Excited State Dynamics of Brightly Fluorescent Second Generation Epicocconone Analogues

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Supporting Information

ABSTRACT: The natural product epicocconone, owing to its unique fluorescence properties, has been developed into a range of products used in biotechnology, especially proteomics. However, its weak green fluorescence in its native state, while advantageous for proteomics applications, is a disadvantage in other applications that require two-color readouts. Here we report the photophysical characterization of two brightly fluorescent analogues of epicocconone. These analogues, with naphthyl or pyridyl groups replacing the heptatriene chain, resulted in bright fluorescence in both the native state and the long Stokes shifted enamine. Time-resolved fluorescence studies and DFT calculations were carried out to understand the excited state processes involved in fluorescence. Results showed the p-chloro group on the pyridyl is responsible for the high fluorescence of the native fluorophore. The application of one of these compounds for staining electrophoresis gels is exemplified.

1. INTRODUCTION

Fluorescence based biological assays require large extinction coefficients and quantum yields in order to achieve the best possible sensitivity. In addition, a large Stokes shift is desirable, to avoid interference from Rayleigh scattering and minimize inner filter effects.1 Design and elucidation of the photophysics of new fluorescent probes is an area which continues to be of considerable interest, as the discovery of new fluorescent frameworks has the potential of broadening the range of utility of application of small molecules in biology.2−6 One approach, in this context, is the discovery of new fluorophores from nature.7 Epicocconone (1) is an example of a cell-permeable latent fluorophore isolated from the fungus Epicoccum nigrum using bioassay directed procedures.8 It fluoresces feebly at 530−610 nm in aqueous solutions but reacts reversibly with lysine residues of proteins to yield highly emissive enamines (λem = 610 nm)9,10 that are characterized by high molar absorptivity (ε = 104), strong orange-red fluorescence (ε = 100 nm Stokes shift), and low cytotoxicity.11 These properties have led to a range of commercial applications including cell tracking and two-color staining11−13 when multiplexed with other fluorescent probes,14 detection of proteins in 2D gel electrophoresis (Deep Purple Total Protein Stain; GE Healthcare),15 protein quantification in solution (FluoroProfile; Sigma-Aldrich),16 live cell imaging (LavaCell; Fluorotechnics), as well as in kinetic assays for enzymatic digestion.17

In our earlier studies with epicocconone and its analogues, we elucidated the nonradiative pathways responsible for the poor fluorescence of 1 compared to 4. We found that the green emission of 1 is enhanced in the presence of surfactants such as SDS, CTAB, and Triton X100 and with α- and β-cyclodextrins due to the rigidity provided by the micelles and cyclodextrin cavity, restricting photoisomerization and thus enhancing fluorescence.9,18 In addition, by comparing analogues with an enolizable and unenolizable β-diketone, it was established that photoisomerization of the heptatriene side chain, not the tautomerism of the β-diketone, was the major nonradiative process in 1.19−21 Thus, analogues where the triene side chain of 1 was replaced by the isosteric phenyl or anisyl residue possess significantly higher quantum yields.20,21 The corresponding butylamine adducts showed similar reversible enamine formation with butylamine, as was evident from the red-shifted absorption and emission spectra. However, unlike 1, they did not show the characteristic increase in quantum yield upon reaction with amines, suggesting that heptatriene photoisomerization was not a significant relaxation pathway for the enamine of epicocconone (4). It was also found that intramolecular H-bonding of the enolized β-diketone helped stabilize the excited state of the butylamine adducts.20 Photobleaching, a major issue with 1,22 can be attributed to photoisomerization of the heptatriene chain and/or photo-oxidation of the alcohol. By replacing the triene with more...
stable aromatic rings and the diol of 4 with a tertiary alcohol (as in 5 and 6), it was envisaged that photobleaching and stability would be increased.

The present study is focused on the excited state dynamics of two new epicocconone analogues. In these compounds, the isomerizable heptatriene chain of 1 (Scheme 1, red) is replaced with a naphthyl (2) or pyridyl (3). In addition, the oxidizable CH₂OH group (Scheme 1, blue) is replaced by a gem-dimethyl group. These compounds all reacted with amines, such as butylamine (Scheme 1, magenta), to form enamines (5, 6) in a similar fashion to 1.

