Supporting Information

Ultrafast and Selective Labeling of Endogenous Proteins Using Affinity-based Benzotriazole Chemistry

Xiaoyi Xin,[+,a] Yu Zhang,[+,a] Massimiliano Gaetani,[c] Susanna L. Lundström,[c] Roman A. Zubarev,[c] Yuan Zhou,[b] Dale P. Corkery,[+,a] Yao-Wen Wu*[,a]

[a]Department of Chemistry, Umeå Centre for Microbial Research (UCMR), Umeå University, 90187 Umeå, Sweden.

[b]School of Medical Technology, Xuzhou Medical University, No. 209, Tongshan Road, Xuzhou 221004, China

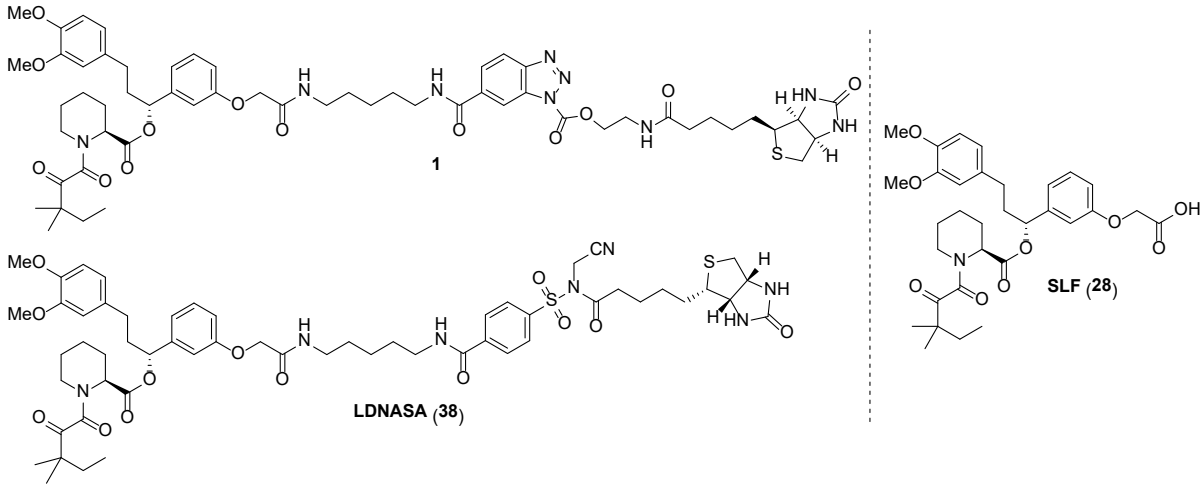
[c]Division of Physiological Chemistry I, Chemical Proteomics Core Facility, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm 17177, Sweden; Chemical Proteomics, Science for Life Laboratory (SciLifeLab), Stockholm 17177, Sweden

[+]These authors contributed equally to this work.

*Correspondence to: yaowen.wu@umu.se

Part I: Biological Experiments

1. FKBP12 Labeling using Compound 1 or LDNASA



FKBP12 Protein Preparation

A pET19_His tag_FKBP12 plasmid containing His tag was used in this study. FKBP12 was expressed in *Escherichia coli* strain BL21 (DE3). Cells were cultured at 37 °C in LB media supplied with 100 μg/mL ampicillin. When the OD600 reached 0.6, 0.1 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression at 20 °C on a shaker (170 rpm) overnight (10 h). Cells were collected in the centrifuge Avanti J20-XP (Beckman Coulter) at 6000 rpm, 4 °C for 20 min, and lysed by a microfluidizer (CONSTANT SYSTEMS. Ltd) in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl pH 8.0, 10% (vol/ vol) glycerol, 2 mM *β*ME, 1 mM PMSF, and 0.1% (vol/vol) Antifoam 204 (Sigma-Aldrich, cat. No.: A6426)]. The cleared lysate was purified by affinity chromatography on a 5 mL

HisTrap™ HP column (GE Healthcare) followed by size-exclusion chromatography on a HiLoad 26/60 Superdex 200 pg column (GE Healthcare) in buffer containing 50 mM HEPES, 50 mM NaCl, pH 7.3 using the NGC system (Bio-Rad).

The purified recombinant FKBP12 protein (5 μM) was incubated with compound **1** (10 μM) or **LDNASA** (**38**) (10 μM) in the absence or presence of **SLF** (**28**) (100 μM) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) at 37 °C. Aliquots at different time points were taken and then quenched by dispensing into the same volume of 2× SDS-PAGE Sample Buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 0.002% bromophenol blue, 5% 2-mercaptoethanol) supplemented with 200 μM **SLF**. The resulting mixture was incubated at 70 °C for 5 min. The samples were subjected to SDS-PAGE and electro-transferred onto a nitrocellulose blotting membrane (Bio-Rad, cat. No.: 1704158) using Trans-Blot® Turbo™ transfer system (Bio-Rad) according to the manufacturer's instructions. The protein transferred to the membrane was stained with Ponceau S [0.1 % (w/v) in 5% acetic acid, Sigma-Aldrich, cat. No.: P7170-1L], then washed with tris-buffered saline with Tween 20 (TBST). The membrane was blocked in 5% skim milk/TBST at room temperature for 1 hour. After washing with TBST (5 min × 3), the membrane was incubated with Streptavidin-HRP (Sigma-Aldrich, cat. No.: RABHRP3-600UL, 1:1,000) in 5% BSA/TBST at room temperature for 1 hour. After washing with TBST (5 min × 3), the signals were visualized using ECL substrate (Bio-Rad, cat. No.: 1705061) by ChemDoc™ MP Imaging System (Bio-Rad) according to the manufacturer's instructions. The images were analyzed using Image Lab software (Bio-Rad) and quantified using ImageJ software.
The assay was carried out in three independent biological replicates.
The representative images are shown in Figures 2a and S1.

2. Determination of Second-order Rate Constant

$$A + B \rightarrow AB$$

[A] = [protein], $[A]_o$= 5 μM; [B]=[probe], $[B]_o$= 10 μM; I = band intensity which is proportional to the product: $[A]_o$-[A]. Given the integrated second-order rate law equation:

$$\ln\frac{[B][A]_0}{[A][B]_0} = k([B]_0 - [A]_0)t$$

and the relationship of the band intensity with the concentration of product:

$$I = I_{max}(1 - \frac{[A]}{[A]_0})$$

It can be derived:

$$I = I_{max}(1 - \frac{1 - \frac{[A]_0}{[B]_0}}{e^{([B]_0 - [A]_0)kt} - \frac{[A]_0}{[B]_0}})$$

where I_{max} = maximal intensity. The band intensity (I) at each time point is plotted against time (t) and fitted to the equation to give the second-order reaction rate constant using GraFit (Erithacus Software Limited).

3. First-order rate constant

The reaction of affinity labeling for covalent protein labeling is:

$$P + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P.R \xrightarrow{k_{chem}} P\text{-}R$$

$$k_{obs} = \frac{k_{chem}}{1 + \frac{K_d}{[R]}}$$

where k_{obs} is the observed reaction rate at a specific concentration of reagent [R]; K_d is the dissociation constant of protein-ligand binding, defined as k_{-1}/k_1; k_{chem} is the rate constant for the irreversible

chemical modification. When [R] >> K_d, $k_{obs} \approx k_{chem}$, which is determined by the intrinsic reactivity of the reactive group (BTA). In the three examples shown in the paper, the affinities range from low nanomolar to submicromolar, which represent typical binding affinities of protein-ligand interactions. Therefore, under the conditions used, the observed ultrafast reaction rate indeed reflects the reactivity of the BTA chemistry driven by proximity effect.

4. Peptide Mapping of the Labeled FKBP12 by compound 1

4.1. Preparation of Labeled FKBP12 Sample

FKBP12 (10 μM) was incubated with **1** (20 μM) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) at 37 °C for 20 min. Then the reaction mixture was purified using PD Spintrap™ G-25 column (GE, cat. No.: 28-9180-04) according the manufactory's instructions.

4.2. Digestion

10 μg (72.4 μL) of the sample was digested without reduction/alkylation with trypsin (1:20 ratio) at 37°C overnight. The digestion reaction was stopped by addition of 5 μL of 20% trifluoroacetic acid (TFA). Finally, the digested peptides were diluted to 150 μL and thereafter desalted using a SPE Pierce C18 Spin Column (Thermo Scientific). These columns were activated by 2 × 200 μL of 50% acetonitrile (ACN) and equilibrated with 2 × 200 μL of 0.5% TFA. The digested peptides were adsorbed to the column using two repeated cycles of 40 μL sample loading and the column was washed using 3 × 200 μL of 0.5% TFA. Finally, the peptides were eluted with 3 × 50 μL of 70% ACN. The solvent was evaporated. Peptides were resolved in 50 μL of 0.1% formic acid and further diluted 4 times prior to nano-LC-MS/MS.

4.3. LC-MS/MS

The nanoLC-MS/MS experiments were performed using a Q-Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. The peptides were separated by C18 reversed phase liquid chromatography using an EASY-nLC 1000 system (Thermo Fisher Scientific). A set-up of pre-column and analytical column was used. The precolumn was a 2 cm EASYcolumn (ID 100 μm, 5 μm particles, Thermo Fisher Scientific) while the analytical column was a 15 cm EASY-column (ID 75 μm, 3 μm particles, Thermo Fisher Scientific). Peptides were eluted with a 35 min optimized gradient: 0-2 min 2% B (B – buffer B, ACN with 0.1% Formic acid), 2-17 min – 2-50% B, 17-22 min – 50-80% B, 22-26 min – 80% B, 26-27 min – 80-100% B, 27-35 min – 100% B at a rate of 300 nL/min. The mass spectrometer was operated in positive ion mode acquiring a survey mass spectrum with resolving power 70,000 (full width half maximum), m/z 400-1750 using an automatic gain control (AGC) target of 3×10^6. The 10 most intense ions were selected for higher-energy collisional dissociation (HCD) fragmentation (30% normalized collision energy) and MS/MS spectra were generated with an AGC target of 5×10^5 at a resolution of 17,500. The mass spectrometer worked in data-dependent mode.

4.4. Data Analysis

The acquired data was processed by pFind Software[1] (version 3.1.5) using a database containing targeted protein sequence FKPB12. The following parameters were used for data processing: maximum 20 ppm error tolerances for both the survey scan and MS/MS analysis. The search parameters were set to Taxonomy: targeted protein, Enzyme: Trypsin, open search mode, maximum of three miss cleavages sites. An FDR of maximum 1% for peptide identification was accepted.

5. FKBP12 Labeling in Cells or Cell Lysate

5.1. FKBP12 Labeling in HeLa Cell Lysate

A mixture of purified recombinant FKBP12 (final 2 μM), HeLa cell lysate (1.5 mg/mL), and compound **1** (final 2.5-10 μM) or LDNASA (final 2.5-10 μM) in the absence or presence of **SLF** (20 μM) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) was incubated for 5 min or the indicated time at 37 °C. Aliquots at different time points were taken and then quenched by dispensing into a same volumn of 2× SDS-PAGE Sample Buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 0.002% bromophenol blue, 5% 2-mercaptoethanol) supplemented with 20 μM **SLF**. The resulting mixture was incubated at 70 °C for 3 min. The samples were subjected to SDS-PAGE and western blot as shown above.
For detection of FKBP12: the membrane was incubated with ananti-FKBP12 antibody (Thermo Fisher, cat. No.: PA1-026A, 1:1,000) in 5% BSA/TBST at 4 ℃ overnight. After washing with TBST (5 min × 3), the membrane was incubated with the secondary antibody Goat Anti-Rabbit IgG, H & L Chain Specific Peroxidase Conjugate (Merck, Calbiochem, cat. No.: 401315-2ML, 1:10,000) in 5% skim milk/TBST at room temperature for 1 hour. After washing with TBST (5 min × 3), the signals were visualized using ECL substrate (Bio-Rad, cat. No.: 1705061) on ChemDoc™ MP Imaging System (Bio-Rad) according to the manufacturer's instructions.
The assay was carried out in three independent biological replicates.
The representative images are shown in Figure 3a, S5.

5.2. Cell Labeling and Immunoprecipitation

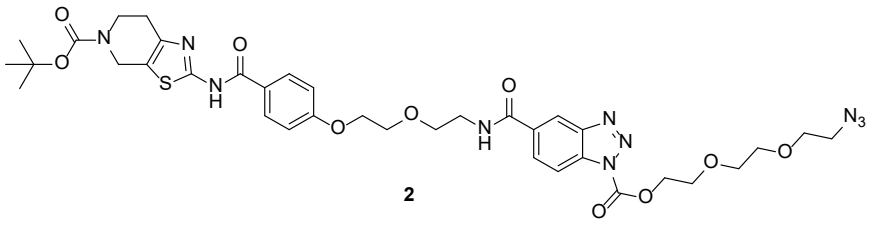
HeLa cells were washed 3 times with PBS and incubated with 1 μM **1** (or 1 μM **1** + 20 μM SLF) in serum free DMEM for 20 minutes. Cells were then washed 3 times with PBS, lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 8, 300 mM KCl, 10% Glycerol, 0.25% Nonidet P-40, 0.5 mM EGTA, 1 mM PMSF, 1× complete protease inhibitor (Roche)), passed 6× through a 21G needle, and cleared by centrifugation (20 min/12,700 rpm/4°C, Eppendorf centrifuge 5427R). Streptavidin beads (Dynabeads™ MyOne™ Streptavidin C1, Thermo Fisher, 65001) were incubated with lysates for 30 minutes at room temperature. Beads were washed 3 times with PBS containing 0.1% BSA and the target antigen eluted by re-suspending in Laemmli sample buffer and heating at 95°C for 10 minutes.
The representative blots are shown in Figure 3b.

6. GRAMD1A_StART Domain Labeling using Compound 2

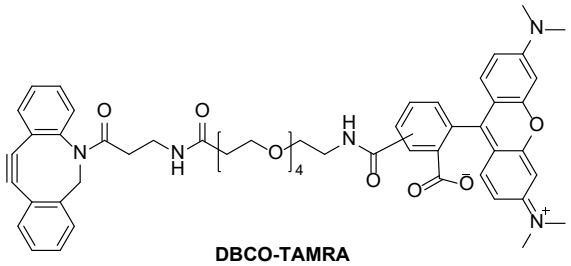
6.1. Protein Preparation

A pMAL_MBP-GRAMD1A 359-547 plamid containing pMAL vector and MBP tag was used in this study. The StART domain (GRAMD1A 359-547) was expressed in Escherichia coli strain BL21 (DE3). Cells were cultured at 37 ℃ in LB media supplied with 100 μg/mL ampicillin. When the OD600 was up to 0.6, 0.1 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression at 20 ℃ on a shaker (170 rpm) overnight (~12 h). Cells were collected in the centrifuge Avanti J20-XP (Beckman Coulter) at 6000 rpm, 4 ℃ for 20 min, and lysed by pressure treatment (CONSTANT SYSTEMS. Ltd) in lysis buffer [50 mM Tris, pH 7.5, 300 mM NaCl, 10% (vol/ vol) glycerol, 2 mM *β*ME, 1 mM PMSF, 0.1% (vol/vol) Triton X-100, and 0.01%-0.1% (vol/vol) Antifoam 204 (Sigma-Aldrich, cat. No.: A6426)]. The cleared lysate was purified by affinity chromatography on a 5 mL HisTrap™ HP column (GE Healthcare) using NGC system (Bio-Rad). The MBP tag was cleaved by 0.1 equivalent of His-MBP-TEV Protease and dialysis against 100 volume of buffer [(50 mM Tris pH 7.5, 300 mM NaCl, 10% (vol/ vol) glycerol)] overnight at rt. Proteins were further purified by affinity chromatography on a 5 mL MBPTrap™ HP column 5 mL (GE Healthcare) in buffer containing 50 mM Tris pH 7.5, 300 mM NaCl, 10% (vol/ vol) glycerol, followed by size-exclusion chromatography on a HiLoad 26/60 Superdex 200 pg column (GE Healthcare) in buffer containing 25 mM Tris pH 7.5, 150 mM NaCl.

6.2. Compounds



2



DBCO-TAMRA

(Jena Bioscience, cat. Nr. CLK-A131)

6.3. Labeling Assay

GRAMD1A_StART (5 μM) was incubated with compound 2 (10 μM) in the assay buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) at 37 °C. Aliquots at different time points were taken and then quenched by 1% SDS. DBCO-TMR (5 eq, Jena Bioscience, cat. Nr. CLK-A131) was added at room temperature for 12 h for copper-free click reaction. Then 5 × SDS-PAGE Sample Buffer was added, and the samples were denatured. The reactions were analyzed by SDS−PAGE and imaged by in-gel fluorescence scanning and subsequent Coomassie blue staining using the ChemDoc™ MP Imaging System (Bio-Rad) according to the manufacturer's instructions. The images were analyzed using Image Lab software (Bio-Rad) and quantified using ImageJ software.
The assay was carried out in three independent biological replicates.
The representative images are shown in Figure 3c.

7. Peptide Mapping of the Labeled GRAMD1A StART Domain by Compound 2

7.1. Preparation of Labeled GRAMD1A_StART Domain Sample

Purified recombinant protein GRAMD1A StART domain (10 μM) was incubated with the labeling compound **2** (20 μM) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) at 37 °C for 15 min or with just buffer for negative controls Then the reaction mixture was purified using PD Spintrap™ G-25 column (GE, cat. No.: 28-9180-04) according to the manufactory's instructions. Each sample was repeated for a total of triplicates for compound-2 labelled and controls.

7.2. Applied Sample Processing and Methods

10 μg of protein per sample were digested without reduction/alkylation with sequencing grad trypsin enzyme (Promega) at 1:20 substrate:enzyme ratio and 37°C, overnight. The digestion reaction was stopped by sample acidification down to pH <3. The digested peptides were thereafter desalted using a sep-Pack C-18 desalting columns or filters (Waters). Eluted peptides were dried by sped-vacuum concentration and dissolved in 20 μL of 0.1% formic acid 2% acetonitrile for subsequent LC-MS/MS analysis

7.3. LC-MS/MS

NanoLC-MS/MS of labelled and unlabelled control protein digests were performed using a system made by a nano-Ultimate 3000 LC coupled to an EASY Spray source as ElectroSpray Ionization (ESI) source and a Q Exactive HF mass spectrometer (Thermo Scientific). Each sample replicate was

injected twice, first with the equivalent of 0.5 µg and then with 3 µg according to estimated protein amount pre-digestion. After being injected, samples were preconcentrated and further desalted in-line using a PepMap C18 nano trap column. Peptide separation was performed using an EASY-Spray C18 reversed-phase nano LC column (Acclaim PepMap RSLC; length, 15 cm; inner diameter, 2 µm; particle size, 2 µm; pore size, 100 Å; Thermo Scientific) at 55 °C and a flow rate of 300 nL/min. Peptides were separated using a binary solvent system consisting of 0.1% (v/v) formic acid (FA), 2% (v/v) acetonitrile (ACN) (solvent A) and 98% ACN (v/v), 0.1% (v/v) FA (solvent B) and eluted with a gradient of 3–26% B in 40 min, 26–95% B in 10 min. Subsequently, the analytical column was washed with 95% B for 5 min before re-equilibration with 3% B for the last 5 minutes. Mass spectra were acquired in a mass-to-charge (m/z) range of 375–1500 with a resolution of 60,000 at m/z 200. Automatic gain control target was set to 1×10^6 with a maximum injection time of 100 ms. The 17 most abundant peptide ions were selected for higher-energy collision dissociation (HCD) with normalized collision energy value set at 28%. The ion abundance threshold was set at 0.1% with charge exclusion of z = 1,>6 ions. The MS/MS spectra were acquired at a resolution of 17,500, with a target value of 4×10^5 ions and a maximum injection time of 120 ms. The fixed first m/z was set to 100, and the isolation window to 2 m/z. The instrument was operated in the positive ion mode for data-dependent acquisition of MS/MS spectra with a dynamic exclusion time of previously selected precursor ions of 45 s. To avoid carry over blanks were run in between each sample.

