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Fast Excited-State Deactivation in N(5)-Ethyl-4a-hydroxyflavin Pseudobase

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ABSTRACT: We present a study of the excited-state behavior of N(5)-ethyl-4a-hydroxyflavin (Et-FIOH), a model compound for bacterial bioluminescence. Using femtosecond pump–probe spectroscopy, we found that the Et-FIOH excited state exhibits multieponential dynamics, with the dominant decay component having a 0.5 ps lifetime. Several possible mechanisms for fast excited-state decay in Et-FIOH were considered: (i) excited-state deprotonation of the –OH proton, (ii) thermal deactivation via $1_n\pi^* \rightarrow 1\pi\pi^*$ conical intersection, and (iii) excited-state release of OH$^-$ ion. These mechanisms were excluded based on transient absorption studies of two model compounds (N(5)-ethyl-4a-methoxyflavin, Et-FIOMe, and N(5)-ethyl-flavinium ion, Et-Fl$^+$) and based on the results of time-dependent density functional theory (TD-DFT) calculations of Et-FIOH excited-states. Instead, we propose that the fast decay in Et-FIOH is caused by $S_1 \rightarrow S_0$ internal conversion, initiated by the excited-state nitrogen planarization ($sp^3 \rightarrow sp^2$ hybridization change at the N(5)-atom of Et-FIOH $S_1$ state) coupled with out-of-plane distortion of the pyrimidine moiety of flavin.

INTRODUCTION

Many bacteria found in seas and oceans are known to cause bioluminescence. While isolated bacteria do not express the genes necessary for bioluminescence, high densities of bacteria produce luminescence that is detectable even by satellites. This cooperative bioluminescence by bacteria is known as quorum sensing. Due to their light-emitting properties, bacteria can form symbiosis with fish and squids. In this symbiotic relationship, the host uses bacterial luminescence to confuse predators and attract prey, while bacteria benefit from available sources of nutrients and oxygen. It is still not known why bacteria luminesce, but there is some evidence that the emitted light allows them to perform light-driven DNA repair in the dark environment.

Bacterial bioluminescence is catalyzed by flavoprotein luciferases and the reaction involves the oxidation of a long-chain hydroperoxide FlHOOH, which has been isolated and characterized by Bruice12,13,20 experiments suggested that the pseudobase is the emitter responsible for chemiluminescence. Pulse radiolysis19,23 and cyclic voltammetry2,13,20 experiments suggested that the pseudobase is the emitter responsible for chemiluminescence. However, further analysis of chemiluminescence mechanism is complicated by the fact that they eliminate water and coverts to FMN; (vi) reduction of FMN to FMNH$_2$ recovers the catalyst.

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that pseudobases derived from N(5)-alkylated flavin salts have extremely low fluorescence quantum efficiencies (less than 10^{-5} at room temperature in aqueous solution). The fluorescence spectrum of the pseudobase was obtained either in glassy solvent at 77 K or by inserting the pseudobase in the luciferase active site.

Despite the fact that flavin pseudobases could be emissive intermediates in bacterial bioluminescence, their ultrafast dynamics have not been previously investigated. To investigate possible origins of fast excited-state decay of flavin pseudobases, we synthesized N(5)-ethyl-4a-hydroxyflavin (Et-FlOH in Scheme 1) and investigated its photophysical behavior using femtosecond pump–probe spectroscopy. The focus of this study is to pinpoint the processes that cause low fluorescence quantum yields of N(5)-alkylated flavin pseudobases, with the goal of developing more chemiluminescent model compounds for bacterial fluorescence.

Our experimental data show that Et-FlOH exhibits complex excited-state behavior, with the most dominant decay component exhibiting a 0.5 ps lifetime. We investigated several possible origins of such fast excited-state behavior in Et-FlOH: (a) excited-state tautomerization to a keto-amine, (b) excited-state release of OH*, (c) deactivation by conical intersection (CI) involving a dark nπ* state, and (d) deactivation by a conical intersection involving excited-state planarization of N(5)-atom and a distortion of the pyrimidine ring of Et-FlOH. Based on our studies with model compounds EthFIOMe and Et-FlOH (Scheme 1) and time-dependent density functional theory (TD-DFT) calculations, we conclude that the mechanism (d) can be used to interpret the experimental findings.

## EXPERIMENT AND THEORY

### Synthesis

N(5)-Ethylflavinium perchlorate (Et-FlO^+), N(5)-ethyl-4a-hydroxyflavin (Et-FlOH), and N(5)-ethyl-4a-methoxyflavin (Et-FIOMe) were synthesized according to the published procedure.

