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The role of N-substituents in radiationless deactivation of aminated derivatives of locked GFP chromophore

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Abstract: We report the creation of novel group of the ABDI-BF₂ fluorescent dyes based on the conformationally locked GFP chromophore. We studied the intramolecular mechanism of radiationless deactivation of ABDI-BF₂ fluorophore by introducing the various substituents at the nitrogen atom. The results of this study and our previous work allowed us to claim that in case of ABDI-BF₂ this deactivation is determined by the formation of the non-fluorescent internal charge transfer excited state with a planar

Introduction

Synthetic analogues of the chromophores of fluorescent proteins have become a subject of extensive research for the last three decades. The syntheses of various substituted benzylidene imidazolones (BDI) allowed to reveal real chromophores structures and led to better understanding of the fluorescent proteins spectral behavior and their maturation mechanism.^[1] The other investigations revealed a broad diversity of spectral and photochemical properties of synthetic BDI derivatives and provoked several attempts of their practical applications.^[2] Thus, various BDI derivatives have been used as two-photon^[3] and polymeric dyes,^[4] ion sensors^[5] and aggregation-induced emitters.^[6] Also, unique properties of BDI allowed to use them as fluorogenic labels for RNA^[7] and proteins.^[8]

Recently we have proposed a strategy for dramatic improvement of fluorescence quantum yields (FQY) of various BDI derivatives based on introducing of difluoroboryl group, which provides the conformational lock at the benzylidene part of the molecule responsible of non-radiative relaxation of the excited state. This technique allowed us to synthesize several fluorescent analogues of GFP^[9], CFP^[10] and Kaede chromophores.^[11] We also demonstrated that the introducing of amino group (giving ABDI-BF₂ core) provides an improvement of spectral properties of the BDI derivatives by making them red-shifted and pH-independent in the physiological range.^[11,12]

However, the possible formation of non-fluorescent excited state with internal charge transfer (ICT) of the ABDI-BF₂ dyes led to the solvent-dependence of FQY and impedes their usage as the common fluorescent markers.

Here, we present the creation of a novel group of ABDI-BF₂

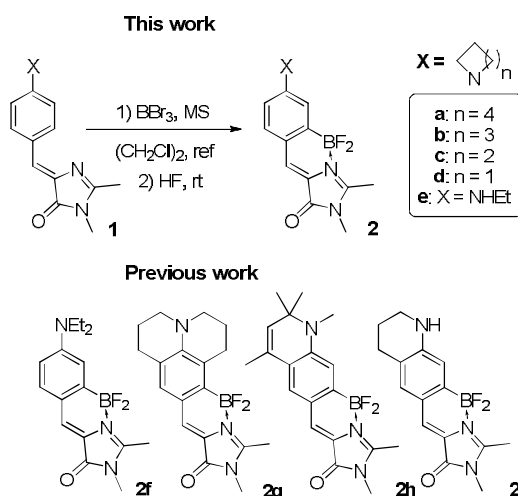
quinoidal structure. The electronic effects have a greater impact on the radiationless deactivation than conformational. Thus, the electron-donating group introduction is more effective than the rigid-derivatives creation. The presented dyes are characterized by high fluorescence quantum yields and pH-independence in the physiological pH range making them promising candidates for a wide spectrum of fluorescent labeling applications.

dyes substituted with differently sized nitrogen-containing rings as well as non-rigid derivative with one flexible substituent. Study of the optical properties of these compounds and comparison with our previous results made it possible to better understand the influence of conformational and electronic effects of the substituents on formation of non-fluorescent ICT state.

Results and Discussion

Synthesis

Conformationally locked aminated GFP chromophore analogues (ABDI-BF₂, **2**) were synthesized from corresponding imidazolones **1** by coordination-assisted borylation reaction^[13] which was previously used in the synthesis of conformationally locked derivatives of the fluorescent proteins chromophores^[9-12] and other dyes^[14] (Scheme 1). The detailed synthetic procedures and characterization of compounds are presented in Supporting Information.



Scheme 1. Various ABDI-BF₂ **2** and their synthesis

The yield of compounds **2a-c,e** varied within the range of 50-70%. At the same time, compound **2d** was obtained with a yield

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of only 10%, since the borylation of the azetidene derivative **1d** was accompanied by opening of the four-membered ring.

