized, phenoxazine system, i.e., it is a phenoxazinium; the conspicuous difference between this and Nile Red **2** is that the latter is neutral. Both dyes have a 9-diethylamino substituent to donate electron density across the ring, but Nile Red **2** and Blue **3** have different electron acceptors, a carbonyl and an iminium group, respectively (Fig. 7).

One obvious consequence of the difference in charges for Nile Red **2** and Blue **3** is that water-solubility of Nile Blue is significantly better. Like its Red cousin, the fluorescence maxima and intensity of Nile Blue are good indicators of the polarity of the dye's environment. This solvatochromic effect gives a red-shift in polar media, as would be expected for stabilization of a more charged excited state. The intensity of the fluorescence of **3** in water is about 0.01; this is a small value but, in comparison with the lack of fluorescence of Nile Red in aqueous media, this is significant.

4.2. Syntheses

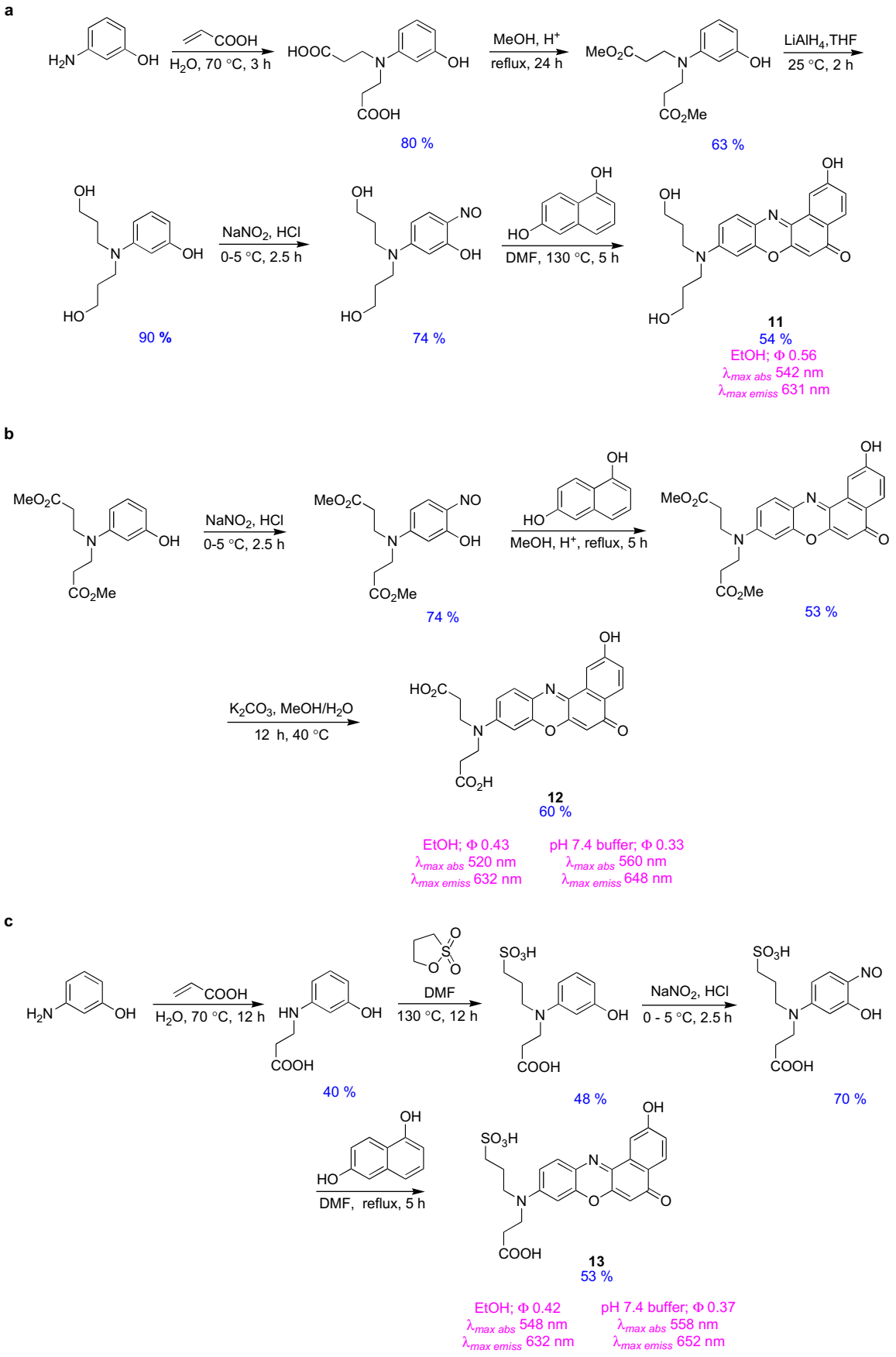
The original synthesis of Nile Blue[2] involved condensation of 5-amino-2-nitrosophenol with 1-aminonaphthalene in acetic acid, but the yield was only 3%. However, use of perchloric acid in ethanol gives a significantly higher yield (reaction 2).[35]



3
31 %

(2)

De novo syntheses of Nile Blue derivatives involve use of similar, but substituted starting materials. Scheme 8 describes syntheses of dyes **17**–**20** that incorporate 8-hydroxy julolidine.[35] This amine is a 'privileged fragment' in dye syntheses because it holds the nitrogen lone pair in



Scheme 7. Synthesis of 2-hydroxy Nile Red derivatives featuring the following functionalities: (a) a diol; (b) a dicarboxylic acid; and (c) a carboxylic acid sulfonic acid combination.

