Synthesis and Characterization of Model Compounds for the Neutral Green Fluorescent Protein Chromophore

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Abstract: Two new derivatives of the green fluorescent protein (GFP) chromophore each containing a charged substituent (CH2CH2NH3+ and CH2CH2NMe3+, respectively) on the imidazolone ring have been prepared. These compounds are model systems for the neutral GFP chromophore with an unprotonated phenol moiety. The ammonium groups allow for electrospray ionization and the possibility to study the optical properties of the isolated chromophores in the gas phase. A derivative containing a CH2CH2OH substituent arm was also prepared.

Key words: amides, amines, chromophores, heterocycles, phenols

The green fluorescent protein (GFP) is an important phototoxic protein that is widely employed in molecular and cell biology owing to its fluorescent properties.1 The chromophore moiety sits at the center of a β-barrel that consists of 238 amino acids in a single chain. The absorption spectrum of GFP shows two maxima, one at 479 nm which is ascribed to the excitation of a deprotonated (phenolate) chromophore and a main peak at 397 nm which supposedly correlates to a protonated (neutral phenol) chromophore.1 Yet, different mutants show spectral behavior that cannot be explained by ionization of the phenol group alone suggesting other ionizable sites in the immediate surroundings of the chromophore.2 In order to elucidate how the absorption maxima are perturbed by the protein environment, the gas-phase absorption properties of the isolated chromophore in its neutral charge state (i.e., as a phenol) was recently investigated by state-of-the-art experiments in a heavy-ion storage ring.3 Comparison between gas-phase and protein absorption data provides directly the extent to which the protein tunes the chromophore properties, while one would have to correct for solvent effects when comparing protein data to solution data. Compound 1 was chosen as a suitable model system for the neutral chromophore. In order for the chromophore to be transferred to the gas phase by electrospray ionization, an ammonium group was attached as a so-called spectator group. Thus, the chromophore moiety is still neutral while the molecule as a whole is charged. In the absence of a charged spectator group, the chromophore moiety itself would become protonated or ionized under the conditions of electrospray ionization. Yet, the spectator group may nevertheless perturb the optical properties of the chromophore. Thus, we have recently investigated the strong influence which a charged spectator group exerts on the neutral retinal Schiff base chromophore.4 In compound 1, one of the ammonium hydrogen atoms can in fact form a hydrogen bond to the imidazole carbonyl oxygen, which is likely to disturb the optical properties. For this reason, we have designed a new and better model system for the neutral chromophore, namely compound 2, in which the three ammonium hydrogen atoms are substituted by three methyl groups (Figure 1). Here we present the synthesis of these two model systems, the chloride salt 1 and the iodide salt 2, and absorption properties in solution. Moreover, we have prepared a model system containing CH2CH2OH as R group.

Figure 1  GFP chromophore and model compounds

Our synthesis starts from 4-[(Z)-4-acetoxybenzylidene]-2-methylazol-5(4H)-one (3) (Scheme 1), which was prepared from N-acetylglycine and 4-hydroxybenzaldehyde.5 Conversion of the oxazol-5(4H)-one ring into an imidazol-5(4H)-one ring has previously been accomplished by treatment with an amine in the presence of K2CO3.6 In a modified procedure, we first treated 3 with ethylenediamine and isolated the ring-opened diamide 4. Moreover, deacetylation had occurred under these condi-
tions to afford the unprotected phenol group. The isolated product contained minor impurities that could not be removed at this stage as purification was limited to repeated dissolution/precipitation steps. Formation of the imidazolone ring was next accomplished by treatment with K₂CO₃ in refluxing ethanol. Subsequent acidification with hydrochloric acid resulted in protonation of both the phenolate and amino groups to provide the product as its chloride salt 1 (precipitates upon addition of diethyl ether), together with traces of the E-isomer. Prolonged exposure to acidic conditions was observed to result in an increase of E/Z ratio. As the workup involved tedious removal of the coprecipitated KCl, we designed another route to obtain the salt 1 in pure form. Thus, we found that simply heating compound 4 in ethanol containing molecular sieves (4 Å) resulted in ring-closure to the imidazolone 5. Upon treatment of this compound with HCl, the pure chloride salt 1 precipitated.

Scheme 1

Next, compound 3 was treated with N,N-dimethylethylendiamine to provide the ring-opened diamide 6 in which deacetylation of the phenol has also occurred. Ring-closure was accomplished by treatment with K₂CO₃ in refluxing EtOH, affording the imidazolone 7, which was purified by column chromatographic workup. This compound was then methylated at room temperature with methyl iodide in dichloromethane. The iodide salt 2 conveniently precipitated in analytically pure form from the solution (Scheme 2). ¹H NMR spectroscopy confirmed that methylation had only occurred at the amine unit.

