A 7-hydroxy derivative of 3-methyleneisoindolin-1-one 1 was synthesized and its properties as a new fluorophore undergoing excited-state intramolecular proton transfer (ESIPT) were investigated. In alcohols and dimethylsulfoxide, 1 exhibited dual emission at ca. 380 and 525 nm when excited at ca. 336 nm, which agreed well with the density functional theory (DFT) and time-dependent (TD)-DFT-calculated emission predictions of 1 and its ESIPT tautomer. In aqueous solutions at near neutral pH, 1 exhibited a broad emission band at ca. 497 nm, presumably caused by the overlap of emissions from 1 and the excited state phenolate species of 1. In binary mixtures of H₂O and EtOH, the wavelength and intensity of fluorescence maxima were dependent on the dielectric constant of the solvent, suggesting that 1 could be applied as a fluorescent probe to monitor aqueous environments.

Key words 3-methyleneisoindolin-1-one; fluorescence; dielectric environment

Fluorophores undergoing excited-state intramolecular proton transfer (ESIPT) have attracted considerable attention and have been used both in solution and in the solid state in many different fields. Upon irradiation, a hydroxyl proton is transferred from the original enol form (E) to form a keto tautomer (K*) as shown in Chart 1. The absorption from the enol form and emissions from both tautomers result in dual fluorescence. Importantly, the emission band of the keto form exhibits a large Stokes shift, which can minimize the background noise. These properties are advantageous in fluorescence detection. The fluorescence of ESIPT dyes has been used to detect anions, which can deprotonate the enol proton and inhibit the ESIPT process. These dyes can also be used to probe the polarity and degree of hydration of their environment. These properties of environment affect the equilibrium between the enol and keto forms and change the ratio of tautomers emission intensity, providing information on the dipole potential of membranes, for example. Hydration levels within micelles and enzymes have also been studied using ESIPT fluorophores, and this array of applications has increased the demand for new ESIPT fluorophores. Herein, we report the synthesis and fluorescent properties of 7-hydroxy-3-methyleneisoindolin-1-one (1, Fig. 1) that undergoes excited-state intramolecular proton transfer.

Recently, we developed 5,6-dimethoxy-3-methyleneisoindolin-1-one, 2 (Fig. 1) for site-specific turn on fluorescent labeling of a lysine residue in a DNA-interacting protein. We studied properties of 2 and found that it was a useful fluorophore; its fluorescence emission was blue-shifted compared to that of the known fluorophore, 4,5-dimethoxyphthalimide 3, and its intensity was similar to that of 3. We found that 2 exhibited higher stability in buffer at pH 8 than 3, which made 2 advantageous for fluorescent labeling studies of biomolecules in aqueous conditions. These results prompted us to study the properties of 3-methylenisoindolin-1-one derivatives with substituents at various positions as new fluorophores and to compare these derivatives with their carbon isostere phthalimide. Recently, a 3-hydroxyphthalimide (4)-based probe was developed as a fluorescent probe for thiol detection. Its strong fluorescence, with a maximum at 516 nm and a large Stokes shift, resulted in a high sensitivity. On the other hand, it has been reported that 3-hydroxyphthalimide does not lase in the shortwave spectral region. We envisaged that 1 might provide a new ESIPT fluorophore in which a proton of phenolic hydroxyl group might easily migrate to a neighbor.
boring carbonyl group in the excited state (Chart 2); thus, we synthesized 5 and studied its properties in comparison with the corresponding phthalimide, 6, to evaluate the effectiveness of 1 as a new fluorophore.

**Results and Discussion**

**Synthesis** The N-6-hydroxyhexyl derivative 5 was synthesized as depicted in Chart 3. The rhodium-catalyzed ortho acylation\(^{12,13}\) of 2-benzyloxybenzoic acid with acetic anhydride proceeded and the following basic hydrolysis yielded a cyclized product, which was converted to 7 by esterification without purification. After catalytic hydrogenolysis of benzyl group of 7, heating the resultant 8 with 6-amino-1-hexanol in toluene afforded 5. The reference compound 6, which contained N-6-hydroxyhexyl group, was synthesized by a similar procedure.\(^{14}\)

**Photophysical Properties of 5 in Organic Solvents** The photophysical properties of 5 were studied in the solvents listed in Table 1. The positions of absorption maxima did not change significantly. In less polar aprotic solvents, such as CH\(_2\)Cl\(_2\), CHCl\(_3\), and dioxane, the fluorescence spectrum of 5 showed a single emission with a maximum at 528–541 nm when excited at ca. 336 nm (Table 1). In dimethyl sulfoxide (DMSO), emission maxima were observed at 375 and 540 nm (Table 1 and Fig. 2a). The intensity ratio of these emission maxima was ca. 2. Optical properties (absorption and fluorescence spectra) of the N-methyl derivative of 1 in E* and K* forms which contain intramolecular hydrogen bonds between hydroxyl and carbonyl groups were calculated by density functional theory (DFT) and time-dependent (TD) DFT calculations at the B3LYP/DGDZVP level\(^{15}\); and the results showed the fluorescence maxima of E* and K* forms at 354 and 515 nm, respectively (Supplementary Materials).

