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Inhibitor-substituted Boron-dipyrromethene as a Turn-on Fluorescence Probe for Enzymes

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Benzenesulfonamide is a known inhibitor of carbonic anhydrase (CA). 8-[4-(Aminosulfonyl)phenyl]-BODIPY is designed as an inhibitor–dye conjugate for turn-on fluorescence sensing of CA, and binds specifically to bovine carbonic anhydrase in water with $K_a = 1.04 \pm 0.25 \times 10^7\,M^{-1}$ ($K_d = 0.96 \times 10^{-7}$ M) with concomitant increase in the fluorescence intensity of the former by a factor of 2.5–2.8 because of the steric constraint imposed on the probe by the enzyme.

Boron-dipyrromethene, the so-called BODIPY **1** (only the core structure is shown, Chart 1),[1] is a class of fluorescent dyes having a number of attractive features.[2] In particular, the high and micropolarity-independent emission efficiency of BODIPY makes it an important tool in various bioimaging applications.[3] In BODIPY, complexation with the difluoroboron unit effectively locks or rigidifies the dipyrromethene structure, affording a fluorescent chromophore. In this regard, it is interesting to note that the free base dipyrromethenes such as bilins (e.g., bilirubin), which are only weakly fluorescent, at best, in fluid media, become highly fluorescent in frozen solutions[4] or when bound to the target proteins in bacteria.[5]

An interesting feature of 8-phenyl-substituted BODIPY **2** is the dependence of its fluorescence on rigidification. The parent compound **2a** ($R^1 = R^2 = H$) exhibits considerably smaller fluorescence yields compared with 8-unsubstituted BODIPY **1**.[2,6] This is because the rotation of the phenyl ring in **2a** easily converts the conformation of the excited state of the molecule from radiative to nonradiative, i.e., from the bright (radiative), metastable, twisted conformer to the more stable, dark (nonradiative) one exhibiting coplanarity of the phenyl ring and BODIPY framework with higher electron delocalization.[6] Introduction of methyl substituents at the ortho positions of the phenyl ring (**2b**) or on the 1 and 7 positions of the BODIPY core (**2c**) or on both (**2d**) suppresses the nonradiative decay by restricting the internal rotation of the phenyl ring and increases the fluorescence quantum yields,[2,7,8] which approach unity.[9] Viscosity is an external factor that governs the ease of rotation; a relevant derivative **2a-1** is used as a molecular rotor to probe the microviscosity of live cells.[10] We imagine that complexation, e.g., with an enzyme, is another external way to impose steric constraints on the phenyl-BODIPY probe, where the phenyl group, acting as an inhibitor of the enzyme, mediates the interaction (Scheme 1).[11] We report, herein, an example of the fluorescence sensing of enzymes along this line.

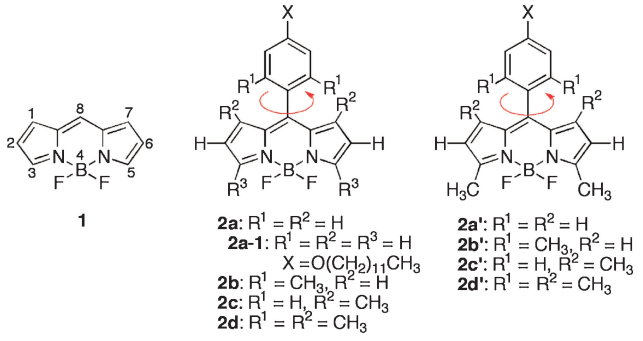
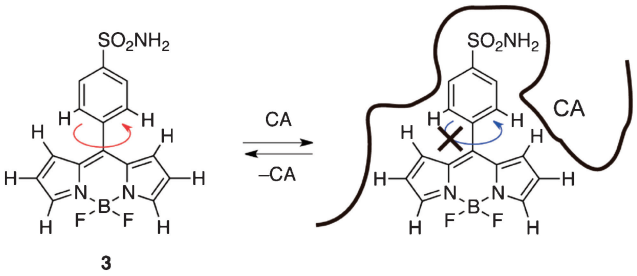


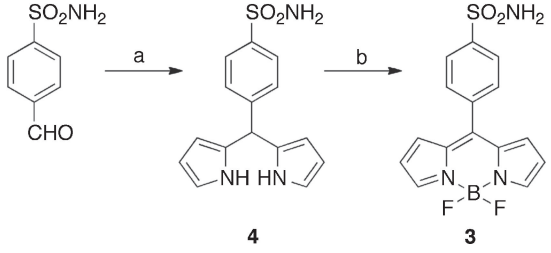
Chart 1.

