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Design and Synthesis of an Enzyme-Cleavable Sensor Molecule for Phosphodiesterase Activity Based on Fluorescence Resonance Energy Transfer

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Abstract: Ratiometric measurement is a technique that can provide precise data and even quantitative detection. To carry out ratiometric measurements, it is necessary that the sensor molecule exhibits a large shift in its emission or excitation spectrum after reaction with the target molecule. Fluorescence resonance energy transfer (FRET) is one mechanism used to obtain a large spectral shift. In this study, our aim was to develop a ratiometric fluorescent sensor molecule for phosphodiesterase activity based on FRET. We designed and synthesized CPF4 with a coumarin donor, a fluorescein acceptor, and two phenyl linkers having the phosphodiester moiety interposed between them. In the emission spectrum of CPF4 in aqueous buffer excited at 370 nm, the emission of the coumarin donor was strongly quenched and the emission of the fluorescein acceptor was observed. This emission spectrum demonstrates that energy transfer from the coumarin donor to the fluorescein acceptor proceeds efficiently. Addition of a phosphodiesterase to an aqueous solution of CPF4 resulted in an increase in the donor fluorescence and a decrease in the acceptor fluorescence. CPF4 exhibited a large shift in its emission spectrum after the hydrolysis of the phosphodiester group by the enzyme. This large shift of the emission spectrum indicates that ratiometric measurements can be made by using CPF4. The method described in this paper for designing enzyme-cleavable sensor molecules based on FRET should be readily applicable to other hydrolytic enzymes.

Introduction

In recent years, many fluorescent sensor molecules have been developed to probe biological phenomena in living cells.[1] A fluorescent sensor is advantageous due to its high sensitivity, but may be influenced by many factors such as photobleaching, changes in the sensor molecule concentration, changes of environment around the sensor molecule (pH, polarity, temperature, and so forth), and stability under illumination. To reduce the influence of such factors, ratiometric measurements are utilized. Ratiometric measurements involve observation of changes in the ratio of the fluorescence intensities of the excitation or the emission at two wavelengths. This technique allows more precise measurement and, with some sensor molecules, quantitative detection is possible. To carry out ratiometric measurements, it is necessary that the sensor molecule exhibits a large shift in its emission or excitation wavelength after reaction with a target molecule. Fluorescence resonance energy transfer (FRET) is one mechanism used to obtain a large spectral shift. FRET is an interaction between the electronic excited states of two fluorophores, in which excitation energy of a donor is transferred to an acceptor without emission of a photon.[2] Recently, some ratiometric fluorescent sensor molecules based on FRET have been developed and widely used, such as the calcium sensor cameleon[3] and the β-lactamase sensor CCF2.[4] In this study, our aim is to develop a ratiometric fluorescent sensor molecule with low molecular weight for phosphodiesterase activity based on FRET.

Phosphodiesterases catalyze the hydrolysis of phosphodiester bonds and their substrates are nucleic acids and cyclic nucleotides.[5] Although phosphodiesterases have many important cellular roles, there is no effective method to monitor their activity in real time with high sensitivity. For example, nucleotide pyrophosphatase/phosphodiesterases[6] (NPPs), one group of phosphodiesterases, have been reported to be implicated

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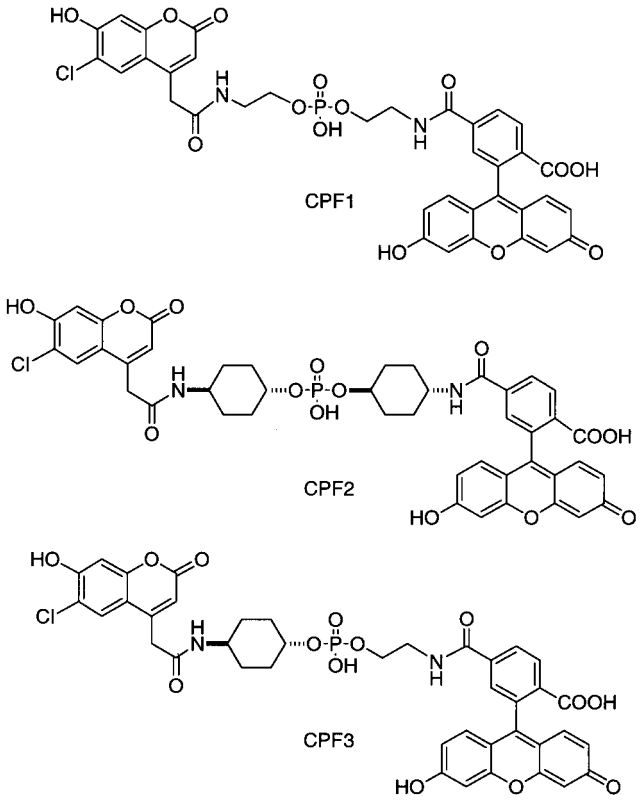
Chart 1. The Structures of CPFs



