

A Fluorogenic Probe for the Copper(I)-Catalyzed Azide–Alkyne Ligation Reaction: Modulation of the Fluorescence Emission via $^3(n,\pi^*)\text{--}^1(\pi,\pi^*)$ Inversion

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Chemoselective ligation reactions represent a powerful approach for labeling of proteins or small molecules without the need of laborious protection and deprotection steps. Recently, two highly selective bioorthogonal ligation strategies have been introduced, both of which utilize the azide moiety as the functional group. The first strategy is based on an intramolecular variant of the Staudinger reaction and has been applied to the labeling of metabolically engineered cell surfaces.¹ The second approach is based on the Cu(I)-catalyzed, Huisgen-like annelation of azides with terminal alkynes,^{2,3} a very robust transformation that has been successfully used for the fluorescent labeling of virus capsid proteins,⁴ the cell-surface labeling of *Escherichia coli*,⁵ and the activity-based protein profiling in live mice.⁶

Here we report a fluorogenic probe (**1**), that takes advantage of the electronic structure changes associated with the triazole ring formed in the Cu(I)-catalyzed azide–alkyne ligation reaction (Scheme 1).⁷ We hypothesized that the electron-donating properties of the triazole ring might effectively modulate the emission response of an electronically coupled coumarin. The fluorescence quantum yield of coumarin is strongly influenced by substituents in the 7-position, which control the relative energy ordering of the two lowest excited states: a nonemissive (n,π^*) state, which originates from the carbonyl-oxygen lone pair, and an emissive locally excited state with (π,π^*) character.⁸ Because substitution with an electron donor increases the charge-transfer character only of the (π,π^*) state but not the (n,π^*) state, the former will be more effectively stabilized in a polar solvent such as water. If the emissive (π,π^*) state lies then sufficiently below the energy level of the nonemissive (n,π^*) state, the molecule should fluoresce with high quantum yield. On the basis of this rationale we designed fluorophore **1**, in which the reactive alkyne moiety is attached at the 7-position of the coumarin framework. To increase the water solubility of the probe, we further functionalized the 4-position with an electronically decoupled carboxylate group (Scheme 1).

The photophysical data for **1** and its ligation product **3** are compiled in Table 1. Under physiological conditions (pH 7.2, 0.1 M KCl) compound **1** exhibits a low fluorescence quantum yield of 1.4% (Figure 1, right). Upon reaction with azide **2** the quantum yield increases to 25%, and consistent with increased charge-transfer character, the peak emission undergoes a bathochromic shift by 27 nm. The UV–vis spectrum of **1** shows two intense absorption bands, which are reminiscent of symmetry-allowed $\pi \rightarrow \pi^*$ transitions (Figure 1, left). Time-resolved emission spectroscopy of **3** revealed a monoexponential decay kinetics with a lifetime of 0.92 ns and a large radiative rate constant, both of which are in agreement with emission from the lowest $^1(\pi,\pi^*)$ state. Furthermore, the time-resolved measurements indicate that the low quantum yield of **1** is primarily due to a large increase of the nonradiative rate constant, while the radiative deactivation rate remains essentially identical within the experimental error (Table 1). Consequently, the radiative

Scheme 1

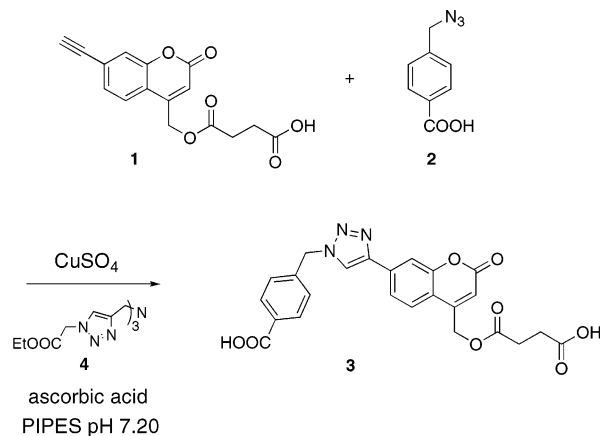


Table 1. Photophysical Properties of Compounds **1** and **3**

	absorption λ_{\max} [nm] ^a	emission λ_{\max} [nm]	Φ ^b	τ ^c [ns]	k_r ^d [10 ⁹ s ⁻¹]	k_{nr} ^e [10 ⁹ s ⁻¹]
1	325 (7100)	388	0.014	<0.1	>1.4	>9.8
3	328 (10 900)	415	0.25	0.92	2.7	0.81

