Dichromic Molecules

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Near-Infrared Dichromic Fluorescent Carbocyanine Molecules**

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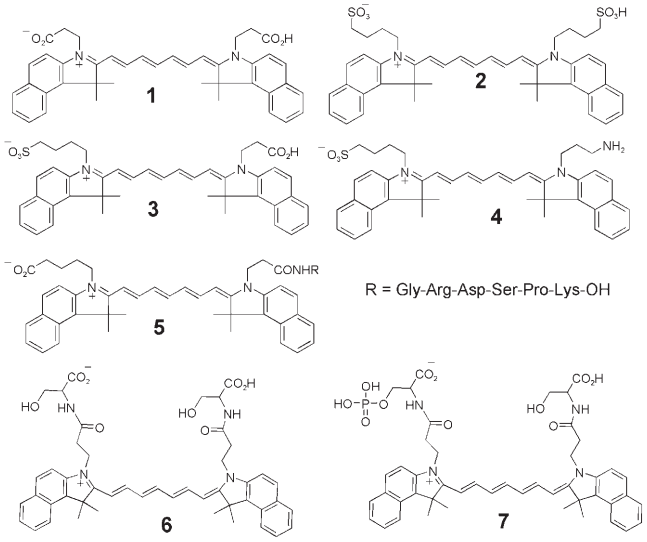
Central to major advances in biochemical assays, molecular sensor technologies, and molecular optical imaging are fluorescent materials that provide high detection sensitivity for molecular processes. In biological optical imaging, the low tissue autofluorescence and the deep penetration of light into the tissues observed at wavelengths between 650 and 900 nm allow the use of near-infrared (NIR) fluorescent dyes as contrast agents in heterogeneous systems.[1,2] The ease of synthesis, biocompatibility, tunable spectral properties, and exceptionally high molar absorptivity of NIR fluorescent carbocyanines provide added incentive for their use in cellular and animal imaging applications.[3,4] For these reasons, a variety of cyanine-based molecular probes are currently used for molecular imaging—including cypate (**1**), which possesses reactive carboxylic acid groups and the nonspecific indocyanine green (ICG, **2**) that incorporates two hydrophilic sulfonate groups (see Scheme 1).[3,5] To improve the hydrophilicity of cypate and minimize potential side reactions caused by the presence of the two carboxylic acid functions, we synthesized a hybrid of cypate and ICG. The new compound, ICG–CO_2H (**3**), has only one reactive carboxylic acid and a water-solubilizing sulfonate group. Similarly, we prepared an analogous amine derivative, namely, ICG-NH_2 (**4**) for labeling peptides or proteins via their activated carboxylic acid groups. In the course of these studies, we observed unusual spectral properties of the cyanine dyes that are amplified by the nonsymmetrical structural features of compounds **3** and **4**.

In previous studies, we and others have consistently reported the characteristic broad absorption and fluorescence emission bands of NIR carbocyanine dyes.[2–5] The spectral profiles of the absorption band typically possess a blue-shifted shoulder, which is generally attributed to the presence of H aggregates or other forms of electronic transitions. Previous literature reports have shown that nonsymmetrical cyanine dyes can generate multiple fluorescence from different excited states. These emissions are generally dependent on the solvents, the pH or the electrolytes used. Here, we present the first examples of NIR dichromic fluorescent molecules based on a carbocyanine dye molecular template. This phenomenon is favored by nonsymmetrical cyanine dye structures and manifested by an increase in the blue-shifted shoulder peak with minimal solvatochromic effect.



Scheme 1. Chemical structures of NIR fluorescent carbocyanines and derivatives.

The absorption and emission spectra of representative compounds studied are shown in Figure 1. In addition to possessing the expected long-wavelength fluorescence, the nonsymmetrical molecular probes **3** and **4** also exhibited a second emission band (at 700 nm), which is blue-shifted relative to the peak at about 800 nm. Initially, we attributed the second peak to the presence of impurities or the half-dye. However, after careful purifications and sample analysis, we confirmed that both emissions originated from the same compound. To further explore the prevalence of this phenomenon in NIR carbocyanine dyes, we prepared and evaluated the spectral properties of the widely used and symmetrical NIR dyes **1** and **2** as well as those of the nonsymmetrical cypate–peptide conjugate **5**.

