Fluorescence Imaging

Pyrromethene Dialkynyl Borane Complexes for "Cascatelle" Energy Transfer and Protein Labeling**

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The design of strongly luminescent probes is a thriving subject which finds applications in electroluminescent devices and fluorescence technology.[1,2] Fluorescent labels and probes have found widespread uses in biomedicine, polymer science, and sensor chemistry.[3,4] One of the most promising classes of dyes is that of cyanines stabilized with BF_2 fragments, such as 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (Bodipy) derivatives.[5] These non-ionic, stable molecules have exceptional optical and luminescence properties,[6] and a wide range of dyes have been engineered by modification of the pyrrole substituents.[7] To date, chemical modification of the BF_2 fragment with ethynylaryl modules has rarely been attempted.[8]

Frequently encountered deficiencies in the use of Bodipy derivatives are the small Stokes shifts ($\Delta\lambda \approx 600\ cm^{-1}$) between the lowest energy absorption band and the emission band, and the quenching of their fluorescence on conjugation to proteins.[5] Larger Stokes shifts would be advantageous in simplifying simultaneous excitation and emission detection, since background interferences are reduced and wide-band excitation and emission filters may be used, thus increasing the intensity of the fluorescence signal. Also desirable is the enhancement of this signal intensity by incorporation of a chromophore active in the region 350–370 nm where Bodipy species do not absorb, pyrene is an example of such a chromophore.[9]

Herein, we describe an elegant method for increasing the Stokes shift of Bodipy derivatives and minimizing fluorescence quenching after bioconjugation. This approach involves functionalization of the Bodipy core by binding ethynylpyrene entities directly to the boron center. Irradiation of the pyrene chromophore results in exclusive emission from the Bodipy core as a result of efficient intramolecular energy

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transfer, so that these new dyes function as a small cascade device or "cascatelle". Further functionalization enables ready attachment of the dyes to biopolymers such as proteins.

While trigonal alkynylboranes are well-known,[10] tetrahedral species are rare[11] and herein we describe the first such compounds in which two ethynyl units are bound to the boron center of a bora-indacene (Figure 1). The syntheses of **1** and **2** involve the displacement of fluoride from boron by 4-lithioethynyltoluene and 1-lithioethynylpyrene, respectively. Both compounds are obtained in satisfactory yield (**1** 62%, **2** 30%), and have been characterized by chemical analyses, full spectroscopic measurements, and crystal structure determinations.

The structure determinations (Figure 1) show an almost exact tetrahedral geometry for the sterically congested boron atoms. In both, the N-B-N angle is 106°, while the C-B-C angle for **1** is 115° and for **2** is 111°. The B–C and C≡C bonds fall in the ranges 1.58–1.60 Å and 1.18–1.20 Å, respectively. The B–C distances are longer than those of trigonal tris(3,3-dimethyl-1-butynyl)borane but similar to those in its tetrahedral pyridine adduct.[12]

