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Synthesis and Spectral and Chemical Properties of the Yellow Fluorescent Protein zFP538 Chromophore†

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ABSTRACT: Members of the green fluorescent protein (GFP) family become chromophoric through a unique pathway based on autocatalytic modifications of their amino acid residues. The yellow fluorescent protein zFP538 from the button polyp *Zoanthus* possesses unique spectral characteristics that are intermediate between those of the green and orange-red fluorescent proteins. In this study, we used chemical synthesis to resolve conflicting data from crystallographic and biochemical analyses of the zFP538 chromophore structure. We synthesized 2-(5-amino-1-oxopentyl)-5-(4-hydroxybenzylidene)-3-methyl-3,5-dihydro-4*H*-imidazol-4-one (**5**), which can spontaneously react intramolecularly to form cyclic imine (**7**). Compound **7** represents the native chromophore structure reported in the crystallographic study. We have also discovered an unusual isomerization of a 2-acylimidazolone to a 2,6-diketopiperazine derivative. The zFP538 chromophore is a complex system with intriguing chemical and spectral behavior, properties that have led to discrepancies in the interpretation of its structure. Our study supports the findings of previous crystallographic work, which postulated a cyclic imine chromophore structure within the native zFP538 protein, and also provides an explanation for experimental results obtained in the biochemical characterization of zFP538-derived chromopeptides.

Members of the green fluorescent protein (GFP)[1] family are now widely used as genetically encoded fluorescent labels (*1*). They enable the visualization of structures and processes in living cells and organisms. The practical use of GFP-like proteins is based on their unique ability to form chromophores inside protein globules via self-catalyzed reactions of post-translational modifications of internal amino acids (*2*). Chromophore maturation requires no additional enzymes or cofactors besides molecular oxygen. As a result, genes encoding GFP-like proteins can be introduced into nearly any cell or organism, leading to the functional expression of fluorescent proteins under the control of a specific promoter. GFP from the jellyfish *Aequorea victoria* was the first fluorescent protein characterized and cloned (*3*, *4*). GFP homologues were subsequently found in corals (*5*), crustaceans (*6*), and even lower chordates (*7*). Coral GFP-like proteins are the most colorful homologues and include cyan, green, yellow, and red fluorescent proteins as well as nonfluorescent purple-blue chromoproteins (*1*, *8*).

Chromophore structures and the mechanisms of their formation in GFP-like proteins are under extensive study; these investigations provide an understanding of the "heart" of fluorescent proteins and suggest possible ways to modify their spectral properties. It has been demonstrated that the green chromophore in GFP is formed by cyclization of the protein backbone in the Ser65-Tyr66-Gly67 region (numbering of *A. victoria* GFP), followed by dehydrogenation of the Cα−Cβ bond of Tyr66. As a result, a bicyclic structure of 5-(4-hydroxybenzylidene)-3,5-dihydro-4*H*-imidazol-4-one is formed: the six-membered aromatic ring of the Tyr66 side chain is linked to an unusual five-membered heterocycle, which itself originates from condensation of the carbonyl carbon of Ser65 with the nitrogen of Gly67.

Further chemical modifications of the green chromophore occur in red-shifted GFP-like proteins. In particular, oxidation of a Cα−N bond results in an acylimine group conjugated to a GFP-like core in DsRed (*9*, *10*). The DsRed-like chromophore is formed within many other proteins with red-shifted absorption and fluorescence (*2*). In some proteins, the acylimine moiety of the DsRed chromophore is further attacked by various nucleophiles to form additional types of red-shifted chromophores. For example, the chromophore in the purple chromoprotein asFP595 is formed by hydrolysis of the acylimine group, resulting in cleavage of the protein backbone and formation of a keto group conjugated to a GFP-like chromophore core (*11*, *12*). In the orange fluorescent proteins mOrange and mKO, nucleophilic addition of Thr65 (in mOrange) or Cys65 (in mKO) side chain groups leads to unusual heterocycles without protein backbone scission (*13*, *14*).

The yellow fluorescent protein zFP538 from the button polyp *Zoanthus* demonstrates unique spectral characteristics that are intermediate between those of the green and orange-red fluorescent proteins ($\lambda_{max,ex}$ = 528 nm, and $\lambda_{max,em}$ = 538 nm) (*5*).

