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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).

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aromatic amino acids (Phe, His, Trp, and Tyr) play key roles in the recognition of receptors and have frequently been replaced by unnatural aromatic amino acids in bioactive peptides.[2]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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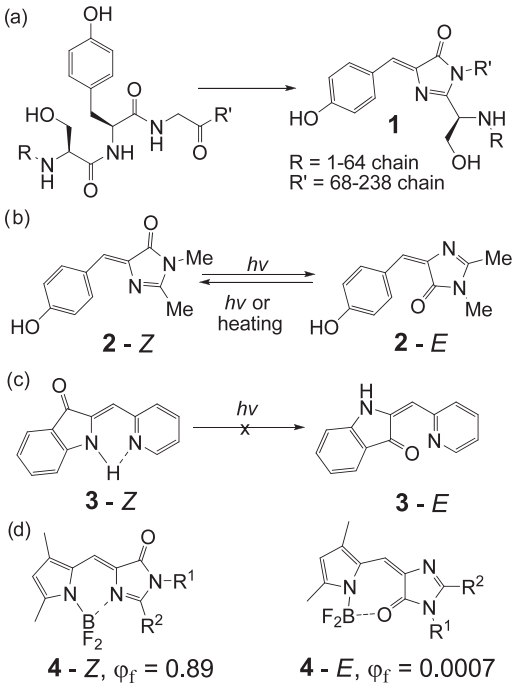
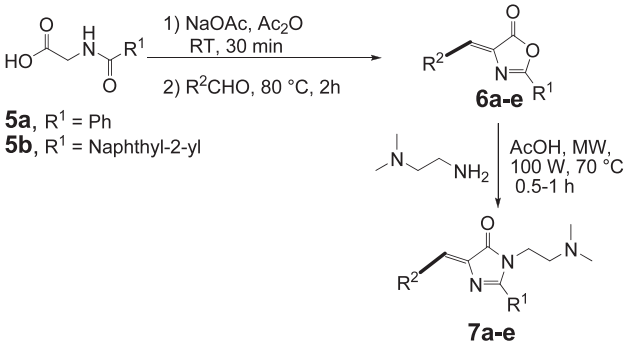


Scheme 1.



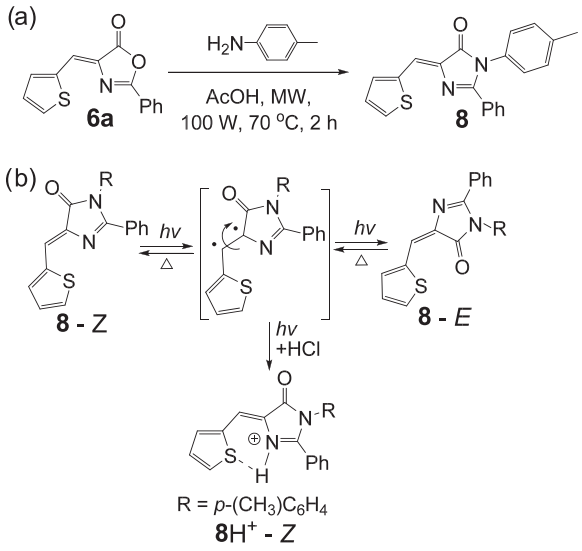
Scheme 2. (For designation of R^1 and R^2 see Table 1).

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.

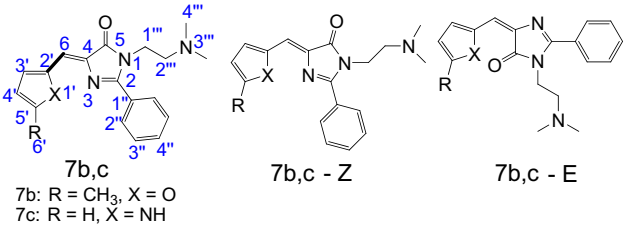
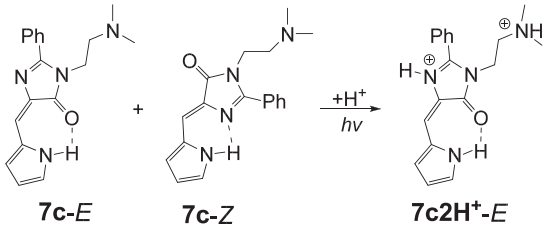


Figure 1.