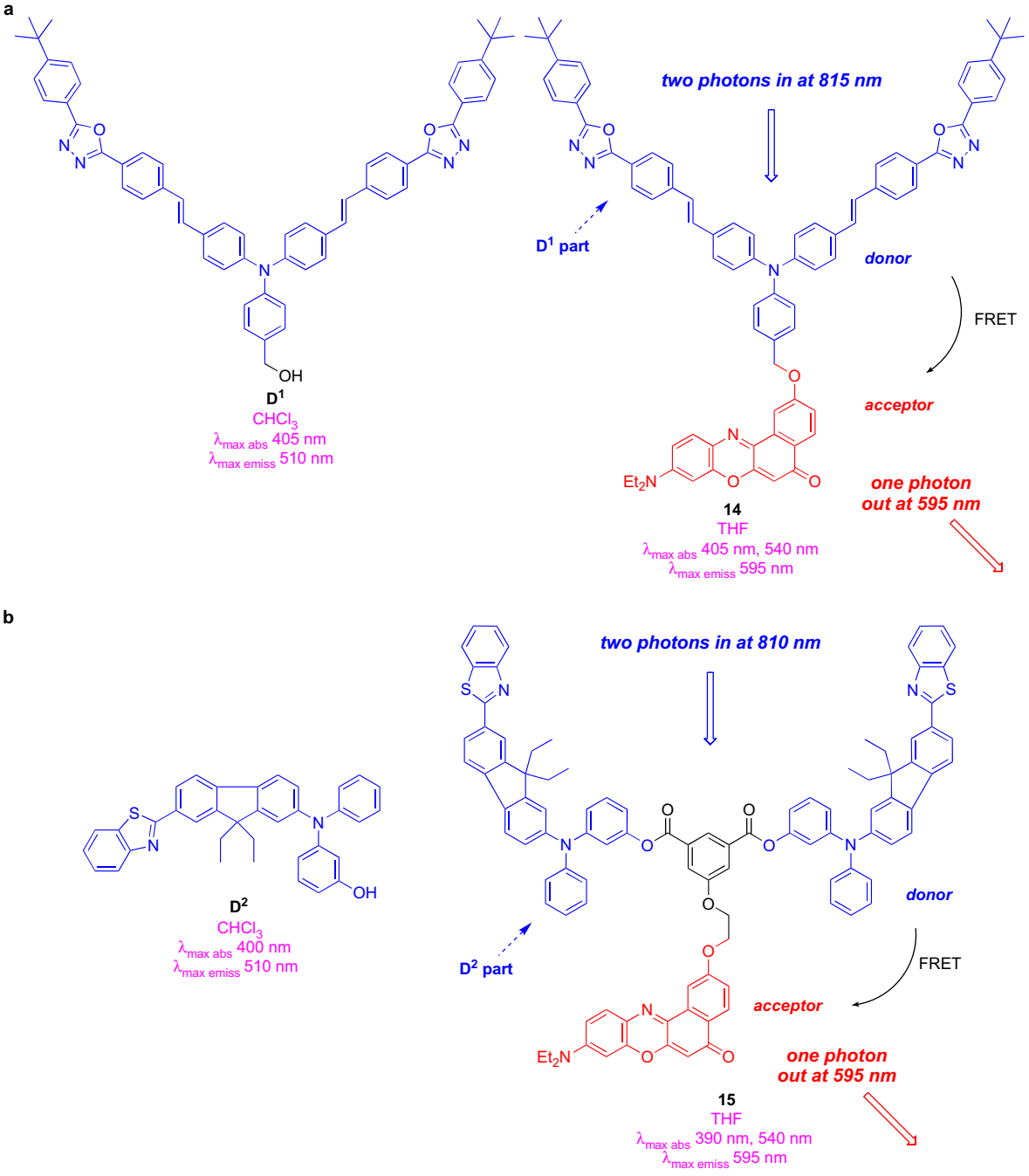


Figure 6. Two-photon-donor acceptor FRET cassettes: (a) **D**^{1} is a two-photon donor used to make cassette **14**; (b) cassette **15** is made from **D**^{2}.

conjugation with the aromatic rings. This alters the reactivity of the starting material; in fact, nitrosylated 8-hydroxyjulolidine was insufficiently reactive in this synthesis so Hartmann and co-workers modified the synthon to include the reactive azo-functionality as shown. Unfortunately, the julolidine fragment is quite hydrophobic too, so the product dyes have very limited solubility in aqueous systems.

An early synthesis of Nile Red featured hydrolysis of a Nile Blue derivative (see Scheme 2b). This implies that Nile Blue derivatives have finite hydrolytic stability, and this could be a drawback in some situations. Dyes **17**–**20** are much less vulnerable to this mode of decomposition because of their fused cyclic structures that hold the amines in place.

Nile Blue derivatives with enhanced water-solubilities and/or groups for bioconjugation can be made from amines with appropriate *N*-substituents. For instance, Scheme 9 shows preparations of dyes **21**,[36,37] **22**/**23**,[38,39] and **24**/**25**[40] in which sulfonic acid groups enhance water solubilities and carboxylic acid groups could potentially be activated and reacted with amines on biological molecules. The syntheses of **24** and **25** are shorter and higher yielding than other syntheses of water-soluble Nile Blue derivatives.

Compounds **22** and **23** are the 'EVO Blue' dyes.[38,39] These have been claimed to be more stable than rhodamine and BODIPY dyes under acidic conditions. This enhanced acid stability was suggested to be useful in the context of high

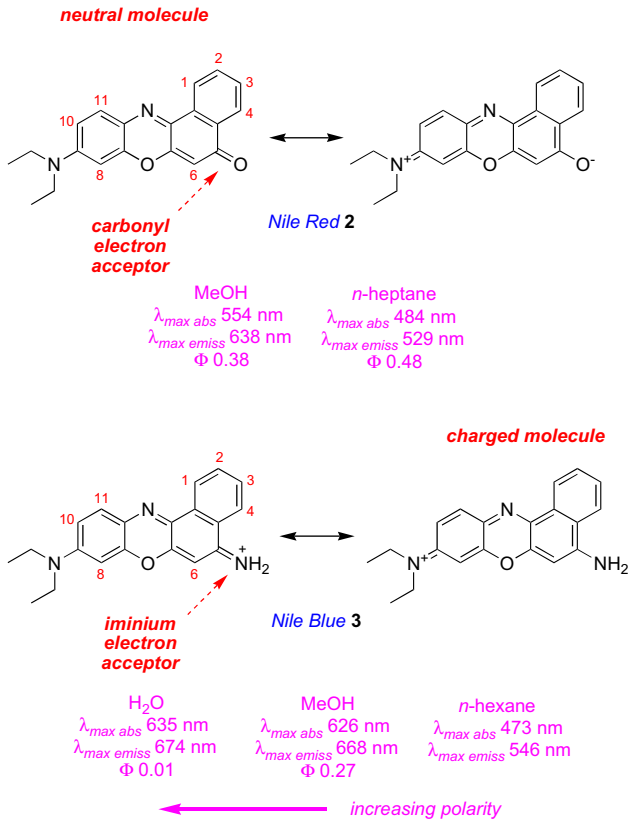


Figure 7. Structures of Nile-Red and Blue contrasted.

throughput screening and solid phase syntheses that involve cleavage from resins by acids.

