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Synthesis and photochemistry of pH-sensitive GFP chromophore analogs

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ABSTRACT

GFP chromophore analogs (**7a**–**e**, **8**, and **10a**,**b**) containing 2-thienyl-, 5-methyl-2-furyl-, 2-pyrryl, and 6-methyl-2-pyridyl-groups were synthesized and their fluorescence spectra recorded in the pH range 1–7. NMR studies showed that protonation of **8** (2-thienyl system) inhibited photoisomerization (*Z*–*E*) about the exocyclic double bond but that protonation of **7c** (*E* + *Z*) (2-pyrryl system) gave only **7cE**. Fluorescence studies revealed enhancement of fluorescence intensity of **7c** and **7b**,**e** (furyl system) below pH 2.5 and gave a similar result for **10a** (pyridyl system) below pH 6. Quantum yields at pH 1 were low, probably due to excited state proton transfer (ESPT).

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1. Introduction

Fluorescent peptide labeling is useful for monitoring biological activity since a fluorophore in a peptide or a protein enables ligands, inhibitors, and antigens to be detected at low concentration.[1] Natural aromatic amino acids (Phe, His, Trp, and Tyr) play key roles in the recognition of receptors and have frequently been replaced by unnatural aromatic amino acids in bioactive peptides.[2]

Green fluorescent protein (GFP) chromophore **1**, Scheme 1a, and similar proteins (CFP or YFP) are well established as fluorescent markers for monitoring biological activity because they have high light emission (quantum yields up to $\Phi_f = 0.8$) and work well both in vitro and living mammalian cells.[3] However, the large size (up to 238 amino acids) of GFP can cause misfolding or other structural changes in target proteins. Unlike the chromophore of wild-type GFP, which is surrounded by its protein sequence (1–64 and 68–238) and stabilized as the *Z*-isomer,[3c,4] the GFP model chromophores of type **2** show only low fluorescence at 20 °C due to *Z*–*E* photoisomerization at the exo-methylene group (Scheme 1b).[5] Arai et al. demonstrated that hemi-indigo derivative **3**[6] exists as the *Z*-isomer stabilized by six-membered ring intramolecular hydrogen bonding, thus preventing or minimizing photoisomerization (Scheme 1c).The GFP chromophore analog **4** is also stabilized as the *Z*-isomer by boron ligation and shows high fluorescent activity ($\Phi_f = 0.89$) compared to low fluorescence of the boron ligated *E*-isomer ($\Phi_f = 0.0007$, Scheme 1d).[7a]

Zelewsky and co-workers showed that the proton, the smallest known cation, can act as a coordinating center and fix bipyridine ligands in a helical conformation.[7b] We reasoned, therefore, that molecules of types **7**, **8**, and **10**, might be stabilized as the *Z*-isomer by protonation of the imidazolinone nitrogen (or pyridine N in the case of **10a**) and subsequent hydrogen bonding with the heteroatom of the adjacent heterocyclic ring. The objective of the work was to test this hypothesis and monitor the effect of protonation on fluorescence activity.

2. Results and discussion

2.1. Preparation of imidazolinone chromophores 7a–e and 8

Azalactones **6a**–**e** were each synthesized by reaction of hippuric acid **5a** or 2-(2-naphthamido)acetic acid **5b** with the appropriate aldehyde in the presence of sodium acetate and acetic anhydride (Scheme 2).[8] Compounds **6a**–**e** reacted under microwave conditions with *N*,*N*-dimethylethylenediamine to give **7a**–**e** in yields of 30–81% (Table 1).

Compound **8** was also synthesized from **6a** and *p*-toluidine in 56% yield (Scheme 3a). Fluorophore **8** was isolated as the *Z*-isomer as revealed by ^{1}H NMR (Fig. S1a, see ESI) which showed an upfield resonance at 6.8 ppm for the olefinic proton analogous to that found in the boron complex of **4**-*Z* and in contrast to the downfield resonance of the olefinic proton of **4**-*E*.[7a] After 1.5-5.5 h under UV light (365 nm) a solution of **8** in DMSO-d_6, revealed the formation of increasing amounts of the *E*-isomer (Fig. S1b and c, see ESI). In the presence of concd HCl, the NMR spectrum showed only the *Z*-isomer even after 16 h under UV irradiation (Fig. S1d, see ESI)

