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Microwave-Assisted Synthesis of Azacoumarin Fluorophores and the Fluorescence Characterization

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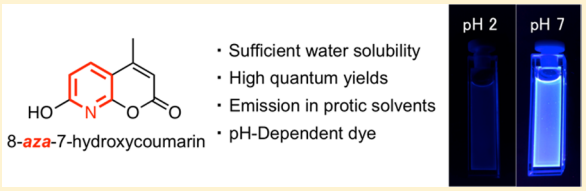
Supporting Information

ABSTRACT: By screening of reaction conditions and evaluation of its fluorescent properties, an environmentally sensitive fluorescent group, 8-aza-7-hydroxy-4-methylcoumarin, was synthesized in 70% yield using $MgBr_2$ as a Lewis acid under microwave irradiation. It has a high fluorescent quantum yield, is adequately soluble in water, and produces fluorescent emission in protic solvents and at neutral pH. Therefore, it could be useful in biosensors that are required to emit in hydrophilic environments such as cells.



Fluorescent imaging has become an indispensable technology in the life sciences to gain an understanding of biological phenomena. It has been especially useful in the discovery, cloning, and characterization of the green fluorescent protein (GFP)[1] because labeling with a fluorescent protein or organic small molecule facilitates observation of the behavior of biological molecules in living cells.[2] In addition to GFP derivatives, there are also near-infrared fluorescent protein derivatives (iRFPs)[3] and fluoresceins, rhodamines, and BODIPYs as fluorescent organic small molecules.[4] Recently, fluorometric sensors that alter the fluorescent property in response to changes in polarity, metal ion concentrations, and pH have been studied, and novel fluorophores have been developed.[5] In particular, benzofurazan and dansylamine analogues have been identified as environmentally sensitive fluorophores that increase the fluorescent intensity in hydrophobic environments but decrease it in hydrophilic environments.[6] Such compounds have been applied as probes in screening and protein detection (Figure 1a).[7] It could be difficult, however, for these fluorophores to detect specific phenomena because absence of fluorescence from a sensor could lead to a false positive and misdetection. From the aspect of accuracy and convenience, fluorophores with opposite fluorescence properties to those of benzofurazan and dansylamine analogues would be more favorable. To date, 7-methoxy-4-methylcoumarin,[8] 8-methoxy-4-methyl-2*H*-benzo[*g*]-chromen,[9] acridine,[10] and pyrene-3-carboxaldehydes[11] have been reported as fluorophores emitting in hydrophilic environments (Figure 1b), but the applicability of these fluorophores is limited by low fluorescence quantum yields, insolubility in aqueous solvents, and difficulties of derivatization. Consequently, the development of a new type of environmentally sensitive fluorophore capable of overcoming these problems remains challenging.

In this report, we document the synthesis and fluorescence characteristics of 8-aza-7-hydroxycoumarin (8-aza-hc), a novel fluorophore that emits with high fluorescence quantum yields in protic solvents and, in contrast, shows decreased fluorescence intensity in aprotic solvents. The fluorescence intensity of 8-aza-7-hydroxycoumarin also depends on the pH of the solution. The newly identified 8-aza-7-hydroxycoumarin-type fluorophores are environmentally sensitive and are potentially useful in biological applications (Figure 1c).

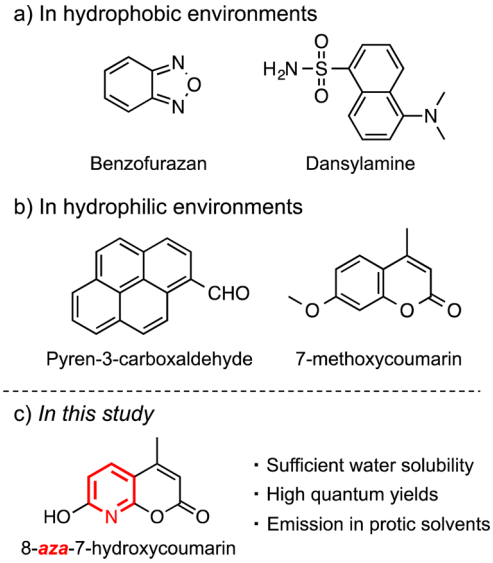


Figure 1. Representative fluorophores emitting (a) in hydrophobic environments and (b) in hydrophilic environments. (c) The 8-azacoumarin fluorophore.