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[1]Abbreviations: asFP595, purple chromoprotein from *Anemonia sulcata*; DMF, dimethyl formamide; DsRed, red fluorescent protein from *Discosoma*; GFP, green fluorescent protein from *A. victoria*; zFP538, yellow fluorescent protein from *Zoanthus*.

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The chromophore-forming residues in zFP538 are Lys-Tyr-Gly. Differing opinions concerning the structure of the zFP538 chromophore have been published (*15*−*17*).

In this study, we used chemical synthesis to resolve conflicting data from crystallographic (*16*, *17*) and biochemical (*15*) analyses related to the zFP538 chromophore structure. This approach allowed us to establish a structural basis for the complex spectral transformations of the zFP538 chromophore described by Zagranichny et al. (*15*).

EXPERIMENTAL PROCEDURES

NMR spectra were recorded with Varian unity 600 MHz, Bruker Avance III 600 MHz, and Bruker DRX-500 MHz instruments. UV−vis spectra were recorded with a Varian Cary 100 spectrophotometer. Fluorescence excitation and emission spectra were recorded with a Varian Cary Eclipse fluorescence spectrophotometer.

2-(5-tert-Butyloxycarbonylaminopentyl)-5-(4-hydroxybenzylidene)-3-methyl-3,5-dihydro-4H-imidazol-4-one (***3***). *N*-Boc-6-aminohexanoylglycine (obtained by standard peptide synthesis) (5.77 g, 0.02 mol) was added to 100 mL of a THF solution containing dicyclohexylcarbodiimide (6.19 g, 0.03 mol). The reaction mixture was stirred overnight and then filtered and concentrated on a rotary evaporator.

The resulting oil was dissolved in 50 mL of acetic anhydride, and sodium acetate (1.64 g, 0.02 mol) and *p*-acetoxybenzaldehyde (4.93 g, 0.03 mol) were added. The resulting mixture was stirred at 40 °C for 3 days until it solidified (indicating the formation of oxazolone **1**). Most of the acetic anhydride was removed in vacuo, and ethanol (150 mL) was added followed by aqueous methylamine (c = 11.6 M, 8.6 mL, 0.1 mol). The mixture was vigorously stirred until the crystals of oxazolone **1** disappeared. Water (200 mL) was added, and crystals of **2** (with a crystalline admixture of dicyclohexylurea) were collected by suction and air-dried. The crude solid obtained was heated to reflux with 2 g of anhydrous cesium carbonate in 100 mL of DMF with vigorous stirring. The course of the reaction was monitored by TLC (SiO_2, 10% chloroform/ethanol). The R_f of **2** was 0.33, while the R_f of **3** was 0.62. A spot-to-spot conversion was observed after 3 min. The reaction mixture was cooled, diluted with water (300 mL), and extracted with ethyl acetate (4 × 100 mL). The extracts were washed with brine, dried with anhydrous sodium sulfate, and evaporated. Column chromatography (SiO_2, 10% chloroform/ethanol) gave pure **3** (4.96 g, 64%) as yellow crystals: ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.43 (s, 9H, Boc), 1.46 (m, 2H, CH_2), 1.57 (m, 2H, CH_2), 1.83 (m, 2H, CH_2), 2.59 (t, 2H, CH_2), 3.16 (m, 5H, N−CH_3 and CH_2−NHBoc), 4.62 (br s, NH-Boc), 6.89 (d, 2H, aromatic), 7.08 (s, 1H, vinylic CH), 7.20 (br s, phenolic OH), 8.05 (d, 2H, aromatic); MS (ESI-MS) calcd for $C_{21}H_{30}N_3O_4^+$ [M + H^+] 388.22, found 388.34.