2. RESULTS AND DISCUSSION

Epicocconone (1) and its butylamine adduct (4), in acetonitrile, showed absorption maxima at 435 and 520 nm, respectively, while their emission maxima appeared at 535 and 615 nm, respectively.¹⁹ The absorption maximum at 415 nm of compounds 2 and 3 was blue-shifted relative to 1 by 20 nm in acetonitrile. In contrast, the butylamine adducts (5 and 6) absorbed at 515 and 520 nm, respectively (Figure 1), similar to 1. The emission maxima were found to be very similar to 1: at 530 nm for 2, at 515 nm for 3, at 610 nm for 5, and at 615 nm for 6, respectively, in acetonitrile (Figure 1). These data suggested that the side chain (red in Scheme 1) has little effect on the chromophore.

In spite of having very similar absorption and emission wavelengths to 1, the fluorescence quantum yields of 2 and 3 were quite different (Figure 2, Table 1) and must be a result of the side chains. This could be ascribed to the absence of the nonradiative decay pathways resulting from photoisomerization in the heptatriene chain of 1. The quantum yields of 2 and 3 were found to be 15× and 21× higher than that of 1 in acetonitrile, respectively, while quantum yields of 2 and 3 were 3× and 5× higher than that of 1 in tert-butanol and 10× and

Table 1. Molar Extinction Coefficients and Quantum Yields of Compounds 1–3 in Different Solvents and of Compounds 4–6 in Acetonitrile

<table>
<thead>
<tr>
<th>Solvent</th>
<th>(\varepsilon) (\text{Lit}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1})</th>
<th>(\phi_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>acetonitrile</strong></td>
<td>1² ²⁵ ³² ³⁵</td>
<td>³⁶ ³⁷ ³⁸ ³⁹</td>
</tr>
<tr>
<td>water</td>
<td>¹² ¹³ ¹⁴ ¹⁵</td>
<td>¹⁶ ¹⁷ ¹⁸ ¹⁹</td>
</tr>
<tr>
<td>tert-butanol</td>
<td>²⁰ ²¹ ²² ²³</td>
<td>²⁴ ²⁵ ²⁶ ²⁷</td>
</tr>
</tbody>
</table>

**Notes:**

- For compounds 1–3, \(\varepsilon\) values are given at 435 nm for 1, 415 nm for 2, and 520 nm for 3.
- For compounds 4–6, \(\phi_f\) values are given for 4 at 355 nm, 5 at 415 nm, and 6 at 515 nm.

²⁰× higher in 20 mM SDS solution. In water, quantum yields were found to be 4× and 5× higher for 2 and 3, respectively, than 1 (Table 1, Figure 2).
Upon formation of the butylamine adduct of 1 (4), the quantum yield increased 5.6× in acetonitrile compared to 1, but the quantum yield of 5 decreased almost 1.7× with respect to 2, while 6 showed almost a 3× lower quantum yield with respect to 3 (Table 1, Figure 2). Large Stokes shifts were observed in all cases, in accordance with putative enamine formation. All the enamines (4–6) actually had quite similar quantum yields, absorption spectra, and Stokes shifts (Figure 2). This observation confirmed that the photophysics/excited state dynamics of the enamines is also dominated by the bicyclic core (Scheme 1; in black) and that the side chain has little if any effect.

The high quantum yield of 3 (>55% cf. epicocconone; <3%) was accompanied by a 2× higher molar absorptivity (Figure 2) in acetonitrile (Table 1). This represents a dramatic improvement in photophysical characteristics (brightness) over 1. However, the butylamine adduct (6) did not maintain these impressive characteristics, with the quantum yield dropping to 17%, quite similar to 4. To better understand these potentially useful photophysical characteristics, we undertook nanosecond and femtosecond decay studies.

The fluorescence decays in the nanosecond time regime were in line with the corresponding quantum yields of 2 and 3 in different solvents. Decays were found to be a single exponential for 2 in acetonitrile and in tert-butanol, while, in water, a bimodal decay was observed (Figure 3A, Table 2). In the case of 3, similar observations were made (Figure 3B, Table 2). A slower decay of 2 in acetonitrile than in tert-butanol was in accordance with the observed higher quantum yield of 2 in acetonitrile. Similar observations were recorded for 3. A dominant faster component in water for 2 was in agreement with its lower quantum yield in this solvent. This faster component disappeared in 20 mM SDS. However, there was an apparent anomaly in water. The dominant decay with a τ value of 1.1 ns (Table 2) implied that 3 should have a higher quantum yield in water than in organic solvent, which was not the case. This observation suggested there might be an ultrafast relaxation pathway that is missed by time-correlated single-photon counting experiments on the nanosecond time scale.

