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Graphical Abstract





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Naphthalene derivatives of a conformationally locked GFP chromophore with large Stokes shifts

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ABSTRACT

The active development of fluorescence microscopy requires an increase in the variety of the dyes and their characteristics. Compounds with a large Stokes shift, i.e. a large difference between the positions of the absorption and emission maxima, have attracted the interest of researchers as a tool that can be used for multicolor labeling. One of the known approaches to increase the Stokes shift is the introduction of additional polycyclic fragments. Herein, we report novel derivatives of a conformationally locked GFP chromophore containing the naphthalene ring. The proposed modification leads not only to the enhancement of the Stokes shift up to 100 nm but also leads to the noticeable red-shift of the emission and absorption spectra in contrast with the corresponding derivatives with one benzene ring.

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Introduction

Fluorescent labeling is one of the most important research technique of modern science.¹ Most commonly, it is used in fluorescence microscopy to visualize biological processes in real time. Various fluorescent molecules play leading roles as a key tool in such experiments.² The active development of fluorescence microscopy requires an increase in the variety of the dyes and their characteristics. Compounds with a large Stokes shift, i.e. a large difference between the positions of the absorption and emission maxima, have attracted the interest of researchers as a tool that can be used for multicolor labeling. One of the known approaches to increase the Stokes shift is the introduction of additional polycyclic fragments (for example, naphthalene) into the dye. This approach has previously been used to create analogs of fluorescein,³ rhodamine,⁴ BODIPY⁵ and other dyes⁶ with a Stokes shift exceeding 100 nm.

Among the diversity of dyes, various derivatives of the GFP chromophore should be noted.⁷ These compounds contain the benzylidene imidazolone (BDI) core,⁸ i.e. they belong to a very wide class of dyes based on the methine motif.⁹ They are characterized by diverse colors and can be synthesized easily;¹⁰ therefore they have been utilised as components of fluorescent labeling systems¹¹ and as fluorescent tags themselves.¹² It was also shown that they often demonstrate low fluorescence quantum yield (FQY) in solution, but their fluorescence can be significantly increased if the benzylidene moiety is conformationally fixed (otherwise, its flexibility can lead to nonradiative dissipation of excitation energy and FQY reduction).¹³ Thus, we have previously synthesized a number of

fluorescent GFP chromophore derivatives with an immobilizing difluoroboryl group.¹⁴

In the present work, in accordance with the aforementioned approach of the Stokes shift increase, we proposed to introduce polycyclic fragments onto the structure of conformationally locked GFP chromophore derivatives (Scheme 1). We showed that the introduction of a naphthalene group to the benzylidene moiety led to a red-shift of the absorption and emission maxima, as well as a significant increase in the Stokes shift to 100 nm or more.



Scheme 1. Previously synthesized derivatives of a conformationally locked GFP chromophore and their analogs with a naphthalene ring.

Results and Discussion

The synthesis of initial non-locked imidazolones was conducted via the standard approach using ethyl((1-

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methoxy)amino)acetate¹⁵ from aldehydes **8**, **11** and **14** (Scheme 2). Introduction of the difluoroboryl group was carried out *via* the sequential addition of boron tribromide and hydrogen fluoride¹⁶ (Scheme 2).



Scheme 2. Synthesis of naphthalene derivatives of a conformationally locked GFP chromophore.

It is interesting to note that the synthesis of derivative **6** requires the introduction of an additional bulky protective group since the action of boron tribromide on an unprotected analog of imidazolone **12** led to formation of the bromo-naphthol derivative (probably resulting from the addition-elimination reaction of BBr₃ with this naphthol).

The study of the optical properties showed that the FQY of all non-locked compounds (**10**, **12** and **15**) was extremely low (less than 0.1%) while the borated derivatives showed sufficiently intense fluorescence in almost all solvents. We found that the introduction of the naphthalene ring led not only to red-shift of the maxima position but also to a noticeable increase in the Stokes shift (Table 1, Fig. 1). The maximum effect was observed for 2,6 substituted naphthalene derivatives, which apparently provide the maximum geometric separation of substituents.

All compounds were also characterized by pronounced solvatochromism (Table 1, Fig. 2). The most long-wave position of the maxima was observed for the most polar and protic solvents, as it was previously shown for other BDL.^{14c} This effect was more pronounced for the positions of emission maxima, that indicates a possible high degree of charge redistribution in the excited state, which is stabilized in polar media.



Figure 1. Absorbance (left) and emission (right) spectra of the original dyes (solid black) and their naphthalene (solid gray) derivatives in acetonitrile.



Figure 2. Absorbance (left) and emission (right) spectra of compounds 4, 5, 6 and 7a in various solvents.

