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Brightly fluorescent purple and blue labels for amines and proteins

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ABSTRACT

Novel amino-reactive phenoxazines were obtained by reasonably simple synthetic protocols and characterized in terms of their use as fluorescent labels for amines, amino acids and proteins in general. Purple labels (alternatives to Texas Red) and blue labels (alternatives to Cy-1) were obtained by this strategy. The absorption/emission maxima, in aqueous solution, are at around 589/630 nm and 648/670 nm, respectively, thus indicating larger Stokes' shifts than those of common cyanine-type of labels. The new labels are compatible with commercial diode laser light sources.

Labeling of biological matter such as amino acids, proteins, cells or viruses with synthetic fluorescent labels has become an indispensible tool in bioanalytical sciences. Applications are ranging from simple immunoassays and histochemical techniques to high-throughput screening and cell biology.[1,2] Fluorescence labeling is also a preferred method in separation techniques such as electrophoresis and chromatography.[3] The widespread use of fluorescent labels can be owed to the remarkable sensitivity of fluorescence detection that can even go down to the single molecule level in case of certain techniques such as laser-assisted fluorometry.[4–6] In recent years, the growing interest in tumor imaging has inspired important and developing fields in fluorescence spectroscopy, for example, laser-induced fluorescence microscopy of (tumor) cells.[7] Fluorescence imaging in the spectral range between 600 nm and 1000 nm is particularly appealing (compared to the UV and shortwave visible part)[8–10] as biomolecules display less background fluorescence and straylight,[11] and for the better penetration properties of longwave radiation into tissue in this regime. Green, yellow and (deep) red laser diodes are preferred light sources for their affordable and compact nature. Such laser diodes can be battery-powered and easily driven with high efficiency in terms of conversion of electrical energy into light.[12] Hence, there is a substantial interest in fluorescent labels for use in bioanalytical sciences and in combination with diode laser light sources.

There is a vast number of fluorescent labels in the literature with excitation maxima above 580 nm, some of them being available from commercial sources (e.g., from Invitrogen; www.probes.-com), Dyomics (www.dyomics.de), Attotec (www.atto-tec.com), ActiveMotif Chromeon (www.chromeon.de) or Amersham (www.amershambiosciences.com). The state of the art of the synthesis and application of oxazine-type labels has been reviewed, for example, by Drexhage,[13] Hartmann,[14] Tung,[15] and Simmonds and Briggs.[16] Unfortunately, some of these methods are either cumbersome or lead to products that are covered by patents. Among the long-wavelength emitting dyes, oxazines and benzoxazines (such as Nile Red and Nile Blue) are particularly attractive for their extraordinary stability and brightness.[17] Oxazine based labels have been used in a wide range of applications, for example, in nucleic acid detection, histochemistry,[18,19] protein labeling[20] or environmental analysis.[21] The deeply colored oxazines possess a mesomeric donor–acceptor chromophoric system.

Oxazinones, like the purple label in Scheme 1, are strongly solvatochromic (e.g., orange and strongly fluorescent in nonprotic solvents, red in methanol, purple and weaker fluorescent in aqueous solution). Diamino-substituted oxazines (the blue oxazine in Scheme 2) are blue and less prone to solvatochromism. Due to their charged nature, they display improved water solubility and often undergo an increase in fluorescence quantum yield upon conjugation. Another feature that attracts attention is the relative ease of structural modification for introducing various functional groups for bioconjugation. Recently, we have reported on the synthesis of clickable (azide or alkyne functionalized) and thiol reactive (maleimide functionalized) oxazine derivatives.[22,23] Herein we present the synthesis of purple and blue oxazine labels that are accessible in a few synthetic steps and to the best of our knowl-

