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Aminoluciferins as Functional Bioluminogenic Substrates of Firefly Luciferase

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Abstract: Firefly luciferase is widely used as a reporter gene in assays to study gene expression, gene delivery, and so on because of its extremely high signal-to-noise ratio. The availability of a range of bioluminogenic substrates would greatly extend the applicability of the luciferin–luciferase system. Herein, we describe a design concept for functional bioluminogenic substrates based on the aminoluciferin (AL) scaffold, together with a convenient, high-yield method for synthesizing N-alkylated ALs. We confirmed the usefulness of ALs as bioluminogenic substrates by synthesizing three probes. The first was a conjugate of AL with glutamate, Glu–AL. When Glu–AL, the first membrane-impermeable bioluminogenic substrate of luciferases, was applied to cells transfected with luciferase, luminescence was not observed; that is, by using Glu–AL, we can distinguish between intracellular and extracellular events. The second was Cy5–AL, which consisted of Cy5, a near-infrared (NIR) cyanine fluorescent dye, and AL, and emitted NIR light. When Cy5–AL reacted with luciferase, luminescence derived from Cy5 was observed as a result of bioluminescence resonance energy transfer (BRET) from AL to Cy5. The NIR emission wavelength would allow a signal to be observed from deeper tissues in bioluminescence in vivo imaging. The third was biotin–DEVD–AL (DEVD = the amino acid sequence Asp-Glu-Val-Asp), which employed a caspase-3 substrate peptide as a switch to control the accessibility of the substrate to luciferase, and could detect the activity of caspase-3 in a time-dependent manner. This generalized design strategy should be applicable to other proteases. Our results indicate that the AL scaffold is appropriate for a range of functional luminophores and represents a useful alternative substrate to luciferin.

Keywords: aminoluciferin · bioluminescent probes · biosensors · firefly luciferase · luminescence

Introduction

Bioluminescence is employed in various highly sensitive in vitro detection systems, as well as for in vivo imaging. One of the most frequently used systems is the North American firefly, *Photinus pyralis*, luciferin–luciferase system, which is used as a reporter in biochemical assays, in cell cultures, and recently in animal models to study gene expression, gene delivery, tumor progression, and so on.[1–4] Characteristic advantages of bioluminescence systems include a high quantum yield (e.g., the recently reported value of $\Phi_{bl} = 0.41$)[5] without excitation light and extremely high signal-to-noise ratios compared with other imaging techniques, such as fluorescence, even in vivo. Recently, bioluminescence systems have been applied to detect other events, such as protein–protein interactions and protease activity, by modification of luciferase.[6–11] On the other hand, functional luciferin analogues, the luminescence properties of which are changed dramatically by specific activity, for instance, β-galactosi-

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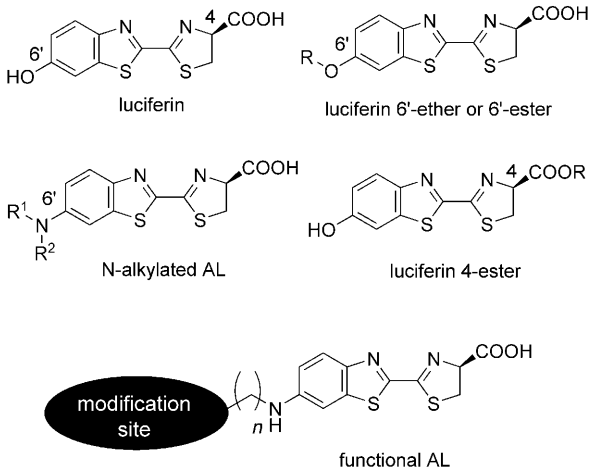
dase,[12] phosphatase,[13] glutathione S-transferase,[14] and lactamase[15] activity, have also been reported as highly sensitive indicators for target molecules. But, because the design principle is based only on using luciferin 6′-ether or 6′-ester derivatives, which have no emission prior to hydrolysis, the range of available chemical modifications is restricted and this approach is applicable to only a limited range of target molecules. To further extend the applicability of the luciferin–luciferase system, we focused on aminoluciferin (AL) as a scaffold to develop functionalized bioluminescent substrates. Herein, we present a novel design concept based on AL modified with various functionalities at the 6′-position (Scheme 1). As a first step, we developed an efficient synthetic method for obtaining N-alkyl derivatives in higher yield than those obtained with the method reported by Klaubert et al.[16] We then confirmed the usefulness of AL as a functional bioluminogenic scaffold by designing and synthesizing the first membrane-impermeable luciferase substrate (Glu–AL), a probe emitting NIR light (Cy5–AL), and a probe for the detection of caspase-3 activity (biotin–DEVD–AL; DEVD = the amino acid sequence Asp-Glu-Val-Asp).



Scheme 1. Chemical structures of firefly luciferin and AL derivatives described herein.

Abstract in Japanese:

firefly の luciferin-luciferase 反応は感度の良さから遺伝子発現等のレポーター酵素として広く利用されている。近年 luciferase を機能化することで、タンパク質間相互作用や酵素活性などの検出が行えることが示されている。一方、luciferin の機能化も行われているが、luciferase の基質特異性の高さから分子設計の制約が大きい。我々は amino 基を有する基質、aminoluciferin（AL）骨格に着目し、amino 基から修飾部位を導入することで様々な機能化が行えるのではないかと考えた。最初に、効率的かつ簡便な N-アルキル化反応を検討した。N-アルキル化反応としては還元的アミノ化反応が有用であることを見出した。次に AL の機能化に着手し、我々は 3 つの異なる基質を設計、合成した。すなわち、水溶性基の導入による細胞膜非透過性の基質の開発（Glu-AL）、近赤外領域に発光波長を有するプローブの開発（Cy5-AL）、最後に luciferase への accesibility の制御を利用した caspase-3 の活性を検出するプローブの開発（biotin-DEVD-AL）である。いずれの場合も設計通りの機能を有し、それぞれ有用な機能をもつプローブとして更なる応用が期待される。これらは AL 骨格を機能性基質の基本骨格に用いることで初めて実現できるものであり、修飾部位を様々に設計することにより今後多くの機能性基質が開発されていくことが期待される。

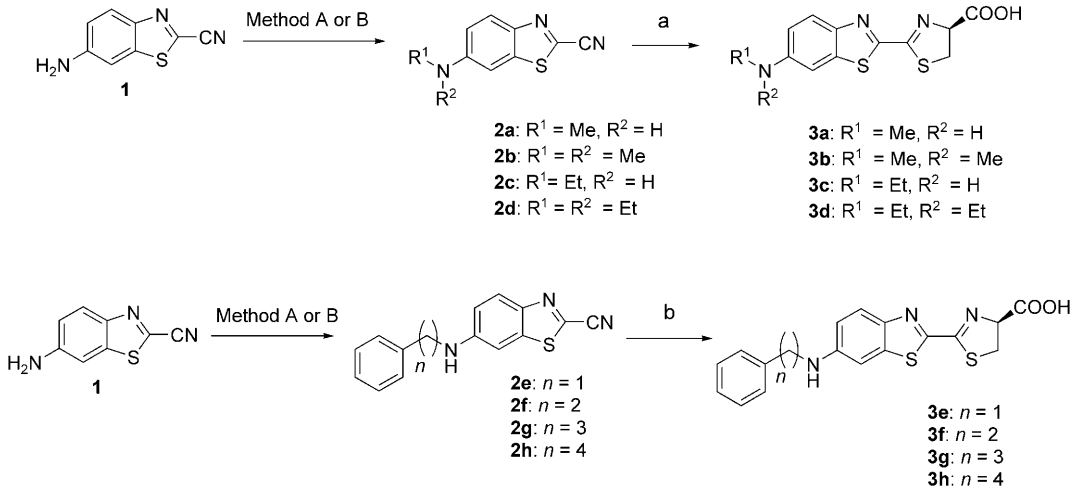
Results and Discussion

Improved Method for N-alkylation and Properties of AL Derivatives

Firefly luciferase has a high specificity for firefly luciferin as a substrate, therefore, it is difficult to introduce functionality, for example, 6′-ethers, 6′-esters, and 4-esters of luciferin lack bioluminescence activity (Scheme 1). We focused on AL as a scaffold because AL derivatives exhibit strong luminescence properties as substrates of luciferase.[16] N-Alkylation by the nucleophilic substitution reaction of the amino group of 6-amino-2-cyanobenzothiazole (**1**) was reported by Klaubert et al.,[16] but the reaction yields were quite low (5–30%) owing to poor nucleophilicity of the amino group and a long reaction time was required. Moreover, only monoalkylation could be achieved and not dialkylation. Thus, we aimed to develop a more effective and convenient reaction. We examined two kinds of reductive amination using the corresponding aldehyde under acidic conditions: method A: acid, H_2SO_4 (aq); reductant, $NaBH_4$; solvent, tetrahydrofuran (THF); method B: acid, acetic acid; reductant, $NaBH_3CN$; solvent, acetonitrile. To examine whether these reactions could be used as a general method for N-alkylation, we synthesized a series of derivatives with alkyl amino moieties at the 6-position of **1**, such as methyl (**2a**), dimethyl (**2b**), ethyl (**2c**), diethyl (**2d**), benzyl (**2e**), phenylethyl (**2f**), phenylpropyl (**2g**), and phenylbutyl (**2h**) groups (Scheme 2). The reaction yields were dramatically improved (39–91%, see Table 1) compared with the previously reported method. By using method A, low reaction yields were observed in some cases, but by using method B the products were obtained in good yields, although $NaBH_3CN$ was required. Additionally, dialkylation of **1** can be performed in reasonable yields (54% for dimethylation, 62% for diethylation). Then, the corresponding AL derivatives (**3a**–**3h**) were obtained by condensation with D-cysteine in aqueous methanol (Scheme 2). Next, we investigated the luminescence properties of the products, especially the bioluminescence emission maximum λ_{max}. We observed luminescence with a longer-wavelength emission peak than that of luciferin or AL for all of the compounds synthesized (Table 1 and Figure S1 in the Supporting Information). The kinetic parameters with luciferase were measured and are summarized in Table 1. The properties of the dialkylated AL derivatives were similar to those of the monoalkylated ALs.