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.

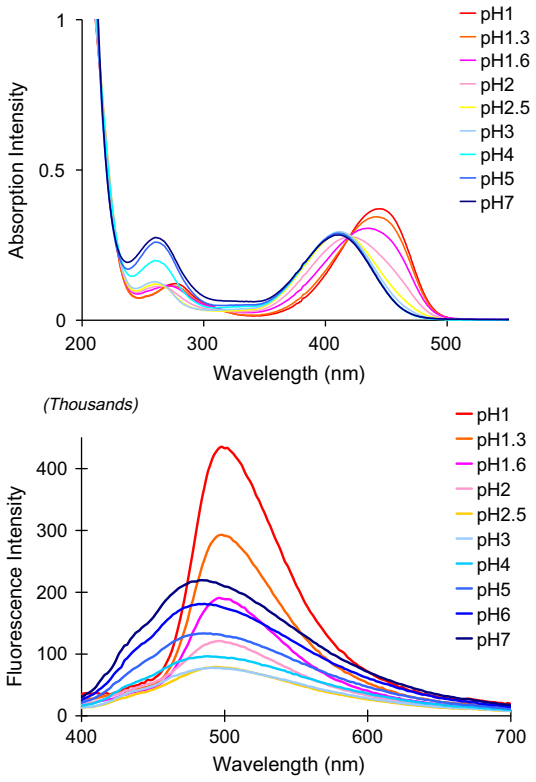
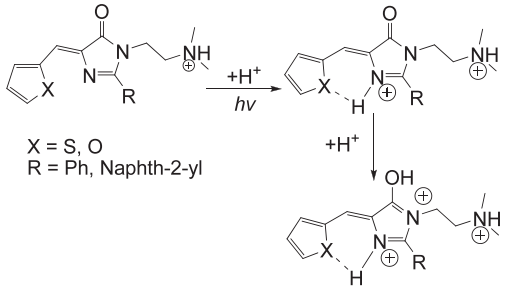


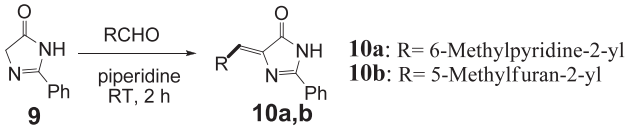
Figure 2. Absorption (top) and emission (bottom) spectra of **7b** at 10^{-5} M in Britton–Robinson Buffer at pH 1-7. (Fluorescent spectra were subjected to Raman peak correction).

oxygen. The coupling of 3.7 Hz between H-6 and C-5 confirms the *Z*-isomer. Compound **7c** exists in $CDCl_3$ as a mixture of *E*- (75%) and *Z*-isomers (25%), but in TFA-*d* only one isomer is found with the ^{15}N chemical shift of N-3 shifted upfield by 95.9 ppm from the *E*-isomer and 82.7 ppm from the *Z*-isomer. We suggest that in this case, the *E*-isomer (**7c2H$^+$**) predominates in the protonated system due to hydrogen bonding between the NH proton of the pyrryl group and the carbonyl oxygen (Scheme 4).

