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A Synthetic GFP-like Chromophore Undergoes Base-Catalyzed Autoxidation into Acylimine Red Form

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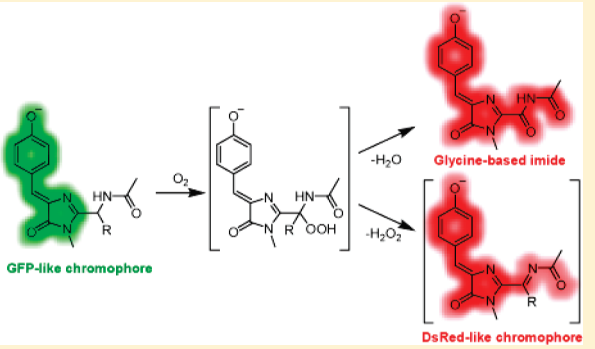
Supporting Information

ABSTRACT: Fluorescent proteins are widely used in modern experimental biology, but much controversy exists regarding details of maturation of different types of their chromophores. Here we studied possible mechanisms of DsRed-type red chromophore formation using synthetic biomimetic GFP-like chromophores, bearing an acylamino substituent, corresponding to an amino acid residue at position 65. We have shown these model compounds to readily react with molecular oxygen to produce a highly unstable DsRed-like acylimine, isolated in the form of stable derivatives. Under the same aerobic conditions an unusual red-shifted imide chromophore—a product of 4-electron oxidation of Gly65 residue—is formed. Our data showed that GFP chromophore is prone to autoxidation at position 65 Cα by its chemical nature with basic conditions being the only key factor required.



INTRODUCTION

Green Fluorescent Protein (GFP) from jellyfish *Aequorea victoria* and numerous homologue proteins from various marine animals attract a keen interest due to their intensive use as genetically encoded fluorescent labels in living systems.[1] The most intriguing feature of GFP-like proteins is self-catalyzed formation of their chromophores. During this multistep process three internal amino acids of fluorescent proteins (Ser65-Tyr66-Gly67 in GFP) undergo chemical modifications resulting in bi- or tricyclic structures with an extended system of conjugated double bonds capable of absorbing and emitting visible light.

In GFP, chromophore is formed by cyclization of the protein backbone (carbonyl group of Ser65 reacts with amide nitrogen of Gly67 with formation of 5-membered heterocycle) and oxidation of the $C_\alpha-C_\beta$ bond of Tyr66. Chromophore formation is a fully self-catalyzed process, no external enzymes and cofactors are required, except molecular oxygen. These reactions result in a so-called GFP-like chromophore: 5-(4-hydroxybenzylidene)-3,5-dihydro-4*H*-imidazol-4-one (Figure 1). Structural studies revealed that all known natural proteins of the GFP family contain the GFP-like chromophore as a core structure. A number of further modifications of the GFP-like core were found in red-shifted proteins.[2] The majority of natural red fluorescent proteins and chromoproteins carry the so-called DsRed-like chromophore, which includes the acylimine moiety formed by dehydrogenation of the $C_\alpha-N$ bond of a residue at position 65 (here and below numbering is according to GFP) by molecular oxygen.[3,4] The acylimine group of the DsRed-like chromophore is stable only within the protein and readily undergoes hydrolysis after protein denaturation.[3] Moreover, this highly reactive group can be further modified within some proteins to produce additional types of chromophores. For example, chromoprotein asFP595 from the sea anemone *Anemonia sulcata* possesses a protein backbone break just before its chromophore that contains a keto group conjugated to the GFP-like core.[5,6] Apparently, this structure is formed by hydrolysis of the acylimino group.

Much controversy exists regarding details of both GFP-like and DsRed-like chromophore formation. A "conventional" maturation scheme of DsRed-like proteins, which was generally accepted for many years, postulates autoxidation of GFP-like intermediate into final DsRed-like chromophore (Figure 1, upper pathway).[4] On the contrary, the recent studies by Subach et al.[7] and Strack et al.[8] give evidence for an unexpected blue-shifted intermediate containing an *N*-acylamino group and a saturated Tyr66 C_β atom (Figure 1, bottom pathway). In these studies the GFP-like chromophore was found to be a byproduct, not an intermediate on the way to the fully mature red protein.

The existing experimental data suggest that both mechanisms of RFP maturation are independently realized in Nature in separate evolutionary branches of fluorescent proteins. Some proteins (including DsRed, hcCP, cgCP, TagRFP, etc.) maturate via a blue-shifted intermediate,[7,8] while others, e.g., z2FP574 and asFP595, undergo maturation with a green GFP-like form clearly being an intermediate.[9,10]

Here we studied one of the possible mechanisms of DsRed-like chromophore formation using chemical synthesis of biomimetic

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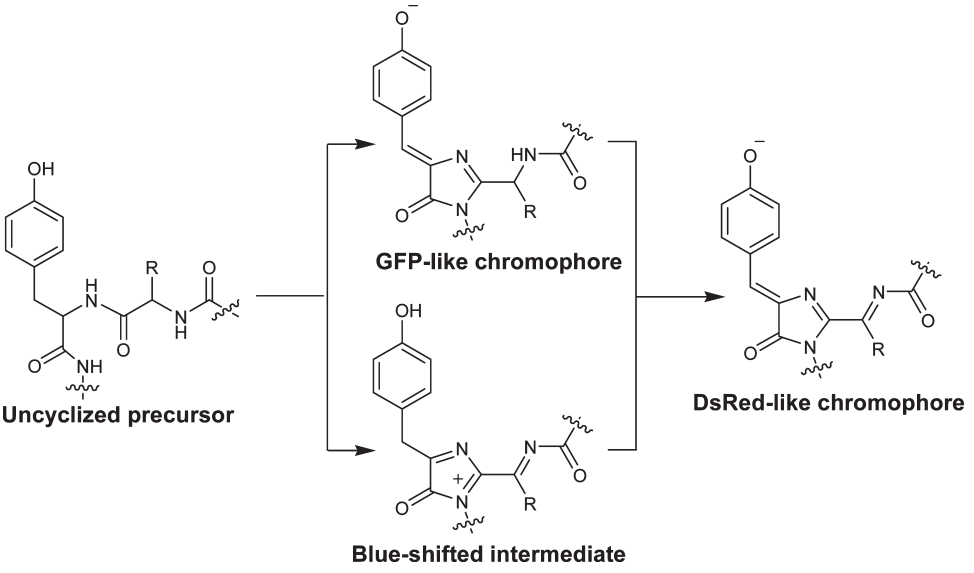

Figure 1. Two routes of DsRed-like chromophore formation.