Research into 8-azacoumarin fluorophores stemmed from our previous development of the 8-azacoumarin-4-ylmethyl type of photolabile protecting groups that efficiently promote photoreactions in aqueous media by a "heavy atom" effect.[12]

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Because it is known that excitation relaxation processes include both fluorescence emission, a radiative deactivation, and a radiationless deactivation or photoreaction, we assumed that 8-azacoumarin chromophores with or without substituents that participate in a photoreaction in aqueous media could also exhibit fluorescence emission in the same conditions. (Figure 2). The synthesis and the fluorescent quantum yield of 8-

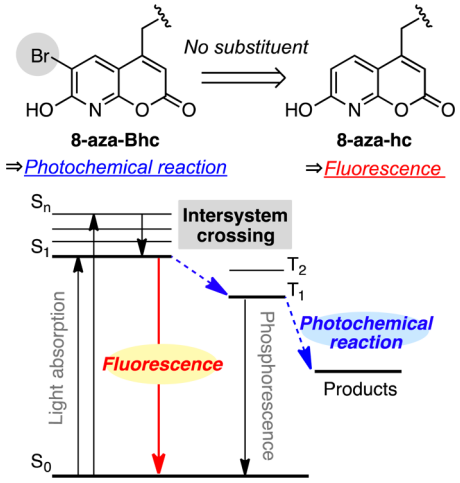
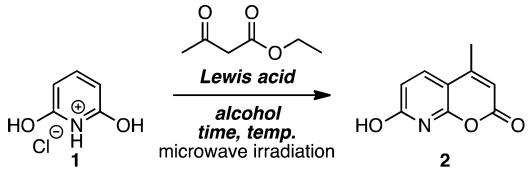


Figure 2. Jablonski diagram of 8-azacoumarin chromophores.

azacoumarins were first reported by Atkins and Bliss in 1978,[13] but the synthetic yield was extremely low, and their fluorescence properties were not investigated in detail. These drawbacks have restricted biological applications of 8-azacoumarins as fluorophores. We investigated the synthesis of 8-aza-hc.

Table 1 showed the results of the screening of the reaction condition. At first, von Pechmann's conditions were used and gave the desired product but in only 7% yield. Inspired by the work of Baxendale et al.[14] and Besson et al.,[14] microwave irradiation was used for the synthesis of azacoumarins under von Pechmann conditions. After 30 min of microwave irradiation, the desired product was generated in less than 5% yield, and unchanged starting material mostly remained. When the irradiation time was increased from 30 min to 3 h, the yield was improved to 23% (Table 1, entry 1). This result showed that the yield could be improved, and thus changes in the solvent, reaction time, Lewis acid, and temperature were investigated. Entries 1−4 showed that isopropanol as solvent gave the best yield among all of the alcohols investigated. Next, reaction times from 3 to 12 h were examined, but no significant improvement was observed (entries 3, 5, and 6). Some Lewis acids proved to be unsuitable for the synthesis of the azacoumarins, but LiBr and $MgBr_2$ slightly improved the yield (entries 9 and 10). In particular, the amount of $MgBr_2$ could be decreased from 3.0 to 1.0 equiv without reducing the yield (entry 11), which indicated that $MgBr_2$ was an optimum Lewis acid for the synthesis of 8-aza-hc-4-CH_3. In addition, the yield was substantially improved by raising the temperature to 120 °C (entry 12). This is perhaps due to the successful activation of ethyl acetoacetate enhancing the electrophilicity through the coordination of $MgBr_2$ to the 1,3-dicarbonyl groups of ethyl acetoacetate.[15] $ZnCl_2$ failed to activate the reagent sufficiently to induce electrophilic attack on the electron-deficient and poorly nucleophilic pyridine ring. From these results, we identified the optimal conditions as follows:

Table 1. Screening of Reaction Conditions[a]



entry	alcohol	time (h)	LA[b]	temp (°C)	yield[c] (%)
1[d]	EtOH	12	$ZnCl_2$	100	7
2	EtOH	0.5	$ZnCl_2$	100	<5
3	EtOH	3	$ZnCl_2$	100	23
4	MeOH	3	$ZnCl_2$	100	12
5	^{i}PrOH	3	$ZnCl_2$	100	27
6	^{n}BuOH	3	$ZnCl_2$	100	23
7	^{i}PrOH	6	$ZnCl_2$	100	29
8	^{i}PrOH	12	$ZnCl_2$	100	30
9	^{i}PrOH	3	$AlCl_3$	100	
10	^{i}PrOH	3	$CaCl_2$	100	
11	^{i}PrOH	3	LiBr	100	30
12	^{i}PrOH	3	$MgBr_2$	100	47
13[e]	^{i}PrOH	3	$MgBr_2$	100	47
14[e]	^{i}PrOH	3	$MgBr_2$	120	70
15[e]	^{i}PrOH	3	$MgBr_2$	140	45

[a]Reaction conditions: **1** (1.2 mmol), ethyl acetoacetate (1.0 mmol), and Lewis acid (3.0 mmol) in the solvent (2.0 mL) were irradiated under microwave and then stirred for 2 h at rt. [b]Lewis acid. [c]Not isolated. [d]Without microwave irradiation. [e]Lewis acid was 1.0 mmol.

