Chemiluminescence

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Chemiluminescent Energy-Transfer Cassettes Based on Fluorescein and Nile Red**

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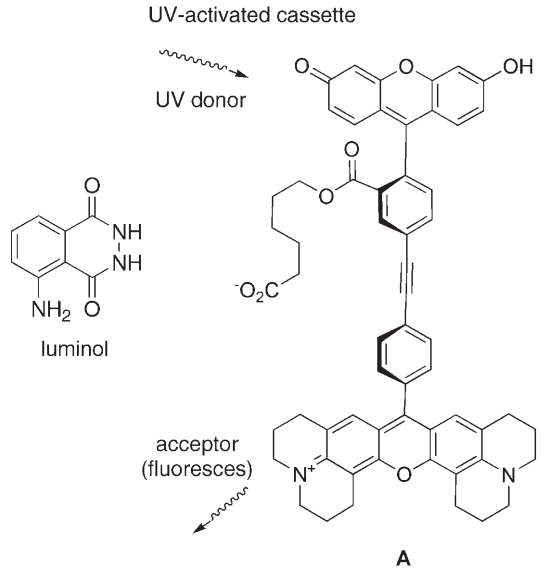
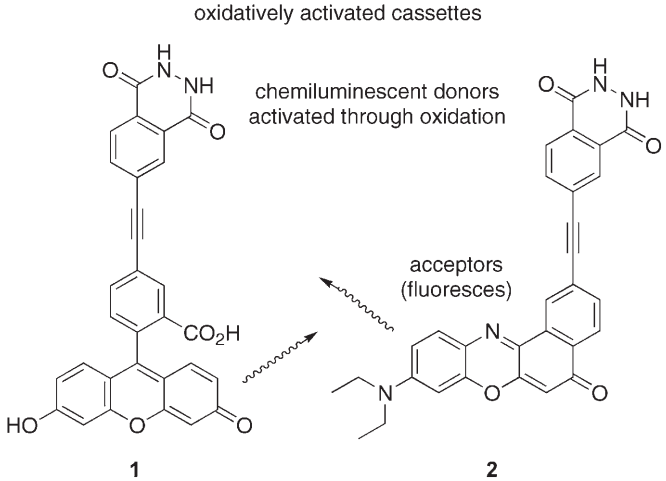
The two most common ways to induce chemiluminescence in purely organic, nonbiological systems are to treat either oxalate esters or luminol derivatives with basic hydrogen peroxide.[1,2] Both these types of mixtures emit light at relatively short wavelengths, which are not ideal for applications in biotechnology. Luminol, for instance, emits in the range 420–450 nm, depending on the solvent media.[3] Intimate mixtures of oxalate esters or luminol,[4] an oxidant, and an acceptor dye give longer wavelength emissions through intermolecular energy transfer. This results in the mesmerizing, long-lived emissions seen in "light-stick devices". However, the options for forming discrete probes for biotechnology that emit at longer, and generally more useful, wavelengths are limited.[5–10]

An ongoing project in our group features twisted, but otherwise conjugated, donor and acceptor cassettes for labeling biomolecules.[10,11] The motivation for this is that energy transfer can occur through bonds as well as through space, hence it can be relatively fast and efficient. All our published research to date features cassettes based on UV-absorbing donors, like compound **A**. We thought it would be intriguing to make cassettes where the donor might be activated chemically instead. Oxalate esters are not useful donors for through-bond energy-transfer cassettes because it is impossible to conjugate an acceptor to the oxalate fragment. Consequently, luminol-based systems were selected. Described herein are the syntheses and spectroscopic properties of the fluorescein- and nile red based, chemically activated cassettes **1** and **2**.

Nearly all luminol derivatives are almost insoluble in most organic media, and this makes them extremely difficult to manipulate. After considerable experimentation, one solution to this problem emerged: bis(*N*-protection) of compounds like **3** with 4-methoxybenzyl (PMB) groups. This approach gave organic-soluble, easily chromatographed intermediates, and the PMB group is removed in the closing stages of the synthesis through treatment with trifluoroacetic acid (TFA). Thus, Scheme 1 shows the syntheses that evolved to form compounds **1** and **2**. In both routes, the cyclic hydrazide **3** was bis-*N*-protected, then elaborated through Sonogashira reactions[12] featuring derivatives of 5-bromofluorescein[13] and 2-hydroxy nile red.[14] The route to the cassettes would have been more convergent if an alkyne derivative of luminol could have been coupled with halogenated/triflated acceptors, but that approach was ineffective.