Other approaches to water-soluble Nile Blue derivatives have involved modification of the parent dye after the heterocyclic framework was assembled. For instance, Scheme 10a shows a patent procedure for alkylation of the 5-amino/iminium substituent; we note that chlorosulfonic acid is an unusual choice for the ester hydrolysis reaction. In the second example, Scheme 10b, an amino anthracene was used to prepare a derivative of Nile Blue that has an extended aromatic system. This modification gave a probe with absorbance and fluorescence emission shifted to the red. Unfortunately, the material **27** was probably not a pure compound since the degree of sulfonation, the regiochemistry, and the chemical/quantum yields were not given.[41]

Direct iodination of Nile Blue is possible, and the 6-iodo derivative **28** can be prepared from this (Scheme 11). However, chromatography was required, the yield of the iodinated product was low, and slight variation of the conditions can lead to formation of other regioisomers. Similar considerations apply to the corresponding bromination reaction, except the product yield was better. Conversely, the *de novo* synthesis of the 2-iodo derivative **30** is unambiguous with respect to the regioisomer formed, but column chromatography is still required and the yield is also low. The diiodide **31** can be formed by a second iodination of the 2-iodide. These halogenated derivatives are potentially useful for forming more sophisticated derivatives, but they were originally prepared as possible photosensitizers to initiate processes that are destructive to carcinogenic cells.[42,43] These photosensitizers promoted to the excited singlet state decays to the triplet state and generates singlet oxygen, which is considered to be responsible for its potent activity toward cancer cells.

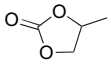
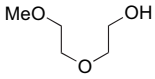
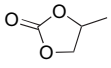
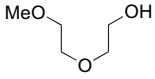
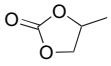
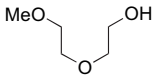
4.3. Spectroscopic properties

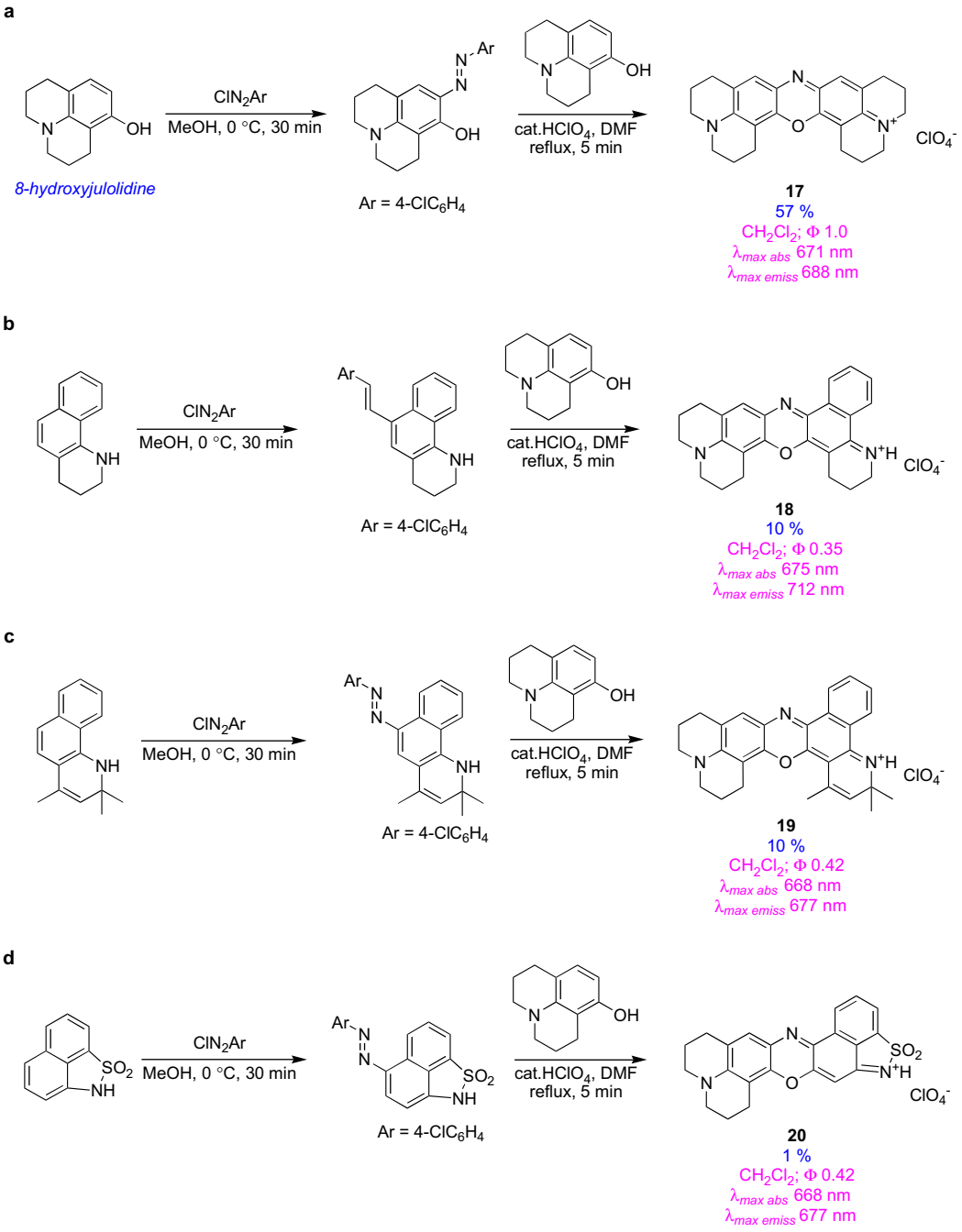
Like Nile Red **2**, Nile Blue shows progressively longer absorption and emission maxima as the solvent polarity is increased. However, the Stokes' shifts observed for the red probe **2** in different solvents is far greater; this parameter for the Blue compound can still be exceptionally high (almost 100 nm), but in polar solvents it is reduced to around 40 nm (Table 4).[44]

