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Synthesis and Spectroscopic Studies of Model Red Fluorescent Protein Chromophores

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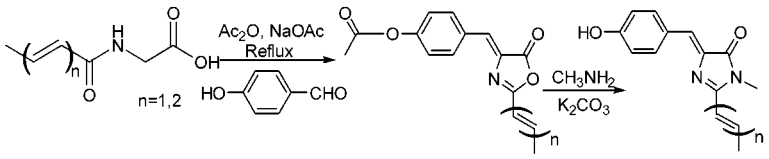
ABSTRACT



Here we describe the synthesis and spectroscopic characterization of two compounds designed to model the chromophore in DsRed, a red fluorescent protein. Comparison with model green fluorescent protein (GFP) chromophores indicates that the additional conjugation in the DsRed models can account, in part, for the red-shifted absorption and emission properties of DsRed compared to those of GFP. In contrast to the GFP models, the DsRed models are fluorescent with quantum yields of 0.002–0.01 in $CHCl_3$.

DsRed is a recently cloned bright red fluorescent protein from coral (*Discosoma* sp.) with great potential as a complement to the widely utilized *Aequorea victoria* green fluorescent protein (GFP)[1] due to its significantly red-shifted excitation and emission maxima (λ_{ex} 558 nm, λ_{em} 583 nm).[2] Although DsRed and GFP share only 23% sequence identity, X-ray crystallography reveals that DsRed also forms the β-can structural motif observed for GFP.[3,4] In addition, like GFP, the chromophore in DsRed is formed by the intramolecular reaction of three amino acid residues (Q66Y67G68). However, while both the DsRed and GFP chromophores share the same 4-hydroxybenzylideneimidazolinone core, the chromophore in DsRed differs from that in GFP by the presence of an acylimino group at the 2-position of the imidazolinone ring (Figure 1). The acylimino group is formed by oxidation of the Q66 C_α−N bond, and the resulting additional conjugation in the chromophore is believed to partly account for the red-shifted spectroscopic properties of DsRed compared to those of GFP.[3−6] To study the role of the protein

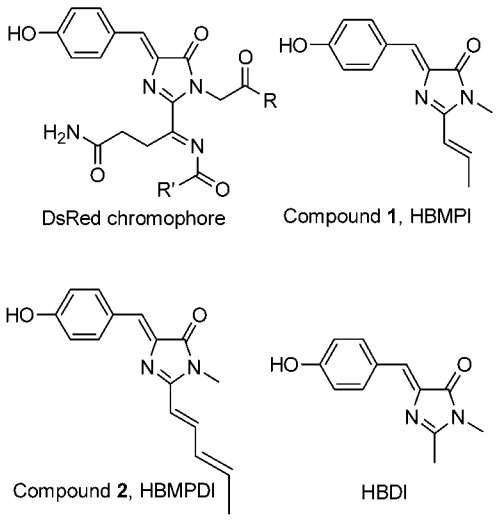


Figure 1. Structure of the DsRed chromophore, HBMPI **1**, HBMPDI **2**, and HBDI.

(1) Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509−544.
(2) Matz, M. V.; Fradkov, A. F.; Labas, Y. A.; Savitsky, A. P.; Zaraisky, A. G.; Markelov, M. L.; Lukyanov, S. A. *Nat. Biotechnol.* **1999**, *17*, 969−973.
(3) Wall, M. A.; Socolich, M.; Ranganathan, R. *Nat. Struct. Biol.* **2000**, *7*, 1133−1138.
(4) Yarbrough, D.; Wachter, R. M.; Kallio, K.; Matz, M. V.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 462−467.

