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Synthesis and Properties of the *p*-Sulfonamide Analogue of the Green Fluorescent Protein (GFP) Chromophore: The Mimic of GFP Chromophore with Very Strong N−H Photoacid Strength

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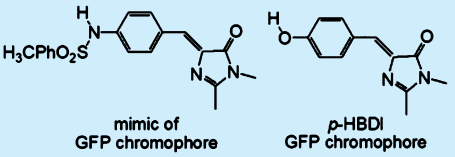
Supporting Information

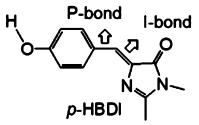
ABSTRACT: The *para*-sulfonamide analogue (*p*-TsABDI) of a green fluorescent protein (GFP) chromophore was synthesized to mimic the GFP chromophore. Its S_1 excited-state pK_a^* value in dimethylsulfoxide (DMSO) is −1.5, which is strong enough to partially protonate dipolar aprotic solvents and causes excited-state proton transfer (ESPT), so it can partially mimic the GFP chromophore to further study the ESPT-related photophysics and the blinking phenomenon of GFP. In comparison with 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) (pK_a = 7.4, pK_a^* = 1.3 in water), *p*-TsABDI (pK_a = 6.7, pK_a^* = −1.5 in DMSO) is a better photoacid for pH-jump studies.



Proton transfer is a cornerstone of acid/base-catalyzed chemical reactions, where the acid/base strength of catalysts plays an important role on the proton transfer.[1] Among acid catalysts, N−H acids are not as common as oxoacids, because N−H acids are much less acidic, such as NH_3 (pK_a = 41)[2a] vs H_2O (pK_a = 31.4)[2b] in dimethylsulfoxide (DMSO), $CH_3C(O)NH_2$ (pK_a = 25.5)[2c] vs CH_3CO_2H (pK_a = 12.6)[2c] in DMSO, and NH_4^+(pK_a = 10.5 in DMSO, 9.2 in H_2O) vs H_3O^+ (pK_a = −1.7 in H_2O).[2d]

Since the pioneering work of Forster[3a] and Weller,[3b] it has been found that the acid strength of some oxoacids can be significantly boosted by photoexcitation. The first singlet (S_1) excited-state acid strength (pK_a^*) of some oxoacids can be several orders of magnitude (ΔpK_a^*) stronger than that of its ground state (pK_a),[4a] such as phenol (pK_a = 9.82, pK_a^* = 4 in water),[4b] 7-cyano-2-naphthol (pK_a = 8.75, pK_a^* = −1.3 in water),[4c] and 8-hydroxypyrene-1,3,6-trisulfonate, HPTS (pK_a = 7.4, pK_a^* = 1.3 in water).[4d] In addition, few N−H acids have been found to be boosted by photoexcitation,[4e] such as tryptophan (pK_a = 9.30, pK_a^* = 7.8 in water).[4f] These photoacids can be initiated by light to generate protons for proton-transfer reactions at a specified instant in time, so a pH-jump study has been one of important applications for these photoacids, and HPTS is one of the best, highly used photoacids for pH-jump studies.[4g,h]

Green fluorescent protein (GFP) and its mutants have attracted interest as fluorescent biological labels in the last two decades.[5] The GFP chromophore is also a photoacid that involves excited-state proton transfer (ESPT).[4a] The wild-type GFP (*wt*GFP) is a globular protein of 238 amino acids that are folded into an 11-stranded *β*-barrel with the *p*-hydroxybenzylideneimidazolinone (*p*-HBDI) chromophore located at the center of the *β*-barrel.[6] The major electronic absorption (395 nm) of the *wt*GFP comes from the neutral form of the *p*-HBDI chromophore. Its minor electronic absorption (475 nm) comes from the electronic absorption of the *p*-HBDI anion.[7] Upon excitation at 395 nm, the neutral *p*-HBDI chromophore undergoes a rapid ESPT, emitting the dominant GFP fluorescence at 508 nm with 80% fluorescence quantum yield.[7] It has been proposed that the hydrogen-bonding network, which is comprised of the neutral *p*-HBDI chromophore, an internal water molecule, Ser205, and Glu222, promotes rapid ESPT from *p*-HBDI.[8a,b] This results in an ESPT phenomenon in GFP where the fluorescence wavelength is increased. This is indirectly proven in the non-ESPT-related blue fluorescent emission of the double mutant S205 V/T203 V of *wt*GFP.[8c] Unfortunately, there remains some ambiguity surrounding the ESPT-related photophysics of the *wt*GFP chromophore, such as charge transfer; thus, further understanding of the GFP chromophore and its analogues is an important issue.