Table 4. UV absorption and fluorescence emission maxima of Nile Blue **3** in different solvents

Solvent	$\lambda_{max\ abs}$ (nm)	$\lambda_{max\ emiss}$ (nm)
Toluene	493	574
4-Chlorobenzene	503	576
Acetone	499	596
DMF	504	598
$CHCl_3$	624	647
1-Butanol	627	664
2-Propanol	627	665
EtOH	628	667
MeOH	626	668
Water	635	674
1.0 N HCl, pH 1.0	457	556
0.1 N NaOH, pH 11.0	522	668
NH_4OH, pH 13.0	524	668

Table 5. Study of effect of different anions on spectral properties of Nile Blue in different solvents

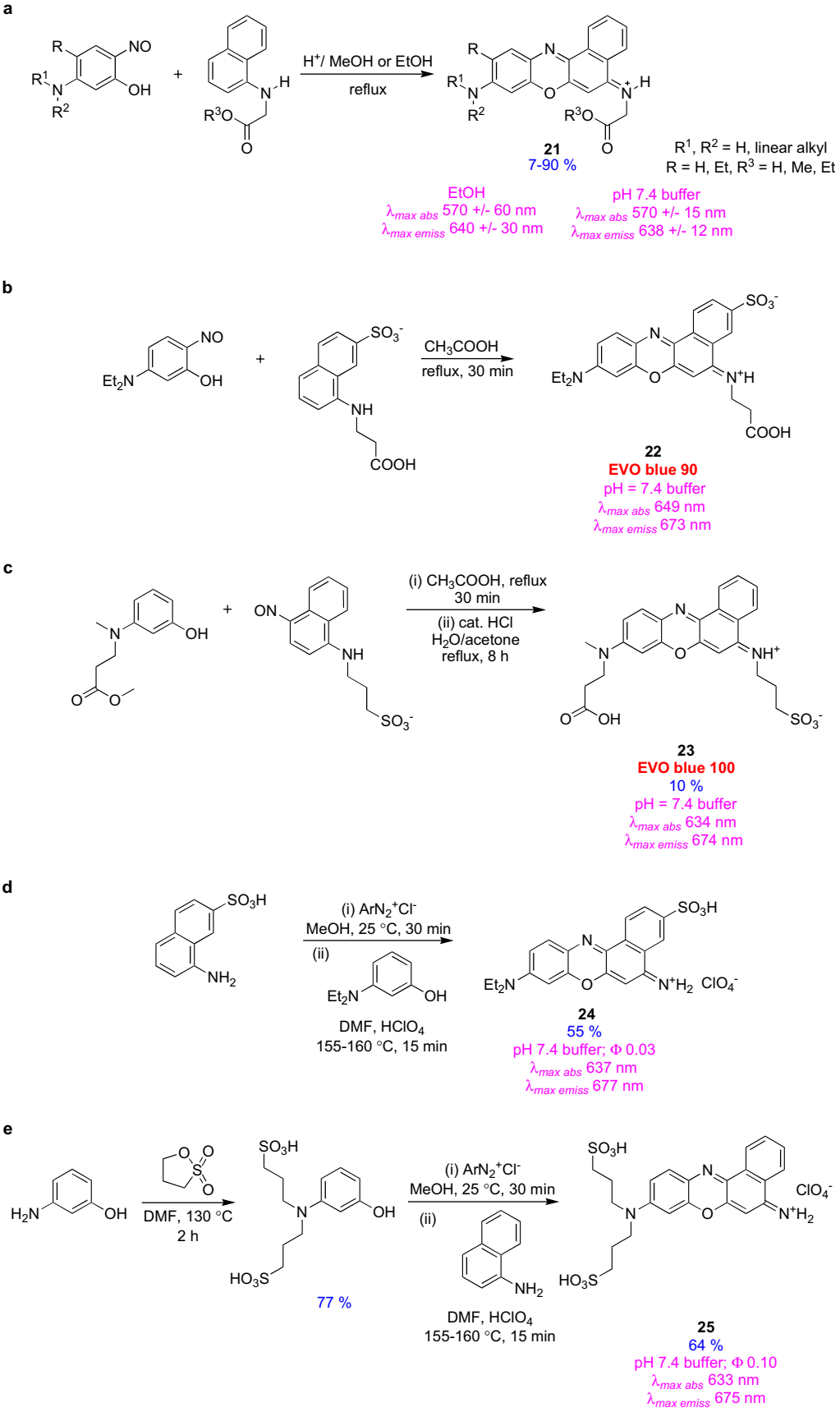
Anion	Solvent	$\lambda_{max\ abs}$ (nm)	$\lambda_{max\ emiss}$ (nm)
Chloride	EtOH	628	689
		632	—
		634	694
Acetate	EtOH	628	689
		632	687
		634	700
Benzoate	EtOH	628	691
		632	690
		634	702
iso-Butanoate	EtOH	628	691
Hydroxide	EtOH	515	661