Carbonic anhydrase was the enzyme of choice here. It is a zinc enzyme, and its enzymatic activity is inhibited by a variety of ligands including benzenesulfonamide. Benzenesulfonamide-BODIPY, or 8-(4-aminosulfonylphenyl)-BODIPY **3** (Scheme 1), was obtained according to the general method of preparing 8-aryl-BODIPY (Scheme 2);[2] the condensation of 4-formylbenzenesulfonamide with 2 mol of pyrrole in the presence of CF_3CO_2H to give dipyrromethane **4** (step a), the oxidation of **4** with DDQ in CH_2Cl_2, the subsequent complexation of the resulting dipyrromethene with BF_3–$O(CH_2CH_3)_2$ and $(CH_3CH_2)_3N$ (step b) to give an inhibitor-substituted BODIPY **3**.[12]

Examination of the emission spectra of benzenesulfonamide-BODIPY **3** in common solvents indicates that they are practically independent of solvent polarities from water (con-



Scheme 1. Complexation of probe **3** with enzyme CA.



Scheme 2. Preparation of probe **3**: a) pyrrole, CF_3CO_2H, Ar, rt, 67%; b) DDQ, CH_2Cl_2, Ar, rt; and then, BF_3–$O(CH_2CH_3)_2$, $(CH_3CH_2)_3N$, Ar, rt, 47%.

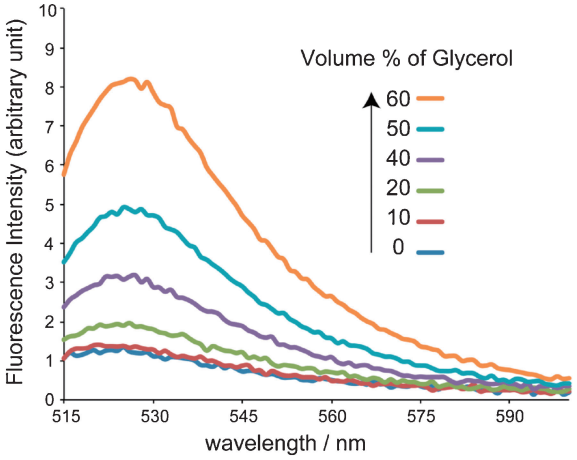


Figure 1. Fluorescence spectra of probe **3** (4.0×10^{-7} M; excitation at 502 nm) in methanol–glycerol mixtures with different volume percentages of glycerol at 35 °C.

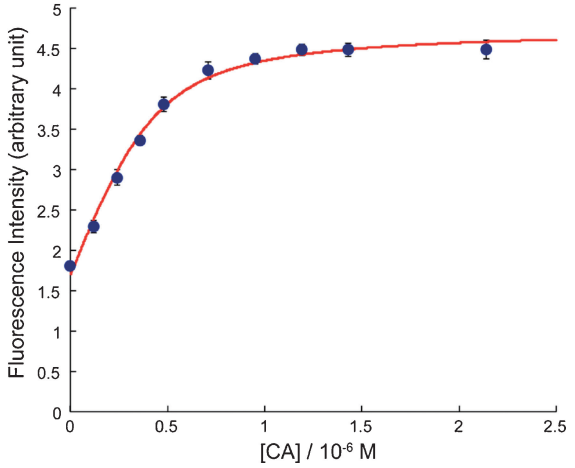


Figure 2. Dependence of fluorescence intensities at 524 nm of probe **3** (4.0×10^{-7} M; excitation at 502 nm) on the concentrations of bovine carbonic anhydrase (CA) in water (HEPES buffer at pH 7.2) containing 1% DMSO at 35 °C. Each point is the average of three measurements, and error bars represent standard deviations.

taining 1% v/v DMSO) and DMF and DMSO to CH_2Cl_2 and $CHCl_3$ (data not shown).[13] However, as expected, they strongly depend on viscosity.[10] Figure 1 shows the spectra of probe **3** in methanol–glycerol mixtures; the fluorescence intensities increase with the increasing volume percentage of glycerol, i.e., with increasing viscosities.