As shown in Figure 1, an excellent overlap of the absorbance spectra of the symmetrical (**1** and **2**) and nonsymmetrical (**3** and **4**) compounds was observed, with **3** and **4** exhibiting a higher absorbance than other compounds at about 700 nm. This trend persisted in different solvents, thus

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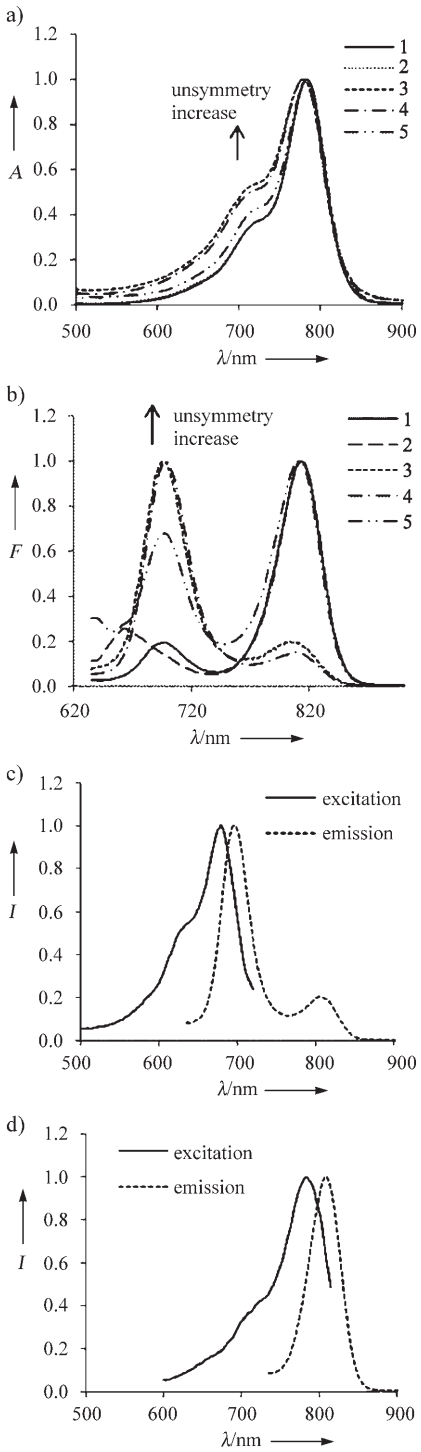

Figure 1. Spectral properties of NIR fluorescent dyes. a) Absorbance and b) dual fluorescence spectra of representative dyes and peptide conjugates. c,d) Fluorescence excitation and emission scans of compound **3** at the c) short (695 nm) and d) long (806 nm) wavelengths in methanol. The excitation was corrected by the lamp intensity (*S*/*N*) (*A* is the normalized absorption, *F* is the normalized fluorescence intensity, and *I* is the normalized absorbance and fluorescence intensity).

suggesting that the peak is an inherent feature of the nonsymmetrical dyes and does not originate from H aggregates (see solvent and pH effects below). In contrast to the absorption spectra, the emission spectral profiles of **1**–**4** were different; for example, the fluorescence of **3** at about 700 nm was much higher than that at about 800 nm, relative to **1** and **2**, upon excitation at 645 nm. Fluorescence excitation scans of **3** at the two emission peaks (namely, at 695 and 806 nm) resulted in calculated S0–S0 transitions, which correspond to 686 and 796 nm, respectively (see Figure 1, bottom). By combining the information from the fluorescence excitation spectra obtained at both wavelengths, we were able to deconvolute the broad absorbance spectra into two distinct absorbance spectra of **3** (Figure 2). The result shows that each

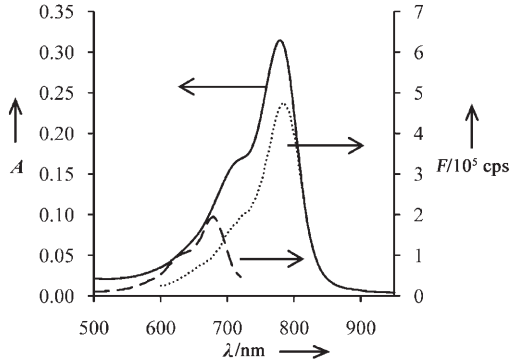

Figure 2. Deconvolution of the absorbance spectra of compound **3** into two distinct bands extracted from fluorescence excitation scans.

absorbance spectrum is characterized by a major peak and a shoulder. The striking similarities between the individual absorbance spectra indicate similar sets of $\pi \rightarrow \pi^*$ and other transitions.