**Table 1. Optical Properties of 5 in Different Solvents\(^a\)**

| Solvent   | \(\lambda_{\text{abs}}\) (log \(e\)) | \(\lambda_{E^*}/\text{nm}\) | \(I_{E^*}\) | \(\lambda_{K^*}/\text{nm}\) | \(I_{K^*}\) |
|-----------|---------------------------------|--------------------------|------------|--------------------------|------------|
| CH\(_2\)Cl\(_2\) | 261 (4.1), 327 (4.0), 343 (4.0) | 529                      | 557        |                          |            |
| CHCl\(_3\)  | 261 (4.1), 328 (4.0), 344 (4.0) | 528                      | 550        |                          |            |
| Dioxane    | 261 (4.1), 327 (4.0), 343 (4.0) | 541                      | 288        |                          |            |
| DMSO       | 263 (3.9), 332 (3.9), 344 (3.9) | 375                      | 221        | 540                      | 106        |
| i-PrOH     | 260 (4.1), 330 (4.0), 342 (4.0) | 380                      | 169        | 525                      | 373        |
| BuOH       | 261 (4.0), 330 (3.9), 343 (3.9) | 382                      | 137        | 527                      | 353        |
| EtOH       | 258 (4.1), 331 (4.0), 343 (4.0) | 382                      | 172        | 526                      | 341        |
| MeOH       | 258 (4.1), 331 (4.0), 343 (4.0) | 384                      | 192        | 525                      | 408        |

\(^a\) 0.75 \(\mu\)M solution, \(\lambda_{\text{abs}}\): the position of absorption maxima, \(\lambda_{E^*}\) and \(\lambda_{K^*}\): the positions of fluorescence maxima attributable to E* and K* bands, \(I_{E^*}\) and \(I_{K^*}\): Maximum fluorescence intensities of the E* and K* bands. b) Excitation at 336 nm except CHCl\(_3\) and DMSO at 338 nm.
Fluorescence spectra in DMSO calculated using DFT/TD-DFT calculations at the PCM/B3LYP/DGDZVP level showed the fluorescence maxima of $E^*$ and $K^*$ forms at 373 and 523 nm. Based on these results, the bands at 375 nm (in DMSO) and 528–541 nm were ascribed to emissions from the $E^*$ and $K^*$ forms, respectively. Dual emission was also observed in alcohols (Fig. 2a), which caused the intensity of $K^*$ and $E^*$ bands to increase significantly and slightly decrease, respectively, compared to those of spectrum in DMSO. The phthalimide 6 showed a single emission at 505–542 nm attributable to its ESIPT tautomer in CH$_2$Cl$_2$, dioxane, DMSO and alcohols (Fig. 2b and Supplementary Materials), showing a clear contrast to the spectra of 5.