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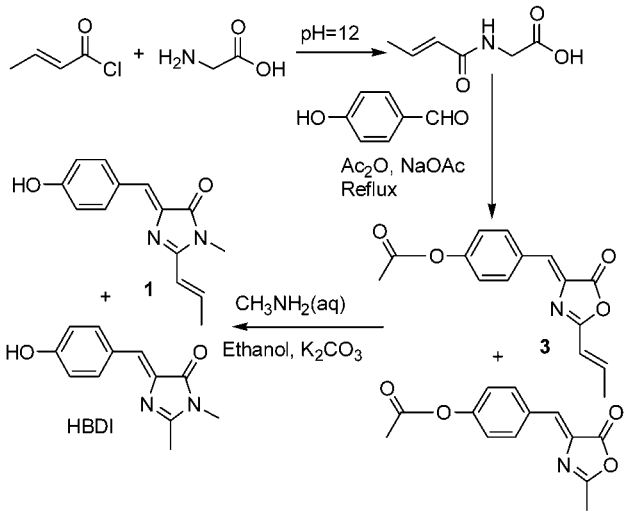
matrix in governing the spectral properties of the DsRed chromophore, we have synthesized 4-hydroxybenzylidene-1-methyl-2-propenyl-imidazolinone (**1**, HBMPI) and 4-hydroxybenzylidene-1-methyl-2-penta-1,3-dien-1-yl-imidazolinone (**2**, HBMPDI) (Figure 1) and performed an initial characterization using absorption, fluorescence, and Raman spectroscopies.

Due to the susceptibility of acylimines to nucleophilic attack,[7,8] we have initially focused on DsRed model compounds containing olefinic substituents on the imidazolinone ring. Two such compounds (**1** and **2**) were prepared via Erlenmeyer azlactone synthesis as shown in Schemes 1 and 2, respectively.[9,10]

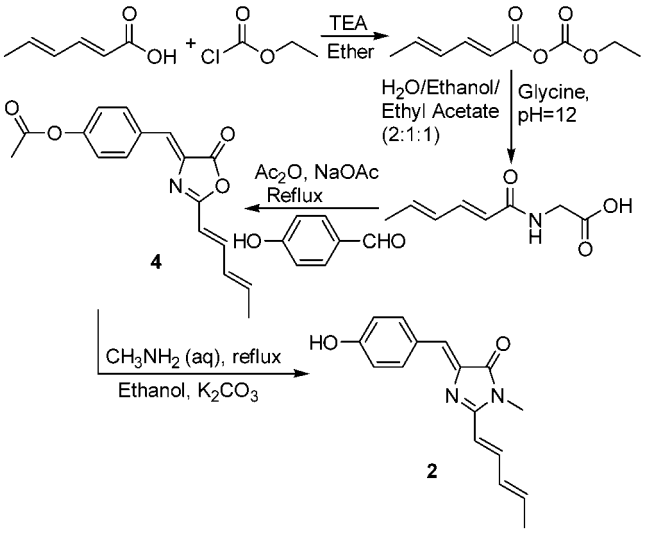
Scheme 1. Synthesis of HBMPI **1**



For compound **1**, glycine was first acylated with crotonic acid under alkaline conditions.[11] Reaction of *N*-crotonylglycine with 4-hydroxybenzaldehyde and anhydrous sodium acetate in acetic anhydride provided the corresponding intermediate azlactone **3** together with a byproduct having a methyl group in place of the crotonyl group at the imidazolinone C2 position. The formation of the byproduct is postulated to result from replacement of the *N*-crotonyl group with an acetyl group derived from the solvent.

^{1}H NMR analysis indicated that the product from the first step of the synthesis contained more than 60% of the desired azlactone, and this was used for the next step of the reaction without purification. Subsequent treatment of **3** with methylamine in the presence of K_2CO_3 afforded HBMPI **1** in

Scheme 2. Synthesis of HBMPDI **2**



20% overall yield. 4-Hydroxybenzylidene-1,2-dimethyl-imidazolinone (HBDI, Figure 1) was also isolated in high yield from the synthesis of **1**. Similarly, HBMPDI **2** was synthesized using the same strategy with 2,4-hexadienoic acid as the initial acylating group. However, the 2-methyl-substituted byproduct, observed in the synthesis of **1**, was not formed, presumably due to steric hindrance in the acetyl exchange reaction. Compound **2** was obtained in 25% overall yield. The absorption spectra of **1** and **2** are shown in Figures 2 and 3, respectively.