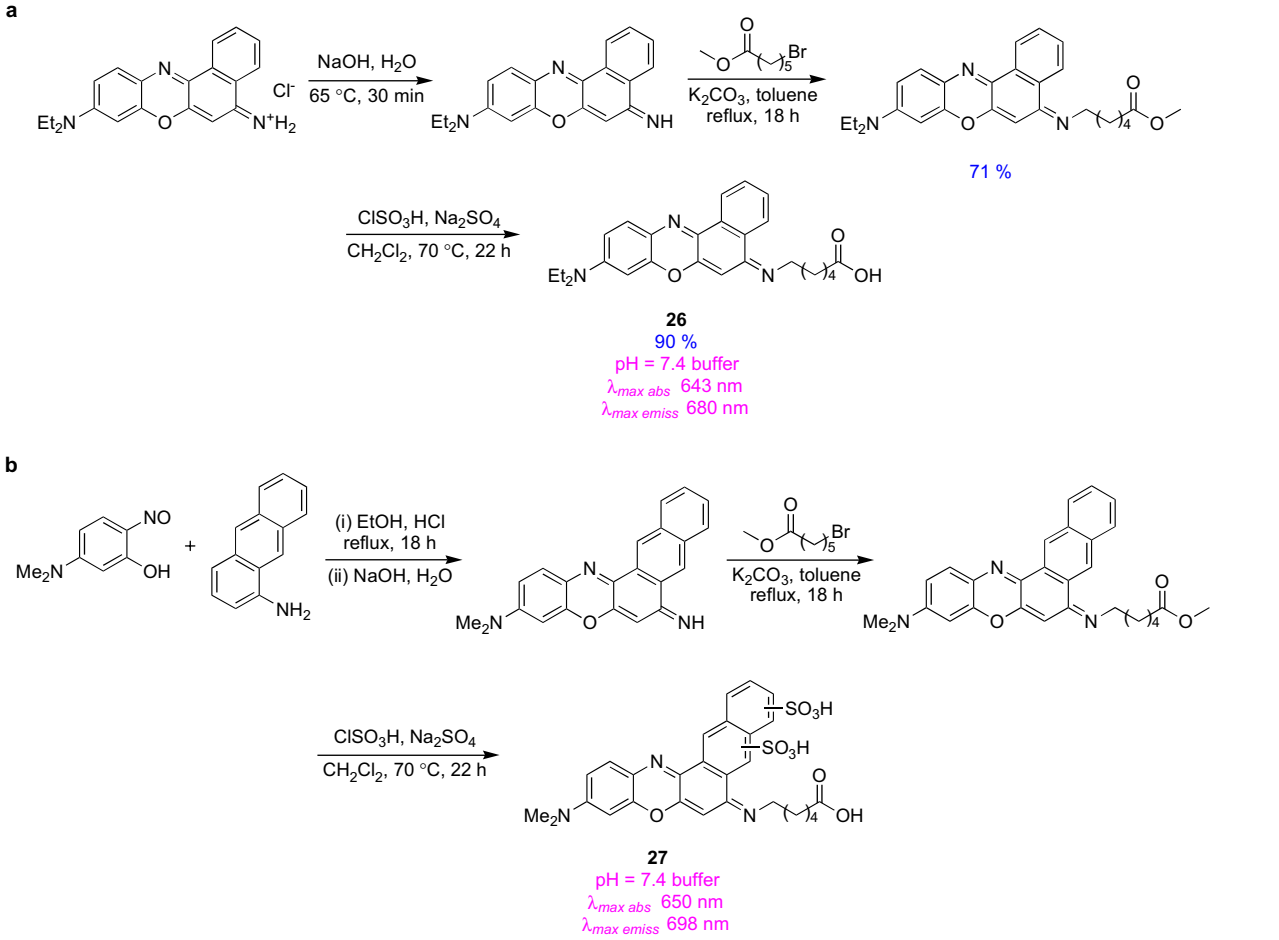
Scheme 8. Syntheses of Nile Blue derivatives from 8-hydroxyjulolidine.

Table 4 also reveals that the spectroscopic characteristics of Nile Blue are pH dependent. This is because under basic conditions the iminium group will be deprotonated, whereas under strongly acidic conditions the 5-amino might even become protonated.[45] Curiously, the spectroscopic properties of Nile Blue are somewhat dependent on the counter ion used.[46] We speculate that this could even be a reflection on intimate ion pairing influencing the solvent sphere of the dye (Table 5).

The fluorescence lifetime of Nile Blue **3** in ethanol has been measured at 1.42 ns. This is shorter than the corresponding value of Nile Red (see above; 3.65 ns). The lifetime of Nile Blue is relatively invariant at dilute concentrations (10^{-3}–10^{-8} mol dm^{-3}) but changes as the concentration is increased, and in different solvents. This is probably an indication of the interdependence of the spectroscopic properties and the degree of aggregation in solution. In support of this assertion, we note that the lifetimes do not seem to be significantly impacted by the viscosity of the medium, but they are temperature dependent.[47] As far as we are aware, the two-photon cross-section of Nile Blue has not been reported.



Scheme 9. Syntheses of Nile Blue derivatives with water solubilizing *N*-substituents.



Scheme 10. Preparation of water-soluble Nile Blue derivatives: (a) with only carboxylic side chain; (b) with carboxylic acid side chain and two additional sulfonic acid groups.

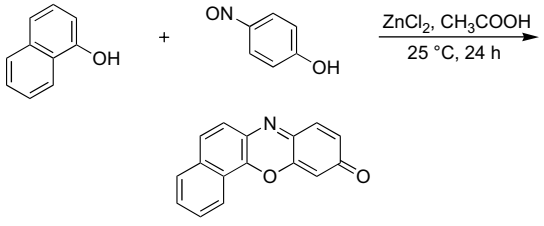
5. Other benzophenoxazine dyes

5.1. Introduction

There is no obvious reason why benzo[*a*]phenoxazines should be more fluorescent than similar compounds with different ring fusion patterns. Our interpretation of the literature is that other phenoxazines with appropriate substituents have certainly been less well studied as fluorescence probes, and are probably less synthetically accessible.