This hypothesis was confirmed by a coupling of 8.5 Hz between H-6 and C-5. Furthermore, the ^1H-^{15}N CIGAR experiment failed to detect $^3J_{H6\text{-}N3}$ coupling which suggests a value < 3.0 Hz and further supports the *E*-isomer assignment. The amide nitrogen of the imidazolinone ring, however, shifted downfield by 9.2 ppm (**7b**), 9.2 ppm (**7c** from *E*-isomer), and by 4.9 ppm (**7c** from *Z*-isomer) which provides evidence for protonation of this nitrogen. For the dimethylamino nitrogen $-N(CH_3)_2$, prominent deshieding was observed in protonated **7b2H$^+$** (+17.5 ppm) and **7c2H$^+$** (+16.0 ppm from *E*-isomer and 16.2 ppm from *Z*-isomer). In contrast to sp^2



Scheme 5.



Scheme 6.

nitrogens, sp^3 nitrogens are known to be deshielded on protonation.[9]

2.3. Fluorescence studies of 7a–e

The absorption and emission spectra of the fluorophores **7a-e** at 10^{-5} M in Britton–Robinson Buffer solution were recorded over the pH range 1–7. From pH 7 to pH 3 there is virtually no change in the absorption spectra of **7a–e** but a distinct decrease in the intensity of the emission spectra by factors that range between 2 and 4 (Fig. 2 for **7b**, S2-4 for **7a**, **7c**, and **7e**, see ESI). The origin of the decrease in fluorescence intensity is clearly not associated with the furyl, thienyl, or pyrryl units or to protonation of N-3 of the imidazolinone ring (pKa ca. 1.8).[10] Compounds **7a** and **7d**, both containing the 2-thienyl group, gave virtually identical absorption and emission spectra (shown only for **7a**, Fig. S2) that revealed a small (ca. 20 nm) bathochromic shift of the absorption spectra below pH 2.5 but no change in the emission spectra. The results are best explained by protonation of N-3 of the imidazolinone ring but only weak H-bonding with sulfur that may allow the thiophene ring to twist out of planarity with the imidazolinone system (Scheme 5).

With compounds **7b** and **7e** containing the 5-methyl-2-furyl unit, the absorption spectra again showed bathochromic shifts (ca. 40 nm) below pH 2.5, but these were accompanied by significant intensity increases of the emission spectra by factors of 7 (Fig. 2 for **7b**) and 10 (Fig. S4 for **7e**). Clearly, protonation of N-3

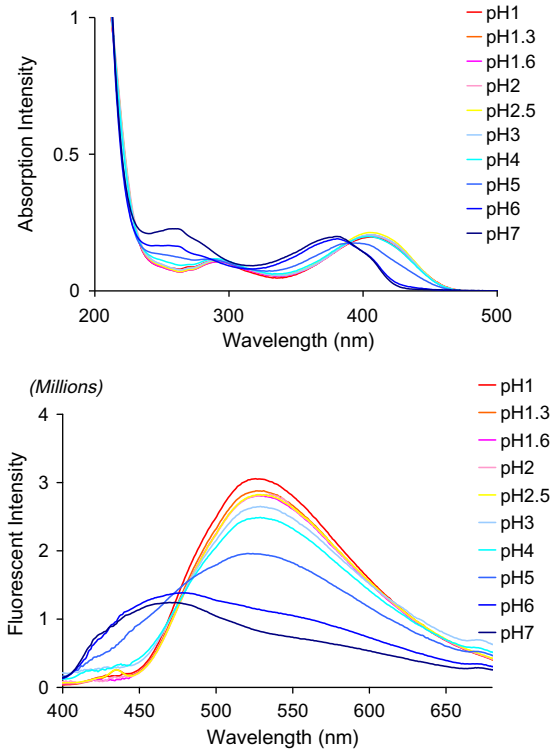
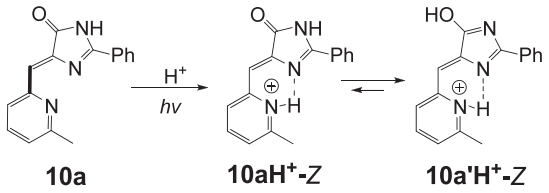


Figure 3. Absorption (top) and emission (bottom) spectra of **10a** at 10^{-5} M in Britton–Robinson Buffer at pH 1–7. (Fluorescent spectra was subjected to Raman peak correction).