### Steady-State Spectroscopy

UV/vis absorption spectra were recorded on a PerkinElmer Lambda 900 spectrometer in a 1 cm quartz cell. Emission spectra were recorded using a Bio-TEK Instruments fluorometer (Photon Technologies Incorporated) in a 1 cm quartz cell. The sample solutions for fluorescence measurements had absorption of 0.1–0.15 at the wavelength of excitation. Fluorescence quantum yield for Et-FlOH was determined using argon-ion laser excitation at 458 nm.

### Femtosecond Pump–Probe Experiment

The laser system for the ultrafast transient absorption measurement was described previously. Briefly, the 800 nm laser pulses were produced at a 1 kHz repetition rate (fwhm = 110 fs) by a mode-locked Ti:sapphire laser (Hurricane, Spectra-Physics). The output from a Hurricane was split into pump (85%) and probe (15%) beams. The pump beam (800 nm) was sent into an optical paramagnetic amplifier (OPA-400, Spectra-Physics) to obtain a 350 nm excitation source. The energy of the pump beam was <1 μJ/pulse. The probe beam (800 nm) was delayed by a delay stage (MM 4000, Newport) and then focused into a rotating CaF_2 crystal for white light continuum generation between 350 and 800 nm. After passing through the cell, the continuum was coupled into an optical fiber and input into a CCD spectograph (Ocean Optics, S2000). The data acquisition was achieved using in-house LabVIEW (National Instruments) software routines. The group velocity dispersion of the probing pulse was determined using nonresonant optical Kerr effect (OKE) measurements. The excitation beam was focused on a sample cell containing carbon tetrachloride (Aldrich Chemical Co.). The sample cell was placed in between a pair of crossed polarizers through which the probe beam was sent. The relative polarization between the incoming pump and probe beams was set at 45°. The induced OKE signal was measured at various time delays. This measurement was used to perform a chirp correction in the collected transient absorption data by a code written with Matlab 7.1 software. First, the temporal evolution of the OKE signal at every wavelength was fitted to a Gaussian function. The value for the peak maximum obtained from the fit was used to construct the wavelength-dependent zero-times. Then, the time arrays from the transient absorption data were chirp-corrected by subtracting the value of zero-time at every wavelength.

### Data Analysis

Transient absorption data were analyzed using SPECFIT/32 Global Analysis System (Spectrum Software Associates, MA, U.S.A.). This program allows a decomposition of transient absorption data using kinetic models. The fitting process returns the predicted absorption spectra of individual colored species involved in the photochemical process along with their decay profiles. The analysis is achieved by a global analysis method that uses a singular value decomposition method to reduce the size of the fitted data. First, we performed global analysis of the data to determine the sufficient number of decay components and obtain the decay-associated difference spectra (DADS). Once we obtained the decay lifetimes, we applied a series of models, and the goodness of the fit was judged using the value of root-mean-square error. This procedure provided species-associated difference spectra (SADS) presented in this manuscript.

### Computational Methods

All calculations were performed at the Ohio Supercomputer Center. Ground-state geometry optimizations of Et-FlOH were performed starting from several input geometries using Gaussian 09 at the B3LYP/6-31+G* level of theory and with consideration of implicit solvation of acetonitrile (using the polarization continuum model, PCM). Tight optimization criteria were used in all calculations. All stationary points were confirmed to be energy minima using vibrational frequency calculations (B3LYP/6-31+G*), which confirmed that all of the computed vibrational frequencies were real (i.e., no imaginary vibrational frequencies). Vertical excitations were then calculated with time dependent DFT at the TD B3LYP/6-31+G* level of theory with consideration of implicit solvation of acetonitrile using the PCM. Difference-density plots were generated from the computed electron densities of the ground and excited states.

## RESULTS AND DISCUSSION

### Absorption and Emission Spectra of Et-FlOH

Figure 1 presents the absorption and fluorescence spectra of Et-FlOH...
The absorption maximum appears at 348 nm, and we assign this band to the $\pi,\pi^*$ transition (based on the TD-DFT calculation presented below). The fluorescence spectrum exhibits a large Stokes shift ($\lambda_{\text{MAX}} = 496$ nm), indicating significant nuclear rearrangements in the excited state. The quantum yield of Et-FlOH fluorescence is only $3 \times 10^{-3}$, suggesting that fast $S_1 \rightarrow S_0$ thermal deactivation or a photochemical reaction occurs in Et-FlOH. A previous report by Tu and co-workers shows that the insertion of Et-FlOH into the luciferase active site leads to a strong increase in its fluorescence intensity and a decrease in the Stokes shift (fluorescence occurs with a maximum at 440 nm). These results demonstrate that the nonemissive deactivation of Et-FlOH occurs at a very fast time scale, with the most dominant decay component having a 500 fs lifetime. Furthermore, the nonemissive deactivation occurs via several emissive transient species, as illustrated by a progressive red-shift in the stimulated emission signal of Et-FlOH as a function of probe delay.