^a Lowest-energy band, extinction coefficient ϵ ($[M^{-1} \text{ cm}^{-1}]$) in parentheses. ^b Fluorescence quantum yield (quinine sulfate as standard). ^c Fluorescence lifetime (instrument resolution 100 ps). ^d Radiative rate constant ($k_r = \Phi/\tau$). ^e Nonradiative rate constant ($k_{nr} = (1 - \Phi)/\tau$).

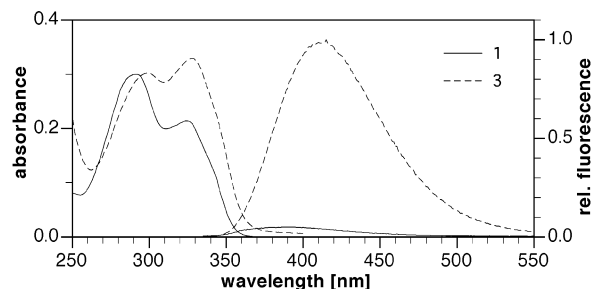


Figure 1. Absorption and fluorescence emission spectra (λ_{ex} 320 nm) of compound **1** (—) and **3** (---) (30 μM , 10 mM PIPES pH 7.20, 25 $^{\circ}\text{C}$).

lifetimes of **1** (<7 ns) and **3** (3.7 ns) are similarly short, suggesting that emission occurs for both compounds from a $^1(\pi,\pi^*)$ and not $^1(n,\pi^*)$ state.⁸

While the kinetics for the [3 + 2] cycloaddition reaction of alkynes with azides is slow, the reaction rate increases strongly in the presence of Cu(I).² Interestingly, Cu(I) complexes of tris-triazole ligands such as **4** are highly effective catalysts for this reaction.⁴ If 5 μM of **1** is converted in the presence of 100 μM azide **2** and 60 μM of the Cu(I) catalyst, the reaction is complete in less than 5

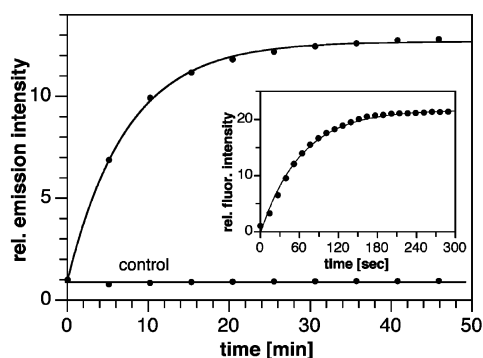


Figure 2. Kinetics for the reaction of **1** with **2** at pH 7.2 (10 mM PIPES, 37 °C). Conditions: 60 μ M **1**, 20 μ M **2**, in situ-formed catalyst: 60 μ M **4**, 60 μ M CuSO₄, 300 μ M ascorbic acid as reducing agent. Control without azide **2**. Inset: identical conditions, but 5 μ M **1**, 100 μ M **2** (λ_{exc} 350 nm, λ_{em} 420 nm).

Table 2. State Energies and Dipole Moments^a

	1a	3a
GS dipole moment [D] ^b	9.3 (7.2)	16.5 (13.0)
π - π^* exptl [eV] ^c	3.82	3.78
π - π^* energy (S ₁) [eV] ^b	3.86 (3.94)	3.70 (3.90)
dipole moment [D] ^b	12.4 (5.1)	21.6 (14.1)
n - π^* energy (S ₂) [eV] ^b	3.92 (3.70)	4.08 (3.80)
dipole moment [D] ^b	4.6 (2.3)	12.40 (8.1)

^a Based on INDO1/s CIS calculations using ground-state geometries optimized at the (B3LYP/6-31G*) level. ^b SCRF solvent model with $a_0 = 4.5$ and 4.9 Å cavity radii for **1** and **3**, respectively. Gas-phase values in parentheses. ^c 0.1 M KCl, pH 7.20 (PIPES 10 mM), 25 °C.

min. To evaluate the ligation kinetics under conditions that would be more typical for biological labeling applications, we reacted 20 μ M of azide **2** in the presence of 60 μ M dye **1**. Within approximately 30 min more than 90% of the azide had been converted, and the reaction is completed in less than 1 h (Figure 2).