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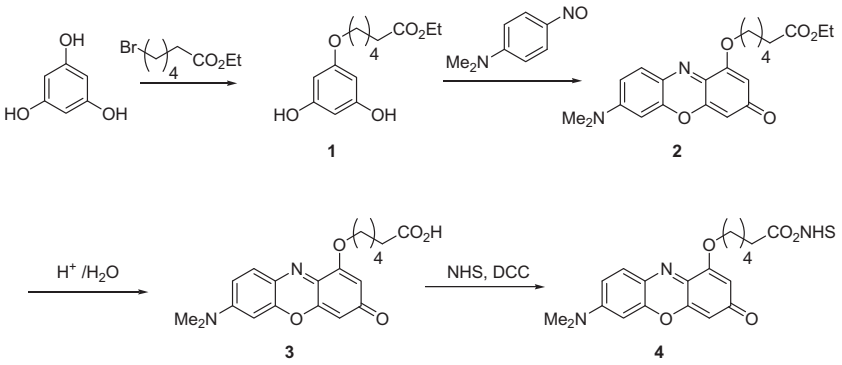
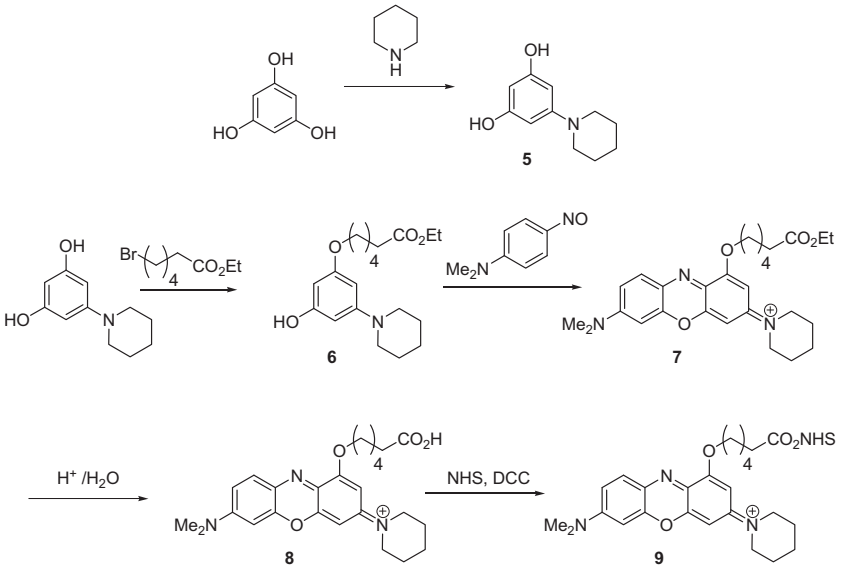


Scheme 1. Synthesis of the purple phenoxazine label **4**.



Scheme 2. Synthesis of the blue phenoxazine label **9**.

edge are not protected by any existing patents. Both can be used in combination with commercial diode lasers and are comparable, in terms of brightness and photostability, with other probes in this spectral range, therefore offering good alternatives to them.

The oxazine framework was chosen on the basis of its synthetic conciseness and spectral properties. We anticipated that the dyes can be synthesized in relatively few steps and that they exert all the aforementioned advantages of labels operating in this highly desirable spectral region. The hydroxyphenoxazine can be easily derivatized with a C-6 linker carrying a carboxylic function. Subsequent in situ activation by converting to its *N*-hydroxysuccinimide ester offers a ready-to-use amine reactive label.[24]

When designing the synthetic routine to reach the purple and the blue phenoxazine labels, we intended to minimize the necessary synthetic steps. Synthesis in both cases started from commercially available phloroglucinol. In case of dye **3**, phloroglucinol was first functionalized with ethyl ω-bromohexanoate to afford monosubstituted intermediate **1**. This intermediate was then condensed with *p*-nitroso-*N*,*N*-dimethylaniline to give phenoxazine **2**, which was subsequently hydrolyzed under acidic conditions to result in the purple carboxylic acid **3**.

As outlined in Scheme 2, the synthesis of the blue phenoxazine **8** also started from phloroglucinol, which was first converted to **5** by reaction with piperidine. Compound **5** was then reacted with ω-bromohexanoate to furnish **6**. Subsequent condensation with *p*-nitroso-*N*,*N*-dimethylaniline provided phenoxazine **7**. Similarly to the previous protocol, acidic hydrolysis of the ester afforded the carboxylic acid functionalized dye **8**. Both carboxy-functionalized dyes can be converted into their ready-to-use active ester form, for example, *N*-hydroxysuccinimide (NHS) esters, using standard in situ protocols prior to their use in amino-group modification reactions.

