Leucine aminopeptidase (LAP) assay

Leu-Abd (10 μM) was dissolved in 100 mM HEPES buffer (pH 7.4) with 10μM $Tb(NO_3)_3$. The reaction buffer was stirred for 1 hour for complexation before the enzyme reaction. The samples were incubated with 0.002 U LAP at 37 °C.

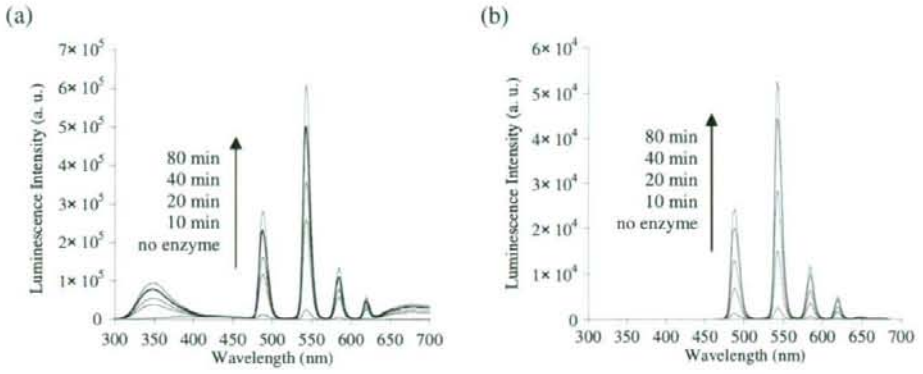

Figure S14. (a) Steady-state and (b) time-resolved (delay time: 60 μs, gate time: 2.0 ms) luminescence spectral change of 10 μM LY-Abd-Tb with LAP. λ_{ex} = 250 nm.

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Communication

Covalent Protein Labeling Based on Noncatalytic #-Lactamase and a Designed FRET Substrate

Shin Mizukami, Shuji Watanabe, Yuichiro Hori, and Kazuya Kikuchi

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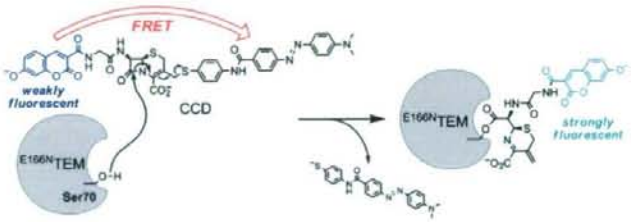

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COMMUNICATIONS

Covalent Protein Labeling Based on Noncatalytic β-Lactamase and a Designed FRET Substrate

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Fluorescence microscopy is one of the most common techniques employed in the field of life science. With the rapid progress that has been achieved with regard to optical systems, fluorescent proteins (FPs) have acquired important roles for fluorescence microscopy experiments. In order to visualize the localization and behavior of particular proteins of interest, FPs such as green fluorescent protein (GFP) have conventionally been used.[1] More recently, techniques for labeling proteins with small molecules have attracted the attention of many life scientists because they can extend the range of natural FPs, for example, by incorporating near-infrared fluorescent dyes, MRI contrast agents, or biofunctional molecules such as biotin. Several approaches for modifying proteins with small molecules have been commercialized, including methods based on the tetracysteine tag,[2] HaloTag,[3] and SNAP-tag.[4] Other protein labeling methods involving the use of biotin ligase,[5] transglutaminase,[6] hexahistidine,[7] tetra-aspartic acid,[8] etc. have also been reported. Among the abovementioned labeling methods, only the tetracysteine tag exhibits fluorogenic properties. In the other labeling methods, it is necessary to wash the treated cells prior to microscopic measurements to eliminate background fluorescence. Thus, new labeling techniques that satisfy the dual criteria of specificity and fluorogenicity are desirable.

In this paper, we report a specific protein labeling system with an off-on fluorescence switch. It involves covalent modification of a genetically engineered hydrolytic enzyme with a rationally designed fluorogenic probe that exploits the principle of fluorescence resonance energy transfer (FRET). Using this system, we can achieve specific and fluorogenic protein labeling under physiological conditions.

First, we designed the tag protein. Plant or bacterial proteins are preferably used to achieve bioorthogonal labeling in mammalian cells. We focused on β-lactamase as the candidate tag because β-lactamases are small bacterial enzymes that hydrolyze antibiotics containing a β-lactam structure and have no endogenous counterpart among eukaryotic cells.[9] β-Lactamase has been widely used as a reporter enzyme for examining gene expression in living mammalian cells.[10] Class A β-lactamases such as the 29 kDa TEM-1[11] have been extensively investigated with regard to their structures, enzyme reaction kinetics, substrate specificity, inhibitors, etc.[12] The reaction of TEM-1 with β-lactams involves acylation and deacylation steps (Scheme 1). In the acylation step, Ser70 attacks the amide bond of the β-lactam ring to form an intermediate acyl-enzyme complex (ES*). In the deacylation step, an activated water molecule hydrolyzes the ester bond of the intermediate to yield the product. Previous studies have shown that Glu166 is essential for the deacylation step[13] and that the E166N mutant of TEM-1 (^{E166N}TEM) accumulates the acyl-enzyme intermediate by markedly slowing deacylation (k_3) relative to acylation (k_2).[14] We hoped to exploit the properties of the ^{E166N}TEM mutant to covalently attach a fluorescent substrate to β-lactamase.

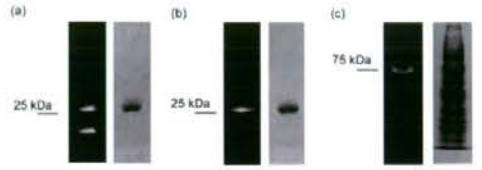
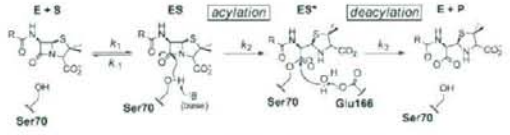
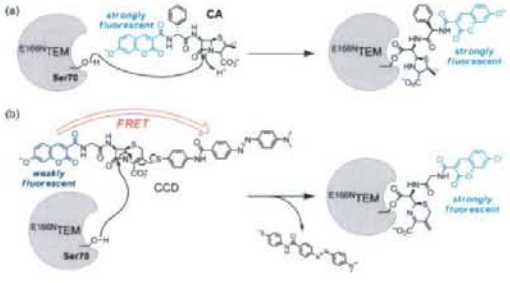


Figure 1. (a, b) Fluorescence (left) and CBB-stained (right) gel images of ^{E166N}TEM after incubation with (a) CA and (b) CCD. (c) Fluorescence and CBB-stained gel image of MBP-^{E166N}TEM mixed with HEK293T cell lysate after incubation with CCD.