The enamine of 3 (5) exhibited a bimodal decay and had a faster component similar to 2 in water but with a significantly lower relative contribution, which explained the higher quantum yield of 5 over 2 in water. The fluorescence decay of 6, on the other hand, was a single exponential with a lifetime comparable with that of 3 in tert-butanol and slightly lower than the average lifetime of 3 in water. Again, the trend in quantum yields did not match with the observed trend in average lifetimes calculated from the nanosecond time-resolved fluorescence experiments of 3 or 6 (Table 2) where 6 showed a much lower quantum yield than 3 in tert-butanol yet had a 4× higher quantum yield than 3 in water (Table 1).

In order to rationalize this anomaly, the dynamics were studied in the femtosecond–picosecond time domain (Figures 4 and 5). In acetonitrile, 2 had the slowest decay (Figure 4A and C, Table 3), but the corresponding enamine (5) showed an ultrafast component of 2–3 ps that made a 20% contribution to the decay. The slower (ns) component was found to be similar to that for 2, and thus, the ultrafast component could be held responsible for the lower quantum yield of 5 compared to 2 in acetonitrile.

In tert-butanol (Figure 4B and D, Table 3), the ultrafast component was not present and could not account for the similar quantum yields of 2 in tert-butanol and 5 in acetonitrile.

As stated above, 2 and 3 possess different excited state dynamics compared to their butylamine adducts (5 and 6) and the aryl group does not appear to participate in the excited state dynamics of the chromophore. However, a sub-picosecond component of ~600 fs is a significant contributor to the overall decay for 2 in water (Figure 4B and D, Table 3). It was found that the photophysics/excited state dynamics in all cases, in accordance with putative enamine formation. All the enamines (4–6) actually had quite similar quantum yields, absorption spectra, and Stokes shifts (Figure 2). This observation confirmed that the photophysics/ excited state dynamics of the enamines is also dominated by the bicyclic core (Scheme 1; in black) and that the side chain has little if any effect.
that the decay of 2 in water was complete within 30 ps (Figure 4B). The origin of this fast decay is yet to be understood clearly, but our earlier experiments with different analogues of 1 indicated that intramolecular H-bonding of the diketone is affected by the intermolecular H-bonding with water and destabilization of the excited state.20 This may be the case with these compounds as well.

In 20 mM SDS, the ultrafast decay of 2 was quite similar to that in tert-butanol (Figure 4B and D), which might be expected considering the similarities in viscosity and dielectric constant of these two solvents.

Decay of 3 was found to be much faster than 2 in acetonitrile primarily due to an ultrafast component with a 3 ps lifetime (Figures 4A and C and 5A and C, Table 3). However, the quantum yield of 3 was higher than that of 2 (Figure 2, Table 1). This anomaly could not be explained from the temporal parameters but could arise from an internal charge transfer (ICT), for example, as observed in earlier studies.20 Compound 6 had similar lifetimes and respective contributions to 5, and a comparison between the temporal parameters of the two accounted for the slightly lower quantum yield of 6 in acetonitrile (Figures 4 and 5, Table 3). The comparison of quantum yields and the lifetime parameters of 5 and 6 with 2 and 3 supports our hypothesis of distinct excited state dynamics of the amine adducts compared to the native compounds. It is also clear that the side chain of the diketo moiety does not contribute to the excited state dynamics to any major degree with the possible exception of the anomaly noted above for 3.