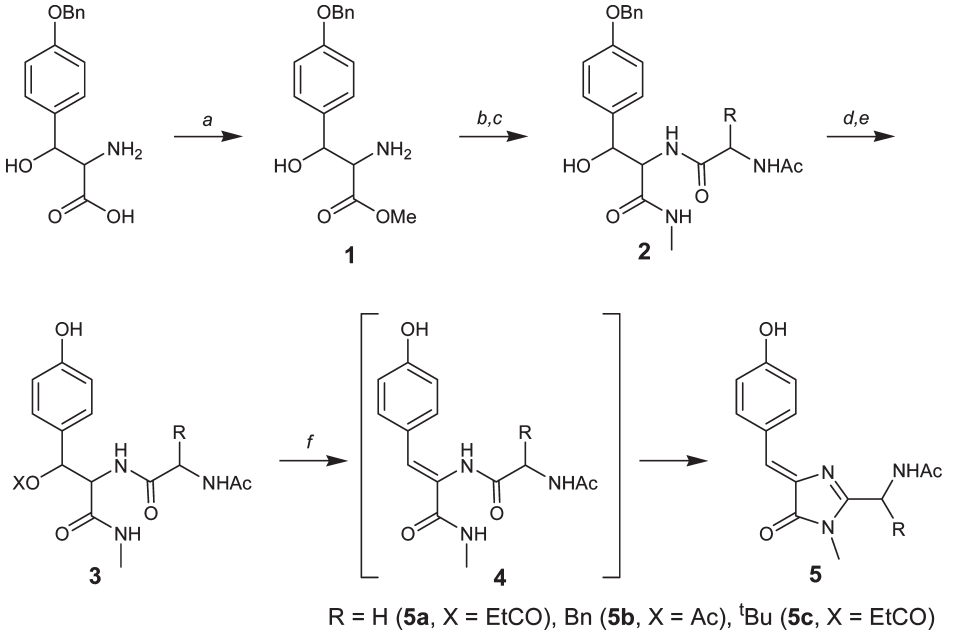

Figure 2. Synthesis of model GFP-like chromophores **5a**−**c**. Reagents and conditions: (a) $SOCl_2$, MeOH; (b) $AcNHCHRCO_2H$/DCC/HOBt; (c) $MeNH_2$; (d) $(EtCO)_2O/ZnCl_2$ or $AcBr/Ac_2O$; (e) H_2, Pd/C; (f) K_2CO_3/DMF.

GFP-like chromophores bearing an α-acetylamino-substituent, which models an amino acid at position 65 responsible for formation of the DsRed-like acylimine group. These model chromophores were found to undergo green-to-red oxidative conversion upon contact with molecular oxygen in basic conditions. This reaction leads to the trapped forms of DsRed-like acylimine and also to a novel chromophore arising from the 4-electron oxidation.

RESULTS

Synthesis of α-Acylamino-Substituted 4-(4-Hydroxybenzylidene)imidazolin-5-ones. N-Acylation of a common O-benzylated precursor **1** with acetylamino acid (Gly, Phe, ^{t}Leu) under standard DCC/HOBt coupling conditions followed by reaction with excess methylamine afforded the desired β-hydroxytyrosine derivative **2** in high yield. The latter was O-acylated and debenzylated under standard catalytic hydrogenation conditions. The resulting **3** was subjected to base-catalyzed elimination/cyclization reaction. In the case of **3c** this reaction was clearly stepwise with a dehydrotyrosine derivative **4c** formed at the first step. For the synthesis of **5a** (Figure 2) a strictly anaerobic environment is required. Even traces of oxygen significantly reduce the yield of the target product (see below). In the case of *tert*-leucine derivatives peptide coupling and cyclization steps are significantly slowed down, presumably due to the bulkiness of the *tert*-butyl group.

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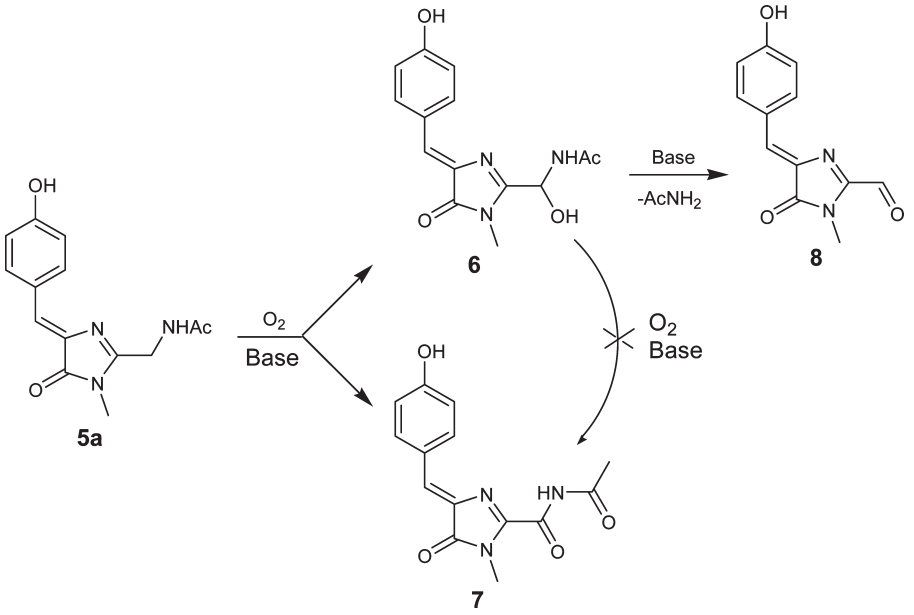

Figure 3. Autoxidation of Gly-derived GFP-like chromophore **5a**.

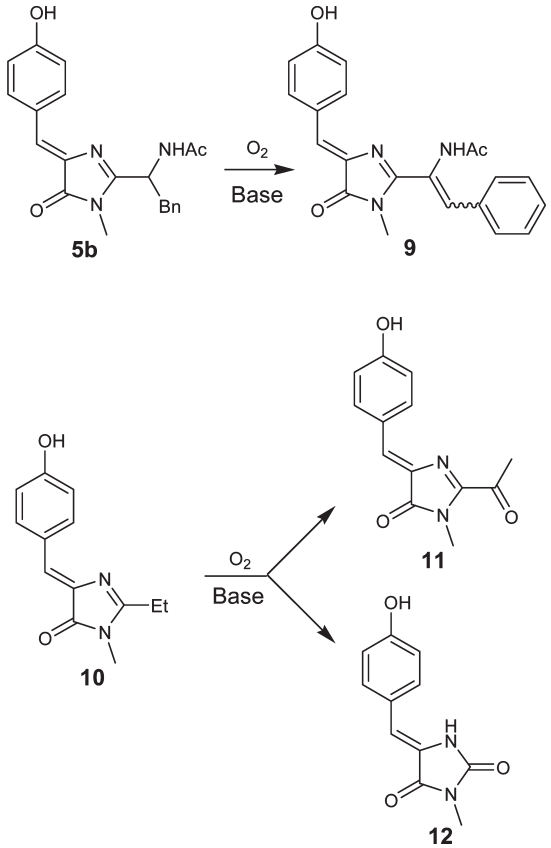

Figure 4. Autoxidation of phenylalanine-derived GFP-like chromophore **5b** and ethyl-substituted chromophore **10**.

Autoxidation of α-Acylamino-Substituted Chromophores 5a−c and 2-Ethyl-Substituted Chromophore 10. Chromophores **5a−c** undergo autoxidation in the presence of bases. The half-life of **5a** in THF at room temperature decreases from more than 8 h to 20 min upon addition of 0.01 M NEt_3. The rate of autoxidation greatly depends on the amino acid residue, decreasing in the following order: Gly-derivative **5a** (half-life <1 min at 100 °C in DMF/Cs_2CO_3), Phe-derivative **5b** (half-life about 3 h under the same conditions), and *tert*-leucine analogue **5c** (almost inert).