To gain further insights into the nature of the fluorescence switching mechanism we performed semiempirical quantum mechanical calculations at the INDO1/s level of theory.⁹ The predicted vertical excitation energies listed in Table 2 are based on the optimized ground-state geometries of the model compounds **1a** and **3a** containing methyl groups in place of the succinyl- and benzyl-substituents. While the calculated gas-phase excitation energies are expectedly higher than the experiment by 0.1–0.2 eV, the SCRF solvent model¹⁰ implemented in the ArgusLab software package⁹ provided a good approximation for the state energies in water.

In agreement with the experimental data, the calculations predict that emission in **1** occurs from a ¹(π , π^*) state (Figure 3). However, the ¹(n , π^*) state lies only slightly above the ¹(π , π^*) state, suggesting a lower ³(n , π^*) energy compared to ¹(π , π^*). According to the El-Sayed rule¹¹ such an energy ordering results in fast intersystem crossing from the ¹(π , π^*) state to the nonemissive ³(n , π^*) state, and typically yields a large nonradiative deactivation rate, which has been indeed observed for **1** (Table 1). In contrast, the triazole-substituent in **3** greatly increases the charge-transfer character of the ¹(π , π^*) excited state, but has the opposite effect on the ¹(n , π^*) state as indicated by the differences of ground- and excited-state dipole moments (Table 2). As a consequence the ¹(n , π^*)–¹(π , π^*) energy gap increases and yields an inverted energy ordering of the ¹(π , π^*) and ³(n , π^*) states. Due to the inherently small singlet–triplet splitting of (n , π^*) states ($\Delta E_{\text{ST}} \approx 0.1$ eV),¹² the ³(n , π^*) level

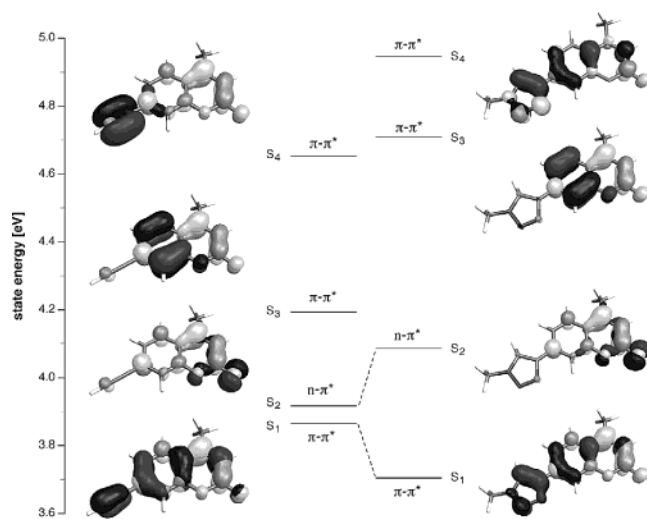


Figure 3. Excited-state energy levels for **1a** and **3a** based on INDO1/s calculations. The surface plots represent densities of the MOs with the largest contribution to the CI-eigenvectors (dark- and light-shaded surfaces refer to occupied and unoccupied MO densities, respectively).

lies now sufficiently above the ¹(π , π^*) state, rendering nonradiative deactivation energetically unfavorable via intersystem crossing to the ³(n , π^*) state.

In conclusion, the electron-donating properties of the triazole ring formed in the azide–alkyne ligation reaction can be effectively utilized for the design of a chemoselective fluorogenic probe that may find a range of applications in biology, analytical chemistry, or material science.

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Supporting Information Available: Experimental details, synthesis, and characterization of compounds **1** and **3** and all intermediates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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