7.4. Data Analysis

The acquired data was processed by PEAKS Software (Bioinformatics Solutions Inc.) using a reduced human proteome database including the protein sequence of the GRAMD1A StART domain. The search parameters, for both labeled and unlabeled sample included: 10 ppm of mass error tolerance for parent ions; 0.05 Da of fragment mass error tolerance; trypsin and partial trypsin enzyme specificity with a maximum of 3 missed cleavages; N and Q deamidation, M oxidation and the labelling modification of 201.07 Da with no specified amino acid specificity as variable modifications. An FDR of maximum 1% for peptide identification was accepted.

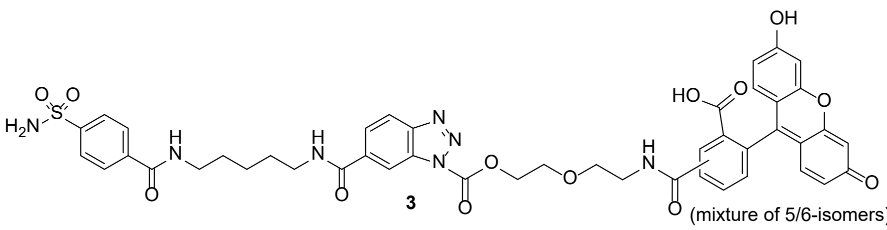
The results are shown in Figure S6.

8. CA12 Labeling in Cells using Compound 3

8.1. Cell Culture

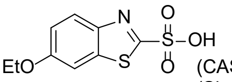
MCF7 (ATCC® HTB-22™) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and non-essential amino acids at 37°C with 5% CO_2. Cells were routinely tested for mycoplasma contamination using the LookOut mycoplasma PCR detection kit (Sigma-Aldrich). Cell lines were authenticated by ATCC with no further authentication carried out after purchase.

8.2. Compounds



3

(mixture of 5/6-isomers)



EZA

(CAS No.: 452-35-7)
(Sigma-Aldrich, cat. No.: 333328-1G)

8.3. Cell Labeling

MCF7 cells were washed 3 times with PBS and incubated with 1 μM **3** (or 1 μM **3** + 100 μM EZA) in serum free DMEM for the indicated length of time. Cells were then washed 3 times with PBS prior to fluorescent microscopy or western blot analysis.

8.4. Fluorescent Microscopy

For live-cell imaging, MCF7 cells were seeded on μ-Slide 8-well glass-bottom chamber slides (Ibidi) and labeled as described above for 30 min. Imaging was performed on a Leica SP8 FALCON inverted confocal system (Leica Microsystems) equipped with a HC PL APO 63x/1.40 oil immersion lens and a temperature-controlled hood maintained at 37°C and 5% CO2. Hoechst 33342 and fluorescein were excited using a 405 nm Diode laser and a tuned white light laser, respectively. Scanning was performed in line-by-line sequential mode. The representative images are shown in Figure 4a.

8.5. Immunoblotting

Cells were washed 3 times with PBS, lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 8, 300 mM KCl, 10% Glycerol, 0.25% Nonidet P-40, 0.5 mM EGTA, 1 mM PMSF, 1× complete protease inhibitor (Roche)), passed 6× through a 21G needle, and cleared by centrifugation (20 min/12,700 rpm/4°C, Eppendorf centrifuge 5427R). Protein concentrations were determined via Bradford assay (Bio-Rad Protein Reagent) and lysates normalized. Lysates were then mixed with 4× sample buffer and boiled for 10 min prior to separation by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) using a Trans-Blot Turbo transfer system (Bio-Rad). After a 1 hour block in TBST with 5% skim milk (at room temperature), membranes were incubated with primary antibody overnight at 4°C [antibodies: anti-CA12 ((Cell Signaling Technology, cat. No.: 5864, 1:1000), anti-fluorescein (Sigma-Aldrich, cat. No.: SAB4600050, 1:1000) and anti beta-actin (Sigma-Aldrich, cat. No.: A2228, 1:10,000)]. Membranes were then washed with TBST and incubated for 1 hour at room temperature with the appropriate HRP-conjugated secondary antibody in blocking buffer. Protein detection was carried out using chemiluminescence (Bio-Rad) and imaged using a ChemiDoc imaging system (Bio-Rad).
The representative blots are shown in Figure 4c.

8.6. Immunoprecipitation

MCF7 cells were labeled with **3** for 30 minutes and lysates harvested, as described above. FITC antibody (Thermo Fisher; 71-1900) was pre-bound to protein A Dynabeads (Thermo Fisher; 10001D) and incubated with lysates for 30 minutes at room temperature. Beads were washed 3 times with PBS-T and the target antigen eluted by re-suspending in Laemmli sample buffer and heating at 70°C for 10 minutes. The representative blots are shown in Figure 4d.

9. Supplementary Figures

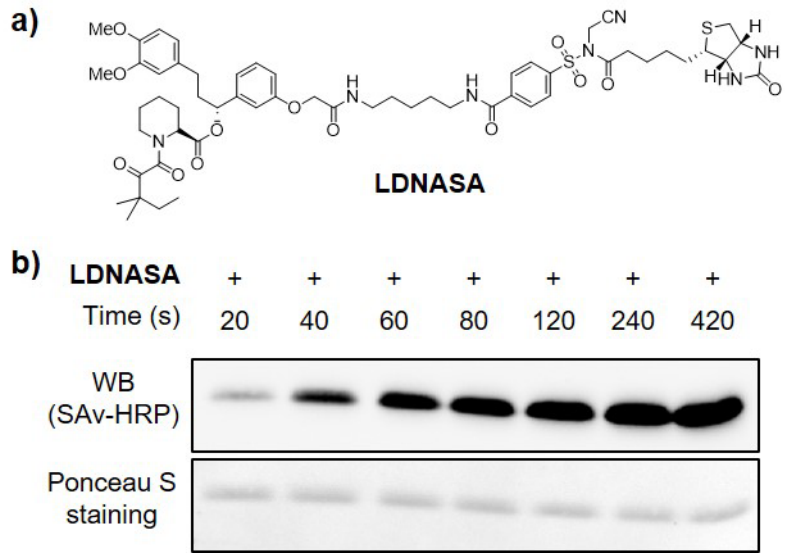


Figure S1. *In vitro* labeling of FKBP12 protein by LDNASA. a) Structure of LDNASA. b) SDS-PAGE and western blotting analysis of labeling reaction of 5 μM FKBP12 with 10 μM LDNASA.

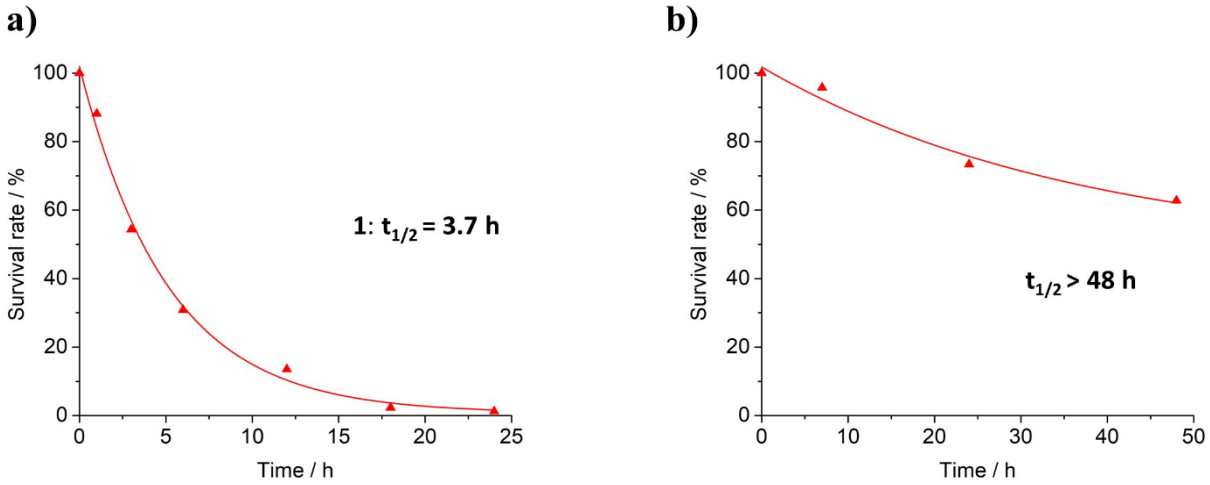


Figure S2. Hydrolysis kinetics of compound **1**. a) Compound **1** (20 μM) was incubated in labeling buffer (50 mM HEPES, 50 mM NaCl, pH 7.3) at 37 °C. b) Compound **1** (200 μM) was incubated in DMSO at 37 °C. Unhydrolyzed reagents at different time points were quantified by RP-HPLC and fitted by a single exponential function to obtain half-life.

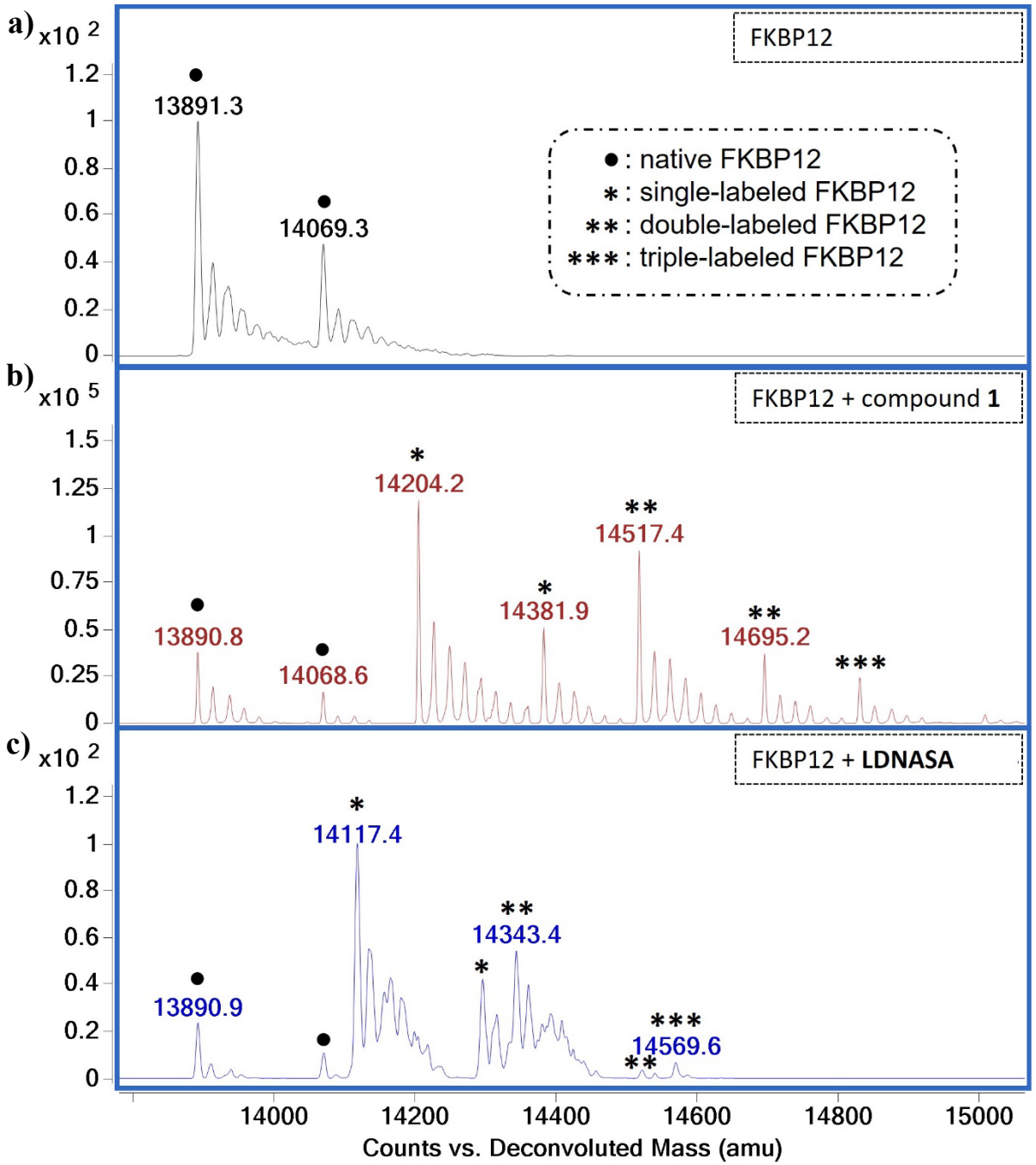
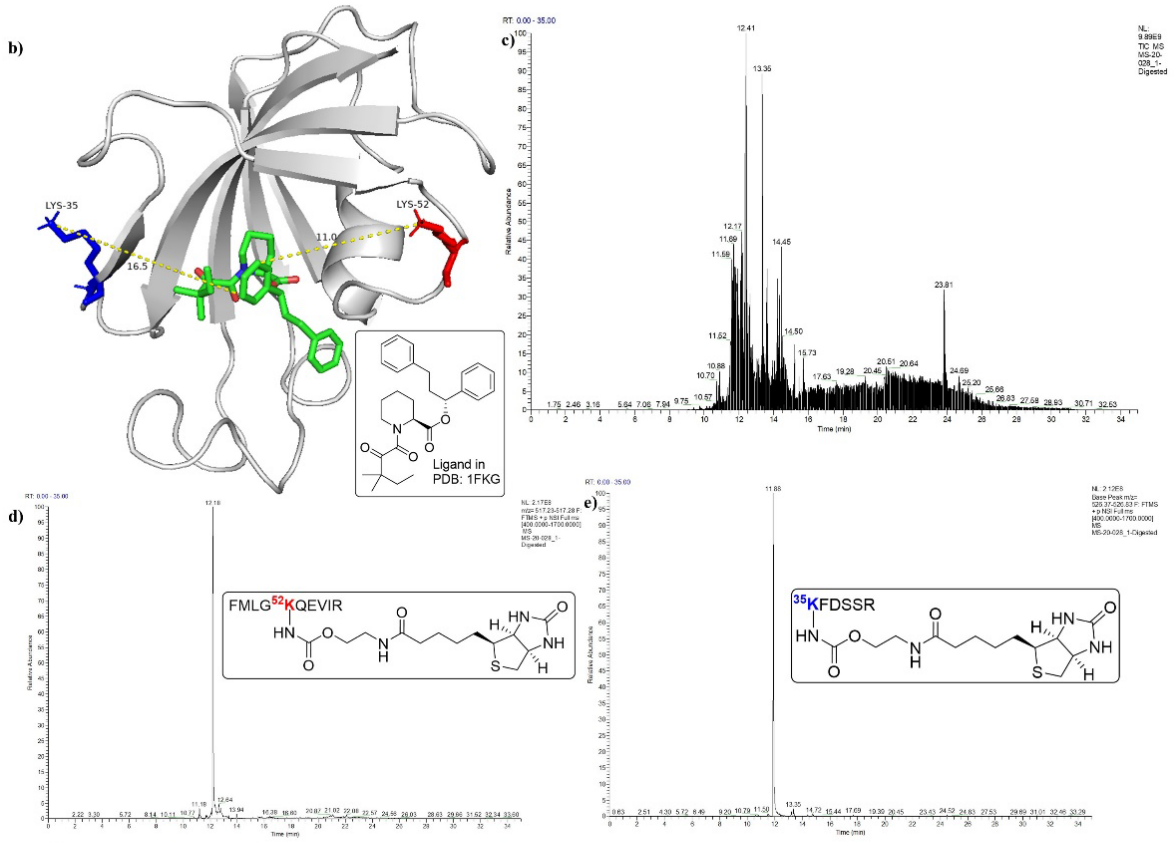
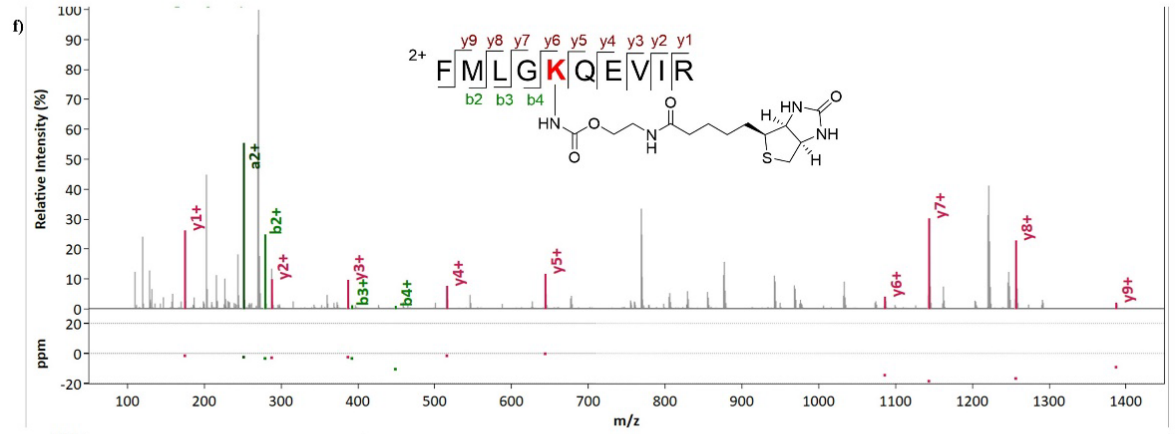


Figure S3. Mass spectra of FKBP12 before and after labeling with **1**. a) ESI-MS of FKBP12. b,c) ESI-MS analysis of the labeling of 5 µM FKBP12 with 10 µM **1** (b) or LDNASA (c) at 37 °C for 15 min.

a)

```
                                  1               11              21
GHHHHHHAENLYFQGHM GVQVETISPG DGRTFPKRGQ TCVVHYTGML
31                41              51              61              71
EDGKKFDSSR DRNKPFKFML GKQEVIRGWE EGVAQMSVGQ RAKLTISPDY
          Peptide 2                Peptide 1
81                91              101
AYGATGHPGI IPPHATLVFD VELLKLE
```





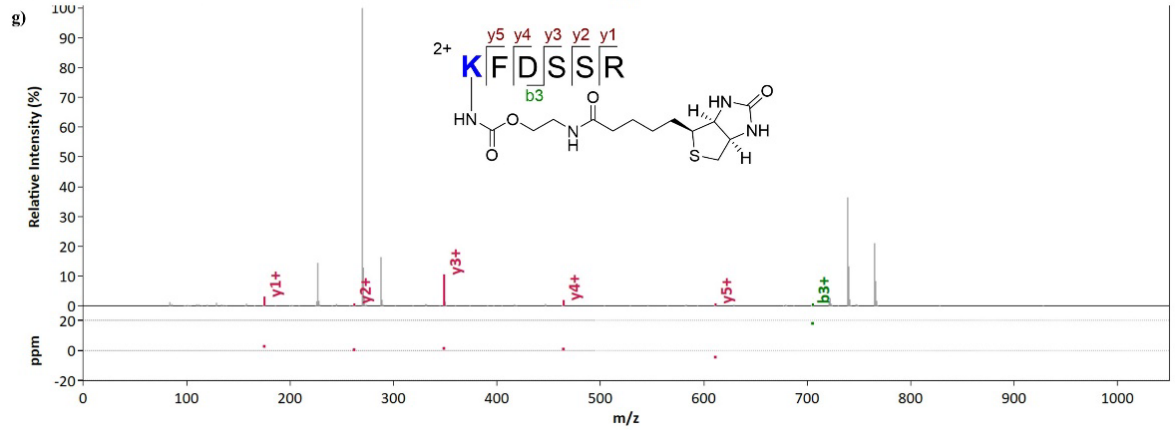


Figure S4. LC-MS/MS analysis of the labeling site of FKBP12 after reaction with **1**. a) The primary sequence of the FKBP12 protein used in this study. Peptide 1 contains the major labeled Lys52 (colored in red) and Peptide 2 contains another (minor) labeled Lys35 (colored in blue) after tryptic digestion. b) The crystal structure of FKBP12-SLF complex (PDB ID: 1FKG). c) Ion chromatogram of the total tryptic digestion products. d,e) Mass spectrum of Peptide 1 (d) and Peptide 2 (e). f,g) MS/MS spectra of the labeled Peptide 1 (f) and Peptide 2 (g). The labeled K52 was colored in red and K35 in blue.