2-(5-tert-Butyloxycarbonylamino-1-oxopentyl)-5-(4-hydroxybenzylidene)-3-methyl-3,5-dihydro-4H-imidazol-4-one (***4***). Imidazolone **3** (1.14 g, 2.94 mmol) and SeO_2 (0.46 g, 4.14 mmol) were placed in dioxane (60 mL) and refluxed for 8 h. The reaction mixture was filtered to remove Se, concentrated on a rotary evaporator, and chromatographed (SiO_2, 10% chloroform/ethanol) to give crude **4** (720 mg). Recrystallization from methanol gave **4** as red needles (602 mg, 51%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.42 (s, 9H, Boc), 1.57 (m, 2H, CH_2), 1.74 (m, 2H, CH_2), 3.10 (t, 2H, CH_2), 3.15 (t, 2H, CH_2), 3.38 (s, 3H, N−CH_3), 6.88 (d, 2H, aromatic), 7.35 (s, 1H, vinylic CH), 8.16 (d, 2H, aromatic); MS (ESI-MS) calcd for $C_{21}H_{28}N_3O_5^+$ [M + H^+] 402.20, found 402.35.

Table 1: ^{1}H and ^{13}C NMR Chemical Shifts in Compounds **5**−**7**, Extracted from ^{1}H, ^{13}C, HMBC, HSQC, COSY, and TOCSY Spectra

	5[b]		**6**[b]		**7**[c]	
atom number[a]	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		160.7		160.0		161.7
2	6.90	115.9	6.95	116.0	6.61	120.3
3	8.00	136.6	8.09	136.7	8.02	137.2
4		125.8		125.6		
5	7.27	137.5	7.69	142.7	7.21	135.5
6				136.4		
7		171.8		164.7		171.5
8	3.27	28.3	3.22	25.3	3.43	29.5
9		152.5		158.7		151.9
10		196.0		156.1		161.8
11	3.04−3.13	25.2	2.79	31.6	2.76[d]	26.1
12	1.66−1.80	21.8, 26.3	1.77−1.83	21.6, 26.0	1.78	19.1
13	1.66−1.80				1.67	22.0
14	3.04−3.13	39.1	3.09	39.2	3.84	50.2

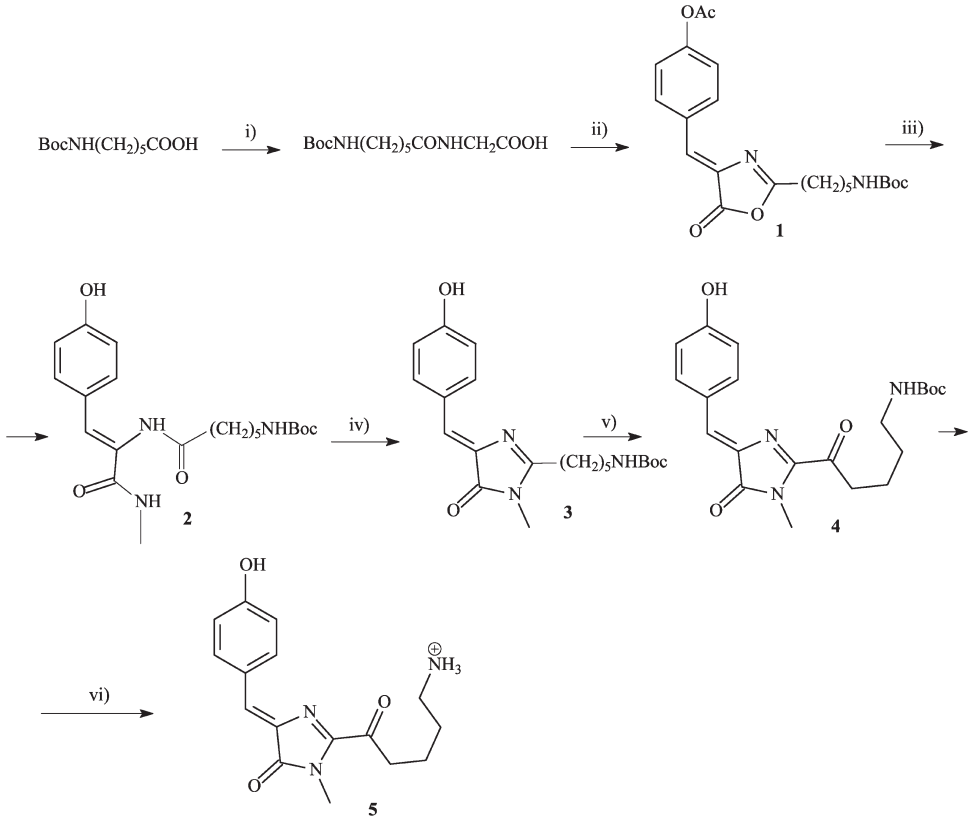
[a] Atom numbering according to Scheme 2. [b] A 3 mg/mL solution in a H_2O/D_2O mixture at pH 3.3 and 20 °C. [c] A 7 mg/mL solution in 2-propanol-d_8, saturated with Cs_2CO_3. [d] Integral intensity lowered ∼10-fold because of exchange with deuterium.