2,6-dihydroxypyridine (**1**) reacting with ethyl acetoacetate in the presence of $MgBr_2$ as a Lewis acid in ^{i}PrOH under microwave irradiation at 120 °C for 3 h gives the desired azacoumarin product (**2**) in 70% yield.

To investigate substituent effects on the azacoumarin platform for fluorescence emissions, we synthesized azacoumarins halogenated at C3 with F, Cl, Br, or I. For the fluorinated and chlorinated azacoumarins, we were able to synthesize the desired compounds (**3** and **4**) in one step using the commercially available ethyl 2-fluoro- or 2-chloroacetoacetate and $ZnCl_2$ rather than $MgBr_2$ in 17 and 16% yield, respectively (Scheme 1a). Unfortunately, when the reaction was carried out using $MgBr_2$, the dehalogenated azacoumarin (**2**) was obtained as a major product. On the other hand, the brominated and iodinated azacoumarins could be prepared from compound **2** (Schemes 1b and c).

The photophysical properties in PBS are shown in Table 2. The maximum wavelengths (λ_{max}) are red-shifted from 351 to 367 nm as the atomic number of the halogen substituent increases, and at the maximum wavelength, these molar absorptivities (ε_{max}) were over 20 000. The results were consistent with the previous report;[13] the λ_{max} is slightly blue-shifted by the introduction of a nitrogen to the coumarin platform because of the increase in the HOMO−LUMO gap (7-hydroxy-4-methylcoumarin (hc): λ_{max} = 356 nm) that could be reasonably supposed to be due to the inductive effect of the nitrogen atom. In contrast, the ε_{max} of azacoumarins was higher than that of coumarins. This property would be preferable in fluorescent dyes. The fluorescence quantum yield of 8-aza-hc was Φ = 0.91 in PBS, higher than Φ in the other halogenated azacoumarins, and the values appeared to decrease as heavy atom substituents, with the exception of the chlorine substituent, increased. In case of the 8-aza-Chc, the energy level change of the orbital involving the photochemical process

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Scheme 1. Synthesis of Halogenated Azacoumarins

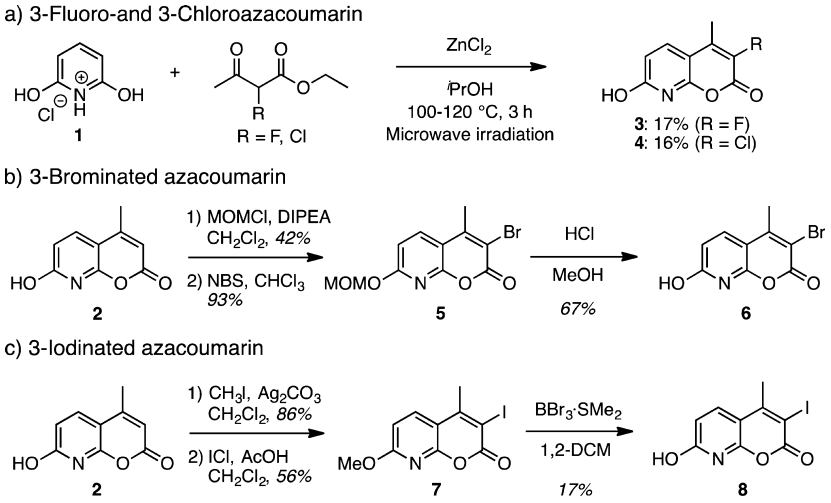

Table 2. Photophysical Properties of 8-Azacoumarin Deriveatives

compound	λ_{abs} (nm)	λ_{em} (nm)	ε_{max} (M^{-1} cm^{-1})	Φ_{fluo} [a]
2	351	411	26232	0.91[b]
3	351	429	22507	0.35
4	361	433	26864	0.67
6	364	435	28151	0.15
8	367	417	22400	0.13

[a]Fluorescent quantum yield in PBS determined by reference substance; quinine sulfate (Φ_{fluo} = 0.577). [b]Literature value = 0.76 in ethanol.

through the substituent electronic effect should lead to an increase in the fluorescent emission.[8]

