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Ylidenemalononitrile Enamines as Fluorescent “Turn-On” Indicators for Primary Amines

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Supporting Information

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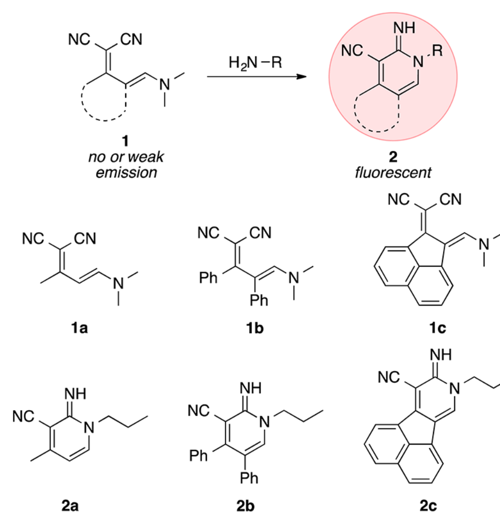
ABSTRACT: Ylidenemalononitrile enamines undergo rapid amine exchange followed by a cyclization with primary amines to yield fluorescent products with emission intensities as high as 900 times greater than the starting materials. After identifying the fluorescent species by X-ray crystallography, we demonstrate that the rate of amine exchange is substrate dependent and that by simple structural variation the fluorescence can be tuned over the entire visible spectrum. We further demonstrate their potential application in biomolecule labeling.

Chromophores and fluorophores reactive toward specific functional groups, such as amines, are valuable for detection and labeling.[1] While many amine reactive labels or dyes exist, they often lack the fluorescence “turn-on” and/or shift in λ_{max} upon reacting that are ideal, and sometimes necessary, for the differentiation of the labeled species and unbound dye.[2] The invariant photophysical properties make these labels less ideal for *in situ* studies where purification of the labeled species and unbound dye is undesired or difficult.[3]

Recently, new probes/labels containing functional groups that react with primary amines to form push−pull anilines,[4] imines,[5] hemiaminals,[6] or enamines[7] have emerged. These next generation compounds yield products with shifted λ_{max} and/or an increased quantum yield relative to the starting material. One example is the commercially available natural product epicocconone that, upon the addition of primary amines, is transformed from a weak green emitter to a bright orange emitter.[7] Despite these successes, most “turn-on” probes possess large molecular weights and are difficult to modify in order to change the emission properties. A broader range of applications could be realized with a smaller, more easily modifiable class of “turn-on” probes.

Here we introduce a new class of weakly fluorescent ylidenemalononitrile enamines (**1**) that undergo a “turn-on” mechanism when reacted with primary amines. The basic elements are shown in Scheme 1 where an amine exchange and cyclization occurs converting virtually nonfluorescent enamines into fluorescent cyclic amidines (**2**). The products exhibit both a spectral shift and as high as 900-fold greater emission intensity enhancement relative to the starting materials. The mechanism for the “turn-on” enables the system to be selective toward alkyl primary amines. Additionally, the fluorescent species can be tuned to yield colors ranging from cyan to orange by altering the enamine substituents.

Scheme 1. Reaction Between 1 and a Primary Amine to Produce 2



Previously, we demonstrated that enamines such as **1a** could be synthesized in moderate to high yields from ylidenemalononitriles and *N*,*N*-dimethylformamide dimethyl acetal in the presence of acetic anhydride and could lead to polysubstituted nicotinitriles after a subsequent Pinner cyclization.[8] We then discovered that species **1a−c** react with primary amines to yield fluorescent species (**2a−c**) (Scheme 1) when we attempted to produce 2-aminopyridines by treating **1a** with propylamine and heat.[9] Analysis by ^{1}H NMR spectroscopy demonstrated that **1a** reacts with propylamine (∼300 min; room temperature) to yield a new species with changes to the vinyl protons (Figure 1a). These resonances suggested that the new product was either the isomerized *cis*-enamine[10] or the acyclic amidines[11] **2a** (Scheme 1).

The ambiguity of the ^{1}H NMR spectra was resolved by obtaining the crystal structures of **2a** and **2b** (Figure 1b,c). Analysis of the structures revealed that the reaction between the enamine and primary amine yields rigid, cyclic amidines. The amidine moieties of **2a** and **2b** are plannar suggesting that each is fully conjugated; however, **2a** exhibits uneven bond-length distribution when compared to **2b** indicating that **2a** is less aromatic than **2b**.