The red-shift of the stimulated emission signal is more clearly observed in the absorption spectra of the components obtained using target analysis of time-resolved data (see data analysis in the experimental section). Component analysis of Et-FlOH transient absorption data was obtained using $S_1^a$ and $S_1^b$ had initial concentration at $t = 0$, set arbitrarily at 1 mM since the extinction coefficients for transient species are not known. Deconvolved transient absorption spectra of $S_1^a$, $S_1^b$, and $S_1^c$ and their lifetimes are shown in Figure 3. We can see that the initially produced $S_1^a$ state exhibits stimulated emission signal at 411 nm and a stimulated emission band at 478 nm. The stimulated emission band appears to red-shift at longer time delays, reaching the value of 585 nm at $t = 10$ ps. The complexity of Et-FlOH excited-state dynamics can also be observed from the 410 nm decay (Figure 2b), which was fit to a multieponential decay function convolved with the instrument response function. The following lifetimes were obtained: $\tau_1 = 0.14$ ps ($A_1 = 0.2$), $\tau_2 = 0.50$ ps ($A_2 = 1.1$), and $\tau_3 = 15$ ps ($A_3 = 0.3$). These results demonstrate that the nonemissive deactivation of Et-FlOH occurs at a very fast time scale, with the most dominant decay component having a 500 fs lifetime. Furthermore, the nonemissive deactivation occurs via several emissive transient species, as illustrated by a progressive red-shift in the stimulated emission signal of Et-FlOH as a function of probe delay.

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**Pump—Probe Data on Et-FlOH.** Transient absorption spectra of Et-FlOH obtained at several time delays exhibit significantly different spectral features (Figure 2a). At $t = 0$ ps, the chirp-corrected spectrum consists of an excited-state absorption band at 406 nm and a stimulated emission band at 478 nm. The stimulated emission band appears to red-shift at longer time delays, reaching the value of 585 nm at $t = 10$ ps. The complexity of Et-FlOH excited-state dynamics can also be observed from the 410 nm decay (Figure 2b), which was fit to a multieponential decay function convolved with the instrument response function. The following lifetimes were obtained: $\tau_1 = 0.14$ ps ($A_1 = 0.2$), $\tau_2 = 0.50$ ps ($A_2 = 1.1$), and $\tau_3 = 15$ ps ($A_3 = 0.3$). These results demonstrate that the nonemissive deactivation of Et-FlOH occurs at a very fast time scale, with the most dominant decay component having a 500 fs lifetime. Furthermore, the nonemissive deactivation occurs via several emissive transient species, as illustrated by a progressive red-shift in the stimulated emission signal of Et-FlOH as a function of probe delay.

**Pump—Probe Data on Et-FlOMe.** One possible explanation of complex Et-FlOH dynamics is the excited state deprotonation of the $\text{OH}$ proton. The $pK_a$ value of Et-FlOH in the ground state is 9.2, and it is possible that the excited-state acidity increases due to the coupling of the $\text{OH}$ group with the flavin chromophore. Similar excited-state deprotonations are observed in...
alcohols and amines that are strongly coupled to the aromatic chromophores, such as 2-naphthol, azaindole, or pyranine. If the proton transfer in Et-FlOH is intramolecular, we could observe excited-state tautomerization from Et-FlOH hemiaminal form to the corresponding amino-ketone isomer (Scheme 2). Similar excited-state tautomerizations are known to occur in many alcohols and amines. In most cases, the intramolecular tautomerization occurs via a 6-membered ring intermediate (salicylic acid, hydroxybenzothiazole, and hypercin), but in several cases the

Table 1. Calculated Vertical Excitation Wavelengths and Oscillator Strengths for the First Four Singlet Excited States of Et-FlOH (TD-B3LYP/6-31+G*, PCM acetonitrile)