Quantum yields of ABDI-BF₂ dyes **2**

Previously,^[12] we had already synthesized a number of various ABDI-BF₂ dyes and found that the derivatives with two flexible alkyl substituents at the nitrogen atom (e.g. compound **2f**, Scheme 1) showed noticeable dependence of FQYs on the solvents nature (Fig 1).

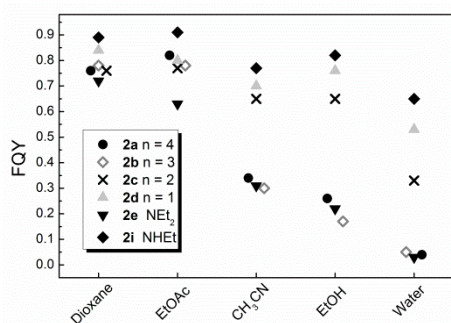
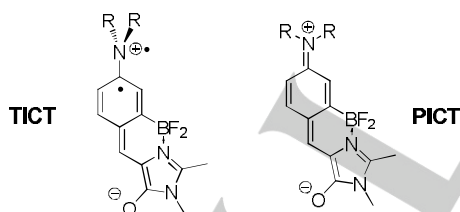


Fig. 1. Fluorescence quantum yields of compounds **2** in different solvents

The similar solvent dependence of FQY has been also described for other fluorescent dyes, comprising dialkylamino substituents, such as Nile red and Nile blue,^[15] Laurdan^[16] and other dyes.^[17] This type of behavior is determined by formation of an additional excited state, characterized by high charge separation – an ICT state.^[18] Formation of ICT state usually leads to a decrease of overall FQY,^[19] and may in several cases produce additional ICT emission band.^[20,16e,17a] It is generally considered, that in case of amino-derivatives, the ICT state can be additionally accompanied by the conformational change to form twisted geometry (TICT – Twisted Intramolecular Charge Transfer, Scheme 2)^[21] or alternatively, forming a quinoidal structure with planar geometry (PICT – Planar Intramolecular Charge Transfer, Scheme 2).^[22]



Scheme 2. Possible structure of planar and twisted forms of ABDI-BF₂ excited ICT state

One of the possible approaches to reduce the formation of ICT state is fixation of substituents at the nitrogen atom in the ortho position of the aromatic system. Thus, we previously created the group of the rigid analogues (Scheme 1, **2g** and **2h**).^[12] However, these compounds also demonstrate the FQY variation, albeit to a lesser degree, than the derivatives with free alkyls. Thus, the ICT state is most likely not twisted since the formation of the twisted geometry is not possible for the compounds **2g** and **2h**.

Another typical approach to avoid the ICT state formation is introduction of cyclic substituents at the nitrogen atom.^[23] This

approach successfully achieved in the design of wide range of the fluorescent dyes.^[23,24] Probably, the introduction of such small cycles impedes the changes of the angle between the C-N-C atoms in the aminogroup, which affects the formation of the ICT state.^[23] In this regard, in the present study we have created a series of ABDI-BF₂ derivatives with differently sized nitrogen-containing rings (ranging from azepane to aziridine, compounds **2a-d**, Fig 1).

As expected, borylation of the corresponding ABDI **1** led to significant increase of FQY (Table 1, Table S2). Yet, noticeable dependence of FQY on the solvents nature of the compounds **2a-d** has been revealed (Table 1, Fig 1, Table S2). It was found, that the most prominent FQY variation was observed for compounds **2a** and **2b** with more than tenfold FQY difference between water and dioxane solutions (Table 1, Table S1), that was not greatly diverse them from the previously obtained derivative **2f** (Table 1, Fig 1). However, for derivatives with smaller cycles (**2c** and **2d**) this effect was much less pronounced. The FQY variations of these compounds were close to that of the rigid ABDI-BF₂ **2g** and **2h** synthesized previously. Thus, for more polar and protic solvents, a decrease in the cycle size led to a noticeable increase in the quantum yield (Fig. 1). Thus, according to the data obtained in this and previous study we can guess that in case of ABDI-BF₂ derivatives ICT state has planar and quinoidal character and its formation cannot be completely suppressed by various kind of amino group substituents fixation, as it was previously reported for N-phenylpyrroles.^[25]

In our previous work we showed that the least variation was observed for rigid ABDI-BF₂ containing hydrogen atoms in the amino group (**2i**, Scheme 1, Table 1).