Autoxidation of **5a** leads to two products **6** and **7** (Figure 3), their ratio depending on the base used, temperature, and the presence of moisture. Thus, in DMF containing $NEtiPr_2$, Cs_2CO_3, and NBu_4F the **6**:**7** ratio was 3:1, 1:1.5, and 1:2, respectively. At elevated temperatures aldehyde **8** appeared in the reaction mixture along with **6** and **7**. The separate experiment where pure **6** was heated in basic DMF showed **8** to result from decomposition of **6**. In strictly anhydrous conditions the yield of **7** in autoxidation of **5a** remained unchanged, whereas only traces of **6** were observed.

Purified **6** remained unchanged when subjected to the same autoxidation conditions suggesting that two independent pathways lead from **5a** to **6** and **7** (Figure 3). Another alternative is the autoxidation of unhydrated acylimine intermediate but it can be ruled out since autoxidation should be much slower than hydration.

Autoxidation of Phe-derivative **5b** leads to a tautomer of DsRed-like chromophore **9** (mixture of cis- and trans-isomers) (Figure 4).

tert-Leucine derivative **5c** is almost inert toward autoxidation even under prolonged boiling in oxygenated DMF/Cs_2CO_3.

Chromophore[6] **10** was studied under the same autoxidation conditions as compounds **5a−c**. The rate of autoxidation was very low (half-life approximately 6 h at 100 °C in DMF/Cs_2CO_3), consistent with reported preparation of **10** not requiring anaerobic environment. The two main products of autoxidation reaction were oxo-derivative[6] **11** and hydantoine **12** (Figure 4).

Details of the autoxidation study of **10** are given in the Supporting Information.

Spectral Properties of 5a−c, 6, 7, and 9. As expected, both **5** and **6** displayed spectral behavior similar to that of the simplest 2-methyl-substituted chromophore[11] (Table 1). In contrast,

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Table 1. Spectral Properties of 5a−c, 6, 7, and 9

	neutral form[a]	anionic form[b]	
compd	abs max, nm (ε, M^{-1} cm^{-1})	abs max, nm (ε, M^{-1} cm^{-1})	emission max, nm
5, 6	376	492	n.d.
7	419 (48000)	577 (77000)	603
9	400 (25000)	514 (46000)	618

[a] Measured in 10 mM AcOH in DMF. [b] Measured in 5 mM Cs_2CO_3 in DMF.

absorption maxima of the chromophores **7** and **9** showed a strong red shift for both neutral and anionic states (Table 1 and Figure 5). Interestingly, absorption maximum of chromophore **9** (514 nm in basic DMF) is strongly blue-shifted with respect to the corresponding Kaede-like chromophore[12] without an *N*-acylamino group (553 nm). This fact is consistent with the lowered polarization of the chromophore due to the cross-conjugation with the acylamino group. Anionic forms of **7** and **9** exhibited weak red fluorescence (fluorescence quantum yield $\sim 5 \times 10^{-4}$). A considerable difference between absorbance and excitation maxima of **9** in basic DMF was observed (Figure 5B). A similar peculiarity was documented for the close structural analogue of **9**—Kaede-like chromophore[12] FYG—as well as for some mutant fluorescent proteins.[13]

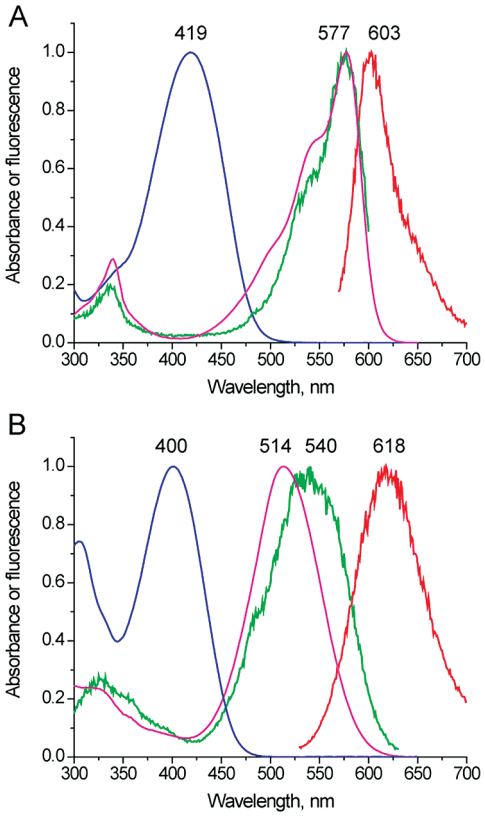

Figure 5. Absorbance and fluorescence spectra of chromophores **7** (A) and **9** (B): normalized absorbance in acidic DMF (blue lines) and basic DMF (magenta lines), excitation in basic DMF (green lines), and emission in basic DMF (red lines)

DISCUSSION

Synthesis of GFP-like Substrates for Autoxidation. Known methods for the synthesis of imidazolones[14] include base-catalyzed cyclization of dehydrotyrosine derivatives,[11] reaction of amidines[15,16] or imidates[17] with suitable 1,2-dielectrophiles, intramolecular aza-Wittig reaction of α-azidoimides,[18] and cross-coupling of boronic acids with thioimidazolone.[19] We developed a synthetic approach to previously unknown α-acetylamino-substituted GFP-like chromophores **5** based on cyclization of dehydrotyrosine derivatives **4** (Figure 2). We found that Erlenmeyer azlactonization usually used for synthesis of dehydrotyrosine derivatives does not tolerate the α-acetylamino group (presumably due to the rearrangement reported elsewhere[20]). Direct N-acylation of O-protected ΔTyr is known to be inefficient,[21] so we used a readily available synthetic equivalent[22] of ΔTyr **1**. Contrary to the previous results[23] peptide **2** underwent fragmentation leading to 4-benzyloxybenzaldehyde at the attempt at acylation in basic conditions. However, we were able to perform acylation of **2** in the presence of a Lewis acid ($(EtCO)_2O/ZnCl_2$ or $AcBr/Ac_2O$). Potassium carbonate was used at a cyclization stage instead of Cs_2CO_3 (proposed earlier[24]) because of complicated separation of the highly polar product **5a** from cesium salts. Inert atmosphere is required for the preparation of **5a** as it reacts with oxygen readily.

Autoxidation Mechanism. We found that the autoxidation rate greatly depends on the basicity of the media and on the nature of the amino acid side chain. In neutral and acidic media (AcOH or TFA in CH_2Cl_2, THF, or DMF) **5a** is subject to very slow autoxidation. Addition of bases leads to a dramatic acceleration of the reaction. Increasing the concentration of the base as well as its strength results in further enhancement of the reaction rate. The bulkiness of the amino acid side chain seems to be another key factor determining the autoxidation rate, consistent with the manifold rate decrease from **5a** to **5c**. The facilitating and directing effect of the *N*-acylamino group on autoxidation is evident from comparison of chromophores **5** with ethyl-substituted chromophore **10**, which is autoxidized very slowly, producing a different set of products (see Figure S1 in the Supporting Information for details).