We evaluated the fluorescent properties of 8-aza-hc (**2**) because it has a remarkable fluorescence quantum yield in PBS and is a promising compound that is fluorescent in water. The absorption and fluorescent spectra of 8-aza-hc in various solvents are shown in Figure 3. The absorption spectra have shoulders: one around 320 nm in aprotic solvents, and the other around 350 nm in protic solvents. This observation implies that different structures exist in protic or aprotic solvents.[16] The fluorescence spectra also have shoulders, but unexpectedly, the fluorescent intensity in aprotic solvents was extraordinarily weak. On the other hand, the fluorescent intensities in protic solvents are remarkably higher than those in aprotic solvents. These results suggested that the proton of the hydroxyl group at the C7-position of 8-azacoumarins might be associated with the fluorescence and that the fluorescent emission in protic solvents occurs when the hydroxyl group is deprotonated. This indicates that the 8-aza-hc is an environmentally fluorescent group with a fluorescence intensity that depends on the surrounding environment.

Next, we researched the effect of pH on the fluorescent properties of 8-aza-hc and investigated whether deprotonation of the hydroxyl group at the C7-position might be required for fluorescence emission. Figure 4 shows the results of the fluorescent spectra in citrate-phosphate buffer from pH 2.55 to 7.17 at 25 °C. The fluorescence intensity was quite low at pH 2.55 but gradually increased as the pH value approached 7. The fluorescence intensity at pH 7.17, a typical intracellular pH, was approximately 30-fold higher than that at pH 2.55, indicating that 8-aza-hc might be a pH-dependent fluorescent group as a result of the deprotonation process (pK_a = 5.96). This property of 8-aza-hc was opposite to that of existing pH-depending fluorescent groups which emit in acidic environments.[17] The difference in the fluorescence intensity of 8-aza-hc between pH 2.55 and 7.50 was 15-fold, while that of 7-hydroxy-4-methylcoumarin (hc) was 1.4-fold (Figure 5). In addition, the saturated concentration in PBS of 8-aza-hc was 9-fold higher than that of hc (8-aza-hc: 5365 μM, hc: 627 μM).[18] This

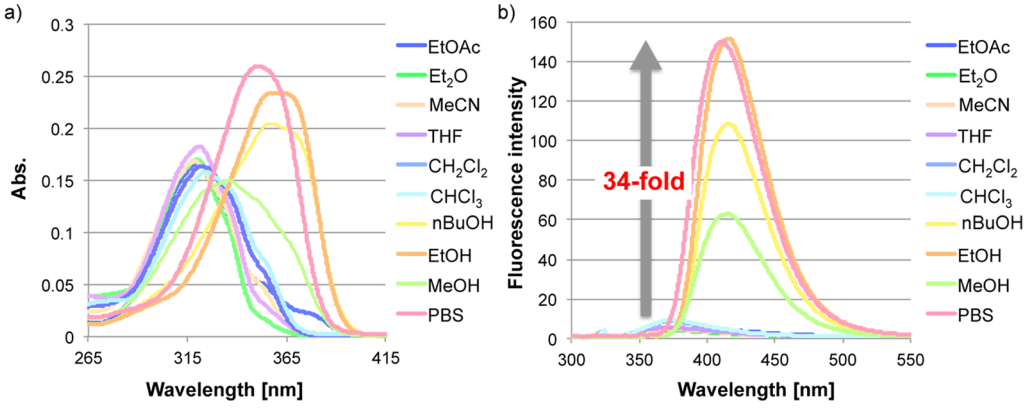

Figure 3. (a) Absorption spectra and (b) fluorescent spectra of the 8-aza-hc in various solvents.