in the regulation of various intra- and extracellular processes, including cell differentiation and motility, bone and cartilage mineralization, and signaling by nucleotides and insulin. Some important proteins categorized in NPPs such as PC-1[7] and Autotaxin[8] have been the focus of much interest in recent years. Since NPPs consist of phosphodiesterase I (EC 3.1.4.1), the probe for phosphodiesterase I activity will be a useful tool for investigating the role of NPPs. Berkessel and Riedl have developed fluorescence reporters for phosphodiesterase I activity, in which a naphthalene moiety acts as the fluorophore and an azobenzene moiety as the quencher.[9] These reporters fluoresce upon addition of phosphodiesterases, but have limitations for biological application because of their short excitation wavelength and weak fluorescence. Also, the change of fluorescence intensity is observed at one wavelength. To obtain a ratiometric fluorescent sensor molecule for phosphodiesterase activity, we previously designed and synthesized intramolecular FRET compounds (CPFs) which have coumarin as a donor, fluorescein as an acceptor, and phosphodiester as a linker (Chart 1).[10] CPF1 with two ethylene linkers and CPF3 with one ethylene and one cyclohexane linker exhibited fluorescence quenching due to the dye-to-dye close contact. If the fluorophores are introduced intramolecularly with a flexible linker, they should form the ground-state dye-to-dye close contact in an aqueous environment and the fluorescence should be quenched.[11,12] CPF2 with two cyclohexane linkers exhibited acceptor fluorescence due to FRET by hindering the close contact of the two fluorophores. So, it is necessary that the structure of the sensor molecule is such as to prevent dye-to-dye close contact in an aqueous environment. However, CPF2 was not hydrolyzed by phosphodiesterases due to the structural features of the molecule. Thus, it is also necessary that the molecule should be recognized as a substrate by phosphodiesterases. None of the first-generation CPFs satisfied both requirements (no close contact of fluorophores in aqueous environments and recognition as a substrate by the enzyme) and worked as a ratiometric fluorescent sensor molecule for phosphodiesterase activity. In this study, we intended to develop an enzyme-cleavable sensor molecule for ratiometric measurement by optimizing the linker such as to satisfy both requirements.

Results and Discussion

Design and Synthesis of CPF4 and Its Spectroscopic Properties. There are several reports[13] about the features of the substrate binding site of phosphodiesterase I based on the specificity and kinetics of some artificial substrates. According to the reports, phosphodiesterase I catalyzes the hydrolysis of a wide range of synthetic phosphodiesters in addition to naturally occurring nucleotide substrates. In particular, the compounds which contain diphenyl phosphate structure [e.g., bis(4-nitrophenyl) phosphate,[13a,b] 4-nitrophenyl phenyl phosphate,[13a] 4-nitrophenyl 4-*tert*-butylphenyl phosphate,[13d] and bis(4-methylumbelliferyl) phosphate[13e]] could be hydrolyzed by phosphodiesterase I. Thus, it was presumed that the phenyl moiety would be appropriate as the enzyme cleavable linker and designed CPF4 with two phenyl linkers. The structure of CPF4 and the sensor mechanism are shown in Scheme 1. In CPF4, coumarin as the donor and fluorescein as the acceptor are held in close proximity, and the absorption spectrum of the acceptor and the emission spectrum of the donor overlap. Therefore FRET takes place and the acceptor fluorescence can be observed. After the hydrolysis of the phosphodiester group by a phosphodiesterase, FRET no longer takes place and the donor fluorescence can be observed because the donor and acceptor diffuse away from one another. Synthesis of CPF4 is described in Scheme 2. The control compounds **1** and **2** were also synthesized to investigate the spectroscopic properties of CPF4.