One of the issues the ESPT-related photophysics of GFP may help to solve is the GFP blinking phenomenon. It was attributed to the temporary conversion to a nonfluorescent form, which has been variously suggested to be triplet formation,[9a,b] proton transfer,[9c,d] or Z/E isomerization.[9e−g] The identity of the nonfluorescent form and the photophysics of GFP blinking phenomenon remain unsolved.[9h] The GFP blinking phenomenon occurs right after the ESPT of GFP. How is the ESPT-related photophysics of GFP linked to the nonfluorescent form where the GFP blinking phenomenon happens? Uncovering the ESPT-related photophysics of GFP may help us to understand the GFP blinking phenomenon, because it is likely due to the ESPT-related photophysics.

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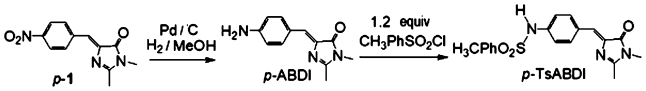
To study ESPT-related photophysics of GFP, *p*-HBDI was attempted to reproduce the ESPT phenomenon of *wt*GFP, but it failed to display ESPT, because the *p*-HBDI excited state undergoes much faster radiationless transitions.[10a] In this paper, we report that the *p*-sulfonamide analogue (*p*-TsABDI) of the GFP chromophore, which displays ESPT, may qualify to be a model for further study of ESPT-related photophysics of the *wt*GFP choromphore. In addition, we also report that the pK_a of *p*-TsABDI can be significantly decreased by 8.2 through photoexcitation at a specified instant in time, and that makes it better than HPTS in pH-jump studies.[4g,h]

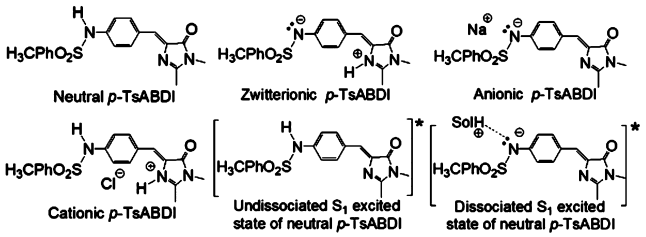
For the synthesis of *p*-TsABDI (Scheme 1), reduction of *p*-**1** to *p*-ABDI with $SnCl_2$ was attempted,[10b] but it was not so successful in our hands. It is better to reduce *p*-**1** to *p*-ABDI with Pd-catalyzed hydrogenation. Then, *p*-TsABDI was prepared by treating *p*-ABDI with 1.2 equiv of tosyl chloride in the presence of NEt_3.

Scheme 1. Preparation of *p*-TsABDI



Because *p*-TsABDI includes both the acidic sulfonamide and the basic amidine moieties, it may exist as a neutral or zwitterionic structure in an aprotic or protic solvent. In order to identify the solution structures, ESPT phenomena, and photoacid strength of *p*-TsABDI in an aprotic or protic solvent, electronic absorption and fluorescent emission spectra of *p*-TsABDI and its cation and anion were measured and compared in a protic solvent (H_2O), aprotic solvents, or mixed CH_3CN(aprotic)−H_2O(protic) solvents (see Table 1).