It is hard to describe in words the spectacular chemiluminescence of these compounds without films of the experi-





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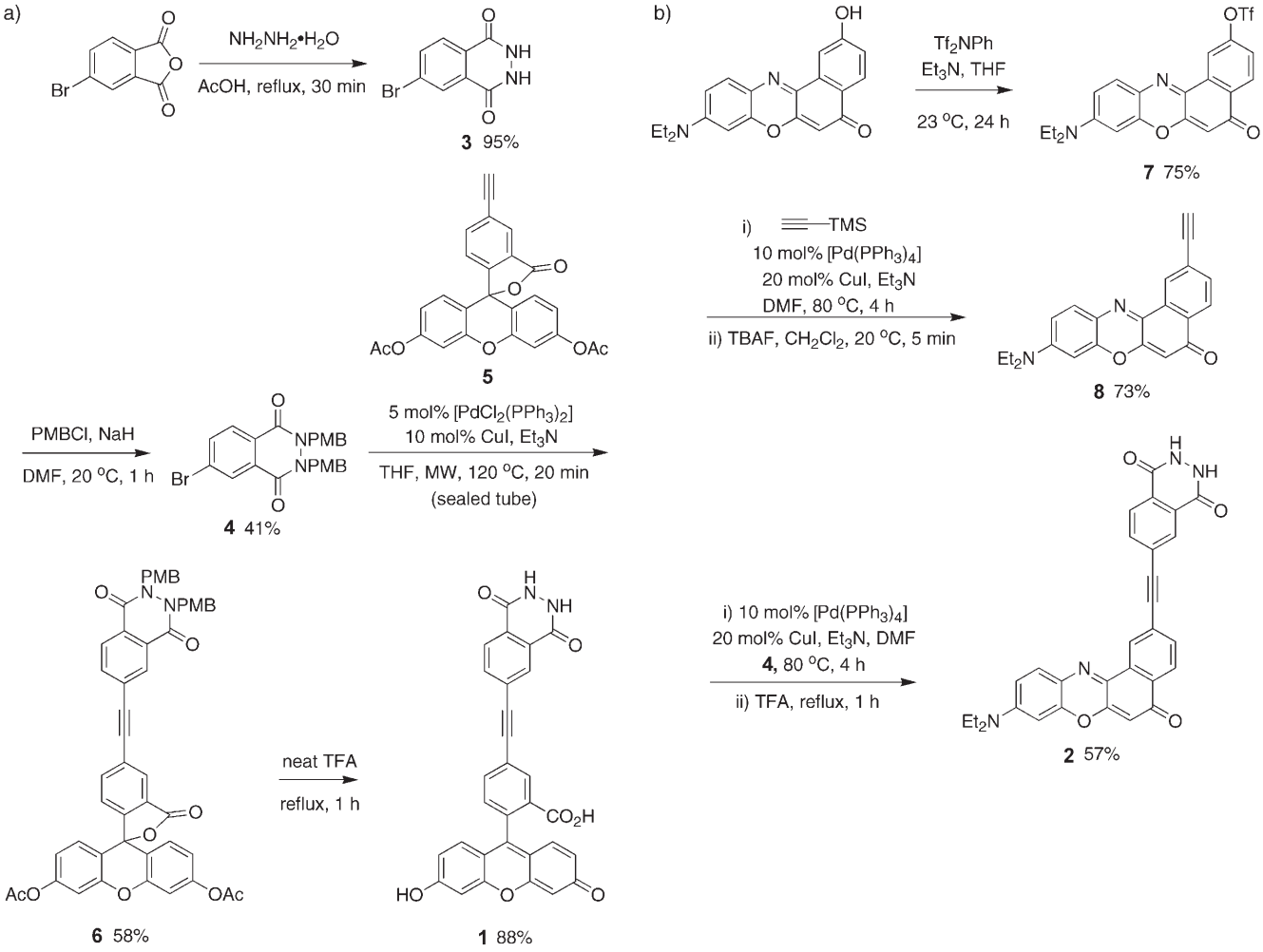


Scheme 1. Preparation of a) the fluorescein cassette **1** and b) the nile red based cassette **2**. MW = microwave treatment, Tf = trifluoromethanesulfonyl, TBAF = tetrabutylammonium fluoride, TMS = trimethylsilyl.

ments (Figure 1). For cassette **1**, 100-μL aliquots of the compound (10^{-5} M, in pH 10 aqueous $Na_2CO_3/NaHCO_3$ buffer solution) were added with stirring to a sample cell containing $CuSO_4$ (1.5×10^{-3} M) and H_2O_2 (2.0×10^{-3} M). Cassette **2** is not very soluble in aqueous media and, in any case, the quantum yield for nile red emission is less than 0.1 in water. Consequently, for **2**, potassium *tert*-butoxide in THF (10^{-2} M) was added to the compound dissolved in anhydrous *N*,*N*-dimethylformamide (DMF; 10^{-5} M). This experiment was done open to the air, and oxygen is presumed to be the oxidant. Luminol, under the conditions used for cassette **1**, gives a bright blue emission. However, if efficient energy transfer occurs for compounds **1** and **2**, then it is expected that yellow/green and red chemiluminescence are emitted, which are characteristic of fluorescein and nile red, respectively. This is exactly what we saw. Cassette **1** gave a bright yellow/green emission, whereas **2** glowed with a less-intense red color. No trace of blue in the emission was seen in either case.