**Fluorescence Spectra of 5 in Aqueous Solutions**

Investigation of fluorescence properties of 5 in aqueous solutions was important for biological applications. The fluorescence spectra of 5 at pH 5.5, 6, and 7 were comparable, each showing fluorescence maxima at ca. 496 nm (Fig. 3a). The maximum fluorescence intensity was larger than that in organic solvents, and the fluorescence quantum yield ($\Phi_{flu}$) was 0.23, which was comparable to that of 6 (0.22; the reported $\Phi_{flu}$ of N-butyl derivative of 4 was 0.27$^{[30]}$). Further increasing pH value caused a blue-shift; the $\lambda_{max}$ at pH 9.1 was 483 nm. The emission band of 5 was broader than the $K^*$ emission band in other solvents, which suggested the possibility that the $K^*$ band might overlap with bands of ionized forms, such as the anionic ($A^-*$) and cationic protonated ($C^+*$) forms, as is the case for 7-hydroxycoumarin. The phenol of 7-hydroxycoumarin is mildly acidic ($pK_a$ ca. 7.7) and 7-hydroxycoumarin exists as an enol form ($E$ form) and an anionic form ($A^-*$ form) in aqueous solutions at near neutral pH in the ground state. In the excited-state, 7-hydroxycoumarin displays strongly acidic behavior ($pK_a$ ca. 0.45) and its fluorescence spectra appear as overlapped emissions from its $E^*$, $K^*$, $A^-*$, and $C^+*$ forms.$^{[30]}$ The absorption spectra of 5 were measured in buffers at pH values ranging from 5.5 to 10.8 to investigate the molecular species present in aqueous solutions at biologically relevant pH. The absorption spectra were comparable at pH 5.5, 6, and 7 (Fig. 3b). At pH 8, 9.1, and 10.8, the intensity of absorption maximum at ca. 335 nm decreased and the intensity of the peak at 374 nm increased. These results indicated that 5 predominantly exists in the enol form at pH below 7 and phenol is deprotonated at pH above 8.$^{[17]}$ When solutions of 5 in buffers at pH 8 and 9.1 were excited at the corresponding absorption maxima (372 and 374 nm), the emission band was observed at 479 nm. Fluorescence spectra of $E^*$, $K^*$, $A^-*$ in water were calculated using DFT/TD-DFT calculations at the PCM/B3LYP/DGDZVP level, predicting the fluorescence maxima of $E^*$, $K^*$, $A^-*$ forms occurred at 373, 523, and 479 nm, respectively (Supplementary Materials). These results indicated that contributions from $K^*$ and $A^-*$ forms may have caused the blue-shifted emission maxima in fluorescence spectra of 5 in aqueous solutions compared to those in other solvents.

Next, we investigated the change in the fluorescence in various EtOH–H$_2$O binary mixtures. In mixed solvents, 5 exhibited a single band and emission from the enol form at ca. 380 nm was not observed clearly. As the proportion of water in the mixture was reduced from 100 to 0%, the wavelength of the fluorescence emission maxima shifted by 29 nm, from 497 to 526 nm (Fig. 4a), which correlated with the dielectric constants (Fig. 4b) and percentage of H$_2$O in the solvent (Supplementary Materials). The intensity of the fluorescence maxima also changed in response to the dielectric constant and percentage of H$_2$O in the mixture changed although a linear dependence was not observed (Fig. 4c and Supplementary Materials). The intensity ratio of the fluorescence maxima in H$_2$O and EtOH was 2.4. As a reference, the intensity ratio of the fluorescence emission maxima of 6 in H$_2$O and EtOH...
was 4.3, and the wavelength shift was 16 nm. Previously, the dielectric nature of the binding site of a DNA-binding protein was successfully studied using fluorescent oligodeoxynucleotides, which exhibit solvatofluorochromic shifts depending on the dielectric constant of their environment. In that study, the wavelength difference in the emission maxima in H$_2$O and EtOH was ca. 30 nm, which is comparable to the shift observed for 5.

In conclusion, we prepared 7-hydroxy-3-methyleneisoindolin-1-one (5) and found that 5 underwent ESIPT. In aqueous solution at pH 7, 5 exhibited a fluorescence band at ca. 500 nm and a large Stokes shift (160 nm). The sensitivity of the fluorescence of 5 in aqueous solutions to the dielectric constant of the medium suggested that 1 can be used to probe the dielectric environment.

**Experimental**

**General Information** NMR spectra were measured on a Bruker Advance (500 MHz for $^1$H-NMR, 125 MHz for $^{13}$C-NMR) at 300 K. Chemical shifts (δ) are referenced to residual proton in the deuterated solvent. UV-Vis spectra were measured on a SHIMADZU UV-2450 spectrometer. Fluorescence spectra were measured on a JASCO FP-6300. Electrospray ionization (ESI)-MS analysis was carried out on a Bruker micrOTOF spectrometer. For solution of 5 at pH ranging from 5.5 to 9.1, the following buffer solutions were used: pH 5.5; 1mM citrate buffer, pH 6, 7, and 8; 1mM phosphate buffer, pH 9.1; 1mM borate buffer. Solution of 5 at pH 10.8 was prepared from phosphate buffer, pH 8 by addition of aqueous NaOH solution.

**Propyl 2-Acetyl-6-benzyloxybenzoate (7)** A 100mL round flask was charged with 2-benzyloxybenzoic acid (4.56 g, 20.0 mmol), potassium fluoride (2.32 g, 40.0 mmol), activated at 120°C for 2 h) and [Rh(cod)Cl]$_2$ (0.15 g, 0.30 mmol) under Ar. Degassed mesitylene (16 mL), acetic anhydride (7.56 mL, 80.0 mmol) were then injected and the mixture was stirred at 145°C for 14 h. After cooling to room temperature, 6.25m NaOH solution was added and the solution was stirred at 100°C for 1 h. The reaction mixture was acidified with conc. HCl (pH<4). The mixture was extracted with ethyl acetate twice. The combined layers were washed with brine, dried over Na$_2$SO$_4$, filtered and evaporated in vacuo. The residue was purified by column chromatography ($n$-hexane–ethyl acetate=4:1 to 1:1) to obtain 7 (2.71 g, 43%) as pale yellow oil.