The emission spectrum of CPF4 in an aqueous buffer solution excited at 370 nm, which is the excitation wavelength of the coumarin moiety, is shown in Figure 1 (spectrum A). The emission of the coumarin donor at around 450 nm was strongly quenched and the emission of the fluorescein acceptor at around 515 nm was observed. This emission spectrum demonstrates that energy transfer from the coumarin donor to the fluorescein acceptor can proceed efficiently. The energy transfer efficiency

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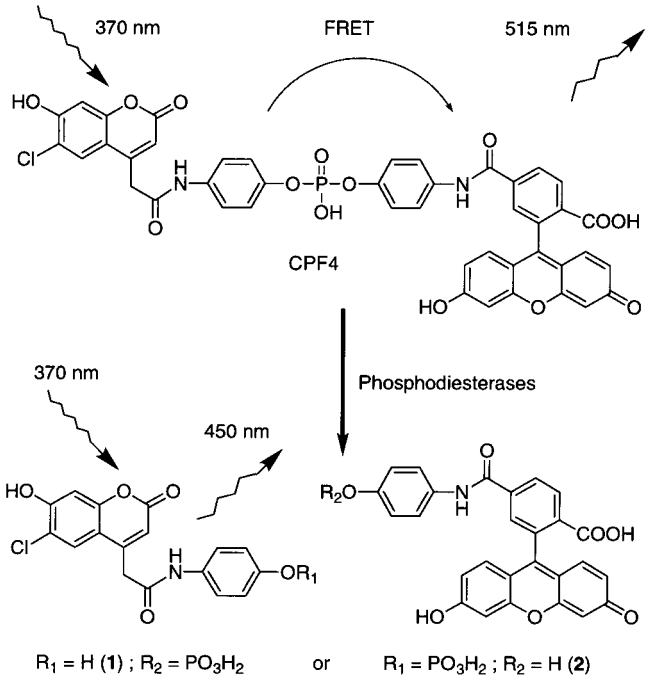
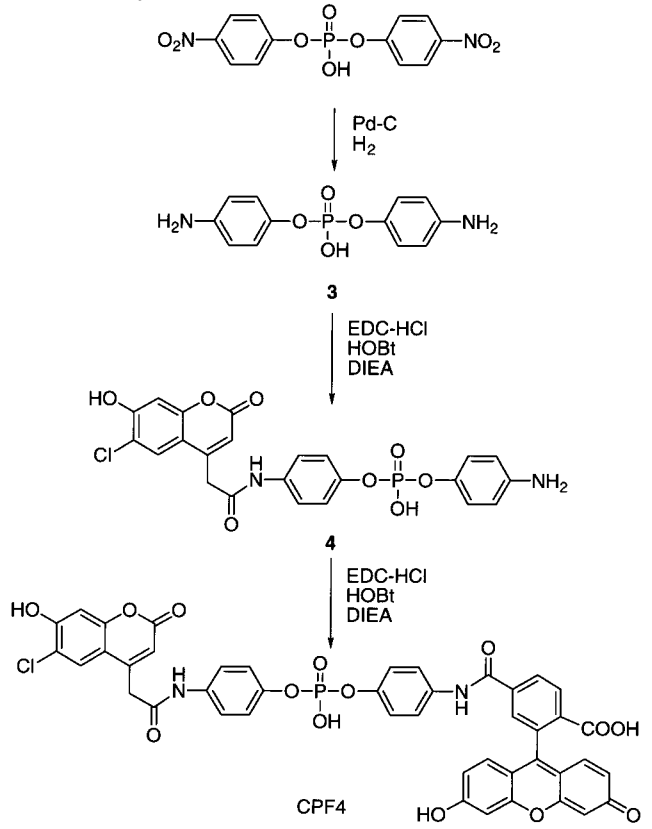
Scheme 1. The Structure of CPF4 and the Sensor Mechanism



Scheme 2. Synthetic Scheme for CPF4



was calculated as 94%. According to the Förster theory,[2] the rate of FRET is inversely proportional to R.[6] So, the energy transfer efficiency is strongly dependent on R when R is near the Förster distance (R_0), which can be defined as the donor−acceptor distance at which the transfer efficiency is 50%. The transfer efficiency abruptly increases to 100% as R decreases below R_0. In CPF4, R was estimated to be shorter than 30 Å by means of the CPK model and R_0 was calculated to be approximately 48 Å from the fluorescence of compound **1** and the absorption of compound **2**. Thus, these values indicate that the transfer efficiency should be close to 100% and the experimentally obtained value for the FRET efficiency of CPF4 (94%) is reasonable. Figure 2 shows the pH profile of fluorescence intensity of the donor and acceptor. Both the donor and acceptor fluorescence strikingly decreased below pH 7. The pK_a values of the donor and acceptor emission were calculated as 5.95 and 6.48, respectively. The pH profile of the fluorescence ratio of the donor and acceptor emission is also shown. The fluorescence ratio value was hardly affected by change of pH in the physiologically possible region.