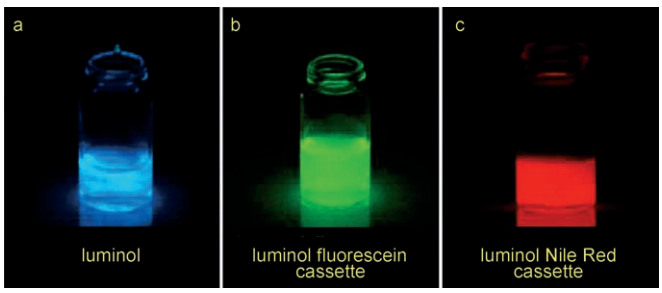
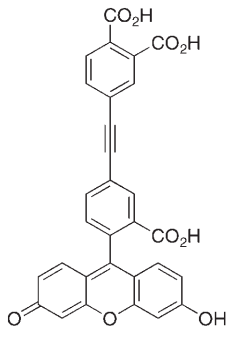


Figure 1. Pictures of a) luminol, b) cassette **1**, and c) cassette **2** when activated with an oxidant.



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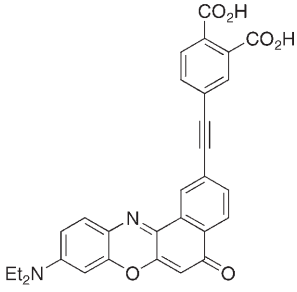

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Compounds **9** and **10** were prepared as controls (see the Supporting Information) as it is thought that the excited species from luminol derivatives involves the corresponding phthalate dianions. Indeed, ESIMS analysis of cassettes **1** and **2** after the oxidative activation revealed the presence of **9** and **10**, respectively. This was confirmed through HPLC analyses in the case of **1**.

Figure 2a shows normalized UV absorption and fluorescence spectra for **9** and **10**. The extent of overlap between the chemiluminescence output of the phthalate derived from luminol and the UV absorption of the acceptor part of cassettes **9** and **10** is shown in Figure 2b. Normalized chemiluminescence emissions for **1** and **2** are shown in Figure 2c. The emissions of **1** and **2** are sharp and characteristic of the acceptors only; no chemiluminescence from the donor was detected.

Sometimes, the eyes can play tricks on the brain and that is partially true in this case. The chemiluminescence from cassette **2** appears to be weaker than that for **1**, but the quantitative data collected in Table 1 indicates that this is not the case. Chemiluminescent quantum yields measured relative to luminol indicate that **2** actually emits more strongly. Probably, chemiluminescence from **2** appears to be weaker than that from **1** because the human eye is about five times more sensitive to light near the emission maximum of fluorescein than it is to light emitted from the nile red acceptors.[15]