Scheme 1. Mechanism of β-Lactam Cleavage by Class A β-Lactamases; (E) Enzyme, (S) Substrate, and (P) Product



Scheme 2. Structures and Labeling Mechanisms of the Fluorescent Probes (a) CA and (b) CCD



To investigate the feasibility of fluorescently labeling ^{E166N}TEM under physiological conditions, we designed and synthesized a penicillin-based fluorescent probe, coumarinyl ampicillin (CA). The labeling scheme is illustrated in Scheme 2a. Since CA contains 7-hydroxycoumarin, successfully labeled ^{E166N}TEM should exhibit cyan fluorescence. ^{E166N}TEM was incubated with CA in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C, and protein labeling was assessed by SDS-PAGE. Fluorescent proteins were detected by irradiating the gels with UV light at 365 nm. When purified ^{E166N}TEM was mixed with CA, a protein band of ~29 kDa was observed that exhibited cyan fluorescence (Figure 1a); Coomassie Brilliant Blue (CBB) staining confirmed that this band corresponded to ^{E166N}TEM. In contrast, when wild-type (WT) TEM-1 was incubated with CA, no cyan fluorescence was seen (Figure S1a). Although CA successfully labels ^{E166N}TEM, other fluorescent bands were also observed on the gel. Since these bands were also seen when only

10.1021/ja8082285 CCC: $40.75

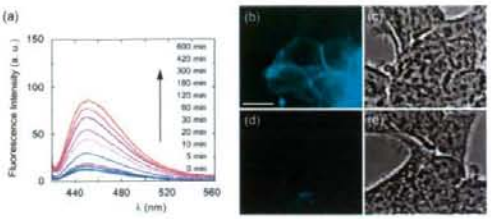


Figure 2. (a) Time-dependent emission spectra (λ_{ex} = 410 nm) of CCD (1 μM) in the presence of ^{E166N}TEM in 100 mM HEPES buffer (pH 7.4) containing 0.1% DMSO at 25 °C. (b–e) Optical microscopic images of CCD-labeled HEK293T cells expressing ^{E166N}TEM-EGFR (b,c) and EGFR (d,e), labeled with 5 μM CCD. (b,d) Fluorescence microscopic images, excitation at 410 nm. (c,e) phase contrast microscopic images. Scale bar: 10 μm.

CA was electrophoresed (Figure S2), a washing procedure should be performed before observation under a fluorescence microscope.

Next, we designed and synthesized CCD (Scheme 2b), a fluorescence off–on labeling probe. This molecule has three main components: 7-hydroxycoumarin, cephalosporin, and 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL). Since the absorption spectrum of DABCYL substantially overlaps with the emission spectrum of 7-hydroxycoumarin, the fluorescence of CCD would be expected to be largely quenched by intramolecular FRET from coumarin to DABCYL. Based on related probes of β-lactamase activity,[10] cleavage of the β-lactam of CCD by ^{E166N}TEM should result in covalent attachment of the coumarin to the protein with concomitant release of the DABCYL moiety as shown in Scheme 2b. After loss of the DABCYL group, the cyan fluorescence of coumarin should be restored by cancelation of FRET.

The fluorescence spectrum of CCD confirmed that the coumarin fluorescence was almost completely quenched because of FRET. The fluorescence quantum yield of CCD was 0.006, which is much lower than that of CA (Φ = 0.40). When CCD was incubated with ^{E166N}TEM, the fluorescence increased considerably in a time-dependent manner (Figure 2a). This indicates that ^{E166N}TEM cleaved the β-lactam of CCD and eliminated the DABCYL group. When the DABCYL group was completely eliminated by WT TEM-1, the fluorescence signal increased approximately 30-fold. The apparent rate of reaction between CCD and ^{E166N}TEM was approximately 80-fold slower than that of the reaction between CCD and WT TEM-1 (Figure S3), probably because the mutation at E166 decreases the acylation rate (k_2) somewhat.[15]

CCD specifically labels ^{E166N}TEM, as demonstrated by incubation of the probe molecule with both ^{E166N}TEM and WT TEM-1 in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C, followed by SDS-PAGE analysis. As shown in Figure 1b, only the band corresponding to ^{E166N}TEM exhibited cyan fluorescence; no fluorescence was associated with WT TEM-1 (Figure S1b). In contrast to CA, unreacted CCD yielded considerably weaker fluorescence on the gel. In MALDI-TOF MS analyses of the samples, the molecular mass peak for the protein–probe adduct was only detected when ^{E166N}TEM was incubated with CCD (Figure S4).

This system can therefore be used to label target proteins in a biological medium. For example, we fused ^{E166N}TEM to maltose binding protein (MBP, 42 kDa), mixed the purified MBP–^{E166N}TEM construct with HEK293T cell lysate, and incubated the mixture with CCD at 25 °C for 45 min. SDS-PAGE analysis revealed that fusion protein was efficiently and selectively labeled with the fluorogenic probe (Figure 1c).

Finally, we investigated specific labeling of target proteins displayed on the surface of living cells. ^{E166N}TEM was fused to the N-terminus of epidermal growth factor receptor (EGFR), a membrane associated protein, and the construct was produced in HEK293T cells. After treatment with CCD (see Supporting Information), the cells were examined under a fluorescence microscope. Only cells producing the ^{E166N}TEM-EGFR fusion protein emitted cyan fluorescence as a consequence of specific labeling by the probe (Figure 2b–e).

In conclusion, we have developed a novel protein labeling system that combines a genetically modified β-lactamase with low molecular weight fluorogenic β-lactam probes. Through appropriate probe design, we succeeded in labeling proteins with a sensitive fluorophore in vitro and on living cells. In principle, this system does not require washing procedures to remove the unreacted probes after labeling. Furthermore, since the ^{E166N}TEM tag protein is absent in mammalian cells, it can be used for the specific labeling of proteins in higher eukaryotes. We anticipate that this labeling system will find wide application in the field of life science.

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Supporting Information Available: Detailed experimental procedures and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA8082285

Supporting Information

Covalent Protein Labeling Based on Non-catalytic β-Lactamase by Using FRET Substrate

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CONTENTS:

1. Materials and Instruments

General Chemicals for organic synthesis were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co, and were used without further purification. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE·HCl) was obtained from Otsuka Chemical Co. Ltd. Restriction endonucleases and PrimeSTAR® HS DNA Polymerase were purchased from Takara Bio Inc. Kanamycin and inorganic chemicals were from Wako Pure Chemical or Nacarai Tesque. Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (Qiagen Inc.).

XL10-Gold (Stratagene) was used as the intermediate host in site-directed mutagenesis experiments. *E. coli* BL21(DE3) (Novagen) was the host for target gene expression. Luria-Bertani (LB) medium or Terrific Broth (TB) was used as the growth medium for all bacterial strains. Bacto Agar (Wako Pure Chemical) was added at a concentration of up to 1.5% for the preparation of solid medium.

NMR spectra were recorded on a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H and at 100.4 MHz for ^{13}C NMR, using tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-700 for FAB, on a Waters LCT-Premier XE for ESI, and on an Applied Biosystems Voyager RP for MALDI. UV-Visible absorbance spectra were measured using a Shimadzu UV1650PC spectrometer. Fluorescence spectra were measured using a Hitachi F4500 spectrometer. Slit width was 2.5 nm for both excitation and emission, and the photomultiplier voltage was 700 V. Fluorescence microscopic images were recorded using IX71 (Olympus) for the fluorescent microscope, Cool Snap HQ (Roper Scientific) for the cooled CCD camera, Polychrome V (TILL Photonics) for the xenon lamp with a monochromator, U-MNV2 DM455 (Olympus) for the dichroic mirror, U-MNV2 BA455 (OLYMPUS) for the emission filters, and MetaMorph (Universal Imaging Corporation) for the imaging software and data analysis. All probes were dissolved in DMSO (biochemical grade, Wako) before fluorescence measurement in order to be solubilized in the aqueous solvents. Silica gel column chromatography was performed using BW-300 (Fuji Silysia Chemical Ltd.).

2. Syntheses of Compounds (Schemes S1 and S2)

Synthesis of I. 2,4-Dihydroxy benzaldehyde (3.0 g, 22 mmol) and diethyl malonate (4.2 g, 26 mmol) were dissolved in anhydrous EtOH (30 mL), then piperidine (370 mg, 4.3 mmol) was added. The reaction mixture was refluxed for 12 h, then cooled in ice bath. The precipitate was collected and washed with cold EtOH to afford **I** (2.5 g, y. 49%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.29 (t, J = 6.9 Hz, 3H), 4.28 (q, J = 6.9 Hz, 2H), 6.69 (d, J = 1.8 Hz, 1H), 6.85 (dd, J = 1.8, 8.7 Hz), 7.74 (d, J = 8.7 Hz. 1H), 8.67 (s, 1H), 11.1 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 15.2, 61.9, 102.9, 111.5, 113.1, 115.1, 133.1, 150.4, 157.5, 158.1, 164.0, 165.2; HRMS (ESI^{+}) *m/z*: 257.0406 (Calcd for $[M+H]^{+}$: 257.0426).

