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Brightly fluorescent purple and blue labels for amines and proteins

Martin Link, Péter Kele †, Daniela E. Achatz, Otto S. Wolfbeis *

Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, D-93053 Regensburg, Germany

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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-

* Corresponding author. Tel.: +49 941 943 4065, fax: +49 941 943 4064.
E-mail addresses: otto.wolfbeis@chemie.uni-r.de, otto.wolfbeis@chemie.uni-regensburg.de (O.S. Wolfbeis).
† Present address: Institute of Chemistry, Eötvös Loránd University, H-1117 Budapest, Hungary

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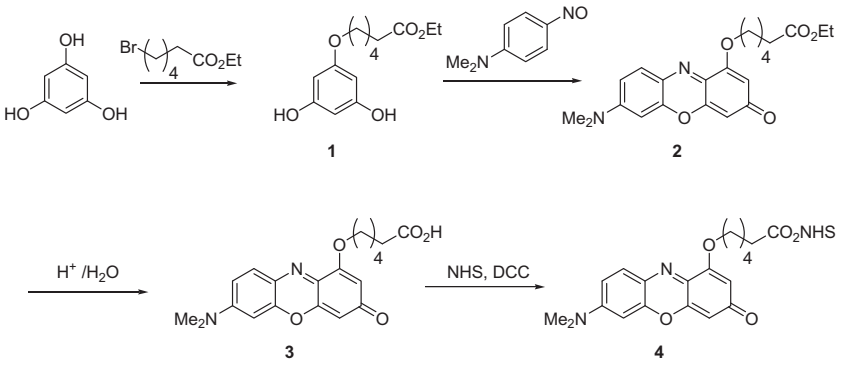
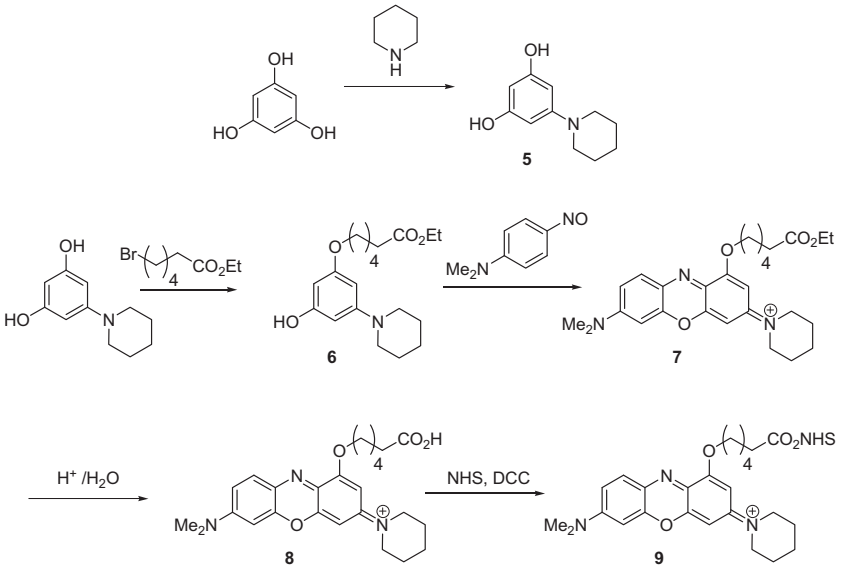


Scheme 1. Synthesis of the purple phenoxazine label **4**.



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