Design and Synthesis of Glu–AL

The above results provide a basis for the design and development of functional bioluminescent substrates by using AL as a scaffold. First, we tried to control the membrane perme-



Scheme 2. Top: synthesis of N-alkylated AL. Reagents and conditions: Method A) 61 (**2a**), 54 (**2b**), 23 (**2c**), and 5% yield (for **2d**); Method B) 91 (**2c**), 62 (**2d**); a) D-cysteine, K_2CO_3, H_2O (pH 8)/MeOH, 78 (**3a**), 90 (**3b**), 83(**3c**), and 79% yield (**3d**). Bottom: synthesis of AL with a benzene ring. Reagents and conditions: Method A) 24 (**2e**), 39 (**2f**), 57 (**2g**), and 53% yield (**2h**); Method B) 57% yield (**2e**), b) D-cysteine, K_2CO_3, H_2O (pH 8)/MeOH, 42 (**3e**), 82 (**3f**), 50 (**3g**), and 52% yield (**3h**). Details of methods A and B are given in the text.

Table 1. Reaction yields of N-alkylation of **2a**–**2h**, along with λ_{max}, K_m, and relative V_{max} data for AL derivatives.

	R^1	R^2	Reaction yield[a] [%]		λ_{max}	K_m	Relative V_{max}
			Method A	Method B	[nm][b]	[μM][c]	
AL	H	H	–	–	596	0.76±0.14	100[d]
3a	Me	H	61	–	615	0.071±0.006	25±1.2
3b	Me	Me	54	–	615	0.059±0.002	16±1.0
3c	Et	H	23	91	613	0.033±0.003	22±1.4
3d	Et	Et	5	62	606	0.12±0.01	16±0.9
3e	benzyl	H	24	57	605	0.041±0.003	1.6±0.09
3f	phenylethyl	H	39	–	604	0.081±0.005	7.3±0.08
3g	phenylpropyl	H	57	–	601	0.42±0.04	39±3.4
3h	phenylbutyl	H	53	–	601	0.34±0.03	2.2±0.09

[a] This represents reaction yields of N-alkylation of **2a**–**2h** by reductive amination; method A: H_2SO_4, $NaBH_4$, THF; method B: AcOH, $NaBH_3CN$, CH_3CN. [b] Obtained from Figure S1 in the Supporting Information. [c] Values of K_m were determined from maximal light intensities after measurements to estimate the initial velocity.[17] [d] The V_{max} value of AL was about 30% that of luciferin.

ability of substrates. Although luciferin has a carboxylic acid moiety at the 4-position, it can permeate through the cell membrane and, to the best of our knowledge, no membrane-impermeable bioluminogenic substrates are available (Figure 1a). We focused on the introduction of negatively charged hydrophilic groups, such as carboxylic and sulfonic acids (Figure 1a). This design strategy could not have been applied to the luciferin scaffold because luciferin does not lose luminescence activity upon the introduction of a functional group at the 6′- or 4-positions. Thus, we prepared the conjugate of 6-carboxymethylamino-2-cyanobenzothiazole (**4**) with glutamate and the corresponding AL derivative (Scheme 3), namely, Glu–AL, which has a total of three carboxylic acid moieties (Figure 1b) and investigated the luminescence properties and membrane permeability. The luminescence properties of Glu–AL were similar to those of N-alkylated AL (Figure S2 and Table S1 in the Supporting Information). We then cultured COS7 cells transfected with luciferase in a 96-well plate. When AL or luciferin was applied to the plate, a signal was clearly detected (Figure 1c and Figure S3b, c in the Supporting Information). On the other hand, in the case of Glu–AL, the luminescence remained almost at the background level; however, strong luminescence was observed in the lysate (Figure 1c and Figure S3a in the Supporting Information). The results indicate that Glu–AL is a membrane-impermeable substrate. Therefore, by using Glu–AL, we can distinguish between intra- and extracellular events. For example, it may be possible to detect adenosine triphosphate (ATP) release sensitively[18] or secretion of luciferase-fused proteins from cells after some stimulation.[19]

Design and Synthesis of Cy5–AL

As mentioned in the Introduction, bioluminescence in vivo imaging (BLI) can be used to sensitively monitor gene expression, gene delivery, and tumor progression. However, for in vivo imaging, emission in the NIR region of $\lambda = 650$–900 nm would be even better than bioluminescence from the firefly luciferin–luciferase system because emission in this region is subject to minimal interference from hemoglobin absorption and offers good tissue penetration.[20,21] So far, the longest wavelength maximum among luciferase variants is about $\lambda_{max} = 630$ nm[22,23] and according to a recent report on the fluorescence properties of oxyluciferin, this wavelength appears to be the upper limit for firefly luciferin.[24] To obtain NIR emission with luciferase, we designed an AL

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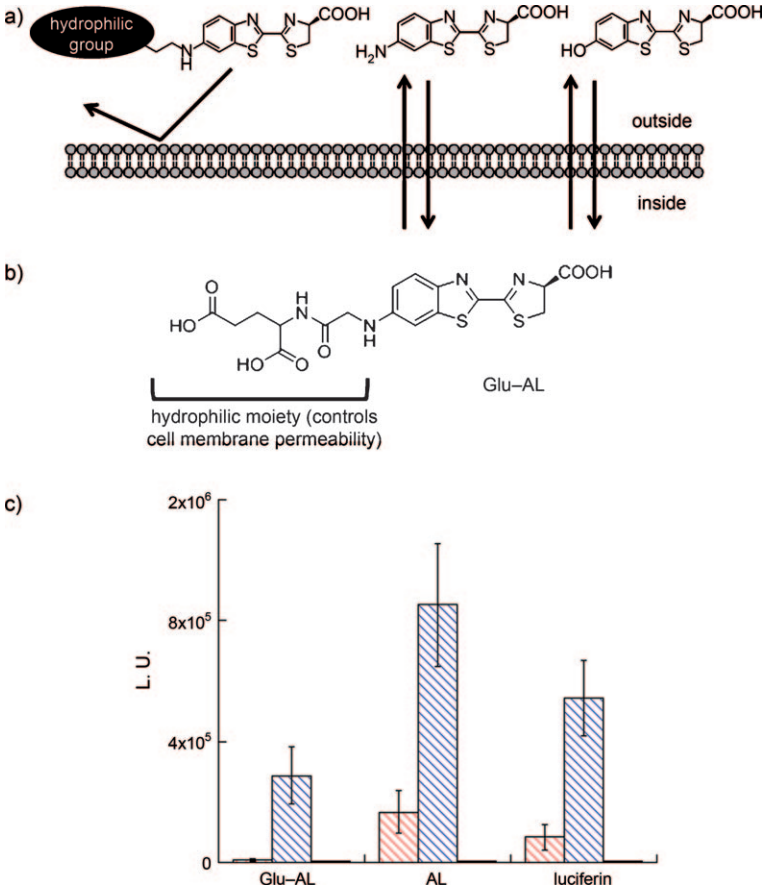
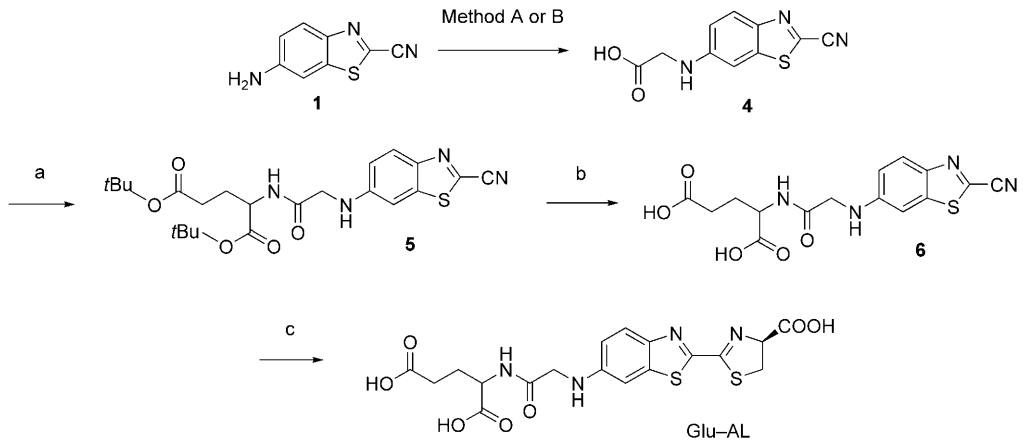