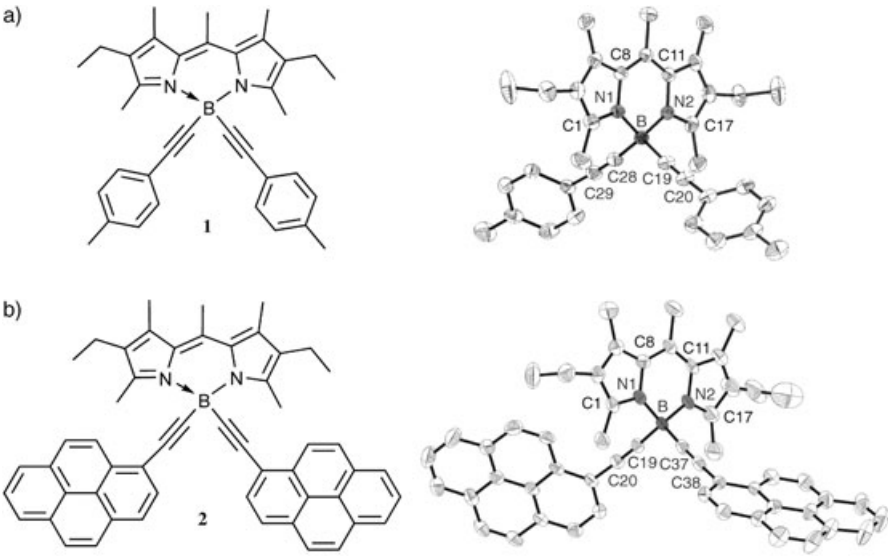


Figure 1. Structures of **1** (a) and **2** (b). Selected bond lengths [Å] and angles [°]: for **1** B-C28 1.589, B-C19 1.595, B-N1 1.562, B-N2 1.557, C28-C29 1.206, C19-C20 1.204, N1-C1 1.350, N1-C8 1.403, N2-C17 1.351, N2-C11 1.401; C28-B-C19 114.76, C28-B-N2 108.67, N1-B-N2 106.05, N2-B-C19 109.62. For **2** B-C19 1.603, B-C37 1.585, B-N1 1.563, B-N2 1.565, C20-C19 1.187, C37-C38 1.194, N1-C1 1.352, N1-C8 1.402, N2-C17 1.344, N2-C11 1.403; C19-B-C37 111.61, C19-B-N1 108.18, N1-B-N2 105.89, N2-B-C37 109.64, N2-B-C19 110.99.

The electronic absorption spectra of **1** and **2** reflect the presence of the separate Bodipy and ethynylaryl units (Figure 2). A strong band at 516 nm is attributed to the $S_0 \rightarrow S_1$ Bodipy transition, while a second weaker and broader band at 371 nm (clearly seen in Figure 2a) is typical of the $S_0 \rightarrow S_2$ transition.[13,14] Peaks observed at higher energies may be confidently assigned to the spin-allowed π–π* transitions of the ethynyl ($\lambda \approx 320$ nm) and toluyl ($\lambda \approx 265$ nm) groups (Figure 2). For **2**, a strong absorption at 330–370 nm, partially overlapping the $S_0 \rightarrow S_2$ Bodipy transition, is assigned to the ethynylpyrene unit.[9] The spectroscopic data are summarized in Table 1.

When excited at 516 nm, both **1** and **2** emit strongly, with high fluorescence quantum yields, in the region 535–540 nm. The radiative rate constants have values close to 1.5×10^8 s^{-1}. For **2**, excitation in the pyrene absorption band did not lead to pyrene emission but instead to emission characteristic of the indacene core. The fluorescence excitation spectrum matches the absorption spectrum, indicating that there is an efficient energy transfer from the pyrene to the Bodipy moiety. The quantum yield measurements are consistent with an efficiency close to 100% for this transfer. The result is that there are virtual Stokes shifts of approximately 10^4 cm^{-1}.

To devise a means of grafting these highly fluorescent units onto biopolymers, we synthesized an indacene derivative bearing an iodophenyl substituent on the *meso* position. Substitution of the iodine by a carboxybutylethynyl unit ultimately led to the activated ester **3d** (Scheme 1), which was expected to be reactive towards protein amino acid side-chain nucleophiles such as the terminal amino group of lysine (Scheme 1).[16]