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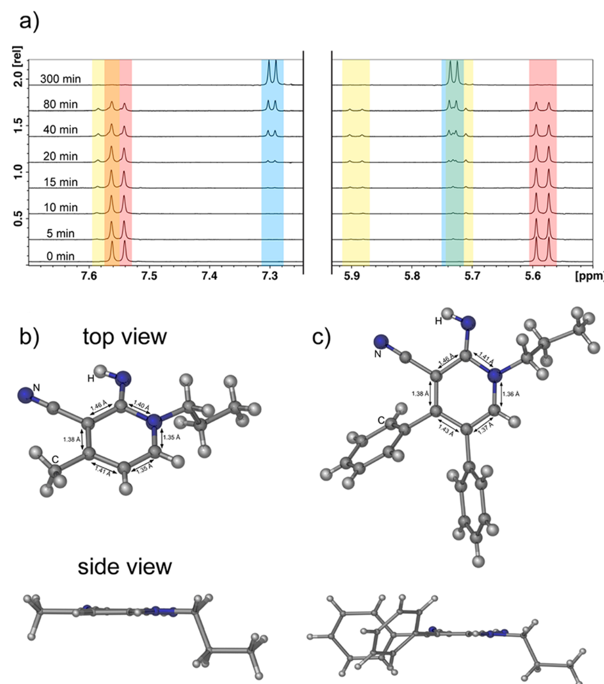

Figure 1. (a) ^{1}H NMR spectra of the reaction between **1a** (100 mM) and propylamine (12 equiv) in acetonitrile-d_3 over time. **1a** is highlighted in red, **2a** is highlighted in blue, and a possible intermediate(s) is highlighted in yellow. Crystal structures of (b) **2a** and (c) **2b**. For clarity, larger images are located in the Supporting Information.

DFT calculations of compounds **2a−c** were performed at the B3LYP/6-31G* level with a static electric field representing CH_2Cl_2. Based on the trend in HOMO−LUMO gap, we anticipated an increasingly lower energy absorption and emission in the order of **2a** > **2b** > **2c**. In accordance with this prediction, a broad range of emission colors can be achieved (Figure 2). When **1a−c** were treated with propylamine in a solution of CH_2Cl_2 or acetonitrile, **2a**, **2b**, and **2c** provided cyan (CIE coordinates: 0.24, 0.38), green (0.33, 0.55), and orange (0.48, 0.51), respectively (Figure 2). The photophysical properties of complexes **1a−c** and **2a−c** are summarized in Table 1.

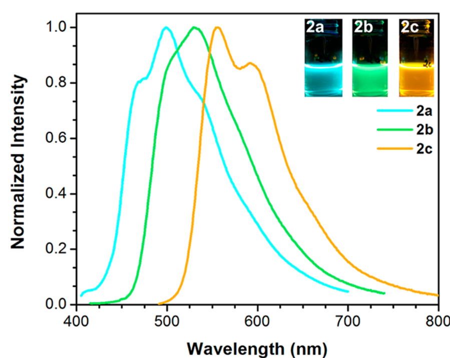

Figure 2. Normalized emission spectra of **2a−c** in CH_2Cl_2 at room temperature. Photographs shown are of 1 mM solutions of **2a−c** in CH_2Cl_2 under 365 nm irradiation.

The emission intensity "turn-on" of the complexes is best exemplified by comparing the fluorescent quantum yields (Φ_{PL}) for **1** and **2** as summarized in Table 1. When comparing **1a** (0.1%) to **2a** (8.0%), **1b** (0.035%) to **2b** (7.0%), and **1c** (0.016%) to **2c** (14.2%), there is an approximate 80-, 200-, and 900-fold increase in Φ_{PL}, respectively. Presumably, the lack of emission in compounds **1a−c** at room temperature is due to torsional relaxation of the dimethylamine or photoinduced electron transfer (PET). At 77 K, in glassy solvent (2-MeTHF), the emission intensity and lifetime of **1a−c** significantly increase suggesting torsional and vibrational relaxations are the primary nonradiative pathways, which is inhibited by the rigid matrix.

The real-time fluorescent "turn-on" time course of **1a** is shown in Figure 3a. A solution of **1a** in acetonitrile was treated with propylamine, and the emission was monitored over time. In 80 min, there is a ∼75-fold increase in emission intensity. The visible emission change associated with **1a** to **2a** is illustrated in Figure 3b.

The reaction rate between propylamine and **1a** (Figure 3a) and **1b** (data not shown) is slow and requires concentrations too high to function within real-time sensors or for *in vivo* studies. On the other hand, the reaction of **1c** to generate **2c** under the same conditions is visibly faster than that observed with **1a** and **1b**. The formation of **2c** is also visible in ambient light, where the color of the solution changes from magenta to orange as **1c** transforms into **2c**. Therefore, the reaction kinetics of **1c** in the presence of propylamine was easily monitored by UV−vis spectroscopy. The absorption spectrum of **1c** (0.045 mM in acetonitrile) was monitored once a second for 300 s after the addition of 355 equiv (16 mM) of propylamine. The spectral changes indicate a transition from **1c** (red) into **2c** (pink) (Figure 4).