In the present study we have created a non-rigid analogue of this compound (**2e**, Scheme 1) and showed that FQY of this compound was the least solvent-dependent among ABDI-BF₂ derivatives synthesized in this work (Table 1, Fig 1, Table S2). Obviously, that formation of the ICT state with the high degree of charge delocalization is governed by the electronic effects of the substituent in amino group. The hydrogen atom is the weaker electron donors than alkyl groups and it less stabilizes the positive charge on the nitrogen atom (Scheme 2) which suppresses the formation of ICT state. Thus, we can guess that in case of ABDI-BF₂ derivatives electronic effects have a greater impact on the formation of ICT state than conformational.

Solvatochromism of ABDI-BF₂ dyes **2**

As reported previously for the other ABDI-BF₂ dyes^[12] substantial dependence of the absorption and emission maxima on the nature of the solvent was observed for **2a-d** (see Fig 2 for **2d** and Table 1 and Fig S1-5 for other compounds).

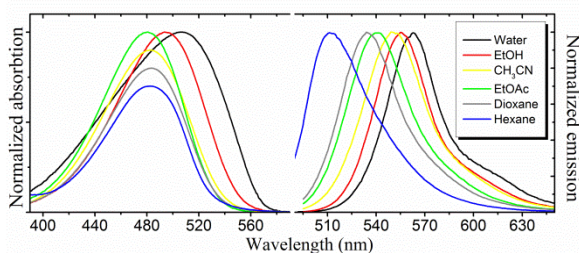


Fig. 2. Fluorescence and absorbance spectra of **2d** in different solvents

A pronounced bathochromic shift was observed in polar and protic solvents, thus the difference between water and hexane reached 30–50 nm. This phenomenon was shown previously for the other ABDIs^[26] and a group of dialkylamino-substituted dyes.^[15–17]

The nature of N-alkyl substituent also influenced on the maxima positions (Fig 3). The derivatives with large cyclic substituents **2a,b** had the positions of the maxima almost identical to the diethylamine derivative **2f**, whereas the maxima of the derivatives with smaller cycles of **2c,d** were slightly (10–20 nm) blue-shifted, which correlates well with the previously reported data.^[23] The compound **2e** was characterized by an even larger blue-shift of absorption and emission spectra (Table 1), which is explained by the less donor nature of the substituent. This effect was previously observed for other fluorescent dyes containing amino groups,^[15,16] as well as other BDI.^[26a,e] and relates to the influence of the substituents nature on the position of the Frontier Orbitals (HOMO and LUMO).

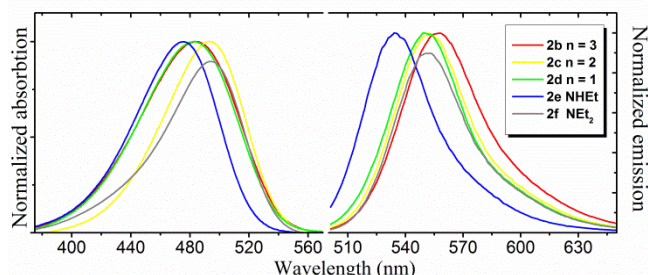


Fig. 3. Fluorescence and absorbance spectra of compounds **2b-d**, **2a**, and **2f** in acetonitrile

Analysis of the emission spectra of compounds **2a-d** in different solvents (Table S3) revealed no unusual emission maxima, as it was shown previously for **2f** and others, which indicates a nonradiative decay of ICT state. This claim is additionally confirmed by the fact that the positions of emission maxima of the ABDI-BF₂ **2** dyes was in a good agreement with a Kamlet-Taft model^[27] as it was shown previously for **2f**.^[12] This model correlates the spectral shift ν of the solute with the solvent parameters that are responsible for the acidic (α), basic (β), and polar solvating (π^*) properties of the latter:

$$\nu (1/\text{cm}) = \nu_0 + p\pi^* + a\alpha + b\beta$$

Multivariable regression of absorption and emission data for compounds **2a,c,d,f** data are summarized in Table 2.

As it was shown previously for **2f** both the dipole moment (parameter p) and acidity (parameter b) increase upon excitation of other ABDI-BF₂ derivatives **2**. An unusual switch of b sign from positive (hypsochromic shift) to negative (bathochromic

shift) upon excitation was also revealed that indicate a dramatic shift in their photoacidity. Herewith the values of all the parameters were not very different for the whole group of compounds, however, the difference between the ground state and the excited one decreased with decreasing cycle size.

