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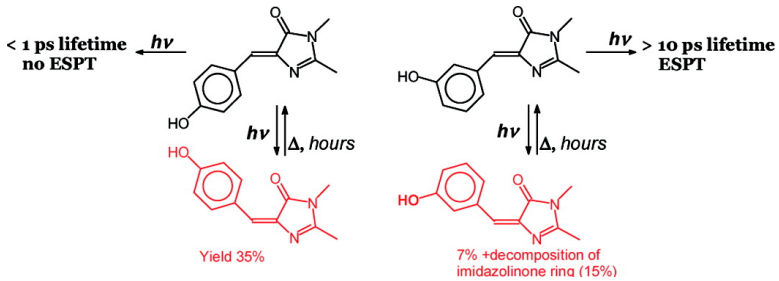

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The Meta-Green Fluorescent Protein Chromophore

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The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has attracted great interest as a biological fluorescence marker.[1,2] The chromophore of this 238 amino acid protein is a *p*-hydroxybenzylideneimidazolinone (*p*-**HBI**) derivative formed by post-translational cyclization and oxidation of the Ser-65, Tyr-66, and Gly-67 residues.[3−6] The GFP crystal structure shows the chromophore in the middle part of the central helix created by an 11-stranded β-barrel, which plays a critical role for the superb fluorescence properties of GFP and its mutants. Such fluorescent proteins are restricted to the toolbox of natural aromatic amino acids which can replace the *p*-phenol moiety, for example, phenyl alanine, tryptophan, and histidine, and we wondered how replacement of the *p*-phenol with its *m*-equivalent might alter its photophysics.

The dimethyl derivative of the GFP chromophore (*p*-**HBDI**) and several of its derivatives have been synthesized, and their photochemistry investigated. Released from the protein β-barrel, *p*-**HBDI** demonstrates ultrafast (<1 ps) deactivation in all organic and aqueous solvents at room-temperature owing to ultrafast conformational relaxation to a nonfluorescent twisted intermediate. Recently, an ortho isomer of **HBDI** was synthesized.[7] A ground-state hydrogen bond between hydroxyl group and imidazolinone nitrogen in this compound resulted in utrafast formation of the tautomer (zwitterion) with an increased fluorescence quantum yield.

The prototropic behavior of the GFP chromophore is responsible for its green fluorescence. This chromophore falls into the general category of hydroxyarene photoacids with which our group has significant experience.[8] Such hydroxyarenes exhibit high excited-state acidities but neutral ground states. In the case of GFP, the imidazolinone ring is an electron-withdrawing substituent, increasing both the ground- and excited-state acidity, but to different extents. We speculated that *m*-hydroxy substitution would have analogous effects on the photophysics to those noted by Lewis in the case of another hydroxystyryl aromatic, hydroxystilbene.[9]

A consequence of the para relationship between the incipient oxyanion center and the electron-withdrawing imidazolinone ring in GFP is that the ground-state acidity is increased. Substitution on the meta position provides no such possibility, while the excited state should show enhanced charge transfer. Thus the meta isomer should show diminished anion formation in the ground state but excellent excited-state acidity. This excited-state reversal of ortho, para directing effects, known as the "meta" effect, has been the subject of numerous theoretical and experimental observations[10] and is shown clearly by examining the corresponding frontier molecular orbitals (highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs)) for both conjugate bases at the AM1 level (see Figure 1), showing greater charge transfer for the meta isomer. The closest analogy in solution chemistry involves *m*- and *p*-hydroxystilbenes.[9] In this case, the para isomer undergoes rapid cis/trans isomerization and sluggish proton transfer, while the meta isomer exhibits the reverse behavior, reflecting the molecular orbital principles we outlined above. Thus the *m*-tyrosine GFP derivative may be much more photochemically robust than the wild-type GFP, and it should certainly exhibit an enhanced excited-state acidity.