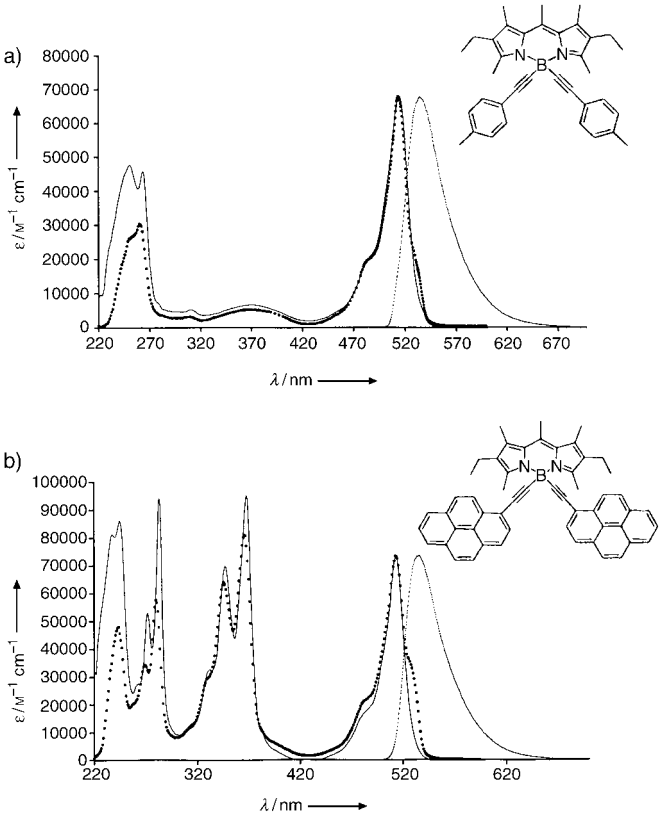
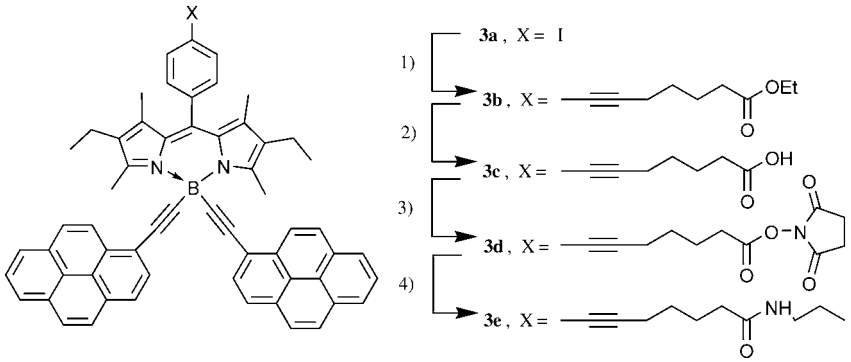


Figure 2. Absorption (——), emission (-----), and fluorescence excitation spectra (•••••) for compounds **1** (a) and **2** (b) in dichloromethane solution. Emission spectra were obtained upon excitation in the ethynyltoluyl moiety absorption (252 nm) for **1** and in the ethynylpyrene moiety absorption (371 nm) for **2**, while fluorescence excitation spectra were measured upon emission at 535 nm.

Table 1: Spectroscopic[a] data at 298 K for the new compounds.

Compound	λ_{abs} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_F [nm]	τ_F [ns]	ϕ_F [b] [%]	η [c] [%]	k_r [$10^8 s^{-1}$]	k_{nr} [$10^6 s^{-1}$]
1	516	67100	537	9.0	95		1.1	5.6
2	516	73000	535	6.2	94		1.5	9.7
	371	95000	535		90	96		
3b	523	50000	539	6.2	90		1.5	16.1
	370	61500	539		85	94		
3e	522	61000	538	5.0	82		1.6	36.0
	370	88000	538		55	67		

[a] Determined in aerated dichloromethane solution. [b] Determined using rhodamine 6G ($\phi_F = 0.76$ in water[15]) as reference. All ϕ_F were corrected for changes in refractive index. [c] Efficiency energy transfer from ethynylpyrene to the indacene moiety, calculated by dividing the quantum yield found by excitation in the chromophores by those found by excitation in the indacene moiety.



Scheme 1. 1) HC≡C$(CH_2)_4$COOEt (1 equiv), [$PdCl_2(PPh_3)_2$] (6% mol), CuI (10% mol), RT, 16 h, 91%. 2) NaOH (10 equiv), ethanol/THF (1:1), 60°C, 12 h, 87%. 3) DMAP (2 equiv), EDCI (2 equiv), *N*-hydroxysuccinimide (2 equiv), RT, 1 h, 54%. 4) *n*-propylamine, RT, 1 h, 74%. DMAP = dimethylaminopyridine, EDIC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

succimimide (NHS) esters.[17] Indeed, it proved possible to attach multiple fluorescent tags to BSA using **3d** in various label/protein ratios (ranging from 2:1 to 10:1), in a 1:1 (v/v) water/DMSO solution. Characterization of the conjugates in terms of label/protein molar ratio was performed by means of UV/Vis absorption spectroscopy, under the assumption that the absorption spectra of the conjugates are the sum of the absorption spectra of BSA and the Bodipy dye and that these spectra coincide with those of the free BSA and **3e**, respectively (Figure 3).