Finally, we obtained compound 8 in a one-pot reaction by simply refluxing compound 3 overnight with ethanol-amine in ethanol (Scheme 3). This compound may be interesting to study as phenolate ion in the gas phase in order to investigate the influence of hydrogen bonding between the hydroxy group and the carbonyl oxygen.

Compounds 1 and 2 exhibit similar absorption maxima in H₂O, λ_max = 368 and 369 nm, respectively, as well as in MeOH, λ_max = 370 and 372 nm, respectively. These values are similar to those observed for related model systems devoid of the ammonium arm, yet blue-shifted relative to the value of 397 nm observed for the protein. Moreover, we studied the absorption behavior in CH₂Cl₂ containing 0.4% DMSO (Figure 2). The neutral amine 5 and the ammonium salt 2 absorb at the same maximum (λ_max = 373 nm) in this medium. Addition of 1 equivalent of HBF₄ to the amine causes a slight broadening of the spectrum. However, addition of 10 equivalents of HBF₄ to the amine causes a significant red-shift in the absorption maximum to 405 nm as now not only the amine group but also the imidazolone nitrogen should be protonated. Previous studies on the protonated 4-hydroxybenzylidene-imidazolone have revealed an absorption maximum at 395 nm in CH₂Cl₂ and at 383 nm in DMSO. The observed red-shift to λ_max 405 nm after protonation of 5 may reflect the influence exerted by a hydrogen bond between the carbonyl oxygen and the ammonium group. This intramolecular hydrogen bond formation is expected at least in the gas phase. The protonated GFP chromophore was previously found to absorb at 406 nm in the gas phase and one would accordingly expect a red-shift from this value for diprotonated 5 in the gas phase. Compound 2 lacks the possibility for forming a hydrogen bond to the carbonyl oxygen, and we expect its gas-phase absorption spectrum to be blue-shifted relative to that of compound 1 (for which λ_max = 415 nm in the gas phase). Gas-phase investigations are currently being undertaken. These nontrivial experiments will allow us to elucidate spectroscopic changes induced by forming a hydrogen bond without in-
terfering solvent effects and to validate calculational methods.

In conclusion, we have obtained several new model systems of the GFP chromophore that are suitable for gas-phase experiments for elucidating the influence of hydrogen bonding on the intrinsic optical properties.

Chemicals were purchased from Aldrich and used as received. TLC was carried out using aluminum sheets pre-coated with silica gel 60F (Merck 5554). The plates were inspected under UV light. Melting points were measured on a Reichert melting point apparatus and are uncorrected. 1H NMR (300 MHz) and 13C NMR (75 MHz) spectra were recorded at 25 °C on a Varian instrument, using the residual solvent (DMSO; δ = 2.5; δc = 39.5) as the internal standard. All chemical shifts are quoted on the 0 scale (ppm), and all coupling constants (J) are expressed in Hz. Fast atom bombardment (FAB) mass spectra were obtained on a Jeol JMS-HX 110 Tandem Mass spectrometer in the positive ion mode using 3-nitrobenzyl alcohol as matrix.

Figure 2 UV-Vis Absorption spectra in CH2Cl2 containing 0.4% DMSO

(Z)-1-(2-Aminoethyl)-4-(4-hydroxybenzylidene)-2-methyl-1H-imidazol-5(4H)-one Hydrochloride (1)

Method 1: Compound 4 (135 mg, 0.51 mmol) was dissolved in absolute EtOH (10 mL), and then K2CO3 (260 mg, 1.88 mmol) was added. After stirring for 16 h at reflux, the mixture was subjected to centrifugation in order to remove the K2CO3 precipitate and then acidified by dropwise addition ofaq 4 M HCl until acidic. Precipitation by addition of Et2O (90 mL) followed by precipitation by addition of Et2O (100 mL). The solvent was decanted off, and the precipitate was washed with Et2O (50 mL) to give, after drying under vacuum (oil pump), 1 (27 mg, ca. 100%) as a yellow powder with traces of EtOH and KCl (116 mg, <80%). Removal of KCl was attempted by redissolving in absolute EtOH, but this was unsuccessful. The compound was instead obtained pure by Method 2.

Method 2: Compound 5 (23 mg, 0.09 mmol) was dissolved in EtOH (10 mL), whereuponaq 4 M HCl was added until pH was reached (2 drops), followed by precipitation by addition of Et2O (90 mL). The solvent was decanted off, and the precipitate was washed with Et2O (50 mL) to give, after drying under vacuum (oil pump), 1 (27 mg, ca. 100%) as a yellow powder with traces of EtOH and Et2O.


