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Merocyanine Dyes with Improved Photostability

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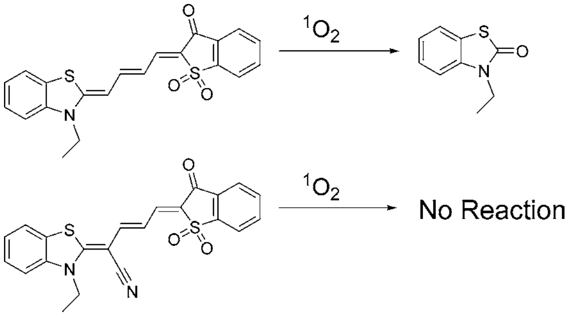
ABSTRACT



Merocyanine dyes have proven valuable for live cell fluorescence imaging applications, but many structures have been limited by rapid photobleaching. We show that photostability is substantially enhanced for merocyanines having a cyano group at a specific position in the central polymethine chain. Evidence is presented that this is due to reduction in reactivity of the dyes with singlet oxygen. These results point toward cyano-substitution as a general strategy for improving dye performance in imaging applications.

Merocyanine dyes are heterocyclic chromophores that are extensively used in a number of areas (i.e., as photographic sensitizers, for nonlinear optics, and in chemotherapy).[1−6] Recently they have also been employed as sensors of protein conformation and protein interactions in live cell imaging.[7,8] The efficacy of merocyanine dyes as components of biosensors depends not only on their fluorescence emission properties, but also on their photostability. Unfortunately, in many cases very useful merocyanine dyes degrade rapidly in light. In our recent work we showed that the major photodegradation path is oxidation of the dyes by photogenerated singlet oxygen.[8] The ground (triplet) state of oxygen is a very effective quencher of the triplet excited state of the dye, with a rate of about 10^9 L/mol·s.[9] Triplet−triplet energy transfer results in the production of highly reactive electrophilic singlet oxygen that reacts with the ground state of the dye and destroys the fluorophore.

Our initial focus was to find the position of singlet oxygen attack in dye molecules. We photobleached methanolic solutions of the dyes **I−SO**[8] and **S−SO**[8] and measured

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product formation using LC-MS. Analysis of the reaction mixtures revealed that the major product was either oxindole (**1**) or benzothiazolone (**2**) (Figure 1). The formation of these products can be explained by the initial attack of singlet oxygen on the most nucleophilic α-carbon of the central polymethine chain, with formation of dioxetane (**4**). Presumably, the zwitterionic intermediate (**3**) is the first addition product, as it was previously reported in singlet oxygen reactions with enamines.[10] The cleavage of the dioxetane results in the formation of two carbonyl fragments, and stable products benzothiazolone or oxindole can then be detected in the reaction mixture.

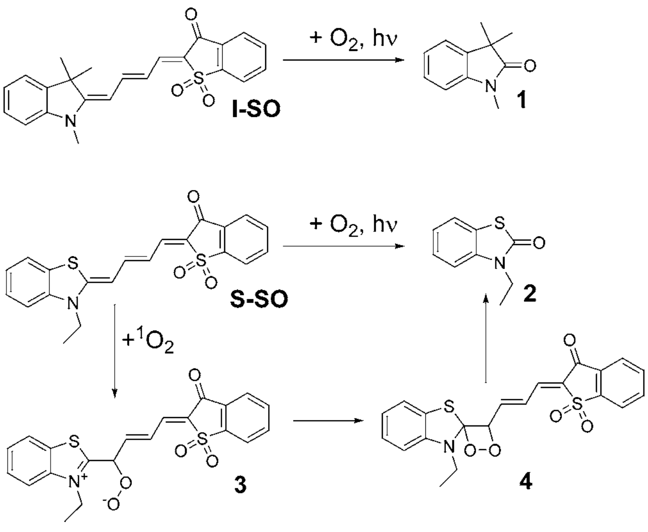
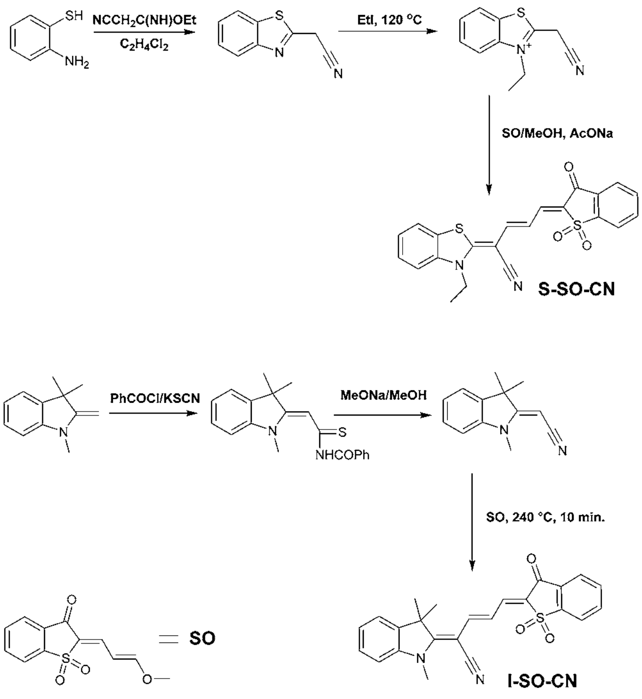