Table 2. Solvatochromic spectral parameters (in 10³/cm) of **2a,c,d** compared to **2e**

Cmpd	a	b	p	ν_0	R
2e Abs ^[12]	-0.5	0.6	-0.4	20.2	0.84
2a Abs	-0.6	0.6	-0.3	20.0	0.87
2c Abs	-0.5	0.6	-0.4	20.1	0.86
2d Abs	-0.6	0.3	-0.6	20.9	0.90
2f Em ^[12]	-0.3	-0.5	-1.2	19.4	0.95
2a Em	-0.3	-0.4	-1.1	19.1	0.97
2c Em	-0.3	-0.5	-1.0	19.2	0.96
2d Em	-0.3	-0.4	-1.0	19.3	0.95

Acid-base behavior of ABDI-BF₂ dyes **2**

The study of aqueous solutions of compounds **2** has shown that an acidification leads to a noticeable bathochromic shift in absorption maxima associated with protonation of the aromatic amino group (Fig 4, S10–13). As it was shown earlier, all compounds **2** in protonated form possessed merely identical spectral characteristics, since conjugation of the amino group lone pair is lost upon protonation leading to the disappearance of the nitrogen atom substituents' influence.

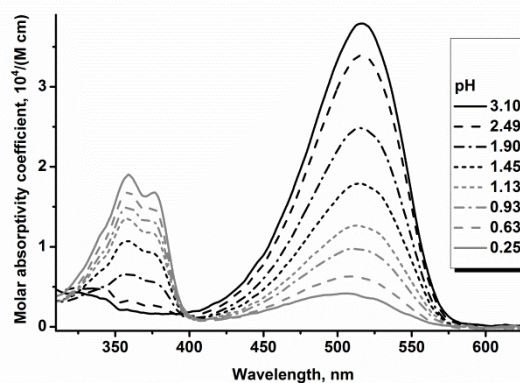


Fig. 4. pH-titration of compound **2c**

All the new compounds **2** obtained in this study were characterized by a fairly low pK_a value (2.5–1.5). At the same time we surprisingly observed no significant differences between pK_a of the cyclic derivatives **2a-d**. All these compounds were characterized by pK_a value close to 2, which is noticeably lower than that of the dialkyl derivative **2f** and its analogues (Table S4, Fig S6–9).

Stability of ABDI-BF₂ dyes **2**

Earlier,^[10] we found out that the borylated derivatives of chromophores are easily destructed in basic conditions. In this

regard, we also have studied the chemical and photo-stability of the derivatives **2**, which were synthesized in this work, as well as a rigid and non-rigid compounds **2g** and **2f** obtained previously.^[12]

We have established that the action of an aqueous solution of methylamine (this reagent was chosen because it does not lead to a decrease in solubility and is also used in the synthesis of fluorescently labeled oligonucleotides) leads to the destruction of all compounds **2**. In this case, the disappearance of fluorescence is also accompanied by loss of color, which is evidence of the previously proposed mechanism of the decomposition associated with the opening of the imidazolone ring.^[10] The rigid derivative **2g** which was obtained by us earlier was the least stable, whereas the cyclic derivatives **2c,d** synthesized in the present study showed much greater stability (Fig 5A, Fig S14-15).

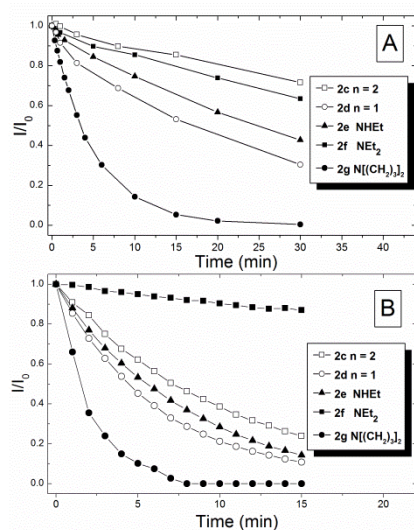


Fig 5. Time-dependent decrease of compounds **2** fluorescence in methylamine solution (12% in water) at the room temperature (A) and upon irradiation (B).