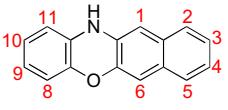
5.2. Syntheses

The benzo[*c*]phenoxazine **32** has been prepared via a route that is similar to those used for the Nile compounds **2** and **3**. 1-Naphthol is used in this synthesis (reaction 3)[46] rather than 2-naphthol, the isomer used for **2** and **3**. This change is necessary to obtain the different ring fusion, but it also may account for the very poor yield. This is because of the well-known reduced reactivity for 1-napthol at the 2-position in electrophilic substitution reactions, relative to the greater tendency for 2-napthol to react at the 1-position. To the best of our knowledge, the fluorescent properties of **32** have not been reported in any depth; indeed, the molecule lacks an electron donor in conjugation with the carbonyl to give it the type of extended oscillating dipole that seems to be common for fluorescent molecules.



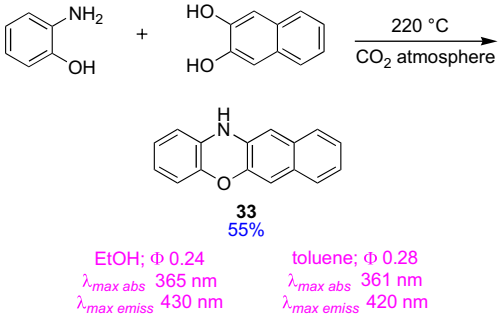
(3)

Benzo[*b*]phenoxazines are linear. Substituted derivatives of these are numbered according to the system shown below.



The linear system **33** has been prepared via the high temperature condensation process shown in reaction 4.[48] This has absorption and fluorescence emission properties that are characteristic of an extended aromatic heterocycle.

Like **32**, this compound does not have substituents that would allow it to be reduced to a phenoxazinone or phenoxazinium form.



(4)

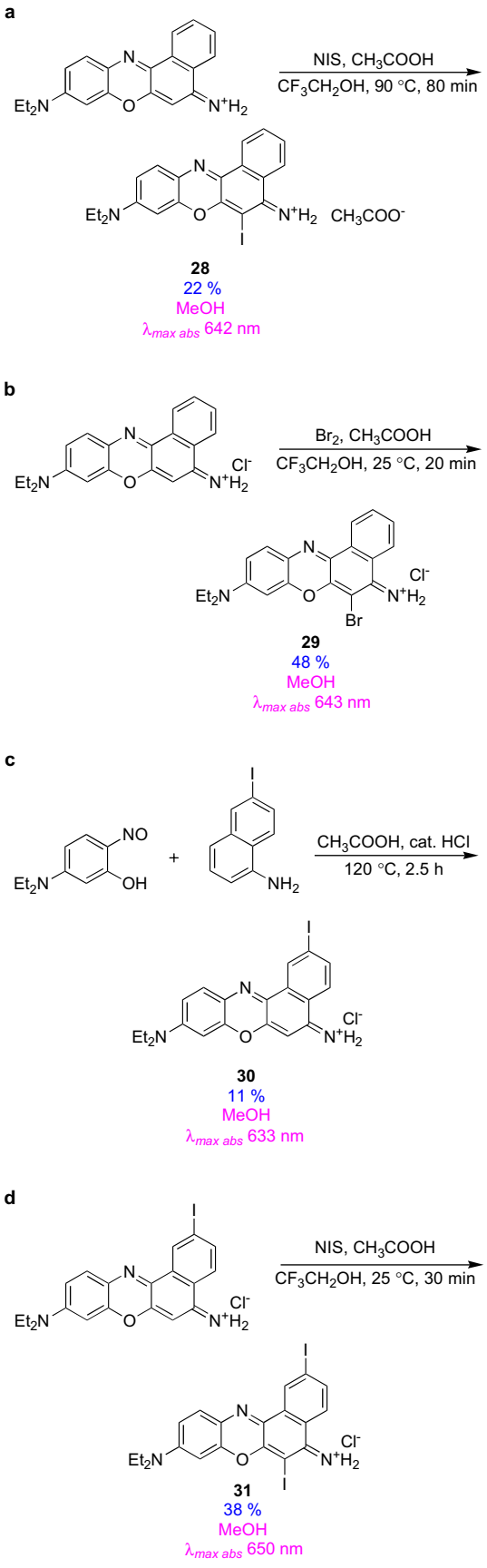
Some nitro and amino derivatives of benzo[*b*]phenoxazines have been reported in literature, and Scheme 12 shows them.[49] Parts a and b show nitrosylation/oxidation reactions that can be used to prepare nitro-substituted derivatives **35** and **36**. Predictably, these are not particularly fluorescent compounds. However, the 1,9-diaminobenzo[*b*]phenoxazinium **37** has the potential to be strongly fluorescent. Unfortunately, it was neutralized to **38** *then* the fluorescence properties were recorded.