		H-derivatives		OH-derivatives						NR ₂ -derivatives		
		1 ^{14a}	4	2 ^{14b} (original)		5		6		3 ^{14c}	7.0	76
		(original)	4	neutral	anionic	neutral	anionic	neutral	anionic	(original)	/a	70
Water	Abs ^a	368	427(-°)	404	485	466(15)	561(56.5)	424(14)	490(55)	511(49.5)	476(-°)	511(-°)
	Em ^b	430(~10)	532(65)	485	520	554(77)	572(40)	564(27)	638(5)	564(5)	670(4)	674(2)
	Brightness	-	_c	-	-	11500	22500	4000	3000	2500	_c	_c
EtOH	Abs ^a	365	436(-°)	416	512	479(20)	565(52.5)	430(14.5)	529(58)	496(46.5)	498(18)	510(26)
	Em ^b	426(~20)	510(30)	483	532	543(65)	573(33)	550(61)	653(9)	555(35)	650(19)	653(13)
	Brightness	-	_c	-	-	13000	17500	9000	5000	16500	3500	3500
CH ₃ CN	Abs ^a	360	425(19)	402	533	458(23)	563(49.5)	422(17.5)	_d	485(45)	487(21)	498(30)
	Em ^b	420(55)	497(86)	469(73)	540	532(80)	571(27)	524(77)	_d	552(48)	641(29)	646(20)
	Brightness	-	16500	-	-	18500	13500	13500	_d	22000	6000	6000
EtOAc	Abs ^a	362	428(19.5)	410	533	467(23.5)	567(48.5)	429(17.5)	_ ^d	484(46.5)	486(22)	498(30)
	Em ^b	421(63)	484(95)	465	543	525(80)	574(19)	511(88)	_d	538(67)	607(40)	617(31)
	Brightness	-	18500	-	-	19000	9000	15500	_d	31000	9000	9500
Dioxane	Abs ^a	365	435(20)	414	529	474(21)	567(50.5)	433(18)	_d	489(52.5)	492(22.5)	508(31.5)
	Em ^b	422(78)	483(98)	464	541	523(86)	576(7)	504(94)	_d	532(78)	595(45)	602(34)
	Brightness	-	19500	-	-	18000	3500	17000	_d	41000	10000	10500

Table 1. Optical properties of compounds 1-7 in various solvents at 3-10 μ M concentrations.

^a peak maximum in nm (extinction coefficient in (mM cm)⁻¹), ^b peak maximum in nm (fluorescence quantum yield in %)), ^c non-soluble enough, ^d anionic form cannot be obtained.

Solvatochromism was especially noticeable for 7 amine derivatives. These compounds were also characterized by a significant variation in FQY depending on the properties of the solvent. The analysis of the behavior of these compounds using the Kamlet-Taft model (see ESI part 3) showed that their solvatochromic properties are similar to the properties of other previously obtained conformationally locked amino BDIs. Thus, both compounds 7 demonstrate the dipole moment and acidity increase upon excitation.

Previously, we suggested that this FQY variation can be explained by the formation of an additional non-fluorescent excited state characterized by a significant degree of charge separation and planar geometry (PICT state). Formation of this state (as well as FQY quenching) cannot be prevented by conformational fixation of the benzylidene moiety, as it is not accompanied by a loss of planarity. But for simple amino-BDI, these changes could be partially blocked by the introduction of rigid substituents at the nitrogen atom, for instance, by the introduction of a pyrrolidine residue.¹⁷ However, the introduction of this group did not significantly affect the variation of FQY in the case of naphthalene derivatives – the characteristics of 7a and 7b derivatives were similar. The introduction of the pyrrolidine fragment allowed shifting the spectral maxima to the long-wave region but the spread of FQY over the solvents remained practically unchanged. Such a variation can make it difficult to use these compounds as common fluorescent dyes but enable their use as fluorogenic dyes - compounds that have weak fluorescence in the free-state but strongly fluoresce when binding to a specific target.11

The study of the spectra in water showed that acidification of solutions of compounds 7 or basification of solutions of compounds 4 and 5 is followed by a noticeable shift of the maxima of absorption and emission. These shifts are explained by the respective protonation of the amino group and deprotonation of the phenol moiety. Deprotonation of phenols 4 and 5 led to a noticeable bathochromic shift and was characterized by pKa 6.0 and 8.3, respectively. Protonation of 7a and 7b led to a hypsochromic shift and occurred at a pH of \sim 3-4 (see ESI part 4, Table S4).

Conclusion

In this paper, we proposed a new group of derivatives of a conformationally locked GFP chromophore, containing a naphthalene residue. The novel dyes are characterized by a noticeable red-shift of emission and absorption spectra in contrast with the corresponding derivatives that contain only one benzene ring. Also, all the obtained compounds are characterized by a significant Stokes shift and most of them have no pH-dependent transformation under physiological conditions. Thus, the presented dyes are excellent candidates for application as novel fluorescent tags, which can potentially be used for multicolor labeling together with other dyes with small Stokes shift. Compounds 7 are noteworthy as they demonstrate not only a large Stokes shift but also a significant variation of FQY, which can be used in actively developing fluorogenic labeling technologies.

Acknowledgments

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- 15. The corresponding aromatic aldehyde (10 mmol) was dissolved in CHCl₃ (50 mL) and mixed with a methylamine solution (40% aqueous, 2.5 mL) and anhydrous Na₂SO₄ (10 g). The mixture was stirred for 48 h at room temperature, filtered and dried over additional Na₂SO₄. The solvent was evaporated, then ethyl((1-methoxy)amino)acetate (1.75 g, 11 mmol) was added. The mixture was stirred for 24 h at room temperature, the solvent was evaporated, and the product was purified by column chromatography.
- 16. The corresponding imidazolone (2 mmol) was dissolved in dry DCE (200 mL), molecular sieves 4 Å (30 g) and 3 Å (30 g) were added, followed by a solution of boron tribromide in DCE (1M, 10 mL, 10 mmol). The reaction mixture was heated at reflux for 4 h (for 4, 5 and 7) or stirred for 14 d (for 6) under an inert atmosphere. The mixture was cooled and filtered; the molecular sieves were washed with ethanol (2 × 25 mL) and DCE (2 × 50 mL). The solution was mixed with aqueous HF (20%, 8 mL) and stirred for 30 min. The mixture was dissolved with EtOAc (100 mL), washed with NaHCO₃ (5%, 2 × 50 mL), water (2 × 50 mL) and brine (2 × 50 mL) then dried over Na₂SO₄. The solvent was evaporated and the product was purified by column chromatography.
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Derivatives of locked GFP chromophore containing the naphthalene ring are presented.

Compounds are characterized by a noticeable redshift of emission and absorption.

Accepting The proposed compounds demonstrate large Stokes shifts.