Supporting Information

New class of bioluminogenic probe based on bioluminescent enzyme-induced electron transfer: BioLeT

Hideo Takakura,[1,2,3, †] Ryosuke Kojima,[1,4, †] Mako Kamiya,[2,5] Eiji Kobayashi,[6] Toru Komatsu,[1,5] Tasuku Ueno,[1] Takuya Terai,[1] Kenjiro Hanaoka,[1] Tetsuo Nagano[7] and Yasuteru Urano[1,2,8,9]*

[1]Graduate School of Pharmaceutical Sciences, [2]Graduate School of Medicine, [7]Open Innovation Center for Drug Discovery, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033 Japan. [8]Basic Research Program, [9]CREST, [5]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan. [6]Division of Development of Advanced Treatment, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi-ken, 329-0498 Japan. Current position: [3]Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA. [4]Department of Biosystems Science and Engineering (D-BSSE), ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland. [†] These authors contributed equally to this work.

*uranokun@m.u-tokyo.ac.jp

Supplementary figures, notes, schemes inventory.

Supplementary figures

Synthesis and characterization

Supplementary Figures

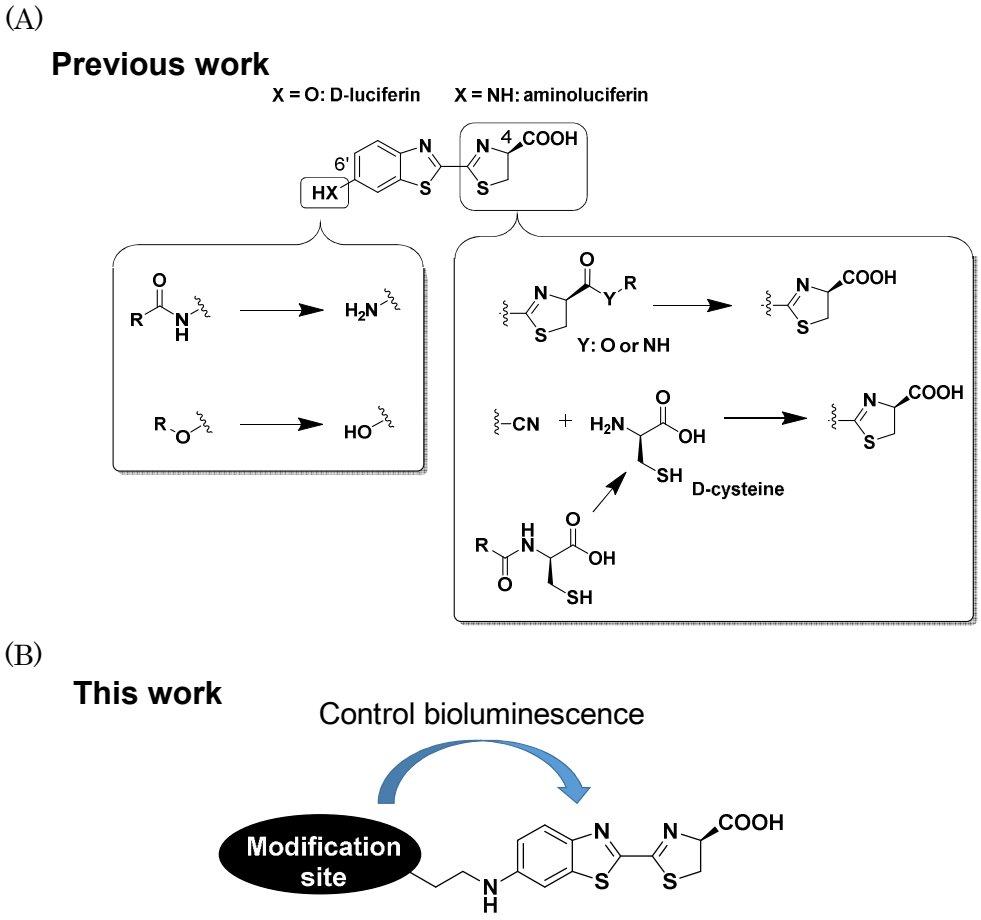


Figure S1. Structures and classification of small-molecule-based bioluminescence probes. (A) Typical structures of firefly luciferin-based bioluminescence probes. D-Luciferin is the native substrate for firefly luciferase. Aminoluciferin (AL) is an amino-substituted analog, one of the most highly luminescent substrates. D-Luciferin 6'-*O*-ether or ester and AL 6'-*N*-amide are non-luminescent substrates and can be used as conventional small-molecule-based bioluminescence probes. Hydrolysis of ester and amide at the 4-position and formation of D-luciferin from D-cysteine and nitrile precursors can also be used as actuation mechanisms of conventional probes. Almost all of these rely on eventual release or generation of D-luciferin or AL after the target reaction. (B) Bioluminogenic probes in our study. We have made it possible to rationally control bioluminescence from a remote modification site, providing a flexible design strategy for bioluminogenic probes.

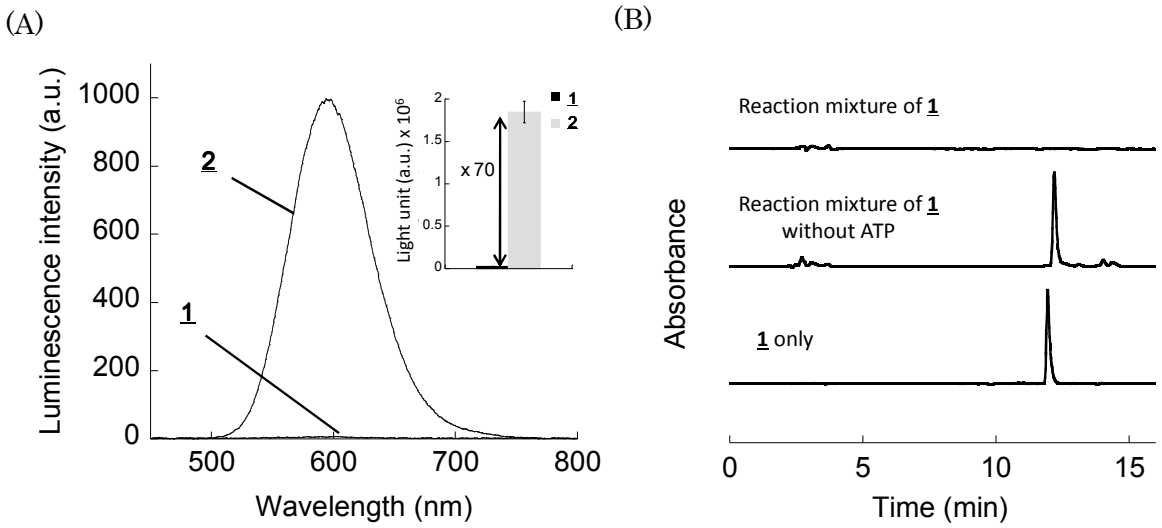
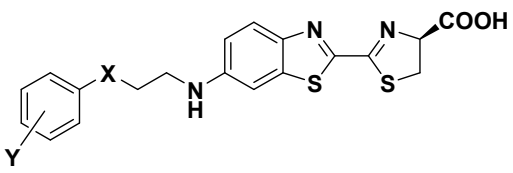


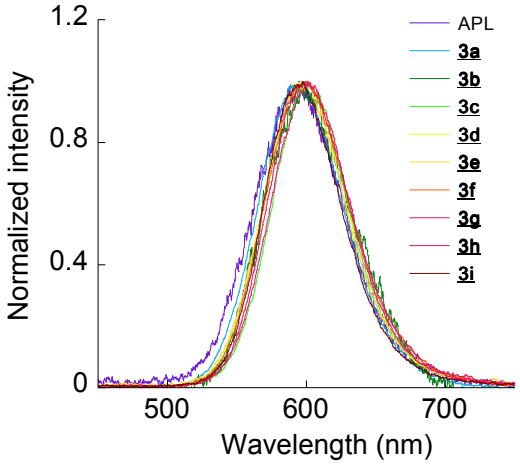
Figure S2. Luminescence properties of compounds **1** and **2**, and their consumption by luciferase. (A) Bioluminescence spectra of **1** and **2**. The luminescence intensities are shown in the inset. (B) HPLC analysis of the enzymatic reaction mixture of **1** was conducted with detection at 380 nm. As a negative control, reaction mixture without ATP was prepared (ATP is required for the bioluminescence reaction). Compound **1** was completely consumed by luciferase, but the bioluminescence of **1** was very weak.

(A)



3a; X=CH_2, Y=4-NMe_2
3b; X=NH, Y=H
3c; X=O, Y=4-Me
3d; X=O, Y=3-Me
3e; X=O, Y=H
3f; X=O, Y=4-Cl
3g; X=CH_2, Y=4-Me
3h; X=CH_2, Y=4-Cl
3i; X=CH_2, Y=3-Cl

(B)



(C)

Compound	HOMO (eV)	K_m (μM)	Relative L.I.
3a	-4.78	0.59 ± 0.03	4.0 ± 0.2
3b	-4.98	0.59 ± 0.07	3.0 ± 0.2
3c	-5.67	0.45 ± 0.01	17 ± 2
3d	-5.79	1.40 ± 0.06	22 ± 0.8
3e	-5.88	0.61 ± 0.01	25 ± 2.3
3f	-6.13	0.77 ± 0.04	29 ± 2.7
3g	-6.16	0.52 ± 0.04	11 ± 2.7
3h	-6.60	0.48 ± 0.03	77 ± 12
3i	-6.73	1.20 ± 0.05	100

Figure S3. Properties of compound **3a-i**. (A) Chemical structures of AL derivatives bearing benzene moieties with various HOMO energy levels. (B) Normalized bioluminescence spectra of **3a-i**. (C) HOMO energy level of the benzene moiety, K_m, and relative intensity of **3a-i**.

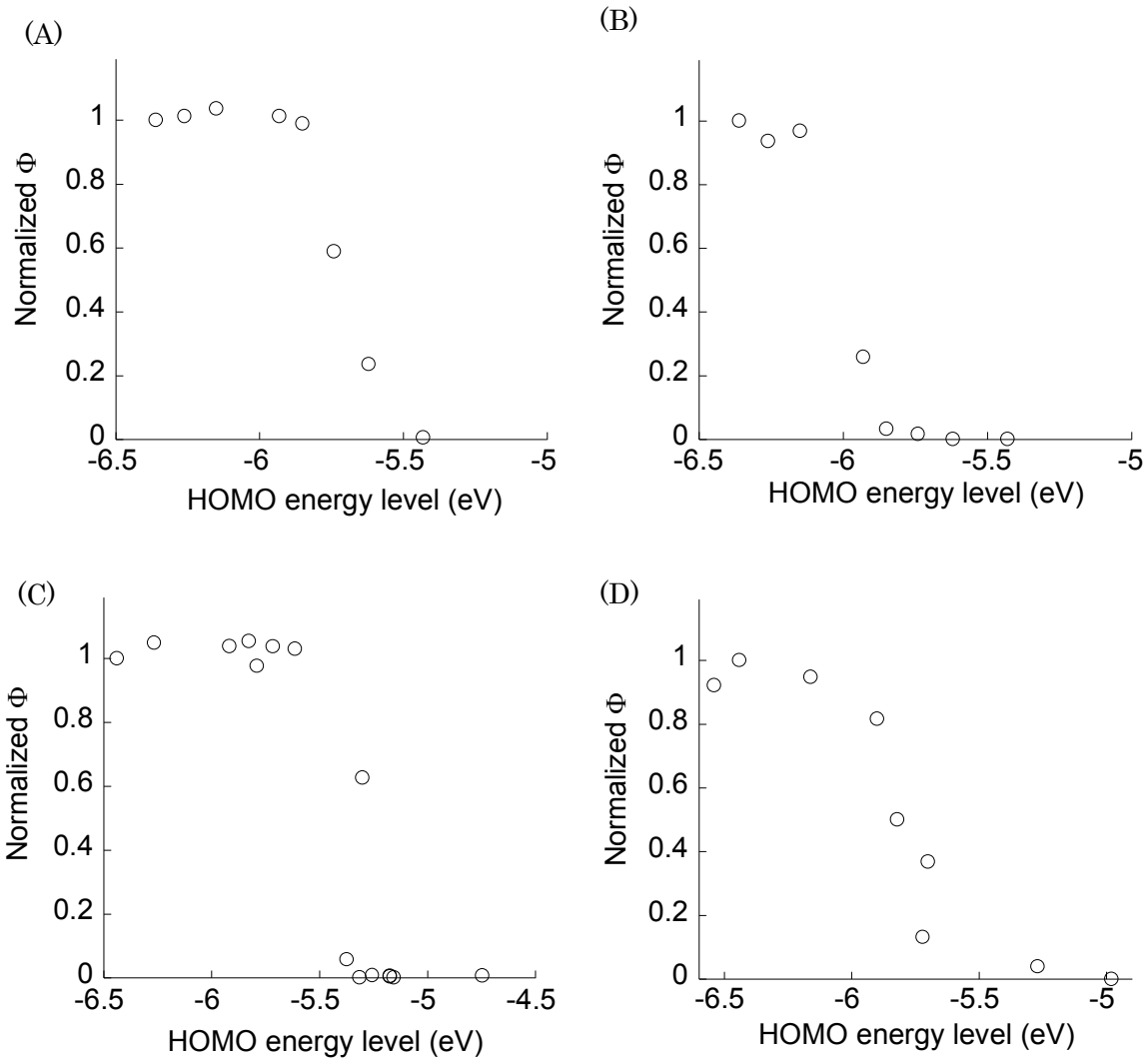
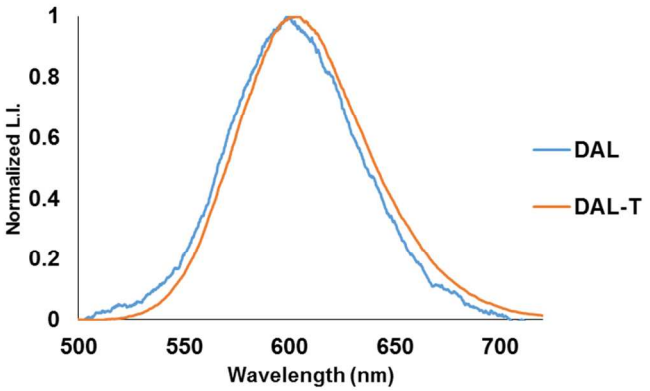


Figure S4. Relationship between HOMO energy level of adjacent benzene moieties and normalized fluorescence quantum yield. (A) Tokyo Green (anion form) [1], (B) Tokyo Green (neutral form) [1], (C) BODIPY[2], and (D) cs-124[3].

(A)



(B)

compound	HOMO (eV)	K_m (μM)	Relative L.I.
DAL	-4.68	1.73 ± 0.15	1
DAL-T	-6.22	2.11 ± 0.66	41.2 ± 3.4

Figure S5. Properties of DAL and DAL-T. (A) Normalized bioluminescence spectra of DAL and DAL-T. (B) HOMO energy level of the benzene moiety, K_m, and relative luminescence intensity (L.I.) of DAL and DAL-T.

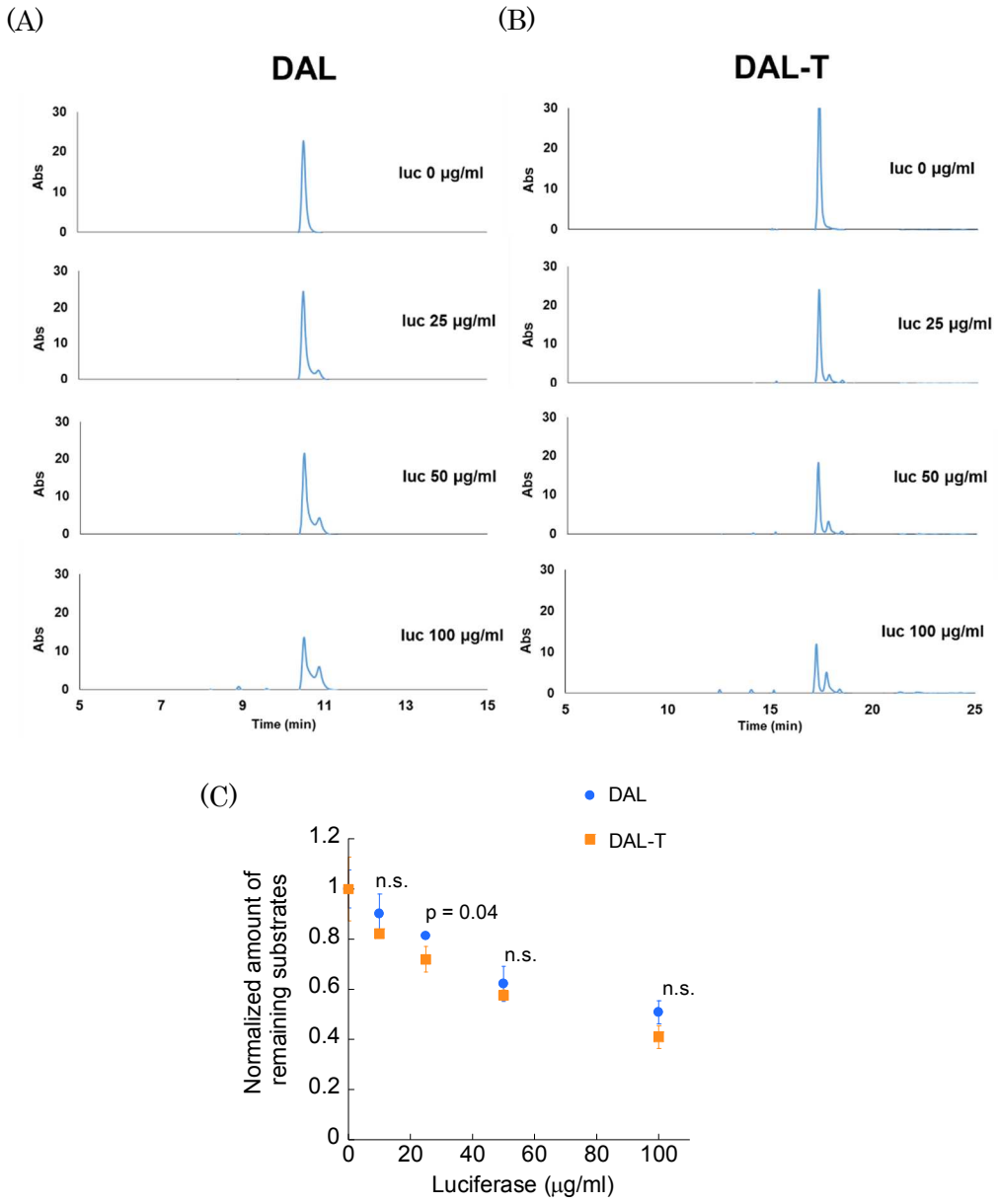
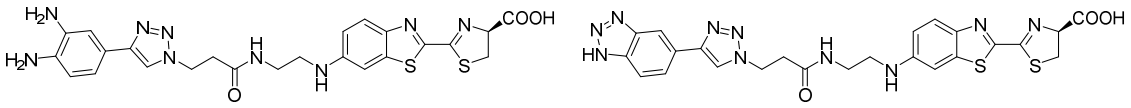


Figure S6. Comparison of the consumption rates of DAL and DAL-T with firefly luciferase. The consumption of (A) DAL and (B) DAL-T was monitored with LC-MS. The presence of each compound was confirmed by measuring the absorbance at 380 nm as well as by detection of the MS (ESI^+) signal in all experiments. (C) Quantification of remaining substrates during reaction with firefly luciferase. Amount of remaining substrate was evaluated from the integral value of the absorbance at 380 nm of the substrate divided by the integral of the absorbance at 490 nm of 2-Me-4-OMe-TG[1] (internal standard) (n = 3). Statistical analysis was done with Student's *t* test (n.s.: not significant, $p > 0.05$).