The absorption spectrum of the purple phenoxazine (free acid) **3** exhibits a broad band cantered at 598 nm with a shoulder at around 565 nm. The emission spectrum shows a maximum at 630 nm (Fig. 1). It was found that dye **3––similar** to other Nile Red type of dyes––displays strong solvatochromism. The absorption/emission maxima in water (589/630 nm) are blue-shifted to 557/619 nm in methanol and 510/580 nm in toluene. We therefore believe that the respective label can also be utilized as an intraprotein (local) polarity probe,[25] comparable in its response to that of certain ketocyanines.[26] The molar absorbance of **3** is 38,000 L/(mol cm) at the peak wavelength. The quantum yield in aqueous solution is 0.05 using Nile Red (QY 0.018)[27] as a reference standard.

Compared to phenoxazine **3**, the absorption and emission spectra of dye **8** are distinctly red-shifted due to the presence of the iminium group (Fig. 2).[28] The absorption maximum is located at 648 nm in aqueous solution, with a shoulder at around 598 nm, while the emission peaks at 670 nm. Compared to **3**, the spectral properties of **8** are much less affected by the polarity of the solvent

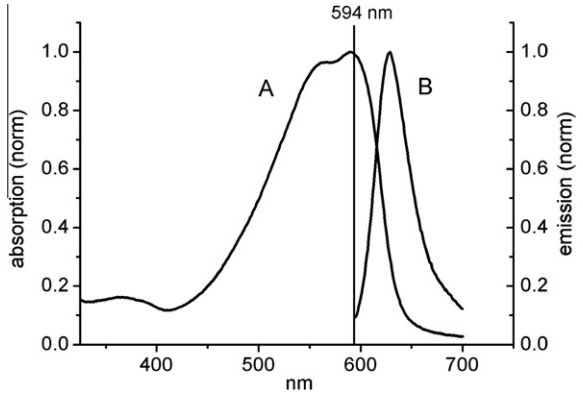


Figure 1. Absorption (A) and emission (B) spectra of the purple dye **3** in water. The line of the 594-nm helium–neon laser is also shown.

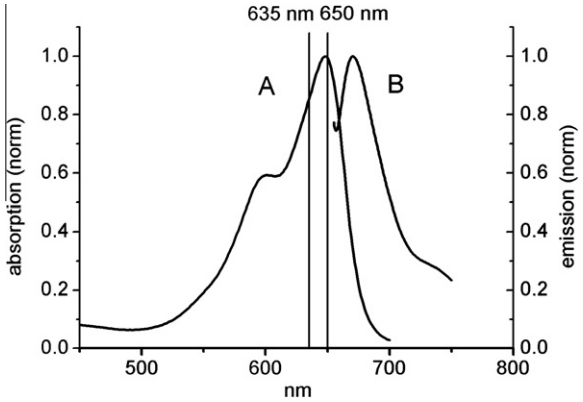


Figure 2. Absorption (A) and emission (B) spectra of the blue dye **8** in water. The lines of the 635-nm and 650-nm diode lasers are also shown.

(e.g., 639/663 nm in methanol). The molar absorbance of **8** is 71,000 L/(mol cm) at the peak wavelength, whilst the quantum yield in water is 0.004 using Nile Blue (QY 0.004[29] in water) as the reference standard.

