Pergamon

Tetrahedron Letters 39 (1998) 5239–5242

TETRAHEDRON LETTERS

Fluorescent Properties of Model Chromophores of Tyrosine-66 Substituted Mutants of *Aequorea* Green Fluorescent Protein (GFP)

Satoshi Kojima, Hiroko Ohkawa, Takashi Hirano, Shojiro Maki, Haruki Niwa,*
Mamoru Ohashi, Satoshi Inouye,† and Frederick I. Tsuji,‡

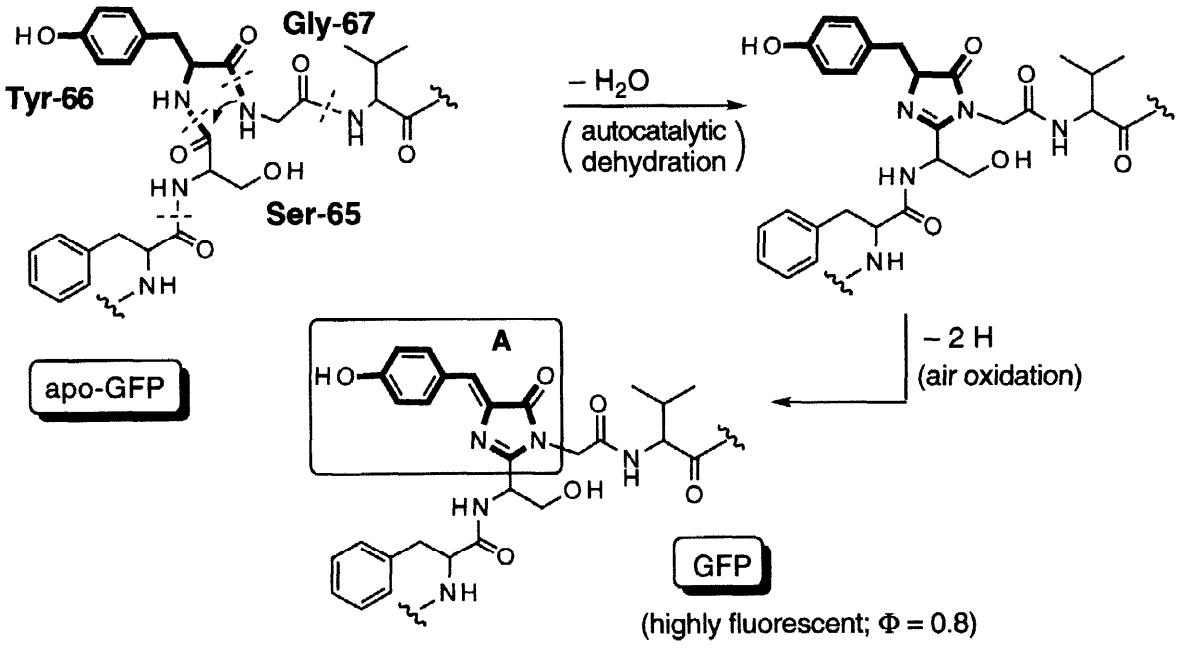
Department of Applied Physics and Chemistry, University of Electro-Communications, Chofu, Tokyo 182, Japan
†Yokohama Research Center, Chisso Corp., Kanazawa-ku, Yokohama, 236, Japan
‡Marine Biology Research Division 0202, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, U. S. A.

Received 10 April 1998; revised 11 May 1998; accepted 15 May 1998

Abstract: In an ethanol-glass marix at 77 K, model compounds of the chromophores of recombinant GFP-Y66F, GFP-Y66W, and GFP-Y66H exhibited fluorescence properties close to those of the corresponding mutant proteins.

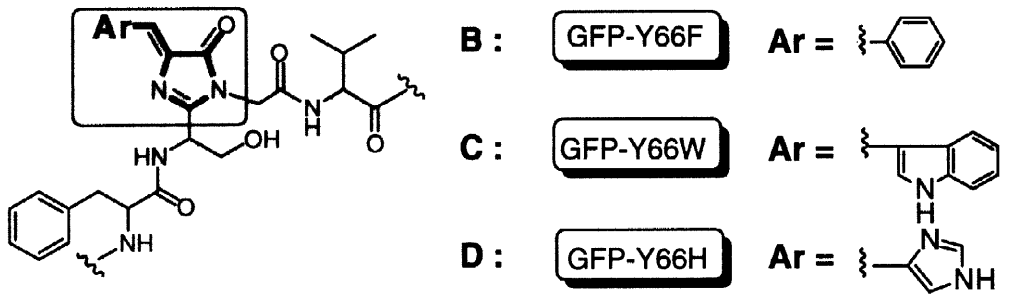
Keywords: Erlermeyer azlactone synthesis; Fluorescence; Mutation; Proteins