Figure 1. a) Schematic illustration of cell membrane permeable and impermeable substrates. b) Chemical structure of Glu–AL. c) Light units (L.U. [a.u.]) of Glu–AL, AL, and luciferin. Red column: COS7 cells transfected with luciferase, blue column: lysate of COS7 cells transfected with luciferase, green column: COS7 cells (not transfected).

derivative linked with a NIR-emitting cyanine (Cy) dye. When this substrate reacts with luciferase, bioluminescence resonance energy transfer (BRET) is expected to occur from the substrate to the NIR dye, thus affording NIR luminescence (Figure 2a). Based on this approach, we synthesized a bioluminogenic substrate consisting of AL and Cy5, namely, Cy5–AL (Figure 2b and Scheme 4), and investigated whether or not NIR luminescence could be observed. First, we confirmed that the absorbance of Cy5 sufficiently overlapped with the luminescence of AL (Figure 2c). As shown in Figure 2d, the bioluminescence of Cy5–AL was seen in the NIR region ($\lambda_{max} = 673$ nm), which was identical to the fluorescence peak of Cy5, and the luminescence of AL was not observed at all, thereby suggesting that highly efficient energy transfer occurred. Thus, by utilizing this luciferin-based BRET strategy, it should be possible to obtain multicolor luminescence in the NIR region. Although a luciferase-based BRET strategy, in which NIR dyes or quantum dots were combined with luciferase, has been reported,[25,26] it lacked generality because the injection of modified luciferase in vivo was required. Our strategy can be applied to conventional systems and it should be possible to obtain signals from deeper tissues in BLI.



Scheme 3. Synthesis of Glu–AL. Reagents and conditions: Method A) glyoxylic acid, 23%; Method B) glyoxylic acid, 90%; a) glutamic acid di-*tert*-butyl ester, 1-hydroxybenzotriazole (HOBT), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophospate (HBTU), NEt_3, *N*,*N*-dimethylformamide (DMF), 67%; b) trifluoroacetic acid (TFA), CH_2Cl_2, 19%; c) D-cysteine, K_2CO_3, H_2O (pH 8)/MeOH, quant. Details of methods A and B are given in the text.

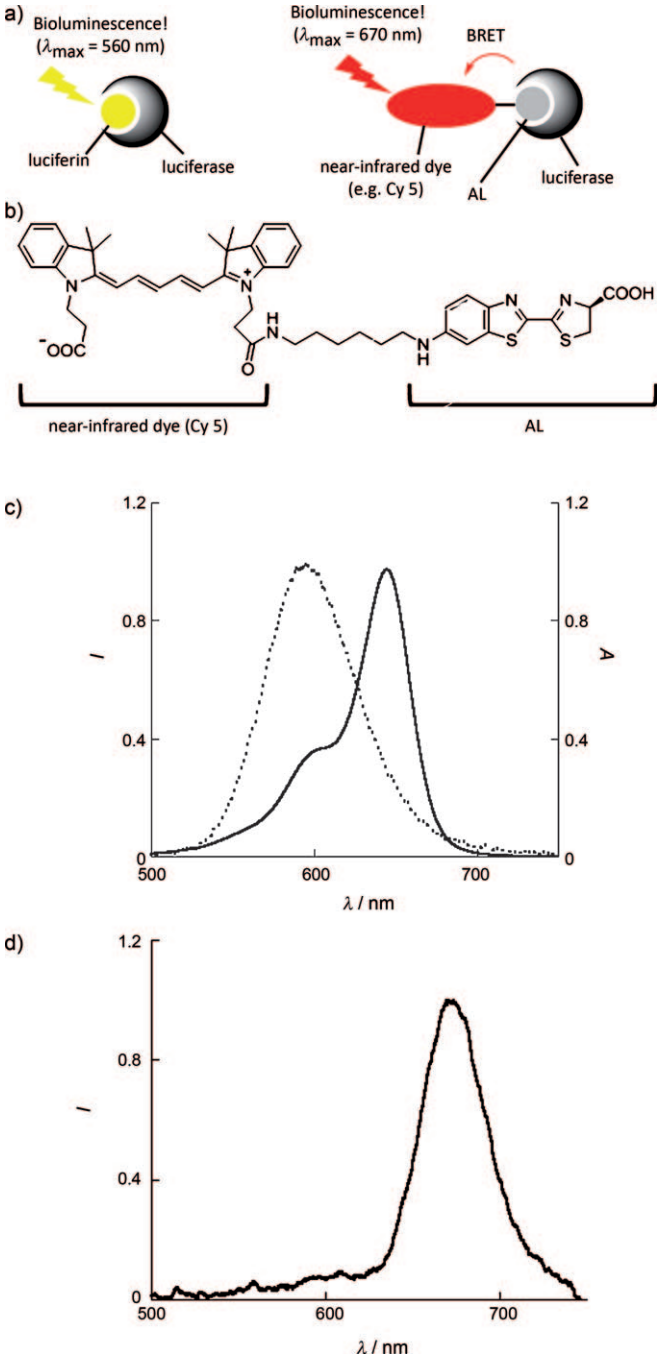


Figure 2. a) Schematic illustration of NIR luminescent substrate based on a BRET strategy. b) Chemical structure of Cy5–AL. c) Absorbance spectrum of Cy5–AL (——) and bioluminescence spectrum of AL (•••••). d) Bioluminescence spectrum of Cy5–AL. I = normalized luminescence intensity.

Design and Synthesis of a Substrate for Which Bioluminescence is Controlled by Accessibility to Luciferase

Finally, we focused on the X-ray crystal structure of the luciferin–luciferase complex. It has been reported that the benzothiazole moiety of firefly luciferin lies deep in the enzymatic active site of luciferase (Figure S4 in the Supporting Information).[27] Therefore, a bulky substituent at the 6′-position of luciferin might block interaction with luciferase. For this purpose, we chose avidin, which is a tetrameric protein containing four identical subunits, each of which binds to biotin with high affinity and specificity. The dissociation constant is $K_D \approx 10^{-15}$ M, which makes avidin–biotin binding one of the strongest known noncovalent interactions. We expected that AL with a biotin moiety, biotin–AL, would exhibit bioluminescence like other AL derivatives, whereas the complex between biotin–AL and avidin might not because avidin would block the access of luciferase to biotin–AL (Figure 3a). Accordingly, we synthesized biotin–AL by conjugating **4** with biotin, followed by condensation with D-cysteine (Figure 3b and Scheme 5). We investigated the luminescence of biotin–AL and the complex with avidin. As shown in Figure 3c, bioluminescence was observed for biotin–AL, but in the presence of avidin, the bioluminescence was lost. This result suggested that a huge molecule located adjacent to the substrate could block interaction with luciferase. We utilized this information to develop a design strategy for a novel luminescence probe consisting of three units: the first is a biotin site that can interact with avidin to block access by luciferase, the second is a peptide linker that contains a cleavage sequence for a target enzyme, and the third is the substrate site (Figure 3b). As proof of concept, we designed a caspase-3 probe with a DEVD sequence, which was cleaved by caspase-3. We synthesized biotin–DEVD–AL by means of solid-phase synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Scheme S1 in the Supporting Information). The complex with avidin did not show bioluminescence, but in the presence of caspase-3, time-dependent bioluminescence appeared (Figure 3d). HPLC analysis of the reaction mixture showed a single peak and the retention time of the reaction product was identical to that of Lys–AL, which was formed from biotin–DEVD–AL by caspase-3 (Figure 3e). The results indicated that biotin–DEVD–AL was degraded by caspase-3 to Lys–AL with release of avidin, and Lys–AL emitted bioluminescence. These findings demonstrate that luminescence can be switched on by controlling the accessibility of the substrate to luciferase. This design strategy should be applicable to not only aminopeptidases and exopeptidases, but also to carboxypeptidases and endopeptidases. It is important to note that the enzymatic reaction of some exopeptidases is known to be influenced by the residue after the scissile bond. For example, in the case of capase-3, charged and bulky residues are poorly tolerated.[28] Conventionally, an anilide group is required at the site, but with our strategy, a wide range of amino acids can be used. Therefore, we envision that this strategy could potentially be used in a wide variety of applications for the detection of the activity of various proteases, including for in vivo imaging.