Synthesis of II. Compound **I** (0.39 g, 1.6 mmol) was dissolved in 2 N NaOH aq. (5 mL) and was stirred at RT for 12 h. The solution was acidified with 2 N HCl aq., then the precipitate was collected and washed with water to afford **II** (0.29 g, y. 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.74 (d, J =

2.1 Hz, 1H), 6.84 (dd, J = 2.1, 8.7 Hz, 1H), 7.74 (d, J = 8.7 Hz, 1H), 8.68 (s, 1H), 11.1 (br, 1H), 12.8 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 102.4, 111.7, 113.6, 115.1, 133.1, 150.5, 158.1, 158.6, 165.0, 165.3; HRMS (ESI$^+$) m/z: 206.0215 (Calcd for [M+H]$^+$: 206.0220).

Synthesis of III. Compound **II** (0.25 g, 1.2 mmol) and *N*-hydroxysuccinimide (0.21 g, 1.8 mmol) were dissolved in DMF (5 mL), then WSCD·HCl (0.35 g, 1.8 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 3 h under Ar. The reaction mixture was diluted with ethyl acetate and washed with 10% citric acid aq., and water, then dried with brine and sodium sulfate. Evaporation yielded compound **III** (0.24 g, y. 66%). ^{1}H NMR (400 MHz, DMSO-d_6) δ2.91 (s, 4H), 6.77 (d, J = 1.8 Hz, 1H), 6.89 (dd, J = 1.8, 8.7 Hz, 1H), 7.87 (d, J = 8.7 Hz, 1H), 9.00 (s, 1H), 11.4 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 102.4, 111.7, 113.6, 115.1, 133.1, 150.5, 158.1, 158.6, 165.0, 165.3; HRMS (ESI$^+$) m/z: 326.0263 (Calcd for [M+H]$^+$: 326.0277).

Synthesis of CA. Ampicillin (0.35 g, 0.99 mmol) was dissolved in DMF (20 mL), then DIEA (one drop) and compound **III** (0.20 g, 0.66 mmol) were added at 0 °C. The mixture was stirred for 10 h. Then 10% citric acid aq. was added and the mixture was extracted by AcOEt. The organic phase was washed with 10% citric acid aq. and water, then was dried with brine and sodium sulfate. Evaporation yielded coumarinyl ampicillin (CA) (0.31 g, y. 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.41 (s, 3H), 1.55 (s, 3H), 4.21 (s, 1H), 5.40 (d, J = 4.0 Hz, 1H), 5.56 (dd, J = 3.0 Hz, 7.7 Hz, 1H), 5.92 (d, J = 7.6 Hz, 1H), 6.83 (s, 1H), 6.88 (d, J = 8.4 Hz, 1H), 7.35 (m, 7H), 7.83 (d, J = 8.43 Hz, 2H), 9.38 (d, J = 7.6 Hz, 2H), 9.62 (d, J = 7.6 Hz, 2H), 11.1 (s, 1H), 13.4 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ26.5, 30.2, 55.5, 58.0, 63.6, 67.0, 70.2, 101.8, 111.0, 112.9, 114.3, 126.5, 127.7, 128.3, 132.1, 138.1, 148.4, 156.3, 160.7, 161.2, 163.8, 168.8, 169.6, 173.1; HRMS (FAB$^+$) m/z: 538.1278 (Calcd for [M+H]$^+$: 538.1284).

Synthesis of 1. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE·HCl) (1.0 g, 2.5 mmol), HOBt monohydrate (760 mg, 4.9 mmol) and Boc-glycine (480 mg, 2.7 mmol) were dissolved in DMF (5 mL), then triethylamine (250 mg, 2.5 mmol) and WSCD·HCl (570 mg, 3.0 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 6 h under Ar. The reaction mixture was diluted with ethyl acetate, and washed with sat. $NaHCO_3$ aq., 10% citric acid, and water, and then the mixture was dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography, elution with hexane / ethyl acetate (1:1) afforded **1** (1.25 g, y. 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 3.48 (d, J = 18.4 Hz, 1H), 3.65 (d, J = 18.4 Hz, 1H), 3.81 (s, 3H), 3.83 (m, 2H), 4.43 (d, J = 12.0 Hz, 1H), 4.54 (d, J = 12.0 Hz, 1H), 4.97 (d, J = 5.2 Hz, 1H), 5.23 (s, 2H), 5.84 (dd, J = 5.2 Hz, 8.8 Hz, 1H), 6.89 (d, J = 8.8 Hz, 2H), 7.34 (d, J = 8.8 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.1, 28.2, 43.2, 44.1, 55.2, 57.5, 58.9, 68.2, 80.5, 113.9, 125.5, 126.2, 126.6, 130.6, 156.0, 159.9, 161.1, 164.6, 170.2, 171.2; HRMS (FAB$^+$) m/z: 526.1426 (Calcd for [M+H]$^+$: 526.1415).

Synthesis of 2. A mixture of **1** (560 mg, 1.1 mmol) and sodium iodide (1.6 g, 11 mmol) was stirred in acetone for 1 h at RT. The reaction mixture was concentrated under reduced pressure, and diluted with ethyl acetate. The organic layer was washed with water, then dried with brine and sodium

sulfate. The light orange amorphous residue was used without purification for the next reaction. The iodide derivative (400 mg, 0.65 mmol) was dissolved in DMF, and $NaHCO_3$ (80 mg, 0.95 mmol) was added. Then, DMF solution of *p*-aminothiophenol (120 mg, 0.95 mmol) was added dropwisely. The mixture was stirred at RT for 5 h under Ar. The mixture was diluted with ethyl acetate, and was washed with *sat.* $NaHCO_3$ aq., 10% citric acid aq., and water, then was dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography, elution with hexane / ethyl acetate (1:1) afforded **2** (260 mg, y. 54% (2 steps)). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 3.33 (d, J = 18.0 Hz, 1H), 3.59 (d, J = 13.6 Hz, 1H), 3.67 (d, J = 18.0 Hz, 1H), 3.75 (br, 2H), 3.81 (s, 3H), 3.84 (m, 2H), 4.17 (d, J = 13.6 Hz, 1H), 4.87 (d, J = 4.9 Hz, 1H), 4.98 (d, J = 11.7 Hz, 1H), 5.07 (d, J = 11.7 Hz, 1H), 5.17 (br, 1H), 5.84 (dd, J = 4.9 Hz, 8.8 Hz, 1H), 6.53 (d, J = 8.3 Hz, 2H), 6.89 (d, J = 8.8 Hz, 2H), 7.04 (d, J = 8.8 Hz, 1H), 7.15 (d, J = 8.3 Hz, 2H), 7.30 (d, J = 8.8 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.3, 28.6, 39.2, 44.3, 55.3, 57.5, 58.7, 67.6, 80.6, 113.9, 115.5, 120.6, 124.0, 127.0, 130.6, 130.7, 136.0, 147.1, 156.0, 159.8, 161.4, 164.4, 170.0; HRMS (FAB^+) *m/z*: 615.1940 (Calcd for $[M+H]^+$: 615.1947).