Figure 1. Mechanism of photoxidation of **I−SO** and **S−SO** dyes.

The proposed mechanism suggests that the rate determining step of the dye oxidation reaction will include charge transfer from the α-carbon atom to a singlet oxygen molecule.[11] Such a reaction could be inhibited by the attachment of an electron-accepting group to partially remove electron density from the α-carbon. The cyano-group (−C≡N) was chosen because it has strong electron-withdrawing ability and a small size; steric hindrance from bigger groups could potentially destroy the planarity of the dye molecule and thereby diminish the fluorescence quantum yields.[12] We synthesized dyes with a cyano group at the first carbon of the chain (**S−SO−CN** and **I−SO−CN**) (Scheme 1).

Scheme 1. Synthesis of Cyano-Substituted Dyes **S−SO−CN** and **I−SO−CN**



The photophysical and photochemical properties of **I−SO−CN** and **S−SO−CN** were compared with those of the unsubstituted analogues **I−SO** and **S−SO** (Table 1). The −CN group greatly improved the photostability of the dyes. Solutions of **I−SO**, **S−SO**, **I−SO−CN**, and **S−SO−CN** dyes were irradiated by a halogen tungsten lamp (150 W). Absorption spectra of the solutions were recorded at different times to monitor dye photobleaching. For all dyes, photobleaching followed first-order kinetics. Rate constants were obtained from a plot of ln *C* vs time (Figure 2, Table 1). As shown in Figure 2, the dyes with cyano groups were significantly more photostable than their unsubstituted counterparts. Cyano substitution decreased photobleaching rates for **I−SO** and **S−SO** dyes by 5.5- and 39-fold, respectively.

Table 1. Properties of the Dyes in Methanol

dye	absorbance λ_{max}/nm (ϵ^a)	emission λ_{max}/nm	Φ^b	photobleaching rate, s^{-1}
I−SO	586 (143 000)	615	0.03	1.33×10^{-6}
S−SO	598 (143 000)	617	0.05	5.23×10^{-6}
I−SO−CN	528 (55 000)	595	0.03	0.242×10^{-6}
S−SO−CN	547 (95 000)	597	0.05	0.135×10^{-6}

[a] Extinction coefficient. [b] Fluorescence quantum yield.

Introduction of the cyano group suppressed the extinction coefficients of the dyes in methanol by 34% (**S−SO**) and 62% (**I−SO**), and led to hypsochromic shifts of both absorption (about 55 nm) and emission (20 nm) wavelength maxima. The fluorescence quantum yields in methanol were unaffected.

We examined the reactivity of the dyes with singlet oxygen to confirm that the −CN substituent affected photobleaching mediated by singlet oxygen. Singlet oxygen was generated