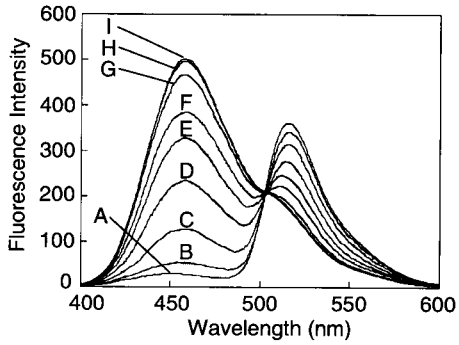


Figure 1. The emission spectra of a 1.0 μM solution of CPF4 in Tris-HCl buffer (100 mM, pH 8.8) at 37 °C: (A) without enzyme; (B) 1 min; (C) 5 min; (D) 10 min; (E) 15 min; (F) 20 min; (G) 30 min; (H) 45 min after addition of 0.05 u of phosphodiesterase I (Crotalus adamanteus venom); (I) a mixture of 1.0 μM **1** and 1.0 μM **2**.

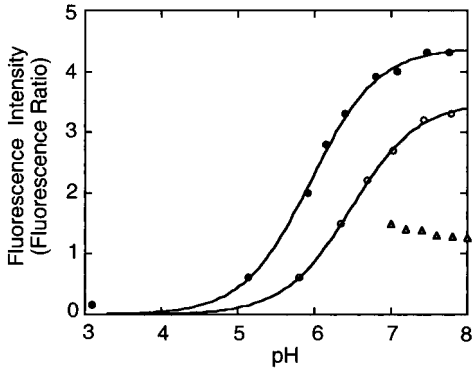


Figure 2. Effect of pH on the fluorescence intensity of CPF4. Closed circle: fluorescence intensity of 1.0 μM compound **1**, λ_{ex} 370 nm, λ_{em} 450 nm. Open circle: fluorescence intensity of 1.0 μM CPF4, λ_{ex} 370 nm, λ_{em} 515 nm. Open triangle: fluorescence ratio of compound **1** and CPF4. Solvent: 100 mM sodium phosphate buffer.

The absorption spectra of CPF4 and the mixture of compounds **1** and **2** were measured in aqueous environment (Figure 3). It was reported that a large red shift in the absorption spectrum is observed if the coumarin and fluorescein moieties form dye-to-dye close contact.[7,8] CPF1, which shows fluorescence quenching due to dye-to-dye close contact, exhibited a large red shift in its absorption spectrum (λ_{max} = 380 nm, 500 nm). No shift in the absorption spectrum of CPF4 (λ_{max} = 372 nm, 495 nm) was observed compared to the control (λ_{max} = 372 nm, 495 nm). So, the absorption spectrum demonstrates that the two fluorophores in CPF4 cannot interact with each other and fluorescence quenching due to dye-to-dye close contact is not observed.

Enzyme Assay. Addition of a phosphodiesterase, for example phosphodiesterase I from Crotalus adamanteus venom,[14] to an

Table 1. Summary of CPFs

CPFs	abs max (nm) donor	abs max (nm) acceptor	acceptor quantum eff[c]	FRET eff[d] (%)	K_m[e] (M)	v_{max}[e] (mol/min/mg)
CPF1[a]	380	500	0.10		2.5×10^{-6}	1.6×10^{-9}
CPF2[a]	372	494	0.83	>95		
CPF3[a]	373	497	0.27			
CPF4	372	495	0.58	94	1.6×10^{-7}	1.8×10^{-9}
control[b]	372	494	0.69			
bis(4-nitrophenyl) phosphate					1.0×10^{-3}	3.4×10^{-7}

[a] Reference 10. [b] A mixture of **1** and **2**. [c] Data were measured in aqueous buffers, pH 7.4 (λ_{ex} = 492 nm, λ_{em} = 515 nm). [d] Data were obtained from the fluorescence quantum yield of donor in the presence and in the absence of acceptor. [e] Data were measured in 100 mM Tris-HCl buffer (pH 8.8) at 37 °C.