Next, we have investigated the pH dependency of the two phenoxazine dyes. Fluorophores **3** and **8** were excited at their respective absorption maxima, and the change of their fluorescence intensity with pH was measured at their emission maxima. Figure 3 depicts the changes of emission intensities at the emission maxima of the new oxazine dyes as a function of pH. The emission intensity of dye

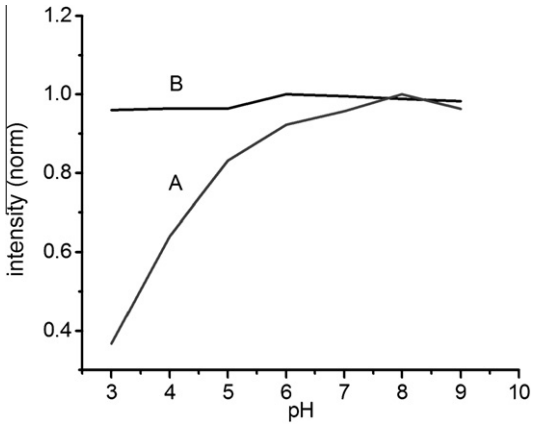


Figure 3. Normalized pH dependencies of the emission of dye **3** (A) and **8** (B).

3 drops drastically at pH values below pH 8, whilst the fluorescence of dye **8** remains virtually unchanged. These findings can be explained by the chemical structure of the two dyes: dye **3** is a slightly basic molecule whose nitrogen atoms (dimethylamino group and heterocycle) are prone to protonation. Upon protonation, the molecule (that is electrically neutral in its nonprotonated form) becomes charged. The resulting positive charge is most likely mesomerically distributed over the whole π-electron system and thus affects emission intensity. Contrary to this, the positive charge present in compound **8** makes it less prone to protonation. These structural differences are reflected in the response of emission intensities to the pH of the environment for the two dyes.

In order to elaborate the labeling potential of the oxazines, dyes **3** and **8** were converted to their active esters using DCC and NHS in dry DMSO (see Schemes 1 and 2 and the supplementary data). The resulting solutions of label **4** and **9**, respectively, were then directly used to label amino acids, peptides, and proteins. The pre-formed active esters were found to be stable if stored dry and can be stored at −18 °C at least for 1 week without any change in activity.

The amino acids L-lysine, L-serine, L-glycine, L-glutamic acid, L-aspartic acid were labeled by treating them with solutions of either **4** or **9** overnight at room temperature.[30] In each case, the labeling process was monitored via TLC. As a model system for peptide labeling we have chosen bradykinin. Treatment of the amine containing media with solutions of labels **4** or **9** overnight at room temperature resulted in efficient staining of the samples as indicated by complete conversion of the starting materials into their red or blue conjugates.

Labeling of proteins was studied on BSA, a 65 kDa protein that often serves as a model protein in labeling experiments. Like in the case of amino acids, labeling was carried out by treatment with the active ester solutions overnight at room temperature. The protein was purified by size exclusion chromatography and submitted to TOF-MS analysis (see data in the Supplementary data). The effect of BSA on the fluorescence intensity of the dyes was checked via BSA titration e.a. 5 μL (c = 0.054 M) of nonreactive dye (**3** or **8**) in buffered medium using BSA in concentrations between 0 mg/L and 1000 mg/L. Results have shown that no significant change in fluorescence intensity or emission maxima could be observed, indicating that there is no unspecific binding of the dyes on the protein surface. Contrary to this, obvious changes in the positions of absorption and emission maxima as well as quantum yields were seen in case of covalent modification of BSA with the reactive dyes. Characteristic photophysical properties of labels and their conjugates to BSA are summarized in Table 1.

Conjugation also resulted in remarkable changes in the shapes of the bands, especially for compound **9** (Fig. 4). This effect is visible only on the covalent attachment of the fluorophore to the protein. The absorption spectra remain unchanged if nonreactive dyes are added to BSA. The changes shown in Figure 4 in the absorption spectra clearly indicate covalent attachment of the labels to BSA.

In order to demonstrate the wider scope of the labels, we have also labeled amino-modified silica nanoparticles (SiNPs) with the purple dye **4**. SiNPs are a new and interesting class of materials. They are readily available in defined sizes and––unlike nonencap-

Table 1
Photophysical properties of the dyes and their BSA conjugates at pH 7.2

	$\lambda_{abs\ (max)}$ (nm)	$\lambda_{em\ (max)}$ (φ)[a] (nm)
4	598 (565)	630 (0.05)
4-BSA	550 (589)	627 (0.04)
9	648 (598)	670 (0.004)
9-BSA	592 (658)	677 (0.003)

[a] Quantum yields were determined in phosphate buffered saline (pH 7.2; 50 mM) using Nile Red and Nile Blue, respectively, as standards.