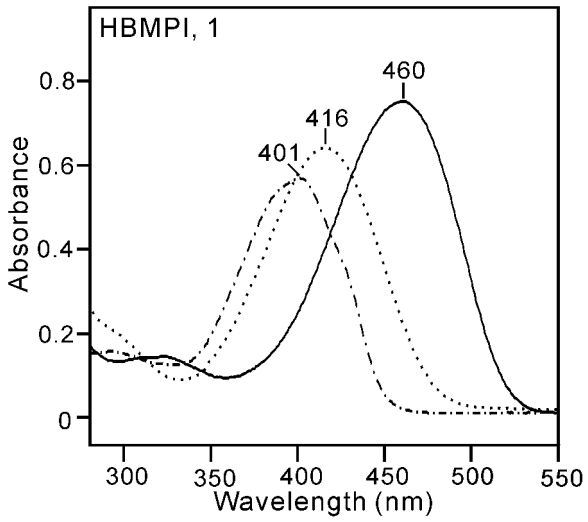


Figure 2. Absorption spectra for the cationic (◆◆◆, 1 M HCl), neutral (—◆—◆, 20 mM sodium acetate pH 5.5), and anionic (——, 1 M KOH) forms of HBMPI **1**.

Due to the extension of the conjugated system compared to HBDI (λ_{max} = 368 and 425 nm for neutral and anionic forms, respectively), the absorption maxima of the neutral

(5) Baird, G. S.; Zacharias, D. A.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A* **2000**, *97*, 11984−11989.
(6) Gross, L. A.; Baird, G. S.; Hoffman, R. C.; Baldridge, K. K.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11990−11995.
(7) Kupfer, R.; Meier, S.; Wurthwein, E. U. *Synthesis* **1984**, 688−690.
(8) Malassa, I.; Matthies, D. *Chemiker-Zeitung* **1987**, *111*, 253−261.
(9) Buck, J. S.; Ide, W. S. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. II, p 55−56.
(10) Kojima, S.; Ohkawa, H.; Hirano, T.; Maki, S.; Niwa, H.; Ohashi, M.; Inouye, S.; Tsuji, F. I. *Tetrahedron Lett.* **1998**, *39*, 5239−5242.
(11) Sheehan, J. C.; Duggins, W. E. *J. Am. Chem. Soc.* **1950**, *72*, 2475−2477.

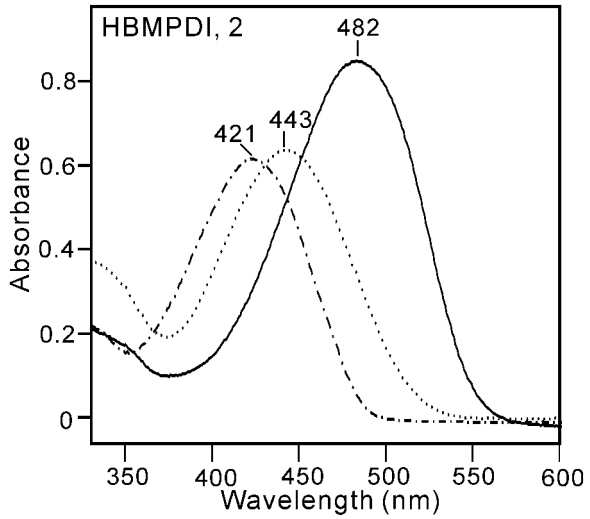


Figure 3. Absorption spectra for the cationic (◆◆◆, 1 M HCl), neutral (—◆—◆, 20 mM sodium acetate pH 5.5), and anionic (— —, 1 M KOH) forms of HBMPDI **2**.

