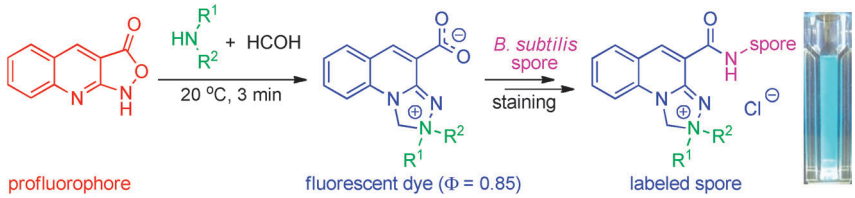

Tandem fluorogenic reaction: A new platform for the direct, selective and sensitive detection of formaldehyde and/or secondary aliphatic amines, based on tandem Mannich–electrophilic amination reaction, is described. Biological applications of hydrophilic, water-soluble fluorescent 2,2-dialkyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium-8-carboxylates (*Safarinium P* probes) are exemplified by labeling of the amine-containing Ac-AKF-NH_2 peptide and *Bacillus subtilis* spores in aqueous solution.

Fluorescent Probes

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The Tandem Mannich–Electrophilic Amination Reaction: a Versatile Platform for Fluorescent Probing and Labeling

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The Tandem Mannich–Electrophilic Amination Reaction: a Versatile Platform for Fluorescent Probing and Labeling

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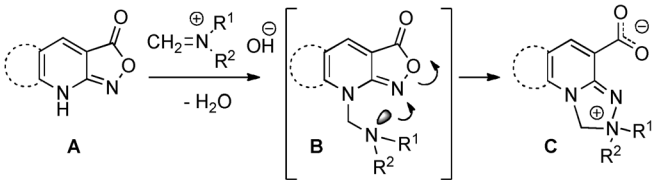
The design and application of fluorogenic reactions in fluorescence bioimaging has attracted increasing attention because of their rapid response as well as high selectivity and sensitivity. Chemodosimeters, a class of sensing system based on analyte-induced chemical reactions, have found valuable applications as probes for environmental and physiological monitoring and assaying of biological systems.[1,2] Further development of optical probe technology requires the design of new fluorophores with advantageous physicochemical and photophysical properties, such as good water-solubility when applied in medical research, high photostability as well as large Stokes shifts for easy separation of excitation and emission to avoid cross-talk with autoluminiscent samples and simplify the signal evaluation.

In this context, it is pertinent to note that formaldehyde[3] and aliphatic amines[4] are both ubiquitous pollutants, with natural and industrial sources, to which humans are chronically exposed by different means. Currently, there are many methods to detect formaldehyde[5] and aliphatic amines[6] which include capillary electrophoresis, electrochemistry, HPLC, gas chromatography, mass spectrometry, electrochemiluminiscence and spectrofluorimetry. Chromatographic methods, however, usually need expensive instruments and sophisticated procedures.[7] Moreover, the above mentioned methods for sampling and determination of both formaldehyde and aliphatic amines have drawbacks of either long sampling and derivatization time, elevated temperature and exclusion of light required for effective derivatization, comparatively high detection limits due to low fluorescence quantum yield of a tagging reagent or pH-dependent fluorescence of the derivatized analyte. Therefore, there is a need for novel, inexpensive, sensitive and rapid analytical technologies for detection and measurement of both formaldehyde and volatile aliphatic amines, for which fluorescence-based techniques are advantageous.

Herein we report a new platform for fluorescent sensing and imaging based on selective tandem Mannich–electrophilic amination reaction of fluorogenic 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one or isoxazolo[3,4-*b*]quinolin-3(1*H*)-one with formaldehyde and secondary amines. From this multicomponent reaction a new class of photostable fluorescent dyes with pyrido-triazolium and quinolino-triazolium core structures, which can serve for environmental monitoring of either formaldehyde or secondary amines, are obtained and upon esterification with *N*-hydroxysuccinimide (NHS) provide fluorescent amine-reactive probes useful for bioimaging purposes.

The general concept underlying this platform is outlined in Scheme 1. We have found previously that cyclic hydroxy-



Scheme 1. The tandem Mannich–electrophilic amination reaction.

guanidine-*O*-sulfonate afforded 1,2,4-triazolium derivatives upon treatment with Eschenmoser's salt by a tandem nucleophilic addition–electrophilic amination reaction.[8] We reasoned that fused isoxazolo-3-ones of type **A** bearing formal *N*-acyloxyamidine group should react analogously with iminium salts, generated in situ from formaldehyde and secondary amines, to give fused 1,2,4-triazolium salts of general formula **C**. We also expected that, analogous to quinolinium salts,[9] these compounds will exhibit a pronounced fluorescence property.