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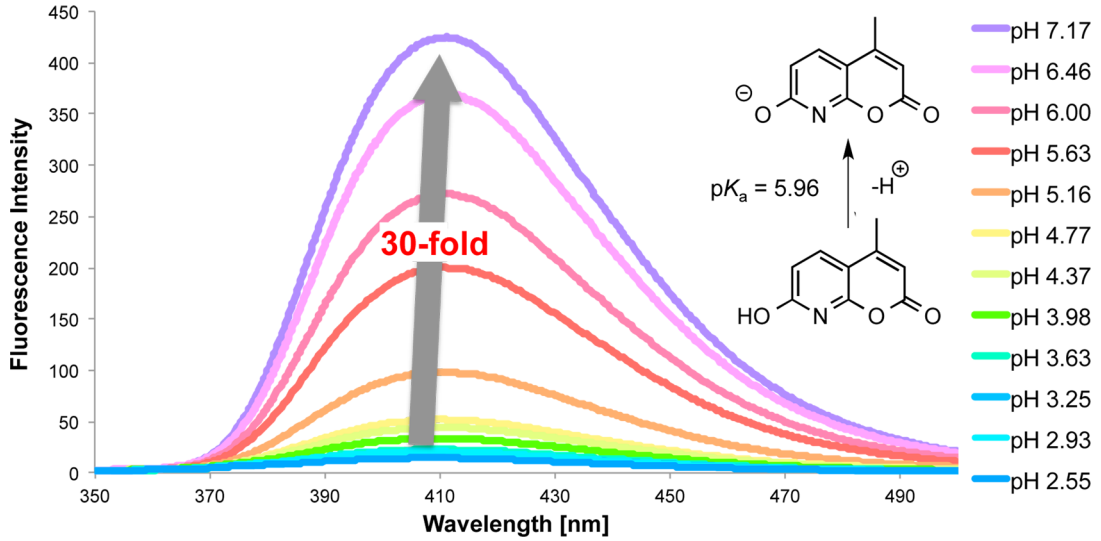

Figure 4. Dependence of the fluorescent intensity of the 8-aza-hc on pH values.

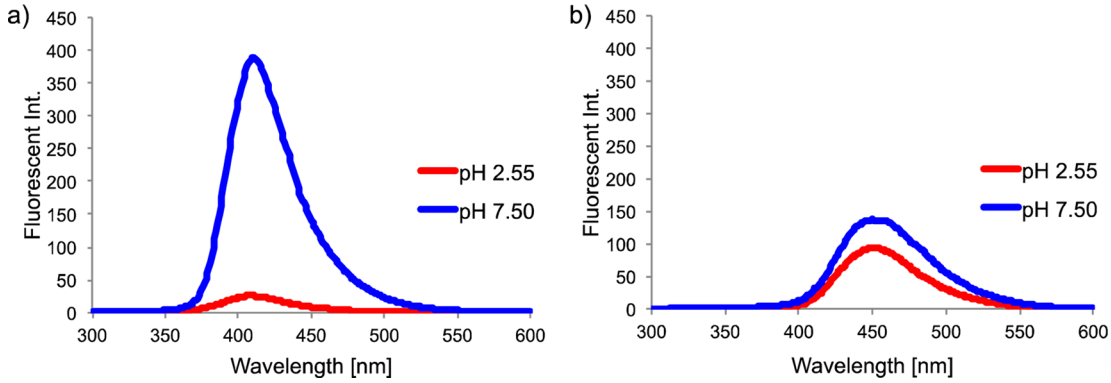

Figure 5. Fluorescence intensities at pH 2.55 and 7.50 of (a) 8-aza-hc and (b) hc.

suggested that the introduction of a nitrogen to a coumarin platform could produce a clear on−off switch and sufficient water solubility. From the viewpoint of these advantages, 8-aza-hc could be useful in elucidation of intracellular events such as abnormality in pH.

In summary, we focused on the 8-azacoumarin backbone, and this led to identification of water-soluble and highly photoreactive photolabile protecting groups and the optimum synthetic method and fluorescent properties for 8-aza-7-hydroxy-4- methylcoumarin (8-aza-hc). In optimized conditions, 2,6-dihydroxypyridine hydrochloride was reacted with ethyl acetoacetate in ^{i}PrOH using $MgBr_2$ as a Lewis acid with microwave irradiation for 3 h at 120 °C, which gave the desired 8-aza-hc with yields up to 70%. The results from the absorption and fluorescence spectra revealed that the fluorescence quantum yield of 8-azacoumarin derivatives was decreased by introduction of a heavy atom at the C3-position. Specifically, the fluorescent quantum yield of unsubstituted 8-aza-hc was 0.91 in PBS, and the maximum absorption was near 320 nm in aprotic solvents and near 350 nm in protic solvents. Furthermore, the fluorescence intensity in aprotic solvents was quite low but increased approximately 30-fold in protic solvents at neutral pH, indicating that the 8-aza-hc fluorophore might emit when the hydroxyl group at the C7-position is deprotonated.

These properties are useful for environmentally sensitive fluorescent groups as sensors that can be detected by emitting fluorescence. The identified 8-aza-hc fluorophore could be useful for finding biologically active compounds. Work designed to clearly demonstrate the utility of labeling bioactive compounds with an 8-aza-hc fluorophore is currently in progress.