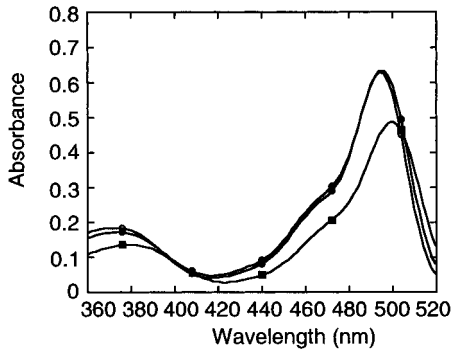


Figure 3. The absorption spectra of a 10 μM solution of CPF4 (open circle), a mixture of 10 μM **1** and 10 μM **2** (closed circle), and a 10 μM solution of CPF1 (closed square) in HEPES buffer (100 mM, pH 7.4) at 25 °C.

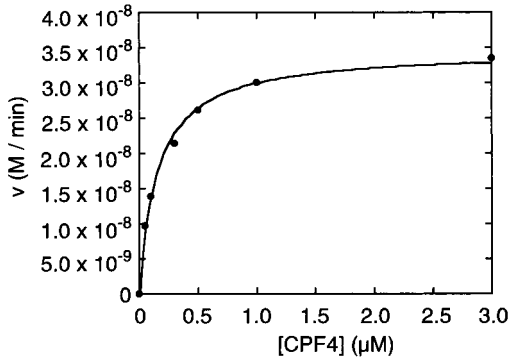


Figure 4. Michaelis−Menten plot of CPF4 hydrolysis by phosphodiesterase I (0.01 u) in 100 mM Tris-HCl buffer (pH 8.8) at 37 °C.

aqueous solution of CPF4 resulted in a rapid increase in the donor fluorescence at around 460 nm and a rapid decrease in the acceptor fluorescence at around 515 nm as shown in Figure 1. The emission intensity of CPF4 asymptotically approached that of an equimolar mixture of **1** and **2** (spectrum I). CPF4 exhibited a large shift in its emission spectrum after the hydrolysis of the phosphodiester group by the enzyme. The emission maximum shifted from 515 to 460 nm. This large shift of the emission spectrum indicates that ratiometric measurements can be feasible by using CPF4. A plot of initial velocity of hydrolysis versus substrate concentration is shown in Figure 4. The values of initial velocity were calculated from the enhancement of the donor emission intensity (λ_{ex} 370 nm, λ_{em} 450 nm). From this Michaelis−Menten plot, K_m and v_{max} were calculated as 160 nM and 1.8×10^{-9} mol/min/mg, respectively. The CPFs data are summarized in Table 1. As a standard, K_m and v_{max} of bis(4-nitrophenyl) phosphate were also measured under the same conditions (Table 1). The kinetic parameters (K_m and v_{max}) of phosphodiesterase I were reported against the various substrates.[13,15] It can be seen that these values vary widely according to the structures of the substrates [e.g., bis(4-nitrophenyl) phosphate:[13b] K_m = 770 μM, v_{max} = 650 nmol/min/mg; thymidine dinucleotide:[13b] K_m = 210 μM, v_{max} = 110 μmol/min/mg; 5′-ATP:[15a] $K_m \ll 200$ μM, v_{max} = 4.6 μmol/min/mg; *p*-nitrophenyl thymidine 5′-phosphate:[15b] K_m = 46 μM, v_{max} = 50 μmol/min/mg]. Comparing the experimentally obtained kinetic parameters of CPF4 with the literature data, K_m and v_{max} values of CPF4 are much lower than that for the other substrates including some native substrates. These observations suggest that CPF4 have a high affinity for phosphodiesterase I though the catalytic rate of CPF4 is relatively slow. In addition to phosphodiesterase I, phosphodiesterases consist of cyclic-nucleotide phosphodiesterases and nucleic acid phosphodiesterases (ribonucleases and deoxyribonucleases). So, we performed the fluorogenic assays for these two enzymes by using CPF4. Consequently, the fluorescence spectrum of CPF4 was not affected by the addition of phosphodiesterase 3′,5′-cyclic-nucleotide (from bovine heart, Sigma) nor nuclease (from staphylococcus aureus, Sigma). These observations indicate that CPF4 should be a specific probe for phosphodiesterase I.