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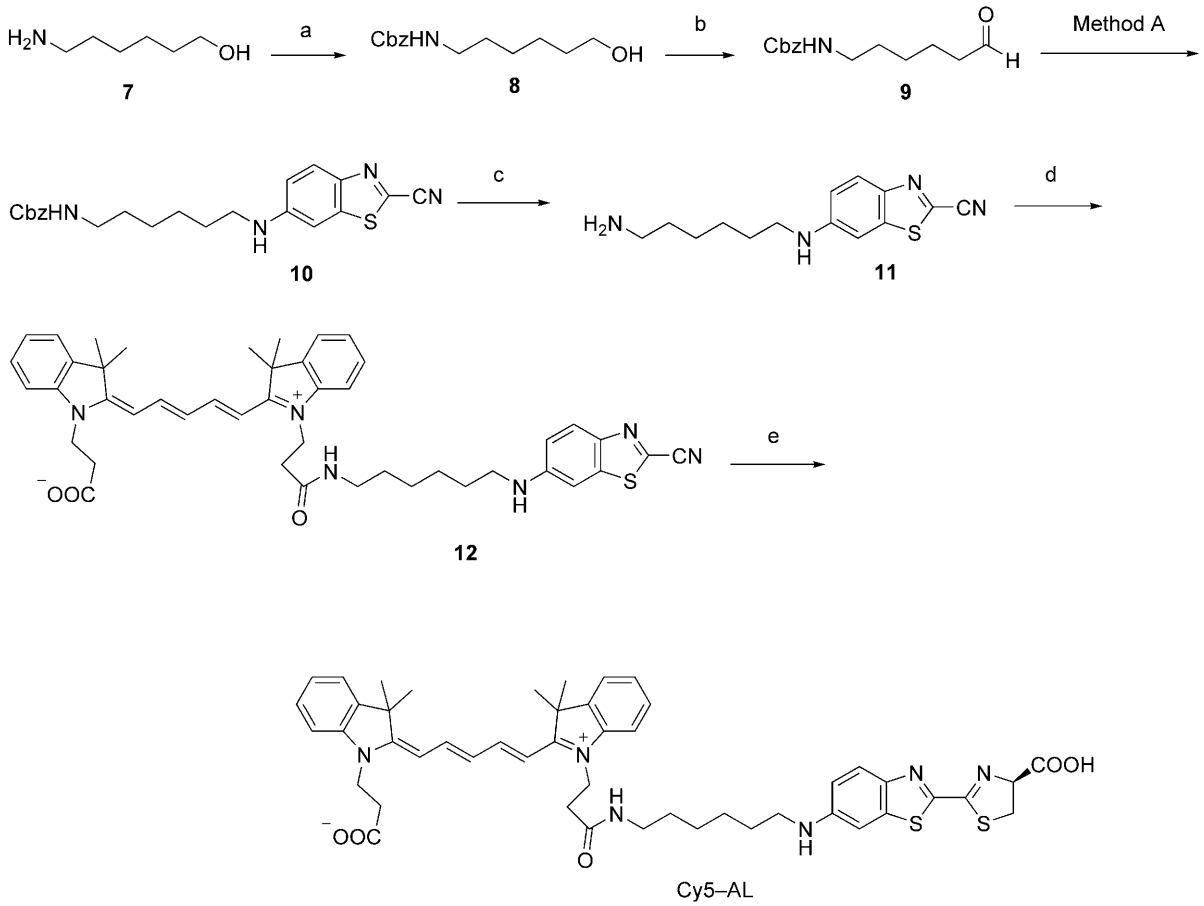


Scheme 4. Synthesis of Cy5–AL. Reagents and conditions: a) benzyloxycarbonyl (Cbz) chloride, $NaHCO_3$, acetone/H_2O, y. 94%; b) pyridinium chlorochromate (PCC), $MgSO_4$, CH_2Cl_2, 16%; Method A) **1**, 56%; c) TFA, CH_2Cl_2, 82%; d) Cy5-SE, NEt_3, DMF, 27%; e) D-cysteine, K_2CO_3, H_2O (pH 8)/MeOH, 47%. Details of method A are given in the text.

Conclusion

We developed an easy, quick, and high-yielding synthetic method for preparing N-alkylated ALs, which enabled us to efficiently synthesize AL derivatives with various functional moieties. This makes it possible to design and develop a wide range of novel functionalized AL-based bioluminogenic substrates by chemical modification. We expected that this approach would be very flexible, for example, allowing modification of bioavailability, conjugation with biomolecules, control of luminescence activity or wavelength, and easy targeting. To confirm the value of this methodology, we introduced three types of functionality into bioluminogenic substrates, demonstrating the feasibility of controlling distribution by the use of a cell membrane impermeable probe, adjusting luminescence emission to the NIR range by the application of BRET, and detecting protease activity by introducing an appropriate protease substrate sequence. These results indicate that the AL scaffold should be suitable for a range of functional luminophores, some of which would not be feasible in a luciferin-based system, and thus, we propose that AL represents a useful alternative substrate to luciferin.

Experimental Section

Materials

In general, chemicals were of the best grade available, supplied by Wako Pure Chemical, Tokyo Chemical Industries, and Aldrich Chemical Company, and were used without further purification. Special chemicals were dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo) and *N,N*-dimethylformamide (DMF) (fluorometric grade, Dojindo). Luciferase from *Photinus pyralis* (molecular weight 120 kDa, E.C. 1.13.12.7) was purchased from Sigma–Aldrich (Tokyo, Japan). Avidin from eggs (molecular weight 66 kDa) was purchased from Pierce. NMR spectra were recorded on a JNM-LA300 (JEOL) instrument at 300 MHz for ^1H and at 75 MHz for ^{13}C nuclei. δ values are given in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with JEOL JMS-T1000LC AccuTOF (ESI) and JEOL SX-102A (EI) instruments. Bioluminescence spectra were obtained on a Hitachi F4500 instrument. Bioluminescence kinetics results were obtained on a Perkin–Elmer Envision instrument. The luminescence signal was detected by using a CCD camera attached to an IVIS instrument (Xenogen). HPLC analyses were performed on an Inertsil ODS-3 (4.6×250 mm) analytical column and an Inertsil ODS-3 (10×250 mm) semipreparative column using a HPLC system composed of a pump (PU-2010, Jasco) and a detector (MD-2080, Jasco).

Synthesis and Characterization

Compound **1** was synthesized according to a reported method[29,30] with some modifications, as described in the Supporting Information (Scheme S3). Cy5 was synthesized as reported.[31]

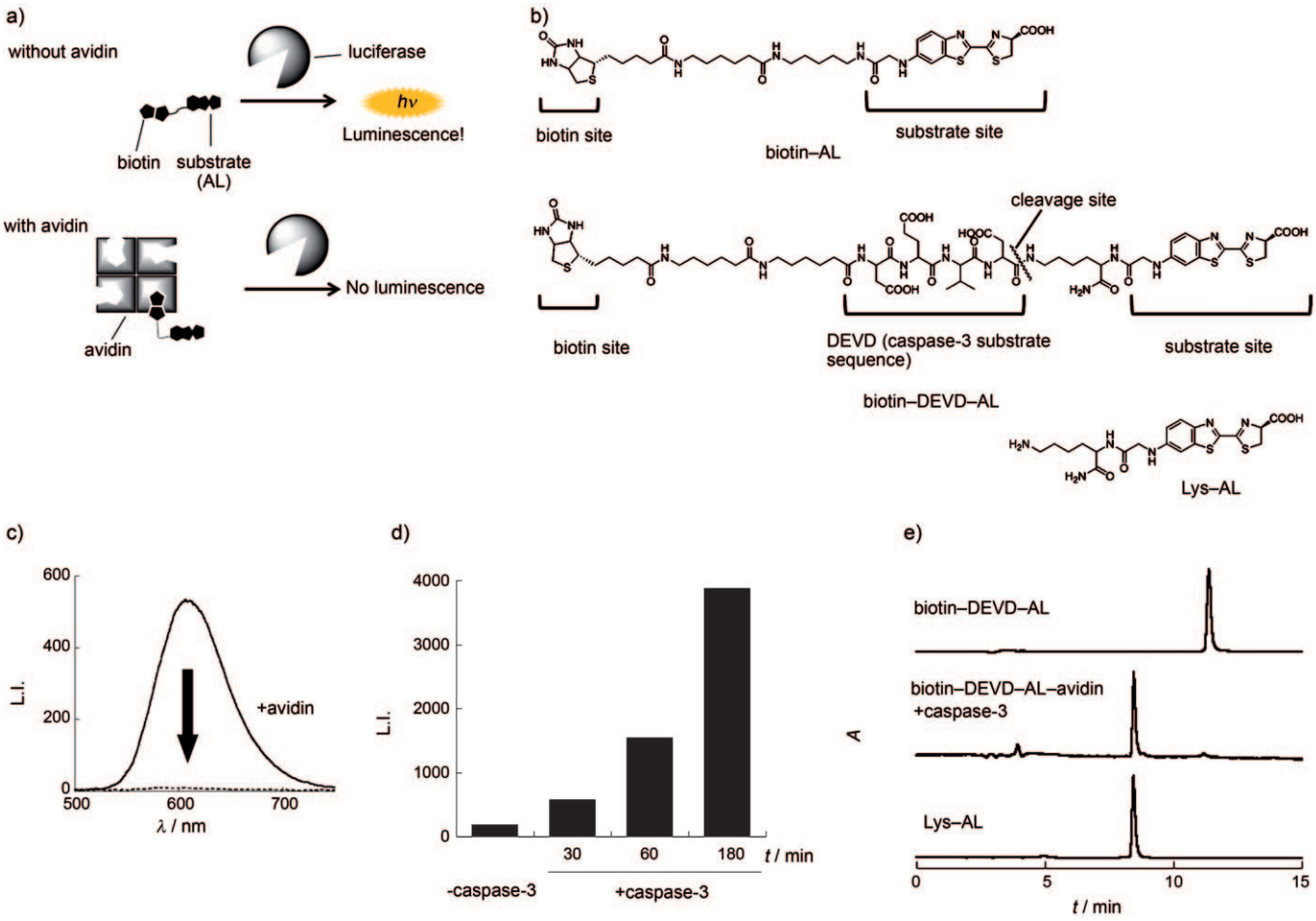
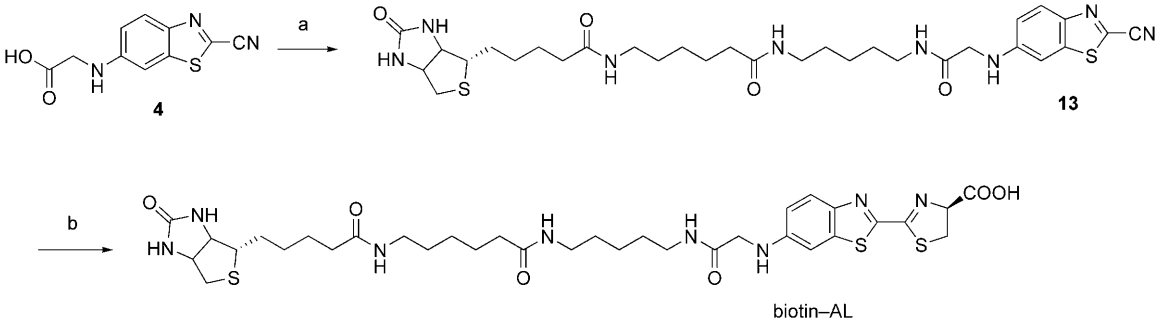