In water and tert-butanol, the fluorescence decay for 3 was found to be slower than 2, which falls in line with a higher quantum yield of 3 over 2 in these solvents. In 20 mM SDS, 3

<table>
<thead>
<tr>
<th>solvent</th>
<th>τ1 (ps)</th>
<th>a1</th>
<th>τ2 (ps)</th>
<th>a2</th>
<th>τ3 (ps)</th>
<th>a3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3CN</td>
<td>45</td>
<td>0.15</td>
<td>1120</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (CH3CN)</td>
<td>2.3</td>
<td>0.20</td>
<td>65</td>
<td>0.15</td>
<td>1050</td>
<td>0.65</td>
</tr>
<tr>
<td>water</td>
<td>0.6</td>
<td>0.30</td>
<td>5</td>
<td>0.50</td>
<td>30</td>
<td>0.20</td>
</tr>
<tr>
<td>20 mM SDS</td>
<td>140</td>
<td>0.15</td>
<td>810</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tert-butanol</td>
<td>15</td>
<td>0.15</td>
<td>850</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH3CN</td>
<td>3.2</td>
<td>0.45</td>
<td>40</td>
<td>0.20</td>
<td>970</td>
<td>0.35</td>
</tr>
<tr>
<td>6 (CH3CN)</td>
<td>2.5</td>
<td>0.15</td>
<td>60</td>
<td>0.10</td>
<td>840</td>
<td>0.75</td>
</tr>
<tr>
<td>water</td>
<td>2.4</td>
<td>0.45</td>
<td>30</td>
<td>0.30</td>
<td>730</td>
<td>0.25</td>
</tr>
<tr>
<td>20 mM SDS</td>
<td>2.0</td>
<td>−0.15</td>
<td>3300</td>
<td>1.15</td>
<td></td>
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<tr>
<td>tert-butanol</td>
<td>33</td>
<td>0.10</td>
<td>1100</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aτ1 is the fluorescence lifetime and a, the relative amplitude.
displayed a rise time of 2 ps, the magnitude of which was almost the same as that of the ultrafast decay found for 3 in water (Figure 5B). The suppression of the ultrafast deactivation process for 3 leads to an increase in the fluorescence lifetime to 3.3 ns (Table 3) in SDS solution and explains the higher quantum yield of 3.

Both 2 and 3 exhibited slower decays in all the solvents studied (Figure 6) with respect to 1. In particular, the ultrafast decay in water, characteristic of epicocconone, was absent in 3 altogether (Figure 6A). This explains the high quantum yield of 3 in water compared to 1. However, the ultrafast dynamics alone is not adequate to explain the higher quantum yield of 3 over 2 in acetonitrile. In polar solvents like water and tert-butanol, though, in the relative quantum yields of 1, 2 and 3 were in line with their picosecond lifetimes.

These data can be rationalized further by comparison with other fluorophores with related functional groups. For example, for 1-aminofluorenone, where there is an intramolecular H-bonding network, there is a 7.5–9 ps excited state intramolecular proton transfer (ESIPT). Polar protic solvents introduce additional solute charge reorganization and solvent reorientation mechanisms that can perturb the excited state.\textsuperscript{23,24} The role of hydrogen bonding networks in ESIPT processes has also been established in N,N-dimethylanilino-1,3-diketone and curcumin; both contain a \( \beta \)-keto-enol group similar to 1–6 and a propensity for charge transfer mediated ESIPT, that is on the 4–5 ps time scale.\textsuperscript{25,26} From these studies, and comparing with our data, it appears that the shorter components (\( \tau_1 \)) in the 2–3 ps regime for 3 in acetonitrile and 20 mM SDS (Table 3) can be associated with its internal charge transfer, while in water this ubiquitous short component in 2 and 3 (0.6/2 ps) is most likely due to intermolecular proton transfer with solvent. Desolvation of water, which takes place in the sub-picosecond time scale,\textsuperscript{27} is unlikely but cannot be ruled out. The slower process (\( \tau_2 \)), which ranges from 15 to 50 ps, can then be ascribed to the conformational relaxation of the intramolecular \( \beta \)-diketo H-bond in aprotic solvents or intermolecular H-bonded species with polar protic solvents.

The longest lifetime decays (\( \tau_3 \)) can be assigned to the emissive \( S_1 \) state.