Table 1. Photophysical Properties of Neutral, Zwitterionic, Anionic, and Cationic *p*-TsABDI



solvent	λ_{abs} (nm)	ε (M^{-1} cm^{-1})	λ_f (nm)	ϕ_F ($\times 10^{-3}$)
		Neutral Compounds		
DMSO[a]	378	1.1×10^4	425	1.1
DMSO[a]	378	1.1×10^4	520	0.06
CH_3CN[a]	372	8.6×10^3	437	0.7
CH_3CN[a]	372	8.6×10^3	512	0.04
THF[a]	375	7.4×10^3	441	1.1
THF[a]	375	7.4×10^3	510	0.01
		Zwitterionic Compounds		
H_2O	408	9.1×10^3	502	0.1
		Anionic Compounds		
H_2O	408	9.1×10^3	502	0.1
DMSO	450	1.2×10^4	501	0.8
CH_3CN	441	1.3×10^4	507	0.9
		Cationic Compounds		
CH_3CN	384	9.0×10^3	437	0.9

[a]The solvent was dried.

For the fluorescent emission spectrum of *p*-TsABDI, it displays dual fluorescence in polar aprotic solvents (see Table 1 and Figure 1) The major peak is located at 425 nm ($\Phi_F = 1.1 \times 10^{-3}$) in DMSO, 437 nm ($\Phi_F = 7 \times 10^{-4}$) in acetonitrile, and 441 nm ($\Phi_F = 1.1 \times 10^{-3}$) in tetrahydrofuran (THF), and the other is a weak shoulder fluorescence at 520 nm ($\Phi_F = 6.0 \times 10^{-5}$) in DMSO, 512 nm ($\Phi_F = 4.0 \times 10^{-5}$) in acetonitrile, and 510 nm ($\Phi_F = 1.0 \times 10^{-5}$) in THF. This is quite unusual.

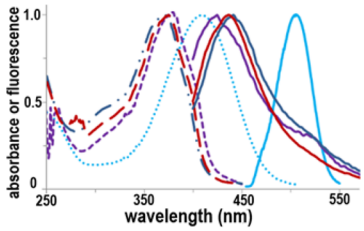


Figure 1. Normalized electronic absorption (dotted line) and fluorescent emission spectra (solid line) of *p*-TSABDI in water (cyan), DMSO (purple), acetonitrile (dark blue), or THF (red).

To check if the weak shoulder fluorescence of *p*-TsABDI is caused by ESPT, it was compared with the fluorescence of the *p*-TsABDI anion. The *p*-TsABDI anion was prepared by titration of *p*-TsABDI in acetonitrile or DMSO with $NaOH_{(aq)}$ with 3 μL of water added (see Figure 2 and Table 1). Its lowest-energy electronic absorption is significantly red-shifted to 450 nm ($\varepsilon = 1.2 \times 10^4$ M^{-1} cm^{-1}) in DMSO and 441 nm ($\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}) in acetonitrile. It is consistent with another similar experimental result that the electronic absorption of the *p*-HBDI anion is red-shifted by ~75 nm, in comparison with that of *p*-HBDI.[10d] This result indicates that the sulfonamide N−H acid of *p*-TsABDI is not dissociated in polar aprotic solvents. The fluorescent emission of the *p*-TsABDI anion is also significantly red-shifted to 501 nm ($\Phi_F = 8.0 \times 10^{-4}$) in DMSO and 507 nm ($\Phi_F = 9.0 \times 10^{-4}$) in acetonitrile, which looks like the weak shoulder fluorescence of *p*-TsABDI. However, according to the electronic absorption spectra of *p*-TsABDI, *p*-TsABDI in dipolar aprotic solvents does not have any trace of the *p*-TsABDI anion (see Figure 3). Hence, we suggest that the weak shoulder fluorescence of *p*-TsABDI in DMSO, acetonitrile, and THF is important evidence for the ESPT of *p*-TsABDI (see Figure 3). We suggest that the weak shoulder fluorescence of *p*-TsABDI comes from its dissociated S_1 excited state while the other