(A)



DAL2 DAL2-T

(B)

Compound	DAL2	DAL2-T
Relative L.I.	1.0	2.2

Figure S7. Comparison of luminescence intensities of DAL2 and DAL2-T. (A) Structures of DAL2 and DAL2-T. (B) Relative luminescence intensity (L.I.) of DAL2 and DAL2-T

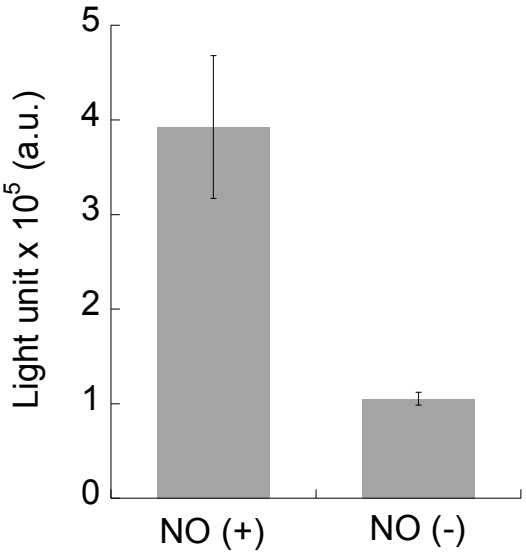


Figure S8. Luminescence increase of DAL after reaction with NO. NO-bubbled solution was added to solution containing DAL, and the luminescence increase was monitored (n = 3).

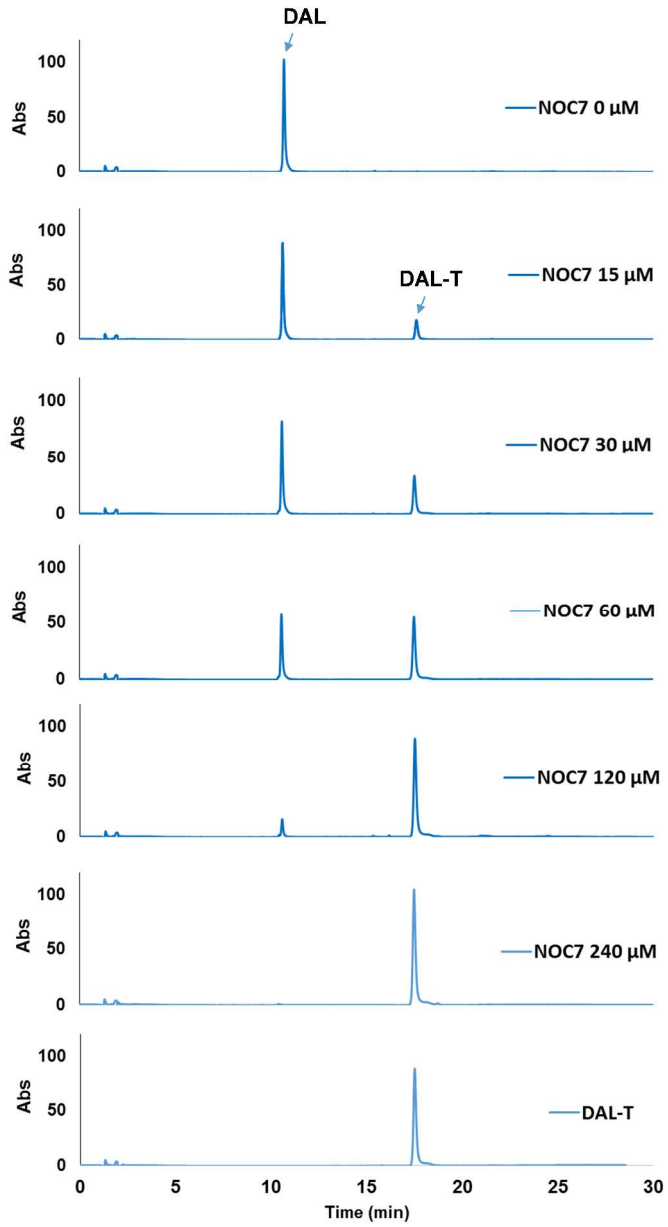


Figure S9. LC-MS analysis of the product after the reaction of DAL with NO released from NOC7. Absorbance at 380 nm was monitored and the MS signal (ESI^+) was also confirmed in all cases.

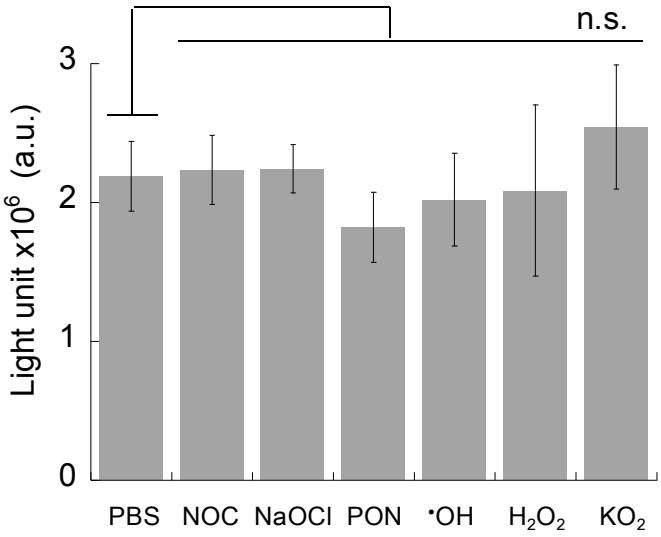


Figure S10. ROS sensitivity of luciferase reaction. Various ROS were added to a solution of AL and luciferase, and the luminescence intensity generated by addition of ATP solution was measured (n = 3). Statistical analysis was done with Student's *t* test (n.s.: not significant, $p > 0.05$). PON: peroxynitrite.

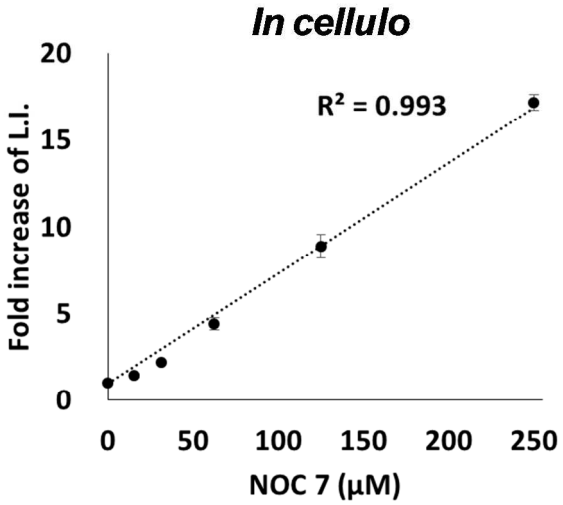


Figure S11. Quantification of NO from NOC7 with DAL in luc2-expressing HEK293 cells.

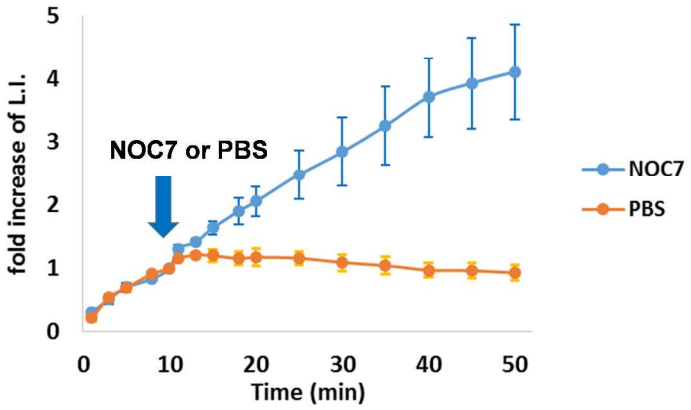


Figure S12. Kinetics of luminescence signal in *in vivo* imaging of NO with DAL in the peritoneal cavity of luc-Tg rat. Kinetics of fold increase of luminescence intensity with 1 μmol DAL upon i.p. injection of 20 μmol NOC7 or PBS. This protocol is the same as for Fig. 4 (error bars represent ±S.E.M. n = 3).

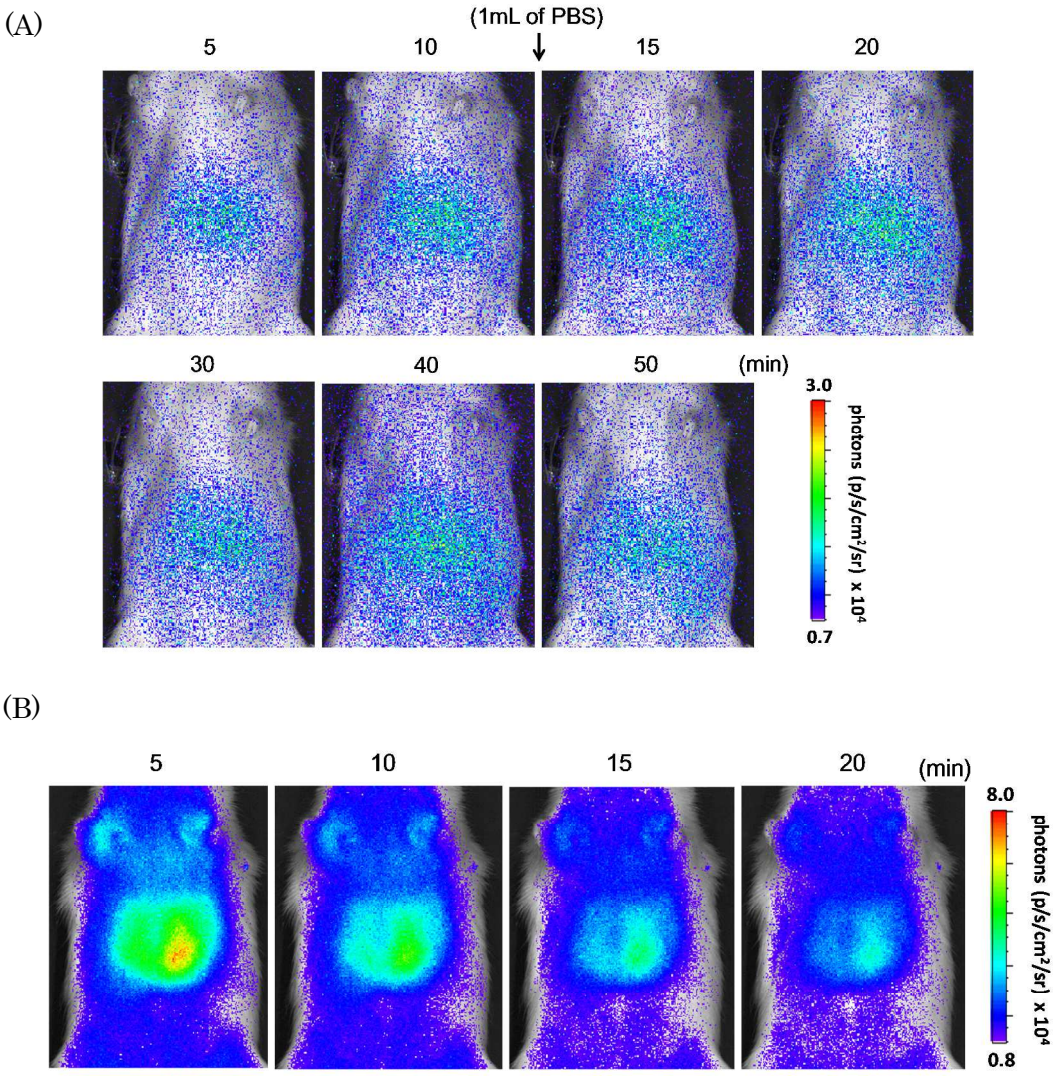


Figure S13. Biodistribution of DAL in luc-Tg rat injected i.p. or i.v. (A) BLI of a luc-Tg rat into which 1 μmol of DAL was injected i.p., followed by i.p. injection of 1 ml of PBS 10 min later. This shows that the i.p. injection of 1 ml of PBS did not affect the distribution of DAL *in vivo*. Note that the image at 50 min in this Fig. S13A is the same as the left image of Fig. 4A, but with a different dynamic range to clearly show the biodistribution. Also, these images were used to prepare the orange line (PBS) of Fig. S12. (B) BLI of a luc-Tg rat to which 0.5 μmol of DAL was injected i.v. through the penile vein.

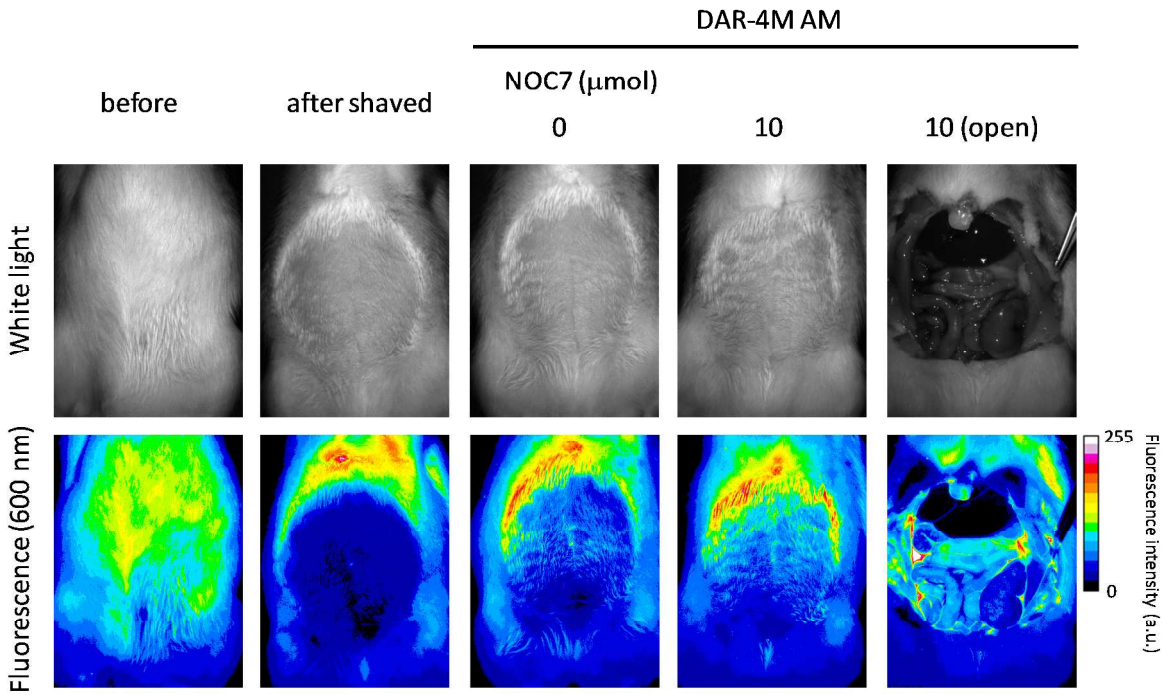


Figure S14. Fluorescence *in vivo* imaging of a rat injected with DAR-4M AM and NOC7 i.p. The images of the rat were taken before and after shaving the abdominal fur, at 10 min after the injcetion of DAR-4M AM (1 μmol) and at 40 min after the injection of NOC7 (10 μmol). After opening of the abdomen, another image was acquired.

Supplementary notes:

Note S1: Discussion of activation mechanism of NAT2 probe

There is a commercially available *N*-acetyltransferase 2 (NAT2) probe[4] that exhibits a distinct bioluminescence increase upon acetylation of alkylamine conjugated to AL[5], but the underlying mechanism of bioluminescence activation is unclear. We do not think the activation mechanism of the NAT2 probe is electron transfer for the following reasons. Typically, the pK_a of protonated alkylamine is 10-11, which means that the amine is predominantly protonated in neutral aqueous solutions. In general, protonated amines do not have much ability to act as electron donors in the process of electron transfer.

Also, when we compared the luminescence intensities of the NAT2 probe and aminoethyl-aminoluciferin (AE-AL), which we synthesized (Scheme S8), the brightness of AE-AL was higher than that of NAT2 probe (Fig. S15). Considering that the efficacy of electron transfer is closely correlated to distance between the electron donor and acceptor (see Fig. S7), this result seems incompatible with the idea that the activation mechanism involves electron transfer. The reason why the NAT2 probe is dark may be related to the substrate specificity of firefly luciferase.