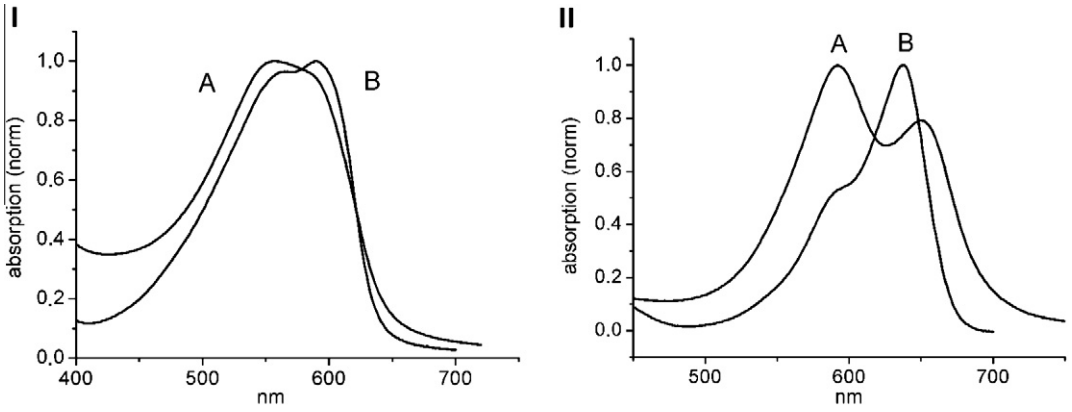


Figure 4. Absorption spectra of dye–BSA conjugates (A) and free dyes (B) for phenoxazine **4** (I) and **9** (II) in PBS (50 mM, pH 7.2).

sulated quantum dots––are biocompatible.[31,32] Fluorescent SiNPs are either prepared by post-synthetic direct tagging of the amino-modified silica surface (method A), or by doping fluorophores into the outer coating of silica nanoparticles during nanoparticle fabrication (method B).[33] In method A (Fig. 5) silica nanopowder was treated with aminopropyltriethoxysilane (APTES) in order to create an amino-modified surface. Next, label **4** was added to a suspension of the amino-modified silica nanopowder in ethanol and the reaction mixture was stirred overnight at rt. The labeled red particles were collected by centrifugation and washed with ethanol to remove unreacted label. Parallel to this, a blank sample was prepared by combining plain silica nanoparticles (without amino groups) with activated fluorophore **4**. The blank samples showed virtually no color after centrifugation and washing, whereas the amino-labeled particles displayed the characteristic red color.

In method B (Fig. 5), plain silica nanoparticles (SiNPs) were first prepared by the Stöber method using tetraethylorthosilicate (TEOS).[34] Parallel to this, a mixture of APTES in ethanol and label **4** were stirred overnight in order to obtain silane **10**. This dye-silane conjugate was then directly used to coat the SiNPs with a dye doped shell around the silica core. The labeled SiNPs were purified by size exclusion chromatography (SEC) on Sephadex® LH-20. The labeled particles were obtained by collecting the first colored fraction. These particles are more homogeneous in terms of size distribution. We find method B to be easier and to give more strongly colored SiNPs, probably because the label is contained in the complete outer shell, and not only on the surface as in SiNPs prepared by method A.