On the basis of the above data we propose α-hydroperoxide **13** (first suggested for the DsRed maturation mechanism[4]) to be a common intermediate in all autoxidation reactions observed (Figure 6). Its fate is determined by the nature of the amino acid residue. Thus, both **13a** and **13b** undergo hydrogen peroxide elimination leading to the highly unstable DsRed-like acylimines **14a** and **14b**. In the case of Phe-derived acylimine **14b** the likely path for its stabilization is tautomerization into acylenamine **9**, whereas the Gly-derived acylimine **14a** is stabilized by addition of a water molecule resulting in hyroxylamide **6**. For Gly-derived hydroperoxide **13a**, an additional decomposition path arises due to the absence of the side chain in the Gly residue. Cleavage of the O−O bond results in a 4-electron oxidation product **7** that is formed independently from a 2-electron oxidation product **6**. The **6**:**7** ratio is thought to depend on relative rates of NH vs CH deprotonation in **13a**. These facts taken together imply that the 2α-position of the chromophore is in fact the site of oxygenation.

Isolation of acylimines **14** corresponding to the DsRed-like chromophore is complicated by their high electrophilicity. Our attempts to prepare sterically hindered and thus less reactive

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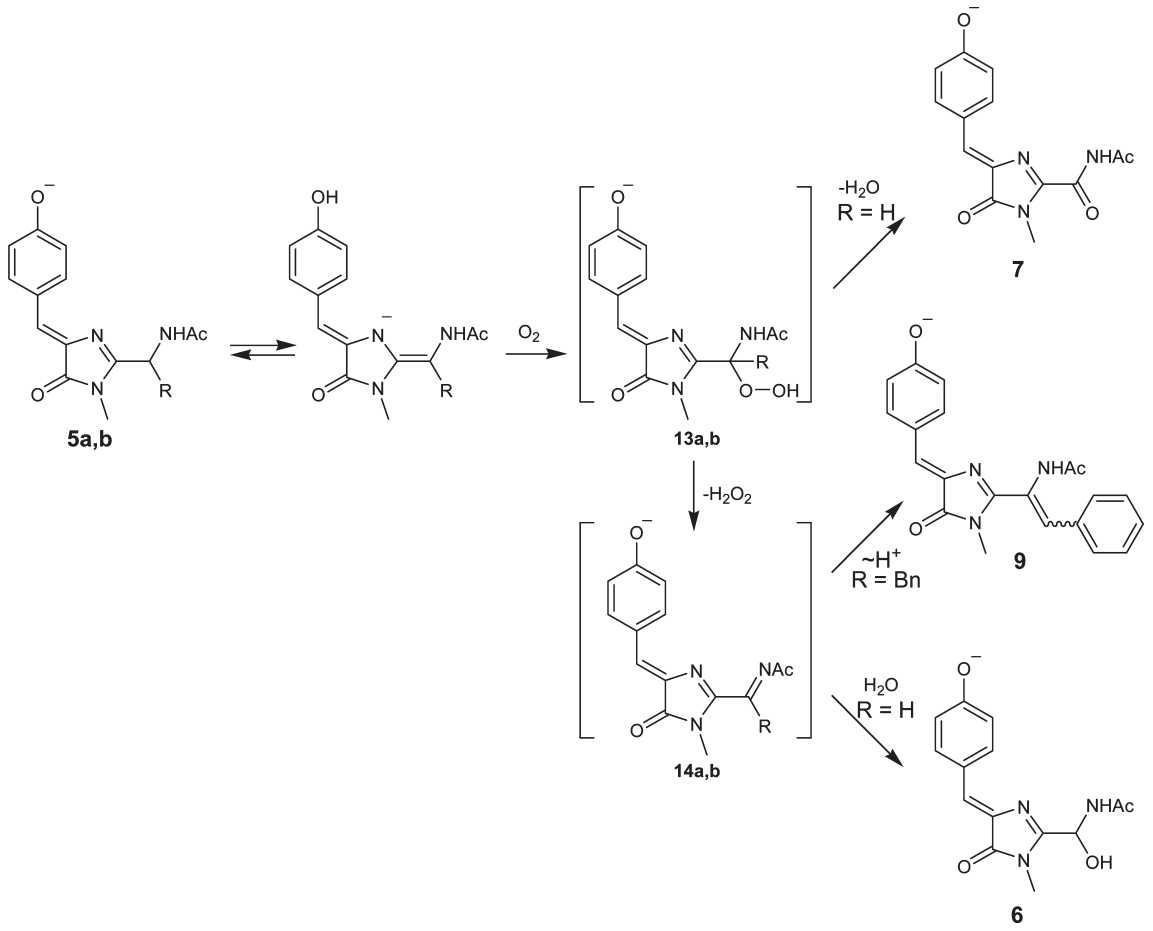

Figure 6. A proposed mechanism of autoxidation of GFP-like chromophores **5a** and **5b** leading to stabilized forms of DsRed-like acylimine **6** and **9** and to an unexpected imide **7**.

tert-butyl-substituted acylimine were unsuccessful because *tert*-Leu derivative **5c** is almost inert toward oxygen (higher temperatures result in decomposition not associated with α-autoxidation). Low reactivity of **5c** is consistent with both the bulkiness and the electron-donating effect of the *tert*-butyl group.

Relevance to Fluorescent Proteins. Our data for the first time show that the GFP chromophore is prone to autoxidation at position 65 Cα by its chemical nature with basic conditions being the only key factor required. It should be emphasized, that the conditions of this autoxidation closely resemble native conditions of maturation of FPs. Indeed, dimethylformamide is known to mimic a polyamide protein shell, and in the case of nonbulky chromophore analogues the autoxidation process took place effectively at room temperature with a half-life of 20 min, which is comparable with or even faster than maturation of red fluorescent proteins.

Therefore, in red FPs maturing through a GFP-like intermediate (such as z2FP574 and asFP595) the role of protein environment can be essentially basic catalysis. Residues acting as a general base in the vicinity of the chromophore may be crucial for red chromophore formation. Notably, our data allow us to propose a new mechanism of z2FP574 maturation. The fully mature z2FP574 carries DsRed-type chromophore formed by Asp65-Tyr66-Gly67 in which Asp65 is decarboxylated.[25,26] It was demonstrated that z2FP574 maturation proceeds through the GFP-like green intermediate and Asp65 is essential for this green-to-red conversion.[9] To explain the observed interconnections between formation of acylimine and Asp65 decarboxylation, Pakhomov and Martynov suggested a "coupled oxidation–decarboxylation reaction".[9] However, in our opinion, green-to-red maturation of z2FP574 evidently consists of at least two consequent irreversible reactions; thus, decarboxylation cannot drive the preceding oxidation. In the present paper we show that oxidation at position 65 requires deprotonation of Cα. Therefore, we suggest that the Asp65 side chain carboxylate can be a base facilitating proton abstraction from Cα-65 (Figure 7). Further oxidation results in β-imino carboxylic acid, which is known to readily undergo decarboxylation. Interestingly, mutation of D65E in z2FP574 results in only partial inhibition of green-to-red maturation and complete inhibition of decarboxylation,[9] which is consistent with our model but inconsistent with the "coupled oxidation–decarboxylation reaction".