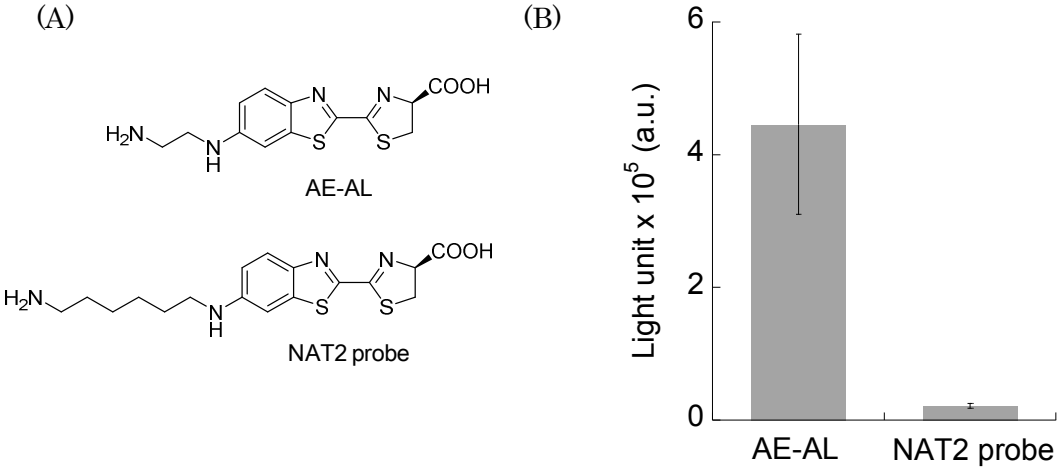


Figure S15. Comparison of luminescence intensity between AL derivatives. (A) Chemical structures of AE-AL and NAT2 probe. (B) Luminescence intensity of AE-AL and NAT2 probe.

Note S2: Discussion of correlation of luminescence intensity with HOMO energy level in Fig. 2B.

The luminescence intensity shown in Fig. 2B is thought to reflect the product of the speed of consumption of substrate and the quantum yield from the singlet excited state, of which the latter can be

quenched by BioLeT. Therefore, when the enzymatic consumption rate is slower, the luminescence intensity would be lower, even if the substrates have the same quantum yield. In order to minimize the influence of consumption rate, we prepared AL substrates having a similar size of benzene moiety for validation of BioLeT. Nevertheless, it is impossible to exclude the effect of the reaction rate completely, because it would depend on multiple factors, such as the bulkiness and lipophilicity of the substrate and specific interactions between the substrate and amino acid residues in the active site of luciferase. Indeed, the correlation between luminescence intensity and HOMO energy level is weaker than that in the case of PeT. Also, there seems to be an outlier (**3g**, HOMO energy level: -6.16 eV) in Fig. 2B; the most likely explanation of this is that the reaction rate of **3g** is relatively low for some reason.

Materials and general information

General chemicals were of the best grade available, supplied by Wako Pure Chemical, Tokyo Chemical Industries, Aldrich Chemical Company and Dojindo, and were used without further purification. NOC7, NOC5, MeOH (fluorometric grade), DMSO (fluorometric grade), DMF (fluorometric grade) were purchased from Dojindo. Luciferase from *Photinus pyralis* (E.C. 1.13.12.7) was purchased from Sigma-Aldrich Japan K.K. ATP disodium salt dehydrate was purchased from MP Biomedicals. NAT2 probe was purchased from Promega. DAR-4M AM was purchased from Sekisui Medical Co., LTD. ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded on a JNM-LA300 or JNM-LA400 (JEOL) instrument at 300 or 400 MHz for ^{1}H-NMR and at 75 or 100 MHz for ^{13}C-NMR. δ values are given in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with a JEOL JMS-T1000LC AccuTOF (ESI) or with a JEOL SX102A (EI). Flash column chromatography was performed with High Flash^{TM} column (Yamazen) fitted on an EPLC AI-580S system (Yamazen). Desalting of the compound was conducted with Sep-Pak C18 cartridges (Vac 6 cc (1g) or Vac 35 cc (10 g)) (Waters). LC-MS analysis was performed with a reverse-phase column (GL Sciences, Tokyo, Japan) (Inertsil ODS-3 2.5 mm × 250 nm) fitted on an Agilent 1200 series/6130 Quadrupole system. HPLC purification and analyses were performed with reverse-phase columns, Inertsil ODS-3 10 mm × 250 mm for purification and Inertsil ODS-3 4.6 mm × 250 mm for analysis (GL Sciences, Tokyo, Japan), fitted on a PU-1587, PU-2010 or PU-2080 (JASCO) system with an MD-2080 detector (JASCO), using eluent A and eluent B as specified for each compound. Bioluminescence spectra were obtained on a fluorescence spectrophotometer F4500 (Hitachi) (excitation light was physically blocked by a black well plate). Bioluminescence kinetics and light units were measured on an EnVision 2103 Multilabel Reader (Perkin Elmer). Bioluminescence images *in cellulo* and *in vivo* were taken with a IVIS Lumina II system (Xenogen). Images taken with the IVIS lumina II were analyzed with Living Image R3.0 software. Fluorescence images of animals were taken with a Maestro In-Vivo imaging system (CRi Inc.). All the experiments were carried out at room temperature (r.t.), unless otherwise specified.

Procedures

Calculation of HOMO energy level of each substrate (Fig. 2A, S3C, S5B): HOMO energy level of each benzene moiety was calculated with B3LYP/6-31G by Gaussian 98W after rough structure optimization by MM2 (Chem3D).

Measurement of relative luminescence intensity of AL derivatives (Fig. 1B, 2B, 3A, S2A, S3C, S5B, S7B, S15):

Fig. 1B, 2B, S2A, S3C: The reaction cocktail, consisting of 30 mM HEPES buffer (pH 7.7, 140 μl) containing 5 mM $MgSO_4$, 5 mM DTT, 714 μM CoA, 0.43 μg/ml luciferase from *Photinus pyralis*, and 0-12.9 μM substrate, was prepared on a 96-well plate. Luminescence measurement was started in an Envision 2103 plate reader (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 30 mM HEPES buffer (pH 7.7, 60 μl) containing 5 mM $MgSO_4$ and 8.67 mM ATP was injected with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 90 sec for each condition. Final concentrations after injection of ATP: 30 mM HEPES buffer, 5 mM $MgSO_4$, 3.5 mM DTT, 500 μM CoA, 0.30 μg/ml luciferase from *Photinus pyralis*, 0-9 μM substrate, and 2.6 mM ATP. The luminescence intensity was obtained by integration of luminescence intensity from the time of injection for 90 sec (Fig. 1B, S2A) or 60 sec (Fig. 2B, S3C). Data shown are mean ± S.D. of three to five independent measurements. Relative luminescence intensity of **1**, and **2** was determined at 0.18 μM. Relative luminescence intensity of **3a-i** was determined at 9 μM.

Fig. 3A, S5B, S7B: The reaction cocktail, consisting of 100 mM NaPi buffer (pH 7.7, 140 μl) containing 0-91.4 μM each substrate, 5.0 mM $MgSO_4$, 0.429 μg/ml luciferase from *Photinus pyralis*, and a cosolvent (less than 0.86% DMSO), was prepared on a 96-well plate. Luminescence measurement was started in the Envision 2103 (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 100 mM NaPi buffer (pH 7.7, 60 μl) containing 8.67 mM ATP, 5.0 mM $MgSO_4$ was added with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 30 sec under each condition. Final concentrations: 0-64 μM substrate, 5.0 mM $MgSO_4$, 0.30 μg/ml luciferase, and less than 0.6% DMSO, 2.6 mM ATP. The luminescence intensity was obtained by integration of luminescence intensity from the time of injection for 25 sec. Data shown are mean ± S.D. of four independent measurements. Relative luminescence intensity of DAL and DAL-T was determined at 8 μM. Relative luminescence intensity of DAL2 and DAL2-T was determined at 32 μM.

Fig. S15: The reaction cocktail, consisting of 30 mM HEPES buffer (pH 7.7, 140 μl) containing 14.3 μM each substrate, 5.0 mM $MgSO_4$ and 0.43 μg/ml luciferase from *Photinus pyralis* was prepared on a

96-well plate. Luminescence measurement was started in the Envision 2103 (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 30 mM HEPES buffer (pH 7.7, 60 μl) containing 8.67 mM ATP, 5.0 mM $MgSO_4$ was added with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 30 sec under each condition. Final concentrations: 10 μM substrate, 5.0 mM $MgSO_4$, 0.30 μg/ml luciferase and 2.6 mM ATP. The luminescence intensity was obtained by integration of luminescence intensity from the time of injection for 25 sec. Data shown are mean ± S.D. of three independent measurements.

K_m of each substrate was determined by using the data collected in these experiments. Luminescence intensity of each substrate was plotted at various concentrations. K_m was determined from a Michaelis-Menten plot.

Snapshot of bioluminescence (Fig. 1B, 3A): In a quartz cell (1 cm × 1 cm), 30 mM HEPES buffer (3 ml) containing 20 μM each substrate, 5 mM $MgSO_4$, and 2.6 mM ATP was prepared. Luciferase (final 20-33 μg/ml) was added in a dark room and the luminescence image was taken with a digital camera (PEN E-P2 (Olympus)). Exposure time was 10-30 sec. (Compounds **1** and **2** were compared under the same conditions. DAL and DAL-T were compared under the same conditions.)

Measurement of luminescence spectra of DAL upon reaction with NO and various ROS. (Fig. 3B): In a quartz cell (1 cm × 1 cm), 100 mM NaPi buffer (pH 7.7, 1.97 ml) containing DAL (12 μM when dissolved in 2 ml, 0.08% DMSO as a cosolvent) and 5 mM $MgSO_4$ was prepared. DAL was reacted with NO and various ROS with stirring under the following conditions.

NO: 8 μl of 30 mM NOC7 in 0.1 N NaOH aq. was added to the solution (120 μM when dissolved in 2 ml), and the solution was stirred at 25 °C for 10 min.

Hypochlorite: 5 μl of 2.4 mM NaOCl in 0.1 N NaOH aq. was added to the solution (6 μM when dissolved in 2 ml), and the solution was stirred at 25 °C for 1 min.

Peroxynitrite: 5 μl of 2.4 mM NaOONO in 0.1 N NaOH aq. was added to the solution (6 μM when dissolved in 2 ml), and the solution was stirred at 25 °C for 1 min.

OH radical: 2 μl of 1 M H_2O_2 in PBS was added to the solution (1 mM when dissolved in 2 ml). Then, 20 μl of 36 mM ferrous perchlorate aq. (20 μl, 360 μM when dissolved in 2 ml) was slowly added with vigorous stirring and the solution was stirred at 25 °C for 1 min.

Hydrogen peroxide: 2 μl of 1 M H_2O_2 in PBS was added to the solution (1 mM when dissolved in 2 ml), and the solution was stirred at 25 °C for 5 min.

Superoxide: 2 μl of 1 M KO_2 dissolved in PBS was added to the solution (1 mM when dissolved in 2 ml; the solution of KO_2 was prepared just before use) and the solution was stirred at 25 °C for 5 min.
Then, 520 mM ATP (2.6 mM when dissolved in 2 ml) in 100 mM NaPi buffer (pH 7.7, 10 μl) was added to the solution. The bioluminescence spectrum was measured with a F4500 fluorescence spectrophotometer immediately after addition of 100 mM NaPi buffer (pH 7.7, 20 μl) containing 1 mg/ml luciferase from *Photinus pyralis* (10 μg/ml when dissolved in 3 ml) with stirring at 25 °C.

Quantification of NO released from a NO donor *in vitro* (Fig. 3C): In wells of a 96-well plate, 800 μM DAL in 100 mM NaPi buffer (pH 7.7, less than 6.0% DMSO as a cosolvent, 4 μl) and 0–9.6 mM NOC7 in 100 mM NaOH aq (2 μl) were spotted separately in the same well. The reaction was started by addition of 100 mM NaPi buffer (pH 7.7, 74 μl) containing 13.3 mM $MgSO_4$. The plate was shaken for 10 sec in the Envision 2103 and was incubated at r.t. for 10 min, then 100 mM NaPi buffer (60 μl) containing 1 μg/ml luciferase from *Photinus pyralis* was added. Luminescence measurement was started in the Envision 2103 (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 100 mM NaPi buffer (pH 7.7, 60 μl) containing 8.67 mM ATP was injected with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 30 sec under each condition. Concentrations during reaction of DAL and NO: 100 mM NaPi buffer (pH 7.7), 40 μM DAL, 0–240 μM NOC7, 12.3 mM $MgSO_4$, total volume 80 μl. Final concentrations after addition of luciferase and ATP: 16 μM DAL, 0–96 μM NOC7, 5.0 mM $MgSO_4$, 0.3 μg/ml luciferase from *Photinus pyralis*, less than 0.12% DMSO, 2.6 mM ATP, total volume 200 μl. The value of luminescence intensity was obtained by integration of luminescence intensity from the time of injection for 25 sec. The Y axis in the figure is the luminescence intensity, and the X axis is the concentration of NOC7 during the reaction (before addition of luciferase and ATP). Data shown are mean ± S.D. (n = 4).

***In vivo* imaging of NO with DAL (Fig. 4, S12, S13A)**: All procedures were carried out in compliance with the *Guide for the Care and Use of Laboratory Animal Resources,* and were approved by the Institutional Animal Care Committee. Details of the luc-Tg rat have been reported.[6] We used male luc-Tg rats (9-14 weeks old, 260-330 g body weight). Luc-Tg rats were given an intraperitoneal (i.p.) injection of 1 μmol DAL dissolved in 900 μl of PBS (containing 11% DMSO as a cosolvent) in the peritoneal cavity under isoflurane anesthesia. Bioluminescence images were acquired at a given time with an IVIS lumina II (gain 1 min, stage D). At 10 min after the injection, the rats were injected i.p. with 20 μmol NOC7 in 1 ml of PBS (control: PBS only). Image processing was performed using imaging software (Living Image). ROI was selected to cover the whole peritoneal cavity. All

luminescence intensity values were normalized by the luminescence intensity at 10 min after injection of the probe (soon before injection of NOC7). The BLI shown in Fig. 4 were taken at 40 min after the injection of NOC7.

Measurement of luminescence spectra of each substrate (Fig. S2A, S3B, S5A):

Fig. S2A, S3B: In a quartz cell (1 cm × 1 cm), a reaction cocktail consisting of 30 mM HEPES buffer (pH 7.7, 3 ml) containing 5 mM $MgSO_4$, 2.6 mM ATP, 3.5 mM dithiothreitol (DTT), 1.5 mM coenzyme A (CoA), and 40 μg/ml luciferase was prepared. Luminescence spectra were measured with a F4500 fluorescence spectrophotometer immediately after the addition of 12 μM (final) substrate with stirring at 25 °C. The slit width was 2.5 or 10 nm for emission and the photomultiplier voltage was 700 V or 950 V. Parameters were chosen so as not to exceed the upper limit of measurement.

Fig. S5A: In a quartz cell (0.5 cm × 0.5 cm), a reaction cocktail consisting of 100 mM NaPi buffer (pH 7.7, 1 ml) 10 μM substrate, 2.6 mM ATP, 5.0 mM $MgSO_4$, and less than 0.58% DMSO as a cosolvent was prepared. Luminescence spectra were measured with a F4500 fluorescence spectrophotometer soon after the addition of 50 μg/ml luciferase (final) with stirring at 25 °C. The slit width was 10 nm or 20 nm for emission and the photomultiplier voltage was 700 V or 950 V. Parameters were chosen so as not to exceed the upper limit of measurement.

HPLC analysis of consumption of compound 1 by luciferase (Fig. S2B): The reaction cocktail consisted of 30 mM HEPES buffer (pH 7.7, 2.88 ml) containing 5 mM $MgSO_4$, 2.6 mM ATP, 3.5 mM DTT, 1.5 mM CoA, and 12 μM substrate **1** (all concentrations are final ones when dissolved in 3 ml). After enzymatic reaction by addition of 1 mg/ml luciferase (120 μl, 40 μg/ml when dissolved in 3 ml), the solution was filtered by centrifugation using an Amicon Ultra-4 (Millipore) and the filtrate was lyophilized. The residue was dissolved in water (50 μl), and an aliquot of the solution was subjected to analytical HPLC. For the negative control, ATP was omitted. Eluent and detection conditions were as follows: eluent A: H_2O/0.1% trifluoroacetic acid (TFA), eluent B: 80% MeCN/20% H_2O/0.1% TFA, A/B = 80/20 to 20/80 (20 min); Flow rate was 1.0 ml/min. Detection wavelength = 380 nm.

LC-MS analysis of the rates of consumption of DAL and DAL-T by firefly luciferase (Fig. S6): DAL and DAL-T were reacted with luciferase under the following conditions at r.t. for 30 min: 15 μM substrate, 0-100 μg/ml luciferase, 2.6 mM ATP, 5.0 mM $MgSO_4$ in 100 mM NaPi (pH 7.7, 150 μl). The reaction was stopped by addition of 1.5 μl of AcOH (1%). Then, 10 μM 2-Me-4-OMe-TG[1] (15 μl, 1 μM when dissolved in 151.5 μl) was added to each reaction solution as an internal standard. An aliquot of

the reaction solution (30 μl out of 166.5 μl) was subjected to LC-MS analysis (Eluent A: H_2O, 0.1% formic acid, B: MeCN:H_2O 4:1, 0.1% formic acid, A:B 90/10 to 20/80 for 20 min). Absorbance at 380 nm and 490 nm was monitored for substrates and 2-Me-4-OMe-TG, respectively, and the MS signal (ESI^+) was also confirmed. The rate of consumption of the substrates was evaluated from the integral value of the absorbance at 380 nm of the substrates/integral of the absorbance at 490 nm of 2-Me-4-OMe-TG. Data shown are mean ± S.D. (n = 3). Statistical analysis was done with Student's *t* test.