We then moved on to the interaction of the inhibitor-substituted BODIPY **3** with bovine carbonic anhydrase (CA). In the presence of CA in water (HEPES buffer at pH 7.2) containing 1% v/v DMSO, the fluorescence intensity at 524 nm of probe **3** (4.0×10^{-7} M) increases, without any notable shift in the emission maxima, in a [CA]-dependent manner, until reaching a plateau at around [CA] = 1.0×10^{-6} M (Figure 2). The saturation behavior observed is consistent with 1:1 **3**–CA complexation with an association constant of $K_a = 1.04 \pm 0.25 \times 10^7\,M^{-1}$ ($K_d = 0.96 \times 10^{-7}$ M) obtained from least-

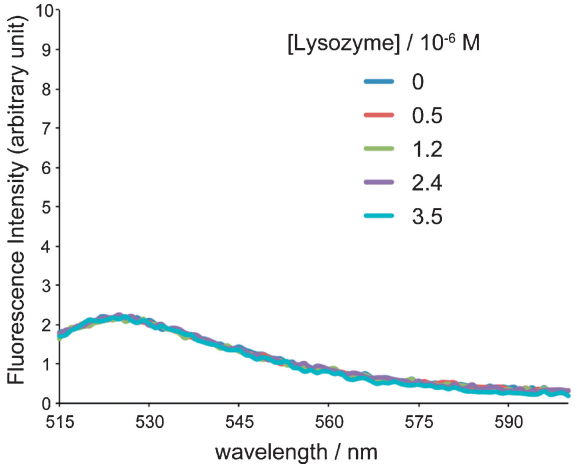


Figure 3. Fluorescence spectra of probe **3** (4.0×10^{-7} M; excitation at 502 nm) in the presence of different amounts of lysozyme in water (HEPES buffer at pH 7.2) containing 1% DMSO at 35 °C.

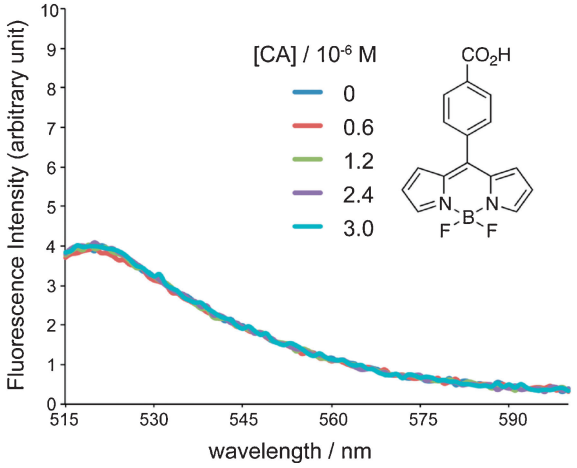


Figure 4. Fluorescence spectra of 8-(4-carboxyphenyl)-BODIPY (4.0×10^{-7} M; excitation at 502 nm) in the presence of different amounts of bovine carbonic anhydrase (CA) in water (HEPES buffer at pH 7.2) containing 1% DMSO at 35 °C.

squares fitting (solid line in Figure 2) and a fluorescence-enhancement or light-up factor $f_{CA(+)}/f_{CA(-)}$ in the range 2.5–2.8.[14]

Control measurements indicate that (1) lysozyme, as the inert reference enzyme, has practically no effect on the fluorescence spectra of probe **3** (Figure 3), (2) the fluorescence spectra of 4-carboxyphenyl-BODIPY, as the inert reference pigment, are hardly affected by enzyme CA (Figure 4), and (3) the CA-induced fluorescence enhancement of probe **3** at [**3**] = [CA] = 4.0×10^{-7} M disappears almost completely (>98%) in the presence of excess benzenesulfonamide, which competes for the binding site in CA. These results confirm that enzyme CA specifically recognizes probe **3** as a benzenesulfonamide derivative and that the **3**–CA complexation is reversible. The dissociation constant, $K_d = 0.96 \times 10^{-7}$ M, for the **3**–CA complex, is roughly one-order of magnitude lower than those reported for the benzenesulfonamide–bovine carbonic anhydrase

complexes; $K_d = 0.91 \times 10^{-6}$ and 0.44×10^{-6} M according to affinity capillary electrophoresis (ACE)[15] and kinetic[16] studies, respectively, and may be in accordance with the observation that *p*-substituted benzenesulfonamides give more stable complexes with CA than the parent (unsubstituted) compound does.[16–20]

The CA-induced light-up of probe **3** is consistent with its rigidification, particularly the rotation of the phenyl ring in the binding pocket of the enzyme (Scheme 1). In light of the fluorescence–viscosity correlation (Figure 1), the light-up factor of $f_{CA(+)}/f_{CA(-)} = 2.5$–$2.8$[14] suggests that the microviscosity felt by probe **3** associated with the enzyme corresponds to that of a 60:40 mixture of methanol and glycerol. Rotation of the phenyl ring or the BODIPY core in the bound probe seems to be moderately prevented. However, we should not delve deeper into this argument, since the restriction of rotation is not the sole factor governing the fluorescence enhancement. Even when the rotation is completely prevented, it is the relative conformation, i.e., the dihedral angle of the phenyl ring and the BODIPY core, that determines the light-up property of the probe.