<table>
<thead>
<tr>
<th>wavelength (nm)</th>
<th>oscillator strength</th>
<th>state</th>
<th>experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>382</td>
<td>0.2143</td>
<td>S₁</td>
<td>347</td>
</tr>
<tr>
<td>312</td>
<td>0.1372</td>
<td>S₂</td>
<td>308</td>
</tr>
<tr>
<td>286</td>
<td>0.0814</td>
<td>S₃</td>
<td>282</td>
</tr>
<tr>
<td>268</td>
<td>0.0005</td>
<td>S₄</td>
<td></td>
</tr>
</tbody>
</table>

*Experimental values were obtained in acetonitrile.
4-membered (cytosine\textsuperscript{43}) and 5-membered (pyridylpyrrole\textsuperscript{44} and hydroxyflavone\textsuperscript{47}) tautomerizations were observed.

Figure 8. Schematic representation of Förster cycle used to estimation the pseudobase pK\textsubscript{a}\textasciitilde of Et-FIOH. The absorption wavelengths for Et-FIOH and Et-Fl\textsuperscript{+} were obtained from the UV/vis absorption spectra in acetonitrile.

To investigate this possibility, we synthesized 4a-methoxy-N(5)-ethylflavin derivative (Et-FIOMe) which lacks a hydroxylic proton and thus cannot tautomerize. Transient absorption spectra of Et-FIOMe were obtained at different time delays after the 350 nm excitation pulse (Figure 4a). Similarly to Et-FIOH, stimulated emission spectra of Et-FIOMe shifted to the lower energy at longer time delays: from 482 nm at 0 fs to 574 nm at 4.2 ps. Figure 4b shows the decay dynamics collected at 413 nm and a fit using a triexponential function (\(\tau_1 = 200\) fs, \(\tau_2 = 2.8\) ps, and \(\tau_3 = 91\) ps). The lifetimes obtained for Et-FIOMe are longer than those obtained for Et-FIOH, possibly due to increased steric effects of –OCH\(_3\) group in the structural rearrangements of excited Et-FIOMe. However, the spectra and lifetimes of Et-FIOMe are qualitatively similar to those of Et-FIOH.

The spectral similarity can also be observed from the component analysis of Et-FIOMe transient absorption data (Figure 5) using S\(_1\)a\rightarrow S\(_1\)b\rightarrow S\(_1\)c\rightarrow S\(_0\) model. The deconvolved spectra of Et-FIOMe are similar to the behavior as Et-FIOH: (i) we observed three decay components with similar red-shift of the stimulated emission and (ii) the lifetimes of Et-FIOMe components are longer than, but comparable to, the corresponding Et-FIOH lifetimes. On the basis of these findings, we exclude excited-state tautomerization as a possible origin of fast Et-FIOH excited-state decay.

**TD-DFT Calculations.** Another possible mechanism of fast Et-FIOH excited-state decay involves internal conversion via the low-lying \(\pi,\pi^*\) state. The similar mechanism was found to occur in pyrimidine DNA bases, where excited-state decay occurs via two conical intersections involving initially excited \(\pi,\pi^*\) state, subsequently populated dark \(\pi,\pi^*\) state and the ground state of pyrimidine bases.\textsuperscript{48,49} To investigate whether \(\pi,\pi^*\) states are involved in the fast excited-state deactivation of Et-FIOH, we studied the excited-states of Et-FIOH using TD B3LYP/6-31+G\(^*\) methodology. In all calculations presented here, we considered only the S-stereoisomer of Et-FIOH, as we do not expect stereochemistry to play a role in the excited-state behavior of Et-FIOH. We considered implicit solvation of acetonitrile using the PCM model. Frequency calculation was conducted to confirm the absence of imaginary frequencies. The front and side views of the optimized Et-FIOH ground-state geometry are shown in Figure 10a (conformer 1). The computed optimized structure suggests that the central ring of the isoalloxazine moiety

Figure 9. (a) Ground state absorption (black) and fluorescence (red) spectra of 2 \times 10^{-5} M N(5)-ethyl flavinium perchlorate (Et-Fl\textsuperscript{+}) in acetonitrile; (b) transient absorption spectrum of 2 mM Et-Fl\textsuperscript{+} in acetonitrile collected 1 ps after the 555 nm excitation pulse. Spectra were obtained from ref 51.

Figure 10. Optimized geometries for conformers 1—3 (B3LYP/6-31+G\(^*\) and a PCM model of acetonitrile). Color code: gray, carbon; blue, nitrogen; red, oxygen. H-atoms are excluded for clarity.
is not planar due to the sp³ type geometry of N5 atom. On the other hand, phenyl and pyrimidine rings of the isoalloxazine moiety maintain their planarity.