Scheme 7.

Table 2
Quantum yields and excitation coefficients of **7a**–**e** and **10a**,**b** at pH 1

Entry	Compound	$\lambda_{abs\ max}$	ε ($M^{-1}\ cm^{-1}$)	$\lambda_{em\ max}$	Φ_f
1	**7a**	409	24313	488	0.0008
2	**7b**	444	22376	502	0.0037
3	**7c**	462	45580	494	0.0017
4	**7d**	410	26913	512	0.0008
5	**7e**	451	28617	511	0.0060
6	**10a**	405	20343	526	0.0293
7	**10b**	449	20046	503	0.0070

in **7b** and H-bonding with furyl oxygen enforces planarity on the *cis* configuration and the ensuing higher degree of conjugation enhances both the fluorescence wavelength and intensity (Scheme 5).

Finally, compound **7c** containing the pyrrole system, shows similar behavior to that of **7b** and **7e** with emission intensity increasing five fold as the pH falls from 2.5 to 1 (Fig. S3, see ESI). With **7c,** however, protonation may lead to the *Z* or *E* isomers as depicted in Scheme 4, either or both of which may account for the increase in fluorescence intensity.

2.4. Preparation and fluorescence of imidazolinone chromophores 10a,b

Compounds **10a** and **10b** were synthesized in yields of 18% and 29%, respectively, from compound **9**[7a] as shown in Scheme 6.

The absorption and emission spectra of **10a** and **10b** were recorded at 10^{-5} M in Britton–Robinson Buffer (Fig. 3, S5, see ESI). Fluorescence of **10a** increased below pH 6 initially due to protonation of the pyridine nitrogen to **10a**H^+-*Z* followed by tautomerism of the amide group to **10a**'H^+-*Z* (Scheme 7) as indicated by the red shift in both absorption and emission spectra. However, fluorescence of **10b** increased only below pH 2.5 thus exhibiting similar behavior to that of **7b** and **7e**.

2.5. Quantum yields (Φ_f) measurements

The molar extinction coefficients (ε, $M^{-1}\ cm^{-1}$) and quantum yields (Φ_f) of **7a**–**e** and **10a**,**b** at pH 1 are shown in Table 2. The best potential fluorescence marker, **10a**, provides only 2.9% of the fluorescence intensity of GFP and then only at pH 1. It seems likely that the presence of protons necessary to enforce planarity and thus enhance conjugation, also provides a pathway for fluorescence decay via the phenomenon of excited state proton transfer.[11]

3. Conclusions

GFP modified pH-sensitive chromophores were synthesized and their fluorescence activity measured in Britton–Robinson Buffer over the pH range 1–7. Chromophores containing five-membered heterocyclic rings (**7b**,**c**,**e,** and **10b**) showed an increase in fluorescence below pH 2.5 but the best quantum yield (observed with **10a**) was only 2.9% of the value found for wild type GFP probably due to excited state proton transfer. The results demonstrate that high increase of fluorescence intensity requires a planar configuration within the protonated conjugated molecular system. In the case of furan-containing systems, NMR studies indicate that protonation of N-3 enforces planarity by H-bonding with furanyl oxygen in the *Z*-configuration. However, protonation of N-3 in the pyrrole system may also create planarity within the *E*-configuration by H-bonding between pyrrole –NH and carbonyl oxygen.

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Supplementary data

Supplementary data (experimental procedures; photoisomerization of **8**; absorption and emission spectra of **7a**,**c**,**e,** and **10b**; ^1H, ^{13}C and ^{15}N NMR chemical shift assignments of **6a**, **7b**, and **7c**; characterization of **56**, **6a**–**e**, **7a**–**e**, **8**, and **10a**,**b**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.12.082.

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