Synthesis of 3. Compound **2** (270 mg, 0.44 mmol) and 4-dimethylaminoazobenzene-4'-carboxylic acid (130 mg, 0.48 mmol) were dissolved in pyridine (5 mL), then $POCl_3$ (74 mg, 0.48 mmol) was dropwisely added at −20 °C for over 5 min. The mixture was stirred at −20 °C for 20 min under Ar. The reaction mixture was diluted with ethyl acetate, and was washed with sat. $NaHCO_3$ aq., 10% citric acid aq., and water, then was dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography, eluted with MeOH / CH_2Cl_2 (0% → 1%) to afford **3** (76 mg, y. 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9H), 3.11 (s, 6H), 3.40 (d, J = 18.0 Hz, 1H), 3.63 (d, J = 18.0 Hz, 1H), 3.80 (m, 3H), 4.15 (d, J = 13.3 Hz, 1H), 4.86 (d, J = 4.8 Hz, 1H), 5.04 (d, J = 11.8 Hz, 1H), 5.08 (d, J = 11.8 Hz, 1H), 5.24 (br, 1H), 5.71 (dd, J = 4.8 Hz, 9.0 Hz, 1H), 6.76 (d, J = 9.2 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 7.09 (d, J = 9.0 Hz, 1H), 7.28 (d, J = 8.6 Hz, 2H), 7.34 (d, J = 8.6 Hz, 2H), 7.54 (d, J = 8.6 Hz, 2H), 7.92 (m, 6H), 8.03 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.3, 28.3, 38.0, 40.3, 44.3, 55.2, 57.5, 58.8, 67.7, 80.6, 111.5, 113.9, 120.6, 122.4, 124.4, 125.5, 126.9, 128.1, 128.4, 129.4, 130.6, 134.3, 134.4, 138.1, 143.6, 152.9, 155.4, 156.0, 159.8, 161.4, 164.3, 165.2, 170.0; HRMS (FAB^+) *m/z*: 866.3000 (Calcd for $[M+H]^+$: 866.3006).

Synthesis of 4. Compound **3** (40 mg, 46 μmol) was dissolved in anhydrous CH_2Cl_2 (4 mL), then thioanisole (800 μL) and TFA (2.4 mL) were added at 0 °C. The mixture was stirred at 0 °C for 4 h, then was poured into cold ether (15 mL). The precipitate was collected and was washed with ether to afford **4** (36 mg, quant.) as the TFA salt. ^{1}H NMR (400 MHz, CD_3OD) δ 3.12 (s, 6H), 3.55 (d, J = 17.6 Hz, 1H), 3.76 (m, 3H), 3.93 (d, J = 13.6 Hz, 1H), 4.33 (d, J = 13.6 Hz, 1H), 5.06 (d, J = 4.9 Hz, 1H), 5.72 (d, J = 4.9 Hz, 1H), 6.85 (d, J = 9.3 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H), 7.70 (d, J = 8.8 Hz, 2H), 7.88 (t, J = 9.3 Hz, 4H), 8.04 (d, J = 9.3 Hz, 2H); ^{13}C NMR (100 MHz, DMF-d_7) δ 28.4, 35.8, 40.2, 41.6, 58.4, 59.9, 112.3, 121.3, 121.4, 122.4, 125.9, 126.0, 129.5, 129.7, 132.2, 133.1, 135.9, 140.0, 143.9, 154.0, 155.5, 162.9, 163.8, 165.0, 165.8, 167.9; HRMS (FAB^+) *m/z*: 646.1915 (Calcd for $[M+H]^+$: 646.1906).

Synthesis of CCD. Compound **4** (10 mg, 13 μmol) was dissolved in DMF (500 μL), then TEA (one

drop) and compound **III** (4.4 mg, 15 μmol) were added at RT. The mixture was stirred for 1 h, then the solvent was evaporated, and 100 mM ammonium formate aq. (15 mL) was added. The precipitate was collected and washed with 100 mM ammonium formate aq. and water thoroughly to afford pure CCD (10 mg, y. 91%). ^{1}H NMR (400 MHz, DMF-d_7) δ 3.13 (s, 6H), 3.61 (d, J = 18.1 Hz, 1H), 3.83 (d, J = 18.1 Hz, 1H), 4.02 (d, J = 13.2 Hz, 1H), 4.27 (d, J = 3.9 Hz, 2H), 4.34 (d, J = 13.2 Hz, 1H), 5.20 (d, J = 4.9 Hz, 1H), 5.83 (dd, J = 4.9 Hz, 8.8 Hz, 1H), 6.87 (d, J = 2.0 Hz, 1H), 6.91 (d, J = 9.3 Hz, 2H), 6.97 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 7.47 (d, J = 8.8 Hz, 2H), 7.90 (m, 7H), 8.20 (d, J = 6.8 Hz, 2H), 8.84 (s, 1H), 9.01 (d, J = 8.8 Hz, 1H), 9.18 (t, J = 5.4 Hz, 1H), 10.5 (s, 1H), 11.5 (brs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.3, 36.9, 39.8, 42.3, 57.7, 58.8, 101.8, 111.0, 111.6, 113.1, 114.4, 120.8, 121.6, 125.2, 128.9, 129.0 (two carbons), 129.6, 131.6, 132.1, 134.9, 138.4, 142.7, 148.3, 152.9, 154.3, 156.4, 160.9, 161.6, 163.0, 163.9, 164.2, 164.9, 169.2; HRMS (FAB$^+$) m/z: 834.2010 (Calcd for [M+H]$^+$: 834.2016).

3. Preparation of Proteins

Plasmid constructs and point mutation. The pET-24a(+)-WT TEM-1 vector was kindly provided by Prof. S. Mobashery (Georgia Institute of Technology).[S1] A point mutation to yield pET-24a(+)-^{E166N}TEM was generated in the pET-24a(+)-WT TEM-1 by using the QuikChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer's protocol. The mutagenic primers GTAACTCGCCTTGATCGTTGGAACCCGGAGCTGAATGAAGCC and GGCTTCATTCA-GCTCCGGGTTCCAACGATCAAGGCGAGTTAC were used. The double-stranded plasmid DNA was sequenced using the Sequenase DNA sequencing kit (BigDye Terminator version 3.1; Applied Biosystems) and a set of custom-made internal primers, according to the manufacturer's protocol.

Purification of β-lactamases. For enzyme purification, *E. coli* BL21(DE3) cells were cultured overnight in LB medium supplemented with 20 μg/mL kanamycin and subsequently diluted 100-fold in Terrific Broth supplemented with 20 μg/mL kanamycin, 500 mM sorbitol, and 2.5 mM betaine. After incubation for 4–5 h at 37 °C with shaking (260–280 rpm); until the optical density of the culture medium was 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM was added to the medium, and the cultures were incubated overnight at 25 °C. The cells were pelleted by centrifugation, and the supernatants containing the enzyme were concentrated and replaced by 10 mM Tris-HCl buffer (pH 7.0) using a Vivaspin centrifugal concentrator (membrane molecular weight cut off: 10,000; Sartorius). The concentrated proteins were loaded onto a DEAE Sephadex column equilibrated with the same buffer. The enzymes were eluted with a stepwise gradient elution (10–100 mM Tris-HCl buffer, pH 7.0).

Expression and purification of MBP-^{E166N}TEM. A DNA fragment of MBP was amplified from pMAL-c2(New England Biolabs) by PCR and was digested with *Nde*I and *Nhe*I. The DNA fragment was ligated to pET-21b(+), which was digested with the same restriction enzymes, to yield pET-21b(+)-MBP. For construction of pET-21b(+)-MBP-^{E166N}TEM fusion gene, the insert of ^{E166N}TEM, which was amplified from pET-24a(+)-^{E166N}TEM by PCR and treated with *Eco*RI and

*Hin*dIII, was ligated to similarly digested pET-21b(+)-MBP. MBP-^{E166N}TEM was expressed in *E. coli* BL21(DE3) cells in LB medium supplemented with 100 μg/mL ampicillin at 25 °C for 20 h after IPTG (100 μM) had been added to the growing cell culture (OD_{600} = 0.6–1.0). The cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris-HCl buffer (pH 7.4), 200 mM NaCl, and 1 mM EDTA), and lysed by sonication. The lysate was applied to an amylose resin column (New England Biolabs), and the fusion protein was eluted using an elution buffer (20 mM Tris-HCl buffer (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 10 mM maltose).