Continuous exposure with light (515 nm diode, Fig 5B, Fig S16) also led to a loss of fluorescence and color of the solutions of compounds **2**. The previously obtained rigid derivative **2g** was also the least photostable. For the other compounds **2** obtained in the present study a pronounced dependence of photostability and FQY (Fig 5B, Fig S16) was observed, which indicates that the destruction of compounds upon irradiation probably occurs from the fluorescent form of the excited state (locally excited state) and not a non-fluorescent ICT state, as far as the less stability appears for more fluorescent substances that last longer in the fluorescent state.

Conclusions

Thus, we report the creation of a novel group of dyes base on the ABDI-BF₂ core. These dyes **2** are characterized by high fluorescence quantum yields and pH-independence of spectra in the physiological range. We introduced substituents with differently sized nitrogen-containing rings in the ABDI-BF₂ core

and studied the impact of the ring size on the formation of non-fluorescent ICT state. The results of this study and our previous work allowed us to claim that in case of ABDI-BF₂ ICT excited state is non-fluorescent and has quinoidal character with non-twisted planar structure (PICT). Additional synthesis of the non-rigid compound **2e** bearing hydrogen atom in amino group demonstrated that electronic effects have a greater impact on the formation of ICT state than conformational. Thus, the electron-donating group introduction is more effective than the rigid-derivatives creation.

Highly-fluorescent cycloalkylamino (**2c,d**) and non-rigid (**2e**) derivatives, demonstrated significantly greater chemical and photo-stability than the rigid-derivatives which we proposed earlier. They are also more chemically accessible compounds and seem to be promising fluorescent dyes for various applications.

Experimental Section

Synthetic procedures, additional spectral data and pH titration curves are presented in Supporting information.

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Keywords: GFP • chromophore • fluorescence • borylation • ICT-state

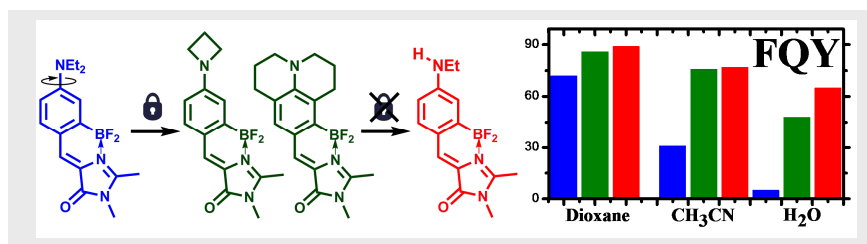
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Table 1. Optical properties of compounds **2** in different solvents

		Water	EtOH	CH ₃ CN	EtOAc	Dioxane	pK(water)
2a	Abs	519(30000)	509(33000)	495(31000)	492(34000)	497(34500)	2.0
	Em	567	558	553	541	538	
	QY	0.04	0.26	0.34	0.82	0.76	
2b	Abs	~505*	500(33000)	484(37000)	481(39000)	487(42000)	-*
	Em	~560*	564	558	546	540	
	QY	~0.05*	0.17	0.30	0.78	0.78	
2c	Abs	516(33000)	508(35500)	493(35500)	491(38500)	496(39500)	1.6
	Em	562	560	552	541	537	
	QY	0.33	0.65	0.65	0.77	0.76	
2d	Abs	508(51500)	493(55500)	482(59000)	480(62500)	484(62000)	2.0
	Em	562	556	551	542	535	
	QY	0.53	0.76	0.70	0.80	0.84	
2e	Abs	495(31500)	500(35500)	475(32500)	477(36000)	481(36000)	2.5
	Em	548	544	536	527	521	
	QY	0.65	0.82	0.77	0.93	0.89	
2f	Abs	520(53500)	505(48000)	494(45500)	492(47000)	497(51000)	3.9
	Em	563	557	554	538	538	
	QY	0.03	0.22	0.31	0.63	0.72	
2i	Abs	515(45000)	512(45000)	487(44000)	492(43500)	494(45000)	2.2
	Em	560	556	547	540	533	
	QY	0.63	0.64	0.67	0.64	0.65	

(* - non soluble enough)



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The role of N-substituents in radiationless deactivation of aminated derivatives of locked GFP chromophore

We report the creation of a group of the fluorescent dyes based on the locked GFP chromophores aminated derivatives. Their properties study allowed us to claim that their fluorescence quantum yield variation is determined by the formation of the non-fluorescent internal charge transfer excited state with a planar quinoidal structure. Thus, the electronic effects have a greater impact on the radiationless deactivation than conformational.

Key Topic: fluorescent dyes