2-(5-Amino-1-oxopentyl)-5-(4-hydroxybenzylidene)-3-methyl-3,5-dihydro-4H-imidazol-4-one Salt with Trifluoroacetic Acid (***5***). Boc-protected chromophore **4** (3 mg) was incubated with 0.2 mL of a 20% trifluoroacetic acid solution in dichloromethane for 30 min at room temperature. The volatiles were then removed on a rotary vacuum concentrator. Re-evaporation with dichloromethane was performed several times until no free trifluoroacetic acid remained. The solid obtained was pure by NMR and could be stored at −20 °C in a tightly closed vessel for 1 month with no detectable decomposition. NMR data are given in Table 1: MS (ESI-MS) calcd for $C_{16}H_{20}N_3O_3^+$ [M + H^+] 302.15, found 302.28.

RESULTS

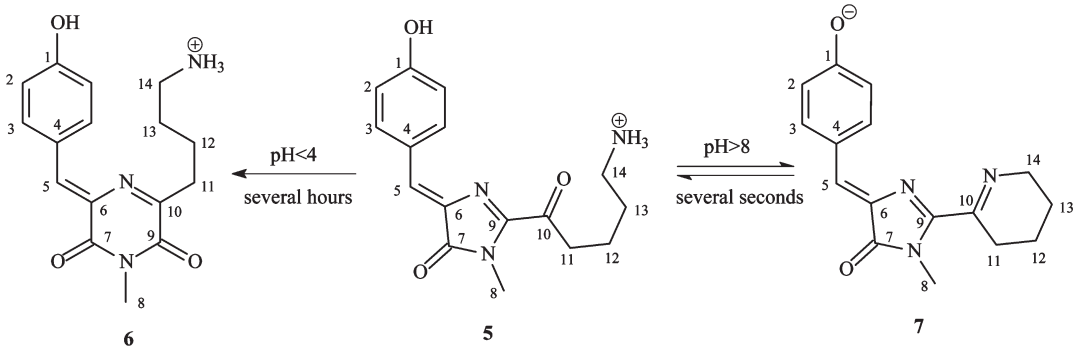
Synthesis. To obtain the target structure **5**, we used an approach utilized in our previous work (*12*) that includes oxidation with SeO_2 at a key stage (Scheme 1). We synthesized *p*-hydroxybenzylidene imidazolone **3**, bearing a Boc-protected ω-aminopentyl substituent at the 2-position of the imidazolone, via a standard (*18*) set of transformations, including Erlenmeyer azlactonization, nucleophilic aminolysis of the azlactone, and cyclization of the dehydrotyrosine derivative **2** under basic conditions to form imidazolone **3**. In this work, we have found optimal conditions for the aforementioned cyclization, namely, heating in DMF with Cs_2CO_3 as a base. These conditions provide quantitative yields, and reaction times are shortened to several minutes [we have tested these reaction conditions on several other substrates (data not shown)]. Previously, this transformation was accomplished by prolonged (several hours) heating in aqueous or alcoholic media with alkaline metal carbonates or hydroxides (*12*, *18*, *19*). When substituents at positions 1 and 2 of the imidazolone are simple alkyls, both methods give good results, but the latter method is strongly preferred when substituents are sterically hindered or when competing side reactions can take place.

Scheme 1: Synthesis of the zFP538 Chromophore (in noncyclized form **5**)[a]



[a] (i) (a) Glycine ethyl ester hydrochloride, ethanolic NaOH, DCC; (b) aqueous NaOH, ethanol, 50 °C, then HCl (78%); (ii) DCC, then *p*-acetoxybenzaldehyde, NaOAc; (iii) $MeNH_2$; (iv) Cs_2CO_3, DMF, reflux for 5 min (64% over three stages); (v) SeO_2, dioxane, reflux (51%); (vi) DCM, TFA, room temperature, 30 min (100%).

