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Effect of the *o*-Acetamido Group on pH-Dependent Light Emission of a 3-Hydroxyphenyl-Substituted Dioxetane Luminophore

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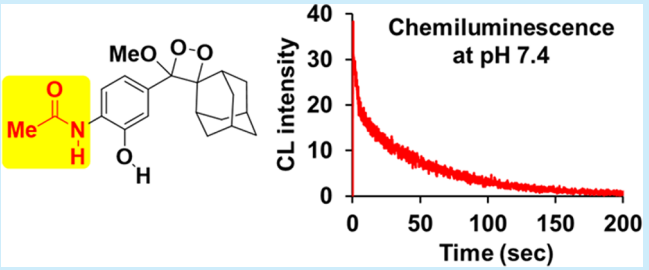
Supporting Information

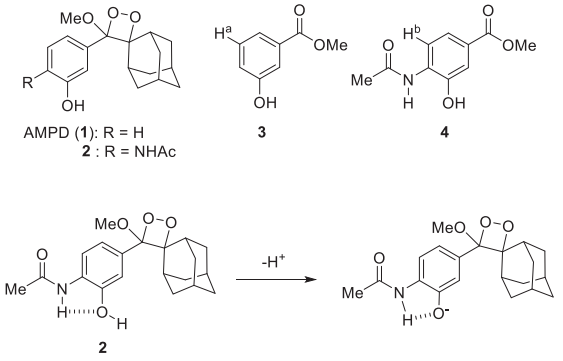
ABSTRACT: A pioneering chemiluminescent molecule reported by Schaap and co-workers, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-hydroxy)phenyl-1,2-dioxetane (AMPD), does not require enzymatic activation but is unsuitable for use under physiological conditions. To overcome this limitation, we have developed a new AMPD derivative that contains an acetamido group at the *ortho* position of the hydroxy group as an intramolecular hydrogen-bonding site in order to lower the pK_a value. This compound exhibits a superior chemiluminescence response to AMPD in the physiologically relevant pH range.



Many fluorescent probes are routinely utilized for investigations of cellular biology, screening for drug discovery, bioimaging, and so on.[1] Analysis based on chemiluminescence (CL) is almost 10 000-fold more sensitive than that based on fluorescence,[2] and detection is very simple because CL does not require excitation with photoirradiation.

The luciferin−luciferase system is widely applied in biological studies, but the requirement for luciferase limits its applicability to intact living cells and its availability for *in vivo* studies. On the other hand, a phenolic moiety directly linked to the 1,2-dioxetane structure can emit light triggered by phenoxy anion formation without enzymatic assistance. Among such compounds, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-hydroxy)phenyl-1,2-dioxetane (AMPD) (**1**), reported by Schaap and co-workers, was a pioneering organic light emitter for chemiluminescence sensing (Scheme 1).[3] Matsumoto et al. have also extensively investigated related 1,2-dioxetanes with greater thermal and chemical stability.[4] Several AMPD derivatives have already been applied in clinical laboratory tests for evaluating enzyme activity.[2]

Scheme 1. Structures of AMPDs 1 and 2 and Methyl 3-Hydroxybenzoates 3 and 4



However, AMPD and its analogues generally have two major problems that limit their usefulness. One is they are only usable over a restricted pH range. The other is the low efficiency of light emission under aqueous conditions,[2,3d−i,5] as compared with organic solvents such as MeCN, DMSO, and so on.[3,4] AMPD emits light under basic conditions because the pK_a of its phenolic hydroxy group is ca. 10, and formation of the phenoxy anion as an intramolecular electron donor is essential for the chemiexcitation process. Therefore, several 1,2-dioxetane compounds having an electron-withdrawing group, such as a chloro group, on the phenolic ring have already been developed in order to lower the pK_a of the phenolic hydroxy group by decreasing the electron density of the phenolic aromatic ring.[5,6a,c−e] Surfactants have been used as enhancers in order to improve the CL quenching of AMPD derivatives in aqueous solutions.[6]