The bioluminescence system of the jellyfish, *Aequorea victoria*, involves two closely associated proteins: a calcium binding photoprotein aequorin[1-3] and a chromophore-containing green fluorescent protein (GFP).[4-6] GFP is made up 238 amino acid residues in a single polypeptide chain and emits a greenish fluorescence (λmax 508 nm, Φ = 0.8) when exposed to long ultraviolet light.[5, 7-9] The GFP chromophore is depicted in formula **A** in Scheme **1**. The chromophore is formed from a tripeptide -Ser^{65}–Tyr^{66}–Gly^{67}- in the primary structure of the expressed protein via a post-translational autocyclodehydration and subsequent autoxidation (Scheme **1**).[10-12] Recently, we reported on the structure, biogenesis and fluorescence of the chromophore in wild-type GFP by means of chemical and spectroscopic methods using model compounds **1** and **2**.[13,14] X-ray crystallograhic analyses of mutant GFPs have been recently published.[15] When the GFP gene is expressed, the characteristic green fluorescence of GFP can be easily detected by simply illuminating with a conventional black light source. An external cofactor is not required. Recent progress in heterologous



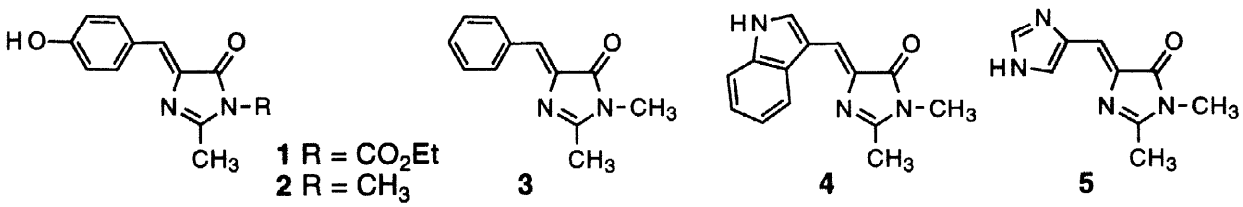
Scheme 1. Biogenesis of Chromophore Moiety **A** in Wild -type GFP

PII: S0040-4039(98)01031-4



Scheme 2. Putative Structures of Mutant Chromophores

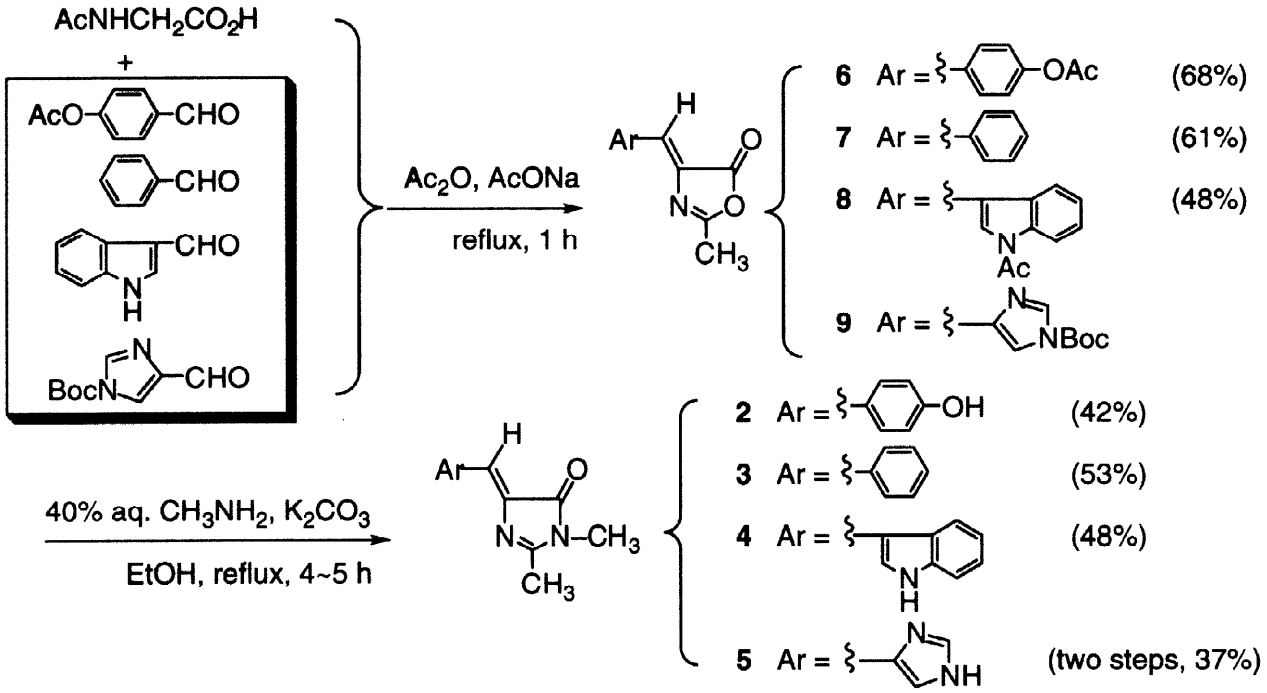
expression of cDNA for GFP in various organisms has led to widespread application of the protein as a reporter in studies involving gene expression, protein localization, protein-protein association, and cell development.[16] Of particular interest would be the creation of a mutant GFP with a red-shifted fluorescence maximum. Such a GFP could serve as a powerful probe, allowing biological events to be visualized in a dual - red and green - color mode. Recently, the fluorescence of the GFP mutants GFP-Y66W, GFP-Y66H and GFP-Y66F (Tyr^{66} in the wild protein sequence replaced by Trp^{66}, His^{66}, and Phe^{66}, respectively) has been reported.[17,18] The GFP-Y66W, GFP-Y66H and GFP-Y66F mutants had a blue-shifted fluorescence, with maxima at around 480, 450 and 440 nm, respectively. The chromophores of GFP-Y66F, GFP-Y66W and GFP-Y66H are shown in **B**, **C**, and **D** in Scheme **2**. In order to study the fluorescent properties of model chromophores of the mutant proteins, we have synthesized and measured the fluorescence of 4-arylmethylideneimidazol-5-ones **2**, **3**, **4**, and **5** which are the chemical analogues of the chromophore moieties of wild-type GFP, GFP-Y66F, GFP-Y66W, and GFP-Y66H, respectively.