The 100-nm shift in the two emission spectra suggests that the excited-state properties of the two fluorescence peaks may be different. This was confirmed by determining the fluorescence-lifetime properties of compounds **1**–**4**, which show that the fluorescence lifetime at 700 nm is more than 1.5 times longer than that at 800 nm (Table 1).

Table 1: Dynamic optical properties of representative cyanine molecules in DMSO.

Compound	Lifetime at 700 nm [ns][a]	Fractional contribution [%]	Lifetime at 800 nm [ns][b]
1	weak signal	99	0.87[6]
2	1.50[c]	99	0.97[6]
3	1.39	92	0.86
4	1.40	90	0.90

[a] Excitation: 650 nm; emission: 700 nm. [b] Excitation: 773 nm; emission: 820 nm. [c] Average of two lifetimes, namely, 1.26 ns (39%) and 1.59 ns (60%).

Interestingly, both symmetrical and nonsymmetrical cyanine dyes exhibit dual fluorescence at about 800 and 700 nm. The symmetrical dyes, such as ICG and cypate, have relatively small contributions of the 700-nm emission in the overall fluorescence spectra (namely, 18 and 24%, respectively). The weak signal may account for the omission of the blue-shifted

peaks in previous reports of the fluorescence properties of these compounds.[7–10] Upon conjugation of the symmetrical dye cypate with a peptide, the resulting nonsymmetrical cypate–peptide conjugate **5** exhibited a 45% increase in the level of the 700-nm emission band (relative to cypate), albeit still lower than the 82% obtained with **3** and **4**. Although the 100-nm blue-shifted emission peak could indicate the elimination of a methine bond from the heptamethine structure,[11] it is unlikely that the emission band at 700 nm resulted from the formation of lower homologues (such as tri- or pentamethines) of cypate or ICG, which typically have similar fluorescence lifetimes of cypate at 800 nm (namely, 0.86 ns in dimethyl sulfoxide, DMSO). Additionally, the fluorescence maximum of a potential contaminant (half-dye) used to prepare **3** and **4** (see the Supporting Information) is 665 nm and a corresponding fluorescence lifetime of only 0.39 ns, which are different from those observed for the dichromic fluorescent dyes. Considering that the ratio of the fluorescence at 700 and 800 nm is independent of the solvent system and the pH value (for values between 2 and 10), we can assume that the emission at 700 nm does not originate from solvatochromism (Figure 3). Similarly, the absorption and

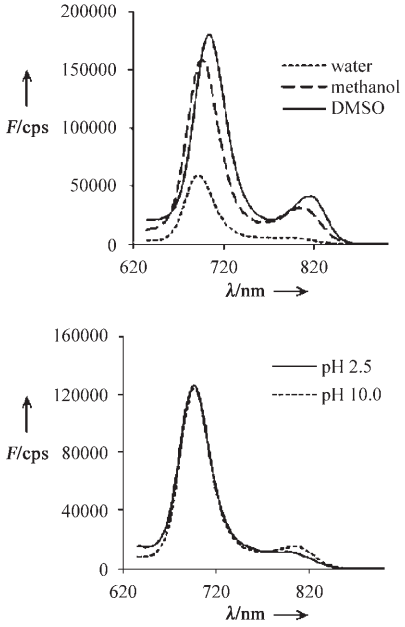

Figure 3. Solvent and pH effects on the fluorescence spectra of **3**.

fluorescence spectral profiles of the blue-shifted peaks are not affected by temperatures between 20 and 80 °C, which further confirms that aggregation is not responsible for the observed increase in the absorption band at 700 nm.