Shabat et al. reported a new class of AMPD derivatives containing acrylate or acrylonitrile as an electron-withdrawing group to improve the emission under aqueous conditions.[2e,f,5,7] These probes exhibit excellent CL properties and are suitable for use under physiological conditions without any enhancer.[2f,5,7,8]

We hit on a different strategy for lowering the pK_a of the phenol moiety. Our group and Ueyama's group separately investigated the effect of intramolecular hydrogen bonding of an amide to a thiolate on the chemical properties of heme, based on the fact that native cytochrome P450 has an NH···S

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hydrogen bond involving the axial thiolate ligand.[9] We were also interested in Ueyama's related report that an acylamido group at the *ortho* position of a phenolic hydroxy group decreases the pK_a value of the phenolic hydroxy group due to the formation of an intramolecular NH···O hydrogen bond.[10]

Alkanoylamido groups are somewhat electron donating[11] and may affect the chemiexcitation step of AMPD derivatives in a different manner from electron-withdrawing groups. Matsumoto and co-workers examined the effect of intramolecular hydrogen bonding between a phenoxy anion and an amidomethyl (e.g., $-CH_2NHCOPh$) or a hydroxymethyl ($-CH_2OH$) group on the CL efficiency under fairly basic conditions ($NaOH/H_2O$−MeCN solvent system), but without considering the effect of pH on the intramolecular hydrogen bonding.[12]

Here we report the design and synthesis of an AMPD derivative **2** containing an acetamido group at the *ortho* position of the hydroxy group as an intramolecular hydrogen bonding site to lower the pK_a of the phenolic hydroxy group (Scheme 1). Notably, the CL properties of **2** containing the acetamido group were significantly improved over those of **1** in the physiologically relevant pH range.

Prior to the synthesis of **2**, we investigated the pK_a values of methyl 3-hydroxybenzoate **3** and its derivative **4** containing an acetamido group (Scheme 1) by means of UV−vis measurements in MeCN/100 mM buffer = 1/9 (v/v) (Figure S1 in Supporting Information).[13] Based on nonlinear least-squares analysis of the pH dependence of absorbance, the pK_a values of **3** and **4** were estimated to be 9.4 ± 0.1 and 8.5 ± 0.1, respectively. Thus, the introduction of the acetamido group on the methyl 3-hydroxybenzoate ester resulted in a decrease of ca. 0.9 units in the pK_a value. Moreover, ^{1}H NMR spectra of **3** and **4** were collected in DMSO-d_6/100 mM carbonate buffer (pD = 11.0) = 1/3 (v/v) (Figure S2 in Supporting Information). Under these conditions, **3** and **4** should be present as anionic forms due to the deprotonation of the hydroxy group. The H^b proton signal of **4** was observed at 8.00 ppm, which is 0.71 ppm downfield from the H^a proton signal of **3** (δ = 7.29 ppm), indicating the deshielding effect of the nearby carbonyl group of the amide on the H^b proton of **4**.[14] Since secondary amides generally adopt *trans* configuration, the proposed conformation of **4** suggests that the neighboring NH proton of the amide forms an NH···O hydrogen bond with the oxyanion of **4** and stabilizes the phenoxy anion.[10] The formation of the NH···O hydrogen bond was also supported by the optimized structure of the anionic form of **4** obtained by DFT calculations (Figure S3a in Supporting Information).