Figure 3. a) Schematic illustration of the concept of blocking access to luciferase with a huge molecule. b) Chemical structures of biotin–AL, biotin–DEVD–AL, and Lys–AL. c) Bioluminescence spectra of biotin–AL with (-----) and without avidin (——). d) Bioluminescence signal of the complex between biotin–DEVD–AL and avidin in the presence and absence of caspase-3. e) HPLC analysis of biotin–DEVD–AL, Lys–AL, and the reaction mixture of the complex between biotin–DEVD–AL and avidin with caspase-3. L.I. = luminescence intensity [a.u.], A = absorbance at 380 nm [a.u.].

General Procedure for N-Alkylation by Method A

The appropriate aldehyde (1.5 equiv for monoalkylation, 10 equiv for dialkylation) was dissolved in THF (20–40 mL). H_2SO_4 (180 mM, 1.5 equiv) was added and the solution was stirred at room temperature for a few minutes. A solution of **1** (50–120 mg, 1 equiv) and sodium borohydride (1.5 equiv) in THF (40–60 mL) was added and the reaction mixture was stirred at room temperature. After 1 h, water and brine were added to the reaction mixture, and the aqueous layer was separated and extracted with ethyl acetate. The organic extract was dried over Na_2SO_4, filtered, and evaporated. The product was purified by silica-gel column chromatography (ethyl acetate/*n*-hexane) or by semipreparative HPLC (eluent A: H_2O/0.1 % TFA, eluent B: 80 % CH_3CN/20 % H_2O/0.1 % TFA).



Scheme 5. Synthesis of biotin–AL. Reagents and conditions: a) biotin X cadaverine, HOBT, HBTU, NEt_3, DMF; b) D-cysteine, K_2CO_3, H_2O (pH 8)/MeOH, 47 % (2 steps).

General Procedure for N-Alkylation by Method B

The appropriate aldehyde (2 equiv for monoalkylation, 10 equiv for dialkylation) was dissolved in CH_3CN (10–20 mL). A solution of **1** (15~30 mg, 1 equiv) and sodium cyanoborohydride (1.5 equiv) in CH_3CN (10–20 mL) was added, and then AcOH (1 mL) was added. The reaction mixture was stirred at room temperature for 30 min. Methanol was added to the reaction mixture to quench excess sodium cyanoborohydride. The mixture was evaporated and the residue was extracted with ethyl acetate. The organic extract was dried over Na_2SO_4, filtered, and evaporated. The product was purified by silica-gel column chromatography (ethyl acetate/*n*-hexane) or by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA).

*2-Cyano-6-methylaminobenzothiazole (**2a**)*

Compound **2a** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:6) to give a yellow solid (61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.92 (d, J = 8.9 Hz, 1H), 6.92 (d, J = 2.4 Hz, 1H), 6.88 (dd, J = 2.4, 8.9 Hz, 1H), 4.27 (brs, 1H), 2.94 ppm (d, J = 5.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 149.9, 144.6, 138.8, 129.5, 125.4, 116.8, 113.9, 99.2, 30.4 ppm; MS (EI$^+$): m/z: 189 $[M]^+$.

*2-Cyano-6-dimethylaminobenzothiazole (**2b**)*

Compound **2b** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:6) to give a yellow solid (54%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.98 (d, J = 9.2 Hz, 1H), 7.06 (dd, J = 2.6, 9.2 Hz, 1H), 7.01 (d, J = 2.6 Hz, 1H), 3.10 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 150.6, 143.8, 138.6, 129.4, 125.1, 114.8, 114.0, 100.7, 40.6 ppm; MS (EI$^+$): m/z: 203 $[M]^+$.

*2-Cyano-6-ethylaminobenzothiazole (**2c**)*

Compound **2c** was prepared by Method A or B and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:4) to give a yellow solid (23% for Method A; 91% for Method B). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.91 (d, J = 9.0 Hz, 1H), 6.92 (d, J = 2.2 Hz, 1H), 6.87 (dd, J = 2.2, 9.0 Hz, 1H), 4.13 (brs, 1H), 3.30–3.18 (m, 2H), 1.33 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 149.0, 144.6, 138.8, 129.6, 125.5, 116.9, 113.8, 99.6, 38.3, 14.4 ppm; HRMS (ESI$^+$): m/z calcd for $C_{10}H_9N_3S$: 204.0595 $[M+H]^+$; found: 204.0555.

*2-Cyano-6-diethylaminobenzothiazole (**2d**)*

Compound **2d** was prepared by Method A or B and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:5) to give a yellow solid (5% for Method A; 62% for Method B). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.95 (d, J = 9.2 Hz, 1H), 7.01 (dd, J = 2.6, 9.2 Hz, 1H), 6.97 (d, J = 2.6 Hz, 1H), 3.46 (q, J = 7.1 Hz, 4H), 1.24 ppm (t, J = 7.1 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.4, 143.3, 139.1, 128.9, 125.5, 114.4, 114.0, 99.9, 45.0, 12.5 ppm; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{13}N_3S$: 232.0908 $[M+H]^+$; found: 232.0872.

*6-Benzylamino-2-cyanobenzothiazole (**2e**)*

Compound **2e** was prepared by Method A or B and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:4) and semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (10 min)) to give a yellow solid (57%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.93 (d, J = 8.7 Hz, 1H), 7.39–7.30 (m, 5H), 6.97–6.91 (m, 2H), 4.61 (brs, 1H), 4.43 ppm (d, J = 5.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.7, 144.8, 138.6, 137.7, 129.9, 128.9, 127.7, 127.3, 125.5, 117.0, 113.8, 100.2, 48.0 ppm; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{11}N_3S$: 266.0752 $[M+H]^+$; found: 266.0759.

*6-Phenethylamino-2-cyanobenzothiazole (**2f**)*

Compound **2f** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:6) to give a yellow solid (39%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.91 (d, J = 9.0 Hz, 1H), 7.38–7.21 (m, 5H), 6.95 (d, J = 2.4 Hz, 1H), 6.85 (dd, J = 2.4, 9.0 Hz, 1H), 4.22 (brs, 1H), 3.49 (td, J = 5.9, 6.9 Hz, 2H), 2.98 ppm (t, J = 6.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.7, 144.6, 138.6, 138.4, 129.5, 128.6, 126.6, 125.4, 117.0, 113.8, 99.8, 44.6, 34.9 ppm; HRMS (ESI$^+$): m/z calcd for $C_{16}H_{13}N_3S$: 280.0908 $[M+H]^+$; found: 280.0876.

*6-(3-Phenylpropylamino)-2-cyanobenzothiazole (**2g**)*

Compound **2g** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:6) to give a yellow solid (57%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.89 (d, J = 9.5 Hz, 1H), 7.36–7.19 (m, 5H), 6.84–6.79 (m, 2H), 4.15 (brs, 1H), 3.22 (td, J = 5.7, 7.2 Hz, 2H), 2.77 (t, J = 7.4 Hz, 2H), 2.02 ppm (tt, J = 7.2, 7.4 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.9, 144.5, 141.0, 138.7, 129.5, 128.5, 128.3, 126.1, 125.4, 117.0, 113.9, 99.6, 43.0, 33.2, 30.4 ppm; HRMS (ESI$^+$): m/z calcd for $C_{17}H_{15}N_3S$: 294.1065 $[M+H]^+$; found: 294.1091.