Using the optimized Et-FIOH structure, we performed TD-DFT calculations to estimate vertical excitation energies and oscillator strengths for the first four singlet excited states of Et-FIOH. The results of our calculations are compared with the experimental absorption spectrum of Et-FIOH in Figure 6 and Table 1. The computed S₁ energy underestimates the experimental value by 0.32 eV, which is well within the acceptable accuracy of TD-DFT method (typical error is in the 0.1–0.5 eV range). The presence of 1nπ⁺ state is usually revealed by the existence of low oscillator strength transition. In the case of Et-FIOH, the first excited state with low oscillator strength is S₄ state (λₐᵣₛ = 268 nm), suggesting this state could have 1nπ⁺ character. Experimentally, we observe strong absorption below 275 nm, which probably arises due to higher energy transitions (S₄ → S₃, where n > 4) that exhibit larger oscillator strengths (we calculated the transition energies only for the first four singlet excited states).

To evaluate the characteristics of calculated excited states, we plotted their difference density plots (DDPs) in Figure 7. Each plot represents a difference in the electronic density between the excited state of interest and the ground state (S₄− S₀). DDPs of the first three Et-FIOH excited states are qualitatively similar and involve density changes across the entire π system of the isoalloxazine moiety, with the charge depletion from the phenyl ring (red color) and accumulation of electronic density on the pyrimidine moiety of the isoalloxazine framework (green). We assign these three state to 1π,π* states with a charge transfer character. The first state with 1π,π* character state is the S₄ state, as can be visualized in Figure 7 as decrease of electronic density from the two imide oxygen atoms and increase in the electron density at the pyrimidine ring. Since this state has very high excitation energy (4.6 eV), we conclude that the 1nπ⁺ state is probably not involved in fast internal conversion of Et-FIOH. In addition, energies of S₂ and S₃ states are also significantly higher than that of S₁ state, and as such are not likely to be involved in internal conversion of Et-FIOH. These results suggest that a single S₁−S₀ conical intersection causes the excited-state deactivation of Et-FIOH, without the mediation by higher excited states.

**Pump–Probe Data on Et-FI⁺.** Another possible deactivation pathway for Et-FIOH excited state involves a release of OH⁻ ion from photoexcited Et-FIOH (Et-FIOH + hν → Et-FI⁺ + OH⁻). The light-induced heterolytic bond cleavage is frequently observed in systems where the cation is stabilized by resonance, such as aminyl alcohols. Pseudobase derivatives are also known to undergo photochemical OH⁻ ion release, as was demonstrated in the case of a pseudobase derived from acridinium ions. The Et-FIOH/Et-FI⁺ equilibrium occurs in the ground state with a pseudobase pKₐ value of 3.6, suggesting that the ground-state release of OH⁻ is thermodynamically unfavorable by 0.61 eV (Figure 8). To estimate whether the release of OH⁻ from photoexcited Et-FIOH is a thermodynamically favored process, we used the Förster cycle presented in Figure 8. On the basis of the UV/vis absorption spectra of Et-FIOH (λₐᵣₛ = 350 nm) and Et-FI⁺ (λₐᵣₛ = 557 nm) and on the ground-state pseudobase pKₐ value of Et-FIOH (pKₐ = 3.6), we find that the Et-FIOH pseudobase pKₐ value changes drastically in the excited state (pKₐ* = 14, AG* = −0.71 eV).

On the basis of the results of this simple analysis, we investigated the photobasic properties of Et-FIOH. Since the photorelease of OH⁻ leads to the formation of Et-FI⁺ in its ground S₀ and/or excited S₁ state, we first indentified the absorption bands of these two states. The S₀ state of Et-FI⁺ exhibits two absorption bands at 420 and 550 nm (black curve in Figure 9a), whereas the S₁ state is characterized by a broad absorption band in the 350–650 nm range and a stimulated emission in the 650–750 nm range (black curve in Figure 9b). By comparing these Et-FI⁺ signature peaks with the spectral features of the transients obtained in the pump–probe experiment using Et-FIOH sample (Figures 2 and 3), we conclude that the formation of Et-FI⁺ does not occur upon excitation of Et-FIOH. Even though it is a thermodynamically favorable process, the release of OH⁻ from photoexcited Et-FIOH does not occur.