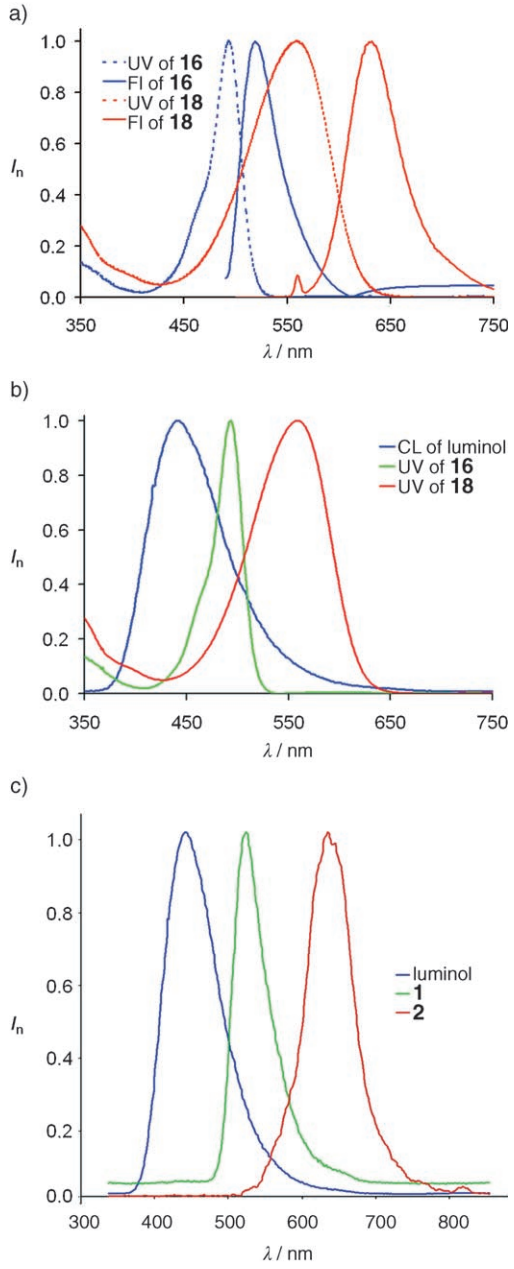


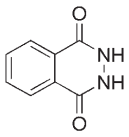
Figure 2. a) Normalized UV/Vis (UV) and fluorescence (Fl) spectra of **9** in pH 10 aqueous sodium carbonate/bicarbonate buffer solution, and of **10** in anhydrous DMF; b) normalized chemiluminescence (CL) spectrum of luminol (blue), UV/Vis absorption bands of compound **9** (green) and compound **10** (red); c) normalized chemiluminescence spectra of luminol (blue), cassette **1** (green), and cassette **2** (red). I_n = normalized intensity.

Table 1: Selected spectroscopic properties of luminol, **1**, **9**, **2**, **10**, and **11**.

Cmpd.	UV	Fluorescence	Chemiluminesence	
	$\lambda_{abs\ max}$ [nm]	$\lambda_{fl\ max}$ [nm]	$\lambda_{chemi\ max}$ [nm]	Rel. Φ_{chemi}
luminol[a]	–	–	442	100
1[a]	494	518	524	61
9[a]	493	519	–	–
2[b]	558	628	634	>100
10[b]	558	628	–	–
11	–	–	412	0.02[c]

[a] In carbonate/bicarbonate buffer solution. [b] In anhydrous DMF. [c] From reference [6].

The relative quantum yields for chemiluminescence that are presented in Table 1 use luminol as a standard. However, the donor fragments of cassettes **1** and **2** do not have the amino substituent of luminol. Small changes to the luminol structure tend to reduce its chemiluminescence dramatically.[3] If the emissions from cassettes **1** and **2** are compared with the hydrazide **11** (which has a much lower absolute quantum yield for chemiluminescence), then the data for cassettes **1** and **2** would appear to be even more impressive.



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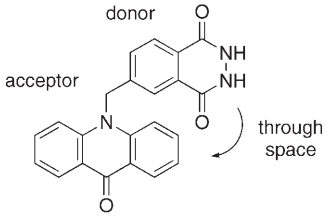
Experimentally, it is extremely challenging to determine the extent of energy transfer through bonds and through space in twisted but otherwise conjugated cassettes. For the UV-activated system **A**, we asserted that through-bond energy transfer must be fast and efficient by considering rates of energy transfer.[16] However, direct observation of rates is hard in chemically activated systems in which excitation of the donor occurs continuously. Further, we have so far been unable to prepare the logical control compounds for comparison: those in which the alkyne linker of cassettes **1** or **2** are replaced by an ethylene fragment. In any case, through-space energy transfer for those controls might differ considerably from that occurring in **1** or **2** because the orientation of the donor and acceptor fragments would be dynamic in the reduced compounds. However, the

through-space energy-transfer cassette **B**, based on luminol, was prepared approximately four decades ago and does provide an interesting comparison.[17–19] The reported relative chemiluminescence quantum yield for this compound (luminol standard) is significantly less than that measured here for cassettes **1** and **2**. It may be that, just as in our UV-activated cassettes like **A**, rapid and efficient energy transfer can occur for the systems that facilitate the possibility of through-bond energy transfer.



B
relative Φ_{chemi} = 8
(luminol = 100)

Calculations of Förster energy transfer for systems that have donor and acceptor fragments arranged within a few Ångstroms are not correct because the theory implies a point dipole approximation that fails when the distance becomes less than the special size of the donor and acceptor charge distributions. Nevertheless, these calculations were performed: the dipole–dipole energy-transfer efficiency was smaller (39 and 42% for **1** and **2**, respectively) than actually observed.

Chemiluminescence provides detection methods that approach the sensitivity of that of radioactivity-based methods.[20] In the context of intracellular imaging, it has the advantage that no excitation irradiation is required. Simple experiments in vitro show that cassettes **1** and **2** can be activated through treatment with peroxidase under physiological conditions. Furthermore, they emit in longer wavelength regions that are more transparent to cellular tissues than the 420–450-nm range in which luminol chemiluminesces. Consequently, there is a possibility that probes based on chemically activated energy transfer can be applied in biotechnology.

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