Construction of the ^{E166N}TEM-EGFR. A DNA fragment of ^{E166N}TEM was amplified from pET-24a(+)-^{E166N}TEM by PCR and was digested with *Nhe*I. The DNA fragment was ligated to pcDNA3.1(+)-EGFR[S3], which was digested with the same restriction enzymes, to yield pcDNA3.1(+)-^{E166N}TEM-EGFR.

4. Labeling Experimental Procedures

Detection of protein labeling by SDS-PAGE. WT TEM-1 or ^{E166N}TEM (20 μM) was added to a solution of CCD (30 μM) in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C. After 30 min, labeled protein was solubilized in 2 × SDS gel loading buffer (100 mM Tris-HCl buffer (pH 6.8), 2.5% SDS, 20% glycerol, and 10% mercaptoethanol) and resolved by SDS-PAGE. Fluorescence images of the gels were then captured using a digital camera under 365-nm illumination by a UV lamp. The gels were stained with Coomassie Brilliant Blue, and images of the stained gels were captured (Figure 1 (a, b), Figure S1). Purified MBP-^{E166N}TEM (4 μM) was mixed with HEK293T cell lysate, which was prepared by a freeze-thaw method. The mixture was added to a solution of CCD (6 μM) in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C. After 30 min, labeled protein was analyzed as described above (Figure 1 (c)).

Labeling of cell surface pcDNA3.1(+)-^{E166N}TEM-EGFR with CCD. HEK293T cells maintained in 10% FBS in DMEM (Invitrogen) at 37 °C under 5% CO2 were transfected with the pcDNA3.1(+)-^{E166N}TEM-EGFR plasmids using Lipofectamine 2000 (Invitrogen). After 5–6 h, the culture medium was replaced with DMEM (without phenol red), and the cells were incubated at 37 °C for 24 h. Then, the cells were washed three times with DMEM and incubated with 5 μM CCD for 1 h in a CO_2 incubator. After the culture medium was replaced, microscopic images were acquired. The fluorescence microscopic images were deconvoluted by using ImageJ 1.38x with a Gaussian 3D point spread function.

Immunofluorescence detection of ^{E166N}TEM-EGFR localization. HEK293T cells were transfected with pcDNA3.1(+)-^{E166N}TEM-EGFR or pcDNA3.1(+)-EGFR by using Lipofectamine 2000 (Invitrogen). After 5–6 h, the cultural medium was replaced with DMEM, and the cells were incubated at 37 °C for 24 h. After one rinse with PBS(−), the cells were fixed at 25 °C for 20 minutes with 3.7% formaldehyde/PBS(−). After two rinses with PBS(−), the cells were blocked at 25 °C for 1

h with blocking buffer (5%Goat Serum (invitrogen)/PBS, 0.05% NaN_3). Anti-EGFR (Calbiochem, GR01) or Anti-β-lactamase (Millipore, AB3738) antibody, followed by fluorescein-conjugated goat anti-mouse IgG (Bethyl, A90-116F) or fluorescein-conjugated goat anti-rabitt IgG (Calbiochem, 401314) were used to stain ^{E166N}TEM-EGFR or EGFR (Figure S5). After mounting, microscopic images were acquired by using a filter set with a 505 nm dichroic mirror and a 510–550 nm emission filter.

MALDI-TOF MS analysis. A total of 0.2 μL CCD stock solution (10 mM) was added to 19.8 μL β-lactamase solution (15 μM) in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C. After incubation at 25 °C for 16 h, unreacted CCD molecules were removed using a Sephadex G-50 Quick Spin column (DNA-grade; GE Healthcare). The samples were subsequently prepared for MALDI-TOF MS by using α-cyano-4-hydroxycinnamic acid as the matrix.

Fluorescence quantum yield. All the fluorescent probes were dissolved in DMSO to obtain 10-mM stock solutions; these solutions were then diluted to the desired final concentrations by using an appropriate buffer. The fluorescence quantum yield of the probes was estimated in 100 mM HEPES buffer (pH 7.4), using a fluorescence standard, quinine bisulfate in 50 mM H_2SO_4 aq. ($\Phi = 0.55$)[S2] as a reference for comparison.

Fluorometric assay. Enzyme assays for the quantification of WT TEM-1 and ^{E166N}TEM were performed in 100 mM HEPES buffer (pH 7.4) at RT. A total of 7 μL purified enzyme (15 μM) was added to 700 μL of buffer containing probes (1 μM). The samples were excited at 410 nm, and the fluorescence intensity enhancement was monitored at 450 nm (Figure 2).

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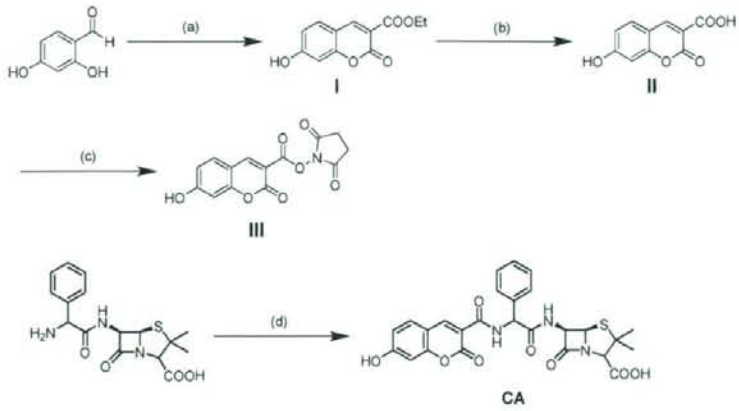
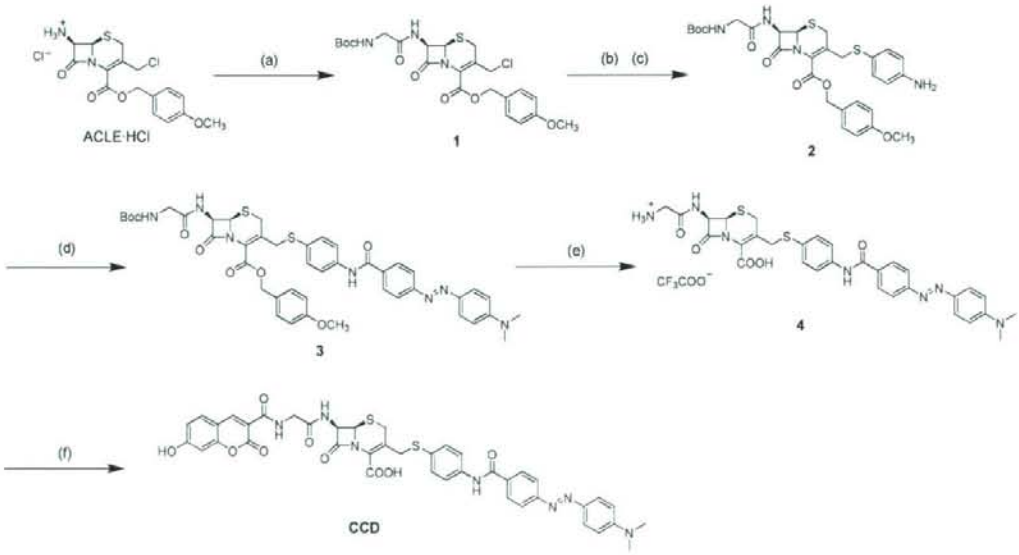
S3) Chen, I.; Howarth, M.; Lin, W. Y.; Ting, A. Y. *Nat. Methods* **2005**, *2*, 99–104.