forms of **1** and **2** are red-shifted to 401 and 421 nm, respectively (Table 1). Similarly, for the anionic forms, the λ_{max} of **1** is shifted to 460 nm, while the λ_{max} of **2** is shifted to 482 nm. The observation that λ_{max} of anionic **1** is red shifted 35 nm compared to that of anionic HBDI is in good agreement with the 34 nm red shift predicted on the basis of ZINDO calculations by Gross et al.[6] for the addition of one ethylenic group to the GFP chromophore. It is worth noting that the anionic form of **1** has a λ_{max} similar to that of alkaline-denatured DsRed (λ_{max} 452 nm). Gross et al. have proposed that alkaline denaturation of DsRed results in hydrolyis of the acylimino group and cleavage of the peptide backbone.[6] The product resulting from acylimine hydrolysis is expected to have a carbonyl substituent at the imidazolinone C2 position, consistent with the observed similarity between λ_{max} for alkaline-denatured DsRed and the anionic form of **1**.

Table 1. Spectroscopic Data of HBMPI (**1**), HBMPDI (**2**), and DsRed

	1			**2**			DsRed
	neutral		anion	neutral		anion	anion
solvent	H_2O	$CHCl_3$	H_2O	H_2O	$CHCl_3$	H_2O	H_2O
λ_{max} (nm)	401	400	460	421	425	482	558
λ_{em} (nm)	460	472	530	523	531	565	583
ϕ^a	0.0005	0.002	0.0006	0.0008	0.01	0.0008	0.7

[a] Quantum yield (ϕ).[12]

The newly synthesized DsRed model chromophores have interesting fluorescence properties as listed in Table 1. While HBDI, the model GFP chromophore, is not detectably fluorescent at room temperature, both HBMPI and HBMPDI have measurable fluorescence spectra with quantum yields

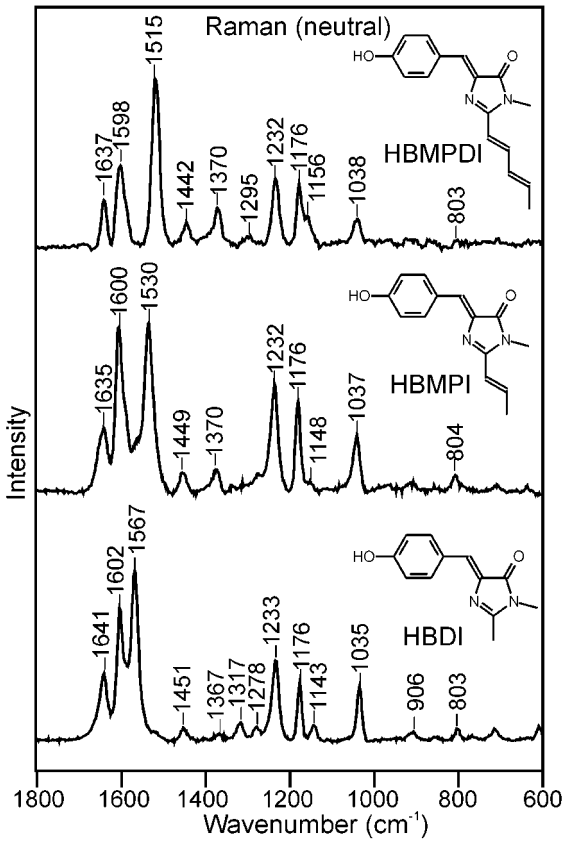


Figure 4. Raman spectra of HBDI, HBMPI **1**, and HBMPDI **2** in sodium acetate buffer (pH 5.5, 20 mM) obtained using 100 mW of 752 nm laser excitation.[15]