There are several examples in the literature where introduction of the COO^- group (which can act as a general base) in the vicinity of Cα-65 resulted in formation of a red chromophore. A single substitution Asn65Asp converted purely green FP zFP506 into a dual-color form where roughly one-third of the protein becomes red fluorescent.[9] Also, it was demonstrated that only Glu and Asp residues at position 65 lead to the appearance of red fluorescence in yellow FP zFP538.[27]

We have found a novel imide-substituted chromophore arising from 4-electron oxidation of the glycine-derived GFP-like

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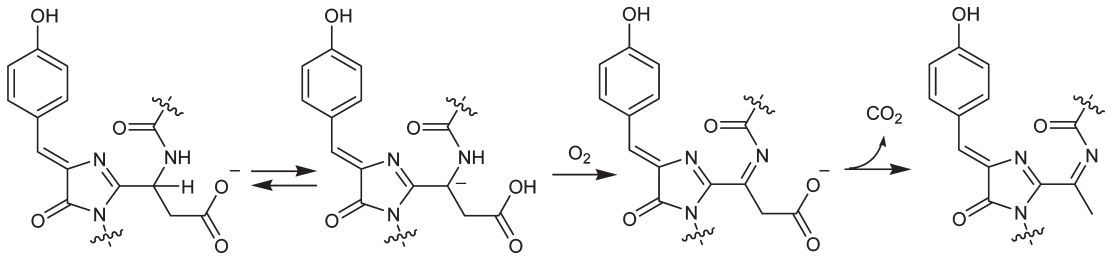


Figure 7. Plausible mechanism of chromophore maturation in z2FP574.

predecessor. In this regard the Gly-Tyr-Gly chromophore-forming sequence is promising as it could produce the novel type of red fluorescent proteins possessing an imide-derived chromophore. This chromophore is not yet found in known fluorescent proteins. Its extended conjugated π-system together with the electron-withdrawing effect of imide substituent make it strongly red-shifted putting it together with known chromophores of DsRed, Kaede, or asFP595 proteins.[6,28] Its formation within the protein could presumably proceed under the same conditions as common maturation of DsRed-like chromophores. Fine tuning of the basic residues near the α-position of Gly65 may be required to enable this pathway.

Our results shed light on the evolution of fluorescent proteins. It was recognized that the color diversity of GFP-like proteins originated independently within different lineages.[29] In particular, multiple independent appearance of RFPs and chromoproteins with the DsRed-like chromophore provides an intriguing example of convergent evolution at the molecular level. Ease of autoxidation at position 65 adjacent to the GFP-like chromophore helps to explain this "mystery", showing that green-to-red maturation is not as complex a process as was previously thought.

Finally, unexpectedly high reactivity of Cα-65 toward oxygen brings up a paradoxical question: Why not all fluorescent proteins are red? Indeed, all green FPs contain GFP-like chromophore with a residue at position 65, which is prone to oxidation. One would expect at least slow conversion of GFPs into the red state, especially when position 65 is occupied by a small residue (e.g., Ser65 in *A. victoria* GFP, Gly65 in copepod[29] and lancelet[30] green FPs). However, it is known that even prolonged (for years) storage of green FPs does not result in the appearance of red fluorescence. It seems that only the absence of strong basic groups around position 65 ensures the stability of green chromophore in GFPs. Thus, a novel approach to generate RFPs from GFPs—introduction of basic residues in proximity to position 65—is emerging. It might be an important step toward the rational design of new fluorescent proteins for the needs of particular applications.

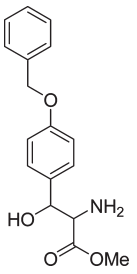
EXPERIMENTAL SECTION

Assignments of ^{1}H and ^{13}C NMR peaks were made based on a combination of COSY, HSQC, and HMBC spectra.

2-Amino-3-(4-(benzyloxy)phenyl)-3-hydroxypropanoic acid,[22] 3,3-dimethyl-2-(*N*-acetylamino)butanoic acid,[31] and 3-methyl-2-ethyl-5-(4-hydroxybenzylidene)dihydroimidazole-5-one[6] (**10**) were prepared according to the known procedures.

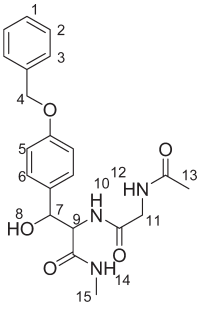
Chromophores **8** and **11** were identical by R_f, UV−vis, and ^{1}H NMR to the samples prepared independently according to the known procedures.[6,12]

Methyl 2-Amino-3-(4-(benzyloxy)phenyl)-3-hydroxypropanoate (1).



$SOCl_2$ (0.1 mol, 7.8 mL) was added dropwise to the stirred suspension of 2-amino-3-(4-(benzyloxy)phenyl)-3-hydroxypropanoic acid (47.3 mmol, 13.6 g) in 80 mL of MeOH at −10 °C. The resulting yellowish solution was refluxed for 2 h. The solvent was evaporated and the residue was suspended in 200 mL of H_2O and thoroughly washed with EtOAc to remove colored byproduct. The suspension was covered with 150 mL of EtOAc and 100 mL of 10% aqueous Na_2CO_3 was added with stirring. The aqueous phase was then extracted twice with 60 mL of EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated. The residue was subjected to the column chromatography ($CHCl_3$−EtOH 10:1) to give **2** (11.1 g, 78%, 3.2:1 mixture of *rel*-(2S,3R) and *rel*-(2S,3S) isomers by NMR) as a colorless waxy solid. Analytical data were consistent with the literature.[32] No separation of isomers is needed for the further steps.

2-(2-Acetamidoacetamido)-3-(4-(benzyloxy)phenyl)-3-hydroxy-*N*-methylpropanamide (2a).



A 1 M solution of DCC in THF (12 mmol, 12 mL) was added at 0 °C to the stirred solution of acetylglycine (12 mmol, 1.40 g), $NEtiPr_2$ (12 mmol, 2.09 mL), and HOBt (12 mmol, 1.62 g) in 30 mL of DMF. After the mixture was stirred at ambient temperature for 1 h a solution of **2** (11 mmol, 3.31 g) in 10 mL of DMF was added and the mixture was left overnight. After addition of 1 mL of AcOH the mixture was stirred for 20 min and filtered, then the filtrate was evaporated in vacuo. The residue was dissolved in 120 mL of EtOAc, washed with water, then 0.5 M HCl, saturated $NaHCO_3$, and brine, and finally dried over Na_2SO_4. After evaporation of EtOAc the crude methyl ester (almost pure by TLC in EtOAc−EtOH 2:1) was redissolved in MeCN (60 mL) and aqueous $MeNH_2$ (40%, 60 mmol, 5.17 mL) was added. The mixture was warmed

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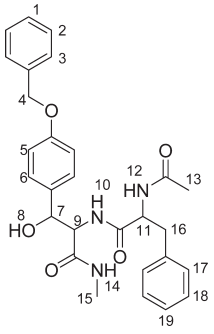
to 40 °C until the reaction was complete by TLC (ca. 3 h). During this time a white precipitate is formed that was found to be pure **2a** (3.03 g, 69% yield for two steps). The mother solution was evaporated and the oily residue was subjected to column chromatography (EtOAc−EtOH 3:1) to give an additional 0.76 g (19%) of **2a**. Total yield: 3.79 g (88% for two steps).