Scheme 2: pH-Dependent Chemical Transformations of the zFP538 Chromophore



Imidazolone **3** was oxidized to the Boc-protected zFP538 chromophore precursor **4** by being heated in a dilute dioxane solution with excess SeO_2. Finally, **4** was smoothly deprotected with trifluoroacetic acid in dichloromethane at room temperature to give **5** as the TFA salt.

Chemical and Spectral Behavior. Although the structure of compound **5** does not correspond to the structure of the mature chromophore within zFP538, we expected that **5** would intramolecularly cyclize to form cyclic imine **7** (Scheme 2). Indeed, we observed this transformation upon basification of the solutions of trifluoroacetate of **5** (pH increased from 5 to 8.5) in water or in 2-propanol. NMR studies clearly indicated the presence of linear aminoketone **5** in acidic D_2O and 2-propanol-d_8. In particular, the ^{13}C chemical shift of C-10 (Scheme 2) was 196.0 ppm (D_2O), which is characteristic of a carbonyl group, and no cross-peak was detected in the C−H HMBC spectrum between CH_2-14 and C-10. In D_2O at pH 8.7, the ^1H NMR spectrum was complex, containing several sets of signals (not shown). Notably, this complexity of the spectrum was not a result of chemical degradation of **5**, since the reappearance of the initial, single set of signals was observed upon acidification to pH 5. This complex behavior (which probably reflects reversible hemiaminal formation in water) impeded unambiguous structure determination. To overcome this problem, we used 2-propanol-d_8 with anhydrous Cs_2CO_3 as a base. In this case, we observed a single set of signals in the NMR spectra corresponding to cyclic imine **7**. In particular, the ^{13}C chemical shift of C-10 became 161.8 ppm, which is characteristic of an imine, and a cross-peak between CH_2-14 and C-10 appeared in the C−H HMBC spectrum. Interestingly, the integral intensity of the signal from CH_2-11 in the ^1H spectrum