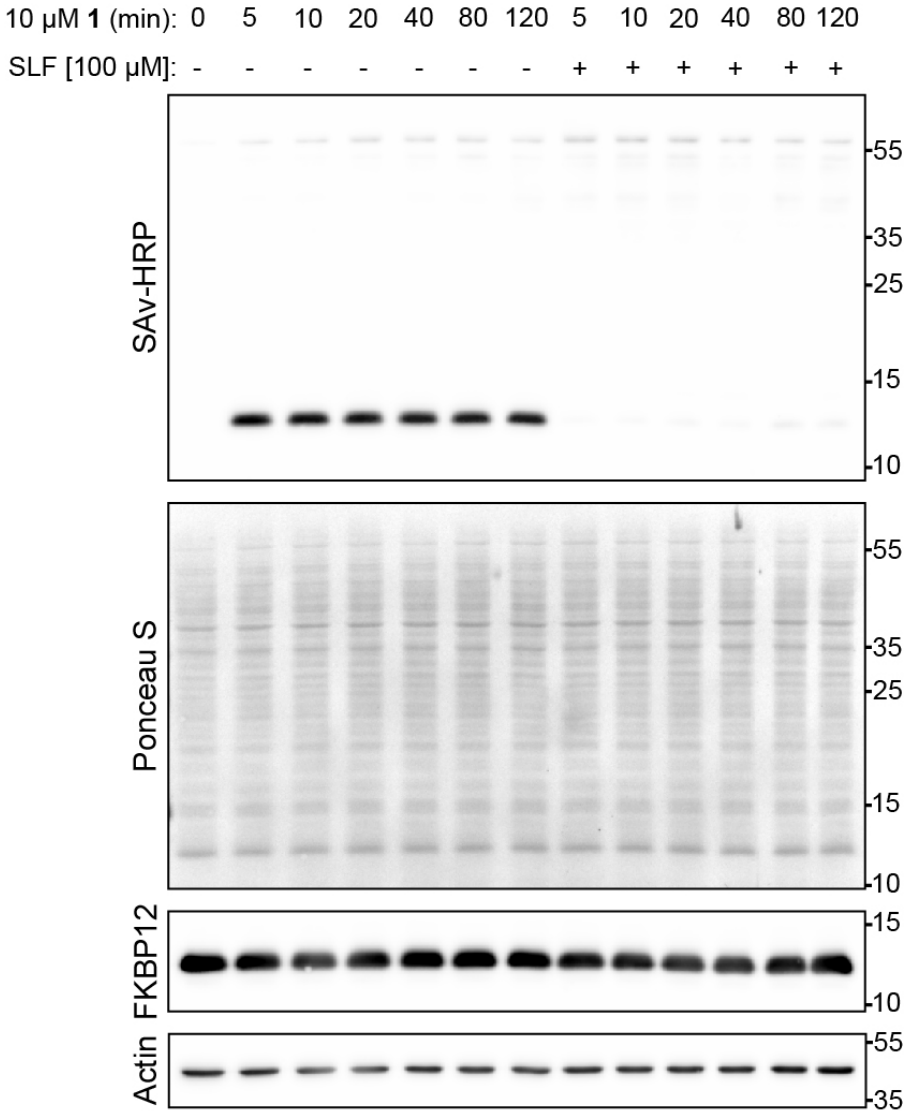


Figure S5. SDS-PAGE and western blotting analysis of FKBP12 labeling with 10 μM **1** for up to 2 h in HeLa cell lysate.

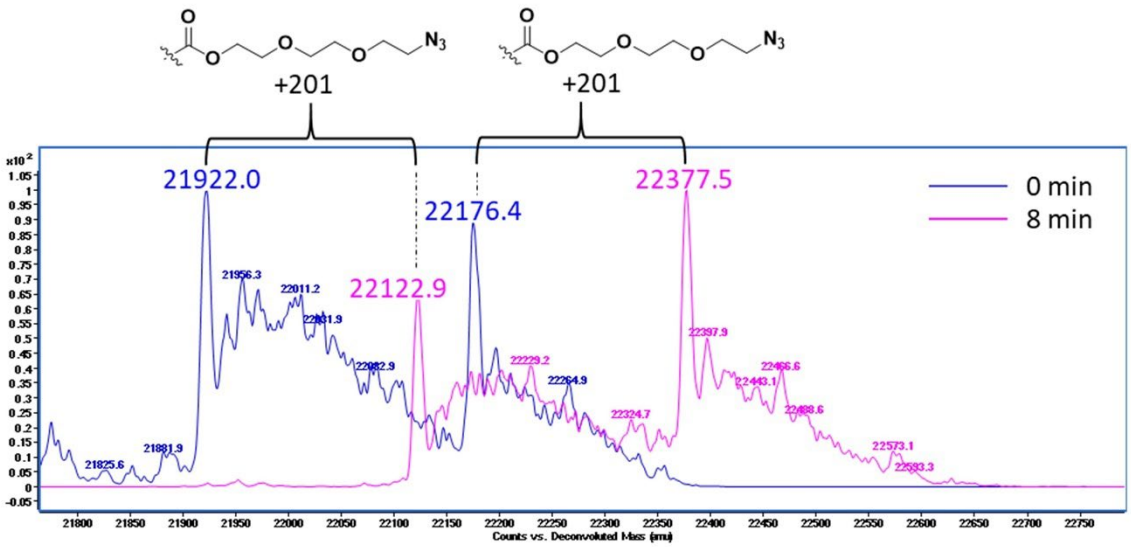


Figure S6. ESI-MS analysis of the labeling of 5 μM GRAMD1A (StART domain) with 10 μM **2**. The deconvoluted mass signals of GRAMD1A (StART domain) are 21922.0 and 22176.4. After labeling by **2** at 37 ℃ for 8 min, the observed mass are 22122.9 and 22377.5.

a)

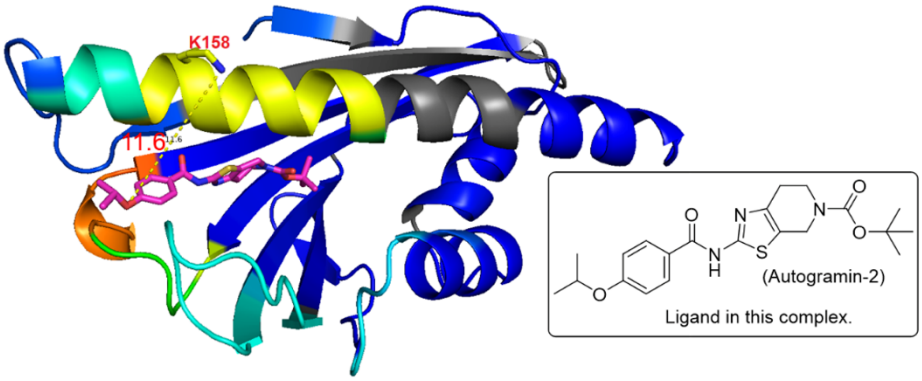
1 GHMLAALLPD LSGRLLINSV FHVGAERLQQ MLFSDSPFLQ GFLQQCKFTD

51 VTLSPWSGDS KCHQRRVLTY TIPISNPLGP KSASVVETQT LFRRGPQAGG

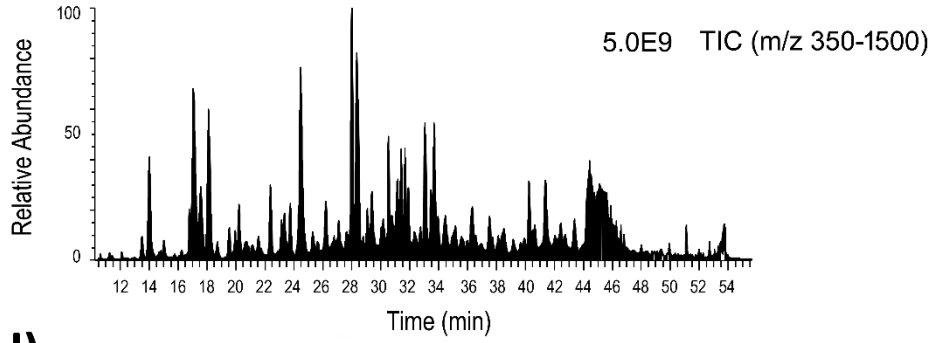
101 CVVDSEVLTQ GIPYQDYFYT AHRYCILGLA RNKARLRVSS EIRYRKQPWS

151 LVKSLIE[158]KNS WSGIEDYFHH LERELAKAEK LSLEEGGKDA RG

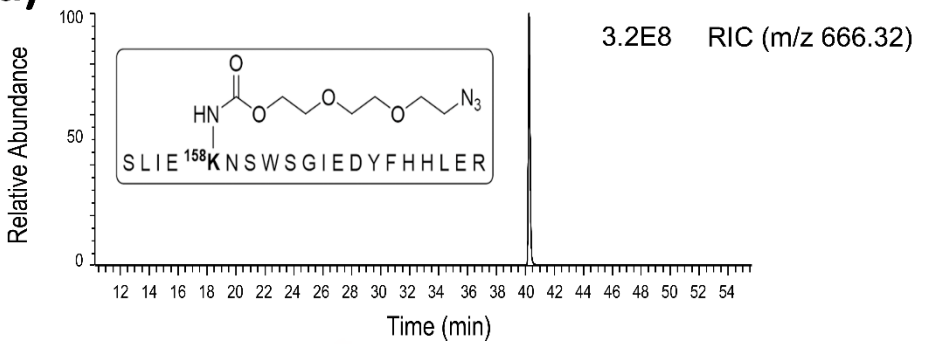
b)



c)



d)



e)

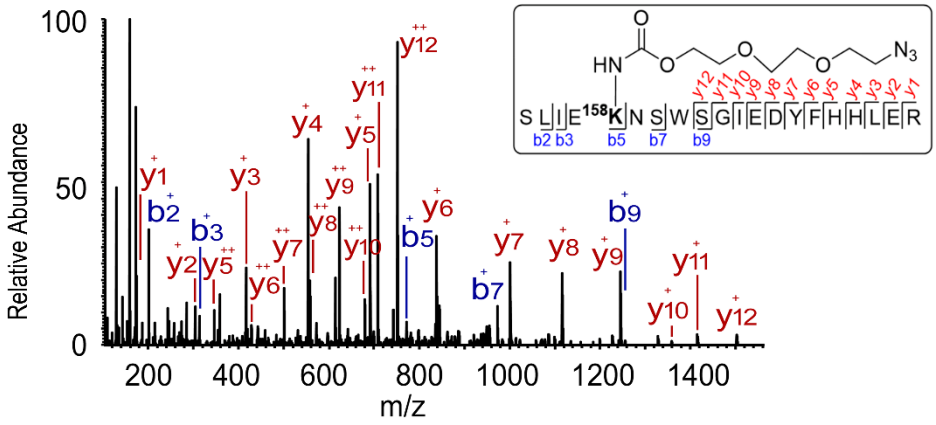


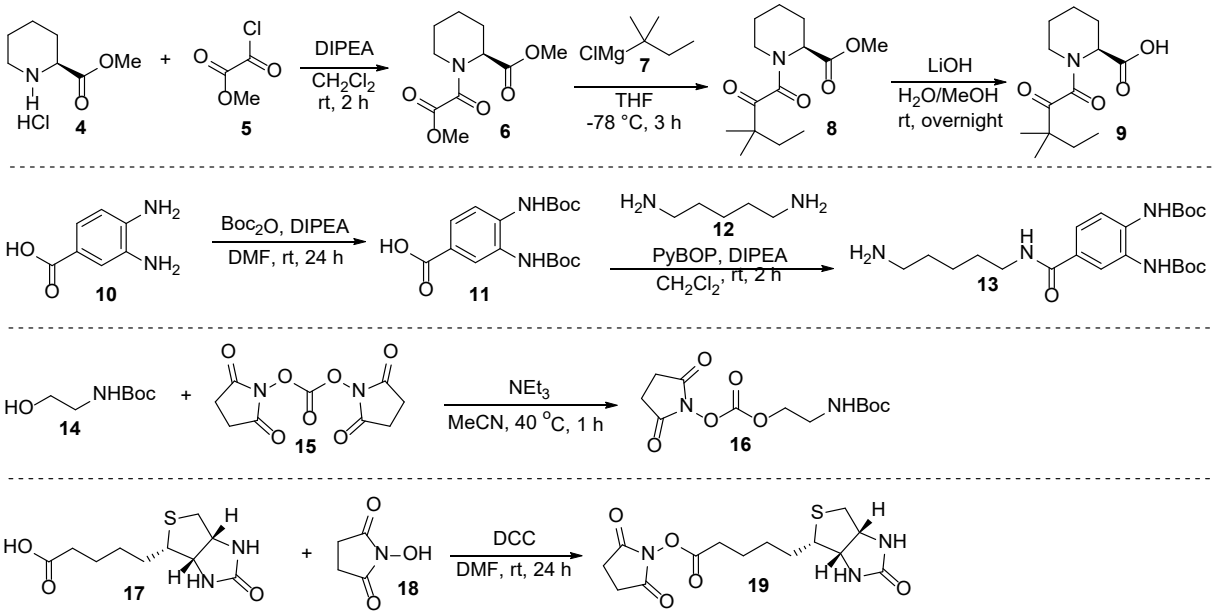
Figure S7. LC-MS/MS analysis of the labeling site of GRAMD1A StART domain after reaction with compound **2**. a) The primary sequence of the recombinant GRAMD1A_StART domain protein used in this study. The underling arrow shows the trypsin-digested peptide fragment containing modified Lys158. b) The homology model of the GRAMD1A StART domain with docked autogramin-2 based on the Hydrogen–Deuterium Exchange Mass Spectrometry (HDX-MS) results (ref. 20). c) LC-MS chromatogram of all peptides contained in the recombinant GRAMD1A_StART domain protein digest, showing the total ion current (TIC) and total ion intensity . d,e) Reconstructed ion chromatogram (RIC) with relative intensity (d) and MS/MS spectrum with fragmentation pattern (e) of the identified peptide fragment labeled on Lys158.

Part II: Synthesis of Compounds

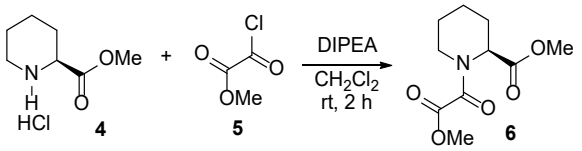
10. General Information

All commercially available compounds were used as provided without further purifications unless otherwise noted. The solvents were treated to anhydrous prior to use according to the standard methods or purchased from commercial sources in anhydrous grade. Flash column chromatography was performed using silica gel (VWR, particle size 40 - 63 μm).
^{1}H, ^{13}C were recorded on a Bruker 400 MHz Avance III, Bruker 500 MHz Avance III, or Bruker 600 MHz Avance III HD using $CDCl_3$, CD_3OD, CD_2Cl_2 or $(CD_3)_2SO$ as solvent at room temperature. ^{1}H and ^{13}C-NMR spectra were calibrated to the solvent signals of $CDCl_3$ (7.26 and 77.16 ppm), CD_3OD (3.31 and 49.00 ppm), CD_2Cl_2 (5.32 and 53.84 ppm) or $(CD_3)_2SO$ (2.50 and 39.52 ppm).[2] The abbreviations *s*, *d*, *t*, *q* and *m* stand for singlet, doublet, triplet, quartet and multiplet in that order.

9. Compound Synthesis and Characterization

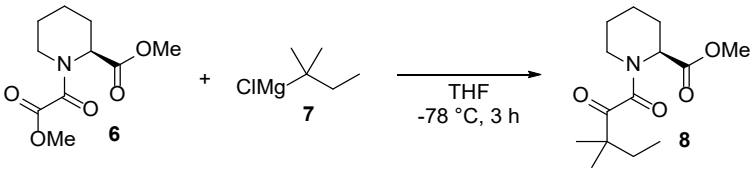


Scheme S1. Synthetic scheme of building blocks **9**, **13**, **16**, and **19**

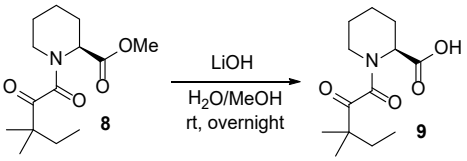


Synthesis of compound **6** following published procedures[3,4]:
N,*N*-Diisopropylethylamine (DIPEA) (12.03 mL, 69.5 mmol, 2.5 equiv.) was added dropwise to a solution of methyl (*2S*)-piperidinecarboxylate hydrochloride **4** (CAS No.: 16850-39-0) (4.992 g, 27.8 mmol, 1 equiv.) in dry CH_2Cl_2 (10 mL) at 0 °C (ice-water bath). Then methyl chlorooxoacetate **5** (CAS No.: 5781-53-3) (3.20 mL, 34.8 mmol, 1.25 equiv) was added dropwise using a syringe. After the addition, the resulting mixture was stirred at room temperature for 2 hours. The mixture was diluted with CH_2Cl_2 (70 mL), washed with water (80 mL × 2), and followed by brine (80 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (eluted with petroleum ether : EtOAc = 2:1) to afford compound **6** (6.033 g, 26.3 mmol, 95% yield) as a colorless oil. This product exists as an approximate 2.5:1 mixture of the *trans* and *cis* conformations. The analytical data matched literature reports.[3,4]
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.22 (d, J = 5.7 Hz, 0.75H), 4.52 (d, J = 5.2 Hz, 0.25H), 4.47 – 4.40 (m, 0.26H), 3.87 (s, 2.21H), 3.83 (s, 0.80H), 3.76 (s, 0.85H), 3.75 (s, 2.15H), 3.62 – 3.50 (m, 0.83H), 3.32 (td, J = 13.1, 3.2 Hz, 0.82H), 2.89 (td, J = 13.3, 3.4 Hz, 0.28H), 2.30 – 2.24 (m, 1.14H), 1.78 – 1.66 (m,

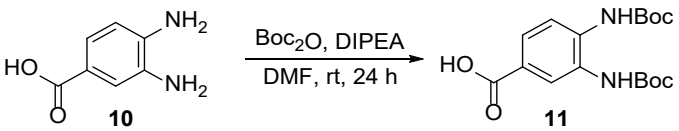
3.43H), 1.57 – 1.46 (m, 1.01H), 1.43 – 1.32 (m, 1.22H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 163.1, 162.9, 161.5, 161.0, 56.8, 52.9, 52.8, 52.7, 52.6, 51.7, 44.4, 39.7, 27.4, 26.5, 25.1, 24.2, 21.1, 20.9.



Synthesis of compound **8** following published procedures[3,4]:
To a solution of **6** (6.028 g, 26.3 mmol, 1 equiv.) in THF (26.3 mL) at -78 ℃ was added dropwise 1,1-dimethylpropylmagnesium chloride **7** (1.0 M in diethyl ether, 34.2 mL, 34.2 mmol, 1.3 equiv.) under argon atmosphere. The resulting mixture was stirred at -78 ℃ for 3 hours, and then was poured into saturated aqueous NH_4Cl (50 mL). The aqueous layer was extracted with EtOAc (50 mL × 3). The combined organic layers were washed with water (50 mL × 2), and followed by brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (eluted with petroleum ether : EtOAc = 10:1) to afford compound **8** (6.326 g, 23.5 mmol, 89% yield) as a colorless oil. This product exists as an approximate 5:1 mixture of the *trans* and *cis* conformations. The analytical data matched literature reports.[3,4]
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 (d, J = 5.7 Hz, 1.00H), 4.48 – 4.43 (m, 0.20H), 4.23 (d, J = 5.2 Hz, 0.21H), 3.76 (s, 0.61H), 3.75 (s, 3.00H), 3.41 – 3.37 (m, 1.03H), 3.21 (td, J = 12.9, 3.2 Hz, 1.02H), 2.91 (td, J = 13.1, 3.5 Hz, 0.21H), 2.33 – 2.27 (m, 1.03H), 2.24 – 2.19 (m, 0.21H), 1.79 – 1.62 (m, 6.38H), 1.56 – 1.34 (m, 2.79H), 1.23 (s, 3.08H), 1.19 (s, 3.08H), 1.18 (s, 0.63H), 1.16 (s, 0.60H), 0.88 (t, J = 7.5 Hz, 3.12H), 0.83 (d, J = 7.5 Hz, 0.63H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.0, 207.8, 171.0, 170.9, 167.7, 166.6, 56.6, 52.7, 52.5, 51.3, 46.9, 44.1, 39.0, 32.8, 32.6, 27.7, 26.5, 25.0, 24.6, 23.8, 23.6, 23.2, 23.1, 21.3, 9.0, 8.9.