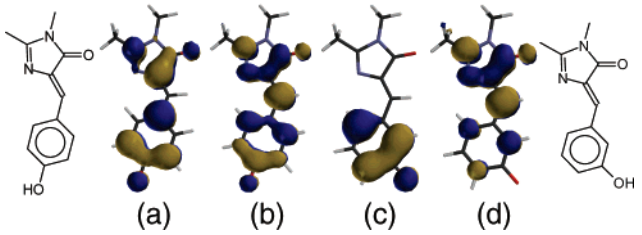

Figure 1. (a) HOMO and (b) LUMO for *p*-**HBDI** (left structure); (c) HOMO and (d) LUMO for *m*-**HBDI** (right structure).

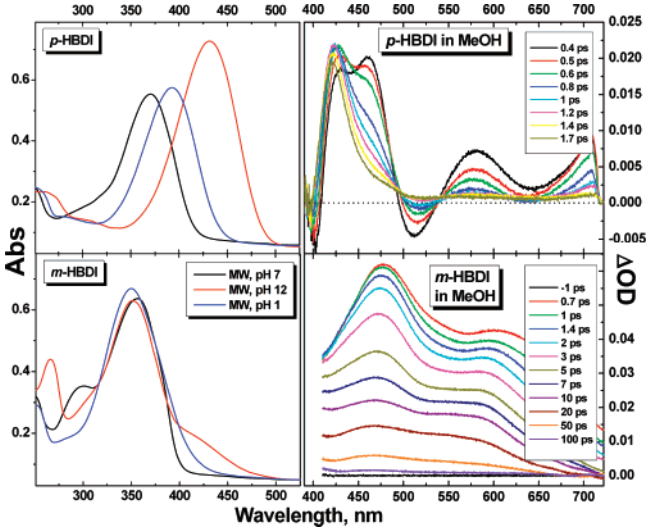

Figure 2. Left column: absorption spectra of *p*-**HBDI** (top) and *m*-**HBDI** (bottom) in methanol/water (MW) 1/1 vol. at various pH. Right column: transient absorption signal of *p*-**HBDI** (top) and *m*-**HBDI** (bottom) in methanol. Pumping occurred with 380 nm, 6 μJ, and 100 fs pulses.

m-**HBDI** was synthesized using our modification[11] of Niwa's procedure.[12] The corresponding methyl ether *m*-**MBDI** was also prepared. It is noteworthy that, despite the previously unknown nature of *m*-**HBDI** itself, several 2-methyl-substituted derivatives exhibiting various biological activities have been synthesized.[13]

While the basicity of the imidazolinone nitrogen (pK_a of 2.1) decreased by only 0.15 pK_a units as compared to *p*-**HBDI**, the pK_a of the hydroxy group (9.5) increased by one pK_a unit, in keeping with the weaker ground-state acidity of the meta isomer and reflecting the localized conjugate base MOs of Figure 1. Moreover, in contrast to the very-well resolved absorbance bands of *p*-**HBDI** in various protonation states (Figure 2), the absorbance of the *m*-**HBDI** cation strongly overlaps with that of neutral species, The

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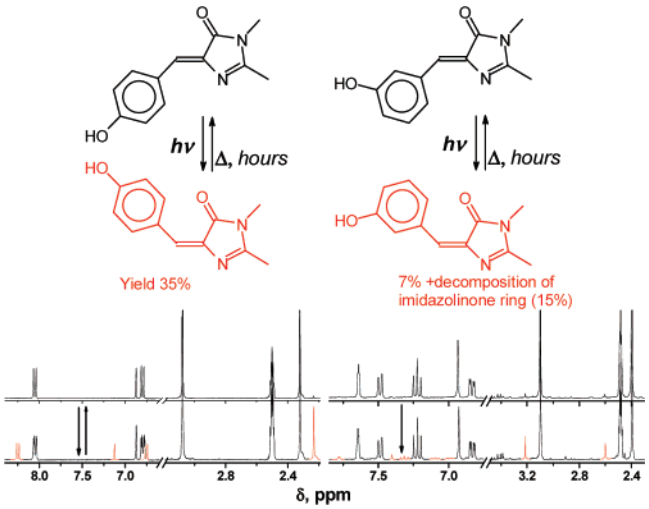