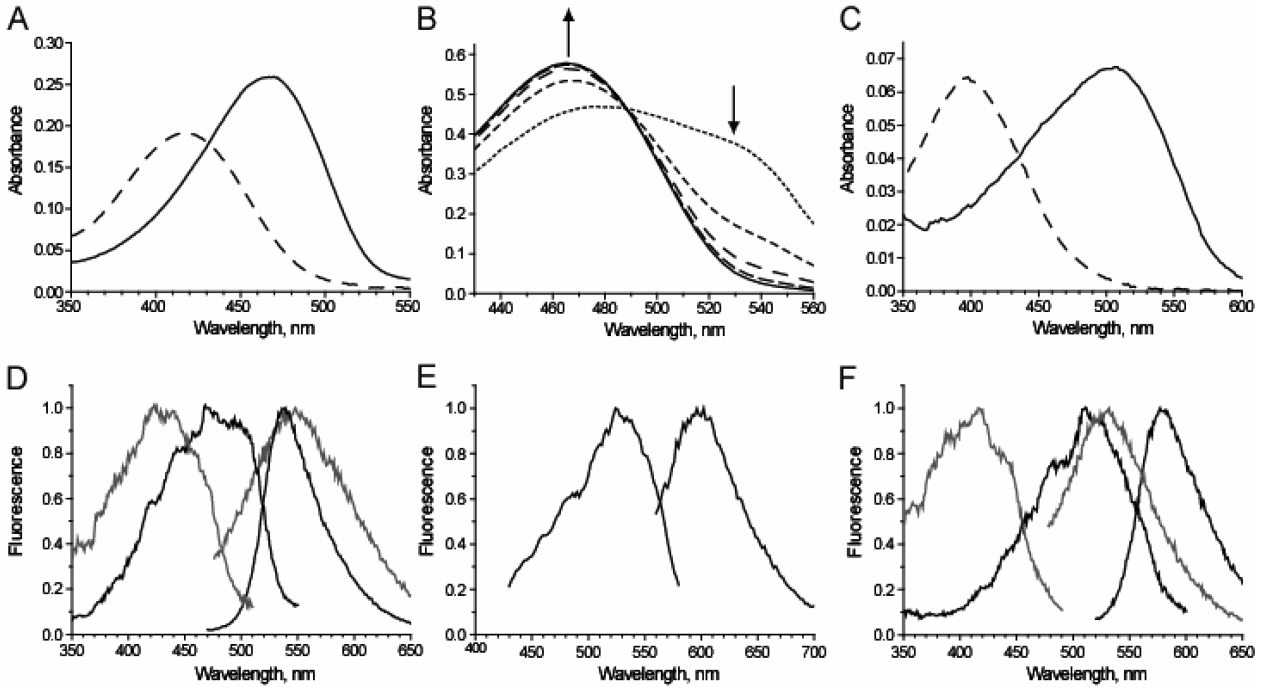


FIGURE 1: Absorption (A−C) and fluorescence (D−F) spectra of model compounds **5**−**7** in water solutions. (A) Absorption spectra of **5** at pH 4.0 (− − −) and **7** at pH 8.9 (—). (B) Change in the absorption spectrum upon basification of the solution of **5** to pH 8.9. Spectra were recorded at 10 s intervals and show the transformation of a short-lived intermediate (deprotonated **5**), absorbing at ~530 nm, into **7**, absorbing at 468 nm. (C) Absorption spectra of **6** at pH 3.0 (− − −) and pH 8.0 (—). (D) Excitation and emission spectra of **5** at pH 4.0 (gray lines) and **7** at pH 8.9 (black line). (E) Excitation and emission spectra of the short-lived intermediate (deprotonated **5**) obtained upon basification of the solution of **5** to pH 8.9. (F) Excitation and emission spectra of **6** at pH 3.0 (gray lines) and pH 8.0 (black lines). In panels D and E, within each pair of lines the emission spectrum is the one at longer wavelengths.

decreased ~10-fold. This effect can be attributed to H−D exchange due to imine−enamine tautomerization.

At pH <5, in both water and 2-propanol, compound **5** unexpectedly underwent a slow conversion to 2,6-diketopiperazine derivative **6**, whose structure was determined by ^{1}H, ^{13}C, HMBC, HSQC, COSY, and TOCSY NMR spectra. These spectra showed that the carbon skeleton remained unchanged while the chemical shift of C-10 moved upfield from 196.0 to 156.1 ppm.

The structural rearrangements described above were found to cause drastic changes in the absorption spectra. When the trifluoroacetate salt of **5** is dissolved in acidic water (pH 4.0), its absorption spectrum peaks at 417 nm (Figure 1A). Upon basification to pH 8.9, the absorption maximum is shifted to 468 nm through a short-lived (half-life of ~8 s) intermediate absorbing at ~530 nm (Figure 1A,B). These spectral transitions can be explained by the deprotonation of **5** (530 nm-absorbing species) and intramolecular formation of cyclic imine **7**, which absorbs at 468 nm in its deprotonated form.

After prolonged incubation of **5** in acidic water, the absorption maximum underwent a blue shift from 417 to 397 nm (Figure 1C), corresponding to the conversion of **5** to **6**. In basic buffer (pH 8.0), the absorption spectrum of **6** peaks at 505 nm, indicating deprotonation of the phenolic hydroxyl.

The fluorescence spectra of **5**−**7** at different pHs were in agreement with the interconversions described in Scheme 2. Compound **5** in acidic water possessed weak fluorescence with excitation and emission maxima at 419 and 545 nm, respectively (Figure 1D). Upon basification, we observed a short-lived form (deprotonated structure **5**) with excitation and emission maxima at 530 and 597 nm, respectively (Figure 1E). This form converted quickly to a blue-shifted form (structure **7**) with excitation and emission maxima at 470 and 538 nm, respectively (Figure 1D). Structure **6** under acidic and basic conditions demonstrated excitation/emission spectra peaks at 405/530 and 510/579 nm, respectively (Figure 1F).

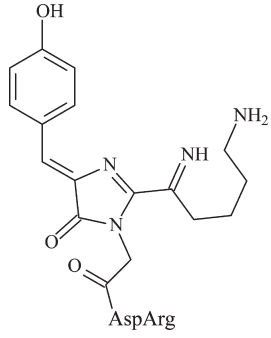


FIGURE 2: Structure ascribed to peptide **III** by Zagranichny et al. (*15*).