Detection of NO with DAL (Fig. S8): A saturated NO solution was prepared by bubbling NO gas for 20 min through water which had been deoxygenated by Ar bubbling for 30 min. In wells of a 96-well plate, 800 μM DAL in 100 mM NaPi buffer (pH 7.7, less than 6.0% DMSO as a cosolvent, 4 μl) and the saturated NO solution or water (16 μl) were spotted separately in the same well. The reaction was started by addition of 100 mM NaPi buffer (pH 7.7, 60 μl) containing 13.3 mM $MgSO_4$. The plate was shaken for 10 sec in the Envision 2103 and incubated at r.t. for 10 min, then 100 mM NaPi buffer (60 μl) containing 1 μg/ml luciferase from *Photinus pyralis* was added. Luminescence measurement was started in the Envision 2103 (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 100 mM NaPi buffer (pH 7.7, 60 μl) containing 8.67 mM ATP was injected with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 30 sec under each condition. Concentrations during reaction of DAL and NO: 100 mM NaPi buffer (pH 7.7), 40 μM DAL, 12.3 mM $MgSO_4$, total volume 80 μl. Final concentrations after addition of luciferase and ATP: 16 μM DAL, 5.0 mM $MgSO_4$, 0.3 μg/ml luciferase from *Photinus pyralis*, less than 0.12% DMSO, 2.6 mM ATP, total volume 200 μl. The value of light unit was obtained by integration of luminescence intensity from the time of injection for 25 sec. Data shown are mean ± S.D. (n = 3).

LC-MS analysis of the products of the reaction of DALs and NO released from NOC7 (Fig. S9): The solution (before addition of luciferase) prepared according to the same protocol as for Fig. 3C was subjected to LC-MS analysis (30 μl). Eluent A: H_2O, 0.1% formic acid, B: MeCN:H_2O 4:1, 0.1% formic acid, A:B 90/10 to 20/80 for 20 min, absorbance at 380 nm was monitored and the MS signal (ESI^+) was confirmed.

Measurement of ROS sensitivity of luciferase (Fig. S10): In wells of a 96-well plate, the following concentration of a ROS or PBS (2 μl) was spotted.
NO: 2 μl of 7 mM NOC5 in 0.1 N NaOH aq. (100 μM when dissolved in 140 μl).

Hypochlorite: 2 μl of 1.4 mM NaOCl in 0.1 N NaOH aq. (20 μM when dissolved in 140 μl).
Peroxynitrite: 2 μl of 1.4 mM NaOONO in 0.1 N NaOH aq. (20 μM when dissolved in 140 μl).
OH radical: 2 μl of 70 mM H_2O_2 in PBS (1 mM when dissolved in 140 μl).
Hydrogen peroxide: 2 μl of 70 mM H_2O_2 in pure water (1 mM when dissolved in 140 μl).
Superoxide: 2 μl of 70 mM KO_2 dissolved in 0.1 N NaOH aq. (1 mM when dissolved in 140 μl).
Then, 100 mM NaPi buffer (pH 7.7, 140 μl) containing 17 μM AL, 5 mM $MgSO_4$, 0.43 μg/ml luciferase and a cosolvent (0.3% DMF) was vigorously added to the well to ensure thorough mixing. In the case of OH radical, 2 μl of 7 mM ferrous perchlorate aq. was added (100 μM when dissolved in 140 μl). The solution was incubated at r.t. for 5 min. Luminescence measurement was started in an Envision 2103 plate reader (mode: luminescence, gain 1 sec). At 5 sec after the start of the measurement, 100 mM NaPi buffer (pH 7.7, 60 μl) containing 5 mM $MgSO_4$ and 8.67 mM ATP was injected with a dispenser fitted to the Envision 2103. In total, measurement was conducted for 30 sec. Final concentrations after injection of ATP: 100 mM NaPi buffer, 5 mM $MgSO_4$, 0.30 μg/ml luciferase from *Photinus pyralis*, 12 μM AL, and 2.6 mM ATP. The luminescence intensity was obtained by integration of luminescence intensity from the time of injection for 25 sec. Data shown are mean ± S.D. of three independent measurements. Statistical analysis was done with Student's *t* test.

Quantification of NO released from a NO donor *in cellulo* (Fig. S11): In wells of a 96-well plate, 0–15 mM NOC7 in 100 mM NaOH aq (1 μl) was spotted. Reaction of DAL and NO was started by addition of PBS (pH 7.4, 59 μl) containing 65.1 μM DAL. The mixture was incubated at r.t. for 15 min. Then, HEK293-luc2 cells (1.0×10^5 cells) suspended in PBS (pH7.4, 60 μl) were added and luminescence from the cells was measured with the IVIS Lumina II system at 1 min after addition of the cells (gain 1 min, stage C). Concentrations during reaction of DAL and NOC7: PBS (pH 7.4) containing 64 μM DAL, 0–240 μM NOC7, total volume 60 μl. Final concentrations after addition of the cells: PBS (pH 7.4) containing 32 μM DAL, 0–120 μM NOC7, 1.0×10^5 cells of HEK293-luc2 cells, total volume 120 μl. The Y axis in the figure is the luminescence intensity, and the X axis is the concentration of NOC7 during the reaction (before addition of HEK293-luc2 cells). Data shown are mean ± S.D. (n = 3).

Observation of biodistribution of substrates in luc-Tg rats (Fig. S13B): Luc-Tg rats (male, 28 weeks old) were given a penile intravenous (i.v.) injection of substrates (0.5 μmol DAL in 500 μl of PBS containing 10% DMSO and 0.01% AcOH as a cosolvent) under isoflurane anesthesia. Bioluminescence images were taken at a given time with an IVIS Lumina II up to 20 min (gain: 5 min, stage D). Image processing was performed with imaging software (Living Image).

Fluorescence i*n vivo* imaging of externally added NOC7 (Fig. S14): We used a male Lew/CrlCrlj rat (8 weeks old, 220 g body weight). The fluorescence images of the rat were taken with a Maestro In-Vivo system using the red filter setting (excitation: 500-560 nm, emission: 580 nm long pass) for DAL-4M AM. The tunable filter was automatically stepped in 10-nm increments from 500 to 720 nm, while the camera sequentially captured images at each wavelength interval. The exposure time was 150 ms. For analysis, 600-nm fluorescence images of DAR-4M AM were exported by Maestro software. During the procedure, the rat was kept under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. First, images were taken before and after shaving of the abdominal fur. Then, the rat was given an i.p. injection of DAR-4M AM (0.1 μmol in 20 μl of DMSO and 880 μl of PBS) and images were acquired at 10 min. Next, the rat was injected i.p. with 10 μmol NOC7 in PBS (1 ml), and images were again acquired at 40 min after the injection of NOC7. The rat was sacrificed by diethylether exposure, then the abdomen was opened, and further images were acquired. The obtained fluorescence images were analyzed using imaging software (ImageJ).

Calculation of limit of detection: The limit of detection (LOD) was determined according to the following equation,[7]

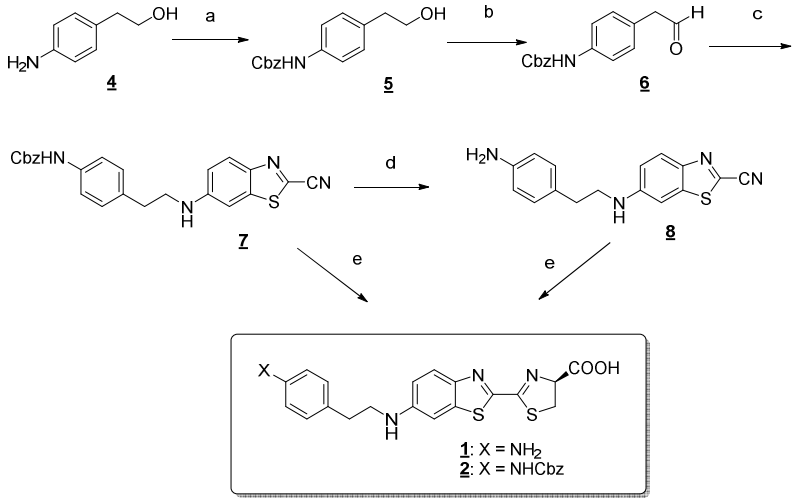
$LOD = 3 \times \sigma/b$

where σ is a standard deviation of the integrated luminescence intensity without ROS and b is the slope.

Cell culture

HEK293-luc2 cells were constructed as reported elsewhere.[8] HEK293 cells and HEK293-luc2 cells were cultured in Dulbecco's modified Eagle's medium (high glucose) (DMEM-HG) (Wako), supplemented with 10% fetal bovine serum (GIBCO). Penicillin (100 units/ml) and streptomycin (100 units/ml) were added to all the culture media. Zeocin (50 μg/ml) was added to culture medium of HEK293-luc2 cells. All the cells were cultured at 37 °C in a CO_2/air (5%/95%) incubator.

Synthesis and characterization



Scheme S1. Synthetic schemes of **1** and **2**. Reagents and conditions: (a) Cbz chloride, TEA, THF, y. 61%; (b) PCC, $MgSO_4$, CH_2Cl_2, y. 13%; (c) 6-amino-2-cyanobenzothiazole, H_2SO_4, $NaBH_4$, THF, y. 29%; (d) TFA, CH_2Cl_2, y. 67%; (e) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 46% (for **1**); y. 81% (for **2**). TEA: triethylamine, THF: tetrahydrofuran, PCC pyridinium chlorochromate, TFA: trifluoroacetic acid.

4-Benzyloxycarbonylaminophenethyl alcohol (5): 4-Aminophenethylalcohol **4** (1.97 g, 14.4 mmol) and triethylamine (2 ml, 14 mmol) were dissolved in 50 ml of tetrahydrofuran. The mixture was stirred for a few minutes, then Cbz chloride (2.5 ml, 17 mmol) was dropped into it, and the whole was stirred overnight at r.t. After 24 hr, the reaction mixture was evaporated. The residue was extracted with CH_2Cl_2, and 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 = 1/2 to 1/1) to give a white solid (2.36 g, y. 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (t, J = 6.1 Hz, 1H), 2.83 (t, J = 6.5 Hz, 2H), 3.83 (td, J = 6.1, 6.5 Hz, 2H), 5.20 (s, 2H), 6.63 (br, 1H), 7.17 (d, J = 8.4 Hz, 2H), 7.29-7.44 (m, 7H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.4, 63.6, 67.0, 119.1, 128.2, 128.3, 128.6, 129.6, 133.7, 136.0, 136.2, 153.4; HRMS (ESI$^+$): *m/z* calcd for $C_{16}H_{17}NNaO_3$: 294.1106 $[M+Na]^+$; found: 294.1097.

General procedure for oxidation of alcohol using PCC (Method A): An alcohol derivative (0.5~3.0 g, 1 equiv.) and $MgSO_4$ (2~4 g) were dissolved in 30~50 ml of CH_2Cl_2. The solution was stirred for a few minutes, and then pyridinium chlorochromate (1.5 equiv.) was added to it. After 5 hr, the reaction mixture was filtered through Celite, and the filtrate was evaporated. The compound was purified by

silica gel column chromatography (ethyl acetate/*n*-hexane).

4-Benzyloxycarbonylaminophenethylaldehyde (6): Compound **6** was synthesized from compound **5** by using Method A. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/6 to 1/4) to give a colorless oil (300 mg, y. 13%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.65 (d, J = 2.2 Hz, 2H), 5.21 (s, 2H), 6.65 (br, 1H), 7.16 (d, J = 8.4 Hz, 2H), 7.34-7.45 (m, 7H), 9.72 (t, J = 2.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 49.5, 66.6, 119.0, 126.3, 127.9, 128.0, 128.3, 129.9, 135.8, 137.1, 153.4, 199.6; HRMS (ESI$^+$): *m/z* calcd for $C_{16}H_{15}NNaO_3$: 292.0950 $[M+Na]^+$; found: 292.0954.

General procedure for *N*-alkylation of 6-amino-2-cyanobenzothiazole (Method B): The *N*-alkylated 6-amino-2-cyanobenzothiazole derivatives were synthesized according to the reported method[4]. The appropriate aldehyde (1.5 equiv) was dissolved in 20~40 ml of tetrahydrofuran. Then 180 mM H_2SO_4 (1.5 equiv) was added, and the solution was stirred at r.t. for a few minutes. A solution of 6-amino-2-cyanobenzothiaozole (50~120 mg, 1 equiv) and sodium borohydride (1.5 equiv) in 40~60 ml of tetrahydrofuran was added and the reaction mixture was stirred at r.t. After 1 hr, 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% acetonitrile/20% H_2O/ 0.1% TFA).

6-(4-Benzyloxycarbonylaminophenethylamino)-2-cyanobenzothiazole (7): Compound **7** was synthesized from compound **6** by using Method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/6 to 1/4) to give a yellow solid (y. 29%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.93 (t, J = 6.9 Hz, 2H), 3.45 (td, J = 6.1, 6.9 Hz, 2H), 4.19 (br, 1H), 5.20 (s, 2H), 6.66 (br, 1H), 6.83 (dd, J = 2.3, 9.0 Hz, 1H), 6.93 (d, J = 2.3 Hz, 1H), 7.16 (d, J = 8.4 Hz, 2H), 7.29-7.43 (m, 7H), 7.90 (d, J = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 34.2, 44.6, 66.8, 99.8, 113.8, 116.9, 119.1, 125.3, 127.9, 128.1, 128.2, 128.4, 129.1, 133.5, 135.9, 136.4, 138.6, 144.5, 148.7, 153.4; HRMS (ESI$^+$): *m/z* calcd for $C_{24}H_{20}N_4NaO_2S$: 451.1205 $[M+Na]^+$; found: 451.1243.

6-(4-Aminophenethylamino)-2-cyanobenzothiazole (8): Compound **7** (92 mg, 220 μmol) was dissolved in 10 ml of CH_2Cl_2 and 60 ml of trifluoroacetic acid (TFA), and the solution was refluxed at 60 °C. After 4 hr, the reaction mixture was evaporated. The residue was extracted with ethyl acetate and

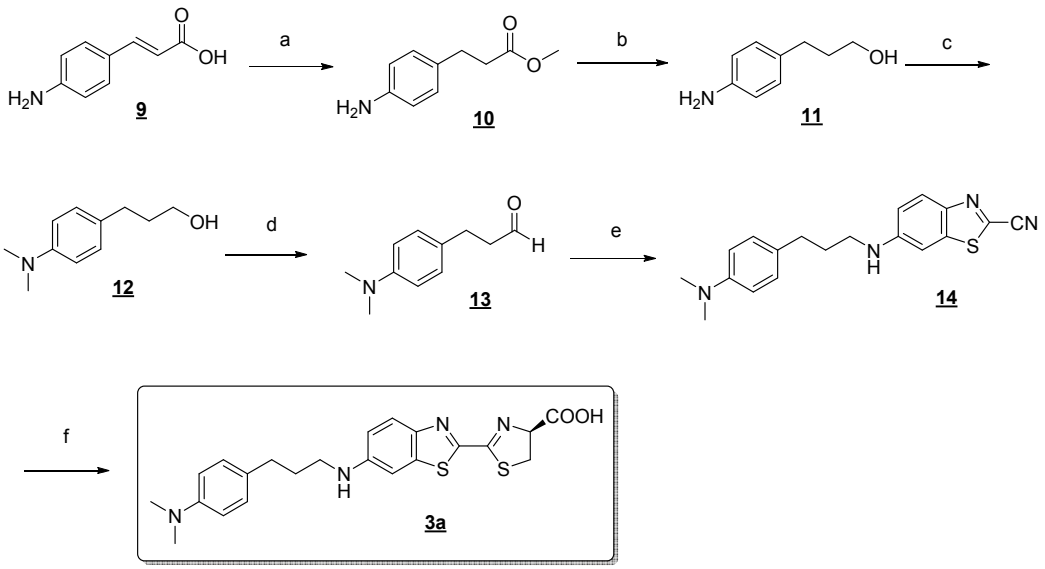
the organic solution was washed with brine, dried over Na_2SO_4, filtered, and evaporated. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3 to 2/1) to give a yellow solid (42 mg, y. 67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.86 (t, *J* = 6.8 Hz, 2H), 3.41 (t, *J* = 6.8 Hz, 2H), 6.66 (d, *J* = 8.4 Hz, 2H), 6.83 (dd, *J* = 2.2, 9.0 Hz, 1H), 6.93 (d, *J* = 2.2 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 2H), 7.89 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 34.1, 44.9, 99.9, 113.8, 115.4, 117.0, 125.4, 128.1, 129.5, 138.7, 144.7, 145.0, 148.9; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{15}N_4S$: 295.1017 $[M+H]^+$; found: 295.1005.

General procedure for cyclization of 2-cyanobenzothiazoles (Method C): D-Cysteine hydrochloride monohydrate (3 equiv) was dissolved in 10 ml of water (Ar bubbling) and the pH of the solution was adjusted to 8 with 0.5 M potassium carbonate. The appropriate 2-cyanobenzothiazole compound (1 equiv) was dissolved in 10~15 ml of methanol with Ar bubbling. The D-cysteine solution was added, and the mixture was stirred at r.t. under an Ar atmosphere in the dark for 1~2 hr. 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% acetonitrile/20% H_2O/0.1% TFA).

D-(−)-2-[6'-(4-Aminophenethylamino)-2'-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (1): Compound **1** was synthesized from **8** by using Method C. The product was purified by semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a red solid (y. 46%). ^{1}H NMR (300 MHz, CD_3OD): δ 2.84 (t, *J* = 7.2 Hz, 2H), 3.33 (t, *J* = 7.2 Hz, 2H), 3.57-3.68 (m, 2H), 5.25 (t, *J* = 9.1 Hz, 1H), 6.77 (dd, *J* = 2.3, 9.0 Hz, 1H), 6.92 (d, *J* = 2.3 Hz, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 7.23 (d, *J* = 8.4 Hz, 2H), 7.64 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, acetone-d_6): δ 35.2, 35.3, 46.2, 79.2, 101.3, 115.4, 116.5, 120.5, 125.4, 128.4, 130.1, 139.8, 146.0, 147.5, 149.8, 166.2, 171.6; HRMS (ESI^+): *m/z* calcd for $C_{19}H_{19}N_4O_2S_2$: 399.0949 $[M+H]^+$; found: 399.0914.

D-(−)-2-[6'-(4-Benzyloxycarbonylaminophenethylamino)-2'-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (2): Compound **2** was synthesized from **7** by using Method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 81%). ^{1}H NMR (300 MHz, CD_3OD): δ 2.80 (t, *J* = 7.4 Hz, 2H), 3.35 (t, *J* = 7.4 Hz, 2H), 3.58-3.71 (m, 2H), 5.06 (s, 2H), 5.27 (t, *J* = 9.0 Hz, 1H), 6.91 (dd, *J* = 2.0, 9.0 Hz, 1H), 7.05-7.13 (m, 3H), 7.17-7.33 (m, 7H), 7.73 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 35.2, 35.3, 45.8, 66.8, 79.1, 101.5, 116.5, 119.3, 125.4, 128.8,

128.9, 129.3, 130.0, 134.8, 137.9, 138.3, 139.8, 146.1, 149.7, 154.3, 155.0, 166.3, 171.5; HRMS (ESI^+): *m/z* calcd for $C_{27}H_{25}N_4O_4S_2$: 533.1317 $[M+H]^+$; found: 533.1365.