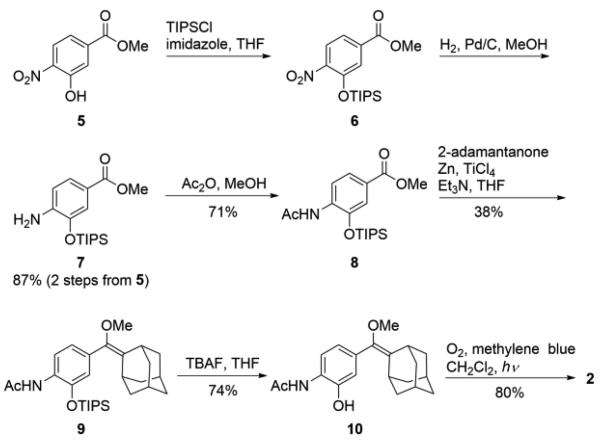
On the other hand, **3** and **4** should be present as neutral forms in DMSO-d_6/100 mM acetate buffer (pD = 5.5) = 1/3 (v/v). The signals of the H^a proton of **3** and H^b proton of **4** were observed at 7.52 and 7.91 ppm, respectively (Figure S4 in Supporting Information). Thus, the deshielding effect of the carbonyl group of the amide on the H^b proton of **4** is also seen in the neutral form of **4**. Therefore, it appears that the formation of the intramolecular NH···O hydrogen bond between the NH proton of the amide and oxygen of the hydroxy group can occur, even though the neutral form of **4** may exist as several conformers in equilibrium.[15]

Taking the reported data[10] and experimental and calculation results together, we consider that the formation of the intramolecular NH···O hydrogen bond in neutral and anionic forms of **4** should decrease the pK_a value of **4** by increasing the acidity of the phenolic hydroxy group of the neutral form and stabilizing the anionic form.[10]

Because the actual CL-emitting moiety of **1** and **2** is the methyl 3-oxybenzoate anion, which is formed by O−O bond cleavage of the 1,2-dioxetane moiety,[3] we designed and synthesized **2** containing the acetamido group at the *ortho* position of the hydroxy group and examined the effect of the acetamido group on the CL behavior at neutral pH.

Compound **2** was synthesized as shown in Scheme 2. The hydroxy group of **5** was protected with triisopropylsilyl

Scheme 2. Synthesis of 2



chloride (TIPSCl), and then the nitro group of **6** was reduced by hydrogenation to give **7**, followed by acetylation to give **8**. The alkene derivative **9** was obtained by the McMurry coupling reaction of 2-adamantanone with **8**.[16] After deprotection of **9** with tetra-*n*-butylammonium fluoride (TBAF), [2 + 2] cycloaddition of singlet oxygen was accomplished by bubbling oxygen through a solution of **10** and methylene blue under visible-light irradiation to afford the desired dioxetane derivative **2**, which was thermally stable enough to permit handling at room temperature.[17]

Next, the pH dependence of the CL of **1** and **2** was examined using freshly prepared sample solutions.[18] The CL spectra of **1** and **2** were measured in MeCN/100 mM carbonate buffer (pH 11.0) = 1/9 (v/v) containing 0.01% AcOH at 25 °C. As shown in Figure 1a, the CL spectrum of **1** at pH 11.0 (emission maximum: ca. 469 nm) is in agreement with reported results.[3g] The emission maximum of the CL spectrum of **2** bearing the acetamido group at pH 11.0 lies at ca. 458 nm which is ca. 11 nm shorter than that of **1**.[19] The CL spectrum of **2** at pH 11.0 overlapped with the fluorescence emission spectrum of the corresponding methyl benzoate ester **4** measured under the same solvent conditions (Figure S9a in Supporting Information). This result suggests that the emitting species of **2** generated by chemiexcitation is the deprotonated form of **4**.

As shown in Figure 1b, negligible CL of **1** was observed in MeCN/100 mM phosphate buffer (pH 7.4) = 1/9 (v/v). On the other hand, the clear CL spectrum of **2** (emission maximum: ca. 454 nm) was observed at the same pH, as expected.