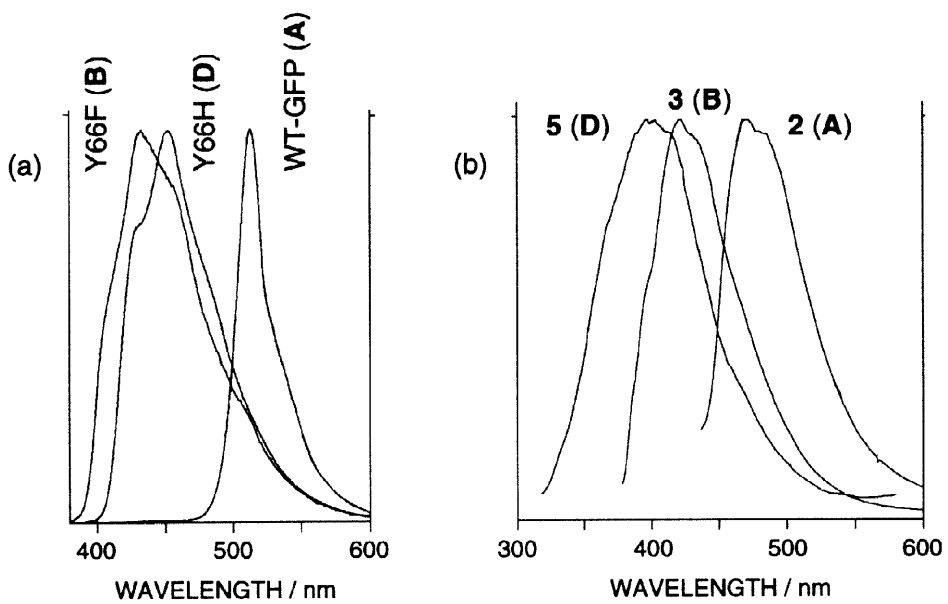
The model chromophores **2**, **3**, **4** and **5** were prepared via Erlermeyer azlactone synthesis[19] as shown in Scheme **3**. Reaction of *N*-acetylglycine, aromatic aldehydes, and anhydrous sodium acetate in acetic anhydride provided the corresponding azlactones **6** (68%), **7** (61%), **8** (48%), and **9** (not isolated), respectively.[20] Reaction of **6**, **7**, **8** and **9** with methylamine in the presence of K_2CO_3 afforded **2** (42%), **3**[21] (53%), **4**[22] (48%), and **5**[23] (two steps, 37%), respectively.[24,25] Recombinant GFP mutants GFP-Y66F, GFP-Y66H and GFP-Y66W were purified as described previously.[8] Figure **1** (a) shows the fluorescence spectra of wild-type GFP, GFP-Y66F and GFP-Y66H. GFP-Y66F and GFP-Y66H had fluorescence maxima near 430 nm and 450 nm, respectively, close to the values previously reported for the mutant proteins.[17,18] Ethanol solutions of **2**, **3**, **4**, and **5** showed only very weak fluorescence at room temperature, as previously found with **1**.[13] However, when the ethanol solutions were frozen in liquid N_2 (77 K) as ethanol-glass, **2**, **3**, and **5** became highly fluorescent with intensities increasing 2000 ~ 3000 fold, as previously reported for **1** [Figure **1** (b)].[13] The fluorescence spectra of GFP-Y66W and **4** are not shown because they were very weak (the intensity of GFP-Y66W was 1% of wild-type GFP). The fluorescence emission maxima of **2**, **3**, **4**, and **5** in ethanol glass were 485 nm (basic condition), 420, 467, and 402 nm (basic condition),[26] respectively.