Scheme S1. Synthetic route to CA. (a) Diethyl malonate, piperidine, EtOH, reflux. (b) 2 N NaOH aq., RT. (c) WSCD·HCl, *N*-hydroxysuccinimide, DMF, 0 °C. (d) **III**, DIEA, DMF, 0 °C.



Scheme S2. Synthetic route to CCD. (a) Boc-glycine, HOBt/H_2O, WSCD·HCl, TEA, DMF, 0 °C. (b) NaI, acetone, RT. (c) 4-Aminothiophenol, $NaHCO_3$, DMF, RT. (d) 4-Dimethylaminoazobenzene-4' -carboxylic acid, $POCl_3$, pyridine, -20 °C. (e) TFA, thioanisole, CH_2Cl_2, 0 °C. (f) Compound **III**, TEA, DMF, 0 °C.

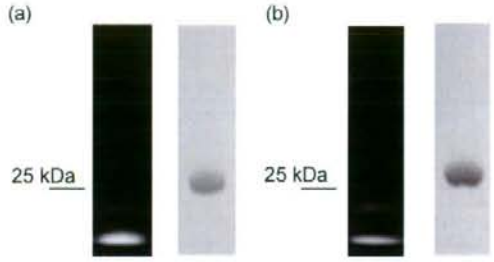


Figure S1. Fluorescence (left) and CBB-stained (right) gel images of WT TEM-1 incubated with (a) CA and (b) CCD.

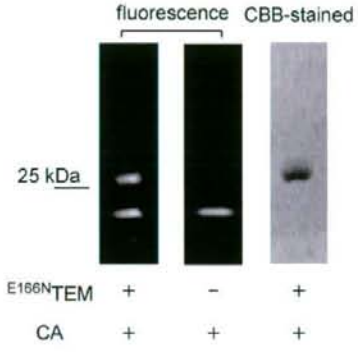


Figure S2. Fluorescence gel images of CA incubated with (left) or without (center) ^{E166N}TEM.

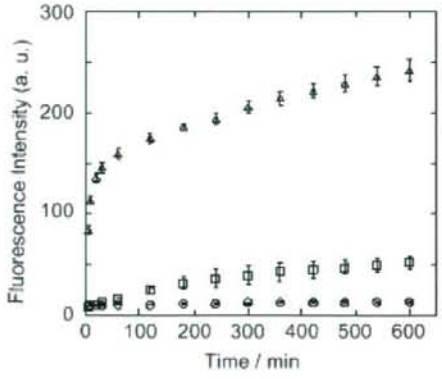
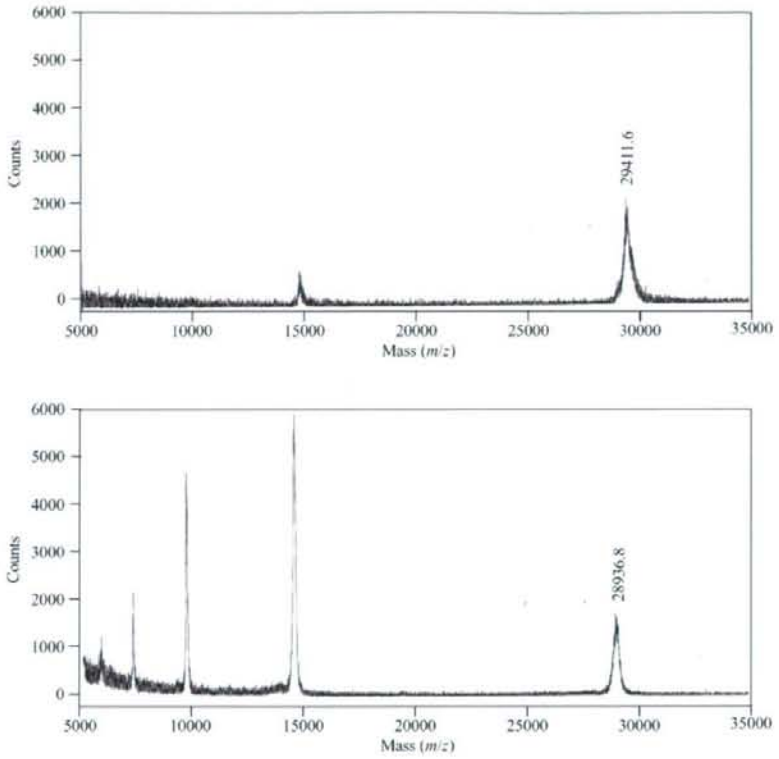


Figure S3. Time-dependent fluorescence intensity of CCD in the presence of ^{E166N}TEM (square), WT TEM-1 (triangle), and in the absence of them (circle).



	Calculated mass	Measured mass
^{E166N}TEM	28934.4	28936.8
^{E166N}TEM + CCD[a]	29407.5	29411.6

[a] After DABCYL group was eliminated.

Figure S4. MALDI-TOF MS of ^{E166N}TEM (upper) and ^{E166N}TEM incubated with CCD (bottom).

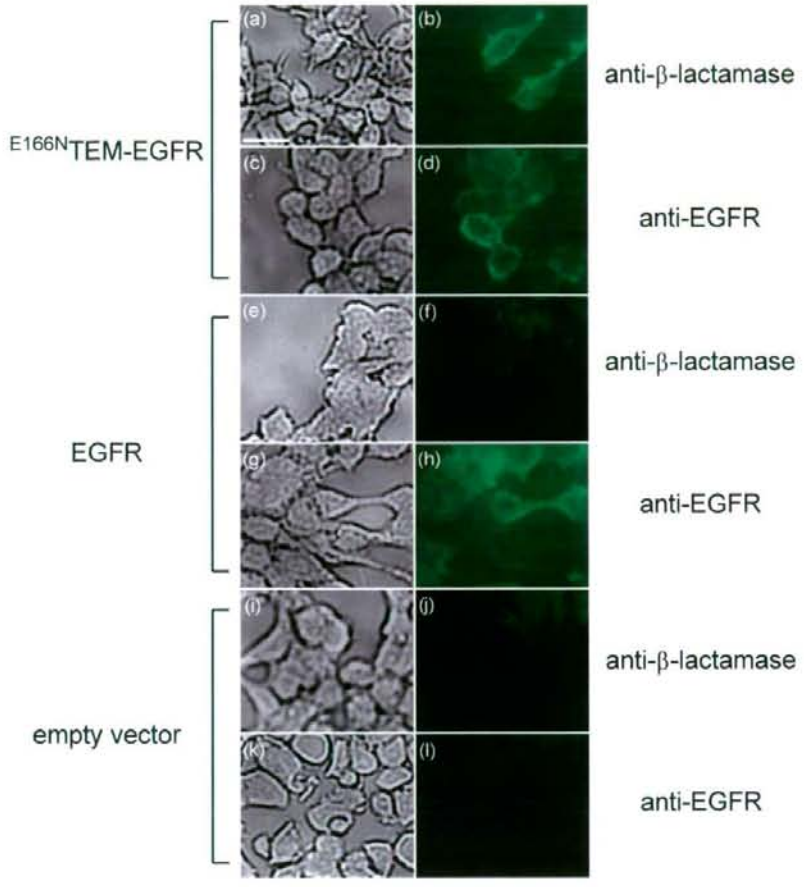


Figure S5. Optical microscopic images of HEK293T cells expressing (a–d) ^{E166N}TEM-EGFR, (e–h) EGFR, and (i–l) transfected with the empty vector, which were immunostained with the primary antibody ((b,f,j) anti-β-lactamase or (d,h,l) anti-EGFR) and the fluorescein-conjugated secondary antibody (scale bar: 20 μm). (b,d,f,h,j,l) Fluorescence microscopic images, excitation at 465 nm. (a,c,e,g,i,k) Phase contrast microscopic images.