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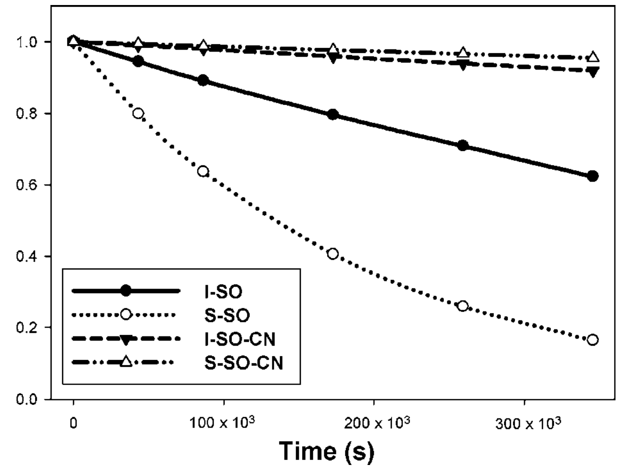

Figure 2. Absorbance decay of **I−SO**, **S−SO**, **I−SO−CN**, and **S−SO−CN** dyes in MeOH upon illumination with light.

by using the photosensitizer Methylene Blue (MB), which has a high quantum yield for singlet oxygen production and allows singlet oxygen to be introduced into the system without excitation of the merocyanine dye.[13] Light from a tungsten lamp was filtered to permit selective excitation of MB. Figure 3 shows that irradiation of the MB resulted in 43% bleaching of **S−SO** but no bleaching of **S−SO−CN**. Under the same conditions, **I−SO** and **I−SO−CN** dyes showed 27% and 0% bleaching. Thus introduction of −CN completely blocked detectable bleaching by singlet oxygen. The greater reactivity of **S−SO** vs **I−SO** toward singlet oxygen can be attributed to the greater electron donating capacity of the benzothiazole relative to the indolenine ring system.

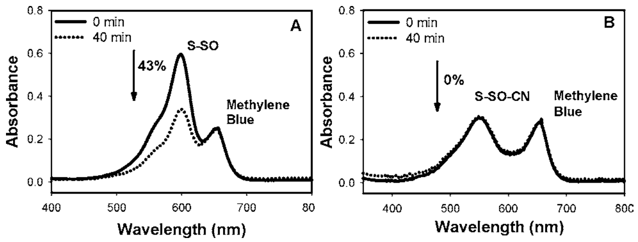

Figure 3. Methylene Blue-sensitized photobleaching of **S−SO** (A) and **S−SO−CN** (B). Samples were irradiated with filtered tungsten lamp light ($\lambda > 630$ nm) for 40 min [dye] = [Methylene Blue] = 3.33 μM.

The relative rates of **S−SO** and **I−SO** bleaching are different when bleaching is solely due to singlet oxygen (Figure 3) or to light (Table 1). The relative bleaching rate, $k_{\mathrm{S-SO}}/k_{\mathrm{I-SO}}$, was 1.78 in the MB solution, compared to 3.93 for light-induced bleaching (Table 1). This is likely because **S−SO** produces singlet oxygen more efficiently than **I−SO**. Heavy atoms, like the additional sulfur in **S−SO**, increase the probability of singlet−triplet intersystem crossing and promote singlet oxygen formation.[14]

Introduction of a cyano group blocked destruction of the dyes by singlet oxygen, yet the cyano-substituted dyes still underwent light-induced bleaching (albeit much more slowly). This likely involves a reactive oxygen species other than singlet oxygen, e.g., superoxide. Superoxide has been detected in solutions of cyanine dyes upon illumination with light, and is generated by electron transfer from the singlet excited state of the dye to an oxygen molecule.[15,16] This would be facilitated by higher energy excited states, consistent with our observation that the dye with shorter excitation wavelengths bleaches more rapidly (Table 1: $k_{\mathrm{S-SO-CN}}/k_{\mathrm{I-SO-CN}} = 0.56$).

Our results are consistent with a report in the literature that the photostability of cyanine dyes is improved by polyfluorination.[17] Electron-accepting fluorine groups were attached to the aromatic rings rather than to the polymethine chain of a benzthiazole-based cyanine dye. The observed effect was moderate: incorporation of eight fluorines in the dye molecule resulted in about a 3.5-fold decrease in the dye photobleaching rate.

These results point toward cyano substitution as a general strategy for improving dye performance in imaging applications. We are currently preparing other merocyanine dyes with cyano substituents in the polymethine chain to further address the mechanism of the photobleaching reaction. Work is in progress to prepare water-soluble analogues of cyano-substituted merocyanine dyes with reactive groups for attachment to proteins. These dyes will be used in the construction of new photostable biosensors for live cell imaging studies. Results of these studies will be reported in due course.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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