The CL kinetic profiles of **1** and **2** at pH 4.0−11.0 are shown in Figures S11 and S12 in the Supporting Information. Figures 2 and 3 show the CL kinetic profiles of **1** (emission at 470 nm) and **2** (emission at 450 nm) at pH 11.0, 8.0, and 7.4. The

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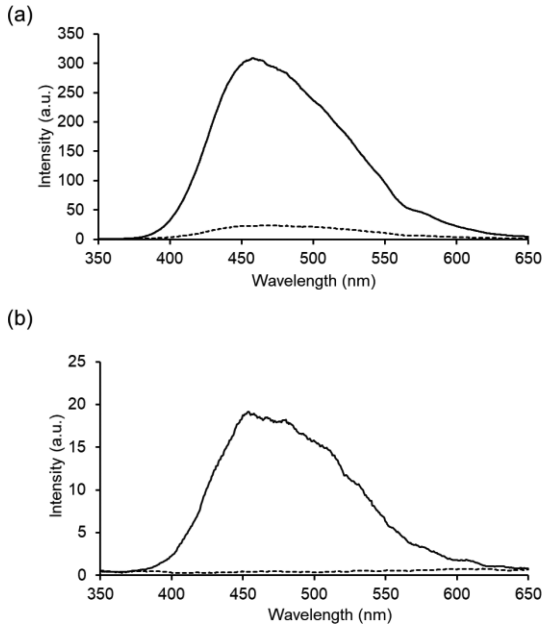

Figure 1. Chemiluminescence spectra of **1** (dotted line) and **2** (solid line) in (a) MeCN/100 mM carbonate buffer (pH 11.0) = 1/9 (v/v) containing 0.01% AcOH and (b) MeCN/100 mM phosphate buffer (pH 7.4) = 1/9 (v/v) containing 0.01% AcOH at 25 °C. [**1**] or [**2**] = 100 μM, scan speed = 60 000 nm/min.

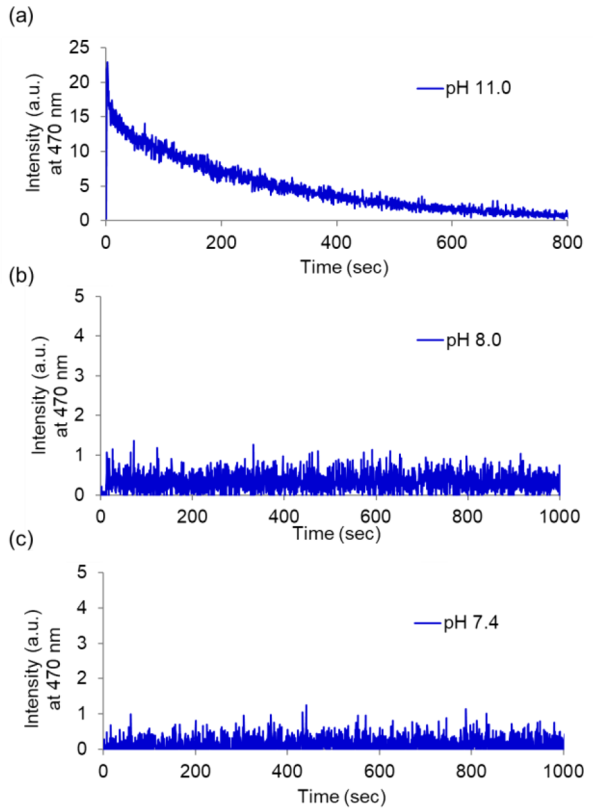

Figure 2. Chemiluminescence kinetic behavior (emission at 470 nm) of **1** (100 μM) at (a) pH 11.0, (b) pH 8.0, and (c) pH 7.4. Conditions: MeCN/100 mM buffer = 1/9 (v/v) containing 0.01% AcOH at 25 °C (pH 11.0: carbonate buffer, pH 8.0 and pH 7.4: phosphate buffer).