*6-(4-Phenylbutylamino)-2-cyanobenzothiazole (**2h**)*

Compound **2h** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:6 to 1:3) to give a yellow solid (53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.89 (d, J = 9.0 Hz, 1H), 7.32–7.16 (m, 5H), 6.88 (d, J = 2.4 Hz, 1H), 6.84 (dd, J = 2.4, 9.0 Hz, 1H), 3.20 (t, J = 6.6 Hz, 2H), 2.69 (t, J = 7.1 Hz, 2H), 1.84–1.66 ppm (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 149.0, 144.5, 141.8, 138.8, 129.4, 128.3, 128.3, 125.9, 125.4, 116.9, 113.6, 99.5, 43.6, 35.4, 28.6, 28.5 ppm; HRMS (ESI$^+$): m/z calcd for $C_{18}H_{17}N_3S$: 308.1221 $[M+H]^+$; found: 308.1175.

General Procedure for Cyclization of 2-Cyanobenzothiazoles

D-Cysteine hydrochloride monohydrate (3 equiv) was dissolved in water (10 mL) under argon bubbling and the pH of the solution was adjusted to 8 with potassium carbonate (0.5 M). The appropriate 2-cyanobenzothiazole compound (1 equiv) was dissolved in methanol (10–15 mL) with argon bubbling and the D-cysteine solution was added. The mixture was then stirred at room temperature under an argon atmosphere in the dark for 1–2 h. The reaction mixture was acidified to about pH 4 with hydrochloric acid and the methanol was evaporated. The residue was extracted with ethyl acetate, and the organic solution was washed with brine, dried over Na_2SO_4, filtered, and evaporated. The product was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA), or recrystallized from ethanol/*n*-hexane.

*D-(−)-2-(6′-Methylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3a**)*

Compound **3a** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) and recrystallized from ethanol/*n*-hexane to give a red solid (78%). M.p. 132–134 °C (dec); ^{1}H NMR (300 MHz, CD_3OD): δ = 7.75 (d, J = 9.0 Hz, 1H), 6.99 (d, J = 2.3 Hz, 1H), 6.88 (dd, J = 2.3, 9.0 Hz, 1H), 5.35 (t, J = 9.1 Hz, 1H), 3.78–3.67 (m, 2H), 2.84 ppm (s, 3H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.3, 167.9, 157.0, 148.5, 147.9, 140.0, 125.7, 118.0, 104.3, 79.2, 35.8, 32.0 ppm; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{11}N_3O_2S_2$: 294.0371 $[M+H]^+$; found: 294.0340; elemental analysis calcd (%) for $C_{12}H_{11}N_3O_2S_2 \cdot 0.25H_2O$: C 48.4, H 3.9, N 14.1; found: C 48.5, H 4.2, N 13.7.

*D-(−)-2-(6′-Dimethylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3b**)*

Compound **3b** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) and recrystallized from ethanol/*n*-hexane to give a red solid (90%). M.p. 177–178 °C (dec); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 13.22 (brs, 1H), 7.98 (d, J = 9.3 Hz, 1H), 7.38 (d, J = 2.5 Hz, 1H), 7.14 (dd, J = 2.5, 9.3 Hz, 1H), 5.44 (dd, J = 8.3, 9.6 Hz, 1H), 3.81 (dd, J = 9.6, 11.4 Hz, 1H), 3.70 (dd, J = 8.3, 11.4 Hz, 1H), 3.10 ppm (s, 6H); ^{13}C NMR (75 MHz, [D$_6$]acetone): δ = 171.5, 166.2, 156.0, 150,9, 146.1, 139.5, 125.3, 115.0, 103.0, 79.2, 41.0, 35.3 ppm; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{13}N_3O_2S_2$: 308.0527 $[M+H]^+$; found: 308.0481; elemental analysis calcd (%) for $C_{13}H_{13}N_3O_2S_2$: C 50.8, H 4.3, N 13.7; found: C 50.6, H 4.4, N 13.4.

D-(−)-2-(6′-Ethylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3c**)

Compound **3c** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) and recrystallized from ethanol/*n*-hexane to give a red solid (83%). M.p. 102–104 °C; ^{1}H NMR (300 MHz, CD_3CN): δ = 7.82 (d, J = 9.0 Hz, 1H), 7.16 (d, J = 2.2 Hz, 1H), 6.96 (dd, J = 2.2, 9.0 Hz, 1H), 5.35 (t, J = 9.2 Hz, 1H), 3.75–3.62 (m, 2H), 3.21 (q, J = 7.2 Hz, 2H), 1.24 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.3, 167.7, 158.0, 148.6, 146.0, 139.8, 125.8, 118.7, 106.3, 79.3, 42.0, 35.9, 13.6 ppm; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{13}N_3O_2S_2$: 308.0527 $[M+H]^+$; found: 308.0483; elemental analysis calcd (%) for $C_{13}H_{13}N_3O_2S_2 \cdot 0.25\,H_2O \cdot 0.25\,EtOH$: C 50.1, H 4.7, N 13.0; found: C 50.2, H 4.6, N 12.7.

D-(−)-2-(6′-Diethylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3d**)

Compound **3d** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) to give a red solid (79%). ^{1}H NMR (300 MHz, CD_3OD): δ = 8.09 (d, J = 9.0 Hz, 1H), 7.94 (d, J = 2.4 Hz, 1H), 7.47 (dd, J = 2.4, 9.0 Hz, 1H), 3.74–3.64 (m, 2H), 3.59 (t, J = 7.2 Hz, 4H), 1.08 ppm (t, J = 7.2 Hz, 6H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.5, 167.7, 155.7, 148.7, 145.7, 140.4, 125.5, 115.1, 102.8, 79.4, 46.1, 35.9, 12.8 ppm; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{17}N_3O_2S_2$: 336.0840 $[M+H]^+$; found: 336.0812.

D-(−)-2-(6′-Benzylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3e**)

Compound **3e** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (20 min)) and recrystallized from ethanol/*n*-hexane to give a red solid (42%). M.p. 108–109 °C; ^{1}H NMR (300 MHz, CD_3OD): δ = 7.77 (d, J = 8.9 Hz, 1H), 7.41–7.20 (m, 5H), 7.02 (d, J = 2.2 Hz, 1H), 6.97 (dd, J = 2.2, 8.9 Hz, 1H), 5.34 (t, J = 9.1 Hz, 1H), 4.41 (s, 2H), 3.78–3.66 ppm (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.3, 167.9, 155.8, 149.3, 146.7, 140.0, 139.9, 129.6, 128.5, 128.2, 125.5, 117.5, 102.9, 79.1, 48.9, 35.7 ppm; HRMS (ESI$^+$): m/z calcd for $C_{18}H_{15}N_3O_2S_2$: 370.0684 $[M+H]^+$; found: 370.0683; elemental analysis calcd (%) for $C_{18}H_{15}N_3O_2S_2 \cdot 0.5\,H_2O$: C 57.1, H 4.3, N 11.1; found: C 57.1, H 4.5, N 10.7.

D-(−)-2-(6′-Phenethylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**3f**)

Compound **3f** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (20 min)) to give a red solid (82%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.79 (d, J = 9.0 Hz, 1H), 7.31–7.19 (m, 5H), 7.13 (d, J = 2.3 Hz, 1H), 6.94 (dd, J = 2.3, 9.0 Hz, 1H), 5.36 (t, J = 9.0 Hz, 1H), 3.79–3.68 (m, 2H), 3.45 (t, J = 7.3 Hz, 2H), 2.94 ppm (t, J = 7.3 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.3, 167.9, 156.0, 148.6, 147.0, 140.5, 140.1, 129.8, 129.5, 127.4, 125.6, 117.6, 103.2, 79.0, 47.0, 35.9, 35.8 ppm; HRMS (ESI$^+$): m/z calcd for $C_{19}H_{17}N_3O_2S_2$: 384.0840 $[M+H]^+$; found: 384.0816.

D-(−)-2-[6′-(3-Phenylpropylamino)-2′-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (**3g**)

Compound **3g** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (20 min)) and recrystallized from ethanol/*n*-hexane to give a red solid (42%). M.p. 128–129 °C; ^{1}H NMR (300 MHz, CD_3OD): δ = 7.71 (d, J = 9.0 Hz, 1H), 7.22–7.12 (m, 5H), 6.98 (d, J = 2.4 Hz, 1H), 6.87 (dd, J = 2.4, 9.0 Hz, 1H), 5.27 (t, J = 9.0 Hz, 1H), 3.70–3.58 (m, 2H), 3.11 (t, J = 7.3 Hz, 2H), 2.65 (t, J = 7.5 Hz, 2H), 1.88 ppm (tt, J = 7.3, 7.5 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 173.3, 167.8, 156.6, 147.4, 142.8, 140.0, 129.4, 127.0, 125.6, 118.0, 104.0, 101.9, 79.1, 45.4, 35.8, 34.1, 31.2 ppm; HRMS (ESI$^+$): m/z calcd for $C_{20}H_{19}N_3O_2S_2$: 398.0997 $[M+H]^+$; found: 398.0959; elemental analysis calcd (%) for $C_{20}H_{19}N_3O_2S_2$: C 60.4, H 4.8, N 10.6; found: C 60.4, H 4.9, N 10.4.