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Molecular Imaging

Detection of Protease Activity using Dual-Function Fluorescence and ^{19}F MRI Spectroscopy**

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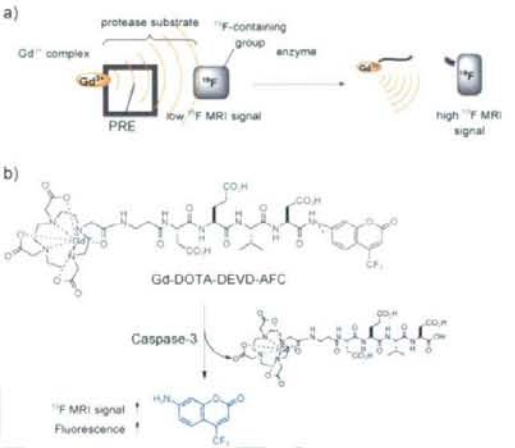
■■Title change ok?■■Noninvasive molecular imaging techniques are important for understanding the actual mechanisms of biological systems. In biological sciences, especially those involving cellular systems, the most widely used imaging technique is fluorescence microscopy, because of its high sensitivity, high spatiotemporal resolution, and simple experimental procedure.[1] On the other hand, magnetic resonance imaging (MRI) is one of the most successful imaging techniques in the field of clinical diagnosis. As MRI can visualize deep regions of animal bodies,[2] application of MRI to in vivo imaging of biomolecules is attracting attention.[3] Several ^{1}H MRI probes have been developed to investigate pH,[4] metal ions,[5] and enzyme activities.[6]

Recently, heteronuclear MRI has been attracting considerable attention as an alternative molecular imaging technique. One of the most promising nuclides for MRI is ^{19}F,[7] which has a high NMR sensitivity that is comparable to that of ^{1}H, and almost no intrinsic ^{19}F signals can be observed in living animals. ^{1}H MRI does not have the drawback of background signals from intrinsic biomolecules, which interfere with the probe signals. Very recently, we developed a novel design strategy for ^{19}F MRI probes that can detect protease activity.[8] We exploited the paramagnetic relaxation enhancement (PRE) effect to achieve off/on switching of the probe MRI signals (Scheme 1a). We were successful in detecting caspase-3 activity with ^{19}F MRI using the PRE mechanism in Gd-DOTA-DEVD-Tfb.■■ok?■■

Although MRI can visualize deep regions of living bodies, its sensitivity is inferior to that of fluorescence measurement. The lower sensitivity requires longer accumulation time for imaging. If the probes are multifunctional, we can choose the appropriate imaging method in accordance with the experimental conditions. A dual-mode probe FSB■■please define FSB■■ was developed, which aggregates to amyloid β(Aβ) plaques for ^{1}H and ^{19}F MRI, and was investigated using fluorescence/MRI measurements■■ok?■■.[9] ^{19}F MRI signals localized on Aβ plaques were observed in living mice in vivo, and fluorescence signals in brain slices ex vivo.[9] As such complementary experiments have resulted in more reliable conclusions, development of multimodal imaging probes is very important.[10] Herein we report a dual-mode probe to detect protease activity by fluorescence measurement and ^{19}F MRI that is based on the development of Gd-DOTA-DEVD-Tfb.[8]



Scheme 1. a) Representation of ^{19}F MRI detection of protease activity. b) Chemical structure of Gd-DOTA-DEVD-AFC and its reaction scheme for detecting caspase-3 activity.

We chose 7-amino-4-trifluoromethylcoumarin (AFC) as a reporter group that is active in both ^{19}F MRI and fluorescence measurement. AFC is strongly fluorescent in polar solvents, and the fluorescence properties of 7-aminocoumarin derivatives depend on the electron-donating ability of the 7-amino group.[11] Usually, the peptide modification on the 7-amino group induces a blue shift of the fluorescence spectrum with a decrease in fluorescence intensity. Thus, AFC has been utilized as the fluorophore for protease activity detection.[12] Furthermore, the ^{19}F NMR spectrum of AFC shows only a singlet peak without any coupling to intramolecular protons. AFC is thus appropriate for ^{19}F MRI.

We designed a bimodal probe Gd-DOTA-DEVD-AFC (Scheme 1), in which the probe consists of mainly three parts:

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Gd^{3+}-DOTA complex, caspase-3 substrate peptide (DEVD), and ^{19}F-containing fluorophore (AFC). When caspase-3 cleaves the C terminus of the DEVD sequence, AFC is produced. After the enzyme is cleaved, the ^{19}F MRI signal is increased in much the same manner as in Gd-DOTA-DEVD-Tfb (Scheme 1a). Simultaneously, the fluorescence spectrum of AFC is increased. Thus, Gd-DOTA-DEVD-AFC is expected to work as a bimodal probe that detects caspase-3 activity.

The Gd-DOTA-DEVD-AFC probe was synthesized using Fmoc solid-phase chemistry, followed by complex formation with Gd^{3+} ions (Scheme 2). The excitation peak of Gd-DOTA-DEVD-AFC is at 340 nm, and irradiation at 400 nm results in lower fluorescence emission. The incubation of the probe with caspase-3 at 37 °C induced the excitation spectral shift toward longer wavelengths. Therefore, when the probe was excited at 400 nm, the emission at around 500 nm was substantially increased (Figure 1). From the fluorescence measurements, the kinetic parameters for hydrolysis of Gd-DOTA-DEVD-AFC by caspase-3 were measured. The V_{max}/K_m value of Gd-DOTA-DEVD-AFC is $7.61 \times 10^{-3}\ s^{-1}$. On the other hand, V_{max}/K_m of Ac-DEVD-AMC, the commercially available fluorescent substrate, is $9.91 \times 10^{-4}\ s^{-1}$. This result indicates that Gd-DOTA complex does not hinder the enzyme reaction at all. Thus, Gd-DOTA-DEVD-AFC can be used as a superior fluorogenic probe for detecting caspase-3 activity.

We measured the ^{19}F NMR spectra of Gd-DOTA-DEVD-AFC and its metal-free analogue■■ok?■■ DOTA-DEVD-AFC using ^{19}F NMR. The NMR signal of Gd-DOTA-DEVD-AFC was broad and weak compared to that of the Gd^{3+}-free DOTA-DEVD-AFC (Supporting Information). This change in peak shape and intensity■■ok?■■ suggests that ^{19}F undergoes an intramolecular PRE effect owing to the presence of Gd^{3+} ions. Longitudinal (T_1) and transverse (T_2) relaxation times of DOTA-DEVD-AFC (250 μM) were (0.479 ± 0.003) s and (0.152 ± 0.006) s, respectively (Table 1). In case of Gd-DOTA-DEVD-AFC, we could not estimate either T_1 or T_2, because these relaxation times were markedly shorter and the ^{19}F NMR signal intensity was low. From molecular modeling, the distance between the Gd^{3+} ion and the ^{19}F atom in the probe was estimated to be less than 25 Å. However, as the substrate peptide is flexible, the Gd^{3+} ion can be distributed in closer proximity to ^{19}F, such that the PRE effect works efficiently.■■ok?■■