In summary, benzenesulfonamide-BODIPY **3** strongly and specifically binds to enzyme CA with a concomitant light-up of the former by a factor of 2.5–2.8. Benzenesulfonamide is the inhibitor that mediates the CA–BODIPY association. BODIPY is a uniquely micropolarity-independent fluorophore.[2] As far as this is true, the present light-up of the probe must be a consequence of the steric constraints imposed on it by the enzyme. It is also important to note that probe **3** shows no sign of nonspecific binding to lysozyme, since nonspecific binding is often a problem in protein sensing.[21] Upon nonspecific (on-surface) binding, the microviscosity as well as the micropolarity of the probe is expected to change. In order to gain a deeper insight of the present selectivity, we need to learn more about the microviscosity of the protein surface and the sensitivity of the conformation of the probe. Additional work is now under way in our laboratories to build up the present strategy used for different inhibitor–enzyme combinations.

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1 IUPAC name for the BODIPY core; 4,4-difluoro-4-bora-3a,4a-*s*-indacene.

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12 Dipyrromethane **4** (42 mg) and DDQ (40 mg) in CH_2Cl_2 (3 mL) was stirred at room temperature for 30 min. Triethylamine (150 μL) and BF_3–$O(CH_2CH_3)_2$ (300 μL) were added and the mixture was stirred for a further 3 h. Workup and chromatography on silica gel with CH_2Cl_2–CH_3OH (60:1) gave compound **3** (22.4 mg, 47%); ^{1}H NMR ($CDCl_3$): δ 6.57 (d, J = 3.2 Hz, 2H), 6.86 (d, J = 3.2 Hz, 2H), 7.73 (d, J = 6.9 Hz, 2H), 7.99 (2H), 8.10 (d, J = 6.9 Hz, 2H); ^{19}F NMR ($CDCl_3$): δ −144.9 (q). HRMS (FAB) m/z: $[M]^+$ found, 347.0714; calcd for $C_{15}H_{12}BF_2N_3O_2S$, 347.0706. UV–vis (H_2O (HEPES buffer with pH 7.2) containing 1% DMSO): $\lambda_{max} = 502$ nm ($\varepsilon = 3.58 \times 10^4$ M^{-1} cm^{-1}).

13 The fluorescence quantum yield of probe **3** in water (HEPES buffer at pH 7.2) containing 1% DMSO is $\Phi = 0.03$ (excitation at 492 nm), obtained in reference to the known quantum yield ($\Phi = 0.85$; C. A. Parker, W. T. Rees, *Analyst* **1960**, *85*, 587) of fluorescein in water (0.1 M NaOH).

14 The light up factor $f_{CA(+)}/f_{CA(-)} = 2.5$ is based on observed fluorescence intensities at $[CA] = 2.14 \times 10^{-6}$ and 0 M. $f_{CA(+)}/f_{CA(-)} = 2.8$ is based on extrapolated fluorescence intensities.

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20 Compared with those for unsubstituted benzenesulfonamide, the association constants of *p*-methylbenzenesulfonamide with bovine CA are 1.7- (ref. 15) or 2.3-times (ref. 17) larger. Those of *p*-nitrobenzenesulfonamide are 6.4- (ref. 15) or 8.6-times (ref. 17) higher. The corresponding affinity-enhancement factors for human CA are 2.9 and 23 for the *p*-methyl and *p*-nitro substituents, respectively (ref. 18).

21 Nonspecific binding of probe **3** to BSA (bovine serum albumin) does occur, resulting in fluorescence enhancement by ≈20% at $[\mathbf{3}] = 4.0 \times 10^{-7}$ M and $[BSA] = 4.0 \times 10^{-6}$ M. This is not surprising, in view of the function of BSA, acting as a nonspecific binder/transporter for various hydrophobic substances.