^{1}H NMR (800 MHz, DMSO-d_6, major isomer/rotamer): δ 1.84 (s, 3H, H^{13}), 2.58 (3H, d, J = 4.7 Hz, H^{15}), 3.60 (1H, dd, J = 5.6 and 16.4 Hz, H^{11}), 3.77 (1H, dd, J = 5.6 and 16.4 Hz, H^{11}), 4.27 (1H, dd, J = 3.2 and 8.8 Hz, H^9), 5.02 (1H, t, J = 3.2 and 4.6 Hz, H^7), 5.06 (2H, s, H^4), 5.60 (1H, d, J = 4.6 Hz, H^8), 6.91 (2H, d, J = 8.6 Hz, H^5), 7.25 (2H, d, 8.6 Hz, H^6), 7.31−7.44 (5H, 3 m, H^1, H^2, H^3), 7.67 (1H, q, J = 4.6 Hz, H^{14}), 7.74 (1H, d, J = 8.8 Hz, H^{10}), 8.07 (1H, t, J = 5.6 Hz, H^{12}).

HRMS (ESI) calcd for $C_{21}H_{26}N_3O_5$ 400.1873, found 400.1844.

2-(2-Acetamido-3-phenylpropanamido)-3-(4-(benzyloxy)-phenyl)-3-hydroxy-*N*-methylpropanamide (2b).

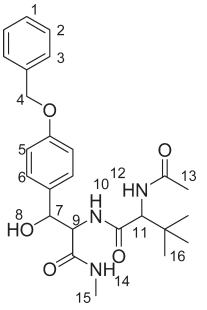


This compound was prepared similarly to **2a**. The product was purified by the column chromatography ($CHCl_3$−EtOH 85:15). Yield: 68% (2 steps).

^{1}H NMR (700 MHz, DMSO-d_6, major isomer/rotamer): δ 1.72 (3H, s, H^{13}), 2.56 (3H, d, J = 4.7 Hz, H^{15}), 2.66 (1H, dd, J = 10.3 and 13.9 Hz, H^{16}), 2.87 (1H, dd, J = 4.9 and 13.9 Hz, H^{16}), 4.24 (1H, dd, J = 2.3 and 8.6 Hz, H^9), 4.59 (1H, m, H^{11}), 5.05 (3H, m, H^4 and H^7), 5.61 (1H, d, J = 4.4 Hz, H^8), 6.89 (2H, d, J = 8.8 Hz, H^5), 7.05 (1H, d, J = 7.1 Hz, H), 7.18−7.41 (5H, m, H^1, H^2, and H^3), 7.22 (2H, d, J = 8.8 Hz, H^6), 7.53 (1H, q, J = 4.7 Hz, H^{14}), 7.65 (1H, d, J = 8.6 Hz, H^{10}), 8.09 (1H, d, J = 8.3 Hz, H^{12}).

HRMS (ESI) calcd for $C_{28}H_{32}N_3O_5$ 490.2342, found 490.2325.

2-Acetamido-*N*-(1-(4-(benzyloxy)phenyl)-1-hydroxy-3-(methylamino)-3-oxopropan-2-yl)-3,3-dimethylbutanamide (2c).

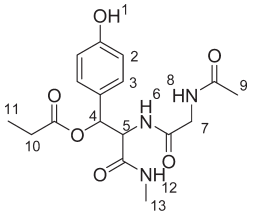


This compound was prepared similarly to **2a**, except the mixture was stirred for 2 h before addition of amine and for 24 h after that. Product was purified by the column chromatography ($CHCl_3$−EtOH 10:1). Yield: 73% (for two steps, mixture of isomers).

^{1}H NMR (700 MHz, $CDCl_3$, major isomer/rotamer): δ 0.67 (9H, s, H^{16}), 1.89 (3H, s, H^{13}), 2.58 (3H, d, J = 4.6 Hz, H^{15}), 4.05 (1H, d, J = 6.8 Hz, H^{11}), 4.30 (1H, dd, J = 2.9 and 8.8 Hz, H^9), 5.07 (2 H, m, H^4), 5.16 (1H, dd, 2.9 and 5.3 Hz, H^7), 5.51 (1H, d, J = 5.3 Hz, H^8), 6.87 (2H, d, J = 8.6 Hz, H^5), 7.21 (1H, q, J = 4.6 Hz, H^{14}), 7.27 (2H, d, J = 8.6 Hz, H^6), 7.30−7.43 (5H, m, H^1, H^2, H^3), 7.73 (1H, d, J = 6.8 Hz, H^{12}), 7.87 (1H, d, J = 8.8 Hz, H^{10}).

HRMS (ESI) calcd for $C_{25}H_{34}N_3O_5$ 456.2499, found 456.2475.

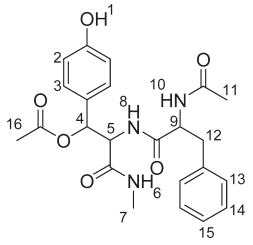
2-(2-Acetamidoacetamido)-1-(4-hydroxyphenyl)-3-(methylamino)-3-oxopropyl Propionate (3a).



A mixture of **2a** (3.45 mmol, 1.38 g, mixture of isomers), propionic anhydride (15 mL), and $ZnCl_2$ (1 mmol, 136 mg) was heated to 90 °C with stirring until the solution became clear and no starting material was detected by TLC (total time approximately 4 h). The excess propionic anhydride and propionic acid were evaporated in vacuo at 70 °C and the crude product was dissolved in 50 mL of EtOH and filtered through a pad of SiO_2. To that solution were added 10% Pd/C (200 mg) and AcOH (5 mL) and the resulting mixture was hydrogenated at ambient temperature for several hours. When the reaction was complete by TLC (ca. 4 h) the mixture was filtered through the paper and evaporated. The product was purified by the column chromatography ($CHCl_3$−EtOH 85:15). Yield: 0.91 g (72% for two steps).

^{1}H NMR (700 MHz, D_2O, major isomer/rotamer): δ 1.24 (3H, t, J = 7.13 Hz, H^{11}), 2.18 (3H, s, H^9), 3.08 (3H, s, H^{13}), 3.71 (2H, q, J = 7.13 Hz, H^{10}), 4.34 (2H, s, H^7), 6.92 (2H, d, J = 8.7 Hz, H^2), 6.95 (1H, s, H^4), 7.86 (2H, d, J = 8.7 Hz, H^3).

2-(2-Acetamido-3-phenylpropanamido)-1-(4-hydroxy-phenyl)-3-(methylamino)-3-oxopropyl Propionate (3b).

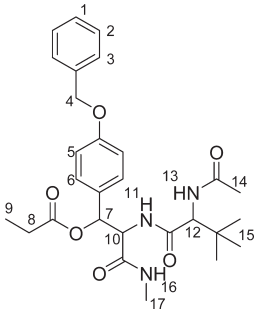


AcBr (2.0 mmol, 0.15 mL in 1.5 mL of Ac_2O) was added to the solution of **2b** (2.0 mmol, 1.0 g) in CH_2Cl_2 (10 mL). After 1 h (100% conversion by TLC $CHCl_3$−EtOH 9:1) the reaction mixture was poured into EtOAc (50 mL), washed with saturated $NaHCO_3$ solution and brine, and dried over Na_2SO_4. After evaporation in vacuo the residue (pure acetylation product by TLC) was resuspended in 30 mL of MeOH. To this solution were added 150 mg of Pd/C, 100 μL of TFA, and the resulting mixture was hydrogenated at ambient pressure until 100% conversion of the substrate by TLC ($CHCl_3$−EtOH 7:1). The clear solution was filtered through the paper and evaporated to dryness. The product was purified by the column chromatography ($CHCl_3$−EtOH 8:1). Yield: 0.65 g (74% for two steps).