D-(−)-2-[6′-(4-Phenylbutylamino)-2′-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (**3h**)

Compound **3h** was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (20 min)) to give a red solid (82%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.85 (d, J = 9.0 Hz, 1H), 7.28–7.12 (m, 6H), 7.05 (dd, J = 2.4, 9.0 Hz, 1H), 5.37 (t, J = 9.1 Hz, 1H), 3.80–3.71 (m, 2H), 3.24 (t, J = 7.0 Hz, 2H), 2.67 (t, J = 7.2 Hz, 2H), 1.82–1.66 ppm (m, 4H); ^{13}C NMR (75 MHz, [D_6]acetone): δ = 173.3, 167.8, 157.8, 148.6, 146.0, 143.2, 139.8, 129.4, 129.3, 126.8, 125.8, 118.8, 106.3, 79.2, 47.1, 36.4, 35.9, 29.7, 28.6 ppm; HRMS (ESI$^+$): m/z calcd for $C_{21}H_{21}N_3O_2S_2$: 412.1153 $[M+H]^+$; found: 412.1113.

6-Carboxymethylamino-2-cyanobenzothiazole (**4**)

Compound **4** was prepared by Method A or B and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:3 to 1:1) and semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) to give a yellow solid (23% for Method A; 90% for Method B). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.87 (d, 1H, J = 9.9 Hz), 7.08–7.04 (m, 2H), 3.99 ppm (s, 2H); ^{13}C NMR (75 MHz, [D_6]acetone): δ = 171.7, 150.2, 145.5, 139.6, 130.3, 126.0, 118.2, 114.6, 101.2, 45.2 ppm; HRMS (ESI$^+$): m/z calcd for $C_{10}H_7N_3O_2S$: 234.0337 $[M+H]^+$; found: 234.0300.

2-Cyano-6-[2-(1,3-di-tert-butoxycarbonylpropylamino)-2-oxoethylamino]benzothiazole (**5**)

1-Hydroxybenzotriazole (HOBT; 23 mg, 150 μmol) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophospate (HBTU; 60 mg, 160 μmol) were added to a solution of **1** (11 mg, 48 μmol) in DMF (2 mL) cooled on an ice bath. After a few minutes, glutamic acid di-*tert*-butyl ester (38 mg, 130 μmol) and ten drops of NEt_3 were added, then the solution was stirred for 1 h at room temperature. Water was added and the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated. The product was purified by silica-gel column chromatography (ethyl acetate/*n*-hexane = 1:3) and semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (10 min)) to give a yellow solid (15 mg, 67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.98 (d, 1H, J = 9.7 Hz), 7.10–7.04 (m, 1H), 7.02–6.95 (m, 2H), 4.58–4.52 (m, 1H), 3.93 (s, 2H), 2.30–2.20 (m, 2H), 2.18–2.05 (m, 1H), 1.95–1.82 (m, 1H), 1.43 (s, 9H), 1.41 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.3, 171.2, 169.1, 147.8, 145.7, 138.4, 131.2, 125.8, 117.2, 113.6, 101.2, 82.7, 81.1, 52.3, 47.8, 31.4, 28.0, 28.0, 27.3 ppm; HRMS (ESI$^+$): m/z calcd for $C_{23}H_{30}N_4O_5S$: 497.1835 $[M+Na]^+$; found: 497.1822.

2-Cyano-6-[2-(1,3-dicarboxypropylamino)-2-oxoethylamino]benzothiazole (**6**)

TFA (2 mL) was added to a solution of **5** (15 mg, 32 μmol) in CH_2Cl_2 (2 mL) and the reaction mixture was stirred for 2 h at room temperature. The reaction mixture was evaporated with toluene to give the crude product. The product was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 80:20 to 20:80 (20 min)) to give a yellow solid (2 mg, 19%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.80 (d, J = 9.7 Hz, 1H), 7.00–6.95 (m, 2H), 4.44–4.35 (m, 1H), 3.84 (s, 2H), 2.21 (t, J = 7.7 Hz, 2H), 2.15–2.05 (m, 1H), 1.90–1.76 ppm (m, 1H); HRMS (ESI$^-$): m/z calcd for $C_{15}H_{14}N_4O_5S$: 361.0607 $[M-H]^-$; found: 361.0595.

D-(−)-2-[6′-[2-(1,3-dicarboxypropylamino)-2-oxoethylamino]-2′-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (Glu–AL)

Glu–AL was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B = 50:50 to 0:100 (20 min)) to give a red solid (quant). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.81 (d, J = 9.0 Hz, 1H), 7.04 (d, J = 2.2 Hz, 1H), 6.96 (dd, J = 2.2, 9.0 Hz, 1H), 5.36 (t, J = 9.1 Hz, 1H), 4.52–4.48 (m, 1H), 3.91 (s, 2H), 3.78–3.68 (m, 2H), 2.30 (t, J = 6.6 Hz, 2H), 2.25–2.16 (m, 1H), 2.00–1.90 ppm (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 176.3, 174.6, 173.4, 173.3, 167.7,

156.5, 149.6, 147.0, 139.9, 125.5, 117.1, 102.5, 79.4, 52.9, 48.3, 35.8, 31.0, 27.7 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{18}H_{18}N_4O_7S_2$: 467.0695 $[M+H]^+$; found: 467.0722.

*6-(Benzyloxycarbonylamino)-1-hexanol (**8**)*

Compound **7** (2.89 g, 25 mmol) was dissolved in water (120 mL) and acetone (240 mL), and $NaHCO_3$ (2.3 g, 28 mmol) was added to the solution. Cbz chloride (4.71 g, 28 mmol) was slowly added over 5 min and the reaction mixture was stirred at room temperature. After 24 h, acetone was evaporated and the precipitate was collected by filtration, washed with water, and dried under high vacuum to afford a white solid without purification (5.84 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.38–7.31 (m, 5H), 5.10 (s, 2H), 4.75 (brs, 1H), 3.63 (td, J=5.9, 6.4 Hz, 2H), 3.20 (td, J=6.1, 6.8 Hz, 2H), 1.62–1.50 (m, 4H), 1.45–1.27 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=156.4, 136.5, 128.5, 128.0, 66.5, 62.6, 40.8, 32.5, 29.9, 26.3, 25.2 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{21}NO_3$: 274.1419 [M+Na]$^+$; found: 274.1394.

*6-(Benzyloxycarbonylamino)-1-hexanal (**9**)*

Pyridinium chlorochromate (PCC; 7.45 g, 35 mmol) was dissolved in CH_2Cl_2 (120 mL) and $MgSO_4$ (3.1 g) was added. A solution of **8** (5.77 g, 23 mmol) in CH_2Cl_2 (50 mL) was also added and the reaction mixture was stirred at room temperature for 4 h. The mixture was filtered, the filter was washed with CH_2Cl_2, and the filtrate was evaporated. The residue was purified by silica-gel column chromatography (ethyl acetate/*n*-hexane=1:2) to give a colorless oil (0.88 g, 16%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=9.76 (s, 1H), 7.40–7.28 (m, 5H), 5.10 (s, 2H), 4.75 (brs, 1H), 3.20 (td, J=6.4, 6.6 Hz, 2H), 2.44 (t, J=7.5 Hz, 2H), 1.73–1.29 ppm (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=202.4, 156.3, 136.4, 128.3, 127.9, 66.3, 43.5, 40.6, 29.5, 26.0, 21.4 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{19}NO_3$: 272.1263 [M+Na]$^+$; found: 272.1235.

*6-[6-(Benzyloxycarbonylamino)hexylamino]-2-cyanobenzothiazole (**10**)*

Compound **10** was prepared by Method A and purified by silica-gel column chromatography (ethyl acetate/*n*-hexane=1:5 to 1:2) to give a yellow solid (56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.90 (d, J=8.9 Hz, 1H), 7.38–7.31 (m, 5H), 6.90 (d, J=2.3 Hz, 1H), 6.87 (dd, J=2.3, 8.9 Hz, 1H), 5.10 (s, 2H), 4.74 (brs, 1H), 4.25 (brs, 1H), 3.28–3.14 (m, 4H), 1.67 (q, J=7.0 Hz, 2H), 1.59–1.34 ppm (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=156.4, 149.1, 144.4, 138.7, 136.5, 129.2, 128.4, 128.0, 128.0, 125.3, 116.9, 113.9, 99.4, 66.5, 43.4, 40.7, 29.8, 28.8, 26.4, 26.2 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{22}H_{24}N_4O_2S$: 431.1518 [M+Na]$^+$; found: 431.1510.

*6-(6-Aminohexylamino)-2-cyanobenzothiazole (**11**)*

Compound **10** (70 mg, 170 μmol) was dissolved in CH_2Cl_2 (5 mL) and TFA (20 mL) was added to the solution. The reaction mixture was stirred at room temperature for 16 h, then evaporated, and the residue was dissolved in 1N HCl (aq). The aqueous solution was washed with CH_2Cl_2 and extracted with ethyl acetate after neutralization. The organic layer was washed with brine, dried over Na_2SO_4, evaporated, and dried under high vacuum to afford a red solid (38 mg, 82%). ^{1}H NMR (300 MHz, CD_3OD): δ=7.81 (d, J=9.0 Hz, 1H), 7.04 (d, J=2.3 Hz, 1H), 6.99 (dd, J=2.3, 9.0 Hz, 1H), 3.18 (t, J=7.0 Hz, 2H), 2.92 (t, J=7.6 Hz, 2H), 1.73–1.61 (m, 4H), 1.54–1.42 ppm (m, 4H); ^{13}C NMR (75 MHz, CD_3OD): δ=151.7, 145.1, 140.4, 129.7, 125.9, 118.4, 114.9, 100.1, 44.2, 40.7, 29.6, 28.5, 27.7, 27.3 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{18}N_4S$: 275.1330 $[M+H]^+$; found: 275.1293.