Spectral changes could be satisfactorily fit with the SPECFIT/32 global analysis software package based on a model with two consecutive first-order reactions, A → B → C.[12] The rate constant for the first step ($k_{A\rightarrow B} = 0.034\ s^{-1}$) is three times slower than the second ($k_{B\rightarrow C} = 0.109\ s^{-1}$). Under the assumption that the amine exchange occurs first (yielding a propylamine enamine intermediate), the reaction is rate limited by the amine exchange, which is then followed by a faster cyclization step. The enhanced reaction rate of **1c**, relative to **1a** and **1b**, may be due to the increased conjugation stabilizing the formation of the intermediate. An intermediate species, B, was also observed when the reaction with **1a** was monitored by ^{1}H NMR spectroscopy (Figure 1a).

The reactivity and selectivity of emission "turn-on" with a variety of amines were then investigated (Figure 5). Steric effects play a significant role in the rate of "turn-on." Where propylamine (*i* in Figure 5) is nearly complete at 5 min, isopropylamine (*ii*), cyclohexylamine (*v*), and benzylamine (*vi*) take at most 2 h to induce a full "turn-on". With very bulky amines, such as *t*-butylamine (*iii*), there was a nominal increase in emission intensity even after 24 h. We also investigated the effect a tethered alcohol or amine would have on the "turn-on". The ethanolamine (*iv*) reaction is nearly complete in 5 min indicating that the alcohol group does not greatly affect the reaction kinetics or emission yield when compared to propylamine (*i*). The tethered amine present in 3-(dimethylamine)-1-propylamine (DMAPA) (*vii*), however, causes a large decrease in emission intensity. Since the amine exchange was confirmed to be complete within minutes by ^{1}H NMR, this decrease may be due to PET between the dimethylamine and the fluorophore. Nonalkyl primary amines show no significant increase in emission (*viii*, *ix*, *x*, and *xi*). Having a mixture of a

Table 1. Photophysical Properties of 1a−c and 2a−c at Room Temperature and 77 K

		emission at room temperature^a					emission at 77 K^d	
compound	absorbance λ (nm) (ε, $\times 10^4$ M^{-1} cm^{-1})^a	λ_{max} (nm)	τ (ns)	Φ_{PL} (%)	k_r (10^7s^{-1})^b	k_{nr} (10^8s^{-1})^c	λ_{max} (nm)	τ (ns)
1a	388 (4.9), 377 (4.6)	410	–	0.1^e	–	–	410	2.0
1b	395 (2.9), 296 (1.0), 261 (1.0)	560	–	0.035^e	–	–	540	6.4
1c	541 (1.3), 520 (1.2), 356 (2.8), 300 (1.5), 267 (1.6)	605	–	0.016^e	–	–	609	12.3
2a	390 (4.8)	500	8.0	8.0^f	1.00	1.15	465	18.0
2b	425 (0.5), 276 (2.4)	530	3.3	7.0^f	2.12	2.81	490	16.1
2c	511 (0.4), 481 (0.5), 390 (1.0), 368 (0.9), 341 (2.9), 325 (2.0)	555	8.5	14.2^f	1.67	1.01	541	17.7

^{a}In CH_2Cl_2. $^b k_r = \Phi/\tau$. $^c k_{nr} = (1 - \Phi)/\tau$. ^{d}In 2-MeTHF. ^{e}Relative quantum yields calculated with the corresponding amidine as a reference. ^{f}Obtained by using an integrating sphere.

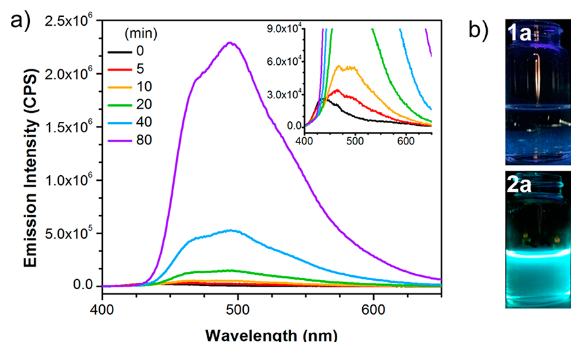

Figure 3. (a) Emission spectra of **1a** (100 mM) in acetonitrile at 0, 5, 10, 20, 40, and 80 min after the addition of propylamine (12 equiv). An aliquot of the reaction was diluted to 1 mM before obtaining the emission spectra. (b) Solutions of **1a** and **2a** (1 mM) in CH_2Cl_2 under 365 nm irradiation.