Scheme S2. Synthetic scheme of **3a**. Reagents and conditions: (a) see supporting reference 9. (b) $LiAlH_4$, THF, y. 51%; (c) formaldehyde, H_2SO_4, $NaBH_4$, THF, y. 66%; (d) SO_3-pyridine, DMSO, TEA, CH_2Cl_2, y. 79%; (e) 6-amino-2-cyanobenzothiazole, H_2SO_4, $NaBH_4$, THF, y. 42%; (f) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 67%.

Methyl 3-(4-aminophenyl)propionate (10): Compound **10** was synthesized from 4-aminocinnamic acid **9** as reported elsewhere.[9] ^1H NMR (300 MHz, $CDCl_3$): δ 2.57 (t, *J* = 7.8 Hz, 2H), 2.84 (t, *J* = 7.8 Hz, 2H), 3.58 (br, 2H), 3.66 (s, 3H), 6.62 (d, *J* = 8.5 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.9, 35.8, 51.2, 115.0, 128.8, 130.0, 144.6, 173.3; MS (EI^+): *m/z* 179 $[M]^+$.

General procedure for reduction reaction using $LiAlH_4$ (Method D): Lithium aluminium hydride (4 equiv.) was dissolved in 10 ml of tetrahydrofuran under an Ar atmosphere on ice. A solution of an ester derivative (0.2~0.5 g, 1 equiv.) in 5 ml of tetrahydrofuran was added, and the reaction mixture was stirred at 0 °C for 30 min. The mixture was quenched with ethyl acetate, and the precipitate was removed by filtration with Celite. The filtrate was evaporated and, the residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane).

3-(4-Aminophenyl)propanol (11): Compound **11** was synthesized from **10** by using method D. The

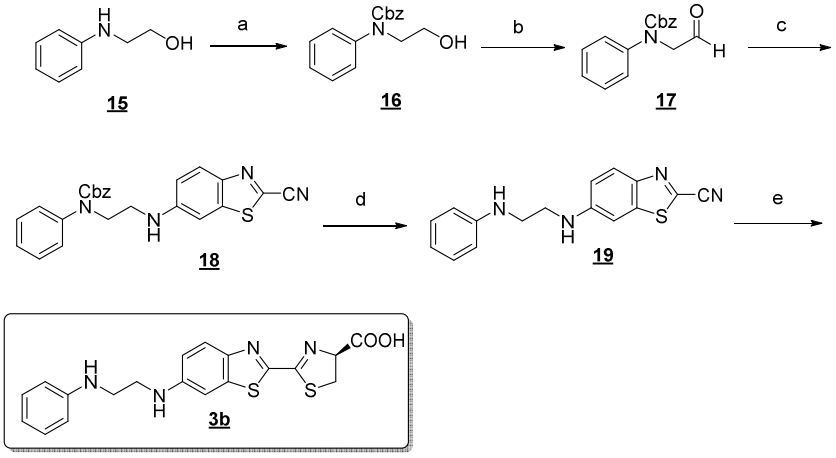
product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 3/1) to give a colorless oil (y. 51%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.31 (br, 1H), 1.77-1.85 (m, 2H), 2.60 (t, *J* = 7.6 Hz, 2H), 3.56 (br, 2H), 3.66 (t, *J* = 6.3 Hz, 2H), 6.63 (d, *J* = 8.3 Hz, 2H), 6.99 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.9, 34.2, 61.8, 115.2, 128.9, 131.8, 143.9; MS (EI^+): *m/z* 151 $[M]^+$.

3-(4-Dimethylaminophenyl)propanol (12): Compound **12** was synthesized from **11** using the same procedure as method B except for an excess amount of formaldehyde. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/2 to 1/1) to give a colorless oil (y. 66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (t, *J* = 5.3 Hz, 1H), 1.81-1.90 (m, 2H), 2.62 (t, *J* = 7.6 Hz, 2H), 2.90 (s, 6H), 3.64-3.70 (m, 2H), 6.70 (d, *J* = 8.7 Hz, 2H), 7.08 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.7, 34.1, 40.6, 61.5, 112.9, 128.6, 130.1, 148.7; MS (EI^+): *m/z* 179, $[M]^+$; HRMS (ESI^+): *m/z* calcd for $C_{11}H_{18}NO$: 180.1388 $[M+H]^+$; found: 180.1369.

3-(4-Dimethylaminophenyl)propionaldehyde (13): Compound **12** (89 mg, 490 μmol) was dissolved in 2 ml of CH_2Cl_2, and then triethylamine (0.5 ml, 3.6 mmol) and dimethyl sulfoxide (1 ml, 14 mmol) were added to the solution. Next, sulfur trioxide pyridine complex (330 mg, 2.1 mmol) was added on ice, and the reaction mixture was stirred at 0 °C for 30 min. Saturated aqueous NH_4Cl and ethyl acetate were added to the reaction mixture. The organic layer was separated, 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 to 1/2) to give a colorless oil (69 mg, y. 79%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.73 (t, *J* = 7.5 Hz, 2H), 2.80-2.91 (m, 8H), 6.69 (d, *J* = 8.7 Hz, 2H), 7.07 (d, *J* = 8.7 Hz, 2H), 9.81 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.1, 40.7, 45.5, 112.9, 128.1, 128.8, 149.2, 202.1; MS (EI^+): *m/z* 177 $[M]^+$.

2-Cyano-6-[3-(4-dimethylaminophenyl)propylamino]benzothiazole (14): Compound **14** was synthesized from compound **13** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) and semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a yellow solid (y. 42%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.92-2.02 (m, 2H), 2.69 (t, *J* = 7.4 Hz, 2H), 2.96 (s, 6H), 3.20 (t, *J* = 7.0 Hz, 2H), 6.77-6.83 (m, 4H), 7.10 (d, *J* = 8.8 Hz, 2H), 7.88 (d, *J* = 9.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.6, 32.2, 41.6, 43.0, 99.6, 113.9, 114.1, 117.0, 125.4, 129.2, 129.5, 131.1, 138.8, 144.6, 148.1, 149.0; HRMS (ESI^+): *m/z* calcd for $C_{19}H_{21}N_4S$: 337.1487 $[M+H]^+$; found: 337.1480.

D-(−)-2-{6'-[3-(4-Dimethylaminophenyl)propylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3a): Compound **3a** was synthesized from compound **14** by using method C. The product was purified by semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a red solid (y. 67%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.92-2.05 (m, 2H), 2.83 (t, *J* = 7.6 Hz, 2H), 3.18 (t, *J* = 6.9 Hz, 2H), 3.26 (s, 6H), 3.67-3.79 (m, 2H), 5.35 (t, *J* = 9.0 Hz, 1H), 6.89 (dd, *J* = 2.2, 9.0 Hz, 1H), 6.96 (d, *J* = 2.2 Hz, 1H), 7.42-7.53 (m, 4H), 7.75 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 31.3, 33.2, 35.3, 43.5, 45.4, 79.1, 101.2, 116.5, 120.2, 125.4, 130.8, 139.7, 142.0, 144.1, 146.0, 149.9, 154.9, 166.2, 171.5; HRMS (ESI$^+$): *m/z* calcd for $C_{22}H_{25}N_4O_2S_2$: 441.1419 $[M+H]^+$; found: 441.1402.



Scheme S3. Synthetic scheme of **3b**. Reagents and conditions: (a) Cbz chloride, pyridine, y. 44%; (b) (i) $(COCl)_2$, DMSO, CH_2Cl_2, (ii) TEA, y. 57%; (c) 6-amino-2-cyanobenzothiazole, H_2SO_4, $NaBH_4$, THF; (d) TFA, CH_2Cl_2, y. 8% (2 steps); (e) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 66%.

2-(Benzyloxycarbonyl)(phenyl)aminoethanol (16): 2-(Phenylamino)ethanol **15** (1.4 g, 10 mmol) was dissolved in 10 ml of pyridine, and Cbz chloride (1.9 ml, 13 mmol) was dropped into the solution on ice. The reaction mixture was stirred at r.t. After 4 hr, it was evaporated. The residue was extracted with ethyl acetate, and 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 = 1/4 to 1/2) and semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a colorless oil (1.2 g, y. 44%). ^{1}H NMR (300 MHz, acetone-d_6): δ 3.68 (t, *J* = 6.0 Hz, 2H), 3.73 (s, 1H), 3.79 (t, *J* = 6.0 Hz, 2H), 5.12 (s, 2H), 7.20-7.38 (m, 10H); ^{13}C NMR (75 MHz, acetone-d_6): δ 54.0, 60.8, 68.2, 127.9, 128.8, 129.1, 129.7,

130.2, 138.3, 143.7, 156.8; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{17}NNaO_3$: 294.1106 $[M+Na]^+$; found: 294.1109.

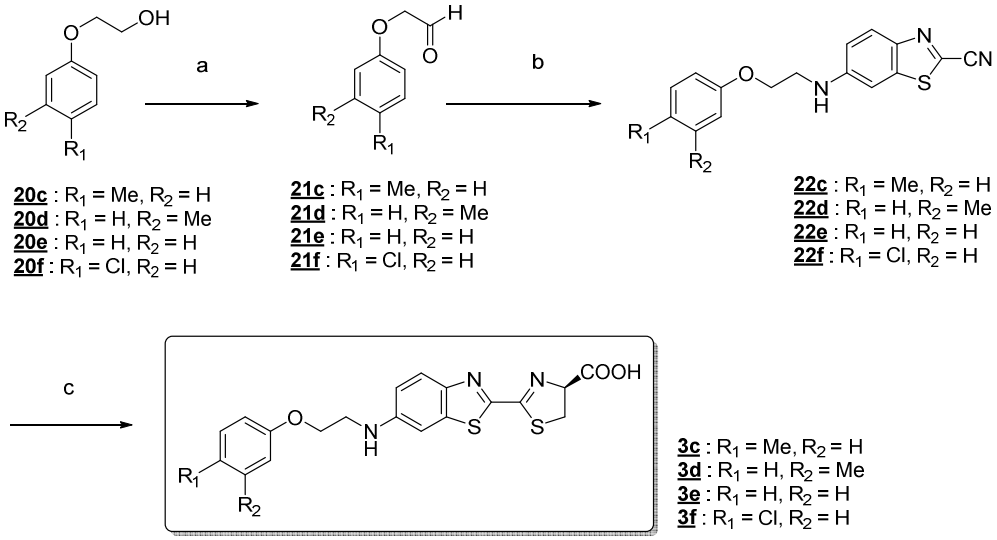
General procedure for Swern oxidation (Method E): Dimethyl sulfoxide (1.0 ml) was added to 10 ml of a solution of 2 M oxalyl chloride in CH_2Cl_2 at −78 °C under an Ar atmosphere. After 5 min, 15 ml of a solution of an ethanol derivative (0.1~1.0 g) and 1 ml of dimethyl sulfoxide in CH_2Cl_2 were added dropwise at −78 ℃, and the reaction mixture was stirred for 30 min. Then 10 ml of triethylamine was added. Water was further added, and the organic layer of the reaction mixture was separated, washed with 1 N hydrochloric acid and 5% aqueous $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and evaporated. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane) or semipreparative HPLC (eluent A: H_2O/0.1% TFA, eluent B: 80% acetonitrile/20% H_2O/0.1% TFA).

2-(Benzyloxycarbonyl)(phenyl)aminoacetaldehyde (17): Compound **17** was synthesized from compound **16** by using method E. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) to give a colorless oil (y. 57%). ^1H NMR (300 MHz, acetone-d_6): δ 4.53 (s, 2H), 5.15 (s, 2H), 7.21-7.39 (m, 10H); ^{13}C-NMR (75 MHz, acetone-d_6): δ 61.0, 67.8, 127.3, 128.3, 128.6, 129.1, 129.3, 129.6, 137.5, 143.3, 155.7, 198.5; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{15}NNaO_3$: 292.0950 $[M+Na]^+$; found: 292.0944.

2-Cyano-6-[2-(phenylamino)ethylamino]benzothiazole (19): Compound **18** was synthesized from **17** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) and semipreparative HPLC (A/B = 50/50 to 0/100 (10 min)) to give a yellow solid. Then, **18** was dissolved in 5 ml of CH_2Cl_2, and 15 ml of trifluoroacetic acid was added to the solution. The reaction mixture was stirred at 60 °C for 30 hr. After evaporation, the residue 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 to 1/1) to give a yellow solid (y. 8% (2 steps)). ^1H NMR (300 MHz, CD_3OD): δ 3.36-3.42 (m, 4H), 6.58-6.69 (m, 3H), 7.01 (dd, *J* = 2.2, 9.0 Hz, 1H), 7.06-7.14 (m, 3H), 7.82 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 43.6, 43.8, 100.4, 114.1, 114.8, 118.3, 118.5, 126.0, 130.1, 130.2, 140.3, 145.4, 149.9, 151.6; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{15}N_4S$: 295.1017 $[M+H]^+$; found: 295.1012.

D-(−)-2-{6'-[2-(Phenylamino)ethylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3b): Compound **3b** was synthesized from **19** by using method C. The product was purified by

semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a red solid (y. 66%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.36-3.59 (m, 4H), 3.59-3.68 (m, 2H), 5.27 (t, *J* = 9.1 Hz, 1H), 6.88 (dd, *J* = 2.2, 9.0 Hz, 1H), 7.04 (d, *J* = 2.2 Hz, 1H), 7.06-7.12 (m, 3H), 7.31 (t, *J* = 7.9 Hz, 2H), 7.72 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 35.3, 43.2, 43.8, 79.2, 101.5, 114.1, 116.6, 118.3, 125.5, 130.0, 139.7, 146.2, 148.8, 149.8, 155.1, 166.2, 171.5; HRMS (ESI^+): *m/z* calcd for $C_{19}H_{19}N_4O_2S_2$: 399.0949 $[M+H]^+$; found: 399.0906.



Scheme S4. Synthetic schemes of **3c-f**. Reagents and conditions: (a) (i) $(COCl)_2$, DMSO, CH_2Cl_2, (ii) TEA, y. 9% (for **21c**); y. 6% (for **21d**); y. 23% (for **21e**); y. 52% (for **21f**); (b) 6-amino-2-cyanobenzothiazole, H_2SO_4, $NaBH_4$, THF, y. 11% (for **22c**); y. 16% (for **22d**); y. 29% (for **22e**); y. 12% (for **22f**); (c) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 80% (for **3c**); y. 77% (for **3d**); y. 57% (for **3e**); y. 66% (for **3f**).

2-(4-Methylphenoxy)acetaldehyde (21c): Compound **21c** was synthesized from **20c** by using method E. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/5 to 1/3) and semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a colorless oil (y. 9%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30 (s, 3H), 4.55 (d, *J* = 1.1 Hz, 2H), 6.80 (d, *J* = 8.5 Hz, 2H), 7.11 (d, *J* = 8.5 Hz, 2H), 9.86 (t, *J* = 1.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.5, 72.9, 114.4, 130.2, 131.3, 155.6, 199.8; MS (EI^+): *m/z* 150 $[M]^+$.

2-(3-Methylphenoxy)acetaldehyde (21d): Compound **21d** was synthesized from **20d** by using method

E. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/5 to 1/3) and semipreparative HPLC (A/B = 80/20 to 20/80 (20 min)) to give a colorless oil (y. 6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.34 (s, 3H), 4.56 (d, *J* = 1.1 Hz, 2H), 6.66-6.74 (m, 2H), 6.84 (d, *J* = 7.7 Hz, 1H), 7.20 (t, *J* = 7.7 Hz, 1H), 9.87 (t, *J* = 1.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.5, 72.6, 111.4, 115.4, 122.8, 129.5, 139.9, 157.6, 199.8; MS (EI^+): *m/z* 150 $[M]^+$.

2-Phenoxyacetaldehyde (21e): Compound **21e** was synthesized from **20e** by using method E. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3 to 1/2) to give a colorless oil (y. 23%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.58 (d, *J* = 1.1 Hz, 2H), 6.87-6.94 (m, 2H), 6.99-7.07 (m, 1H), 7.28-7.36 (m, 2H), 9.88 (t, *J* = 1.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 72.4, 114.3, 121.7. 129.5, 157.4, 199.1; MS (EI^+): *m/z* 136 $[M]^+$.

2-(4-Chlorophenoxy)acetaldehyde (21f): Compound **21f** was synthesized from **20f** by using method E. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3 to 1/1) to give a colorless oil (y. 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.82 (s, 2H), 6.98 (d, *J* = 9.2 Hz, 2H), 7.32 (d, *J* = 9.2 Hz, 2H), 9.79 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 73.5, 116.8, 126.5, 130.0, 157.5, 198.5; MS (EI^+): *m/z* 170 $[M]^+$.

2-Cyano-6-[2-(4-methylphenoxy)ethylamino]benzothiazole (22c): Compound **22c** was synthesized from compound **21c** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) and semipreparative HPLC (A/B = 50/50 to 0/100 (10 min)) to give a yellow solid (y. 11%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30 (s, 3H), 3.59 (t, *J* = 5.2 Hz, 2H), 4.20 (t, *J* = 5.2 Hz, 2H), 6.83 (d, *J* = 8.3 Hz, 2H), 6.95 (dd, *J* = 2.3, 9.0 Hz, 1H), 7.01 (d, *J* = 2.3 Hz, 1H), 7.10 (d, *J* = 8.3 Hz, 2H), 7.94 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.5, 43.2, 66.1, 100.3, 113.7, 114.4, 117.2, 125.6, 130.1, 130.2, 130.8, 138.6, 145.0, 148.7, 156.2; HRMS (ESI^+): *m/z* calcd for $C_{17}H_{16}N_3OS$: 310.1014 $[M+H]^+$; found: 310.1018.