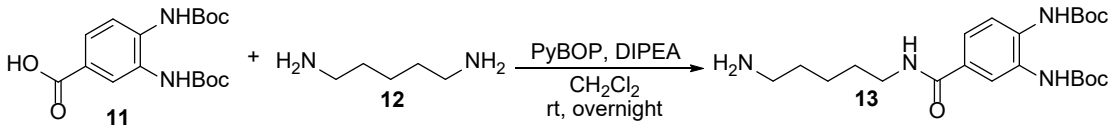


Synthesis of compound **9** following published procedures[3,4]:
To a solution of compound **8** (5.925 g, 22.0 mmol, 1 equiv.) in MeOH (88 mL) at 0 ℃ was added 33 mL of 1 N LiOH aqueous dropwise over a period of 15 min. The resulting mixture was stirred at 0 ℃ for 1 hour, and then warmed to room temperature and stirred at room temperature overnight. The mixture was acidified with 1 N HCl and extracted with CH_2Cl_2 (100 mL × 3). The combined organic layers were washed with water (50 mL), and followed by brine (50 mL), dried over anhydrous Na_2SO_4, filtered. The solvent was removed under reduced pressure to afford compound **9** (4.961 g, 19.4 mmol, 88% yield) as a white solid. This product exists as an approximate 5:1 mixture of the *trans* and *cis* conformations. The analytical data matched literature reports.[3,4]
^{1}H NMR (400 MHz, $CDCl_3$) δ 11.00 (broad, s, 1.20H), 5.32 (d, J = 5.5 Hz, 1.00H), 4.48 (d, J = 13.6 Hz, 0.19H), 4.27 (d, J = 5.5 Hz, 0.19H), 3.43 – 3.38 (m, 1.14H), 3.24 (td, J = 12.8, 3.1 Hz, 1.01H), 2.93 (td, J = 13.2, 3.3 Hz, 0.19H), 2.34 (d, J = 13.8 Hz, 1.02H), 2.26 (d, J = 14.1 Hz, 0.19H), 1.83 – 1.61 (m, 6.35H), 1.58 – 1.38 (m, 2.62H), 1.23 (s, 3.21H), 1.19 (s, 4.07H), 0.88 (t, J = 7.5 Hz, 3.58H).

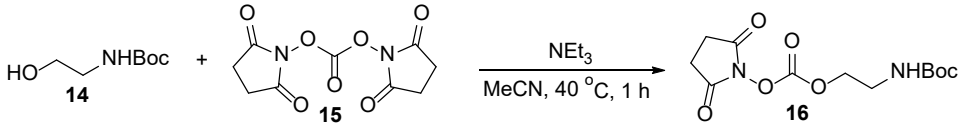


Synthesis of compound **11** following published procedures[5]:
To a solution of di-*tert*-butyl dicarbonate (CAS No.: 24424-99-5) (3.275 g, 15.0 mmol, 3.0 equiv.) in DMF (5 mL) was added 3,4-diaminobenzoic acid **10** (CAS No.: 619-05-6) (761.0 mg, 5.0 mmol, 1.0 equiv.) followed by diisopropylethylamine (DIPEA) (1.039 mL, 6.0 mmol, 1.2 equiv.). The reaction mixture was stirred at room temperature under nitrogen for 24 hours and then poured into 50 mL of

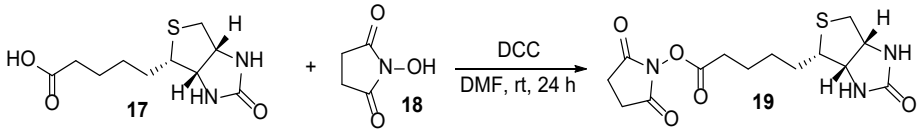
water. The solution pH was adjusted to 6 using 1 M HCl and extracted with EtOAc (20 mL × 4). The combined organic layers were washed with water (20 mL × 4) and brine (20 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was dried under vacuum to give **11** (1.390 g, 3.94 mmol, 79% yield) as a brown solid. The analytical data matched literature reports.[5]
^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 12.78 (br s, 1H), 8.75 (s, 1H), 8.70 (s, 1H), 8.09 (s, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.64 (d, J = 8.3 Hz, 1H), 1.48 (s, 18H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 166.8, 153.2, 152.7, 134.4, 128.9, 125.6, 125.3, 125.1, 122.1, 80.0, 79.7, 28.1, 28.0.



Synthesis of compound **13** following published procedures[6]:
To a solution of 1,4-diaminopentane **12** (CAS No.: 462-94-2) (511.0 mg, 5.0 mmol, 5.0 equiv) in CH_2Cl_2 (15 mL), was added dropwise a solution of compound **11** (352.4 mg, 1.0 mmol, 1.0 equiv), PyBOP® (CAS No.: 128625-52-5) (572.4 mg, 1.1 mmol, 1.1 equiv), and DIPEA (225 μL, 1.3 mmol, 1.3 equiv) in CH_2Cl_2 (10 mL) at room temperature. The resulting mixture was stirred at room temperature overnight. After being concentrated, the residue was purified by silica gel column chromatography (eluted with CH_2Cl_2: MeOH = 5:1) to afford compound **13** (371.7 mg, 0.8514 mmol, 85% yield) as a white solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.48 (d, J = 8.3 Hz, 1H), 7.36 (d, J = 8.3 Hz, 1H), 7.01 (s, 1H), 3.33 – 3.28 (m, 3H), 3.19 – 3.15 (m, 1H), 2.69 (t, J = 6.9 Hz, 2H), 1.90 – 1.87 (m, 1H), 1.50 (s, 9H), 1.48 (s, 9H), 1.34 – 1.29 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.2, 154.5, 153.8, 134.2, 130.5, 129.3, 124.3, 123.9, 123.0, 81.3, 81.2, 41.1, 39.9, 31.0, 29.1, 28.39, 28.36, 24.0; HRMS (ESI): calcd for $C_{22}H_{37}N_4O_5^+$ $[M + H]^+$ 437.2758, found: 437.3084.

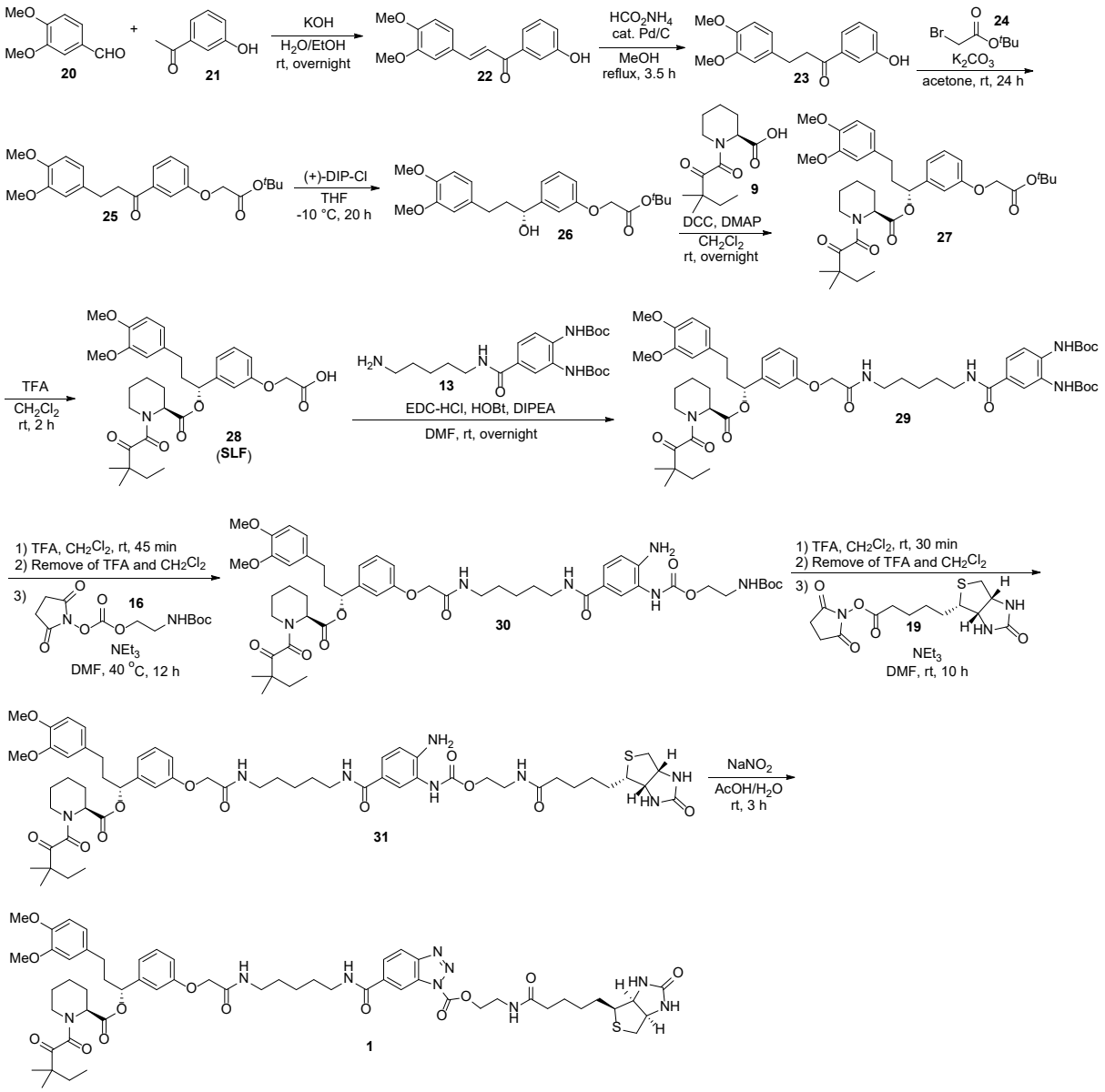


Synthesis of compound **16** following published procedures[7]:
To a solution of 2-(*tert*-butoxycarbonylamino)-1-ethanol **14** (CAS No.: 26690-80-2) (1.612 g, 10 mmol) in dry acetonitrile (50 mL), *N*,*N'*-disuccimidyl carbonate **15** (CAS No.: 74124-79-1) (5.252 g, 20.5 mmol, 2.05 equiv.) and NEt_3 (2.85 mL, 20.5 mmol, 2.05 equiv.) were added and the resulting mixture was stirred at 40 °C for 1 hour. The solvent was removed under reduced pressure, the residue was dissolved in EtOAc (100 mL), washed with saturated aqueous solution of $NaHCO_3$ (50 mL × 2) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (eluted with CH_2Cl_2: MeOH = 30:1) to afford compound **16** (1.786 g, 5.91 mmol, 59% yield) as a colorless oil. The analytical data matched literature reports.[7]
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.98 (br s, 1H), 4.35 (t, J = 5.2 Hz, 2H), 3.46 (q, J = 5.7 Hz, 2H), 2.82 (s, 4H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 155.8, 151.5, 80.0, 70.6, 39.3, 28.4, 25.6.

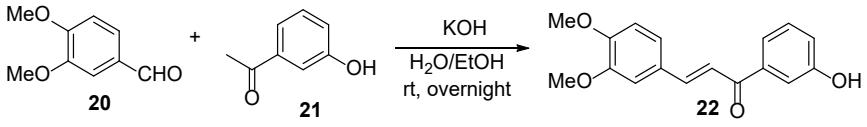


Synthesis of compound **19** following published procedures[8]:
To a solution of biotin **17** (CAS No.: 58-85-5) (488.6 mg, 2.0 mmol) and *N*-hydroxysuccinimide **18** (CAS No.: 6066-82-6) (230.2 mg, 2.0 mmol, 1.0 equiv) in DMF (15 mL), *N*,*N'*-dicyclohexylcarbodiimide (DCC, CAS No.: 538-75-0) (495.1 mg, 2.4 mmol, 1.2 equiv.) was added. The mixture was stirred overnight at room temperature during which time a white precipitate was formed. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue solid was washed with petroleum ether (30 mL × 3) and dried under vacuum to give compound **19** (670.2 mg, 1.963 mmol, 98% yield) as a white solid. The analytical data matched literature reports.[8]
^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 6.42 (s, 1H), 6.36 (s, 1H), 4.32 – 4.29 (m, 1H), 4.15 (t, J = 5.9 Hz, 1H), 3.13 – 3.08 (m, 1H), 2.86 – 2.81 (m, 5H), 2.67 (t, J = 7.3 Hz, 2H), 2.58 (d, J = 12.4 Hz, 1H), 1.73

– 1.60 (m, 4H), 1.55 – 1.44 (m, 2H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 170.3, 168.9, 162.7, 61.0, 59.2, 55.2, 39.8, 30.0, 27.8, 27.6, 25.5, 24.3.



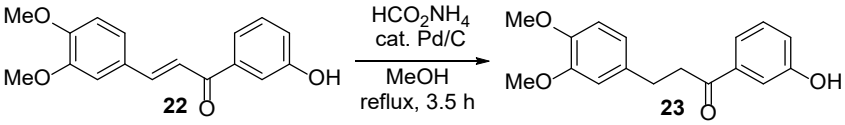
Scheme S2. Synthetic scheme of compound **1**



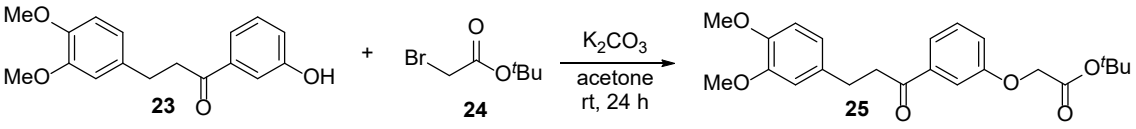
Synthesis of compound **22** following published procedures[3,4]:

To a solution of compounds **20** (16.620 g, 0.1 mol) and **21** (13.620 g, 0.1 mol, equiv.) in EtOH (200 mL) at 0 ℃, was added an ice-water cooled 2 N KOH aqueous (200 mL) dropwise. The mixture was stirred at 0 ℃ for 30 min, and then at room temperature overnight. The mixture was diluted with water (200 mL), and cooled down to 0 ℃, then acidified to pH 6 using concentrated HCl. A yellow solid precipitated. Two workup procedures for aqueous layer and the solid: 1) The aqueous layer was extracted with EtOAc ((150 mL × 3), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2 : MeOH = 10 : 1) to give 4.766 g of **22** as yellow solid. 2) The solid was dissolved in EtOAc (200 mL), washed with water (150 mL × 2), brine (150 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was

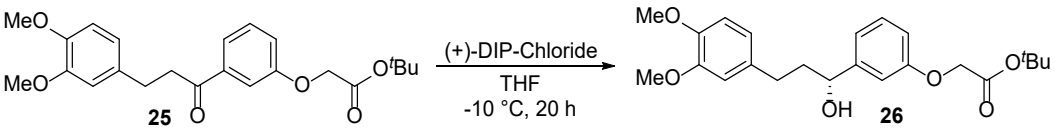
washed with CH_2Cl_2 (50 mL) and petroleum ether (50 mL × 2) to give 17.34 g of **22** as yellow solid. Total **22**: 22.106 g (77.8 mmol, 78% yield). The analytical data matched literature reports.[3,4]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 15.6 Hz, 1H), 7.69 – 7.65 (m, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.40 – 7.33 (m, 2H), 7.21 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.12 (dd, *J* = 7.8, 2.1 Hz, 2H), 6.88 (d, *J* = 8.3 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.1, 156.7, 151.7, 149.3, 145.9, 139.8, 130.0, 127.8, 123.5, 120.9, 120.5, 119.9, 115.4, 111.3, 110.3, 56.14, 56.11.



Synthesis of compound **23** following published procedures[9,10]:
To a suspension of palladium (10 wt%) on carbon (1.706 g) and ammonium formate (5.679 g, 90 mmol, 1.5 equiv.) in MeOH (80 mL) was added compound **22** (17.058 g, 60 mmol). The resulting black suspension was stirred under reflux for 3.5 h. After being cooled to room temperature, the reaction mixture was filtered through a pad of Celite® 545, and the Celite® 545 pad was washed with MeOH (40 mL). The combined filtrate was heated to 50 °C, and water (120 mL) was added. The resulting precipitate suspension was stirred at room temperature overnight. The solid was collected via filtration, and was washed with water (50 mL × 2), and petroleum ether (100 mL × 2), and dried under vacuum to give compound **23** (12.728 g, 44.5 mmol, 74% yield) as a white solid. The analytical data matched literature reports.[9,10]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 – 7.46 (m, 2H), 7.29 (t, *J* = 8.1 Hz, 1H), 7.05 (dd, *J* = 8.0, 1.8 Hz, 1H), 6.78 – 6.75 (m, 3H), 4.74 (broad s, 1H), 3.84 (s, 3H), 3.84 (s, 3H), 3.25 (t, *J* = 7.6 Hz, 2H), 2.99 (t, *J* = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.3, 156.7, 149.0, 147.5, 138.3, 133.9, 130.0, 120.8, 120.5, 120.3, 114.6, 112.0, 111.5, 56.1, 56.0, 40.9, 30.0.



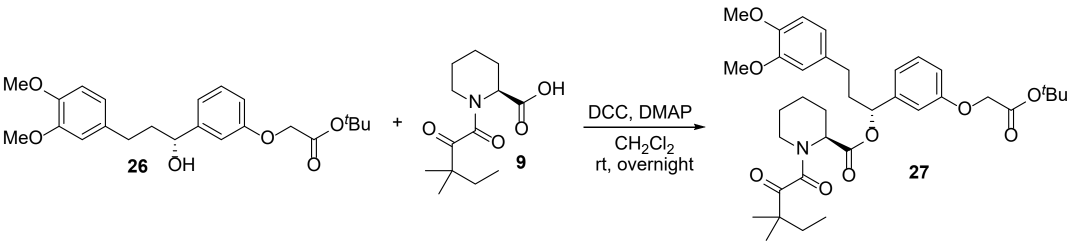
Synthesis of compound **25** following published procedures[9]:
To a suspension of **23** (7.158 g, 25 mmol, 1 equiv.) and K_2CO_3 (6.910 g, 50 mmol, 2 equiv.) in acetone (50 mL), was added *tert*-butyl bromoacetate **24** (7.335 mL, 50 mmol, 2 equiv.) dropwise over a period of 20 min. The resulting mixture was stirred at room temperature for 24 hours. The resulting white solid was filtered off and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (petroleum ether : EtOAc = 5 : 1) to give **25** (7.806 g, 19.5 mmol, 78% yield) as a colorless oil. The analytical data matched literature reports.[9]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, *J* = 7.6 Hz, 1H), 7.46 (t, *J* = 1.9 Hz, 1H), 7.36 (t, *J* = 7.9 Hz, 1H), 7.12 (dd, *J* = 8.3, 2.7 Hz, 1H), 6.81 – 6.76 (m, 3H), 4.55 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.25 (t, *J* = 7.6 Hz, 2H), 3.00 (t, *J* = 7.6 Hz, 2H), 1.48 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.9, 167.7, 158.3, 149.0, 147.5, 138.4, 133.9, 129.8, 121.6, 120.3, 120.2, 113.2, 111.9, 111.5, 82.7, 65.8, 56.1, 56.0, 40.9, 29.9, 28.2.