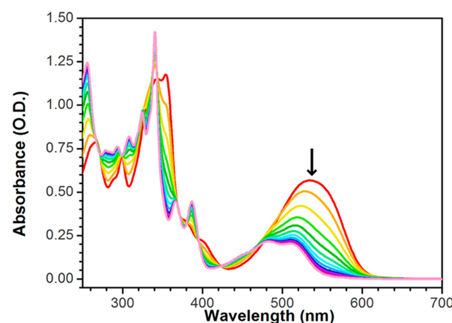

Figure 4. Changes in the absorption spectra of **1c** (0.045 mM in acetonitrile) after the addition of propylamine (16 mM, 355 equiv). Spectra were acquired once a second for 300 s (0 s (red) to 300 s (pink)). Only every 10th spectra is included in the figure for clarity. Product generation is 90% complete in approximately 40 s.

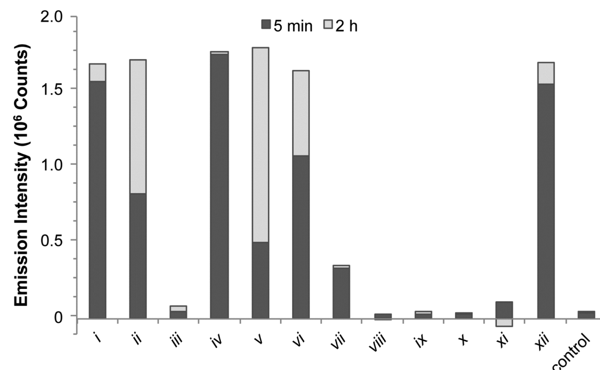

Figure 5. Relative emission intensities at 625 nm depicting the "turn-on" selectivity of **1c** toward alkyl primary amines when excited at 482 nm at 5 min and 2 h. Samples were prepared by treating a solution of **1c** (0.9 mM, acetonitrile) with the appropriate amine(s) (12 equiv, 11 mM). Samples were diluted (0.025 mM) before obtaining emission spectra. Amines are as followed: (*i*) propylamine, (*ii*) isopropylamine, (*iii*) *t*-butylamine, (*iv*) ethanolamine, (*v*) cyclohexylamine, (*vi*) benzylamine, (*vii*) DMAPA, (*viii*) aniline, (*ix*) pyrrolidine, (*x*) TEA, (*xi*) pyridine, (*xii*) 1:1 mixture of TEA and propylamine.

nonreactive amine, triethylamine (TEA), with propylamine (*xii*) has no effect on the "turn-on" process.

While emission "turn-on" upon reaction with common amines is applicable toward detecting chemical leakage or food spoilage,[4,5b,13] smaller, longer-wavelength emitting fluorescent molecules could have great impact as protein labeling reagents.[3b] To demonstrate the enamines' ability to react and label relevant biomolecules, human transferrin glycoprotein (310 μM, 50:50 vol % DMSO/PBS buffer pH = 8.5) was labeled with **1c** (3.1 mM). Labeled transferrin conjugates are very useful as endosome markers for cellular studies.[14] However, purification of the labeled conjugates from the unlabeled dye is known to be difficult,[3c] and the "turn-on" dye could eliminate the need for purification. Within 4 h of the reaction, the magenta solution turned yellow signifying **1c** was consumed. Denaturing of the protein and long reaction times were not necessary for sufficient labeling, which is ideal for maintaining the initial function of most proteins. The labeled transferrin was easily purified by size-exclusion chromatography before the emission spectrum was obtained to ensure the protein was labeled (Figure 6).

In conclusion, we have shown that ylidenemalononitrile enamines can act as fluorescent "turn-on" indicators toward alkyl primary amines. This acyclic to cyclic "turn-on"

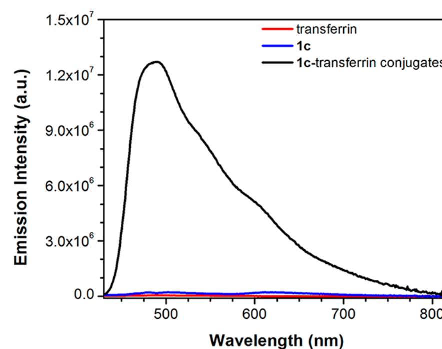

Figure 6. Emission spectra of transferrin, **1c** (30 μM, 50:50 vol % DMSO/PBS buffer, pH = 7), and purified **1c**-transferrin conjugates (~30 μM after purification, PBS buffer, pH = 7) at room temperature. Excitation at 420 nm.

mechanism is unique, and we suggest that is the origin of the excellent primary amine selectivity observed. We also demonstrate that the emission maximum and the reaction kinetics are easily tuned by making changes to the position flanking the ylidenemalononitrile. Having a series of primary amine reactive probes with different exchange rates might have application in both real-time biosensing and array-based sensor strategies, as we have already demonstrated their ability to label the transferrin glycoprotein.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, spectroscopy data, and CIF files. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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