Since the enhancement of the emission band at 700 nm is prominent in nonsymmetrical structures, we attribute the dichromic properties of compounds **3** and **4** to the nonequivalence of their resultant resonance structures (Figure 4). According to the cyanine limit theory, the spectral properties of push–pull polyenes, such as carbocyanines, depend on the strength of the substituents on either side of the heterocyclic chains.[12] At the cyanine limit, the charges are spread along

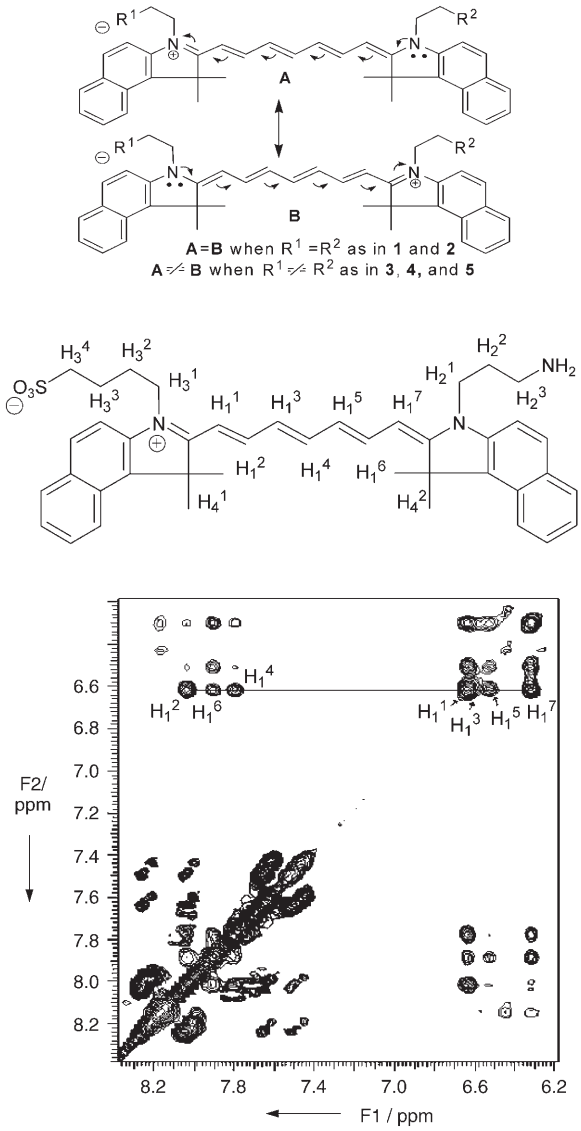

Figure 4. Structural features of nonsymmetrical cyanine dyes and a representative 2D TOCSY NMR spectrum (DMSO, 600 MHz) of **4** showing the seven vinyl protons in the polymethine bridge and their correlation.

the polymethine chain. In the nonsymmetrical cyanines (such as **3** and **4**), the resonance structures are not identical, thus creating a degree of ground-state polarization. According to Kuhn's equation (derived from quantum mechanical analysis to predict the spectral properties of polyenes),[11] the contribution of structural asymmetry represented by the 700-nm emission band corresponds to 0.22 eV. The effects of the structural asymmetry may also be amplified by the long heptamethine chain, as suggested by Tolbert and Zhao.[13] The dependence of dichromic fluorescence on structural asymmetry is not clearly understood at present.

The nonsymmetrical nature of these compounds was supported by 2D NMR analysis, using **4** as a representative compound (see Figure 4 and the Supporting Information). Resonances of the indole N-substituents (H_2^1–H_2^3 and H_3^1–H_3^4) and the polymethine bridge (H_1^1–H_1^7) were identified by