*Synthesis of **12***

Cy5 (34 mg, 68 μmol) was dissolved in DMF (5 mL), and 1,3-dicyclohexylcarbodiimide (DCC; 18 mg, 88 μmol) and *N*-hydroxysuccinimide (NHS; 10 mg, 89 μmol) were added to the solution. The reaction mixture was stirred at room temperature and the progress of the reaction was monitored by HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B=50:50 to 0:100 (10 min)). After 5 h (Cy5/Cy5-SE/Cy5-diSE=0.8:1:0.2; SE=succinimidyl ester), a solution of **11** in DMF (2 mL) and NEt_3 (12 μL) was added to the reaction mixture, and stirring was continued at room temperature for 2 h. The mixture was purified with semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B=50:50 to 0:100 (10 min)) to give a blue solid (18 mg, 27% as a zwitterion). ^{1}H NMR (300 MHz, CD_3OD): δ=8.24 (t, J=13.0 Hz, 2H), 8.15 (brm, 1H), 7.77 (d, J=8.6 Hz, 1H), 7.46–7.20 (m, 8H), 6.98–6.91 (m, 2H), 6.60 (t, J=13.0 Hz, 1H), 6.31 (d, J=13.0 Hz, 2H), 4.39–4.30 (m, 4H), 3.13–3.04 (m, 4H), 2.78 (t, J=7.1 Hz, 2H), 2.64 (t, J=6.3 Hz, 2H), 1.75–1.66 (m, 16H), 1.64–1.51 ppm (m, 4H); HRMS (ESI$^+$): *m/z* calcd for $C_{45}H_{50}N_6O_3S$: 755.3743 $[M]^+$; found: 755.3748.

Synthesis of Cy5–AL

Cy5–AL was purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B=50:50 to 0:100 (20 min)) to give a red solid (47% as a zwitterion). ^{1}H NMR (300 MHz, CD_3OD): δ=8.21 (d, J=13.0 Hz, 2H), 7.71 (d, J=9.0 Hz, 1H), 7.45–7.19 (m, 8H), 6.95 (d, J=2.3 Hz, 1H), 6.87 (dd, J=2.3, 9.0 Hz, 1H), 6.58 (t, J=13.0 Hz, 1H), 6.30 (d, J=13.0 Hz, 2H), 5.35 (t, J=9.1 Hz, 1H), 4.39–4.31 (m, 4H), 3.76–3.66 (m, 2H), 3.14–3.04 (m, 4H), 2.77 (t, J=7.1 Hz, 2H), 2.63 (t, J=6.2 Hz, 2H), 1.70–1.65 (m, 12H), 1.63–1.52 (m, 2H), 1.42–1.30 (m, 4H), 1.29–1.19 ppm (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ=174.9, 174.9, 173.8, 173.1, 172.1, 167.7, 155.8, 155.2, 150.5, 146.1, 143.2, 143.1, 142.5, 142.5, 140.2, 129.7, 127.1, 126.4, 126.4, 125.4, 123.4, 123.3, 117.0, 112.1, 109.1, 104.9, 104.6, 101.3, 79.3, 50.6, 44.5, 41.8, 41.0, 40.6, 35.8, 35.0, 32.6, 30.2, 29.8, 28.0, 27.9, 27.8, 27.6 ppm; HRMS (ESI$^+$): *m/z* calcd for $C_{48}H_{54}N_6O_5S_2$: 859.3675 $[M]^+$; found: 859.3671.

*Synthesis of **13***

HOBT (5 mg, 30 μmol) and HBTU (12 mg, 30 μmol) were added to a solution of 4 (7 mg, 30 μmol) in DMF (2 mL) cooled in an ice bath. After a few minutes, biotin X cadaverine (10 mg, 20 μmol) and a few drops of NEt_3 were added and the solution was stirred for 1 h at room temperature. Water was added, and the reaction mixture was evaporated. The product was used for the next reaction without further purification. HRMS (ESI$^-$): *m/z* calcd for $C_{31}H_{44}N_8O_4S_2$: 655.2849 $[M-H]^-$; found: 655.2897.

Synthesis of Biotin–AL

Purified by semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% CH_3CN/20% H_2O/0.1% TFA, A/B=50:50 to 0:100 (20 min)) to give a red solid (47% (2 steps)). HRMS (ESI$^+$): *m/z* calcd for $C_{34}H_{48}N_8O_6S_3$: 783.2757 [M+Na]$^+$; found: 783.2787.

Measurement of Bioluminescence Kinetics (Figure 1c)

The COS7 cell line was purchased from RIKEN BioResource Center and maintained in Dulbecco's modified eagle medium (DMEM; Sigma, cat. no.: D5796) containing 10% fetal bovine serum (FBS; Gibco, cat. no.: 10082) and 1% penicillin/streptomycin (Gibco, cat. no.: 15140). Luciferase plasmid was kindly provided by Professor Ozawa (Department of Chemistry, School of Science, The University of Tokyo). COS7 cell line was transfected with the plasmid by using Lipofectamine 2000 (Invitrogen), and after 24 h the assay was carried out. COS7 cells transfected with luciferase were cultured on 96-well plates. Measurements were conducted with a plate reader following injection of 100 μM (final) substrate. In the lysate assay, the measurements were conducted after cell lysis (Glo lysis, Promega) for 5 min. The light unit is defined as luminescence accumulation for 10 min from injection of substrates.

Measurement of Absorbance Spectrum (Figure 2c)

3 μM Cy5–AL was dissolved in 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.7) and the absorbance spectrum was measured.

Measurement of Bioluminescence Spectra (Figure 2d)

The reaction cocktail (30 mM HEPES buffer, 5 mM $MgSO_4$, 2.6 mM ATP, 3.5 mM dithiothreitol (DTT), 25 μg mL^{-1} luciferase, pH 7.7) was prepared. Measurements were taken immediately after the addition of 5 μM (final) Cy5–AL at 25°C. The solution contained up to 0.5% (v/v) DMF as a co-

solvent. The slit width was 20 nm for emission and the photomultiplier voltage was 950 V.

Measurement of Bioluminescence Spectra (Figure 3c)

The reaction cocktail (30 mM HEPES buffer, 5 mM $MgSO_4$, 2.6 mM ATP, 3.5 mM DTT, 1.5 mM coenzyme A (CoA), 40 $\mu g mL^{-1}$ luciferase, pH 7.7) was prepared. Measurements were done immediately after the addition of 12 μM (final) biotin–AL with stirring at 25 °C. The solution contained up to 0.4 % (v/v) DMSO as a cosolvent. The complex of biotin–AL and avidin was formed by mixing the solutions (3 mM biotin–AL in DMSO (20 μL) and avidin (1 mg) in 30 mM HEPES buffer (0.98 mL), pH 7.7) for 10 min. The complex was purified by gel filtration (Prepacked Disposable PD-10 Columns, GE Healthcare) based on the exclusion of unbound biotin–AL and eluted with the reaction buffer (30 mM HEPES buffer, 5 mM $MgSO_4$, pH 7.7). The solution contained approximately 15 μM AL derivative (data not shown). 2.6 mM ATP, 3.5 mM DTT, and 1.5 mM CoA (final, respectively) were added to the solution and the measurements were initiated immediately after the addition of luciferase (40 $\mu g mL^{-1}$ (final)) with stirring at 25 °C. The slit width was 2.5 nm for emission and the photomultiplier voltage was 700 V.

Caspase-3 Assay and Bioluminescence Assay (Figure 3d)

The complex of biotin–DEVD–AL and avidin was obtained by mixing the solutions (3 mM biotin–DEVD–AL (15 μL) and avidin (1 mg) in 30 mM HEPES buffer (485 μL), pH 7.7) for 10 min. The complex was purified by gel filtration (PD MiniTrap G-25, GE Healthcare) based on exclusion of unbound probe, and eluted with caspase reaction buffer (30 mM HEPES, 5 mM $MgSO_4$, 1 % glycerol, 1 mM NaCl, 0.1 % CHAPS, pH 7.7). The complex was reacted with caspase-3 (80 mU/mL) in the reaction buffer containing 3.5 mM DTT for 30, 60, and 180 min at 37 °C. Final concentrations of 2.6 mM ATP and 1.5 mM CoA were added to the reaction mixture and then the bioluminescence reaction was initiated immediately after the addition of luciferase (40 $\mu g mL^{-1}$ (final)) with stirring at 25 °C. The slit width was 20 nm for emission and the photomultiplier voltage was 950 V.

HPLC Analysis (Figure 3e)

The enzymatic assay mixtures were retrieved and filtered by centrifugation. The solution was analyzed by HPLC (eluent A: H_2O/0.1 % TFA, eluent B: 80 % CH_3CN/20 % H_2O/0.1 % TFA, A/B = 80:20 to 20:80 (20 min)). The absorbance detection wavelength was 380 nm.

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