The difference in fluorescence quantum yields between the two analogues in different solvents can also be explained by the interplay between radiative and nonradiative decay rates. The latter, calculated from the average lifetime (\( \tau = \sum a_i \tau_i \) from upconversion experiments and \( \phi_i \) values, according to the relations \( k_R = \phi_i/\langle \tau \rangle \) and \( k_{NR} = (1 - \phi_i)/\langle \tau \rangle \), are listed in Table 4. In acetonitrile, compound 3 had a 4\times higher radiative and 2\times higher nonradiative rate constant than 2. The higher quantum yield of 3 in acetonitrile (Figure 2, Table 1) could thus be rationalized by the higher radiative rate of 3. Similarly, in water, the nonradiative rate constant was \( \sim 19 \times \) faster for 2 than for 3, while the radiative rate constant for 2 is \( \sim 14 \times \) times faster than that for 3 (Table 4), resulting in the dominance of nonradiative decays leading to a lower quantum yield for 2 (Table 1). Depression of radiative as well as nonradiative decays was observed for both 2 and 3 in 20 mM SDS with respect to that in water. This effect was strongest for the nonradiative decays and was in line with these molecules being incorporated inside the SDS micelles, that have a higher viscosity and lower dielectric constant, resulting in higher quantum yields.

Titration of 2 and 3 with SDS (Figure 7) showed an increase in quantum yield above the critical micelle concentration (CMC), confirming that these molecules are readily incorporated into micelles. The increase in quantum yield was also reflected in an increase in lifetimes with increasing SDS, indicating that the excited state is also more stable inside the hydrophobic and/or viscous environment of the micelle. The appearance of a 2 ps rise time for 3 in 20 mM SDS, which was similar to the ultrafast lifetime found for 3 in water, suggests that the ICT from the chloro-pyridyl group to the epicocconone nucleus is unaffected by the hydrophobic environment of SDS micelles.

In the case of 3, a charge transfer from the pyridyl group could play a stabilizing role in acetonitrile by providing more of a double bond character to the C43–C28 bond (Scheme 2) while keeping the intramolecular H-bonding of the \( \beta \)-diketone

![Table 4. Average Lifetimes and Radiative and Non-Radiative Rate Constants for Compounds 2 and 3 and Compounds 5 and 6](image)
Figure 7. Quantum yield of (A) 2 and (B) 3 in response to SDS concentration. The CMC for SDS is 8.2 mM.

Scheme 2. Resonance Hybrids of 3 for HOMO (left) and LUMO (right) Orbitals Below

system intact. This would lead to a higher radiative rate in acetonitrile where intramolecular H-bonding is not affected and explains the higher quantum yield of 3 in acetonitrile over tert-butanol.

To test this hypothesis, high level DFT calculations (RI-DFT-D3//BP86/TZVPP) using a continuum solvent model (COSMO) for acetonitrile were carried out on 3. The HOMO and the LUMO of the ground state supported an ICT where the HOMO does not involve the pyridine group but the LUMO does (Scheme 2). Furthermore, bond lengths were measured for the ground state (S0) and the geometry optimized singlet first excited state (S1) (Tables S4 and S5, Supporting Information). It was found that removing either the chlorine or nitrogen had little effect on the bond lengths in S0 (Δr ≤ 0.3 pm). However, in S1, the C43–C28 bond length was ~4.8 pm shorter for 3 compared to 3 with no chlorine. In compensation, C26–C28 is longer by ~4.2 pm, suggesting that C43–C28 has more of a double bond character in the excited state when a chlorine is present (Scheme 2) that would inhibit rotation around C43–C28. This could explain the higher quantum yield of 3 in all solvents compared to 1 (Figure 2). Replacement of the nitrogen with CH had less of an effect.

A similar inference can be drawn between 5 and 6 (Table 4), where a somewhat higher quantum yield for 5 was found to be a result of the interplay between the higher radiative rate constant and lower nonradiative rate constant for 5. Similar radiative and nonradiative rates for 5 and 6 suggest that the excited state dynamics of the butylamine adducts is not a function of the substituent on the acyl side chain but depends on the heteronuclear ring system that is ubiquitous in all epicocconone analogues.