The emission spectra of the labeled SiNPs and of the unreacted reagent were acquired in ethanolic colloidal solution. The peak of the emission of the labeled SiNPs is red-shifted (λ_{em} = 626 nm) in comparison to the maximum of the unreacted label in ethanol (λ_{em} = 619 nm), indicating its presence in a more polar microenvironment. A blank sample was also prepared to elucidate unspecific binding of the fluorophore to the particle surface. The alcosol containing the particles was diluted with ethanol and treated with an ethanolic solution of nonactivated dye **3** (free acid). The mixture

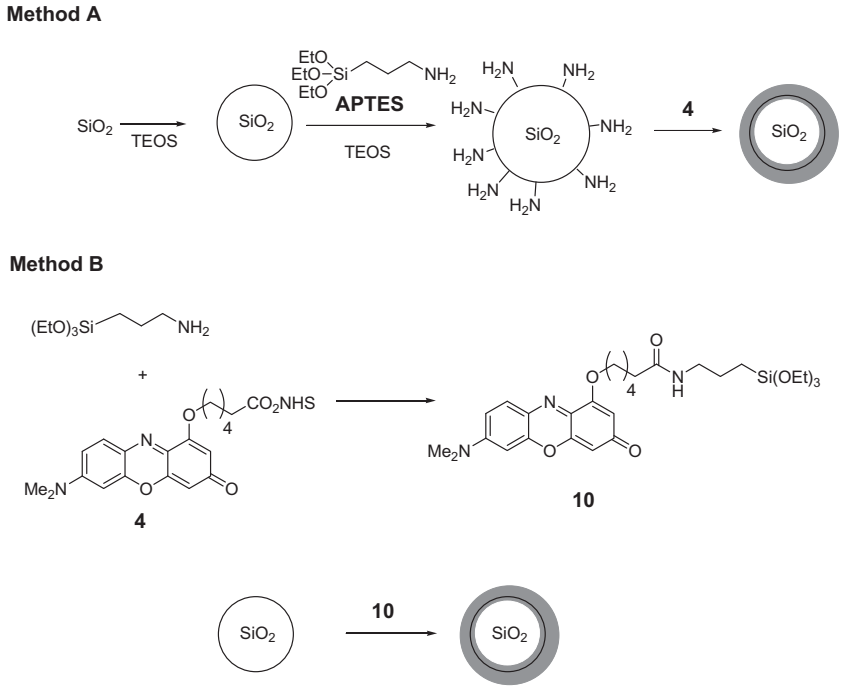


Figure 5. Modification of SiNPs with reactive label **4**.

was stirred overnight and purified by SEC as described above. The SiNPs obtained exhibited no significant fluorescence. See the Supplementary data for spectra and further details of the labeling procedures.

In conclusion, we have synthesized two functionalized phenoxazine dyes that were used as amino-reactive labels in model reactions. The absorption and emission maxima of the compounds are located in the longwave visible and NIR region of the electromagnetic spectrum. They exhibit high absorption coefficients and adequate solubility in aqueous media. The blue phenoxazine (**8**) can be excited by the 635-nm laser diode which is a small, affordable and stable light source widely used in fluorescence instrumentation such as cell sorters and imagers. The absorption coefficients of 38,000 L/(mol cm) for the purple phenoxazine **3** and of 71,000 L $mol^{-1}cm^{-1}$ for the blue phenoxazine **8** are comparable to those of Nile Red (ε = 38,000 L $mol^{-1}cm^{-1}$)[35] and Nile Blue (ε = 76,000 L $mol^{-1}cm^{-1}$)[36] which are well-established stains for biological samples.[37,38] BSA was chosen as an easily accessible and well-known protein for demonstrating the utility of the two amino reactive dyes. At this juncture, activated label **4** and especially label **9**, which is positively charged, have been shown suitable for use in aqueous media. Similar labels were described by the group of Briggs[39] and in a patent.[40] However, the dyes prepared by Briggs et al. are related to the purple label **4** and distinctly larger in size. We also applied our strategy to synthesize label **9** which extends the set of oxazine labels into the longwave part of the visible spectrum. Compared to the blue phenoxazines described in a patent,[40] our labels are not functionalized at the push and pull system of the chromophoric core, which can be disadvantageous in terms of spectral properties of the label as this often results in lower molar absorbance and brightness (*Bs*; defined as the product of ε and quantum yield). Unlike many other labels, the ones reported here do not undergo an increase in brightness on conjugation.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.133.

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