2-Cyano-6-[2-(3-methylphenoxy)ethylamino]benzothiazole (22d): Compound **22d** was synthesized from **21d** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) and semipreparative HPLC (A/B = 50/50 to 0/100 (10 min)) to give a yellow solid (y. 16%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.34 (s, 3H), 3.60 (t, *J* = 5.2 Hz, 2H), 4.21 (t, *J* = 5.2 Hz, 2H), 6.71-6.77 (m, 2H), 6.81 (d, *J* = 7.7 Hz, 1H), 6.95 (dd, *J* = 2.3, 9.0 Hz, 1H), 7.01 (d, *J* = 2.3 Hz, 1H), 7.19 (t, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.5, 43.1,

65.8, 100.3, 111.3, 113.7, 115.3, 117.2, 122.2, 125.6, 129.4, 130.2, 138.6, 139.7, 145.0, 148.7, 158.3; HRMS (ESI^+): *m/z* calcd for $C_{17}H_{16}N_3OS$: 310.1014 $[M+H]^+$; found: 310.1018.

2-Cyano-6-(2-phenoxyethylamino)benzothiazole (22e): Compound **22e** was synthesized from **21e** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) to give a yellow solid (y. 29%). ^1H NMR (300 MHz, $CDCl_3$): δ 3.58-3.68 (m, 2H), 4.23 (t, *J* = 5.1 Hz, 2H), 4.65 (br, 1H), 6.89-7.04 (m, 5H), 7.27-7.35 (m, 2H), 7.95 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 43.1, 65.9, 100.2, 113.7, 114.4, 117.2, 121.4, 125.6, 129.6, 130.1, 138.6, 145.0, 148.7, 158.3; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{14}N_3OS$: 296.0858 $[M+H]^+$; found: 296.0867.

6-[2-(4-Chlorophenoxy)ethylamino]-2-cyanobenzothiazole (22f): Compound **22f** was synthesized from **21f** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4 to 1/2) and semipreparative HPLC (A/B = 50/50 to 0/100 (10 min)) to give a yellow solid (y. 12%). ^1H NMR (300 MHz, CD_3OD): δ 3.60 (t, *J* = 5.4 Hz, 2H), 4.18 (t, *J* = 5.4 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 7.06 (dd, *J* = 2.2, 9.0 Hz, 1H), 7.17 (d, *J* = 2.2 Hz, 1H), 7.23 (d, *J* = 9.0 Hz, 2H), 7.84 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 43.5, 67.5, 100.7, 114.7, 117.0, 118.2, 126.0, 129.8, 130.1, 139.8, 145.3, 150.9, 158.6; HRMS (ESI^-): *m/z* calcd for $C_{16}H_{11}ClN_3OS$: 328.0311 $[M-H]^-$; found: 328.0303.

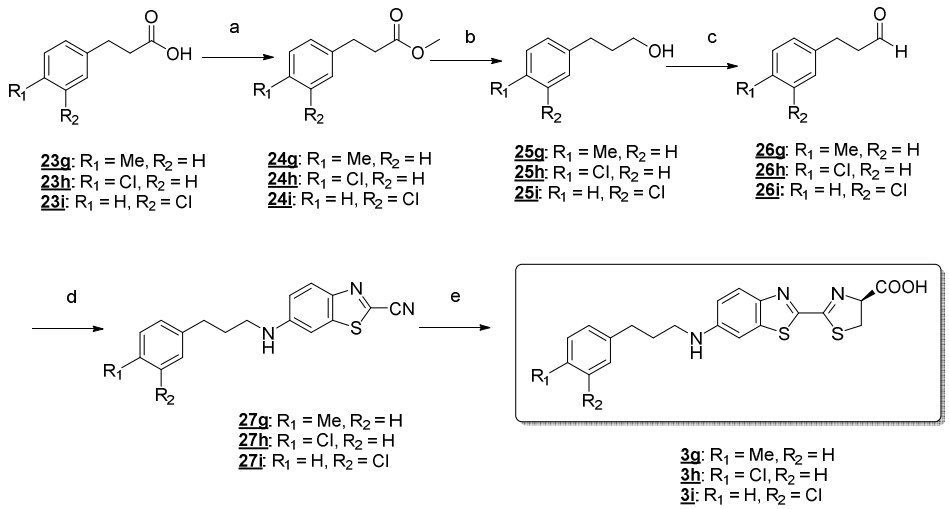
D-(−)-2-{6'-[2-(4-Methylphenoxy)ethylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3c): Compound **3c** was synthesized from **22c** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 80%). ^1H NMR (300 MHz, CD_3OD): δ 2.15 (s, 3H), 3.47 (t, *J* = 5.5 Hz, 2H), 3.59-3.69 (m, 2H), 4.06 (t, *J* = 5.5 Hz, 2H), 5.26 (t, *J* = 9.1 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 2H), 6.87 (dd, *J* = 2.2, 9.1 Hz, 1H), 6.96 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 2.2 Hz, 1H), 7.68 (d, *J* = 9.1 Hz, 1H); ^{13}C NMR (75 MHz, acetone-d_6): δ 20.5, 35.3, 43.7, 67.3, 79.3, 101.5, 115.3, 116.6, 125.5, 130.6, 130.7, 139.7, 146.2, 149.8, 155.2, 157.7, 166.1, 171.7; HRMS (ESI^+): *m/z* calcd for $C_{20}H_{20}N_3O_3S_2$: 414.0946 $[M+H]^+$; found: 414.0929.

D-(−)-2-{6'-[2-(3-Methylphenoxy)ethylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3d): Compound **3d** was synthesized from **22d** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 77%). ^1H NMR (300 MHz, CD_3OD): δ 2.19 (s, 3H), 3.48 (t, *J* = 5.4 Hz, 2H), 3.59-3.69 (m, 2H), 4.07 (t, *J* = 5.4 Hz, 2H), 5.25 (t, *J* = 9.1 Hz, 1H), 6.60-6.69 (m, 3H), 6.87 (dd, *J* = 2.3, 9.0 Hz, 1H), 7.00-7.07 (m, 2H), 7.68 (d, *J* = 9.0 Hz,

1H); ^{13}C NMR (75 MHz, acetone-d_6): δ 21.5, 35.3, 43.7, 67.0, 79.2, 101.5, 112.3, 116.1, 116.6, 122.4, 125.5, 130.0, 139.7, 140.1, 146.2, 149.7, 155.2, 159.8, 166.2, 171.6; HRMS (ESI$^+$): *m/z* calcd for $C_{20}H_{19}N_3O_3S_2$: 414.0946 $[M+H]^+$; found: 414.0927.

D-(−)-2-[6'-(2-Phenoxyethylamino)-2'-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (3e): Compound **3e** was synthesized from **22e** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 57%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.49 (t, *J* = 5.5 Hz, 2H), 3.58-3.69 (m, 2H), 4.10 (t, *J* = 5.5 Hz, 2H), 5.25 (t, *J* = 9.1 Hz, 1H), 6.78-6.91 (m, 4H), 7.05 (d, *J* = 2.3 Hz, 1H), 7.10-7.18 (m, 2H), 7.68 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, acetone-d_6): δ 35.3, 43.7, 67.1, 79.3, 101.5, 115.4, 116.2, 121.6, 125.5, 130.3, 139.7, 146.2, 149.8, 155.2, 159.8, 166.1, 171.7; HRMS (ESI$^+$): *m/z* calcd for $C_{19}H_{18}N_3O_3S_2$: 400.0790 $[M+H]^+$; found: 400.0797.

D-(−)-2-{6'-[2-(4-Chlorophenoxy)ethylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3f): Compound **3f** was synthesized from **22f** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 66%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.49 (t, *J* = 5.4 Hz, 2H), 3.58-3.67 (m, 2H), 4.08 (t, *J* = 5.4 Hz, 2H), 5.26 (t, *J* = 9.2 Hz, 1H), 6.80-6.90 (m, 3H), 7.05 (d, *J* = 2.2 Hz, 1H), 7.14 (d, *J* = 9.2 Hz, 2H), 7.68 (d, *J* = 9.2 Hz, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 35.3, 43.6, 67.7, 79.2, 101.6, 116.6, 117.0, 125.5, 126.0, 130.1, 139.7, 146.2, 149.7, 155.3, 158.7, 166.1, 171.7; HRMS (ESI$^+$): *m/z* calcd for $C_{19}H_{17}ClN_3O_3S_2$: 434.0400 $[M+H]^+$; found: 434.0425.



Scheme S5. Synthetic schemes of **3g-i**. Reagents and conditions: (a) TMS-Cl, MeOH, y. 96% (for **24g**); y. 94% (for **24h**); y. quant (for **24i**); (b) $LiAlH_4$, THF, y. 38% (for **25g**); y. 28% (for **25h**);y. 12% (for **25i**); (c) PCC, $MgSO_4$, CH_2Cl_2, y. 65% (for **26g**); y. 76% (for **26h**); y. 67% (for **26i**); (d) 6-amino-2-cyanobenzothiazole, H_2SO_4, $NaBH_4$, THF, y. 43% (for **27g**); y. 22% (for **27h**); y. 12% (for **27i**); (e) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 43% (for **3g**); y. 85% (for **3h**); y. 94% (for **3i**).

General procedure for esterification reaction (Method F): A carboxylic compound (0.5~1.0 g) was dissolved in 20 ml of methanol. Five drops of chlorotrimethylsilane were added, and the solution was stirred at r.t. When the starting material disappeared, the reaction mixture was evaporated. The product was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane).

Methyl 3-(4-methylphenyl)propionate (24g): Compound **24g** was synthesized from **23g** by using method F. The product was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane = 1/2) to give a colorless oil (y. 96%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.31 (s, 3H), 2.61 (t, *J* = 7.8 Hz, 2H), 2.91 (t, *J* = 7.8 Hz, 2H), 3.67 (s, 3H), 7.09 (s, 4H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 20.6, 30.2, 35.4, 51.0, 127.8, 128.8, 135.2, 137.1, 172.8; MS (EI^+): *m/z* 178 $[M]^+$.

Methyl 3-(4-chlorophenyl)propionate (24h): Compound **24h** was synthesized from **23h** by using method F. The product was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane = 1/2) to give a colorless oil (y. 94%). ^{1}H NMR (300 MHz, acetone-d_6): δ 2.63 (t, *J* = 7.6 Hz, 2H), 2.91 (t, *J* = 7.6 Hz, 2H), 3.61 (s,3H), 7.24-7.34 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.7, 34.8, 50.9, 128.0, 129.2, 131.4, 138.6, 172.2; MS (EI^+): *m/z* 198 $[M]^+$.

Methyl 3-(3-chlorophenyl)propionate (24i): Compound **24i** was synthesized from **23i** by using method F. The product was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane = 1/2) to give a colorless oil (y. quant). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.62 (t, *J* = 7.8 Hz, 2H), 2.93 (t, *J* = 7.8 Hz, 2H), 3.68 (s, 3H), 7.08 (td, *J* = 1.9, 6.7 Hz, 1H), 7.15-7.24 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.0, 34.7, 51.0, 126.0, 126.1, 128.0, 129.3, 133.7, 142.2, 172.2; MS (EI^+): *m/z* 198 $[M]^+$.

3-(4-Methylphenyl)propanol (25g): Compound **25g** was synthesized from **24g** by using method D. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/2 to 1/1) to give a colorless oil (y. 38%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (t, *J* = 5.3 Hz, 1H), 1.82-1.94 (m, 2H), 2.32 (s, 3H), 2.67 (t, *J* = 7.6 Hz, 2H), 3.63-3.72 (m, 2H), 7.10 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.3, 31.5, 34.2, 61.9, 128.2, 128.9, 135.1, 138.6; MS (EI^+): *m/z* 150 $[M]^+$.

3-(4-Chlorophenyl)propanol (25h): Compound **25h** was synthesized from **24h** by using method D. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3 to 1/2) to give a colorless oil (y. 28%). ^{1}H NMR (300 MHz, acetone-d_6): δ 1.74-1.84 (m, 2H), 2.65-2.72 (m, 2H), 3.51-3.58 (m, 2H), 7.20-7.32 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.1, 33.8, 61.4, 128.2, 129.5, 131.2, 140.1; MS (EI^+): *m/z* 170 $[M]^+$.

3-(3-Chlorophenyl)propanol (25i): Compound **25i** was synthesized from **24i** by using method D. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3 to 1/1) to give a colorless oil (y. 12%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.26 (t, *J* = 7.0 Hz, 1H), 1.83-1.95 (m, 2H), 2.70 (t, *J* = 7.7 Hz, 2H), 3.63-3.71 (m, 2H), 7.08 (td, *J* = 1.7, 7.0 Hz, 1H), 7.15-7.24 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.6, 33.8, 61.8, 126.0, 126.6, 128.5, 129.5, 134.0, 143.8; MS (EI^+): *m/z* 170 $[M]^+$.

3-(4-Methylphenyl)propionaldehyde (26g): Compound **26g** was synthesized from **25g** by using method A. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4) to give a colorless oil (y. 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.32 (s, 3H), 2.76 (tt, J = 1.5, 7.5 Hz, 2H), 2.93 (t, J = 7.5 Hz, 2H), 7.09 (s, 4H), 9.82 (t, J = 1.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.8, 27.6, 45.3, 128.0, 129.1, 135.7, 137.1, 201.6; MS (EI+): m/z 148 [M]+.

3-(4-Chlorophenyl)propionaldehyde (26h): Compound **26h** was synthesized from **25h** by using method A. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4)

to give a colorless oil (y. 76%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.75 (tt, *J* = 1.2, 7.3 Hz, 2H), 2.91 (t, *J* = 7.3 Hz, 2H), 7.11 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 9.78 (t, *J* = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.3, 44.9, 128.5, 129.6, 131.9, 138.8, 200.9; MS (EI^+): *m/z* 168 $[M]^+$.

3-(3-Chlorophenyl)propionaldehyde (<u>26i</u>): Compound **<u>26i</u>** was synthesized from **<u>25i</u>** by using method A. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/4) to give a colorless oil (y. 67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.79 (tt, *J* = 1.2, 7.2 Hz, 2H), 2.94 (t, *J* = 7.2 Hz, 2H), 7.08 (td, *J* = 1.8, 6.8 Hz, 1H), 7.15-7.24 (m, 3H), 9.82 (t, *J* = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.6, 44.9, 126.5, 126.5, 128.4, 129.8, 134.3, 142.4, 200.8; MS (EI^+): *m/z* 168 $[M]^+$.

2-Cyano-6-[3-(4-methylphenyl)propylamino]benzothiazole (<u>27g</u>): Compound **<u>27g</u>** was synthesized from **<u>26g</u>** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/6) to give a yellow solid (y. 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94-2.04 (m, 2H), 2.34 (s, 3H), 2.72 (t, *J* = 7.3 Hz, 2H), 3.17-3.25 (m, 2H), 4.17 (br, 1H), 6.77-6.84 (m, 2H), 7.08-7.15 (m, 4H), 7.89 (d, *J* = 9.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.0, 30.5, 32.7, 43.0, 99.6, 113.8, 117.0, 125.4, 128.2, 129.0, 129.2, 135.6, 137.9, 138.7, 144.5, 149.0; HRMS (ESI^+): *m/z* calcd for $C_{18}H_{18}N_3S$: 308.1221 $[M+H]^+$; found: 308.1222.

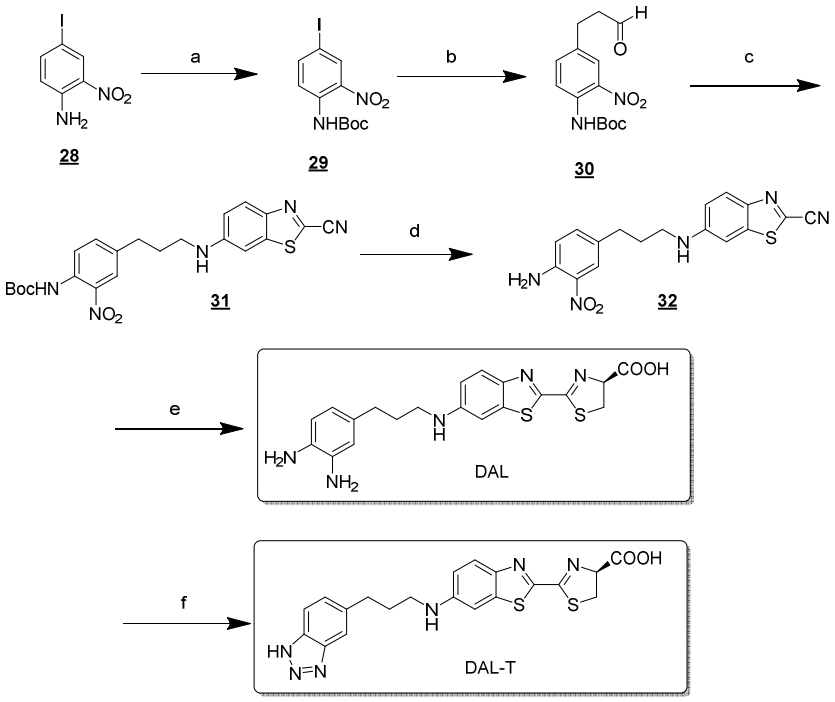
6-[3-(4-Chlorophenyl)propylamino]-2-cyanobenzothiazole (<u>27h</u>): Compound **<u>27h</u>** was synthesized from **<u>26h</u>** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/6 to 1/4) and semipreparative HPLC (A/B = 50/50 to 0/100 (10 min)) to give a yellow solid (y. 22%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.90-2.00 (m, 2H), 2.74 (t, *J* = 7.7 Hz, 2H), 3.17 (t, *J* = 7.0 Hz, 2H), 6.95-7.10 (m, 2H), 7.17-7.28 (m, 4H), 7.81 (d, *J* = 9.7 Hz, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 31.2, 33.1, 43.4, 100.1, 114.8, 118.1, 125.9, 129.2, 129.3, 131.0, 132.0, 139.9, 141.7, 145.0, 151.1; HRMS (ESI^-): *m/z* calcd for $C_{17}H_{13}ClN_3S$: 326.0519 $[M-H]^-$; found: 326.0492.