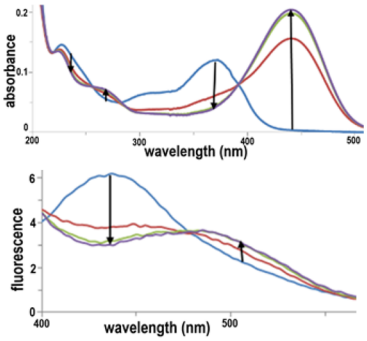


Figure 2. Change in the electronic absorption (top) and in the fluorescent emission spectra (bottom) for the titration of *p*-TsABDI (1.4×10^{-5} M) in CH_3CN with $NaOH_{(aq)}$ (0−9×10^{-4} M) with 3 μL of water added.

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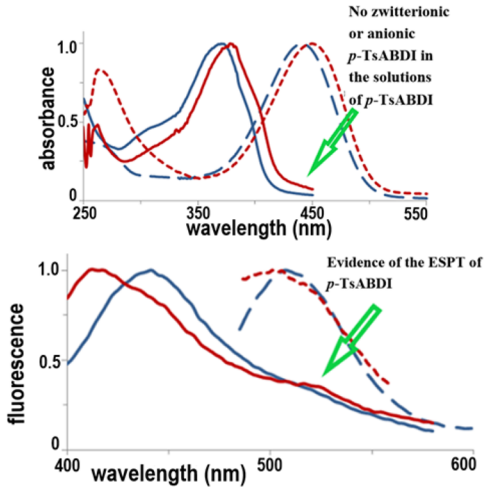
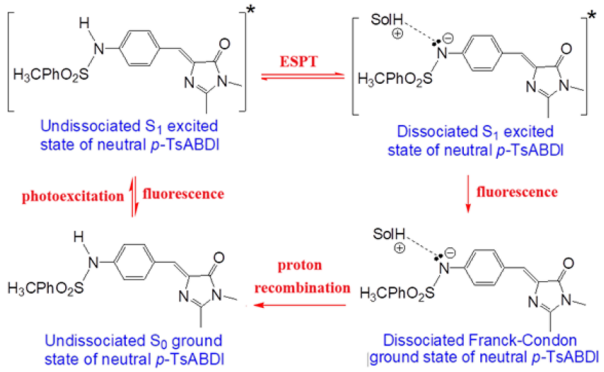


Figure 3. Evidence of the ESPT of *p*-TsABDI. The electronic absorptions (top) and the fluorescent emission spectra (bottom) of the neutral *p*-TsABDI (solid line) and the *p*-TsABDI anion (dotted line) in CH_3CN (dark blue) or DMSO (red).

major fluorescence of *p*-TsABDI is from its undissociated S_1 excited state (see Scheme 2).

Scheme 2. Proposed Photoexcitation, Fluorescence, ESPT and Proton Recombination of *p*-TsABDI



To check if *p*-TsABDI exists as a neutral or zwitterionic structure in an aprotic solvent, its electronic absorption was compared with that of the *p*-TsABDI cation. The *p*-TsABDI cation was prepared by titration of *p*-TsABDI in acetonitrile with $HCl_{(aq)}$ with 3 μL of water added (see Figure 4 and Table 1). Its lowest-energy electronic absorption is slightly red-shifted to 384 nm (ε = 9.0 × 10^3 M^{-1} cm^{-1}) and its fluorescent emission is also ~437 nm (Φ_F = 9.0 × 10^{-4}). It is consistent with another experimental result that the electronic absorption of the *p*-HBDI cation is red-shifted by ~20 nm, in comparison with that of *p*-HBDI.[10d] This result confirms that *p*-TsABDI in CH_3CN exists in the neutral structure, instead of the zwitterionic structure. If *p*-TsABDI in CH_3CN exists in the zwitterionic structure, protonation of *p*-TsABDI would make its electronic absorption blue-shifted. The *p*-TsABDI cation is supposed to have a proton added on the amidine nitrogen, according to the experimental result that the pK_a value for the amidinium N−H proton of the *p*-HBDI cation is 1.4 in water.[10e]