The observed fluorescence spectra had similar shapes to those of the corresponding GFPs (Figure 1).[17] However, the fluorescence maximum was significantly shifted to a lower wavelength in the case of **5**.

In conclusion, the observed results strongly suggest that the fluorescence properties of wild-type and mutant GFPs are significantly influenced by the environment around the chromophore and that formulas **B**, **C** and **D** are the correct chemical structures for the chromophores in the recombinant proteins.



Scheme 3. Preparation of Model Chromophores **2**, **3**, **4** and **5**



Figrue 1. (a) Fluorescence emission spectra of wild-type GFP, GFP-Y66F, and GFP-Y66H in 0.1 M ammonium bicarbonate, pH 8, at room temperature. For each curve, the amplitude is normalized to a maximum value of 1.0.

(b) Fluorescence emission spectra of **2** (5 x 10^{-7} M containing 1 M NaOH, 2.5% v/v), **3** (5 x 10^{-7} M, neutral), and **5** (5 x10^{-7} M containing 1 M NaOH, 2.5% v/v) in ethanol-glass at 77 K. For each curve, the amplitude is normalized to a maximum value of 1.0.

Acknowledgment: Grant-in-Aid for Scientific Research in Priority Area from the Ministry of Education, Science, and Culture of Japan [Grants 06240221 and 06239106 (M. O.)] is gratefully acknowledged.

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19. Previous synthesis of **2** was inefficient.[14] In this study, we prepared **2** via azulactone **5**: Buck, J. S.; Ide, W. S. *Org. Synthesis*, **1943**, *Coll.Vol. 2*, 55–56.
20. Satisfactory IR, ^{1}H NMR, and high resolution mass spectra were obtained for these compounds.
21. **3**: yellow needles; mp 108–109 °C (ether); UV (EtOH) λmax 372 nm (ε 25400); IR (KBr) 1770, 1650, 1600, 1580, 1445 cm^{-1}; ^{1}H NMR (270 MHZ, $CDCl_3$) δ 2.41 (3 H, s), .3.21 (3 H, s), 7.12 (1 H, s), 7.37–7.46 (3 H, m), 8.13 (2 H, m); EIMS (70 eV) *m/z* (relative intensity) 200 (M^+, 100). HREIMS: found *m/z* 200.0943 (M^+); calcd for $C_{12}H_{12}N_2O$ 200.0950.
22. **4**: yellow needles; mp 200 °C (dec) ($CHCl_3$); UV (EtOH) λmax 372 nm (ε 25400); IR (KBr) 3600–3000, 1680, 1630, 1510 cm^{-1}; ^{1}H NMR (500 MHZ, acetone-d_6) δ 2.35 (3 H, s), 3.15 (3 H, s), 7.16-7.23 (2 H, m), 7.33 (1 H, s), 7.48–7.51 (1 H, m), 8.13–8.16 (1 H, m), 8.51 (1 H, d, J = 2.6Hz), 10.98 (1 H, br t, J = 2.6; NH); EIMS (70 eV) *m/z* (relative intensity) 239 (M^+, 31), 56 (100). HREIMS: found *m/z* 239.1049 (M^+); calcd for $C_{14}H_{13}N_3O$ 239.1060.
23. **5**: yellow needles; mp 192 °C (dec) ($CHCl_3$); UV (EtOH) λmax 287 nm (ε 19200); IR (KBr) 3160 (br), 1710, 1640, 1590, 1510 cm^{-1}; ^{1}H NMR (270 MHZ, CD_3OD) δ 2.23 (3 H, s), 2.84 (3 H, s), 7.08 (I H, S), 7.82 (1 H, S), 9.01 (1 H, S); EIMS (70 eV) *m/z* (relative intensity) 190 (M^+, 100), 56 (59). HREIMS: found *m/z* 190.0865 (M^+); calcd for $C_9H_{10}N_4O$ 190.0855.
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26. Under acidic conditions **5** exhibited two comparable fluorescence maxima (357 and 402 nm), whereas the fluorescence maximum at 358 nm was observed under neutral conditions.