To prove this concept, we have initially subjected 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one[10] (**1**) to the reaction with formaldehyde in the presence of secondary amines, as depicted in Scheme 2. Indeed, when heated in sealed tube at 60 °C for 1 h in methanolic solution, the non-fluorescent compound **1** was converted into the highly fluorescent zwitterionic pyrido-triazolium compounds **2a–f** (*Safirinium P*) in quantitative yields. Then, on treatment of **2b**

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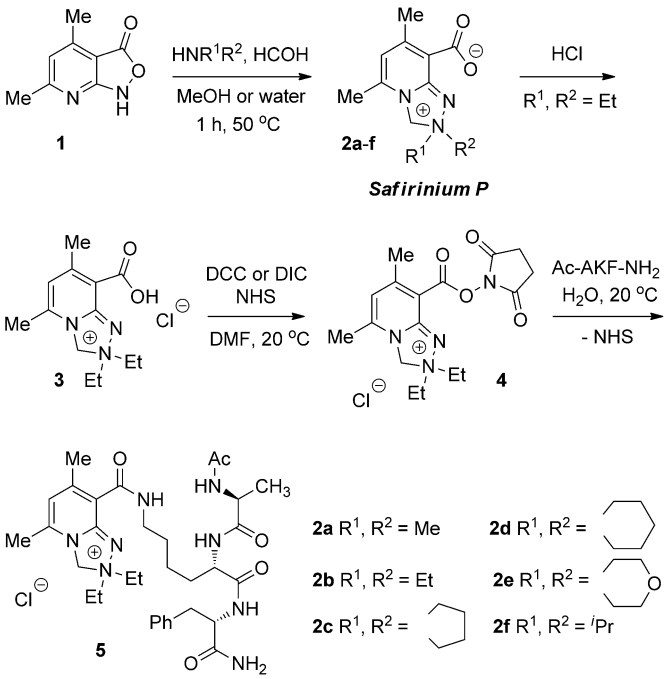


Scheme 2. Synthesis of fluorescent 1,2,4-triazolo[4,3-*a*]pyridin-2-ium-8-carboxylates (*Safirinium P* probes).

with hydrochloric acid, the corresponding salt **3**, with a carboxylic functionality available for esterification, was obtained in quantitative yield. Following the routine synthesis procedure, we then obtained the *N*-hydroxysuccinimidate (NHS) ester **4**, which is an amine-reactive fluorophore, and was subsequently coupled to the lysine-containing tripeptide Ac-AKF-NH_2. Usually, amine-reactive dyes are hydrophobic molecules and, for conjugation purposes, they must be dissolved in anhydrous DMF or DMSO. The NHS ester **4**, however, is water soluble and resistant to hydrolysis at ambient temperature, and therefore, the conjugation process was carried out in aqueous solution at 20 °C to give fluorescent tripeptide **5** modified at the lysine residue.

To produce intrinsically more sensitive indicators, we synthesized quinoline analogues of **2**, which are dyes of type **9** that should have superior fluorescence properties (Scheme 3). First, a two-step procedure was used for the synthesis of fluorogenic isoxazolo[3,4-*b*]quinolin-3(1*H*)-ones (**8a**,**b**). The method is based on our previous finding that 2-chloroquinolines react smoothly with hydroxylamine-*O*-sulfonic acid (HOSA) giving rise to the formation of 2-(sulfooxyamino)quinolines.[11] As shown in Scheme 3, the 2-(sulfooxyamino)quinoline-3-carboxylic acids **7a**,**b** were prepared from corresponding 2-chloroquinoline-3-carboxylic acids **6a**,**b**, and then, upon treatment with aqueous solution of Et_3N at room temperature, compounds **7** were transformed into the desired isoxazolo[3,4-*b*]quinolin-3(1*H*)-ones **8a**,**b**. The most striking property of compounds of type **8** was their ability to react with equimolar amounts of formaldehyde and secondary amine at 20 °C to give the expected fluorescent dyes **9a**–**j** (*Safirinium Q*). *Safirinium Q* dyes form within 3 min in quantitative yields and their formation could be monitored by rapid disappearance of the red color accompanied by appearance of bright greenish-blue fluorescence. The zwitterionic compound **9b** (R^1 = H, R^2, R^3 = Et) was then converted via quinolino-triazolium salt **10** into the reactive labeling agent **11**. Again, coupling of **11** with the lysine-containing tripeptide Ac-AKF-NH_2 confirmed the applicability of these newly prepared quinolino-triazolium probes for bioimaging purposes. However, the reagent **11** proved more susceptible to hydrolysis than the corresponding pyridine-containing reagent **4**. Hence, conjugation of **11** with tripeptide was carried out in anhydrous DMF solution.