EXPERIMENTAL SECTION

General Methods. All reactions were carried out in dried glassware and round-bottomed flasks under nitrogen atmosphere using commercially distilled solvents and reagents unless otherwise noted. CH_2Cl_2 dried over CaH_2 was distilled and stored over molecular sieves. The reactions were monitored by analytical thin-layer chromatography (TLC) precoated silica gel $60F_{254}$ using UV light, phosphorolybdic acid stain, *p*-anisaldehyde stain, or ninhydrin stain. Purification was performed by flash column chromatography using silica gel 60 N. Microwave reactions were carried out in a Biotage microwave reaction kit (sealed vials) in an Initiator (Biotage). The wattage was automatically determined to maintain the desired temperature for the desired period time.

Characterization Data. ^{1}H NMR spectra were recorded at 400 or 500 MHz; ^{13}C NMR spectra were recorded at 125 MHz. $CDCl_3$ or DMSO-d_6 was used as a solvent to obtain NMR spectra. Chemical shifts are given in parts per million (ppm) relative to Me_4Si (δ 0.00 for ^{1}H NMR in $CDCl_3$) or the solvent peak (δ 2.49 for ^{1}H NMR and δ 77.0 for ^{13}C NMR in DMSO) as internal standard. IR spectra were recorded as wavenumber (cm^{-1}). High-resolution mass spectra

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(HRMS) were recorded on a TOF (ESI-MS) spectrometer under positive (ESI^+) or negative (ESI^-) electrospray ionization condition.

Experimental Procedures. *7-Hydroxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**2**).* The microwave reaction kit was charged with 2,6-dihydoxypyridine hydrochloride (999 mg, 6.77 mmol), ethyl acetoacetate (752 mg, 5.78 mmol), magnesium bromide (1.04 g, 5.64 mmol), and 2-propanol (11.3 mL). The reaction mixture was stirred at 120 °C for 3 h under microwave irradiation, and then the reaction mixture was stirred at room temperature for 1.5 h to provide a red precipitate. The precipitate was collected by filtration, washed with Et_2O, and dried over vacuum to give the title compound **2** (718 mg, 70% yield) as a red powder: ^1H NMR (500 MHz, DMSO-d_6) δ 2.35 (d, J = 1 Hz, 3H), 6.15 (d, J = 1 Hz, 1H), 6.61 (d, J = 8.5 Hz, 1H), 8.01 (d, J = 8.5 Hz, 1H), 12.15 (br, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 17.9, 106.4, 108.1, 110.2, 137.9, 153.7, 157.3, 160.0, 164.6; IR (ATR, cm^{-1}) ν 1715 (C═O); HRMS (ESI) m/z calcd for $C_9H_8NO_3$ $[M + H]^+$ 178.0504, found 178.0502.[13]

*3-Fluoro-7-hydroxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**3**).* The microwave reaction kit was charged with 2,6-dihydoxypyridine hydrochloride (289 mg, 1.96 mmol), ethyl 2-fluoroacetoacetate (258 mg, 1.72 mmol), zinc chloride (796 mg, 5.83 mmol), and 2-propanol (3.30 mL). The reaction mixture was stirred at 100 °C for 3 h under microwave irradiation, and then the reaction mixture was stirred at room temperature for 1.5 h to provide a yellow precipitate. The precipitate was collected by filtration, washed with Et_2O, and dried over vacuum to give the title compound **3** (56.3 mg, 17% yield) as a yellow powder: ^1H NMR (500 MHz, DMSO-d_6) δ 2.31 (s, 3H), 6.72 (d, J = 8.5 Hz, 1H), 8.07 (d, J = 8.5 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 10.4, 106.6, 108.6, 132.3, 138.7. 141.2, 143.1, 154.9, 164.4; IR (ATR, cm^{-1}) ν 2922 (OH), 1728 (C═O); HRMS (ESI) m/z calcd for $C_9H_7FNO_3$ $[M + H]^+$ 196.0410, found 196.0407.

*3-Chloro-7-hydroxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**4**).* The microwave reaction kit was charged with 2,6-dihydoxypyridine hydrochloride (290 mg, 1.97 mmol), ethyl 2-chloroacetoacetate (283 mg, 1.72 mmol), zinc chloride (796 mg, 5.76 mmol), and 2-propanol (3.30 mL). The reaction mixture was stirred at 100 °C for 3 h under microwave irradiation, and then the reaction mixture was stirred at room temperature for 1.5 h to provide a yellow precipitate. The precipitate was collected by filtration, washed with Et_2O, and dried over vacuum to give the title compound **4** (57.8 mg, 16% yield) as a yellow powder: ^1H NMR (500 MHz, DMSO-d_6) δ 2.49 (s, 3H), 6.70 (d, J = 8.5 Hz, 1H), 8.14 (d, J = 8.5 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 16.0, 108.5, 115.7, 138.7, 138.7, 149.0, 155.5, 156.7, 164.8; IR (ATR, cm^{-1}) ν 2923 (OH), 1716 (C═O); HRMS (ESI) m/z calcd for $C_9H_7ClNO_3$ $[M + H]^+$ 212.0114, found 212.0109.