Conclusion

The phenyl linkers were sufficiently rigid to prevent dye-to-dye close contact and the diphenyl phosphate could be recognized as a substrate by phosphodiesterase I. Thus, we succeeded in developing a ratiometric fluorescence sensor molecule for phosphodiesterase I activity by introducing two phenyl linkers between the donor and acceptor moieties. Since CPF4 allows more precise and quantitative detection of phosphodiesterase I activity, it should be useful for studies on the biological functions of phosphodiesterases. For example, CPF4 will be applied as a ratiometric probe for the molecules that possess the phosphodiesterase I activity such as NPPs. The method described in this paper for designing enzyme-cleavable sensor molecules based on FRET should be readily applicable to other hydrolytic enzymes.

Experimental Section

Instruments. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Mass spectra (MS) were measured with JEOL SX-102A and JEOL JMS-700T mass spectrometers.

(14) Phosphodiesterase I (from Crotalus adamanteus venom), Sigma. Definition of activity by the suppliers: 1 unit hydrolyzes 1.0 μmol of bis(*p*-nitrophenyl)phosphate per min at pH 8.8 and 37 °C.

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Synthesis of Bis(4-aminophenyl) Phosphoric Acid (3). Bis(4-nitrophenyl)phosphoric acid (1.0 g, 2.9 mmol) was dissolved in ethanol−water (2:1) (30 mL). After the addition of 10% Pd−C (300 mg), the mixture was stirred vigorously under a H_2 atmosphere for 3 h. The Pd−C was filtered off and washed with water. The residue after evaporation of the filtrate was purified by column chromatography with use of HP-20SS (Mitsubishi Chemical Corporation, Tokyo, Japan) resin, eluted with H_2O. The appropriate fractions, after evaporation to dryness, gave 600 mg of **3** (yield 73%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.35 (s, 4H); 6.51 (d, 4H, J = 8.8 Hz); 6.82 (d, 4H, J = 8.8 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 119.0; 120.5; 135.3; 148.0. MS (FAB): 181 ($M + H^+$).

Synthesis of 2-Aminophenyl 6-Chloro-7-hydroxycoumarin-4-acetamidophenyl Phosphoric Acid (4). Compound **3** (150 mg, 0.52 mmol) and 6-chloro-7-hydroxycoumarin-4-acetic acid (140 mg, 0.52 mmol), which had been prepared from chlororesorcinol and acetonedicarboxylic acid, were dissolved in DMF. Diisopropylethylamine (DIEA) (0.10 mL, 580 μmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC−HCl) (190 mg, 1.0 mmol), and hydroxybenzotriazole (HOBt) (130 mg, 0.96 mmol) were added to the solution and the mixture was stirred at 0 °C overnight. The reaction mixture was poured into 2 N HCl (50 mL) and the resulting precipitate was removed by filtration. The mother liquor was evaporated and the residue was purified by column chromatography with use of HP-20SS resin, eluted with H_2O to MeOH/H_2O = 1/1. The appropriate fractions, after evaporation to dryness, gave 24 mg of **4** (yield 8.9%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.87 (s, 2H); 6.30 (s, 1H); 6.91 (s, 1H); 6.98 (d, 2H, J = 8.4 Hz); 7.06 (d, 2H, J = 9.0 Hz); 7.08 (d, 2H, J = 8.4 Hz); 7.41 (d, 2H, J = 9.0 Hz); 7.85 (s, 1H); 10.2 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 103.5; 112.3; 113.0; 116.9; 119.9; 120.3; 120.8; 121.8; 125.6; 126.4; 133.6; 149.1; 150.2; 150.8; 153.2; 156.4; 159.8; 166.1. MS (FAB): 517 ($M + H^+$).