Synthesis of compound **26** following published procedures[9,11]:
(+)-DIP-Chloride™ (CAS No.: 112246-73-8) (9.624 g, 30 mmol, 2 equiv.) in THF (15 mL) was added dropwise to a solution of **25** (6.008 g, 15 mmol, 1 equiv.) in THF (30 mL) at -20 °C. The solution was stirred at -10 °C for 20 hours. The solvent was removed under reduced pressure. The residue was diluted with diethy ether (50 mL) followed by addition of diethanolamine (CAS No.: 111-42-2) (15.765 g, 150 mmol) and stirred at room temperature overnight. The mixture was diluted with EtOAc (100 mL), filtered through Celite® 545, and the filtrate was concentrated. The residue was purified by flash column

chromatography on silica gel (petroleum ether : EtOAc = 5 : 1) to give **26** (3.028 g, 7.5 mmol, 50% yield) as a colorless oil. The analytical data matched literature reports.[11]

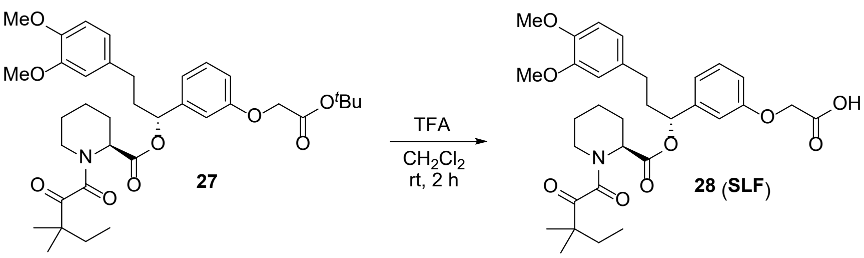
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (t, *J* = 7.9, 1H), 6.96 – 6.91 (m, 2H), 6.80 – 6.77 (m, 2H), 6.73 – 6.70 (m, 2H), 4.65 (dd, *J* = 7.8, 5.2 Hz, 1H), 4.50 (s, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 2.72 – 2.56 (m, 2H), 2.00 – 1.93 (m, 2H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.1, 158.2, 148.9, 147.3, 146.6, 134.4, 129.6, 120.3, 119.2, 113.7, 112.3, 111.8, 111.3, 82.5, 73.8, 65.7, 56.0, 55.9, 40.7, 31.7, 28.1.



Synthesis of compound **27** following published procedures[4]:

To a solution of compound **26** (2.576 g, 6.4 mmol) and compound **9** (1.797 g, 7.04 mmol, 1.1 equiv.) in CH_2Cl_2 (25.6 mL) at 0 °C, was added dicyclohexylcarbodiimide (DCC) (1.452 g, 7.04 mmol, 1.1 equiv.) and 4-dimethylamino)pyridine (DMAP) (86.0 mg, 0.704 mmol, 0.11 equiv.). The mixture was stirred at 0 °C for 2 hours, and then at room temperature overnight. The mixture was diluted with EtOAc (100 mL), filtered through Celite® 545, and the filtrate was concentrated. The residue was purified by flash column chromatography on silica gel (petroleum ether : EtOAc = 4 : 1) to give **27** (3.986 g, 6.23 mmol, 97% yield) as a colorless oil. The analytical data matched literature reports.[4]

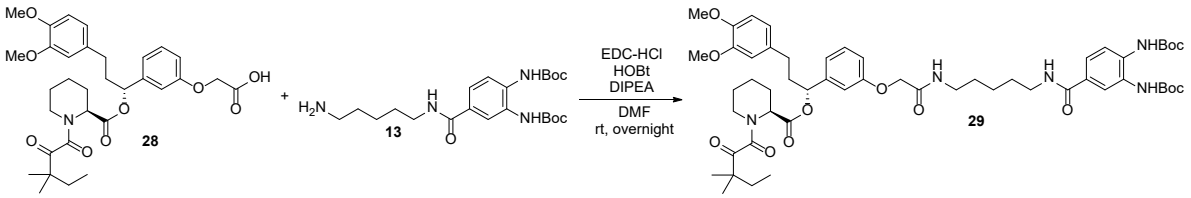
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (t, *J* = 7.9 Hz, 1H), 6.97 – 6.94 (m, 1H), 6.92 – 6.90 (m, 1H), 6.86 – 6.81 (m, 1H), 6.79 – 6.76 (m, 1H), 6.69 – 6.66 (m, 2H), 5.79 – 5.75 (m, 1H), 5.31 (d, *J* = 5.1 Hz, 1H), 4.52 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.35 (d, *J* = 13.3 Hz, 1H), 3.14 (td, *J* = 13.0, 3.0 Hz, 1H), 2.62 – 2.47 (m, 2H), 2.36 (d, *J* = 14.1 Hz, 1H), 2.28 – 2.19 (m, 1H), 2.09 – 2.05 (m, 1H), 1.77– 1.60 (m, 6H), 1.48 (s, 9H), 1.23 (s, 3H), 1.20 (s, 3H), 1.11 (d, *J* = 5.8 Hz, 1H), 0.88 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.9, 169.8, 168.0, 167.4, 158.2, 149.0, 147.4, 141.5, 133.6, 129.8, 120.3, 120.0, 114.4, 113.4, 111.8, 111.4, 82.5, 65.9, 56.0, 56.0, 51.4, 46.8, 44.3, 38.1, 32.6, 31.3, 28.2, 26.6, 25.1, 23.7, 23.2, 21.3, 8.9.



Synthesis of compound **28** (**SLF**) following published procedures[4]:

To a solution of compound **27** (3.986 g, 6.23 mmol) in CH_2Cl_2 (62.3 mL) at 0 °C was added trifluoroacetic acid (TFA) (20 mL). The mixture was stirred at room temperature for 2 hours. The volatile materials was removed under reduced pressure, and the residue was co-evaporated with toluene (5 mL × 2). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2 : MeOH = 30 : 1) to give **28** (3.574 g, 6.12 mmol, 98% yield) as a colorless foam. The analytical data matched literature reports.[4]

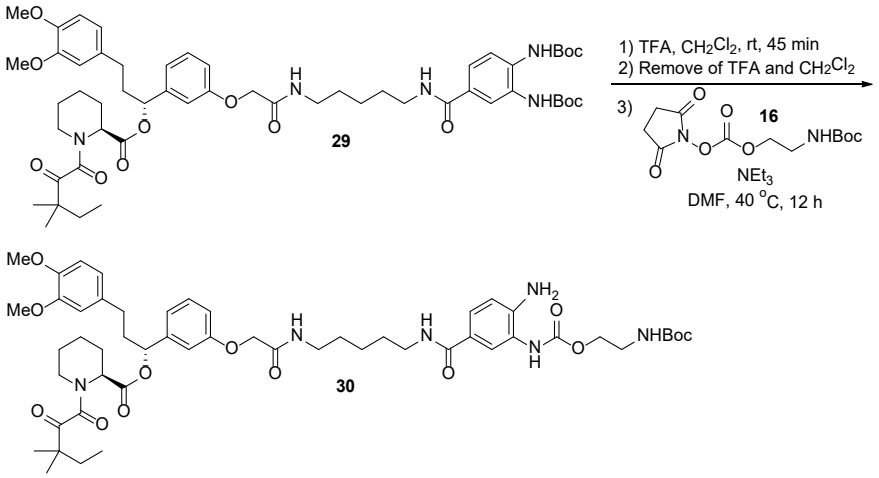
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.92 (broad s, 1H), 7.23 (d, *J* = 8.2 Hz, 1H), 6.91 (d, *J* = 7.7 Hz, 1H), 6.86 – 6.82 (m, 2H), 6.74 (d, *J* = 8.1 Hz, 1H), 6.65 (d, *J* = 2.3 Hz, 1H), 6.63 (s, 1H), 5.76 – 5.70 (m, 1H), 5.28 – 5.25 (m, 1H), 4.66 (d, *J* = 2.7 Hz, 2H), 3.82 (s, 3H), 3.81 (s, 3H), 3.32 (d, *J* = 12.9 Hz, 1H), 3.15 (td, *J* = 13.0, 2.9 Hz, 1H), 2.62 – 2.47 (m, 2H), 2.35 (d, *J* = 13.6 Hz, 1H), 2.24 – 2.15 (m, 1H), 2.07 – 1.98 (m, 1H), 1.77 – 1.58 (m, 5H), 1.48 – 1.43 (m, 1H), 1.37 – 1.30 (m, 1H), 1.17 (s, 3H), 1.15 (s, 3H), 0.83 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.7, 172.7, 169.5, 167.7, 157.8, 149.0, 147.4, 141.8, 133.5, 130.0, 120.4, 120.2, 115.0, 111.9, 111.8, 111.5, 65.1, 56.1, 56.0, 51.8, 46.9, 44.5, 38.2, 32.6, 31.4, 26.6, 25.0, 23.6, 23.1, 21.2, 8.8.



Synthesis of compound **29**:

To a solution of compound **28** (233.5 mg, 0.4 mmol, 1.0 equiv.) in DMF (2 mL) was added compound **13** (192.1 mg, 0.44 mmol, 1.1 equiv.), EDC-HCl (115.0 mg, 0.46 mmol, 1.5 equiv.), HOBt·xH_2O (91.9 mg, 0.6 mmol, 1.5 equiv.), and DIPEA (208 μL, 1.2 mmol, 3.0 equiv.). The mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc (40 mL), washed with water (10 mL) and brine (10 mL × 3), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2 : MeOH = 10 : 1) to give **29** (310.3 mg, 0.31 mmol, 77% yield) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.77 – 7.64 (m, 2H), 7.51 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.31 (t, *J* = 7.9 Hz, 1H), 7.10 – 6.93 (m, 4H), 6.88 – 6.84 (m, 1H), 6.82 – 6.79 (m, 1H), 6.73 – 6.69 (m, 2H), 5.79 (dd, *J* = 8.1, 5.4 Hz, 1H), 5.33 – 5.31 (m, 1H), 4.49 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.40 – 3.32 (m, 5H), 2.63 – 2.55 (m, 2H), 2.39 (d, *J* = 13.4 Hz, 1H), 2.30 – 2.21 (m, 1H), 2.12 – 2.08 (m, 1H), 1.81 – 1.57 (m, 10H), 1.52 (s, 9H), 1.51 (s, 9H), 1.39 – 1.35 (m, 3H), 1.24 (s, 3H), 1.23 (s, 3H), 1.14 (d, *J* = 2.4 Hz, 1H), 0.90 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.7, 170.9, 169.4, 167.9, 167.1, 166.6, 166.3, 157.3, 155.9, 154.0, 153.2, 148.6, 147.1, 141.6, 133.2, 130.3, 129.8, 128.7, 123.9, 120.0, 119.8, 113.7, 113.3, 111.6, 111.2, 80.5, 76.3, 67.1, 60.2, 55.7, 55.6, 51.1, 46.5, 44.0, 39.6, 38.7, 37.9, 32.3, 29.0, 28.8, 28.1, 24.7, 23.9, 23.2, 23.0, 21.0, 20.8, 14.0, 8.6; HRMS (ESI): calcd for $C_{54}H_{76}N_5O_{13}^+$ $[M + H]^+$ 1002.5434, found: 1002.5530.

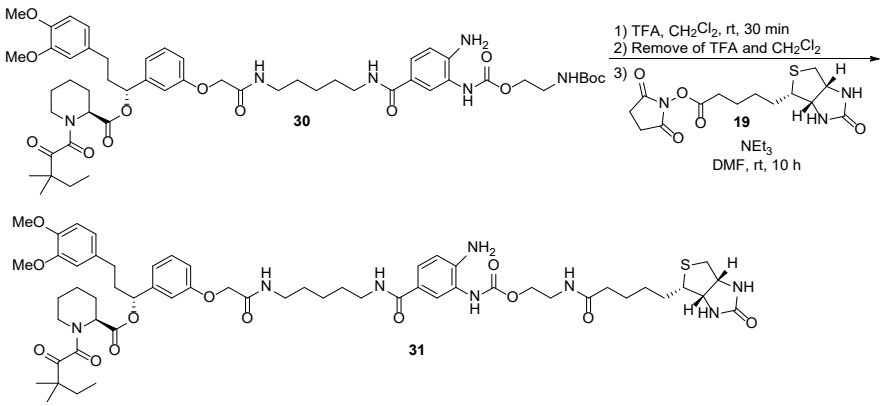


Synthesis of compound **30**:

To a stirred solution of compound **29** (200.4 mg, 0.2 mmol, 1.0 equiv.) in CH_2Cl_2 (4 mL) was added trifluoroacetic acid (TFA, 2 mL) at room temperature. The reaction solution was stirred for 45 min at room temperature. CH_2Cl_2 and TFA was removed under reduced pressure, and further removed by co-evaporation with toluene (1 mL × 2). To a stirred solution of this residue in dry DMF (2 mL) was added compound **16** (66.5 mg, 0.22 mmol, 1.1 equiv.), and NEt_3 (83.4 μL, 0.6 mmol, 3 equiv.). The mixture was stirred at 40 °C for 12 hours. The mixture was diluted with EtOAc (20 mL), washed with water (10 mL) and brine (10 mL × 3), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (eluted using EtOAc with 1% NEt_3) to give compound **30** (135.5 mg, 0.137 mmol, 68% yield) as white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (s, 1H), 7.42 (d, *J* = 9.0 Hz, 1H), 7.20 (d, *J* = 6.9 Hz, 1H), 6.90 (d, *J* = 7.9 Hz, 1H), 6.85 (d, *J* = 2.4 Hz, 1H), 6.78 – 6.68 (m, 3H), 6.60 (dd, *J* = 7.5, 3.9 Hz, 3H), 5.69 (dd, *J* = 8.2, 5.5 Hz, 1H), 5.23 (d, *J* = 5.5 Hz, 1H), 4.38 (s, 2H), 4.09 (t, *J* = 5.4 Hz, 2H), 3.77 (s, 3H), 3.77 (s, 3H), 3.45 – 3.16 (m, 8H), 2.53 – 2.45 (m, 2H), 2.29 (d, *J* = 13.8 Hz, 1H), 2.24 – 2.06 (m, 2H), 1.97 (s, 2H), 1.65 – 1.45 (m, 10H), 1.36 (s, 9H), 1.30 – 1.22 (m, 3H), 1.14 (s, 3H), 1.13 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.0, 169.8, 169.7, 168.3, 168.2, 167.4, 167.2, 166.6, 157.5, 156.2, 155.0, 148.9,

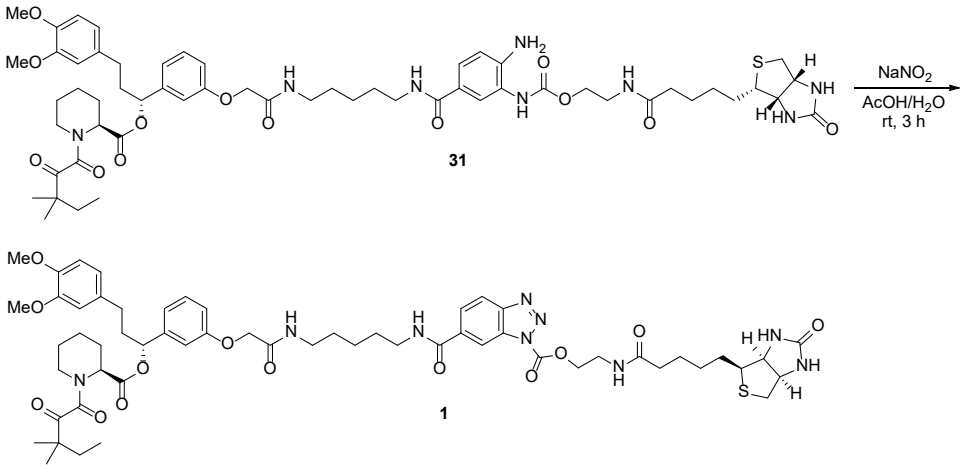
147.4, 144.5, 141.9, 133.4, 130.1, 124.7, 122.5, 120.2, 120.1, 114.0, 113.5, 111.8, 111.4, 79.6, 67.3, 64.8, 56.0, 55.9, 51.4, 46.8, 44.2, 39.7, 38.8, 38.2, 32.5, 31.3, 29.3, 29.1, 28.5, 26.5, 25.0, 24.0, 23.5, 23.3, 21.2, 14.3, 8.8; HRMS (ESI): calcd for $C_{52}H_{73}N_6O_{13}^+$ [M + H]$^+$ 989.5230, found: 989.5406.



Synthesis of compound **31**:

To a stirred solution of compound **30** (69.2 mg, 70 μmol, 1.0 equiv.) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA, 1 mL) at room temperature. The reaction solution was stirred at room temperature for 30 min. CH_2Cl_2 and TFA was removed under reduced pressure, and further removed by co-evaporation with toluene (1 mL × 2). To a stirred solution of this residue in dry DMF (2 mL) was added compound **19** (26.3 mg, 77 μmol, 1.1 equiv.), and NEt_3 (48.7 μL, 350 μmol, 5 equiv.). The mixture was stirred at room temperature for 10 hours. The volatile was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 10 : 1 with 1% NEt_3) to give compound **31** (58.6 mg, 50.5 μmol, 72% yield) as white solid.

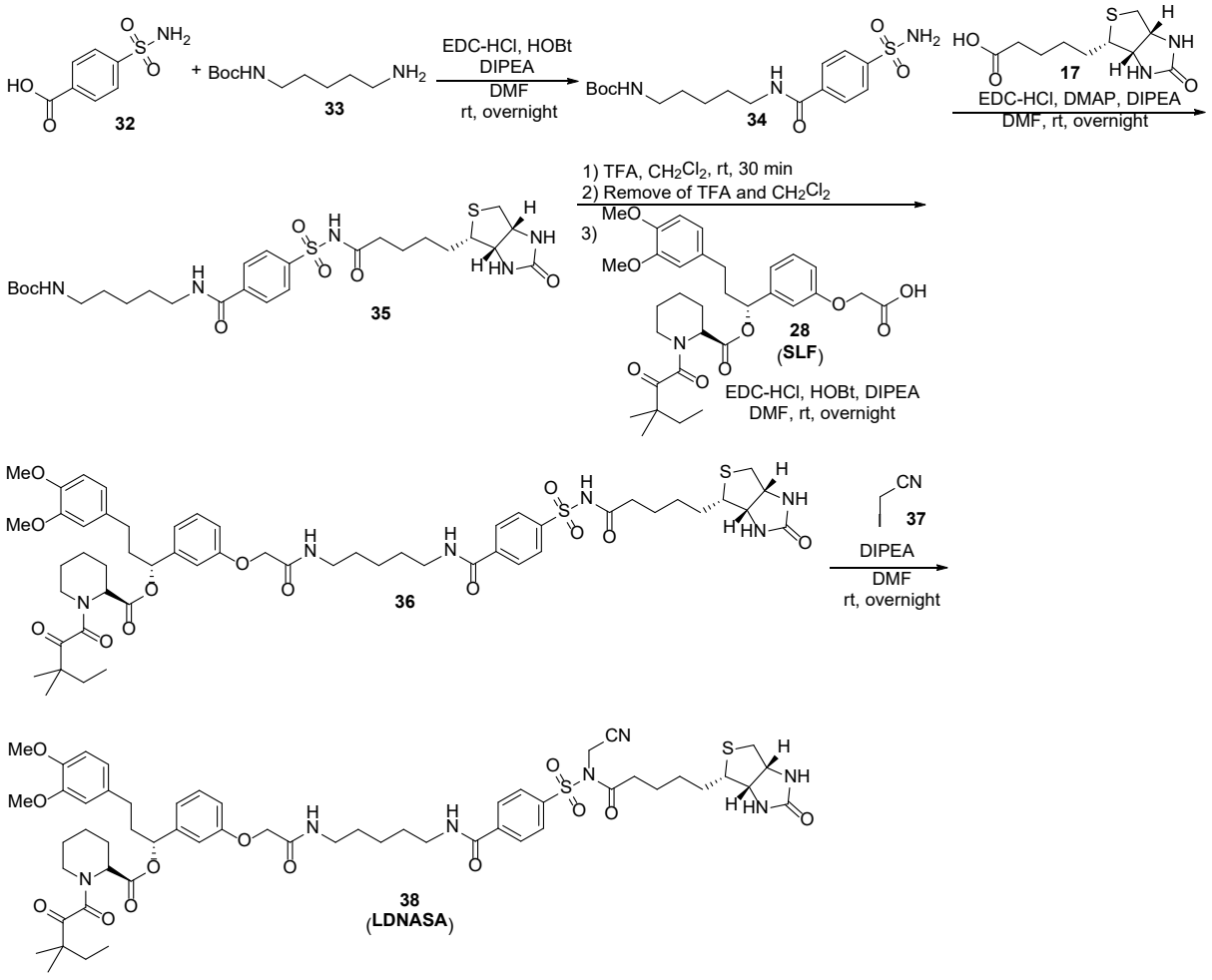
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.22 (br s, 1H), 7.69 (br s, 1H), 7.51 – 7.35 (m, 2H), 7.25 (t, *J* = 7.9 Hz, 1H), 7.03 – 6.88 (m, 3H), 6.80 (d, *J* = 8.4 Hz, 1H), 6.74 (d, *J* = 8.4 Hz, 1H), 6.64 (d, *J* = 5.6 Hz, 3H), 6.46 (br s, 1H), 6.02 (br s, 1H), 5.73 (t, *J* = 7.0 Hz, 1H), 5.26 (d, *J* = 5.1 Hz, 1H), 4.80 (br s, 1H), 4.43 (s, 2H), 4.33 (br s, 1H), 4.13 (s, 2H), 3.81 (s, 3H), 3.80 (s, 3H), 3.47 – 3.19 (m, 11H), 3.14 (t, *J* = 12.9 Hz, 1H), 2.98 (s, 1H), 2.62 – 2.45 (m, 5H), 2.36 – 1.93 (m, 4H), 1.78 – 1.40 (m, 15H), 1.34 – 1.27 (m, 6H), 1.18 (s, 3H), 1.17 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.9, 174.9, 174.2, 169.6, 168.2, 167.5, 167.3, 166.5, 164.3, 157.4, 155.5, 148.8, 147.3, 144.8, 141.8, 133.3, 130.0, 123.6, 122.4, 120.2, 119.9, 115.5, 114.0, 113.4, 111.7, 111.3, 76.5, 67.2, 64.1, 61.7, 60.2, 55.9, 55.8, 51.3, 50.3, 46.7, 44.2, 40.5, 39.8, 38.9, 38.8, 38.1, 35.6, 32.4, 31.2, 29.3, 29.1, 28.0, 26.4, 25.4, 24.9, 24.1, 23.4, 23.2, 21.1, 8.7; HRMS (ESI): calcd for $C_{57}H_{79}N_8O_{13}S^+$ [M + H]$^+$ 1115.5482, found: 1115.5639.