6. Conclusions

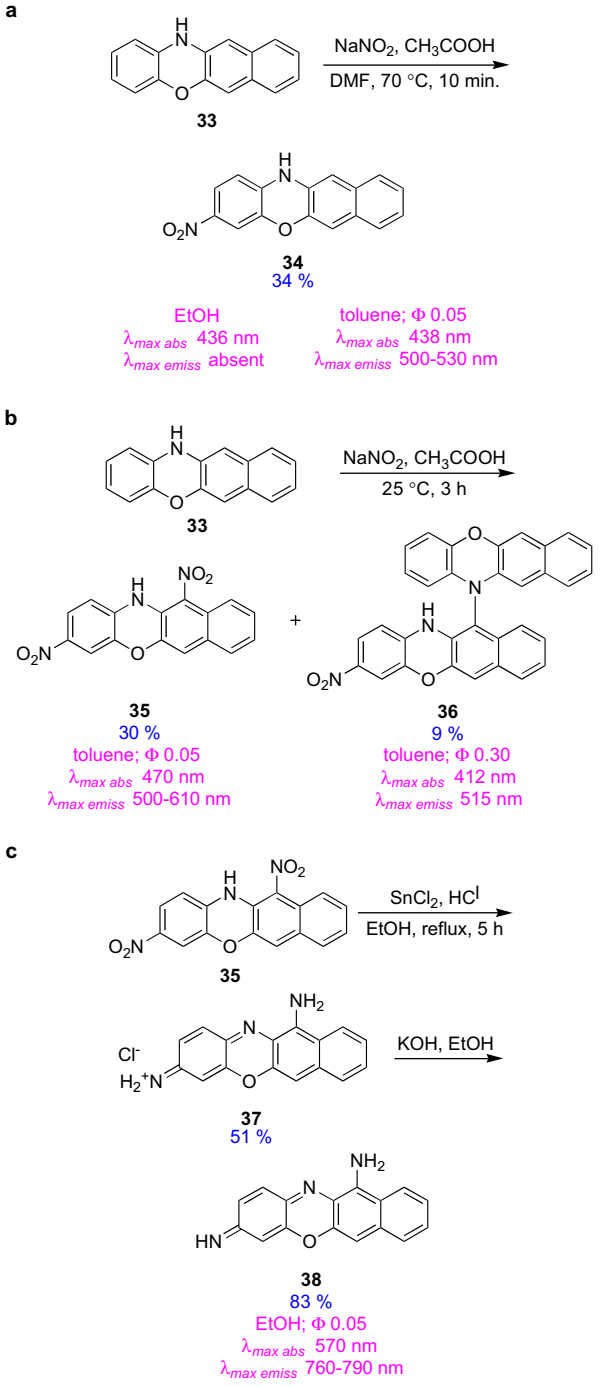
Current knowledge of fluorescent benzophenoxazine-derived probes is based almost on the benzo[*a*]phenoxazine ring fusion series. Almost all the syntheses feature high temperature condensation methods, and not contemporary synthetic methods like, for example, Buchwald–Hartwig couplings to introduce amine substituents. In fact, many of the synthetic methods reported are based on the procedures that are now over a century old. This, and the prevalence of patent literature in this area, mean that many of the experimental procedures presented are difficult to follow, and complete spectroscopic data is rarely recorded.

Nile Red and its derivatives have some interesting spectroscopic properties (long wavelength emissions, large Stokes' shifts) but most compounds in this series have limited water solubilities. There are, however, some recent efforts to make modified compounds to redress this. Nile Blue and its derivatives tend to be more water soluble.

Relatively little work has been done to modify benzophenoxazine dyes so that they emit even further to the red: the longest wavelength emission in the existing probes is about 700 nm. To this end, it would be useful to have access to more functionalized compounds that can be prepared easily on a gram scale, like 2-hydroxy Nile Red **6**. Other modifications might be used to give derivatives with enhanced extinction coefficients (these tend to be 10,000 or less), or improved two-photon cross-sections. These types of developments would be facilitated by more detailed studies of fundamental reactions that can be used to modify these compounds, e.g., halogenation and nitration.



Scheme 11. Preparation of halogenated Nile Blue derivatives: (a) 6-iodo; (b) 6-bromo; (c) 2-iodo; and (d) 2,6-diiodo.



Scheme 12. Syntheses of benzo[*b*]phenoxazine derivatives: (a) 9-nitro; (b) 1,9-dinitro and 9-nitro-1,12-bis(benzo[*b*]phenoxazine); and (c) 1-amino-9-iminobenzo[*b*]phenoxazine.

As far as we can see, there is no good reason why virtually all fluorescent dyes in this series are benzo[*a*]phenoxazine derivatives, and not any other ring fusion. This appears to be merely a question of synthetic availability.

Overall, benzophenoxazine-based probes are an intriguing subset of the fluorescent dye toolbox. They have some obvious drawbacks for applications in biotechnology, but the developments that need to be made to make more useful labels of this type are reasonably well defined.

Acknowledgements

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References and notes

1. Okafor, C. O. *Dyes Pigm.* **1986**, *7*, 103–131.
2. Mohlau, R.; Uhlmann, K. *Justus Liebigs Ann. Chem.* **1896**, *289*, 94–128.
3. Wichmann, C. F.; Liesch, J. M.; Schwartz, R. E. *J. Antibiot.* **1989**, *42*, 168–173.
4. Greenspan, P.; Fowler, S. D. *J. Lipid Res.* **1985**, *26*, 781–789.
5. Okamoto, A.; Tainaka, K.; Fujiwara, Y. *J. Org. Chem.* **2006**, *71*, 3592–3598.
6. Briggs, M. S. J.; Bruce, I.; Miller, J. N.; Moody, C. J.; Simmonds, A. C.; Swann, E. *J. Chem. Soc., Perkin Trans. 1* **1997**, *7*, 1051–1058.
7. Weissleder, R.; Ntziachristos, V. *Nat. Med.* **2003**, *9*, 123–128.
8. Kubota, L. T.; Gouvea, F.; Andrade, A. N.; Milagres, B. G.; de Oliveira Neto, G. *Electrochim. Acta* **1996**, *41*, 1465–1469.
9. Abayomi, L. A.; Terry, L. A.; White, S. F.; Warner, P. J. *Biosens. Bioelectron.* **2006**, *21*, 2176–2179.
10. Ignacio, D. V.; Matias, I. R.; Arregui, F. J.; Claus, R. O. *IEEE Sens. J.* **2005**, *5*, 365–371.
11. Alonso Lomillo, M. A.; Ruiz, J. G.; Pascual, F. J. M. *Anal. Chim. Acta* **2005**, *547*, 209–214.
12. Forrow, N. J.; Sanghera, G. S.; Walters, S. J.; Watkin, J. L. *Biosens. Bioelectron.* **2005**, *20*, 1617–1625.
13. Kerman, K.; Oezkan, D.; Kara, P.; Karadeniz, H.; Oezkan, Z.; Erdem, A.; Jelen, F.; Oezsoez, M. *Turk. J. Chem.* **2004**, *28*, 523–533.
14. Meldola, R. *J. Chem. Soc.* **1879**, 2065–2066.
15. Ottawa, N.; Schafer, G. U.S. 3,655,601, 1972.
16. Eggers, H.; Dieckmann, H. *Biochem. Z* **1942**, *310*, 233–254.
17. Zinger, B.; Shier, P. *Sens. Actuators, B* **1999**, *B56*, 206–214.
18. Jose, J.; Burgess, K. *J. Org. Chem.* **2006**, in press.
19. Long, J.; Wang, Y.-M.; Matsuura, T.; Meng, J.-B. *J. Heterocycl. Chem.* **1999**, *36*, 895–899.
20. Simmonds, A.; Miller, J. N.; Moody, C. J.; Swann, E.; Briggs, M. S. J.; Bruce, I. E. WO9729154, 1997.
21. Alekseev, N. N.; Gorelenko, A. Y.; Vasil'ev, N. N. SU1109393, 1984.
22. Gerasimova, T. N.; Kolchina, E. F.; Kargapolova, I. Y.; Fokin, E. P. *Russ. J. Org. Chem.* **1997**, *33*, 735–739.
23. Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2001**, *124*, 6063–6076.
24. Nakanishi, J.; Nakajima, T.; Sato, M.; Ozawa, T.; Tohda, K.; Umezawa, Y. *Anal. Chem.* **2001**, *73*, 2920–2928.
25. Sherman, D. B.; Pitner, J. B.; Ambroise, A.; Thomas, K. J. *Bioconjugate Chem.* **2006**, *17*, 387–392.
26. Nakaya, T.; Tajima, A.; Saikawa, T.; Takano, S.; Yamauchi, T.; Mori, H. WO2003062213, 2003.
27. Nakaya, T.; Tajima, A.; Tobita, M.; Saikawa, T.; Gao, Y.; Okubo, K. WO2004067519, 2004.
28. Schultz, C.; Wichmann, O.; Black, S. EP1595872, 2005.
29. Cser, A.; Nagy, K.; Biczok, L. *Chem. Phys. Lett.* **2002**, *360*, 473–478.