*7-(Methoxymethoxy)-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**S1**).* To a suspension of compound **2** (354 mg, 2.00 mmol) in CH_2Cl_2 (5.60 mL) was added *N*,*N*-diisopropylethylamine (1.74 mL) at 0 °C; the mixture was stirred at 0 °C for 10 min. MOMCl (304 μL) was added at 0 °C, and the reaction mixture was stirred at room temperature for 1.5 h. The reaction mixture was diluted with CH_2Cl_2, washed with saturated NH_4Cl aq., and dried over Na_2SO_4. Filtration and concentration under reduced pressure gave the crude compound. The crude was purified by flash column chromatography over silica gel with *n*-hexane:EtOAc (1:1) to give the title compound **S1** (186 mg, 42% yield) as a white solid: ^1H NMR (500 MHz, $CDCl_3$) δ 2.40−2.41 (m, 3H), 3.55−3.56 (m, 3H), 5.59 (s, 2H), 6.20 (m, 1H), 6.79 (d, J = 8.5 Hz, 1H), 7.88 (d, J = 8.5 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 18.3, 57.7, 93.0, 108.5, 109.0, 113.0, 136.6, 151.7, 157.6, 160.4, 163.4; IR (ATR, cm^{-1}) ν 1722 (C═O); HRMS (ESI) m/z calcd for $C_{11}H_{12}NO_4$ $[M + H]^+$ 222.0766, found 222.0761.

*3-Bromo-7-(methoxymethoxy)-4-methyl-2H-pyrano[2,3-b]-pyridin-2-one (**5**).* A mixture of compound **S1** (112 mg, 0.508 mmol) and *N*-bromosuccinimide (181 mg, 1.02 mmol) was dissolved with $CHCl_3$ and stirred at room temperature for 24 h. After the reaction mixture was concentrated under reduced pressure, the residue was extracted with $CHCl_3$, washed with water, and dried over Na_2SO_4. Filtration and concentration under reduced pressure gave the crude product, which was purified by flash column chromatography over silica gel with *n*-hexane:EtOAc (1:1) to give the title compound **5** (102 mg, 67% yield) as a white solid: ^1H NMR (500 MHz, $CDCl_3$) δ 2.52 (s, 3H), 3.48 (s, 3H), 5.50 (s, 2H), 6.76 (d, J = 8.5 Hz, 1H), 7.89 (d, J = 8.5 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 19.2, 57.6, 93.0, 108.7, 109.2, 110.4, 137.1, 150.3, 155.8. 156.6, 163.4; IR (ATR, cm^{-1}) ν 1732 (C═O); HRMS (ESI) m/z calcd for $C_{11}H_{11}BrNO_4$ $[M + H]^+$ 299.9871, found 299.9873.

*3-Bromo-7-hydroxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**6**).* Compound **5** (65.6 mg, 0.220 mmol) was dissolved with 2 M HCl/MeOH and stirred at room temperature for 1 h. After the reaction mixture was concentrated under reduced pressure to provide the crude compound, methanol was added, and the white precipitate was filtered. The precipitate was washed with cold methanol and dried under vacuum to give the title compound **6** (38.3 mg, 68% yield) as a white solid: ^1H NMR (500 MHz, DMSO-d_6) δ 2.54 (s, 3H), 6.69−6.71 (m, 1H), 8.16−8.18 (m, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 19.5, 31.1, 107.6, 109.1, 138.9, 152.4, 156.5, 157.0, 165.6; IR (ATR, cm^{-1}) ν 2922 (OH), 1712 (C═O); HRMS (ESI) m/z calcd for $C_9H_6BrNO_3$ $[M + H]^+$ 255.9609, found 255.9611.