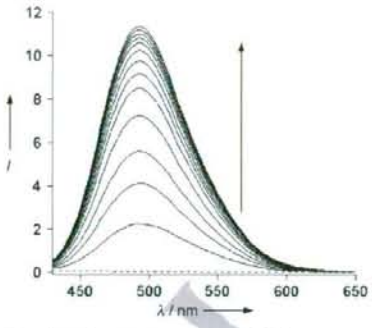


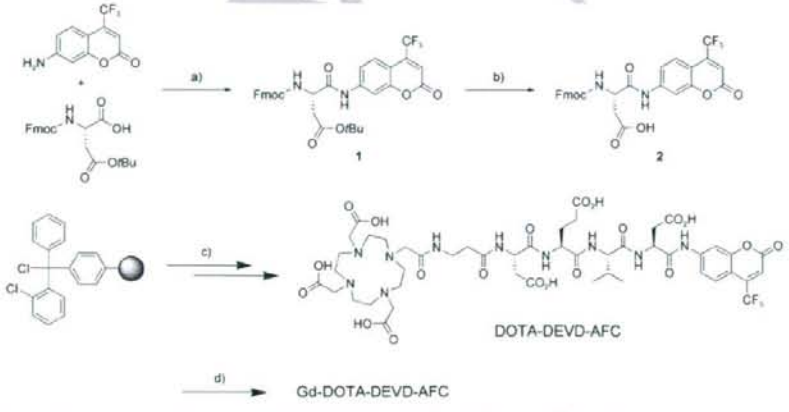
Figure 1. Time-dependent emission spectra of Gd-DOTA-DEVD-AFC (10 μM) with casapse-3 (0.84 mU) in the reaction buffer (pH 7.4) at 37 °C. The spectra were measured every 2 min after the addition of the enzyme. The dotted line indicates no caspase-3. The excitation wavelength: 400 nm. Reaction buffer: HEPES (pH 7.4, 50 mM) containing glycerol (10%), NaCl (100 mM), DTT (10 mM), EDTA (1 mM), and CHAPS (0.1%).

Table 1: Longitudinal and transverse relaxation times of synthesized probes.

	t_1 [s][a]	t_2 [s][a]
DOTA-DEVD-AFC	0.479(3)	0.152(6)
Gd-DOTA-DEVD-AFC	–[b]	–[b]
Gd-DOTA-DEVD-AFC + caspase-3	0.38(4)[c]	0.097(4)[c]

[a] Parenthesis denotes standard deviation ($n = 3$). [b] The relaxation time was too short to be determined. [c] The relaxation times were measured after the enzyme (250 μM) reaction was complete.

We have also performed an enzyme assay using ^{19}F NMR. When Gd-DOTA-DEVD-AFC was treated with caspase-3 in the reaction buffer at 37 °C, a sharper and a more intense ^{19}F NMR signal was observed, with a slight downfield shift (Figure 2). T_1 and T_2 of the cleaved product (250 μM) were elongated to 0.38 ± 0.04 s and 0.097 ± 0.004 s, respectively (Table 1). This finding indicates that the intramolecular PRE effect from the Gd^{3+} ion to the ^{19}F



Scheme 2. Synthetic route to Gd-DOTA-DEVD-AFC. a) $POCl_3$, pyridine. b) trifluoroacetic acid. c) Fmoc peptide synthesis: **2**, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-β-Ala-OH, tris-tBu-DOTA, deprotection. d) $GdCl_3 \cdot 6H_2O$, 100 mM HEPES buffer (pH 7.4).

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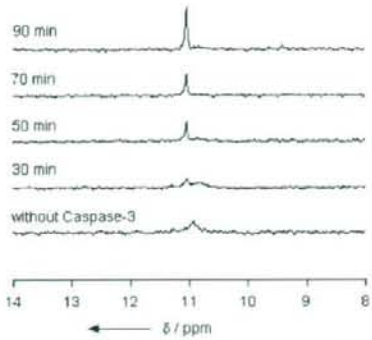

Figure 2. Time-dependent ^{19}F NMR spectra of Gd-DOTA-DEVD-AFC (250 μM) after addition of caspase-3 (1.25 mU) at 37 °C. Reaction buffer: As in Figure 1 plus D_2O (5%)■■ok?■■.

atom was cancelled owing to the cleavage of the probe. After complete cleavage by caspase-3 (confirmed by HPLC), the relaxation times T_1 and T_2 were lower than those observed for the metal-free ligand. These shorter relaxation times are most likely due to the intermolecular PRE of the cleaved Gd-DOTA (Supporting Information, Figure S4).

Finally, we attempted to visualize caspase-3 activity using a ^{19}F MRI phantom with Gd-DOTA-DEVD-AFC. Because of the extremely short relaxation time T_2, the ^{19}F MRI of Gd-DOTA-DEVD-AFC had no signals. When caspase-3 was added to the solution of Gd-DOTA-DEVD-AFC, augmentation of the ^{19}F MRI signal of the probe was observed (Figure 3)■■ok?■■.

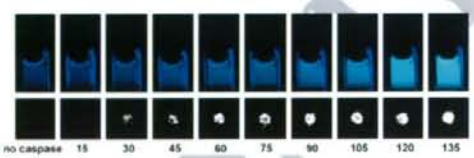

Figure 3. Time-dependent fluorescence images (top, λ_m: 400 nm) and ^{19}F MR phantom images (bottom, diameter: approximately 2 mm) of Gd-DOTA-DEVD-AFC (10 μM for fluorescence measurement and 1 mM for ^{19}F MRI) with caspase-3 (60 nU for fluorescence measurement and 2 mU for ^{19}F MRI) at 37 °C. Reaction buffer: As in Figure 1■■ok?■■. For ^{19}F MRI, [D_6]DMSO (20%) was introduced in the reaction buffer.

In conclusion, we developed a novel dual-modal probe, Gd-DOTA-DEVD-AFC, which detects caspase-3 activity by an increase in both fluorescence and in the ^{19}F MRI signals. Because fluorescence measurement and MRI provide complementary information, such dual-mode probes should be quite useful for various biological experiments. Although several multimodal probes, such as fluorescence measurement and MRI have been developed recently,[13] most probes are constructed by simple attachment of reporter moieties such as fluorescence dyes or MRI contrast agents. In contrast, multimodal probes accompanying plural signal enhancement have been scarcely reported. Such multimodal smart probes would be the next-generation probes in multimodal imaging for detecting enzyme activity.

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Keywords: fluorescence · magnetic resonance imaging · molecular imaging · multimodal spectroscopy · protease

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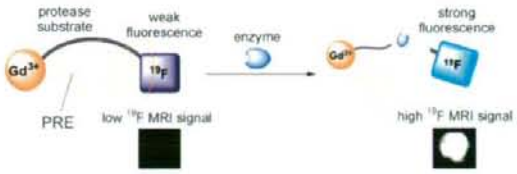
Communications

Molecular Imaging

S. Mizukami, R. Takikawa, F. Sugihara, M. Shirakawa, K. Kikuchi* ____ ▮▮▮▮–▮▮▮▮

Detection of Protease Activity using Dual-Function Fluorescence and ^{19}F MRI Spectroscopy



Dynamic duo: MRI can visualize deep regions of living bodies, whereas fluorescence measurement offers excellent sensitivity. These methods thus offer signal enhancement potential for detecting enzyme activities. A dual-mode off/on probe to detect caspase-3 activity by fluorescence and ^{19}F MRI spectroscopy is presented.

Molekulare Bildgebung

S. Mizukami, R. Takikawa, F. Sugihara, M. Shirakawa, K. Kikuchi* ____ ▮▮▮▮–▮▮▮▮

Detection of Protease Activity using Dual-Function Fluorescence and ^{19}F MRI Spectroscopy

Dynamisches Duo: Die Kernspintomographie (MRI) kann tiefliegendes Gewebe in vivo visualisieren, während Fluoreszenzmessungen ausgezeichnete Empfindlichkeiten bieten. Die kombinierte Verwendung beider Methoden ermöglicht Signalverstärkungen, die zur Detektion von Enzymaktivitäten ausreichen. Eine Zweikanalsonde zur Detektion der Caspase-3-Aktivität durch Fluoreszenzspektroskopie und ^{19}F-MRI wird vorgestellt.

シリーズ脳科学 5

甘利俊一◇監修 | 古市貞一◇編

分子・細胞・シナプスからみる脳