Synthesis of compound **1**:

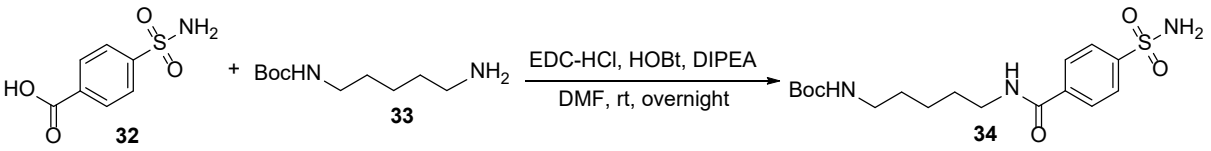
95 μL of compound **31** (21 mM in 1,4-dioxane) was dissolved in AcOH (0.5 mL) and water (0.365 mL). To this solution was added 40 μL of $NaNO_2$ (50 mM in water, 1 equiv.). The mixture was incubated at room temperature for 30 min. Then the reaction was purified by reversed-phase HPLC to give **1** (1.4 mg, 63% yield) as a white powder.

Note: This product exists as an approximate 6.5:1 [based on the ratio of 2.6H (δ 0.85, t) and 0.4H (δ 0.80, t)] mixture of the *trans* and *cis* conformations due to the epimerization of the N-C bond in SLF.[3]
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.52 (s, 1H), 8.14 – 8.12 (m, 1H), 8.09 – 8.04 (m, 2H), 7.91 (s, 1H), 7.29 – 7.27 (m, 1H), 6.96 (d, *J* = 7.8 Hz, 1H), 6.89 (s, 1H), 6.86 (t, *J* = 6.0 Hz, 1H), 6.82 – 6.80 (m, 1H), 6.79 – 6.76 (m, 1H), 6.69 – 6.66 (m, 3H), 5.73 (dd, *J* = 8.1, 5.4 Hz, 1H), 5.30 – 5.29 (m, 1H), 4.70 (t, *J* = 6.4 Hz, 1H), 4.66 – 4.64 (m, 1H), 4.54 – 4.49 (m, 2H), 4.07 – 4.02 (m, 1H), 3.91 – 3.88 (m, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 3.54 – 3.47 (m, 2H), 3.40 – 3.34 (m, 2H), 3.20 (td, *J* = 13.2, 3.0 Hz, 1H), 2.96 – 2.90 (m, 1H), 2.88 – 2.83 (m, 1H), 2.77 –2.73 (m, 1H), 2.62 – 2.58 (m, 1H), 2.55 – 2.50 (m, 1H), 2.40 (d, *J* = 13.6 Hz, 1H), 2.26 – 2.20 (m, 1H), 2.14 (q, *J* = 7.0 Hz, 1H), 2.07 – 2.02 (m, 1H), 1.81 – 1.59 (m, 14H), 1.55 – 1.45 (m, 5H), 1.42 – 1.23 (m, 4H), 1.20 (s, 3H), 1.19 (s, 3H), 1.12 (s, 1H), 0.85 (t, *J* = 7.4 Hz, 2.60H), 0.80 (t, *J* = 7.5 Hz, 0.40H).
^{13}C NMR (151 MHz, $CDCl_3$) δ 208.2, 174.2, 169.9, 168.7, 167.6, 166.5, 157.5, 154.9, 149.0, 148.8, 147.5, 147.1, 142.0, 137.2, 133.4, 131.2, 130.2, 126.4, 120.3, 120.0, 113.9, 113.6, 112.3, 111.90, 111.86, 111.5, 76.7, 68.8, 67.4, 59.9, 58.7, 56.1, 56.0, 54.5, 51.5, 46.9, 44.3, 40.4, 39.0, 38.4, 35.6, 35.3, 32.6, 31.4, 29.3, 28.8, 27.6, 27.4, 26.6, 25.3, 25.1, 24.2, 23.5, 23.4, 21.3, 8.9.
HRMS (ESI): calcd for $C_{57}H_{75}N_9NaO_{13}S^+$ $[M + H]^+$ 1148.5097, found: 1148.4877.

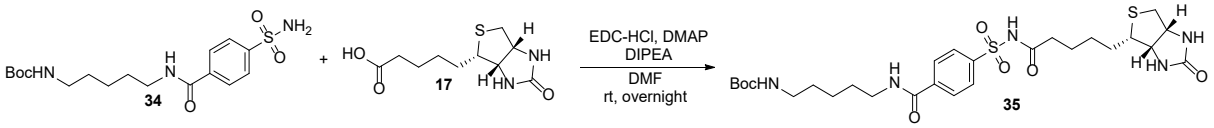


Scheme S3. Synthetic scheme of **LDNASA** (**38**)

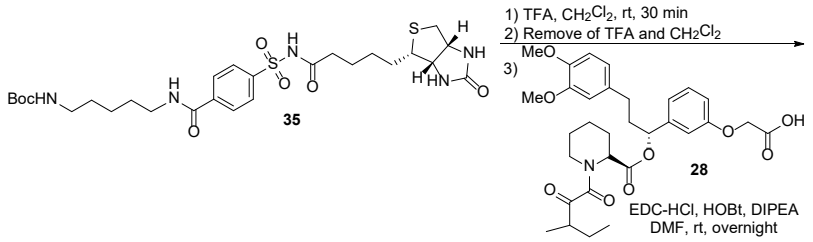
LDNASA (38) was synthesized according reference 12.

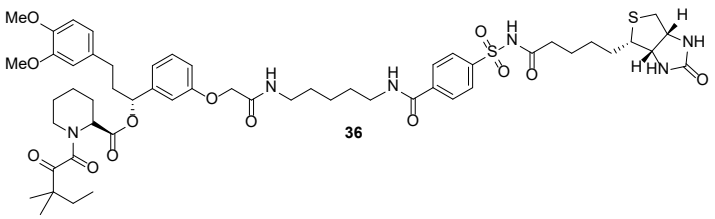


Synthesis of compound **34** following published procedures[12]:
To a stirred solution of 4-sulfamoylbenzoic acid **32** (140.8 mg, 0.7 mmol) in dry DMF (2.8 mL), was added compound **33** (141.6 mg, 0.7 mmol, 1 equiv.), EDC-HCl (201.3 mg, 1.05 mmol, 1.5 equiv.), HOBt·xH_2O (160.8 mg, 1.05 mmol, 1.5 equiv.) and DIPEA (242 µL, 1.4 mmol, 2 equiv.). The mixture was stirred at room temperature overnight. The solution was dissolved with EtOAc (25 mL), and washed with brine (25 mL × 3). The organic layer was dried over Na_2SO_4, filtrated and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 20:1) to give compound **34** (184.1 mg, 0.478 mmol, 68% yield) as white solid. The analytical data matched literature reports.[12]
^{1}H NMR (600 MHz, CD_3OD) δ 7.97 (d, *J* = 8.6 Hz, 2H), 7.94 (d, *J* = 8.4 Hz, 2H), 3.42 – 3.37 (m, 2H), 3.05 (t, *J* = 7.0 Hz, 2H), 1.65 (p, *J* = 7.3 Hz, 2H), 1.52 (p, *J* = 7.2 Hz, 2H), 1.43 – 1.39 (m, 11H); ^{13}C NMR (151 MHz, CD_3OD) δ 168.9, 158.6, 147.6, 139.2, 128.9, 127.3, 79.8, 41.2, 41.0, 30.6, 30.1, 28.8, 25.2.



Synthesis of compound **35** following published procedures[12]:
To a stirred solution of compound **34** (38.6 mg, 0.1 mmol) in dry DMF (2 mL) was added biotin **17** (40.3 mg, 0.165 mmol, 1.65 equiv.), EDC-HCl (57.5 mg, 0.3 mmol, 3 equiv.), 4-dimethylaminopyridine (DMAP) (4.0 mg, 0.033 mmol, 0.33 equiv.) and DIPEA (52 µL, 0.3 mmol, 3 equiv.). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 10:1) to yield compound **35** (51.2 mg, 83.7 µmol, 84 %) as a white solid. The analytical data matched literature reports.[12]
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08 – 7.74 (m, 2H), 4.42 (m, 1H), 4.24 – 4.13 (m, 1H), 3.77 – 3.52 (m, 2H), 3.50 – 2.53 (m, 8H), 2.42 – 2.13 (s, 2H), 1.60 – 1.11 (m, 18H).

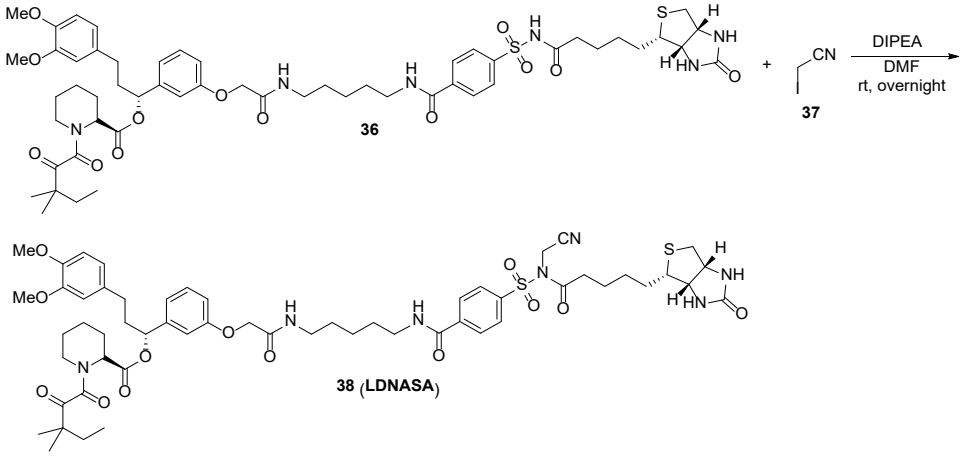




Synthesis of compound **36** following published procedures[12]:
To a stirred solution of compound **35** (50.8 mg, 83 µmol) in CH_2Cl_2 (4 mL) was added trifluoroacetic acid (TFA) (2 mL) at room temperature. The reaction solution was stirred at room temperature for 30 min. TFA and CH_2Cl_2 was removed under reduced pressure and further removed by co-evaporation with toluene (1 mL × 2). The residue was dissolved in dry DMF (0.83 mL), add compound **28** (48.4 mg, 83 µmol, 1 equiv.), EDC-HCl (23.9 mg, 124.5 µmol, 1.5 equiv.), HOBt·xH_2O (19.1 mg, 124.5 µmol, 1.5 equiv.) and DIPEA (72 µL, 415 µmol, 5 equiv.). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography

on silica gel (CH_2Cl_2 : MeOH = 10:1) to yield compound **36** (83.5 mg, 77.5 μmol, 93%) as colorless oil. The analytical data matched literature reports.[12]

^{1}H NMR (600 MHz, $CDCl_3$) δ 10.25 (br s, 3H), 7.93 (s, 4H), 7.81 (s, 1H), 7.20 (t, *J* = 7.9 Hz, 1H), 6.99 – 6.79 (m, 3H), 6.76 (d, *J* = 8.3 Hz, 1H), 6.72 – 6.66 (m, 1H), 6.61 – 6.58(m, 2H), 5.67 (t, *J* = 7.0 Hz, 1H), 5.19 (d, *J* = 5.8 Hz, 1H), 4.39 (s, 2H), 3.76 (s, 3H), 3.74 (s, 3H), 3.60 – 3.56 (m, 8H), 3.31 – 3.18 (m, 4H), 2.79 – 2.60 (m, 2H), 2.55 – 2.42 (m, 2H), 2.30 – 2.14 (m, 4H), 1.98 – 1.94 (m, 2H), 1.71 – 1.45 (m, 15H), 1.12 (d, *J* = 4.9 Hz, 6H), 0.87 – 0.67 (m, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.9, 169.5, 168.2, 167.2, 166.2, 161.4, 157.3, 148.8, 147.2, 141.7, 139.1, 133.3, 129.9, 128.0, 127.8, 120.1, 119.9, 117.7, 115.8, 113.9, 113.3, 111.7, 111.3, 76.4, 67.1, 57.9, 55.8, 55.8, 51.2, 50.1, 46.6, 44.1, 39.9, 38.8, 38.0, 32.3, 31.1, 29.1, 28.7, 26.3, 24.8, 24.0, 23.29, 23.27, 23.1, 21.0, 18.4, 18.2, 18.2, 17.2, 8.6.

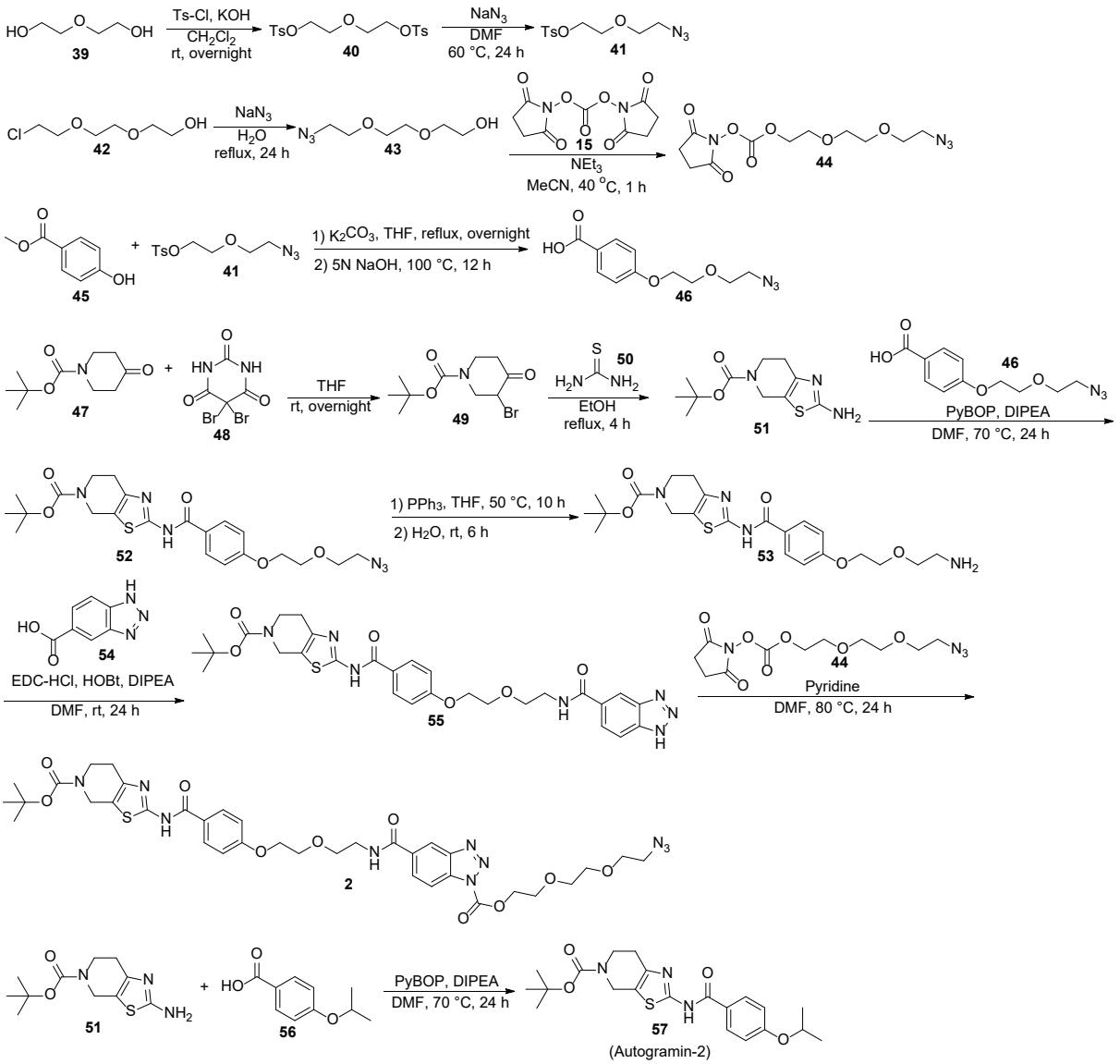


Synthesis of compound **38** (**LDNASA**) following published procedures[12]:

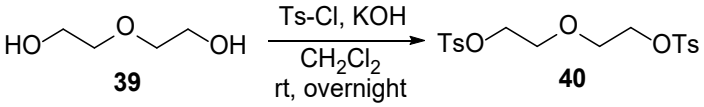
To a stirred solution of compound **36** (83.5 mg, 77.5 μmol) in dry DMF (1 mL) was added iodoacetonitrile **37** (56.1 μL, 775 μmol, 10 equiv.) and DIPEA (67 μL, 387.5 μmol, 5 equiv.). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 20:1) to yield compound **38** (38.4 mg, 34.4 μmol, 44% yield) as colorless oil. The analytical data matched literature reports.[12]

^{1}H NMR (600 MHz, $CDCl_3$) δ 9.12 (br s, 1H), 8.13 (d, *J* = 8.5 Hz, 2H), 8.00 (d, *J* = 8.5 Hz, 2H), 7.79 (t, *J* = 5.6 Hz, 1H), 7.29 – 7.26 (m, 1H), 6.96 (d, *J* = 7.7 Hz, 1H), 6.93 – 6.92 (m, 1H), 6.89 (t, *J* = 6.3 Hz, 1H), 6.82 – 6.80 (m, 1H), 6.77 – 6.75 (m, 1H), 6.68 – 6.65 (m, 2H), 5.76 – 5.73 (m, 1H), 5.67 – 5.61 (m, 2H), 5.28 – 5.27 (m, 1H), 4.79 (d, *J* = 2.6 Hz, 2H), 4.47 (t, *J* = 6.6 Hz, 1H), 4.44 (s, 2H), 4.25 – 4.18 (m, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.78 – 3.72 (m, 1H), 3.68 (q, *J* = 7.0 Hz, 2H), 3.43 (s, 1H), 3.42 – 3.29 (m, 5H), 3.18 – 3.13 (m, 2H), 3.05 – 3.02 (m, 1H), 2.88 – 2.84 (m, 1H), 2.72 – 2.49 (m, 5H), 2.35 (d, *J* = 13.7 Hz, 1H), 2.26 – 2.18 (m, 2H), 2.07 – 2.01 (m, 1H), 1.68 – 1.43 (m, 8H), 1.40 – 1.34 (m, 3H), 1.19 – 1.16 (m, 6H), 0.85 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.0, 171.9, 169.7, 168.4, 167.4, 165.4, 163.7, 157.4, 148.9, 147.4, 141.9, 140.7, 140.1, 133.4, 130.1, 129.0, 127.8, 120.2, 120.1, 115.1, 113.9, 113.5, 111.8, 111.4, 76.5, 67.3, 61.9, 60.3, 56.0, 55.9, 54.7, 51.3, 46.7, 44.2, 40.5, 40.1, 38.7, 38.2, 35.4, 33.4, 32.5, 31.3, 29.2, 28.6, 28.0, 27.8, 26.5, 25.0, 23.9, 23.5, 23.3, 21.2, 8.8;

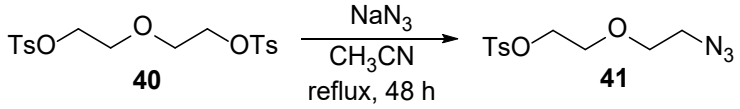
HRMS (ESI): calcd for $C_{56}H_{73}N_7O_{13}S_2Na^+$ $[M + Na]^+$ 1138.4600, found: 1138.4640.