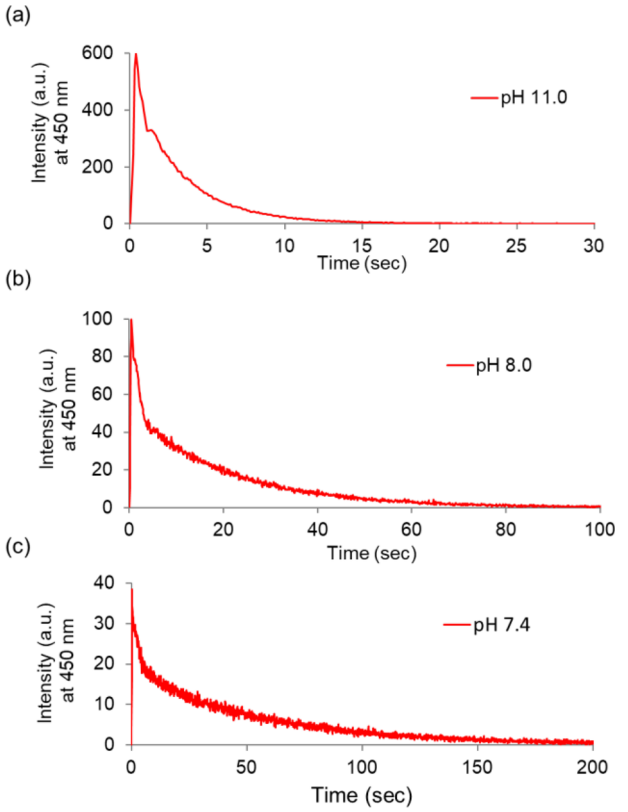

Figure 3. Chemiluminescence kinetic behavior (emission at 450 nm) of **2** (100 μM) at (a) pH 11.0, (b) pH 8.0, and (c) pH 7.4. Conditions: MeCN/100 mM buffer = 1/9 (v/v) containing 0.01% AcOH at 25 °C (pH 11.0: carbonate buffer, pH 8.0 and pH 7.4: phosphate buffer).

maximum emission intensities of **1** at 470 nm and **2** at 450 nm at pH 4.0−11.0 are summarized in Figure 4a.

At pH 11.0, **1** exhibits an initial increase in emission intensity to a maximum, followed by a decrease to zero (Figure 2a). In contrast, a significant increase in the emission intensity of **2** at 450 nm was observed at pH 11.0 (Figures 3a and 4a). The maximum emission intensity of CL of **2** at pH 11.0 is ca. 23-fold higher than that of **1** (Figure 4a). Because the hydroxy groups of **1** and **2** should be mostly deprotonated at pH 11.0, the difference of pK_a values between **1** and **2** should be eliminated at this pH. Therefore, it is suggested that the significant increase in the maximum emission intensity of **2** is caused by the electron-donating effect of the acetamido group.[11,20,21] Moreover, the emission intensity of **2** at both pH 8.0 and 7.4 is significantly higher than that of **1** at the same pH values (Figure 2b vs 3b, Figure 2c vs 3c, and Figure 4a).[22] The CL of **2** was detectable at pH > 6.4 (Figure S12 in Supporting Information).

The areas obtained from the emission decay curve of **1** at 470 nm (0 to 2940 sec) and **2** at 450 nm (0 to 240 sec) in solutions of various pH values are shown in Figure 4b. At pH > 8.4, the emission area of **1** was larger than that of **2** due to the longer-lived emission, even though the emission intensity of **1** is very weak. Based on the CL data at pH 11.0, the CL quantum yield (Φ^{CL}) of **2** was determined to be 2.7×10^{-6}, which is ca. 3-fold lower than that of **1**.[23,24] On the other hand, a larger emission area of **2** was observed at pH 6.4−8.0, compared to that of **1**.