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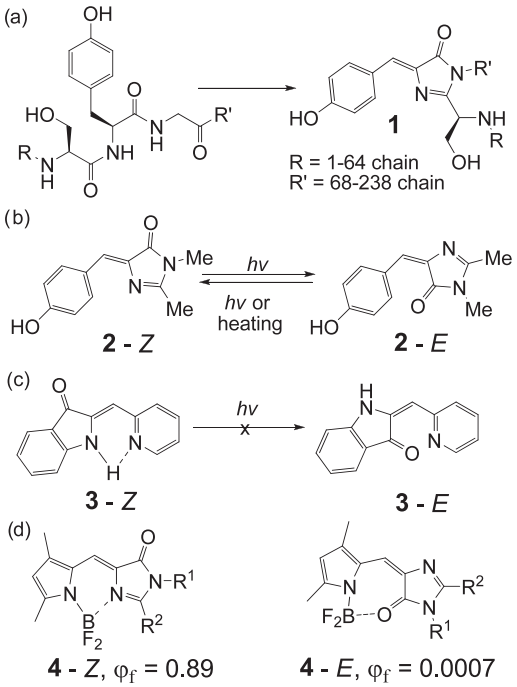
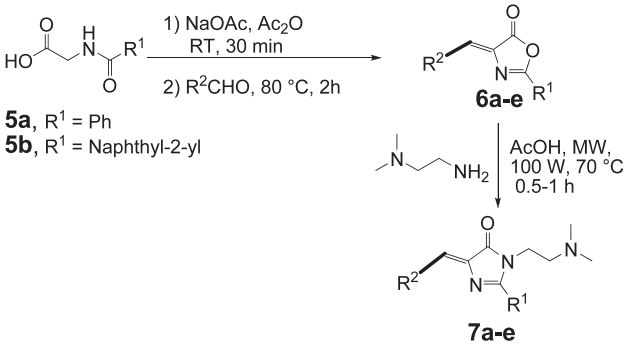


Scheme 1.



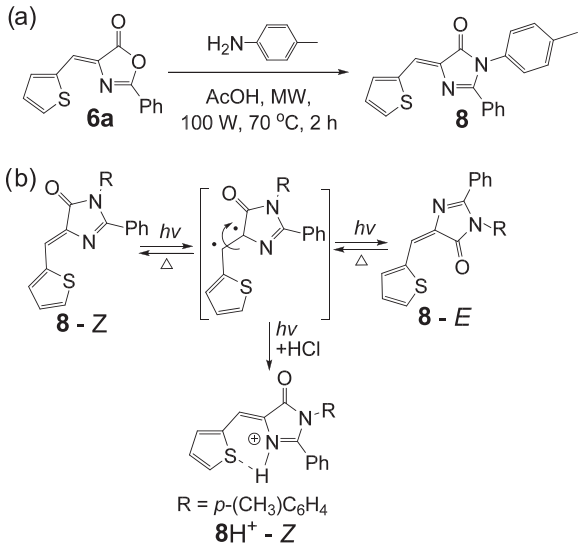
Scheme 2. (For designation of R^1 and R^2 see Table 1).

demonstrating stabilization of the *Z*-isomer by intramolecular hydrogen bonding (Scheme 3b).

2.2. ^{1}H, ^{13}C, ^{15}N NMR study of 7b and 7c

^{1}H and ^{13}C chemical shifts were assigned based on the ^{1}H–^{1}H, one-bond, and long-range ^{1}H–^{13}C couplings, of the gDQCOSY, gHMQC, and gHMBC spectra. Protonation of **7b** and **7c** was studied by ^{15}N NMR in trifluoroacetic acid-*d* (TFA-*d*) using ^{1}H–^{15}N CIGAR-gHMBC experiment (see Fig. 1 for numbering in **7b** and **7c**).

The ^{15}N chemical shift of N-1 was identified by long range correlation with the two methylene groups 1‴ and 2‴. The ^{15}N NMR chemical shift of N-3 was identified by three bond correlation to H6. The dimethylamino nitrogen (N-3‴) chemical shift was revealed by long range correlation with the protons of the two methyl groups (H-4‴) and the two methylene groups (H-1‴ and H-2‴). The data for **7b** and **7c** are reported in Supplementary data (Table S1). In TFA-*d* the ^{15}N chemical shift of N-3 in **7b** moves upfield by 90.5 ppm consistent with protonation and formation of the *Z*-isomer of **7b** by intramolecular hydrogen bonding with furyl



Scheme 3.

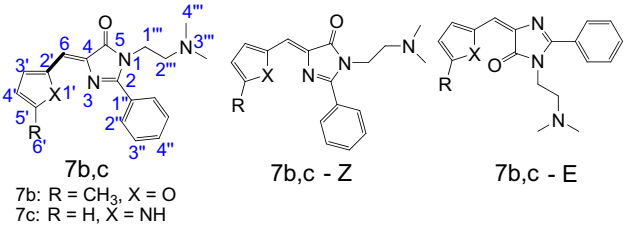
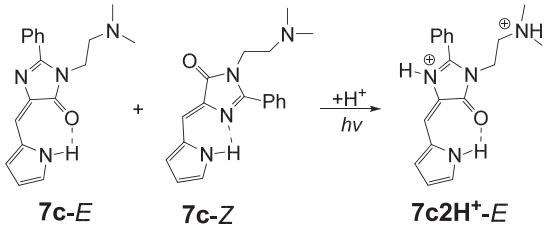


Figure 1.



Scheme 4.

Table 1
R^1 and R^2 designation for **6a–e** and **7a–e**

Entry	R^1	R^2	Compd (Yield)[a]	Compd (Yield)[a]
1	Ph	2-Thienyl	**6a** (68%)	**7a** (33%)
2	Ph	5-Methyl-2-furyl	**6b** (59%)	**7b** (81%)
3	Ph	2-Pyrryl	**6c** (35%)	**7c** (55%)
4	Naphth-2-yl	2-Thienyl	**6d** (50%)	**7d** (51%)
5	Naphth-2-yl	5-Methyl-2-furyl	**6e** (40%)	**7e** (30%)

[a] Isolated yield.

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