The experimentally determined label/protein molar ratios of the conjugates were in good agreement with the label/protein molar ratio used in the conjugation reaction (Table 2), indicating a high reaction yield. These BSA conjugates essentially maintain the spectroscopic characteristics of the model compound **3e** (Scheme 1 and Table 1). The fluorescence emission of aqueous solutions of the conjugates shows maximum intensity at 544 nm (Figure 4), being slightly red-shifted in comparison to that of compound **3e** in dichloromethane. All the protein conjugates remain strongly fluorescent, with an efficiency of energy transfer from the ethynylpyrene chromophore to the indacene moiety similar to that found for **3e** (see Figure 3). The efficiency of the energy transfer from the pyrene to the indacene moiety drops for **3e** and the labeled BSA conjugates when compared to that of **3b**, which has an ester function. This drop is likely to be due to the presence of the amide function in **3e** and the labeled conjugates which provides a non-radiative channel for the deactivation process.[18]

The fluorescence quantum yield decreases slightly with the increase in the degree of labeling of the conjugates,

The demonstration of the suitability of derivative **3d** for protein labeling and the evaluation of the spectroscopic properties of its protein conjugates were performed using bovine serum albumin (BSA) as a model protein which has 59 lysine residues that can potentially react with *N*-hydroxy-

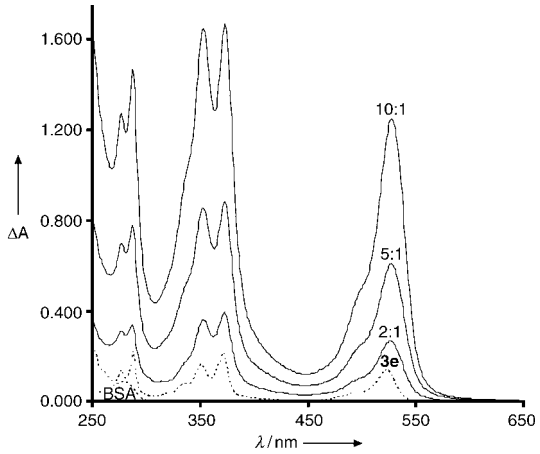


Figure 3. UV/Vis absorption spectra of the BSA conjugates of **3d** in water at a concentration of about 2.3×10^{-6} M and the BSA:**3d** ratios indicated; for comparison, the absorption spectra of BSA in water and of **3e** in dichloromethane at the same concentration are also shown (•••••).

Table 2: Label/protein molar ratios evaluated by UV/Vis spectroscopy and fluorescence properties of the BSA conjugates of **3d** in aqueous solution.

Conjugate	Label/protein molar ratio[a]	ϕ_F [b] [%]	η [c] [%]
2:1	1.9–1.8:1	75[d], 45[e]	61
5:1	4.3–5.2:1	63[d], 38[e]	61
10:1	8.9–9.9:1	49[d], 32[e]	67

[a] The values are the label/protein molar ratios of the conjugates evaluated from the absorption at 528 nm and from the comparison of the absorption of the conjugate at 280 nm with those of free BSA and **3d**. [b] Determined using rhodamine 6G ($\phi_F = 0.76$ in water[15]) as reference. [c] Efficiency of the energy transfer from the ethynyltoluyl or ethynylpyrene chromophore to the indacene moiety, calculated by dividing the quantum yield found by excitation in the chromophores by those found by excitation in the indacene moiety. [d] Upon excitation in the indacene moiety absorption at 528 nm. [e] Upon excitation in the ethynylpyrene chromophore absorption at 370 nm.

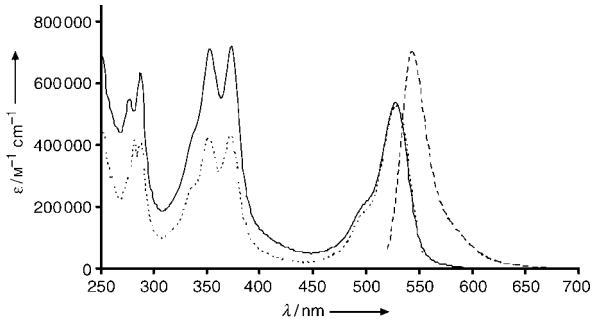


Figure 4. Absorption (——) and emission (-----) spectra of the 10:1 BSA conjugate of **3d** in water solution recorded upon excitation in the ethynylpyrene moiety absorption at 370 nm, and the fluorescence excitation spectra (••••) measured upon emission at 544 nm.

indicating a slight degree of self-quenching. However, the overall fluorescence intensity of the conjugates, as evaluated on the basis of their molar extinction coefficients and fluorescence quantum yields, still increases with the degree of labeling (Figure 5 and Table 2).