Synthesis of CPF4. Compound **4** (16 mg, 31 μmol) and 6-carboxyfluorescein (23 mg, 62 μmol), which had been prepared according to the procedure reported by Rossi and Kao,[16] were dissolved in DMF. DIEA (10 μL, 58 μmol), EDC−HCl (19 mg, 0.1 mmol), and HOBt (14 mg, 0.1 mmol) were added to the solution and the mixture was stirred at 0 °C overnight. The reaction mixture was poured into 2 N HCl (5 mL) and the resulting precipitate was collected by filtration. This precipitate was purified by column chromatography with use of HP-20SS resin, eluted with H_2O. The appropriate fractions, after evaporation to dryness, gave 2.1 mg of compound CPF4 (yield 7.7%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.68 (s, 2H); 5.98 (d, 2H, J = 2.0 Hz); 6.06 (dd, 2H, J = 2.0, 9.1 Hz); 6.61 (d, 2H, J = 9.1 Hz); 7.12 (d, 4H, J = 9.0 Hz); 7.46 (d, 2H, J = 9.0 Hz); 7.49 (s, 1H); 7.61 (d, 2H, J = 9.0 Hz); 7.75 (s, 1H); 8.09 (s, 2H). ^{13}C NMR (75 MHz, D_2O) δ 39.9; 105.1; 106.3; 108.5; 108.6; 113.3; 118.5; 121.8; 122.0; 123.7; 124.2; 124.3; 124.5; 125.3; 130.0; 132.3; 132.7; 134.3; 134.4; 135.6; 144.3; 150.1; 150.2; 152.7; 155.9; 158.2; 159.7; 166.2; 168.6; 169.0; 170.5; 175.3; 181.9. HRMS (FAB^+): calcd for ($M + H^+$), 875.1045; found, 875.1080.

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Synthesis of 6-Chloro-7-hydroxy-4-(4-hydroxyphenylcarbamoylmethyl)coumarin (1). 4-Aminophenol (420 mg, 3.9 mmol) and 6-chloro-7-hydroxycoumarin-4-acetic acid (100 mg, 0.39 mmol) were dissolved in DMF. EDC−HCl (150 mg, 0.77 mmol) and HOBt (100 mg, 0.74 mmol) were added to the solution and the mixture was stirred at 0 °C overnight. The reaction mixture was diluted with 2 N HCl and was extracted with ethyl acetate. The organic phase was washed with 2 N HCl and brine and dried over sodium sulfate. The residue after the removal of solvent was chromatographed on silica gel, eluted with dichloromethane to dichloromethane/MeOH = 19/1. The appropriate fraction gave 80 mg of **1** (yield 59%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.83 (s, 1H); 6.29 (s, 1H); 6.68 (d, 2H, J = 8.6 Hz); 6.91 (s, 1H); 7.33 (d, 2H, J = 8.6 Hz); 7.69 (m, 1H); 7.86 (s, 1H); 9.21 (s, 1H); 10.0 (s, H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 39.3; 103.4; 112.2; 112.8; 115.5; 116.9; 121.2; 126.3; 130.3; 150.3; 153.2; 153.6; 156.4; 159.7; 165.7. HRMS (EI^+): calcd for M^+, 345.0404; found, 345.0425.

Synthesis of 6-(2-Hydoxyphenylcarbamoyl)fluorescein (2). 4-Aminophenol (390 mg, 3.6 mmol) and 6-carboxyfluorescein (100 mg, 0.27 mmol) were dissolved in DMF. EDC−HCl (160 mg, 0.84 mmol) and HOBt (110 mg, 0.81 mmol) were added to the solution and the mixture was stirred at 0 °C overnight. The reaction mixture was diluted with 2 N HCl and extracted with ethyl acetate. The organic phase was washed with 2 N HCl and brine, and dried over sodium sulfate. The residue after the removal of solvent was chromatographed on silica gel, eluted with dichloromethane/MeOH = 19/1 to dichloromethane/MeOH = 9/1. The appropriate fraction gave 70 mg of **2** (yield 56%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.56 (dd, 2H, J = 3.0, 8.6 Hz); 6.61 (d, 2H, J = 8.6 Hz); 6.69 (d, 2H, J = 3.0 Hz); 6.69 (d, 2H, J = 9.2 Hz); 7.42 (d, 2H, J = 9.2 Hz); 7.80 (s, 1H); 8.11 (d, 1H, J = 7.9 Hz); 8.25 (d, 1H, J = 7.9 Hz); 9.27 (s, 1H); 10.1 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 83.4; 102.2; 109.1; 112.7; 114.9; 122.6; 122.7; 124.8; 128.2; 129.2; 129.7; 129.9; 141.2; 151.8; 152.6; 154.1; 159.6; 163.1; 168.0. HRMS (FAB^+): calcd for ($M + H^+$), 468.1083; found, 468.1115.

Fluorometric Analysis. A fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) was used. The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. CPF4 was dissolved in DMSO to make a 10 mM stock solution, which was diluted to the required concentration for measurement.

Absorption Analysis. A spectrometer (UV-1600, Hitachi, Japan) was used. All samples were prepared from 10 mM stock solutions in DMSO.

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