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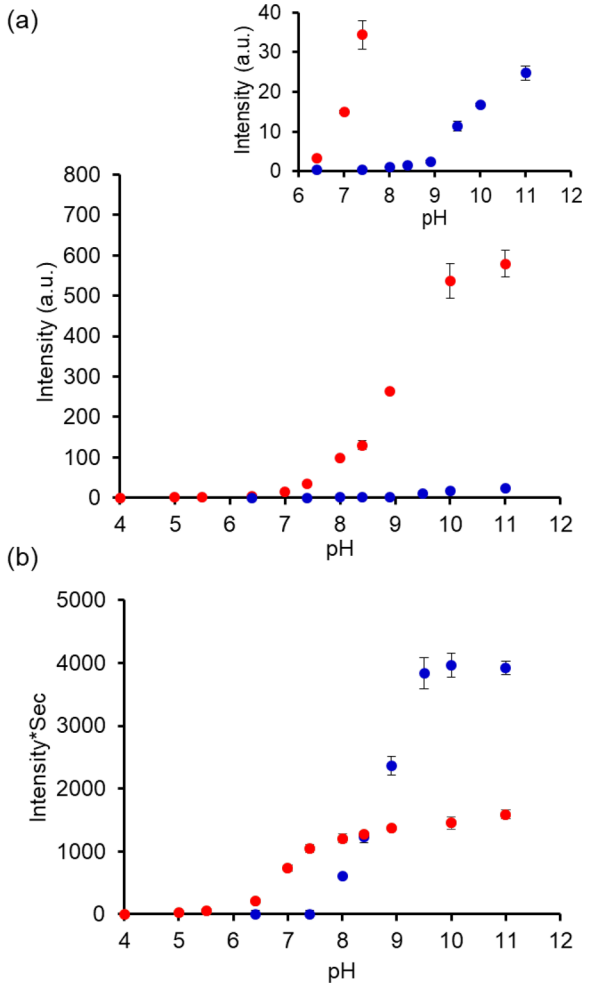

Figure 4. (a) Maximum emission intensity of **1** at 470 nm (blue circles) and **2** at 450 nm (red circles) at various pH values. Inset: expanded portion of the weak emission intensity region. (b) Areas under the emission decay curves of **1** (intensity at 470 nm*sec) (blue circles) and **2** (intensity at 450 nm*sec) (red circles) at various pH values. Conditions: MeCN/100 mM buffer (pH 4.0–11.0) = 1/9 (v/v) containing 0.01% AcOH at 25 °C. Error bars represent standard deviation of three independent experiments.

Based on the results of Figures 2–4, it appears that the introduction of the acetamido group as an intramolecular hydrogen-bonding site at the *ortho* position of the hydroxy group worked as expected, lowering the pK_a value and leading to an increased contribution of the phenoxy anion species in the physiological pH range. The much greater emission intensity per unit time of **2**, compared to **1**, suggests that **2** will serve as a more effective chemiluminescence probe than **1**. Furthermore, the shorter lifetime of the deprotonated form of **2** should provide a higher time resolution in measuring analyte concentration changes. The 1,2-dioxetane decomposition reaction is believed to be initiated by intramolecular electron or charge transfer from the negatively charged phenolate oxygen to the O–O bond.[2−4] Therefore, the faster kinetics of **2** than **1** may reflect not only the increased formation of the phenoxy anion species but also the electron-donating effect of the acetamido group[11] on the phenoxy anion, resulting in enhancement of the chemiexcitation rate.[25] However, other factor(s) may also be involved in accelerating light emission.

In conclusion, we have designed and synthesized an AMPD derivative **2** in which the pK_a of the phenolic hydroxy group is lowered by the introduction of an acetamido group at the *ortho* position of the hydroxy group. To our knowledge, this work is the first to show that intramolecular hydrogen bonding can lower the pH profile of light emission of a phenolic chemiluminogenic compound. The CL properties of **2** in aqueous medium at physiological pH range are superior to those of **1**, supporting the practical value of this approach. Further photophysical studies and improvements (e.g., enhancement of the CL signal and color tuning) should afford useful CL probes. We are currently working on the development of CL probes based on a combination of dioxetane–fluorophore conjugation[26] and our concept of utilizing intramolecular hydrogen bonding.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03913.