To compare the performance of the new Bodipy derivative with that of conventional fluorescent labels, fluorescence-imaging experiments were conducted. Figure 5 shows fluorescence images of spots containing the same amount of the BSA conjugates of **3d** or a fluorescein-labeled rabbit immunoglobulin G (IgG) with a 2.3:1 fluorescein/antibody labeling ratio (the fluorescein-labeled IgG and the 2:1 conjugate spots thus contain comparable amounts of fluorescent label molecules). The comparison of the fluorescence intensities of the spots indicates that, upon excitation in the UV spectral region, the BSA conjugates of **3d** are more efficient emitters than the fluorescein-labeled immunoglobulin (Figure 5a). The fluorescence emission of the BSA conjugates is stronger than that of the fluorescein-labeled IgG even when the latter is measured using fluorescein-specific excitation and emission filters (Figure 5b); the BSA conjugates are only weakly fluorescent using fluorescein filters because such filters do not match the absorption and emission bands of **3d**). This observation demonstrates that the peculiar absorption and emission properties of **3d** could be successfully exploited also using the conventional excitation sources used in fluorescence microscopy, such as mercury arc lamps. Indeed, their most intense emission line of such lamps (365–366 nm) is close to the maximum of the absorption band of the ethynylpyrene chromophore, whereas fluorescein has only a weak absorption at this wavelength.

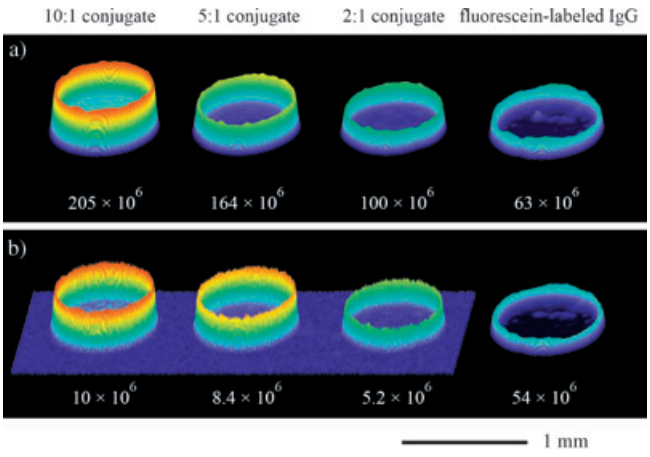


Figure 5. Pseudocolored 3D plots of fluorescence images of spots (diameter ≈ 800 μm) containing approximately 5×10^{-15} mol of the BSA conjugates of **3d** or a fluorescein-labeled rabbit immunoglobulin G. Spots were obtained using a manual microarrayer device, which employs an array of pins to transfer small volumes (ca. 3 nL) of solution onto microscope glass slides. Images were acquired with a standard epifluorescence microscope using either a) a wide-band UV excitation filter and a visible light emission filter or b) fluorescein-specific excitation and emission filters (because of their relatively low intensity, the images of BSA conjugate spots in (b) are shown using a different intensity scale). The images represent the actual fluorescence intensity of each spot, evaluated by integrating the signal over the whole spot area and expressed in arbitrary units.

The key feature of the present molecular design is the introduction of a supplementary chromophore linked by an ethynyl bridge to a tetrahedral boron atom. Such "Bodipyrene" dyes have three outstanding features: 1) relative ease of synthesis; 2) very large Stokes shifts resulting from an efficient, spin-allowed energy transfer from the excited pyrene subunit to the emitting state of the indacene center (a "cascatelle" process); 3) convenient functionalization to introduce an activated ester group suitable for grafting the dye to biopolymers. Very importantly, attachment of the dyes to a protein appears to produce only minor quenching effects and the singular other properties of the dyes are maintained. Fluorescence-imaging microscopy shows that these new labels are considerably superior to conventional fluorophores. The notion of a cascatelle process, clearly substantiated by the present work, is open to many avenues of development, leading to possibilities such as the multichromatic patterning of biomaterials using a single excitation source.

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