The increase in fluorescence associated with increasing SDS and the marked shift in fluorescence emission upon enamine formation suggested that these compounds could be used to stain proteins in SDS polyacrylamide electrophoresis (PAGE). Comparison of the protein staining ability in 1D protein gel electrophoresis (Figure 8) shows comparable results between 1 and 3. Specifically, both dyes stained α-lactoglobulin (lowest band) the best and soybean trypsin inhibitor (second last band) the least. The limits of detection for the six proteins are quite similar and in the range 0.4–1.5 ng/band. However, 3 was not as sensitive as the natural product 1 for 1D PAGE staining, but this may be due to other factors than just photophysical characteristics. For example, in the gel after fixation, there is a high concentration of SDS around the proteins and the pH is around 2.5, which may suit 1 more than 3. In addition, the higher background of gels stained with 3 is a result of 3 being an order of magnitude more fluorescent than 1 (Table 1).

3. CONCLUSIONS

In our previous studies, we established photoisomerization of the heptatriene side chain as the major nonradiative process in epicocconone (1), resulting in low to very low quantum yields. In order to design and synthesize analogues with higher quantum yields, the heptatriene chain needed to be reengineered to eliminate photoisomerization, while the keto–enol moiety needed to be conserved in order to preserve the large Stokes shift for the butylamine adducts. This was achieved in the design and synthesis of 2 and 3 that had significantly higher fluorescence in their native states than epicocconone with quantum yields up to 56% for 3 compared to <3% for epicocconone (1) under the same conditions. The analogues retained the characteristic reaction with amines of 1 to form red fluorescent enamines. The fact that replacement of the isomerizable heptatriene with unusomerizable and bulky naphthyl and p-chloropyridyl groups increased quantum yields demonstrated the importance of photoisomerization in the nonradiative pathways for this class of fluorophores. In addition, we have demonstrated the importance of intramolecular H-bonding in the β-keto–enol group, which helps stabilize the excited states and thus leads to large increases in quantum yields in hydrophobic and viscose environments such as in cellular membranes or around proteins in SDS-PAGE. This keto–enol internal H-bond becomes less important in water, resulting in an order of magnitude lower quantum yields, which is recovered in 20 mM SDS. DFT calculations showed that, in the excited state, the chloro group of 3 was critical for stabilizing the β-keto–enol by inhibiting rotation of the pyridyl group, another potential source of nonradiative decay of the excited state.

The butylamine adducts (4–6) display similar dynamics, which is quite different from that of the native fluorophores.
(1–3). The pyridyl analogue (3) is much more fluorescent than the others, due to hyperconjugation to the Cl atom. This was particularly evident from our computational studies and is supported by experimental results presented here. These results and titrations with SDS suggested 2 and 3 could be useful synthetic alternatives to epicoconone for protein staining and as dual-stains in biotechnology, emitting strongly in the green in their native states and equally strongly in the red once conjugated to amines such as lysine residues in proteins. This is quite unlike the natural product that is essentially non-fluorescent in its native state. The pyridyl analogue (3) in particular is highly fluorescent in both the native and conjugated forms but fluoresces different colors (green and red, respectively), making this compound potentially useful in two-color or turn-on/turn-off applications.

Staining of proteins in electrophoresis gels showed comparable performance between the commercial product (Deep Purple; containing 1) and analogue 3 without protocol optimization. This study points the way to the design of improved analogues with electron donating groups that are currently underway and will be reported in due course.

4. EXPERIMENTAL SECTION

The synthesis of the compounds (2, 3) is described elsewhere.28 A solution of each compound in DMSO (1 mg/mL) was used as the stock solution for each experiment. Spectroscopy grade acetonitrile (Spectrochem, Mumbai, India) was distilled over CaH2 and the distillate passed over activated neutral alumina prior to each experiment. tert-Butanol (spectroscopy grade, Spectrochem, Mumbai, India) was used as received. n-Butylamine (Qualigens, Mumbai, India) is dried with solid KOH and then refluxed with and distilled from P2O5.

The absorption and fluorescence spectra have been recorded on a JASCO V 530 spectrophotometer and a Varian Cary Eclipse fluorimeter, respectively. The emission spectra were recorded with excitation at 400 nm with excitation and emission slit widths of 5 nm and at 500 nm excitation for the butylamine adducts. The absorbencies of the solutions were kept below 0.1 to prevent inner filter effects for steady state measurements and for picosecond–nanosecond time-resolved experiments. The absorbencies of the solutions were kept at ∼1.0 for the upconversion experiments. The absorption and emission spectra were recorded before and after the femtosecond fluorescence decays in order to verify that the samples do not photodegrade. Fluorescence quantum yields (ϕf) of the compounds in neat solutions were calculated after correction for changes in absorbance using Lucifer Yellow CH (ϕf = 0.21) as the reference.29 Fluorescence quantum yields (ϕf) of the butylamine adducts were calculated after correction for changes in absorbance using rhodamine 6G (ϕf = 0.94) as the reference.30 Time-resolved fluorescence in the picosecond–nanosecond time regime were measured using a picosecond pulsed diode laser based time-correlated single photon counting (TCSPC) instrument (IBH, United Kingdom) set at the magic angle with λex = 406 nm.31