of 0.0005−0.0008 in H_2O, consistent with the studies by McCapra et al. on a related GFP model compound.[13] In $CHCl_3$, the quantum yields rise to 0.002 and 0.01 for the neutral forms of HBMPI and HBMPDI, respectively, similar to the values observed for the model GFP chromophores bearing a phenyl substituent on the imidazolinone ring.[14] Most relevant for studies of DsRed is the anionic form of compound **2**, which has an excitation maximum at 482 nm and an emission maximum at 565 nm. While the quantum yields for **1** and **2** are still significantly lower than observed for DsRed (0.7),[6] the similarity in λ_{em} for the anionic form of **2** and the DsRed protein (λ_{em} 583 nm) indicates that the additional conjugation in **2** compared to that in the GFP model HBDI can account for a large part of the red shift observed between GFP and DsRed. However, the Stokes shift in compound **2** is 83 nm, whereas it is only 25 nm in the protein. The smaller Stokes shift for the protein compared to the models indicates that less energy is needed to reorganize the environment to accommodate the excited-state structure in the protein than in the solvent.

(12) White, C. E.; Argauer, R. J. *Fluorescence Analysis*; *A Practical Approach*; Dekker: New York, 1970.
(13) McCapra, F.; Razavi, Z.; Neary, A. P. *J. Chem. Soc., Chem. Commun.* **1988**, 790−791.
(14) You, Y. J.; He, Y. K.; Borrows, P. E.; Forrest, S. R.; Petasis, N. A.; Thompson, M. E. *Adv. Mater.* **2000**, *12*, 1678−1681.

Raman spectroscopy has provided valuable information on the structure of the chromophore in GFP model compounds and proteins.[15−18] To provide a basis for interpreting the Raman spectra of wild-type and mutant DsRed proteins, we have undertaken a Raman analysis of compounds **1** and **2**.

Figure 4 contains the Raman spectra of **1** and **2** together with that of HBDI, a GFP model compound that has been studied in detail using ab initio calculations and isotopic labeling.[19] While there are many similarities in the spectra of the three compounds, reflecting the 4-hydroxybenzylideneimidazolinone structure common to each molecule, two important differences can be observed in the model compound series. Specifically, the 1567 cm^{-1} band in neutral HBDI is shifted to 1530 and 1515 cm^{-1} in neutral **1** and **2**, respectively. This band has been assigned in HBDI to a mode delocalized over the imidazolinone ring and exocyclic C=C bond.[19] The decrease in wavenumber for this band is consistent with increased delocalization as conjugation increases through the series of model compounds. In HBMPI and HBMPDI, the equivalent mode may also involve contributions from the olefinic substituents. In addition, the 1143 cm^{-1} band in HBDI, which corresponds to an imidazolinone C−C single-bond stretching mode, is shifted to 1148 and 1156 cm^{-1} for **1** and **2**, respectively, indicating the strengthening of the single bonds in the conjugated system.

In conclusion, chromophores **1** and **2** provide very useful models for improving our understanding of the structure and optical properties of the chromophore within DsRed. While some differences are expected between model and protein data due to the use of an ethylenic substituent, the results suggest that a significant portion of the red shift in absorption and emission properties between GFP and DsRed can be accounted for by the extended conjugation in the DsRed chromophore. Like GFP, the DsRed protein matrix plays a major role in preventing nonradiative deactivation of the chromophore's excited state.

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Supporting Information Available: Procedures for the preparation of **1** and **2** and related analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Bell, A. F.; He, X.; Wachter, R. M.; Tonge, P. J. *Biochemistry* **2000**, *39*, 4423−4431.
(16) Esposito, A. P.; Schellenberg, P.; Parson, W. W.; Reid, P. J. *J. Mol. Struct.* **2001**, *569*, 25−41.
(17) Schellenberg, P.; Johnson, E.; Esposito, A. P.; Reid, P. J.; Parson, W. W. *J. Phys. Chem. B* **2001**, *105*, 5316−5322.
(18) Tozzini, V.; Nifosi, R. *J. Phys. Chem. B* **2001**, *105*, 5797−5803.
(19) He, X.; Bell, A. F.; Tonge, P. J. *J. Phys. Chem. B*, submitted for publication.