bond spin propagation at the fragment in total correlation spectroscopy (TOCSY) experiments. The correlated proton resonances at $\delta = 4.20$ and 4.26 ppm were unambiguously assigned to H_2^1 and H_3^1, respectively, thus suggesting the loss of orbital degeneracy of the two indolium nitrogen atoms. Proton connectivities were confirmed by vicinal J coupling in the correlated spectroscopy (COSY) experiments (see the Supporting Information). All the connections between adjacent protons were observed. A continuous path indicates the segment H_1^1–H_1^7 at the vinyl bridge as well as H_2^1–H_2^3 and H_3^1–H_3^4 at the methylene chains of the indole N-substituents. Assignments of the H_1^1 and H_1^7 resonances at $\delta = 6.64$ and 6.32 ppm, respectively, were further confirmed by the observed H_1^1–H_3^1 and H_1^7–H_2^1 nuclear Overhauser effect (NOE) cross-peaks (not shown). In addition, the presence of the H_1^6–H_4^2 and H_1^2–H_4^1 NOE peaks clearly indicates that $\delta =$ 7.91 and 8.03 ppm for the H_1^6 and H_1^2 resonances, respectively (which is consistent with the assignments in the COSY spectra). In previous studies, we found that the two *gem*-dimethyl groups in symmetrical cypate showed a singlet upfield.[8] However, the corresponding peak for nonsymmetrical **4** splits into two singlets. In addition, the vinyl protons at $\delta = 6$–7 ppm, which correspond to H_1^1, H_1^3, H_1^5, and H_1^7, are doublets in cypate but distorted in **4**. The ensemble of these results suggests that one of the structures responsible for the emission band at 700 nm consists of a preferentially localized positive charge on one of the indole N atoms in the ground state, which could further be stabilized by the neighboring sulfonate anion. It is therefore likely that the long-wavelength emission arises from the conventional π–π* electronic transition that takes place when the charge is transiently evenly distributed. These two structural dispositions could be associated with different molecular orbital diagrams, thus resulting in different HOMO and LUMO levels. Details of the mechanism of the dual emission are beyond the scope of this study.

A host of applications of this phenomenon can be envisaged; for example, the dual emission wavelengths provide a mechanism to quantify fluorescence signals by ratiometric imaging, without the need to label bioactive molecules with two or more dyes. It also facilitates the design of fluorescence resonant energy transfer (FRET) systems by using another dye to selectively quench the fluorescence of any one of the dual emission bands. It is particularly exciting to note that the dual fluorescence concept can be used to detect a host of enzyme activities by transforming the molecule from a symmetrical to a nonsymmetrical form; for example, kinases are known to phosphorylate serine and threonine residues in proteins and peptides. To demonstrate the potential to monitor the activities of serine kinases, we prepared di-serine-cypate (**6**) and the monophosphate diserine-cypate (**7**). As shown in Figure 5, phosphorylation generated a nonsymmetrical compound, with a corresponding change in the 700-to-800-nm emission ratio from 0.36 to 2.5 (a sevenfold increase). Such a large increase allows the detection of the kinase activity with a high sensitivity.

In conclusion, we discovered a new approach to develop dual NIR fluorescence-emitting molecules through the structural asymmetry of carbocyanine dyes. To the best of our knowledge, this is the first report of NIR dichromic fluorescence properties of nonsymmetrical carbocyanine dyes. Evaluation of the applications in chemistry and biology is in progress and will be reported elsewhere.

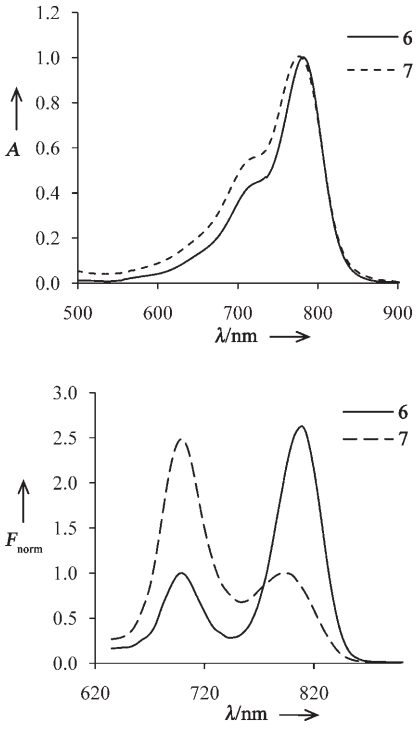

Figure 5. Normalized absorption and emission spectra of diserine-cypate (**6**) and monophosphate diserine-cypate (**7**) in methanol.

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