(Z)-1-(2-Aminoethyl)-4-(4-hydroxybenzylidene)-2-methyl-1H-imidazol-5(4H)-one (6)

Compound 3 (325 mg, 1.5 mmol) was dissolved in absolute EtOH (25 mL) and then ethylenediamine (2.5 mL, 37.4 mmol) was added. After stirring for 15 min at r.t., the mixture was evaporated in vacuo at 30 °C. The orange-red oil was dissolved in hot EtOH (10 mL). A solid was precipitated by the addition of Et2O (100 mL). This procedure was repeated three times followed by drying the solid under vacuum for 2 h. Compound 4 was obtained as a yellow powder containing residual EtOH and minor impurities (520 mg, ca. 90%); mp 130 °C (dec.).

IR (KBr): 3266 (s), 2683 (m), 2594 (m), 1656 (s), 1604 (s), 1510 (s), 1371 (s), 1274 (s), 1208 (s), 1172 (s), 1110 (m), 977 (m), 916 (m), 833 (s), 720 (m), 632 (m), 603 (m), 534 cm⁻¹ (s).

1H NMR (300 MHz, DMSO-d6): δ = 8.42 (br s, 3 H, NH3), 8.07 (d, J = 8.2 Hz, 2 H, Ar), 7.00 (s, 1 H, CH=), 6.88 (d, J = 8.2 Hz, 2 H, Ar), 3.89 (m, 2 H, CH2), 3.06 (m, 2 H, CH2), 2.48 (s, 3 H, CH3).

13C NMR (75 MHz, DMSO-d6): δ = 169.6, 165.5, 158.7, 131.3, 128.2, 127.0, 124.7, 115.6, 42.3, 41.0, 23.0.

vent was decanted off, and this procedure was performed three times, whereupon 6 was isolated as a yellow solid (2.16 g, 82%). This product was used in the next step without further purification; mp 90 °C.

$^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 9.26$ (s, 1 H, ArOH), 7.38 (d, $J = 8.5$ Hz, 2 H, Ar), 7.01 (s, 1 H, CH=), 6.77 (d, $J = 8.5$ Hz, 2 H, Ar), 3.20 (q, $J = 6.8$ Hz, 2 H, NCH$_2$), 2.30 (t, $J = 6.8$ Hz, 2 H, NCH$_2$), 1.25 (s, 6 H, N(CH$_3$)$_2$), 1.99 (s, 3 H, CONHCH$_3$).

HRMS-FAB: $m/z$ [M + H]$^+$ calcd for C$_{4}H$_$_3$N$_2$O$_5$: 292.1661; found: 292.1663.

(Z)-1-[2-(Dimethylamino)ethyl]-4-[4-(hydroxybenzylidene)-2-methyl-1H-imidazol-5-yl]-one (7)

Compound 6 (2.835 g, 9.73 mmol) was dissolved in absolute EtOH (150 mL), and then K$_2$CO$_3$ (4.5 g, 33 mmol) was added. After stirring for 22 h at reflux, the mixture was filtered and concentrated in vacuo to give a red oil. The residue was subjected to column chromatography (SiO$_2$, 10% MeOH in CH$_2$Cl$_2$) to give the product as a yellow solid (1.23 g, 46%); mp 218 °C (dec.).

IR (KBr): 3094 (s), 3012 (s), 1690 (s), 1651 (s), 1603 (s), 1580 (s), 1556 (m), 1515 (m), 1454 (s), 1415 (s), 1365 (m), 1312 (w), 1302 (w), 1285 (s), 1255 (m), 1235 (m), 1172 (s), 1144 (s), 1105 (w), 1066 (m), 1035 (w), 1019 (w), 974 (w), 922 (w), 852 (w), 834 (m), 765 (w), 723 (w), 663 (w), 633 (w), 600 (w), 535 (w), 513 (w), 486 (m), 442 cm$^{-1}$ (w).

$^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 10.07$ (br s, 1 H, ArOH), 8.08 (d, $J = 8.6$ Hz, 2 H, Ar), 6.93 (s, 1 H, CH=), 6.83 (d, $J = 8.6$ Hz, 2 H, Ar), 4.94 (t, $J = 5.6$ Hz, 1 H, OH), 3.61 (t, $J = 5.3$ Hz, 2 H, CH$_2$), 3.51 (m, 2 H, CH$_2$), 1.79 (s, 3 H, CH$_3$).

$^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 169.9, 162.5, 159.5, 136.2, 134.0, 125.4, 125.3, 115.7, 58.7, 42.8, 15.5.

HRMS-FAB: $m/z$ [M + H]$^+$ calcd for C$_{15}$H$_{20}$N$_3$O$_5$: 247.1083; found: 247.1095.

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