To check if *p*-TsABDI exists as a neutral or zwitterionic structure in a protic solvent, the electronic absorption and

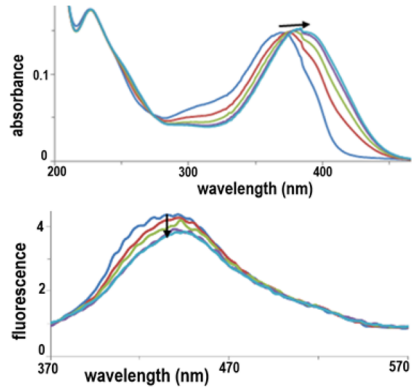


Figure 4. Change in the electronic absorption (top) and in the fluorescent emission spectra (bottom) for the titration of *p*-TsABDI (1.7×10^{-5} M) in CH_3CN with $HCl_{(aq)}$ ($0–1.2 \times 10^{-4}$ M) with 3 μL of water added.

fluorescent emission spectra of *p*-TsABDI were compared with those of the *p*-TsABDI anion in various ratios of mixed $CH_3CN–H_2O$ solvents (see Figure 5). Upon increasing the

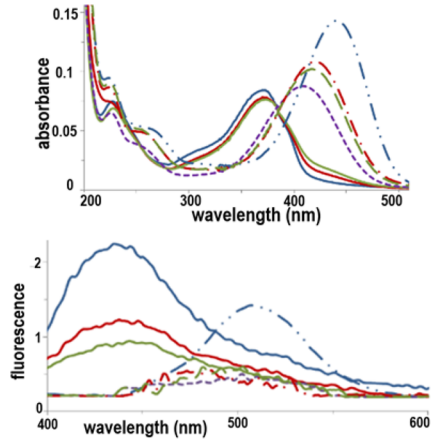


Figure 5. Electronic absorptions (top) and the fluorescent emission (bottom) spectra of the neutral *p*-TsABDI (solid line) and the *p*-TsABDI anion (dotted line) in mixed $CH_3CN–H_2O$ solvents (dark blue for acetonitrile, red for 2:1 $CH_3CN–H_2O$, lime green for 1:2 $CH_3CN–H_2O$, and purple for water).

water percentage of mixed $CH_3CN–H_2O$ solvents, the electronic absorption at 372 nm decreases with a simultaneous increase of the electronic absorption at 408 nm for *p*-TsABDI, while the lowest-energy electronic absorption of the *p*-TsABDI anion is blue-shifted with gradual migration from 441 nm to 408 nm. It means that *p*-TsABDI in mixed $CH_3CN–H_2O$ solvents exists only in two structures: the undissociated neutral structure for the electronic absorption at 372 nm and the zwitterion structure for the electronic absorption at 408 nm. However, *p*-TsABDI in CH_3CN and DMSO exists only in the undissociated neutral structure, as shown in Figure 3. However, the *p*-TsABDI anion always exists in the anionic structure, whereas the ground state of the *p*-TsABDI anion is highly stabilized by various percentages of water through hydrogen bonding, causing blue-shifted migration of the electronic absorption. When the water percentage of mixed CH_3CN-H_2O solvents nears 100%, the lowest-energy electronic absorptions of *p*-TsABDI and the *p*-TsABDI anion are both located at 408 nm, indicating that *p*-TsABDI in H_2O exists only

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in the zwitterion structure and the electronic absorption spectra of the zwitterionic *p*-TsABDI and the *p*-TsABDI anion are almost the same.