Structures of all newly prepared compounds have been confirmed by elemental analyses, IR, ^{1}H NMR, ^{13}C NMR and MS spectra as well as single crystal X-ray analysis of zwitterionic **9g** and the 1,2,4-triazolium salt **10** (see the Supporting Information).

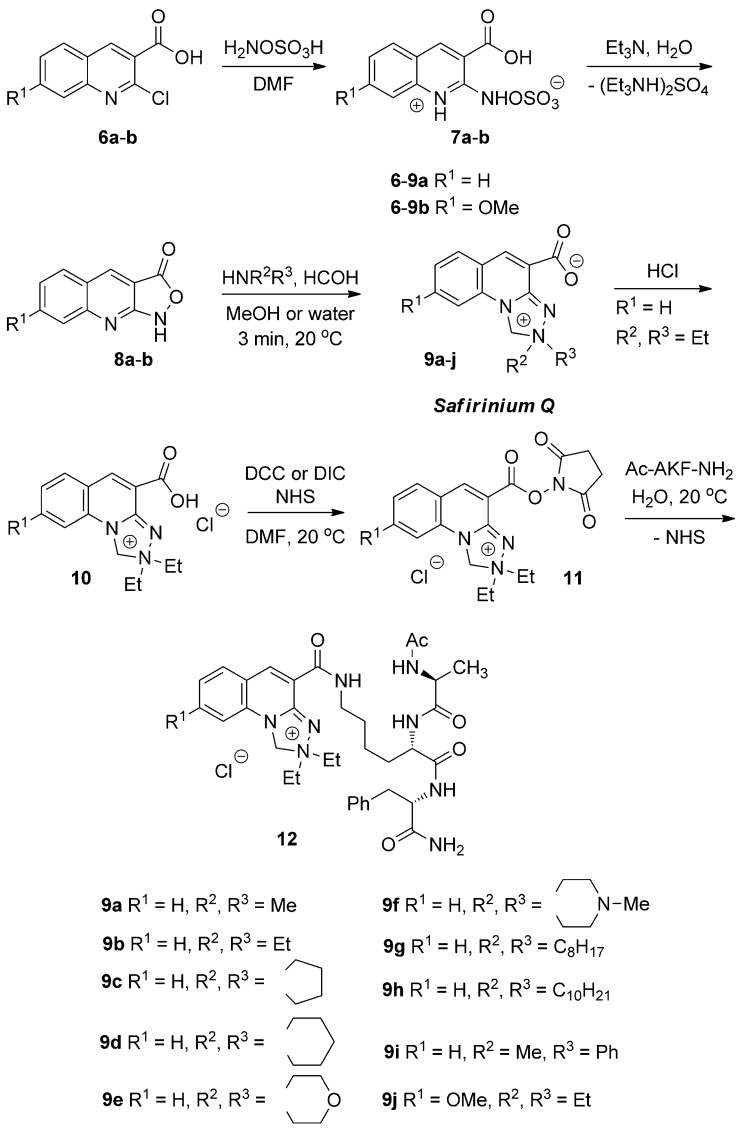
The absorption and fluorescence properties of the compounds **2b**, **9b**, 7-methoxy analogue **9j** and corresponding tripeptide conjugates **5** and **12** were studied in water. The absorption (λ_{abs}) and emission (λ_{em}), molar absorption coefficients (ε) and fluorescence quantum yields (Φ) are presented in Table 1.

Table 1. Spectroscopic data for selected compounds.

	Absorption λ_{exct} [nm]	ε_{exct}	Emission λ_{max} [nm]	Stokes shift [cm^{-1}]	Quantum yield [Φ]	Sensitivity $\varepsilon\Phi$ ($\times 10^{-2}$)
2b	343	4450	463	7556	0.755	33.6
9b	368	3525	463	6575	0.855	30.1
9j	377	9700	456	4595	0.682	66.1
5	310	2810	399	7185	0.093	2.6
12	352	3180	442	5785	0.122	3.9

Zwitterions **2** and **9** show high quantum yields of fluorescence in the range 0.682–0.855. The introduction of the methoxy group at position 7 of quinoline ring (compound **9j**) apparently favors nonradiative deactivation pathways leading to a slight decrease in fluorescence quantum yield (Φ = 0.682 vs. 0.855). As fluorescence intensity is directly proportional to both Φ and ε, the product of these values provides a useful indication of the relative sensitivity offered by a particular fluorescing analyte. As shown in Table 1, the 7-methoxy derivative **9j** offers a twofold increase in sensitivity compared to unsubstituted quinoline compound **9b** and pyridine dye **2b**.