**Propyl 2-Acetyl-6-hydroxybenzoate (8)** 10% Pd/C (1.0 g) in ethyl acetate (5 mL) was stirred at room temperature for 1 h under H$_2$ atmosphere. Then, a solution of 7 (2.71 g, 8.67 mmol) in ethyl acetate (12 mL) was added. The mixture was stirred at room temperature for 20 h under H$_2$ atmosphere. Then reaction mixture was filtered through Celite and evaporated in vacuo. The residue was purified by column chromatography ($n$-hexane–ethyl acetate=8:1 to 1:1) to obtain 8 (1.18 g, 62%);
7-Hydroxy-3-methyleneisoeindolin-1-one (5) A mixture of 8 (290mg, 1.30mmol) and 6-amino-1-hexanol (306mg, 2.60mmol) in toluene (6.5mL) was stirred at 135°C for 14h. After cooling to room temperature, saturated aqueous NaHCO3 solution was added to the reaction mixture and the mixture was extracted with ethyl acetate twice. The combined organic layers were washed with brine, dried over Na2SO4, evaporated in vacuo. The residue was purified by column chromatography (n-hexane–ethyl acetate=2:1 to 1:1) to give 5 (144mg, 42%) as white powder; 1H-NMR (CDCl3) δ: 1.42 (4H, m), 1.45 (1H, brs), 1.58 (2H, m), 1.69 (2H, m), 3.65 (2H, t, J=6.4Hz), 3.75 (2H, t, J=7.3Hz), 4.89 (1H, d, J=2.4Hz), 5.24 (1H, d, J=2.4Hz), 6.91 (1H, d, J=8.2Hz), 7.18 (1H, d, J=7.6Hz), 7.43 (1H, dd, J=8.2, 7.6Hz), 8.10 (1H, s), 13C-NMR (CDCl3) δ: 25.4 (t), 26.6 (t), 28.3 (t), 39.0 (t), 62.8 (t), 90.8 (t), 111.8 (d), 113.9 (s), 116.4 (d), 134.2 (d), 136.8 (s), 142.1 (s), 155.0 (s), 168.9 (s), HR-MS-ESI (m/z): Calcd for C15H19NO3 (M+Na)+ 284.1237. Found 284.1257.

N-6-Hydroxyhexaphthalimide (6) A solution of 3-hydroxyphthalic anhydride (0.89g, 5.42mmol) in toluene (290mg, 1.30mmol) and 6-amino-1-hexanol (306mg, 2.60mmol) in toluene (6.5mL) was stirred at 135°C for 14h. After cooling to room temperature, saturated aqueous NaHCO3 solution was added to the reaction mixture and the mixture was extracted with ethyl acetate twice. The combined organic layers were washed with brine, dried over Na2SO4, evaporated in vacuo. The residue was purified by column chromatography (CHCl3-MeOH=18:1) to give 6 (1.28g, 90%) as white powder; 1H-NMR (CD2OD) δ: 1.32–1.44 (4H, m), 1.53 (2H, m), 1.66 (2H, m), 3.53 (2H, t, J=6.6Hz), 3.61 (2H, t, J=7.2Hz), 7.14 (1H, d, J=8.5Hz), 7.30 (1H, d, J=7.3Hz), 7.58 (1H, dd, J=7.3, 8.5Hz), 13C-NMR (CD2OD) δ: 26.2 (t), 27.4 (t), 29.2 (t), 33.2 (t), 38.2 (t), 62.5 (t), 64.4 (d), 116.2 (s), 124.5 (d), 134.9 (s), 137.0 (d), 156.5 (s), 169.5 (s), 169.8 (s), HR-MS-ESI (m/z): Calcd for C17H17NO3 (M+Na)+ 286.1050. Found 286.1044.

Fluorescence Quantum Yields of 5 and 6 Relative fluorescence quantum yields of 5 and 6 in 1mm phosphate buffer at pH 7 were measured with quinine sulfate as a reference known Φ0=0.55.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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18) The solution of 5 in buffer at pH 10.8 was acidified to pH 6.3 2h after UV measurement and UV spectrum of the sample was measured again. The spectrum was comparable to that of the solution at pH below 7 indicating 5 was stable during UV and fluorescent measurement in this study.
19) The solutions of 5 were excited at 336 nm which was between absorption maxima at pH 5.5 and 9.1, 330 and 375 nm, and was the excitation wavelength used for most of measurements in this study.
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