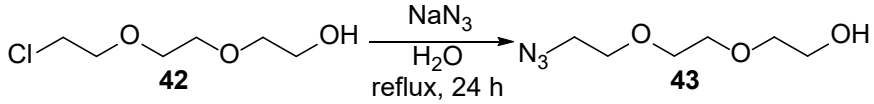
Scheme S4. Synthetic scheme of compounds **2** and Autogramin-2 (**57)**



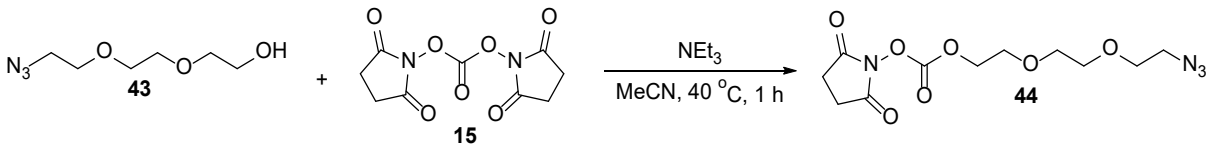
Synthesis of compound **40** following published procedures[13]:
Powdered potassium hydroxide (44.88 g, 0.8 mol) was added portionwise to a solution of diethylene glycol **39** (10.610 g, 0.1 mol, 1 equiv.) and *p*-toluenesulfonyl chloride (38.140 g, 0.2 mol, 2 equiv.) in CH_2Cl_2 (100 mL) at 0 °C. The reaction mixture was stirred at from 0 °C to room temperature overnight. Water (200 mL) was then added and the water phase was extracted with CH_2Cl_2 (50 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4, filtrated and concentrated under reduced pressure to afford **40** (39.415 g, 95.1 mmol, 95% yield) as a white solid. The analytical data matched literature reports.[13]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 8.4 Hz, 4H), 7.34 (d, J = 8.1 Hz, 4H), 4.11 – 4.05 (m, 4H), 3.63 – 3.57 (m, 4H), 2.44 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.1, 133.0, 130.0, 128.1, 69.1, 68.9, 21.8.



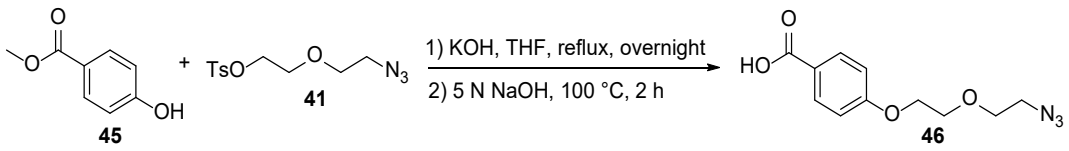
Synthesis of compound **41** following published procedures[14]:
Sodium azide (650.0 mg, 10 mmol) was added portionwise to a solution of **40** (4.145 g, 10 mmol) in acetonitrile (25 mL) at room temperature. The reaction mixture was stirred under reflux for 48 hours. After cooling down to room temperature, the white solid was filtered off. The filtrate was concentrated under reduced pressure and purified by flash chromatography on silica gel (petroleum ether : EtOAc = 5:1) to give compound **41** (1.411 g, 4.946 mmol, 49% yield) as colorless liquid. The analytical data matched literature reports.[15]
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 7.5 Hz, 2H), 7.34 (d, *J* = 7.9 Hz, 2H), 4.21 – 4.12 (m, 2H), 3.70 (t, *J* = 4.0 Hz, 2H), 3.60 (t, *J* = 4.3 Hz, 2H), 3.32 (t, *J* = 4.5 Hz, 2H), 2.44 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.0, 133.0, 130.0, 128.1, 70.3, 69.3, 68.8, 50.7, 21.8.



Synthesis of compound **43** following published procedures[16]:
To a solution of NaN_3 (2.600 g, 40 mmol) in water (80 mL) was added compound **42** (3.372 g, 20 mmol) at room temperature. The reaction mixture was stirred under reflux for 24 hours. The crude mixture was extracted with CH_2Cl_2 (40 mL × 3). The combined organic layers were washed with brine (20 mL) and dried over Na_2SO_4, filtered and evaporated to dryness to obtain compound **43** (3.172 g, 18.1 mmol, 91% yield) as colorless oil with was pure enough for further usage without purification. The analytical data matched literature reports.[16]
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 – 3.71 (m, 2H), 3.70 – 3.65 (m, 6H), 3.64 – 3.59 (m, 2H), 3.40 (t, *J* = 5.0 Hz, 2H), 2.16 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 72.6, 70.8, 70.5, 70.2, 61.9, 50.8.



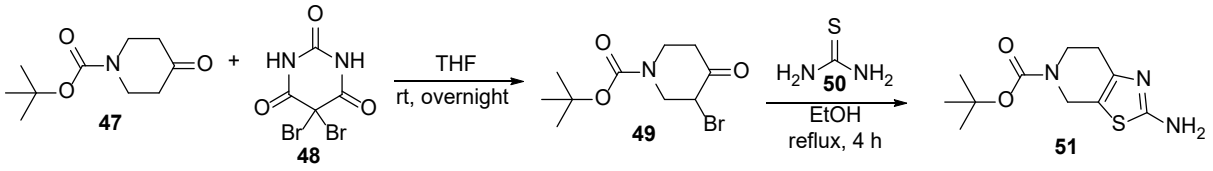
Synthesis of compound **43** following published procedures[17]:
To a solution of compound **43** (1.402 g, 8 mmol) in dry acetonitrile (40 mL), *N*,*N'*-disuccimidyl carbonate **15** (4.099 g, 16 mmol, 2 equiv.) and NEt_3 (2.230 mL, 4 mmol, 2 equiv.) were added and the resulting mixture was stirred at 40 °C for 1 hour. The solvent was removed under reduced pressure, the residue was dissolved in EtOAc (50 mL), washed with saturated $NaHCO_3$ aqueous (50 mL), water (25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (eluted with CH_2Cl_2: MeOH = 30:1) to afford compound **44** (1.454 g, 4.6 mmol, 57% yield) as a colorless oil. The analytical data matched literature reports.[18]
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.49 – 4.43 (m, 2H), 3.81 – 3.76 (m, 2H), 3.68 (d, *J* = 3.7 Hz, 6H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.83 (s, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 151.8, 71.1, 70.8, 70.33, 70.26, 68.5, 50.8, 25.6.



Synthesis of compound **50** following published procedures[19]:
To a solution of methyl 4-hydroxybenzoate **45** (CAS No.: 99-76-3) (228.3 mg, 1.5 mmol, 1.0 equiv.) in THF (7.5 mL) was added K_2CO_3 (629.1 m, 4.5 mmol, 3 equiv.) and compound **41** (427.9 mg, 1.5 mmol, 1.0 equiv.). The mixture was stirred under reflux overnight. After being cooled to room temperature, the mixture was dilutes with EtOAc (20 mL), washed with water (20 mL). The aqueous layer was extracted

with EtOAc (10 ml). The combined organic layers were washed with brine (10 mL), dried over anhydrous Na_2SO_4, filtrated and concentrated under reduced pressure. To the residue was added 10 mL of 5 N NaOH aqueous, stirred at 100 °C for 2 hours. The mixture was cooled to room temperature, washed with CH_2Cl_2 (10 mL × 2), neutralized with concentrated HCl. The resulting precipitate was collected by filtration to give **46** (197.3 mg, 0.785 mmol, 52% yield) as white solid. The analytical data matched literature reports.[19]

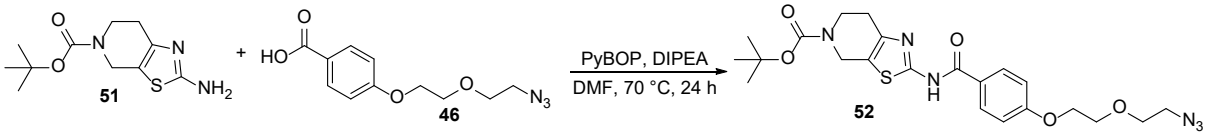
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 4.22 (t, J = 4.7 Hz, 2H), 3.99 – 3.84 (m, 2H), 3.76 (t, J = 5.0 Hz, 2H), 3.42 (t, J = 5.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 163.3, 132.5, 122.1, 114.5, 70.5, 69.7, 67.7, 50.8.



Synthesis of compound **56** following published procedures[20]:

5,5-Dibromobarbituric acid **48** (CAS No.: 511-67-1) (1.001 g, 3.50 mmol, 0.6 equiv.) was added to a solution of *N*-Boc-4-piperidone **47** (CAS No.: 79099-07-3) (1.162 g, 5.83 mmol, 1.0 equiv.) in dry THF (15 mL). The mixture was stirred at room temperature overnight, during which time a white precipitate formed. This precipitate was filtered off, washed with THF (10 mL). The solvent in the filtrate was removed under reduced pressure to yield the bromopiperidinone **49**, which was used without further purification. Crude **49** was dissolved in EtOH (15 mL), and thiourea **50** (487.8 mg, 6.41 mmol, 1.1 equiv.) was added. The mixture was stirred under reflux for 4 hours. The pale yellow precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc (50 mL), washed with saturated $NaHCO_3$ aqueous (25 mL). The aqueous was extracted with EtOAc (10 mL × 2). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered, and the solvent removed under reduced pressure to yield a pale yellow solid. This was washed with petroleum ether/EtOAc (10:1, total 11 mL) to yield **51** (546.8 mg, 2.14 mmol, 37% yield over two steps) as a white solid. The analytical data matched literature reports.[20]

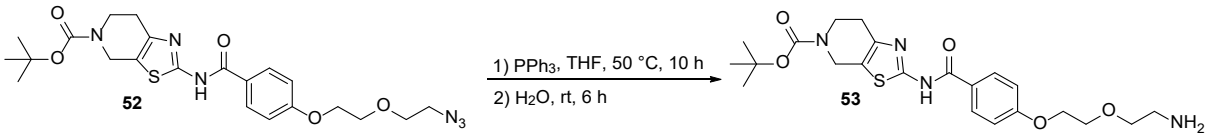
^{1}H NMR (400 MHz, CD_3OD) δ 4.37 (s, 2H), 3.68 (t, J = 5.8 Hz, 2H), 2.55 – 2.51 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 170.1, 156.5, 144.2, 113.5, 81.7, 42.3, 42.0, 28.6, 27.4.



Synthesis of compound **52** following published procedures[20]:

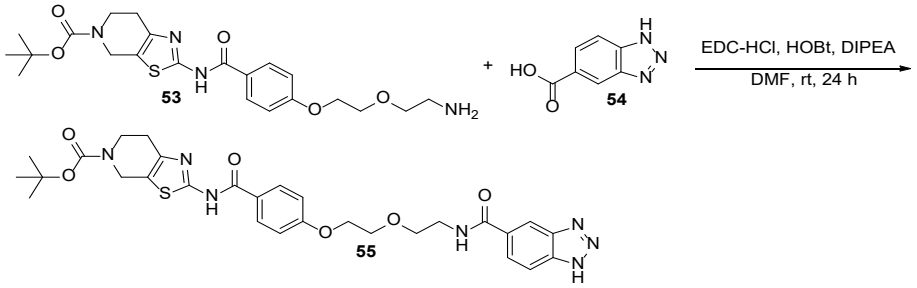
To a solution of compound **51** (199.1 mg, 0.78 mmol, 1.0 equiv.) and compound **46** (195.9 mg, 0.78 mmol, 1.0 equiv.) in DMF (7.8 mL) was added DIPEA (405 μL, 2.34 mmol, 3 equiv.) and PyBOP® (CAS No.: 128625-52-5) (811.8 mg, 1.56 mmol, 2.0 equiv.). The mixture was stirred at 70 °C for 24 hours. After being cooled to room temperature, the mixture was dilutes with EtOAc (30 mL), washed with water (15 mL × 3), brine (15 mL), dried over anhydrous Na_2SO_4, filtrated and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (petroleum ether : EtOAc = 1 : 1) to give compound **52** (243.0 mg, 0.4973 mmol, 64% yield) as colorless oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.91 (d, J = 8.9 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 4.53 (s, 2H), 4.16 – 4.14 (m, 2H), 3.83 – 3.81 (m, 2H), 3.71 – 3.67 (m, 4H), 3.37 – 3.35 (m, 2H), 2.63 (t, J = 5.5 Hz, 2H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 166.4, 163.8, 158.9, 156.2, 144.2, 131.0, 128.8, 125.6, 115.5, 81.6, 71.3, 70.4, 68.8, 51.6, 42.9, 42.1, 28.7, 27.3; HRMS (ESI): calcd for $C_{22}H_{29}N_6O_5S^+$ [M + H]$^+$ 489.1915, found: 489.1913.

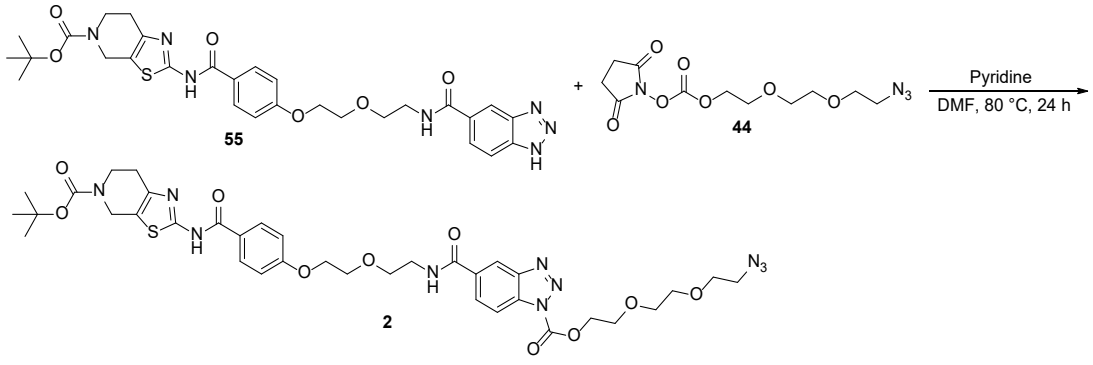


Synthesis of compound **53**:

A solution of compound **52** (242.8 mg, 0.497 mmol, 1.0 equiv.) and triphenylphosphine (156.4 mg, 0.596 mmol, 1.2 equiv.) in THF (2 mL) was stirred at 50 °C for 10 hours under argon atmosphere. After being cooled to room temperature, water (44.7 μL, 2.49 mmol, 5 equiv.) was added and stirred at room temperature for additional 6 hours. The mixture was concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (petroleum ether : EtOAc = 1 : 1) to give compound **53** (153.2 mg, 0.3315 mmol, 67% yield) as a white solid.
^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (d, *J* = 8.5 Hz, 2H), 7.07 (d, *J* = 8.5 Hz, 2H), 4.58 (s, 2H), 4.24 (t, *J* = 4.5 Hz, 2H), 3.86 (t, *J* = 4.5 Hz, 2H), 3.75 (t, *J* = 5.8 Hz, 2H), 3.61 (t, *J* = 5.3 Hz, 2H), 2.84 (t, *J* = 5.3 Hz, 2H), 2.74 – 2.71 (m, 2H), 1.49 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 185.5, 167.3, 163.8, 156.4, 147.7, 144.1, 131.1, 126.4, 115.5, 81.72, 81.69, 78.8, 73.2, 70.5, 68.9, 42.0, 28.7, 27.5; HRMS (ESI): calcd for $C_{22}H_{31}N_4O_5S^+$ $[M + H]^+$ 463.2010, found: 463.2018.

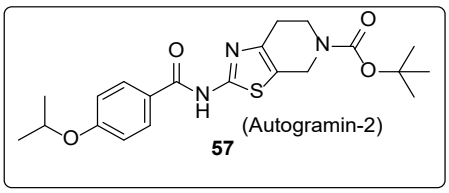


Synthesis of compound **55**:
To a stirred solution of compound **53** (83.2 mg, 0.18 mmol) in dry DMF (1.8 mL) was added benzotriazole-5-carboxylic acid **54** (CAS No.: 23814-12-2) (35.2 mg, 0.216 mmol, 1.2 equiv.), EDC-HCl (CAS No.: 25952-53-8) (51.8 mg, 0.27 mmol, 1.5 equiv.), 1-hydroxybenzotriazole hydrate (HOBt·xH_2O, CAS No.: 123333-53-9) (41.3 mg, 0.27 mmol, 1.5 equiv.), and DIPEA (155.8 μL, 0.9 mmol, 5 equiv.). The mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc (20 mL), washed with water (20 mL) and brine (20 mL × 3), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 20:1) to give compound **55** (30.1 mg, 49.5 μmol, 28% yield) as a white foam.
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.82 (s, 2H), 7.78 (d, *J* = 8.5 Hz, 1H), 7.70 – 7.64 (m, 1H), 7.33 (s, 1H), 6.79 (s, 2H), 4.51 (s, 2H), 3.82 – 3.64 (m, 10H), 2.54 (s, 2H), 1.46 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 167.6, 164.6, 162.8, 162.4, 158.0, 154.8, 142.7, 132.3, 132.2, 132.1, 129.9, 128.8, 128.7, 124.4, 119.5, 114.6, 80.6, 69.8, 69.4, 67.7, 40.1, 36.7, 31.6, 28.5, 14.3; HRMS (ESI): calcd for $C_{29}H_{34}N_7O_6S^+$ $[M + H]^+$ 608.2286, found: 608.2282.



Synthesis of compound **2**:
A solution of compound **55** (30.1 mg, 49.5 μmol), compound **44** (78.3 mg, 247.5 μmol, 5 equiv.), and pyridine (39.9 μL, 495 μmol, 10 equiv.) in dry DMF (1 mL) was stirred at 80 °C for 24 hours. The volatile material was removed under reduced pressure. The residue was purified by Preparative-HPLC [(column: Macherey-Nagel, NUCLEODUR C18 HTec, 5 μm, 250×21 mm; mobile phase: CH_3CN (containing 0.1% formic acid) : H_2O (containing 0.1% formic acid) = 20 : 80 → 90 : 10 (linear gradient over 40 min); flow rate: 18 mL/min; detection UV wavelength: 220 nm)]. The combined collected fractions were lyophilized to give **2** (3.0 mg, 7% yield) as a white powder.