*7-Methoxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**S2**).* To a suspension of compound **2** (178 mg, 1.01 mmol) and Ag_2CO_3 (382 mg, 1.39 mmol) in CH_2Cl_2 was added CH_3I (623 μL, 10.0 mmol), and the mixture was stirred at room temperature for 21 h. The reaction mixture was filtered through Celite and washed with Et_2O, and the filtrate was concentrated under reduced pressure to provide the crude compound. The crude product was purified by flash column chromatography over silica gel with *n*-hexane:EtOAc (1:1) to give the title compound **S2** (143 mg, 74% yield) as a white solid: ^1H NMR (500 MHz, $CDCl_3$) δ 2.40 (m, 3H), 4.02 (s, 3H), 6.18−6.19 (m, 1H), 6.73−6.75 (m, 1H), 7.83−7.85 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 18.3, 54.4, 108.1, 108.4, 112.3, 136.0, 152.0, 157.9, 160.5, 165.0; IR (ATR, cm^{-1}) ν 1713 (C═O); HRMS (ESI) m/z calcd for $C_{10}H_{10}NO_3$ $[M + H]^+$ 192.0661, found 192.0658.

*3-Iodo-7-methoxy-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**7**).* To a solution of compound **S2** (82.8 mg, 0.433 mmol) in CH_2Cl_2 under N_2 were added 1 M ICl/CH_2Cl_2 (0.644 mL, 0.644 mmol) and AcOH (97.0 μL), and the mixture was stirred at room temperature for 26 h. The reaction mixture was quenched with saturated $Na_2S_2O_3$ aq. and extracted with CH_2Cl_2. The extract was washed with saturated NaCl aq. and dried over Na_2SO_4. Filtration and concentration under reduced pressure gave the crude product, which was purified by flash column chromatography over silica gel with *n*-hexane:EtOAc (3:1) to give the title compound **7** (76.7 mg, 56% yield) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) δ 2.65 (s, 3H), 4.03 (s, 3H), 6.74 (d, J = 8.8 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 25.0, 54.7, 88.9, 107.6, 109.0, 136.6, 155.7, 157.2, 157.6, 165.3; IR (ATR) ν 1717 (C═O); HRMS (ESI) m/z calcd for $C_{10}H_9INO_3$ $[M + H]^+$ 317.9627, found 317.9625.

*7-Hydroxy-3-iodo-4-methyl-2H-pyrano[2,3-b]pyridin-2-one (**8**).* Compound **7** (63.4 mg, 0.200 mmol) and $BBr_3{\cdot}SMe_2$ (0.644 mL, 0.644 mmol) were dissolved with 1,2-dichloromethane (8.07 mL) and stirred at 85 °C for 23 h. The reaction mixture was quenched with H_2O and further stirred for 20 min. Then, the reaction mixture was extracted with EtOAc and washed with sat. $NaHCO_3$ aq. The aqueous phase was taken up with citric acid, and the extract was dried over Na_2SO_4. Filtration and concentration under reduced pressure gave the crude product, which was purified by flash column chromatography over silica gel with $CHCl_3$:MeOH (20:1) to give the title compound **8** (12.4 mg, 17% yield) as a white solid: ^1H NMR (400 MHz, DMSO-d_6) δ 2.60 (s, 3H), 6.65 (d, J = 8.5 Hz, 1H), 8.172 (d, J = 8.5 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 24.6, 87.3, 106.3, 108.4, 138.7, 156.6, 156.9, 157.5, 164.9; IR (ATR, cm^{-1}) ν 2917 (OH), 1702 (C═O); HRMS (ESI) m/z calcd for $C_9H_7INO_3$ $[M + H]^+$ 303.9471, found 303.9468.

Fluorescent Spectra Experiments. *Measurement of Fluorescent Spectra in Various Solvents.* A sample stock DMSO solution (2 mM) was prepared, and the fluorescence spectra were measured in various solvents (2 μM containing 0.1% DMSO) such as EtOAc, Et_2O, CH_3CN, THF, CH_2Cl_2, $CHCl_3$, *n*BuOH, EtOH, MeOH, and PBS.

Investigation of the pH-Dependence Effect. The pH dependence effect on fluorescent intensity was investigated by use of McIlvaine

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buffer, which has a buffer capacity from pH 2.2 to 8.0. At first, a sample solution (1 mL, 1 μM) was prepared at pH 2.2 and measured the fluorescence spectrum. Subsequently, we added 10 μL of 2 M NaOH aq. to the sample solution to adjust the acidity and measured the pH value by a pH meter (HORIBA, F51) and the fluorescence spectrum. The aliquot addition of NaOH aq. was continued until pH 8.0, and the fluorescence spectrum was measured each time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b02656.

Experimental procedures for the synthesis of 8-azacoumarin derivatives; determination of fluorescent quantum yield, saturated concentration, and pK_a; ^{1}H and ^{13}C NMR spectra of azacoumarin derivatives (PDF)

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Notes

The authors declare no competing financial interest.

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