All fluorescent dyes **2** and **9** as well as conjugates **5** and **12** display large Stokes shifts from absorption to emission wavelengths. The polarity of the solvent does not significantly influence the emission wavelengths of the probes. The observed lack of solvatochromism can be explained by observing that the permanent positive charge is localized on quaternary nitrogen atom, and, from an electrostatic point of view, the ground state and the excited state are roughly equivalent. Both, **2b** and **9b** were stable under prolonged irradiation at their maximum absorption wavelength, and no appreciable photobleaching was observed after several



Scheme 3. Synthesis of fluorescent 1,2,4-triazolo[4,3-a]quinolin-2-ium-4-carboxylates (*Safirinium Q* probes).

hours of exposure. Finally, dyes **2** and **9** showed a different degree of solubility in water: poor solubility for **9g** (R^2,R^3 = C_8H_{17}) and **9h** (R^2,R^3 = $C_{10}H_{21}$), good solubility for spiro-substituted compounds **2e**, **9e** and excellent solubility for **2a**,**b** and **9a**,**b** (R^2,R^3 = CH_3, C_2H_5).

To test the generality of this method, we analyzed the platform for the detection of formaldehyde. Figure 1 shows the fluorescence emission spectra of dye **9c** generated from profluorophore **8a** in the presence of an equimolar amount of pyrrolidine and increasing concentrations of formaldehyde. For this particular dye, the relationship between induced fluorescence and formaldehyde concentration was linear in the 0.5–1.5 μM concentration range and the limit of formaldehyde detection, based on three times the signal-to noise level, was estimated to be about 50 nM.

Of note, fluorogenic isoxazolones **1** and **8** distinguish formaldehyde from other carbonyl compounds such as acetaldehyde, propionaldehyde and acetone. Higher aldehydes reacted with isoxazolo[3,4-*b*]quinolin-3(1*H*)-one (**8a**) to give unstable non-fluorescent products, and no reaction was noticed with acetone. The selectivity of the sensing platform described herein was also determined by examining the fluorescence responses of **8** toward primary and secondary aliphatic amines. The reaction of **8a** with propylamine was much slower compared to the analogous reaction with secondary amines, and required 12 h for its completion and non-fluorescent products were isolated from the reaction mixture. Thus, concentration of 0.05 μM of a secondary amine could be easily detected even in the presence of a tenfold excess of a primary amine.

We further investigated the utility of the probe **4** in *Bacillus subtilis* spore imaging. *B. subtilis* is the most studied spore-forming bacterium, its spore has a central core that contains chromosomal DNA and cellular proteins necessary for the return to vegetative growth. The core is encased by a thick layer of modified peptidoglycan named the cortex. The cortex is surrounded by a protein multilayer named the

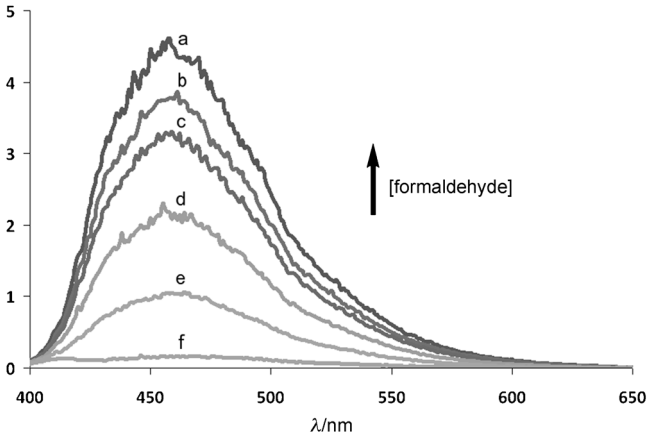


Figure 1. Formaldehyde concentration dependent fluorescence at a) 100 mol%, b) 80 mol%, c) 60 mol%, d) 40 mol%, e) 20 mol%, and f) 0 mol%; formation of compound **9c**. 20% mol HCOH corresponds to 15 μg L^{-1} (0.5 μM).

coat. The spore coat consists of over 70 proteins which become organized in several layers during spore development.[12,13] Importantly, *B. subtilis* is a non-pathogenic bacterium widely used as a probiotic for human and animal consumption.[14] These attributes make *B. subtilis* spores an attractive vehicle for heterologous protein surface display.[15]

However, several questions regarding use of spores as the vehicle remain still unanswered. One of them concerns the fate of ingested spores. It was reported previously that a part of orally administrated spores could successfully produce vegetative cells, which are able to sporulate again inside the gastrointestinal tract (GIT).[16] The possibility for germination inside the GIT may be a factor for immunogenicity of spore-based vaccine.