Detailed experimental procedures, pH-dependent change in absorbance, study of thermal stability, chemiluminescence spectra, fluorescence emission spectra, chemiluminescence kinetic profiles, NMR spectra (PDF)

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Author Contributions

All authors contributed to writing the manuscript and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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(13) The solvent conditions used for UV−vis measurements were MeCN/100 mM buffer 1/9 (v/v) containing 0.01% AcOH. The same conditions were used in CL studies.
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(15) The energy calculation study of the neutral form of **4** suggested that the conformer forming an intramolecular OH···O hydrogen bond between the hydroxy group of the phenolic moiety and the carbonyl oxygen of the amide is more stable than that forming an intramolecular NH···O hydrogen bond (Figure S3b in Supporting Information).
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(17) AMPD derivatives decompose upon heating to give the corresponding methyl 3-hydroxybenzoate and 2-adamantanone (see, ref 3b). The thermal stability of **1** and **2** in C_6D_6 was examined at 60 °C, and production of the corresponding methyl 3-hydroxybenzoate **3** or **4** was monitored by ^{1}H NMR (Figures S5 and S6 in Supporting Information). After heating of **1** in C_6D_6 at 60 °C for 30 h, **3** was obtained in 88% yield (Figure S7 in Supporting Information). On the other hand, the thermal decomposition of **2** afforded **4** in 32% and 63% yields at 30 h and 75 h, respectively. Interestingly, the results indicate that the introduction of the acetamido group at the *ortho* position of the phenolic hydroxy group increases the thermal stability.
(18) Stock solutions of **1** and **2** were prepared in MeCN containing 0.1% AcOH as an additive for stabilization. Namely, the solvent system used in CL studies was MeCN/100 mM buffer = 1/9 (v/v) containing 0.01% AcOH.
(19) The CL spectra of **1** and **2** in MeCN/100 mM Tris buffer (pH 8.9) = 1/9 (v/v) containing 0.01% AcOH at 25 °C are shown in Figure S8 in the Supporting Information. The emission maxima of **1** and **2** at pH 8.9 were observed at ca. 460 nm and ca. 455 nm, respectively.
(20) Although the fluorescence quantum yields of **3** and **4** in aqueous solution should be quite low (see, ref 5), their fluorescence emission spectra in MeCN/100 mM carbonate buffer pH 11.0 = 1/9 (v/v) suggest that the efficiencies of **3** and **4** are roughly similar (Figure S9b in Supporting Information). However, it should be kept in mind that the fluorescence emission maximum of the deprotonated form of **3** in aqueous solution is observed at ca. 415 nm, which is ca. 50 nm shorter wavelength than the CL emission maximum of **1** in the same solvent, although the CL-emitting species of **1** should be **3** (see, ref 3g).
(21) In contrast to the low fluorescence efficiency of **3** and **4** in aqueous media, both **3** and **4** exhibit strong fluorescence emission in MeCN in the presence of TBAF as a base (Figure S10 in Supporting Information). The emission maxima of **3** and **4** were observed at ca. 468 nm and ca. 455 nm, respectively. In MeCN, the fluorescence quantum yield of **4** under the same conditions was determined to be 0.53 which is ca. 1.8-fold higher that of **3**.
(22) The CL kinetic profiles of **2** (Figure 3) appear to show biphasic decay, especially at pH 8.0. Although the acetamido group on **2** may affect the chemiexcited state, the mechanism of CL remains unclear.
(23) Although 1,2-dioxetane molecules exhibit strong light emission in organic solvents, their CL efficiencies markedly decrease in aqueous solutions due to the quenching effect of H_2O molecules (see, refs 2f, 3e, and 3i).
(24) Relative chemiluminescence quantum yield of **2** was determined based on the value for **1** ($\Phi^{CL} = 7.5 \times 10^{-6}$) (see refs3e,[3i] and 4b).
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