Upon increasing the water percentage of mixed CH_3CN-H_2O solvents, both the major and the weak shoulder fluorescent emissions of *p*-TsABDI at 437 and 512 nm and the fluorescent emission of the *p*-TsABDI anion at 507 nm decreases significantly. It is likely because hydrogen bonding with water increases radiationless relaxation of their S_1 excited states.[11] When the water percentage of mixed CH_3CN-H_2O solvents reaches 100%, the fluorescent emissions of *p*-TsABDI and the *p*-TsABDI anion are both located at 502 nm, indicating that the emission spectra of the zwitterionic *p*-TsABDI and the *p*-TsABDI anion are also almost the same.

To understand the ground-state acid strength of *p*-TsABDI, Shi's method[12a] was used, and the ground-state pK_a values of N−H acids of formamide and *p*-TsABDI in DMSO were calculated using the combined methods of B3PW91/6-311++G(3df,2p)//B3LYP/6-31+G(d)//HF//CPCM/UA0. The calculated ground-state pK_a value of formamide in DMSO is 23.1, which is very close to the experimental value of 23.5.[2d] The calculated ground-state pK_a value of *p*-TsABDI in DMSO is 6.7. This is consistent with the experimental result that *p*-TsABDI, which exists as a neutral structure in CH_3CN and DMSO, turns into a zwitterionic structure in water.

According to the thermodynamics of the Forster cycle,[4a] an excited state is a stronger acid than its ground state if the absorption or emission spectrum of the conjugated base is characterized by a red shift, relative to that of the conjugated acid.[4a] Hence, the S_1 excited state of *p*-TsABDI is a stronger acid than its ground state. The Forster equation is

$$pK_a^* = pK_a - N_A\frac{(h\upsilon_1 - h\upsilon_2)}{2.3RT}$$

where frequency (υ) is suggested to be the average of absorption frequency (υ_a) and fluorescence frequency (υ_f) for better accuracy.[12b] In the case of *p*-TsABDI in DMSO,

$$\upsilon_1 = \frac{\left(\frac{c}{\lambda_a(378\text{ nm})} + \frac{c}{\lambda_f(425\text{ nm})}\right)}{2}$$

for *p*-TsABDI and

$$\upsilon_2 = \frac{\left(\frac{c}{\lambda_a(450\text{ nm})} + \frac{c}{\lambda_f(501\text{ nm})}\right)}{2}$$

for the *p*-TsABDI anion (see Table 1). Then, the value of ΔpK_a^* ($\Delta pK_a^* = pK_a^* - pK_a$) is equal to −8.2. The pK_a^* value of the S_1 excited state of *p*-TsABDI in DMSO is −1.5, which is low enough to be able to protonate DMSO, CH_3CN, and THF, causing the ESPT. The pK_a^* value of the S_1 excited state of *p*-HBDI in H_2O is 0.1, based on its ground-state pK_a value of 8.0,[10d] its electronic absorption at 370 nm and the electronic absorption (430 nm) of its anion.[10c] The reason why *p*-TsABDI shows ESPT but *p*-HBDI does not is likely because *p*-TsABDI has a much stronger photoacid strength.

In conclusion, in polar aprotic solvents, such as DMSO, acetonitrile, and THF, *p*-TsABDI exists in the undissociated neutral structure and shows dual fluorescence. The major fluorescence is from the undissociated S_1 excited state, and the weak shoulder fluorescence is from the dissociated S_1 excited state, which is important evidence for the ESPT. In water, *p*-TsABDI exists in the zwitterionic structure, whose electronic absorption and fluorescent emission spectra are almost the same as those of the *p*-TsABDI anion. In comparison with HPTS (pK_a = 7.4, pK_a^* = 1.3 in water),[4d] which is one of the best, highly used photoacids for pH-jump studies, *p*-TsABDI (pK_a = 6.7, pK_a^* = −1.5 in DMSO) is a better photoacid than HPTS for pH-jump studies. Unlike *p*-HBDI, *p*-TsABDI displays ESPT so it qualifies to be a model for further study of the ESPT-related photophysics of GFP. Hopefully, it could be used to uncover the photophysics of the GFP blinking phenomenon as well.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b00257.

Acidity, synthesis, characterization, ^1H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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