Probe **4** can be used as a versatile tool for measuring the percentage of germinating spores inside the GIT depending on bacterial strain variation and animal host. The results obtained with probe **4** will be more accurate because use of antibiotic resistant markers as a selection tool may be imprecise due to the fact that *B. subtilis* has the ability to naturally uptake DNA from the environment. Taking into account information that *B. subtilis* is a part of the natural GIT bacterial flora[17] it is possible that a number of antibiotic resistant counts isolated from feces are a mixture of administrated strain and transformed ones from the GIT. Use of probe **4** will overcome this problem, as the fluorescence label of the coat cannot be transmitted.

The spore suspension was diluted with water to the concentration of $10^5\,mL^{-1}$. The water soluble probe **4** was dissolved immediately before use in conjugation and diluted with buffer (100 mM carbonate/bicarbonate buffer, pH 9.0) to a final concentration of $1\,mg\,mL^{-1}$. An aliquot (10 μL) of the solution of probe **4** was added to 1 mL of spore suspension, mixed well and incubated at room temperature in the dark for 1 h. Microscope observation of spores labeled with probe **4** are presented in Figure 2 (see Supporting Information for details).

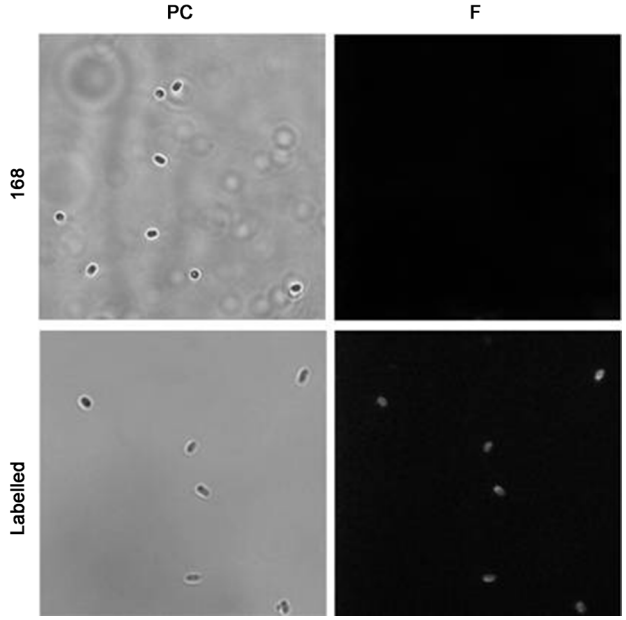


Figure 2. Microscope observation of spores labeled with dye **4**. PC = phase contrast (bright field), F = fluorescence.

The proposed staining method is quick and easy to perform in comparison to the methods published previously.[18] Moreover, fluorescently labeled spores can be analyzed by flow cytometry which make it suitable for detailed analysis of spore population.[19] Additionally, our dye can bind directly to the spore crust or coat without any additional molecules, such as antibodies.

In summary, we have demonstrated that the fluorogenic tandem Mannich–electrophilic amination reaction of non-fluorescent isoxazolo[3,4-*b*]quinolin-3(1*H*)-ones (**8**) leading to fluorescent 1,2,4-triazolo[4,3-*a*]quinolin-2-ium-4-carboxylates (**9**, *Safirinium Q*) can be used as a platform for fast, sensitive and selective detection of formaldehyde and/or secondary aliphatic amines. On the other hand, low cost and easy production of 1,2,4-triazolo[4,3-*a*]pyridin-1-ium-8-carboxylates (**2**, *Safirinium P*) and their corresponding succinimidate esters **4**, which are soluble and stable in aqueous solution, makes them promising cationic, amine-reactive probes for fluorescence bioimaging purposes.

Experimental Section

For the experimental details, see the Supporting Information.

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Keywords: amination · dyes/pigments · fluorogenic reaction · mannich bases · tandem reaction

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