Figure 3. ^{1}NMR spectra of *p*-**HBDI** (left) and *m*-**HBDI** (right) in DMSO-d_6 before (top spectra) and after (bottom spectra) 2 h irradiation at 370 nm. The photolysis resulted in the new features shown in red.

anion of *m*-**HBDI** is characterized by two bands, with the stronger one practically indistinguishable from that of the neutral. The analogous absorbance band splitting of *m*-hydroxystilbene anion is attributed to the mesomeric effect of the phenolate ion on two different charge-transfer transitions.[9b]

The fluorescence lifetimes of *p*-**HBDI** in various protonation states and in various solvents were less than 1.5 ps[14,15] and thus could not be determined by the time-correlated single-photon counting method. However the lifetimes of the meta isomer in some nonaqueous solvents were long enough to be measured. The highest fluorescent quantum yield (FQY 0.0023) and the longest fluorescence lifetimes (biexponential decay with 15 and 90 ps lifetimes, amplitude ratio 10)[16] were detected in DMSO ($\lambda_{max} = 440$ nm). At the same conditions the FQY of *p*-**HBDI** was 10 times smaller. The significantly longer fluorescence lifetime of *m*-**HBDI** can be associated with slower cis/trans photoisomerization, reminiscent of Lewis' observations with the corresponding hydroxystilbenes.[9] However, despite this slower kinetics, isomerization remains the major nonradiative decay pathway in nonaqueous solvents. ^{1}NMR measurements following irradiation of *p*-**HBDI** revealed the photoconversion of the initial *Z*-isomer to the *E*-isomer to the extent of about 35% in DMSO (Figure 3), a result qualitatively similar to Tonge's observations in methanol;[17] however, in latter case the yields were not reported. This isomerization was thermally reversible. In contrast, under the same irradiation conditions *m*-**HBDI** produced much less isomer (7%), and irreversible decomposition was also observed.

A dramatic difference between the meta and para isomers of **HBDI** in methanol has been also demonstrated in preliminary fs pump−probe spectroscopic results: the meta isomer shows a much longer lived excited state (Figure 2), in agreement with fluorescence decay results. Very similar time-resolved spectra of *p*-**HBDI** in water at various pH values were deconvoluted by Vengris et al.[15] into transient absorption and stimulated emission of excited- and ground-state intermediates, including photoionization products. In contrast, the transient absorption signal of *m*-**HBDI** consisted of a very broad absorption signal best fit by a biexponential decay function with 5 and 31 ps components, and a $^3/_2$ amplitude ratio.[16] In addition to slower photoisomerization, we have found that the quantum yields for solvated electrons and **HBDI** radical-ions formed in the result of photolysis were much lower for the meta isomer. The formation of such radical cations, followed by electron transfer from a glutamate carboxylate, may account for the E222 decarboxylation observed for GFP[18] and thus provide for much less efficient decarboxylation in a protein having a *m*-hydroxyaromatic chromophore.

In solution, the decay rate of *p*-**HBDI** is about 1 ps, and no excited-state proton transfer (ESPT) is observed for the free chromophore in all solvents. Nor is ESPT to methanol solvent observed for the meta chromophore (Figure 2). However, in mixed water−methanol solution, slower photoisomerization allows the excited-state protolytic reaction of the free meta chromophore to compete. Our preliminary results[16] demonstrate that, for *m*-**HBDI**, an interesting sequential ESPT is observed, similar to that for hydroxyquinolines.[19] First, the hydroxyl group dissociates in 0.7 ps, and then the reprotonation of imidazolinone nitrogen occurs at 3.1 ps. Curiously, the second proton-transfer step is nonadiabatic and leads to fluorescence quenching. In water-rich solution *m*-**MBDI** shows ultrafast kinetics, probably associated with proton abstraction from water. This selective modulation of acid−base behavior in chromophores, together with solvent variation, including pH change, serves as a context for separation of elementary proton-transfer steps in these systems. Studies of such model unconventional *m*-GFP chromophores will help unravel the complex photochemistry and photophysics of fluorescent prototropic proteins.

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Supporting Information Available: Synthetic details for *m*-**HBDI** and *m*-**MBDI** and pH-titration data. This material is available free of charge via the Internet at http://pubs.acs.org.

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