^{1}H NMR (800 MHz, DMSO-d_6, major isomer/rotamer): δ 1.91 and 2.05 (6H, 2s, H^{11} and H^{16}), 2.63 (3H, d, J = 4.9 Hz, H^7), 2.85 and 3.00 (2H, 2 m, H^{12}), 4.63 (m, H^9), 4.74 (1H, dd, J = 6.1 and 8.8 Hz, H^5), 6.15 (1H, d, J = 6.1 Hz, H^4), 6.71−7.26 (8H, m, H^3, H^{10}, H^{13}, H^{14}, and H^{15}), 6.74 (2H, d, J = 8.6 Hz, H^2), 6.84 (1H, q, J = 4.9 Hz, H^6), 7.09 (1H, d, J = 8.8 Hz, H^8).

2-(2-Acetamido-3,3-dimethylbutanamido)-1-(4-(benzyl-oxy)phenyl)-3-(methylamino)-3-oxopropyl Propionate (3c).

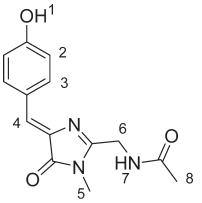
dx.doi.org/10.1021/jo200150b |*J. Org. Chem.* 2011, 76, 2782–2791



^{1}H NMR (700 MHz, $CDCl_3$, major isomer/rotamer): δ 0.86 (9H, s, H^{15}), 0.98 (3H, t, J = 7.5 Hz, H^9), 1.87 (3H, s, H^{14}), 2.27 (2H, dq, J = 2.4 and 7.5 Hz, H^8), 2.42 (3H, d, J = 4.7 Hz, H^{17}), 4.33 (1H, d, J = 9.5 Hz, H^{12}), 4.70 (1H, dd, J = 6.9 and 8.8 Hz, H^{10}), 5.06 (2H, s, H^4), 6.85 (1H, d, J = 6.9 Hz, H^7), 6.92 (2H, d, J = 8.8 Hz, H^5), 7.19 (2H, d, J = 8.8 Hz, H^6), 7.33−7.43 (5H, m, H^1, H^2, H^3), 7.73 (1H, d, J = 9.5 Hz, H^{13}), 7.78 (1H, q, J = 4.7 Hz, H^{16}), 8.07 (1H, d, J = 8.8 Hz, H^{11}).

ESI-MS: m/z 512 ($[M + H]^+$), 438 ($[M - C_2H_5CO_2H + H]^+$), 534 ($[M + Na]^+$).

***N*-((4-(4-Hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-2-yl)methyl)acetamide (5a).**



4a (0.60 mmol, 220 mg), K_2CO_3 (1.28 mmol, 176 mg), and DMF (20 mL) together with a stirring bar were placed into the flask equipped with a septum. Argon was bubbled through the solution via needle for 15 min. The mixture was heated to 110 °C and stirred at this temperature for 40 min. After cooling aqueous NH_4Cl (1.5 mmol, 80 mg, 5 mL of H_2O) was added and the resulting orange solution was evaporated in vacuo at 60 °C (the color changes to yellow as neutralization of chromophore occurs). The residue was extracted with EtOAc−EtOH (20:1) and the extract was subjected to column chromatography (EtOAc−EtOH 15:1). Yield: 121 mg (74%).

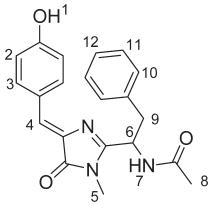
^{1}H NMR (800 MHz, DMSO-d_6, Z-isomer): δ 1.94 (3H, s, H^8), 3.08 (3H, s, H^5), 4.32 (2H, d, J = 5.6 Hz, H^6), 6.85 (2H, d, J = 8.8 Hz, H^2), 6.99 (1H, s, H^4), 8.11 (2H, d, J = 8.8 Hz, H^3), 8.41 (1H, t, J = 5.6 Hz, H^7), 10.16 (1H, br, H^1).

HSQC ^{1}H−^{13}C (800 MHz, DMSO-d_6): 23.4 (H^8), 26.5 (H^5), 37.5 (H^6), 116.0 (H^2), 127.3 (H^4), 134.7 (H^3).

HSQC ^{1}H−^{15}N (800 MHz, DMSO-d_6): cross-peak at 8.41 (H^7).

HRMS (ESI) calcd for $C_{14}H_{16}N_3O_3$ 274.1192, found 274.1167.

***(Z)*-*N*-(1-(4-(4-Hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-2-yl)-2-phenylethyl)acetamide (5b).**

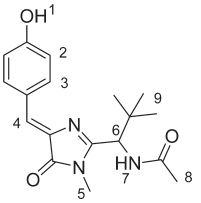


^{1}H NMR (700 MHz, DMSO-d_6, Z-isomer): δ 1.78 (3H, s, H^8), 2.97 (3H, s, H^5), 3.07 (1H, dd, J = 8.6 and 13.7 Hz, H^9), 3.29 (1H, dd, J = 6.4 and 13.9 Hz, H^9), 5.09 (1H, ddd, J = 6.4, 8.3, and 8.6 Hz, H^6), 6.86 (2H, d, J = 8.8 Hz, H^2), 7.00 (1H, s, H^4), 7.19−7.31 (5H, m, H^{10}, H^{11}, and H^{12}), 8.13 (2H, d, J = 8.8 Hz, H^3), 8.53 (1H, d, J = 8.6 Hz, H^7), 10.17 (1H, s, H^1).

HSQC ^{1}H−^{13}C (800 MHz, DMSO-d_6, Z-isomer): 22.7 (H^8), 26.8 (H^5), 37.7 (H^9), 48.0 (H^6), 116.1 (H^2), 126.6 (H^{12}), 127.8 (H^4), 128.4−129.7 (H^{11}−H^{12}), 135.0 (H^3).

HRMS (ESI) calcd for $C_{21}H_{22}N_3O_3$ 364.1661, found 364.1683.

***(Z)*-*N*-(1-(4-(4-Hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-2-yl)-2,2-dimethylpropyl)acetamide (5c).**



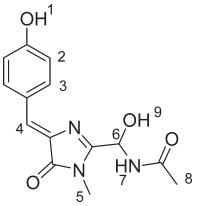
This compound was prepared similarly to **5a**, but the temperature was 140 °C and the reaction was clearly stepwise by TLC with the chromophore formed at the second step.

^{1}H NMR (700 MHz, $CDCl_3$, Z-isomer): δ 1.10 (9H, s, H^9), 2.08 (3H, s, H^8), 3.26 (3H, s, H^5), 4.94 (1H, d, J = 9.25 Hz, H^6), 6.66 (1H, d, J = 9.25 Hz, H^7), 6.86 (2H, d, J = 8.6 Hz, H^2), 7.12 (1H, s, H^4), 7.99 (2H, J = 8.6 Hz, H^3), 8.26 (1H, br, H^1).