6-[3-(3-Chlorophenyl)propylamino]-2-cyanobenzothiazole (<u>27i</u>): Compound **<u>27i</u>** was synthesized from **<u>26i</u>** by using method B. The product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/6) to give a yellow solid (y. 12%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.95-2.05 (m, 2H), 2.75 (t, *J* = 7.4 Hz, 2H), 3.20-3.26 (m, 2H), 4.17 (br, 1H), 6.80-6.90 (m, 2H), 7.08 (td, *J* = 2.0, 6.8 Hz, 1H), 7.19-7.24 (m, 3H), 7.90 (d, *J* = 9.3 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.3, 32.9, 43.0, 99.8, 113.8, 117.0, 125.6, 126.5, 126.6, 128.5, 129.8, 129.9, 134.4, 138.8, 143.1, 144.8, 148.8; HRMS (ESI^-): *m/z* calcd for $C_{17}H_{13}ClN_3S$: 326.0519 $[M-H]^-$; found: 326.0473.

D-(−)-2-{6'-[3-(4-Methylphenyl)propylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3g): Compound **3g** was synthesized from **27g** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 43%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.78-1.89 (m, 2H), 2.20 (s, 3H), 2.60 (t, *J* = 7.5 Hz, 2H), 3.06 (t, *J* = 7.0 Hz, 2H), 3.58-3.68 (m, 2H), 5.26 (t, *J* = 9.1 Hz, 1H), 6.79 (dd, *J* = 2.2, 9.0 Hz, 1H), 6.84 (d, *J* = 2.2 Hz, 1H), 6.99 (s, 4H), 7.65 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, acetone-d_6): δ 21.0, 31.6, 33.4, 35.3, 43.6, 79.2, 101.1, 116.5, 125.4, 129.2, 129.8, 135.9, 139.7, 139.8, 145.9, 150.0, 154.8, 166.2, 171.6; HRMS (ESI^+): *m/z* calcd for $C_{21}H_{22}N_3O_2S_2$: 412.1153 $[M+H]^+$; found: 412.1153.

D-(−)-2-{6'-[3-(4-Chlorophenyl)propylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3h): Compound **3h** was synthesized from **27h** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 85%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.91-2.02 (m, 2H), 2.74 (t, *J* = 7.7 Hz, 2H), 3.22 (t, *J* = 7.2 Hz, 2H), 3.68-3.80 (m, 2H), 5.37 (t, *J* = 9.2 Hz, 1H), 7.02 (dd, *J* = 2.4, 9.0 Hz, 1H), 7.15-7.28 (m, 5H), 7.84 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 30.9, 33.3, 35.8, 46.2, 79.0, 105.7, 118.6, 125.9, 129.5, 131.1, 132.8, 140.0, 141.5, 146.6, 148.3, 157.4, 168.0, 173.3; HRMS (ESI^+): *m/z* calcd for $C_{20}H_{18}ClN_3NaO_2S_2$: 454.0427 $[M+Na]^+$; found: 454.0427.

D-(−)-2-{6'-[3-(4-Chlorophenyl)propylamino]-2'-benzothiazolyl}-Δ^2-thiazoline-4-carboxylic acid (3i): Compound **3i** was synthesized from **27i** by using method C. The product was purified by semipreparative HPLC (A/B = 50/50 to 0/100 (20 min)) to give a red solid (y. 94%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.83-1.92 (m, 2H), 2.66 (t, *J* = 7.7 Hz, 2H), 3.09 (t, *J* = 7.0 Hz, 2H), 3.58-3.69 (m, 2H), 5.26 (t, *J* = 9.1 Hz, 1H), 6.82 (dd, *J* = 2.3, 9.0 Hz, 1H), 6.90 (d, *J* = 2.3 Hz, 1H), 7.06-7.10 (m, 2H), 7.12-718 (m, 2H), 7.67 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 31.5, 33.8, 35.8, 44.1, 79.3, 101.8, 117.2, 125.5, 127.0, 128.0, 129.5, 130.9, 135.2, 140.2, 145.6, 146.4, 150.0, 155.5, 167.8, 173.5; HRMS (ESI^+): *m/z* calcd for $C_{20}H_{19}ClN_3O_2S_2$: 432.0607 $[M+H]^+$; found: 432.0585.



Scheme S6. Synthetic scheme of DAL and DAL-T. Reagents and conditions: (a) (i) NaH, DMF (ii) Boc_2O, crude; (b) allyl alcohol, $Pd(OAc)_2$, Bu_4NBr, $NaHCO_3$, DMF, y. 43% (2 steps); (c) 6-amino-2-cyanobenzothiazole, $NaBH_3CN$, MeCN, AcOH y. 58%; (d) TFA, CH_2Cl_2, y. 83%; (e) (i) $Na_2S_2O_4$, K_2CO_3, DMF, H_2O (ii) D-cysteine, K_2CO_3, MeCN/H_2O (pH 8), y. 78%. (f) NOC7, 100 mM NaPi buffer (pH 7.7), DMSO, y. 53%

***tert*-Butyl (4-iodo-2-nitrophenyl)carbamate (29):** 4-Iodo-2-nitroaniline (**28**, 1.59 g, 5.68 mmol) was dissolved in DMF (10 ml). NaH (270 mg, 60% oil dispersion, 6.75 mmol) was added, and the mixture was stirred at r.t. for 5 min. Boc_2O (1.90 g, 8.70 mmol) was added and the mixture was stirred at r.t. for 2 hr. Sat.NH_4Cl aq (50 ml) was added, and the whole was extracted with ethyl acetate (500 ml). The combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was roughly purified by flash silica gel column chromatography (mixed solvent of ethyl acetate and *n*-hexane; the gradient was determined automatically by the EPLC AI-580S system), affording crude **29** (1.53 g).

***tert*-Butyl (2-nitro-4-(3-oxopropyl)phenyl)carbamate (30):** Crude **29** (466 mg) was dissolved in DMF (3 ml). $NaHCO_3$ (208 mg, 2.47 mmol), Bu_4NBr (479 mg, 1.49 mmol), allyl alcohol (153 µl, 2.24 mmol), and $Pd(OAc)_2$ (20.5 mg, 0.0913 mmol) were added. The mixture was stirred at 50 °C for 8 hr. Ethyl

acetate (100 ml) was added, and the combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by flash silica gel column chromatography (mixed solvent of ethyl acetate and *n*-hexane; the gradient was determined automatically by the EPLC AI-580S system), affording **30** (220 mg, y. 43%, 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94 (s, 9H), 2.84 (t, *J* = 6.9 Hz, 2H), 2.97 (t, *J* = 6.9 Hz, 2H), 7.46 (d, *J* = 8.7 Hz, 1H), 8.01 (s, 1H), 8.46 (d, *J* = 8.7 Hz, 1H), 9.54 (s, 1H), 9.83 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.7, 28.0, 44.5, 81.6, 120.9, 124.9, 134.0, 134.4, 135.8, 135.8, 152.1, 200.3.

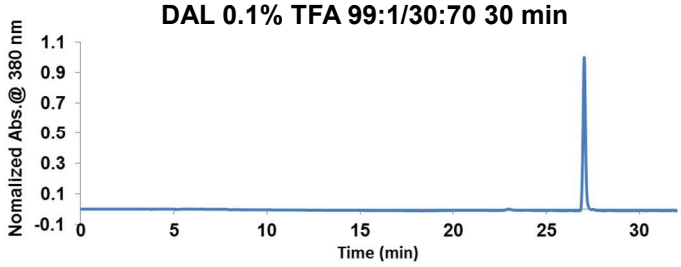
***tert*-Butyl (4-(3-((2-cyanobenzo[d]thiazol-6-yl)amino)propyl)-2-nitrophenyl)carbamate (31):** Compound **30** (220 mg, 0.748 mmol) and 6-amino-2-cyanobenzothiazole (131 mg, 0.748 mmol) were dissolved in MeCN (8 ml). AcOH (400 μl) and $NaBH_3CN$ (100 mg, 1.59 mmol) were added sequentially. The mixture was stirred at r.t. for 10 min. Sat.$NaHCO_3$ aq (30 ml) was added, and the whole was extracted with ethyl acetate (200 ml). The combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by flash silica gel column chromatography (mixed solvent of ethyl acetate and *n*-hexane; the gradient was determined automatically by the EPLC AI-580S system), affording **31** (197 mg, y. 58%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.54 (s, 9H), 1.97-2.07 (m, 2H), 2.97 (t, *J* = 6.9 Hz, 2H), 3.20 -3.27 (m, 2H), 4.30 (t, *J* = 5.4 Hz, 1H), 6.84-6.89 (m, 2H), 7.45 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.89 (d, *J* = 9.0 Hz, 1H), 8.02 (d, *J* = 2.1 Hz, 1H), 8.48 (d, *J* = 8.7 Hz, 1H), 9.56 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.2, 30.2, 32.0, 42.9, 81.8, 99.8, 113.8, 116.9, 121.0, 124.9, 125.5, 129.8, 134.1, 135.1, 135.8, 138.7, 144.7, 148.8, 152.2; HRMS (ESI^-): *m/z* calcd for $C_{22}H_{22}N_5O_4S$: 452.1393 $[M-H]^-$; found: 452.1356.

6-((3-(4-Amino-3-nitrophenyl)propyl)amino)benzo[d]thiazole-2-carbonitrile (32): Compound **31** (170 mg, 0.375 mmol) was dissolved in CH_2Cl_2 (8 ml). TFA (4 ml) was added, and the mixture was stirred for 30 min. Sat. $NaHCO_3$ aq (30 ml) was added, and the whole was extracted with ethyl acetate (150 ml). The combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated, affording **32** (109 mg, y. 83%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.82-1.90 (m, 2H), 2.61 (t, *J* = 7.6 Hz, 2H), 3.07-3.12 (m, 2H), 6.76 (br, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 7.03 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.13 (d, *J* = 2.0 Hz, 1H), 7.31-7.34 (m, 3H), 7.81 (s, 1H), 7.88 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.6, 31.1, 42.0, 99.0, 114.3, 117.2, 119.3, 123.7, 124.7, 127.5, 128.8, 129.9, 136.6, 138.9, 143.1, 144.7, 150.0.

(S)-2-(6-((3-(3,4-Diaminophenyl)propyl)amino)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carbox

ylic acid (DAL): Compound **32** (35.0 mg, 0.0990 mmol) was dissolved in DMF (2 ml). $Na_2S_2O_4$ (120 mg, 0.689 mmol) and K_2CO_3 (42.5 mg, 0.724 mmol) dissolved in H_2O (3 ml) were added. The mixture was stirred at r.t. for 15 min, then H_2O (20 ml) was added, and the whole was extracted with ethyl acetate (100 ml). The combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was dissolved in MeCN (2 ml). D-cysteine (120 mg in the form of hydrochloride monohydrate) dissolved in H_2O (2 ml, pH was adjusted to 8.0 with 0.5 M K_2CO_3 aq.) was added. The mixture was stirred under Ar for 20 min. TFA (200 µl) was added to fix the form of the compound, which was then purified by means of HPLC with eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) (A/B = 90/10 to 30/70 for 20 min), affording DAL (33.0 mg, y. 78%). *For final assays, the compound was purified again in order to completely exclude any remaining impurity that might influence the luminescence intensity, by means of HPLC with eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) (A/B = 99/1 for 5 min, then 99/1 to 30/70 for 30 min). ^{1}H NMR (400 MHz, DMSO-d_6, 0.02% AcOH): δ 1.80-1.92 (m, 2H), 2.59 (t, J = 7.6 Hz, 2H), 3.09 (t, J = 6.8 Hz, 2H), 3.62-3.66 (m, 1H), 3,79-3.76 (m, 1H), 5.36 (t, J = 9.2 Hz, 1H), 6.68 (d, J =7.6 Hz, 1H), 6.79 (s, 1H), 6.91 (dd, J = 8.8, 2.2 Hz, 1H), 6.98 (d, J = 8.8 Hz, 1H), 7.03 (d, J = 2.2 Hz, 1H), 7.80 (d, J = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6, 0.02% AcOH): δ 29.9, 32.0, 34.5, 42.2, 78.0, 99.8, 115.6, 118.4, 120.7, 121.8, 124.3, 138.3, 144.0, 148.9, 153.0, 158.0, 158.4, 164.3, 171.3; HRMS (ESI$^+$): *m/z* calcd for $C_{20}H_{22}N_5O_2S_2$: 428.1215 $[M+H]^+$; found: 428.1173.

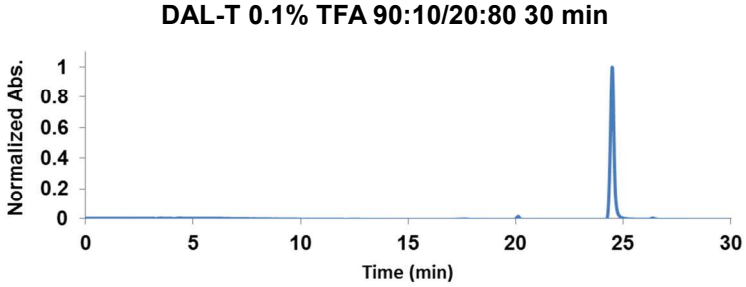
HPLC analysis of DAL: eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) , A/B = 99/1 to 30/70 for 30 min.

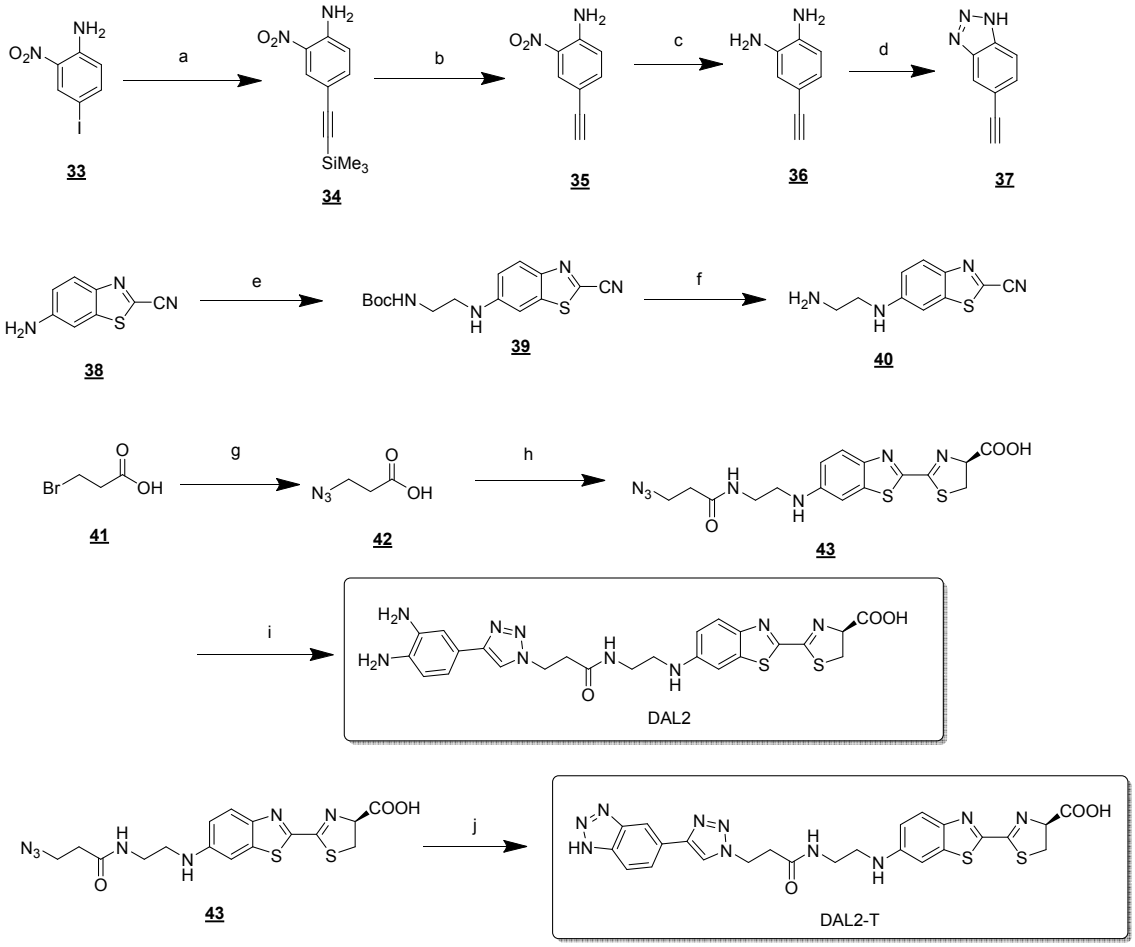


(S)-2-(6-((3-(1H-Benzo[d][1,2,3]triazol-5-yl)propyl)amino)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (DAL-T): DAL (3.0 mg, 0.00702 mmol) was dissolved in DMSO (100 µl). NOC7 was dissolved in 100 mM NaPi buffer (pH7.7, 800 µl) and was soon added to the solution of DAL. The mixture was stirred at r.t. for 15 min and then subjected to HPLC with eluent A (H_2O, 0.1 M TEAA) and

eluent B (80% MeCN, 20% H_2O 0.1 M TEAA) (A/B = 90/10 to 20/80 for 20 min). The product was desalted with a Sep-Pak C18 cartridge, affording DAL-T (2.0 mg, y. 53%). As TEA salt; ^{1}H NMR (400 MHz, CD_3OD): δ 1.30 (t, *J* = 7.2 Hz, 9H, TEA), 2.01-2.09 (m, 2H), 2.96 (t, *J* = 7.2 Hz, 2H), 3.16-3.23 (m, 10H, including TEA), 3.66-3.71 (m, 2H), 5.16 (t, *J* = 9.2 Hz, 1H), 6.88 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.96 (d, *J* = 2.4 Hz, 1H), 7.39 (d, *J* = 8.8 Hz, 1H), 7.67 (s, 1H), 7.73 (d, *J* = 8.8 Hz, 1H), 7.82 (d, *J* = 8.8 Hz, 1H); HRMS (ESI^+): *m/z* calcd for $C_{20}H_{19}N_6O_2S_2$: 439.1011 $[M+H]^+$; found: 439.1005.

HPLC analysis of DAL-T: eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA), A/B = 90/10 to 20/80 for 30 min.