DISCUSSION

In 2004, Zagranichny and co-workers reported the isolation and characterization of chromopeptides derived from zFP538 (*15*). Enzymatic digestion of zFP538 gave three chromopeptides, designated peptides **I**−**III**. Peptide **I** corresponds to an immature GFP-like chromophore, whereas peptides **II** and **III** are derived from the mature yellow chromophore. Peptide **II** at pH 3.0 and 8.0 possesses absorption maxima at 412 and 512 nm with weak fluorescence at 538 and 579 nm, respectively. Peptide **III** demonstrates more complex spectral behavior. At pH 3.0, its absorption maximum is at 420 nm. Upon basification, an intermediate form absorbing at 529 nm appears and then quickly

Table 2: Spectral Properties of Synthetic Compounds **5**−**7** Compared to zFP538-Derived Chromopeptides **II** and **III** (data from ref *15*)

	absorbance (nm)		excitation (nm)		emission (nm)	
compound	acid	base	acid	base	acid	base
6 (peptide **II**)	397 (412)	505 (512)	405 (412)	510 (512)	530 (538)	579 (579)
5 (peptide **III**, initial and intermediate)	417 (420)	530 (529)	419	530 (528)	545	597 (595)
7 (peptide **III**, final)		468 (470)		470 (475)		538 (538)

transforms into a form absorbing at 470 nm. These species produce weak fluorescence at 595 and 538 nm, respectively.

On the basis of optical spectra and incomplete NMR data, the following structures were ascribed. Peptide **II** was stated to be a linear aminoketone identical to our structure **5**. An unusual and apparently highly unstable structure **8**, an unsubstituted aminoketimine (Figure 2), was attributed to peptide **III**, and it was thought to represent the chromophore structure within the native zFP538 protein. In contrast, X-ray crystallographic studies of zFP538 revealed a tricyclic chromophore comprising a six-membered cyclic imine **7** (*16*, *17*).

Our work resolves an apparent conflict between biochemical and crystallographic data. We found that the spectral behaviors of peptides **II** and **III** from ref *15* are virtually identical to those of compounds **5**−**7** in terms of a close coincidence of absorbance positions and fluorescence maxima as well as the characteristic appearance of the intermediate red-shifted form after basification of both peptide **III** and compound **5** (Table 2). We suggest the following alternative interpretation of the experimental results reported in ref *15*. During the course of zFP538 protein denaturation, enzymatic digestion, and purification procedures, the native tricyclic chromophore (analogous to **7**) first undergoes hydrolysis to its linear form (analogous to **5**) to give peptide **III**. In a stronger acid, it further converts to peptide **II**, possessing a six-membered heterocycle (analogous to **6**). Indeed, the amount of peptide **II** greatly increased as more acidic conditions were used in the denaturation and isolation procedures (*15*). The absence of key signals in the two-dimensional NMR spectra of the chromopeptides led to the incorrect structural interpretation of zFP538 chromophore behavior.

In this work, we describe for the first time an unusual isomerization of a 2-acylimidazolone to a 2,6-diketopiperazine derivative (conversion of **5** to **6**). Interestingly, **6** contains the 2,6-diketopiperazine skeleton that occurs in flutimide and related metabolites from several fungus species (*20*−*22*). The described synthesis of 2-acylimidazolones (*12*), followed by spontaneous, acid-induced conversion to 2,6-diketopiperazines, could represent a novel and efficient synthetic pathway to flutimide analogues. These analogues could be potent antiviral and antimicrobial drug candidates (*20*). Notably, a 2-acylimidazolone-based chromophore can be found in natural proteins, namely, in purple chromoprotein asFP595 (*11*, *12*, *23*). Thus, we can propose that isomerizations analogous to the conversion of **5** to **6** may occur in some native GFP-like proteins.

Our study supports the cyclic imine chromophore structure within the native zFP538 postulated in the crystallographic works (*16*, *17*) and provides an explanation for experimental results obtained in biochemical characterization of zFP538-derived chromopeptides (*15*). The zFP538 chromophore has proven to be a complex system with intriguing chemical and spectral behavior which has led to discrepancies in the interpretation of its structure.

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