HRMS (ESI) calcd for $C_{18}H_{24}N_3O_3$ 330.1818, found 330.1826.

General Procedure for Autoxidation of 5a−c. Base (triethylamine, DBU, AcONa, or Cs_2CO_3) was added to a 0.1 M solution of **5a**−**c** in CH_2Cl_2, THF, or DMF and oxygen gas was slowly bubbled through the reaction mixture at constant temperature. Formation of products was determined by UV−vis, NMR spectroscopy, and TLC.

***N*-(Hydroxy(4-(4-hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-2-yl)methyl)acetamide (6).**



A solution of **5a** in wet THF containing 10 mM NEt_3 is oxygenated (see General Procedure) at 30 °C for ca. 45 min. When the consumption of **5a** approaches 90% by TLC the mixture is evaporated in vacuo and subjected to column chromatography (EtOAc−EtOH 10:1). Hydroxyamide **6** and imide **7** are formed in these conditions in ca. 40% and 25% yields, respectively.

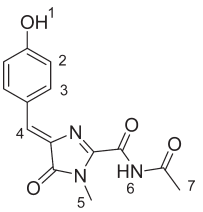
^{1}H NMR (800 MHz, DMSO-d_6, Z-isomer): δ 1.95 (3H, s, H^8), 3.14 (3H, s, H^5), 6.19 (1H, dd, J = 6.1 and 8.6 Hz, H^6), 6.78 (1H, d, 6.1 Hz, H^9), 6.84 (2H, d, J = 8.8 Hz, H^2), 7.06 (1H, s, H^4), 8.15 (2H, d, J = 8.8 Hz, H^3), 8.81 (1H, d, J = 8.6 Hz, H^7), 10.19 (1H, br, H^1).

HSQC ^{1}H−^{13}C (800 MHz, DMSO-d_6): 23.4 (H^8), 27.3 (H^5), 68.9 (H^6), 116.0 (H^2), 128.7 (H^4), 135.2 (H^3).

HSQC ^{1}H−^{15}N (800 MHz, DMSO-d_6): cross-peak at 8.82 (H^7).

HRMS (ESI) calcd for $C_{14}H_{16}N_3O_4$ 290.1141, found 290.1125.

***N*-Acetyl-4-(4-hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazole-2-carboxamide (7).**



Imide **7** is formed along with hydroxyamide **6** during autoxidation of **5a** in 25% yield (see above).

Also, imide **7** can be obtained by oxidation of **5a** or **6** with selenium dioxide by using the following procedure. Selenium dioxide (0.6 mmol,

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67 mg) was added to a stirred solution of **5a** or **6** (0.40 mmol) in dioxane (4 mL). After the mixture was stirred at room temperature for 40 min the solvent was evaporated and the product was purified by column chromatography (EtOAc). Yields are 75% and 60% from **5a** and **6**, respectively.

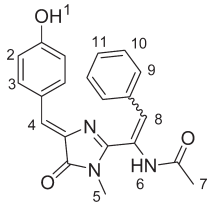
^{1}H NMR (700 MHz, $CDCl_3$): δ 2.61 (3H, s, H^7), 3.56 (3H, s, H^5), 6.96 (2H, d, J = 8.7 Hz, H^2), 7.45 (1H, s, H^4), 8.09 (2H, J = 8.7 Hz, H^3), 9.67 (1H, br, H^1).

^{13}C NMR (200 MHz, DMSO-d_6): δ 25.9, 28.6, 116.7, 125.2, 134.4, 134.8, 136.6, 152.0, 158.7, 162.0, 169.9, 171.5.

HSQC ^{1}H−^{13}C (700 MHz, DMSO-d_6): 25.9 (H^7), 28.6 (H^5), 116.7 (H^2), 134.8 (H^4), 136.7 (H^3).

HRMS (ESI) calcd for $C_{14}H_{14}N_3O_4$ 288.0984, found 288.0983.

***N*-(1-(4-(4-Hydroxybenzylidene)-1-methyl-5-oxo-4,5-dihydro-1*H*-imidazole-2-yl)-2-phenylvinyl)acetamide (9).**



Dry dioxigen gas was passed through the mixture of **5b** (0.30 mmol, 110 mg), Cs_2CO_3 (0.2 mmol, 65 mg), and DMF (5 mL) at 115 °C for 45 min (almost total conversion of **5b**). The mixture was poured into EtOAc−0.1 M HCl, then the aqueous layer was extracted with additional portions of EtOAc. The combined extracts were washed with water and brine and dried over Na_2SO_4. After evaporation the only colored product was purified by chromatography twice (column chromatography: hexane−EtOAc 1:3, then preparative TLC: pure EtOAc). Yield: 21 mg (20%) as a mixture of inseparable isomers.

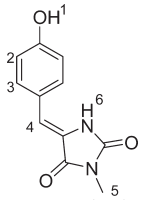
^{1}H NMR (800 MHz, DMSO-d_6, major isomer/rotamer): δ 2.06 (3 H, s, H^7), 3.07 (3H, s, H^5), 6.84 (2H, d, J = 8.8 Hz, H^2), 6.90 (1H, s, H^8), 7.01 (1H, s, H^4), 7.40−7.66 (5H, m, H^9, H^{10}, and H^{11}), 8.10 (2H, d, J = 8.8 Hz, H^3), 10.06 (1H, br, H^1).

Characteristic HSQC ^{1}H−^{13}C cross-peaks: 2.06−22.8, 3.07−28.1, 6.90−127.2, 6.84−116.2, 7.01−127.4, 8.10−134.8.

Characteristic HMBC ^{1}H−^{13}C cross-peaks: 2.06−169.7; 3.07−160.5 and 170.4; 6.90−130.0 and 160.5; 8.10−116.2, 127.4, 134.7, and 160.1.

HRMS (ESI) calcd for $C_{21}H_{20}N_3O_4$ 362.1505, found 362.1492.

5-(4-Hydroxybenzylidene)-3-methylimidazolidine-2,4-dione (12).



Et-HBI **10** was added to a saturated solution of Cs_2CO_3 in DMF and the resulting mixture was oxygenated at 80 °C for 3 h. The mixture was partitioned between EtOAc and 0.1 M HCl, then the aqueous layer was extracted twice with EtOAc. The combined extracts were washed with water and brine, dried over Na_2SO_4, and evaporated. Column chromatography ($CHCl_3$−EtOH 10:1) gave **11**[3] (21%) and **12** (48%)

^{1}H NMR (700 MHz, $CDCl_3$): δ 2.96 (3H, s, H^5), 6.47 (1H, s, H^4), 6.81 (2H, d, J = 8.5 Hz, H^2), 7.50 (2H, d, J = 8.5 Hz, H^3), 9.86 (1H, s, H^6), 10.52 (1H, br, H^1).

HRMS (ESI) calcd for $C_{11}H_{11}N_2O_3$ 219.0770, found 219.0756.

■ ASSOCIATED CONTENT

Supporting Information. Autoxidation study of 2-ethyl-substituted GFP-chromophore, 1D and 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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