30. Magde, D.; Brannon, J. H. *J. Phys. Chem.* **1979**, *83*, 696–699.
31. Albota, M.; Beljonne, D.; Bredas, J.-L.; Ehrlich, J. E.; Fu, J.-Y.; Heikal, A. A.; Hess, S. E.; Kogej, T.; Levin, M. D.; Marder, S. R.; McCord-Maughon, D.; Perry, J. W.; Rockel, H.; Rumi, M.; Subramaniam, G.; Webb, W. W.; Wu, X.-L.; Xu, C. *Science* **1998**, *281*, 1653–1656.
32. Brousmiche, D. W.; Serin Jason, M.; Frechet Jean, M. J.; He Guang, S.; Lin, T.-C.; Chung Sung, J.; Prasad Paras, N. *J. Am. Chem. Soc.* **2003**, *125*, 1448–1449.
33. Brousmiche, D. W.; Serin, J. M.; Frechet, J. M. J.; He, G. S.; Lin, T.-C.; Chung, S.-J.; Prasad, P. N.; Kannan, R.; Tan, L.-S. *J. Phys. Chem. B* **2004**, *108*, 8592–8600.
34. Dias, L. C. J.; Custodio, R.; Pessine, F. B. T. *Chem. Phys. Lett.* **1999**, *302*, 505–510.
35. Kanitz, A.; Hartmann, H. *Eur. J. Org. Chem.* **1999**, 923–930.
36. Frade, V. H. J.; Goncalves, M. S. T.; Moura, J. C. V. P. *Tetrahedron Lett.* **2005**, *46*, 4949–4952.
37. Frade, V. H. J.; Goncalves, M. S. T.; Moura, J. C. V. P. *Int. Electron. Conf. Synth. Organic Chem., 5th, 6th*, 2004, 121–123.
38. Fries, J.; Lopez-Calle, E. EP10236, 2002.
39. Lopez-Calle, E.; Fries, J. R.; Mueller, A.; Winkler, D. *Innovation Perspect. Solid Phase Synth. Comb. Libr: Collect. Pap., Int. Symp. 7th*, 2002, 129–136.
40. Ho, N.-H.; Weissleder, R.; Tung, C.-H. *Tetrahedron* **2006**, *62*, 578–585.
41. Yan, X.; Yuan, P. M. U.S. 6,465,644, 2002.
42. Becker, R. S.; Chakravorti, S.; Das, S. *Photochem. Photobiol.* **1990**, *51*, 533–538.
43. Foley, J. W.; Cincotta, L. U.S. 4,962,197, 1990.
44. Basting, D.; Ouw, D.; Schaefer, F. P. *Opt. Commun.* **1976**, *18*, 260–262.
45. Krihak, M.; Murtagh, M. T.; Shahriari, M. R. *J. Sol-Gel Sci. Technol.* **1997**, *10*, 153–163.
46. Afanas'eva, G. B.; Pashkevich, K. I.; Postovskii, I. Y. *Khim. Geterotsikl. Soedin.* **1971**, *7*, 742–745.
47. Grofcsik, A.; Kubinyi, M.; Jones, W. J. *J. Mol. Struct.* **1995**, *348*, 197–200.
48. Kehrmann, F.; Neil, A. A. *Ber.* **1914**, *47*, 3102–3109.
49. Alekseeva, V. I.; Marinina, L. E.; Savvina, L. P. *Chem. Heterocycl. Compd.* **1995**, *31*, 112–115.

Biographical sketch

Jiney Jose was born in Kerala, India. He received his B.Tech in Chemistry (2002) from U.I.C.T., Mumbai, India. He joined the group of Prof. Kevin Burgess at Texas A & M University, Department of Chemistry in 2003. His research is focused on syntheses, derivatization and photophysical studies of water-soluble benzophenoxazine dyes.

Kevin Burgess is a professor at Texas A & M University, where he has been since September 1992. His research interests focuses on peptidomimetics for mimicking or disrupting protein–protein interactions via combinatorial chemistry and via asymmetric catalysis, and fluorescent dyes for multiplexing in intracellular imaging of protein–protein interactions. Motivation to write this review came from a need for water-soluble Nile Red derivatives in the design and syntheses of through-bond energy transfer cassettes for use in the imaging project.