Scheme S7. Synthetic scheme of DAL2 and DAL2-T. Reagents and conditions: (a) Ethynyltrimethylsilane, $PdCl_2(PPh_3)_2$, CuI, DIEA, THF, y. quant; (b) K_2CO_3, MeOH, CH_2Cl_2, y. 85%; (c) $Na_2S_2O_4$, K_2CO_3, MeOH, H_2O, y. 87%; (d) $NaNO_2$, AcOH, MeOH, H_2O, y. 77%; (e) *N*-Boc-2-aminoacetaldehyde, $NaBH_3CN$, MeCN, AcOH, y. 65%; (f) TFA, CH_2Cl_2, y. 94%; (g) NaN_3, MeCN, y. 59%; (h) (i) WSCD, HOBt, DMF (ii) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 29%.; (i) Compound **36**, $CuSO_4 \cdot 5H_2O$, Na ascorbate, MeOH, H_2O, y. quant; (j) Compound **37**, $CuSO_4 \cdot 5H_2O$, Na ascorbate, MeOH, H_2O, y. 59%

2-Nitro-4-((trimethylsilyl)ethynyl)aniline (34): 4-Iodo-2-nitroaniline (**33**, 5.32 g, 20 mmol) was dissolved in THF (20 ml). $PdCl_2(PPh_3)_2$ (154 mg, 0.22 mmol), CuI (90 mg, 0.47 mmol) and DIEA (10.5 ml, 60 mmol) was added to this solution. The flask was purged with Ar, and ethynyltrimethylsilane (3.1 ml, 22 mmol) was added under an Ar atmosphere. The mixture was stirred at r.t. for 2.5 hr, then H_2O (30 ml) was added, and the whole was extracted with ethyl acetate (50 ml). The combined organic layer was

washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by means of silica gel column chromatography (ethyl acetate/*n*-hexane = 3/7), affording **34** (4.70 g, y. quant). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.22 (s, 9H), 6.32 (br, 2H), 6.74 (d, *J* = 8.7 Hz, 1H), 7.36 (dd, *J* = 8.7, 1.5 Hz, 1H), 8.20 (d, *J* = 1.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 0.0, 93.5, 103.3, 111.8, 118.9, 129.9, 131.3, 138.5, 144.7.

4-Ethynyl-2-nitroaniline (35): Compound **34** (4.65 g, 20 mmol) was dissolved in MeOH/CH_2Cl_2 (50 ml/50 ml) and K_2CO_3 (4.15 g, 30 mmol) was added to the solution. The mixture was stirred at r.t. for 5 hr, then H_2O (100 ml) was added, and the whole was extracted with CH_2Cl_2 (100 ml). The combined organic layer was washed with brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by means of silica gel column chromatography (CH_2Cl_2/*n*-hexane = 7/3), affording **35** (2.65 g, y. 85%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.35 (s, 1H), 6.91 (d, *J* = 8.4 Hz, 1H), 7.37 (dd, *J* = 8.4, 2.1 Hz, 1H), 8.12 (d, *J* = 2.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 79.2, 82.3, 108.1, 119.8, 129.0, 129.6, 137.9, 146.2.

4-Ethynylbenzene-1,2-diamine (36): Compound **35** (162 mg, 1.0 mmol) was dissolved in MeOH (6 ml). $Na_2S_2O_4$ (1.74 g, 10 mmol, 10 eq.) and K_2CO_3 (1.38 g, 10 mmol) dissolved in H_2O (10 ml) were added. The mixture was stirred at r.t. for 5 min, then H_2O (100 ml) was added, and the whole was extracted with ethyl acetate (100 ml). The combined organic layer was washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by flash silica gel column chromatography (mixed solvent of ethyl acetate and *n*-hexane; the gradient was determined automatically by the EPLC AI-580S system), affording **36** (115 g, y. 87%). *This compound was used for the next step immediately, because it was unstable. ^{1}H NMR (300 MHz, CD_3OD): δ 3.17 (s, 1H), 6.59 (d, *J* = 8.1 Hz, 1H), 6.75 (dd, *J* = 8.1, 2.1 Hz, 1H), 6.81 (d, *J* = 2.1 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 75.3, 85.9, 113.3, 116.6, 120.7, 125.0, 135.2, 137.5; HRMS (ESI^+): *m/z* calcd for $C_8H_9N_2$: 133.0766 $[M+H]^+$; found: 133.0815.

5-Ethynyl-1H-benzo[d][1,2,3]triazole (37): Compound **36** (229 mg, 1.73 mmol) was dissolved in MeOH/AcOH/H_2O (7.5 ml/1.5 ml/7.5 ml), and $NaNO_2$ (200 mg, 2.90 mmol) was added to it. The mixture was stirred for 5 min, then H_2O (10 ml) was added, and the whole was extracted with ethyl acetate (100 ml). The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4 and evaporated. The residue was purified by flash silica gel column chromatography (mixed solvent of ethyl acetate and *n*-hexane; the gradient was determined automatically by the EPLC AI-580S system),

affording **37** (192 mg, y. 77%). ^{1}H NMR (400 MHz, CD_3OD): δ 3.61 (s, 1H), 6.75 (dd, *J* = 8.8, 1.6 Hz, 1H), 7.84 (d, *J* = 8.8 Hz, 1H) 8.00 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 80.8, 83.4, 115.3 (br), 118.6, 119.1 (br), 128.9, 138.4 (br).

***tert*-Butyl (2-((2-cyanobenzo[d]thiazol-6-yl)amino)ethyl)carbamate (39):** 6-Amino-2-cyanobenzothiazole (**38**, 475 mg, 2.7 mmol) and *N*-(tert-butoxycarbonyl) aminoacetaldehyde (500 mg, 3.1 mmol, 1.2 eq.) were dissolved in MeCN (30 ml), and acetic acid (1 ml) was added. After the reaction solution became clear, $NaBH_3CN$ (250 mg, 4.0 mmol, 1.5 eq.) was added. The mixture was stirred at r.t. for 10 min, then H_2O (20 ml) was added, and the whole was extracted with CH_2Cl_2 (50 ml). The combined organic layer was washed with sat. $NaHCO_3$ aq and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by means of silica gel column chromatography (CH_2Cl_2, 1.5% MeOH), affording **39** (560 mg, y. 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (s, 9H), 3.31-3.48 (m, 4H), 5.09 (br, 2H), 6.85-6.90 (m, 2H), 7.87 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.3, 39.6, 44.8, 79.9, 99.3, 113.8, 116.9, 125.4, 129.3, 138.7, 144.5, 149.0, 156.9; HRMS (ESI^+): *m/z* calcd for $C_{15}H_{18}N_4NaO_2S$: 341.1048 $[M+Na]^+$; found: 341.1043.

6-((2-Aminoethyl)amino)benzo[d]thiazole-2-carbonitrile (40): Compound **39** (225 mg, 0.71 mmol) was dissolved in CH_2Cl_2 (10 ml), and trifluoroacetic acid (TFA) (2 ml) was added. The reaction solution was stirred at r.t. for 1 min. After evaporation of the solvent, the residue was purified by means of HPLC with eluent A (H_2O 0.1% TFA) and eluent B (MeCN 80%, H_2O 20% 0.1% TFA) (A/B = 80/20 to 20/80 for 20 min), affording **40** (144 mg, y. 94%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.24 (t, *J* = 6.6 Hz, 2H), 3.57 (t, *J* = 6.6 Hz, 2H), 7.05 (dd, *J* = 2.2, 8.8 Hz, 1H), 7.15 (d, *J* = 2.2 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 39.4, 41.7, 101.0, 114.7, 118.6, 126.1, 130.9, 140.0, 145.8, 150.5); HRMS (ESI^+): *m/z* calcd for $C_{10}H_{11}N_4S$: 219.0704 $[M+H]^+$; found: 219.0655.

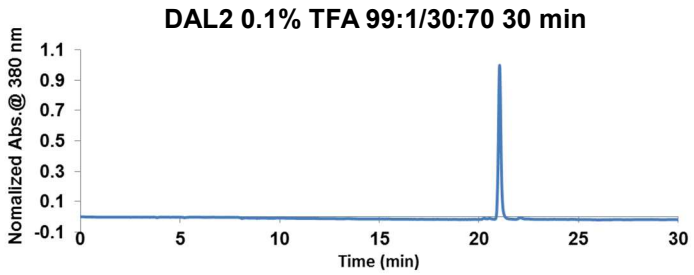
3-Azidopropanoic acid (42): 3-Bromopropanoic acid (**41**, 1.95 g, 12.7 mmol) was dissolved in MeCN (18 ml). NaN_3 (1.50 g, 13.0 mmol) was added and the mixture was refluxed for 4 hr. Then 0.4 N HCl (20 ml) was added and the whole was extracted with ethyl acetate (150 ml). The combined organic layer was washed with 0.4 N HCl and brine (slightly acidified with 0.4 N HCl). The organic layer was dried over Na_2SO_4 and the solvent was evaporated, affording **42** (870 mg, y. 59%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.64 (t, *J* = 6.6 Hz, 2H), 3.59 (t, *J* = 6.6 Hz, 2H), 9.76 (br, 1H)**;** ^{13}C NMR (75 MHz, $CDCl_3$): δ 33.6, 46.3, 176.9.

(S)-2-(6-((2-(3-Azidopropanamido)ethyl)amino)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (43): Compound **42** (19 mg, 0.087 mmol) was dissolved in DMF (300 μl), then **40** (20 mg, 0.091 mmol), WSCD (35 mg, 0.18 mmol), and HOBt (25 mg, 0.185 mmol) were added and the mixture was stirred at r.t. for 2 hr. Ethyl acetate (30 ml) was added and the combined organic layer was washed with H_2O, sat. $NaHCO_3$ aq., and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was dissolved in MeCN (500 μl). D-Cysteine (40 mg in the form of hydrochloride monohydrate) dissolved in H_2O (1.5 ml, pH was adjusted to 8.0 with 0.5 M K_2CO_3 aq.) was added. The mixture was stirred under Ar for 20 min, then a mixture of H_2O (6.0 ml) and MeCN (1.5 ml) was added. TFA (180 μl) was added just before injection into the HPLC column to fix the form of the compound, which was purified by means of HPLC with eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) (A/B = 90/10 to 0/100 for 30 min). The fraction containing the target compound was added to 100 mM NaPi buffer (pH 7.7, 5 ml) and desalted with a Sep-Pak C18 cartridge, affording **43** as the sodium salt (11.0 mg, y. 29%). ^{1}H NMR (400 MHz, CD_3OD): δ 2.46 (t, J = 6.4 Hz, 2H), 3.32 (t, J = 6.4 Hz, 2H), 3.44 (t, J = 6.4 Hz, 2H), 3.55 (t, J = 6.4 Hz, 2H), 3.64-3.74 (m, 2H), 5.17 (t, J = 9.2 Hz, 1H), 6.88 (dd, J = 8.8, 2.4 Hz, 1H), 7.07 (d, J = 2.4 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 36.3, 37.1, 39.8, 43.8, 82.7, 82.9, 101.5, 116.7, 125.3, 140.0, 146.2, 150.0, 156.6, 165.5, 173.4, 177.3; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{18}N_7O_3S_2$: 420.0913 $[M+H]^+$; found: 420.0910.

(S)-2-(6-((2-(3-(4-(3,4-Diaminophenyl)-1H-1,2,3-triazol-1-yl)propanamido)ethyl)amino) benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (DAL-2): Compounds **43** (3.2 mg, Na salt, 0.0068 mmol) and **36** (3.6 mg, 0.027 mmol) were dissolved in DMSO (300 μl). Na ascorbate (2.0 mg, 0.010 mmol) in H_2O (50 μl) and $CuSO_4 \cdot 5\ H_2O$ (3.0 mg, 0.012 mmol) in H_2O (150 μl) were added. The mixture was stirred at r.t. under Ar for 2 hr, then a mixture of H_2O (2.0 ml) and MeCN (0.5 ml) was added. TFA (50 μl) was added just before injection into the HPLC column to fix the form of the compound, which was purified by means of HPLC with eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) (A/B = 99/1 to 30/70 for 30 min), affording DAL-2 (4.0 mg, y. quant). ^{1}H NMR (300 MHz, CD_3OD, 0.05% AcOH): δ 2.88 (t, J = 6.3 Hz, 2H), 3.19 (t, J = 6.3 Hz, 2H), 3.40 (t, J = 6.3 Hz, 2H), 3.72-3.78 (m, 2H), 4.72 (t, J = 6.3 Hz, 2H), 5.38 (t, J = 9.3 Hz, 1H), 6.81 (dd, J = 8.7, 2.1 Hz, 1H), 6.94 (d, J = 2.1 Hz, 1H), 7.02 (d, J = 8.7 Hz, 1H), 7.31 (dd, J = 8.7, 2.1 Hz, 1H), 7.49 (d, J = 2.1 Hz, 1H), 7.68 (d, J = 8.7 Hz, 1H), 8.15 (s, 1H); HRMS (ESI^+): *m/z* calcd for $C_{24}H_{26}N_9O_3S_2$: 552.1600 $[M+H]^+$; found: 552.1592.

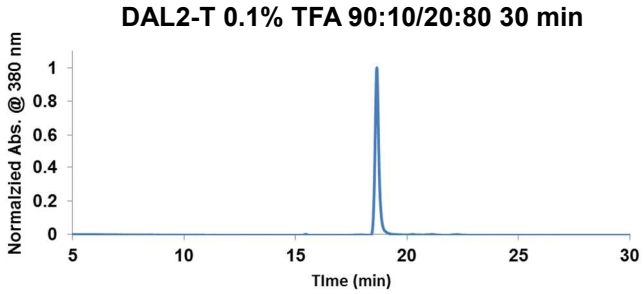
HPLC analysis of DAL2: eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA),

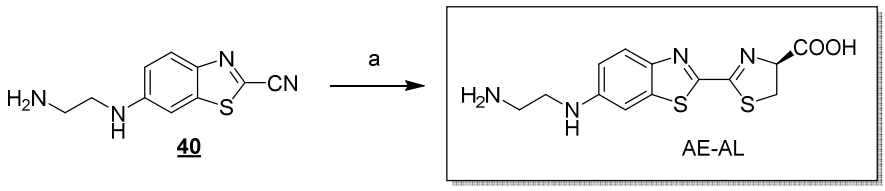
A/B = 99/1 to 30/70 for 30 min.



(S)-2-(6-((2-(3-(4-(1H-Benzo[d][1,2,3]triazol-5-yl)-1H-1,2,3-triazol-1-yl)propanamido)ethyl) amino)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (DAL2-T): Compounds **43** (2.0 mg, Na salt, 0.0045 mmol) and **37** (2.0 mg, 0.014 mmol) were dissolved in DMSO (200 µl). Na ascorbate (1.5 mg, 0.0076 mmol) in H_2O (30 µl) and $CuSO_4 \cdot 5\ H_2O$ (2.0 mg, 0.0080 mmol) were added. The mixture was stirred at 50 °C under Ar for 2 hr, then a mixture of H_2O (2.0 ml) and MeCN (0.5 ml) was added. TFA (50 µl) was added just before injection into the HPLC column to fix the form of the compound, which was purified by means of HPLC with eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA) (A/B = 90/10 to 0/100 for 20 min), affording DAL2-T (1.5 mg, y. 59%). ^{1}H NMR (300 MHz, CD_3OD): δ 2.91 (t, *J* = 6.3 Hz, 2H), 3.21 (t, *J* = 6.0 Hz, 2H), 3.41 (t, *J* = 6.0 Hz, 2H), 3.71-3.76 (m, 2H), 4.76 (t, *J* = 6.3 Hz, 2H), 5.36 (t, *J* = 9.0 Hz, 1H), 6.80 (dd, *J* = 9.0, 2.1 Hz, 1H), 6.96 (d, *J* = 2.1 Hz, 1H), 7.64 (d, *J* = 9.0 Hz, 1H), 7.82-7.86 (m, 2H), 8.22 (s, 1H), 8.36 (s, 1H); HRMS (ESI^+): *m/z* calcd for $C_{24}H_{23}N_{10}O_3S_2$: 563.1396 $[M+H]^+$; found: 563.1391.

HPLC analysis of DAL2-T: eluent A (H_2O, 0.1% TFA) and eluent B (80% MeCN, 20% H_2O, 0.1% TFA), A/B = 90/10 to 20/80 for 30 min.





Scheme S8. Synthetic scheme of AE-AL. Reagents and conditions: (a) D-cysteine, K_2CO_3, MeOH/H_2O (pH 8), y. 43%.

D-(−)-2-[6'-(4-Aminoethylamino)-2'-benzothiazolyl]-Δ^2-thiazoline-4-carboxylic acid (AE-AL): AE-AL was synthesized from compound **40** by using Method C. The product was purified by semipreparative HPLC, neutralized with 100 mM NaPi buffer (pH7.7), desalted, and lyophilized to give the sodium salt as a yellow powder (y. 43%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.20 (t, J = 6.0 Hz, 2H), 3.52 (t, J = 6.0 Hz, 2H), 3.71-3.77 (m, 2H), 5.37 (t, J = 6.0 Hz, 1H), 6.96 (dd, J = 9.0, 2.4 Hz, 1H), 7.15 (d, J = 2.4 Hz, 1H), 7.81 (d, J = 9.0 Hz, 1H); HRMS (ESI$^+$): *m/z* calcd for $C_{13}H_{15}N_4O_2S_2$: 323.0636 $[M+H]^+$; found: 323.0616.

Supporting References

(1) Urano, Y. *et al. J. Am. Chem. Soc.* **127**, 4888-4894 (2005).

(2) Sunahara, H. *et al. J. Am. Chem. Soc.* **129**, 5597-5604 (2007).

(3) Terai, T. *et al. J. Am. Chem. Soc.* **128**, 6938-6946 (2006).

(4) Valley, M. P. *et al. Promega Corporation Web site* (2008).

(5) Woodroofe, C. C. *et al. Biochemistry* **47**, 10383-10393 (2008).

(6) Hakamata, Y. *et al. Translantation* **81**, 1179-1184 (2006).

(7) ACS Committee on Environmental Improvement. *Anal. Chem.* **52**, 2242-2247 (1980).

(8) Takakura, H. *et al. ChemBioChem* **13**, 1424-1427 (2012).

(9) Carsten, M *et al. J. Med. Chem.* **44**, 4691-4703 (2001).