**DFT Calculations on Et-FIOH Conformers.** The fact that none of the above proposed mechanisms accounts for the fast excited-state decay in Et-FIOH led us to postulate that the decay mechanism involves S₁→S₀ conical intersection. To identify the nuclear coordinates responsible for deactivation, we investigated S₀ and S₁ energies of several different Et-FIOH conformers. Using B3LYP/6-31+G* methodology, we optimized S₀ state of Et-FIOH starting from a range of different input geometries. The optimization of all input structures produced one of the three local minima presented in Figure 10. The lowest-energy structure (conformer 1) has two planar rings (xylen and pyrimidine rings), whereas the central pyrazine ring of the isoalloxazine

![Figure 11. Structures and S₁ and S₀ energies of three conformers of Et-FIOH obtained using B3LYP/6-31+G* methodology.](Image)

**Table 2. Calculated S₀ and S₁ Energies for Three Et-FIOH Conformers***

<table>
<thead>
<tr>
<th>conformer</th>
<th>C₄a–N₅–C₅a angle (deg)</th>
<th>S₀ energy (Hartrees)</th>
<th>relative S₀ energy (kcal/mol)</th>
<th>λₐᵣₛ (nm)</th>
<th>orbitals involved</th>
<th>relative S₁ energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>112.7</td>
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<td>0</td>
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<td>HOMO → LUMO</td>
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<tr>
<td>2</td>
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<td>−1105.81976969</td>
<td>3.75</td>
<td>407</td>
<td>HOMO → LUMO</td>
<td>74.00</td>
</tr>
<tr>
<td>3</td>
<td>120.9</td>
<td>−1105.81769071</td>
<td>5.05</td>
<td>434</td>
<td>HOMO → LUMO</td>
<td>70.93</td>
</tr>
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</table>

* Calculations were performed using TD-B3LYP/6-31+G* and a PCM model of acetonitrile.
structure is bent (Figure 10a). In conformers 2 and 3, we observe increased planarity of the central ring and increased bending of the pyrimidine ring (Figure 10b and c).

The $S_0$ energy of these conformers strongly depends on the geometry of the N(5) atom: the closer the C4a–N5–C5a angle is to the sp³-type tetrahedral geometry, the lower is the $S_0$ energy of Et-FIOH conformer. This trend can be clearly observed as changes in C4a–N5–C5a and N1–C10a–C4a–C4 angles (Figure 11). We further performed TD-B3LYP/6-31+G* calculations to estimate energies of $S_1$ states in these three conformers (Figure 11). In contrast to ground-state energies, $S_1$ energies decrease with increasing C4a–N5–C5a angle. Thus, increased planarity of N5-atom leads to $S_0$ state destabilization and $S_1$ state stabilization (Table 2).

Given the high sensitivity of $S_0$ and $S_1$ energies to the degree of hybridization on N(5) atom, we postulate that the cause of fast excited-state deactivation in Et-FIOH is $S_1 \rightarrow S_0$ conical intersection reached by C4a–N5–C5a angle coordinate. It is interesting to note that the increased planarity of the central isoalloxazine moiety (observed as an increase C4a–N5–C5a angle) leads to the decrease in planarity of the pyrimidine ring (observed as an increase in the N1–C10a–C4a–C4 dihedral angle). A similar distortion was found to cause the $S_1 \rightarrow S_0$ conical intersection in cytosine, which is structurally akin to the pyrimidine moiety of Et-FIOH.

**CONCLUSIONS**

In summary, we studied the mechanism of fast excited-state decay in Et-FIOH, a model compound for bacterial bioluminescence. Using femtosecond pump–probe spectroscopy, we found that Et-FIOH exhibits complex excited-state decay dynamics with the highest amplitude decay component exhibiting 500 fs lifetime. We investigated several possible mechanisms of Et-FIOH excited-state deactivation: (i) excited state tautomerization, (ii) deactivation via a dark 'n,π* state, (iii) excited-state release of hydroxide ion, and (iv) thermal deactivation due to $S_1 \rightarrow S_0$ conical intersection. On the basis of our pump–probe data and TD-DFT calculations, we postulate that the $S_1 \rightarrow S_0$ conical intersection along the C4a–N5–C5a/N1–C10a–C4a–C4 coordinates is the reason for the low fluorescence/chemiluminescence efficiency of Et-FIOH. We expect this work to be useful for the development of improved models for bacterial bioluminescence. Furthermore, we and others previously postulated that similar nitrogen inversion in isoalloxazine moiety causes fast excited-state deactivation in reduced flavin cofactors. Thus, the study presented here could be extended to the excited-state behavior of a broad range of reduced flavins.

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