In our femtosecond upconversion setup (FOG 100, CDP), the sample was excited at 400 nm using the second harmonic of a mode-locked Ti:sapphire laser (Tsunami, Spectra Physics) pumped by a 5 W Millennia (Spectra Physics) laser. The fundamental beam (800 nm) was frequency doubled in a nonlinear crystal (1 mm BBO, θ = 25°, ϕ = 90°). The fluorescence emitted from the sample was upconverted in a nonlinear crystal (0.5 mm BBO, θ = 25°, ϕ = 90°) using the fundamental beam as a gate pulse. The upconverted light is dispersed in a monochromator and detected using photon counting electronics. A cross-correlation function obtained using the Raman scattering from ethanol displayed a full width at half-maximum (fwhm) of 300 fs. The femtosecond fluorescence decays were fitted using a Gaussian function of the same fwhm as the excitation pulse. The fluorescence decays were recorded at the magic angle polarization with respect to the excitation pulse on a FOG 100 fluorescence optically gated upconversion spectrometer. The resolution was in appropriate multiples of the minimum step size of the instrument, i.e., 0.78 fs/step. The decays were analyzed by iterative reconvolution using a homemade program.32

Compound 3 was dissolved in DMSO (10 mg/mL) and diluted to 1 mg/mL with water. One vial of GE low molecular
weight markers (GE Healthcare; 17-0446-01, 575 μg of total protein) was diluted with 1× LDS buffer (250 μL of Invitrogen 4X LDS buffer (NP0008), 100 μL of 1 M DTT, and 650 μL of water) to achieve a final concentration of 51.2 μg/mL of soybean trypsin inhibitor (STI). The stock solution was serially diluted (2×) from 12.8 to 0.125 μg/mL (based on STI). Protein solutions (10 μL) and loading buffer (10 μL) were mixed.

12% Bis-Tris Novex NuPage (Invitrogen NP0342BOX), 1 mm thick gels, were loaded with the dilution series (20 μL of each dilution) and run in Xcell SureLock minigel systems (Invitrogen, E10001) at 150 V for approximately 65 min (buffer front just off gel) using MES buffer (50 mM MES, 50 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.3). The gels were then removed and fixed in 15% ethanol (v/v), 1% citric acid (100 mL) on a rocker for 1 h, and then with fresh fixative overnight. The next day, the fixative (100 mL) was replaced and after 1 h drained and stained with either Deep Purple Total Protein Stain (GE Healthcare, RPN6306) or 3 (50 μL of a 1 mg/mL solution in DMSO) in sodium borate buffer (50 mL; 100 mM, pH 10.9). These concentrations resulted in approximately 1 μg/mL active fluorophore in each case. After 1 h of staining, the gels were destained in 15% ethanol (100 mL) for 30 min and transferred to fixative (100 mL; 15% ethanol (v/v), 1% citric acid (w/v)) for 30 min. All gels were imaged using a Typhoon Trio (GE, 63-0055-87) using the 532 nm Nd:YAG laser, 540 PMT, 610BP30 filter, 100 μm resolution, and normal sensitivity.

Density functional theory (DFT) calculations were run using Turbomole 6.5 (Cosmologic, GmbH & Co). Theoretical ground state geometries for all conformations were calculated (RI-DFT-D3/BP86/TZVPP)33–36 in the continuum solvent model for acetanilide (COSMO).37 Ground state and singlet first excited state geometries and energies were compared using RI-DFT//BP86/TZVPP calculations in a vacuum (see the Supporting Information) using the default parameters, except a finer grid size of m358 was used throughout.

**ASSOCIATED CONTENT**

## Supporting Information

Computational results, compound coordinates, steady state spectra, and fluorescence decay data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.5b02190.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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