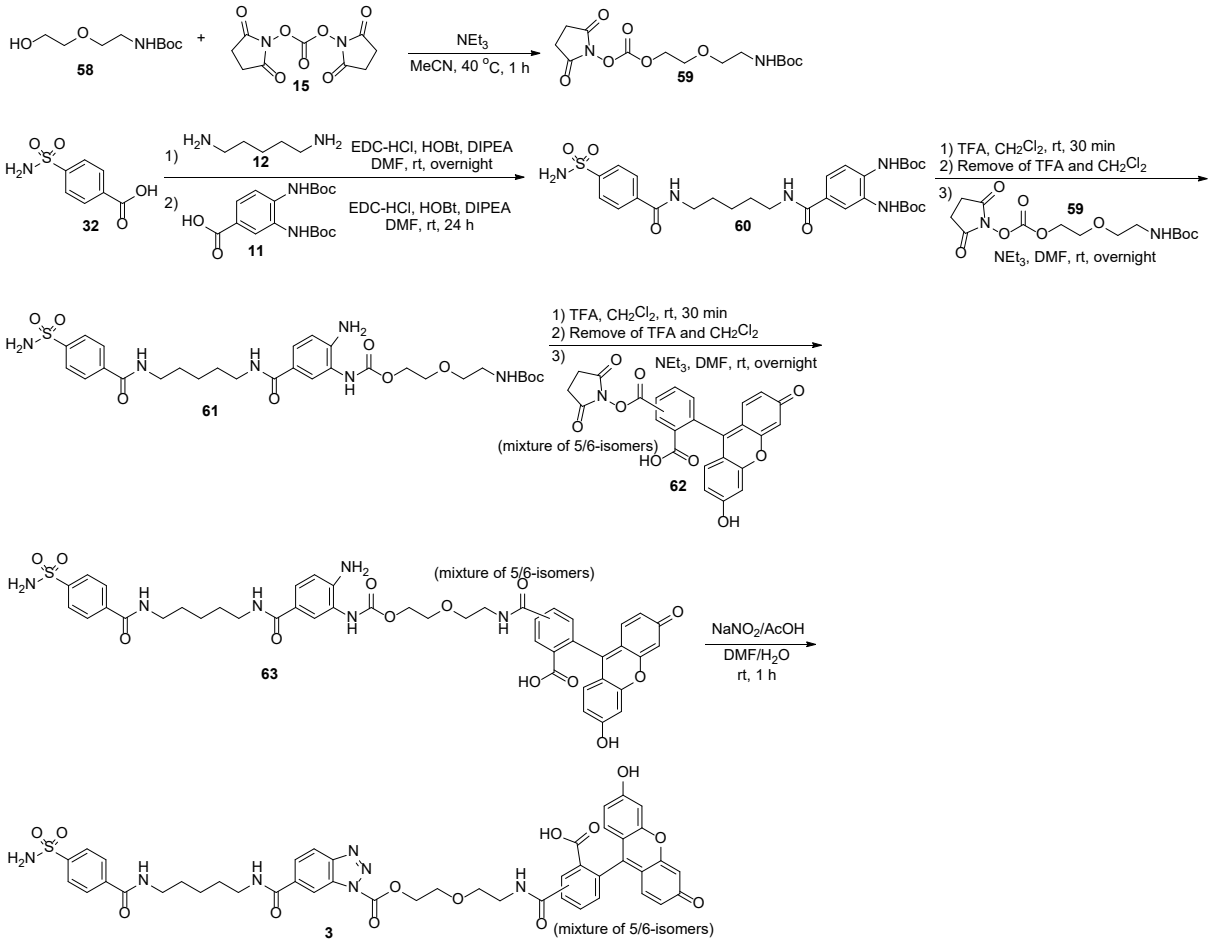
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.11 (d, *J* = 8.6 Hz, 1H), 8.07 (dd, *J* = 8.6, 1.4 Hz, 1H), 7.88 (d, *J* = 8.8 Hz, 2H), 7.01 (d, *J* = 8.6 Hz, 2H), 4.79 – 4.78 (m, 2H), 4.59 (s, 2H), 4.25 – 4.23 (m, 2H), 3.99 – 3.98 (m, 2H), 3.93 – 3.91 (m, 2H), 3.80 – 3.73 (m, 10H), 3.70 – 3.68 (m, 2H), 3.67 – 3.65 (m, 2H), 3.34 (t, *J* = 5.0 Hz, 2H), 2.72 (m, 2H), 1.49 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.2, 165.4, 162.6, 157.1, 154.8, 148.7, 145.9, 133.5, 132.8, 129.7, 129.4, 124.5, 119.3, 118.6, 115.0, 114.0, 100.1, 80.5, 71.0, 70.9, 70.3, 70.0, 69.6, 68.8, 68.2, 67.7, 50.8, 40.1, 28.6; [*Note*: The carbon signals should be 32, but found 29. The sum of -CH_2- and CH_3- signals should be 15, but found 12 (see HSQC data). So three -CH_2- and/or CH_3- carbon signals were overlapping with others.] HSQC (600, 150 MHz, $CDCl_3$) (CH_2 + CH_3 signals part, should be 14, but found 11): {4.78, 68.08}, {4.58, 41.87}, {4.24, 67.52}, {3.98, 68.78}, {3.92, 69.63}, {3.78, 70.10}, {3.74, 40.12}, {3.66, 70.41}, {3.34, 50.78}, {2.72, 26.68}, {1.49, 28.54}; HRMS (ESI): calcd for $C_{36}H_{44}N_{10}O_{10}SNa^+$ $[M + Na]^+$ 831.2855, found: 831.2854.



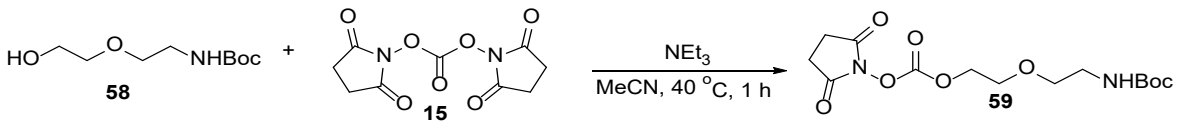
Synthesis of compound **57** following published procedures[20]:

A 10 mL flask was charged with compound **51** (25.5 mg, 0.10 mmol) and 4-isopropoxybenzoic acid **56** (21.6 mg, 0.12 mmol, 1.2 equiv. CAS No.: 13205-46-4) and DMF (1 mL). To this was added DIPEA (51.9 μL, 0.3 mmol, 3 equiv.) and PyBOP® (104.1 mg, 0.2 mmol, 2 equiv. CAS No.: 128625-52-5). The mixture was stirred at 70 °C for 24 hours. The mixture was diluted with EtOAc (10 mL), washed with saturated $NaHCO_3$ aqueous solution (10 mL × 3), saturated NH_4Cl solution (10 mL × 2), and brine (10 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent removed under reduced pressure. The residue was purified by flash chromatography on silica gel (petroleum ether : EtOAc = 5 : 1) to give compound **57** (15.6 mg, 37.4 μmol, 37% yield) as a white solid. The analytical data matched literature reports.[20]

^{1}H NMR (600 MHz, $CDCl_3$) δ 11.29 (br s, 1H), 7.88 (d, *J* = 8.3 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 4.65 – 4.61 (m, 1H), 4.56 (s, 2H), 3.60 (s, 2H), 2.42 (s, 2H), 1.47 (s, 9H), 1.36 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.6, 161.9, 158.1, 154.8, 130.0, 129.8, 123.9, 115.7, 115.6, 80.4, 70.3, 42.0, 41.1, 28.5, 26.3, 22.0.



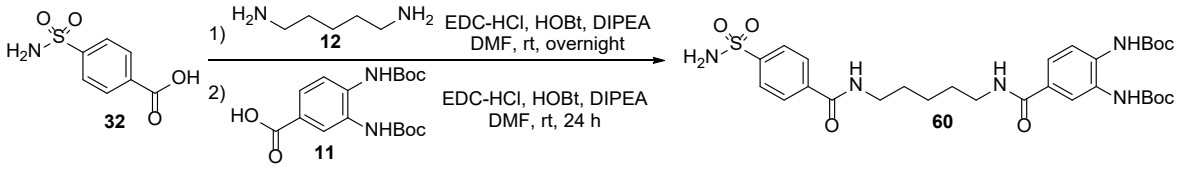
Scheme S5. Synthetic scheme of compound **3**



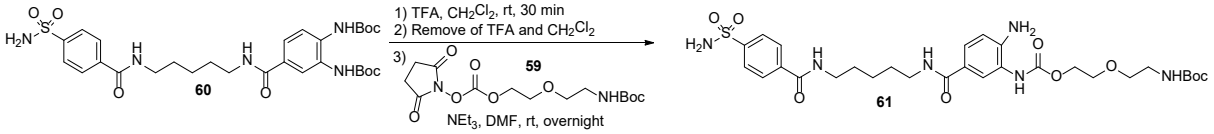
Synthesis of compound **59** following published procedures[17]:

To a solution of compound **58** (410.6 mg, 2 mmol) in dry acetonitrile (16 mL), compound **15** (1.025 g, 4 mmol, 2 equiv.) and NEt_3 (556 μL, 4 mmol, 2 equiv.) were added and the resulting mixture was stirred at 40 °C for 1 hour. The solvent was removed under reduced pressure, the residue was dissolved in EtOAc (30 mL), washed with saturated aqueous solution of $NaHCO_3$ (20 mL), water (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (eluted with CH_2Cl_2: MeOH = 50:1) to afford compound **59** (268.5 mg, 0.775 mmol, 39% yield) as a colorless oil. The analytical data matched literature reports.[17]

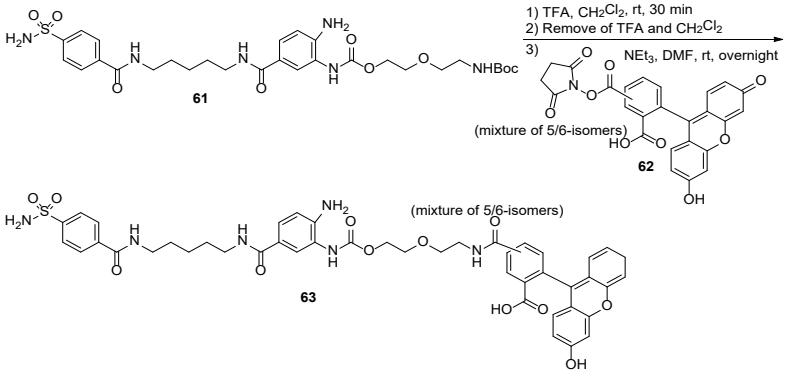
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.28 (s, 1H), 4.49 – 4.39 (m, 2H), 3.74 – 3.67 (m, 2H), 3.52 (t, J = 5.1 Hz, 2H), 3.29 (q, J = 5.5 Hz, 2H), 2.81 (s, 4H), 1.41 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 156.1, 151.7, 79.3, 70.6, 70.0, 68.2, 40.4, 28.5, 25.5.



Synthesis of compound **60**:
To a dry flask, was add compound 4-sulfamoylbenzoic acid **32** (402.4 mg, 2 mmol, 1.0 equiv. CAS No.: 138-41-0), DMF (3 mL), compound **12** (204.4 mg, 2 mmol, 1 equiv.), EDC-HCl (575.1 mg, 3 mmol, 1.5 equiv.), HOBt·xH_2O (459.3 mg, 3 mmol, 1.5 equiv.), and DIPEA (1.039 mL, 6 mmol, 3 equiv.). The mixture was stirred at room temperature overnight. Then compound **11** (704.8 mg, 2 mmol, 1 equiv), EDC-HCl (460.1 mg, 2.4 mmol), HOBt·xH_2O (367.4 mg, 2.4 mmol), and DIPEA (692 μL, 4 mmol) was added. The mixture was further stirred at room temperature for 24 hours. The mixture was diluted with EtOAc (50 mL), washed with water (50 mL × 3) and brine (50 mL × 2), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 20:1) to give compound **60** (166.1 mg, 0.268 mmol, 13% yield) as a white solid.
^{1}H NMR (400 MHz, CD_3OD) δ 7.99 – 7.83 (m, 5H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.56 (dd, *J* = 8.5, 2.1 Hz, 1H), 5.49 (s, 1H), 3.39 (dt, *J* = 10.3, 7.0 Hz, 4H), 1.68 (h, *J* = 6.8 Hz, 5H), 1.53 (s, 9H), 1.52 (s, 9H), 1.49 – 1.39 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 169.3, 168.8, 156.0, 155.3, 147.5, 139.2, 135.6, 135.5, 131.6, 130.7, 128.9, 127.3, 125.2, 123.8, 81.8, 81.7, 41.0, 40.8, 30.1, 30.0, 28.61, 28.59, 25.3; HRMS (ESI): calcd for $C_{29}H_{42}N_5O_8S^+$ $[M + H]^+$ 620.2749, found: 620.2741.



Synthesis of compound **61**:
To a stirred solution of compound **60** (166.1 mg, 0.268 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA, 1 mL) at room temperature. The reaction solution was stirred for 30 min at room temperature. CH_2Cl_2 and TFA was removed under reduced pressure, and further removed by co-evaporation with toluene (1 mL × 2). To a stirred solution of this residue in dry DMF (2 mL) was added compound **59** (139.2 mg, 0.402 mmol, 1.5 equiv.), and NEt_3 (112 μL, 0.804 mmol, 3 equiv.). The mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc (20 mL), washed with water (20 mL × 3) and brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 10:1) to give compound **61** (80.6 mg, 0.124 mmol, 46% yield) as white solid.
^{1}H NMR (400 MHz, CD_3OD) δ 8.01 – 7.91 (m, 4H), 7.73 (s, 1H), 7.49 (d, *J* = 8.5 Hz, 1H), 6.78 (d, *J* = 8.5 Hz, 1H), 4.28 (d, *J* = 4.7 Hz, 2H), 3.70 (s, 3H), 3.54 (s, 3H), 3.37 (dt, *J* = 14.0, 7.0 Hz, 6H), 3.24 (s, 3H), 1.65 (dd, *J* = 12.8, 6.0 Hz, 6H), 1.44 (s, 12H); ^{13}C NMR (100 MHz, CD_3OD) δ 169.7, 168.6, 158.3, 157.2, 147.4, 147.1, 139.0, 128.9, 127.2, 123.9, 123.3, 117.7, 116.6, 116.2, 80.0, 70.9, 70.1, 65.5, 41.2, 40.9, 40.6, 30.2, 29.9, 28.7, 25.3; HRMS (ESI): calcd for $C_{29}H_{43}N_6O_9S^+$ $[M + H]^+$ 651.2870, found: 651.2894.

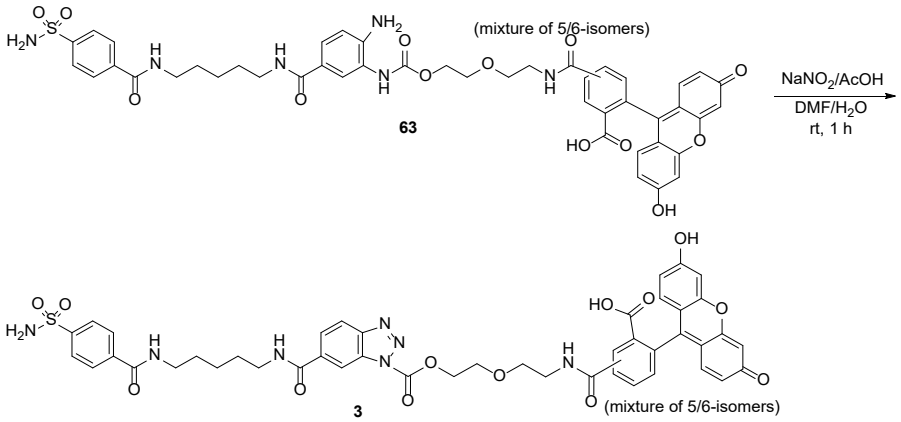


Synthesis of compound **63**:
To a stirred solution of compound **61** (80.6 mg, 0.124 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA, 1 mL) at room temperature. The reaction solution was stirred for 30 min at room temperature. CH_2Cl_2 and TFA was removed under reduced pressure, and further removed by co-evaporation with toluene (1 mL × 2). To a stirred solution of this residue in dry DMF (1.24 mL) was added compound **62**

[NHS-Fluorescein (5/6-carboxyfluorescein succinimidyl ester), mixed isomer. Thermo Scientific, cat. No.: 46410] (58.7 mg, 0.124 mmol, 1 equiv.), and NEt_3 (52 µL, 0.372 mmol, 3 equiv.). The mixture was stirred at room temperature overnight. The mixture was concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (CH_2Cl_2 : MeOH = 5:1) to give compound **63** (91.2 mg, 0.1003 mmol, 81% yield) as yellow solid.

^{1}H NMR (400 MHz, CD_2Cl_2/CD_3OD = 1:1) δ 8.39 (s, 1H), 8.14 – 7.99 (m, 1H), 7.94 – 7.78 (m, 4H), 7.63 – 7.55 (m, 1H), 7.36 (td, *J* = 8.4, 2.0 Hz, 1H), 7.20 – 7.10 (m, 1H), 6.71 – 6.59 (m, 3H), 6.59 – 6.41 (m, 4H), 4.66 (br s, 3H), 4.31 – 4.07 (m, 2H), 3.74 – 3.41 (m, 6H), 3.32 – 3.28(m, 4H), 1.63 – 1.47 (m, 4H), 1.40 – 1.29 (m, 2H).

^{13}C NMR (100 MHz, CD_3OD) δ 170.4, 169.3, 168.2, 167.9, 167.8, 161.8, 156.7, 156.6, 154.0, 153.9, 146.5, 141.4, 138.7, 137.2, 134.7, 130.0, 129.9, 128.6, 126.9, 125.7, 125.0, 124.0, 123.0, 116.2, 113.9, 110.7, 110.7, 103.5, 70.02, 69.99, 65.2, 40.8, 40.6, 40.3, 29.8, 29.5, 24.8; HRMS (ESI): calcd for $C_{45}H_{45}N_6O_{13}S^+$ $[M + H]^+$ 909.2760, found: 909.2812.



Synthesis of compound **3**:

Compound **63** (41.1 mg, 45.2 µmol) was dissolved in DMF (1 mL). To this solution was added a solution of $NaNO_2$ (31.2 mg, 452 µmol, 10 equiv.) in water (0.5 mL), then AcOH (0.5 mL). The mixture was stirred at room temperature for 1 hour. The mixture was directly purified by preparative-HPLC [(column: Macherey-Nagel, NUCLEODUR C18 HTec, 5 µm, 250×21 mm; mobile phase: CH_3CN (containing 0.1% formic acid) : H_2O (containing 0.1% formic acid) = 20 : 80 → 100 : 0 (linear gradient over 40 min); flow rate: 18 mL/min; detection UV wavelength: 220 nm)]. The combined collected fractions were lyophilized to give **3** (16.4 mg, 17.8 µmol, 39% yield) as a yellow powder.

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 10.18 (s, 2H), 8.96 – 8.67 (m, 2H), 8.63 (t, *J* = 5.0 Hz, 1H), 8.51 – 8.37 (m, 1H), 8.33 – 8.00 (m, 3H), 7.98 (dd, *J* = 8.3, 2.2 Hz, 2H), 7.92 (s, 1H), 7.90 – 7.82 (m, 2H), 7.46 (s, 2H), 7.40 – 7.27 (m, 1H), 7.05 – 6.80 (m, 1H), 6.77 – 6.62 (m, 2H), 6.62 – 6.50 (m, 3H), 4.78 – 4.63 (m, 1H), 4.42 – 4.21 (m, 1H), 3.96 – 3.82 (m, 1H), 3.76 – 3.49 (m, 4H), 3.41 (d, *J* = 5.8 Hz, 1H), 3.32 – 3.27 (m, 4H), 1.64 – 1.55 (m, 4H), 1.43 – 1.37 (m, 2H).

^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 165.8, 165.1, 164.9, 164.8, 159.8, 159.6, 151.8, 148.1, 146.2, 146.1, 145.1, 137.6, 132.6, 132.6, 131.3, 129.7, 129.3, 129.2, 127.8, 125.6, 118.9, 113.4, 112.71, 112.66, 109.15, 109.10, 109.08, 102.3, 68.8, 67.9, 67.6, 28.8, 28.8, 24.0. *Note*: three peaks overlap with $(CD_3)_2SO$ (40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89) as shown in peaks of HSQC NMR: {3.34, 40.52}, {3.29, 38.70}, {3.51, 38.57}.

HRMS (ESI): calcd for $C_{45}H_{42}N_7O_{13}S^+$ $[M + H]^+$ 920.2556, found: 920.2561.

11. References

1 H. Chi, C. Liu, H. Yang, W.-F. Zeng, L. Wu, W.-J. Zhou, R.-M. Wang, X.-N. Niu, Y.-H. Ding, Y. Zhang, Z.-W. Wang, Z.-L. Chen, R.-X. Sun, T. Liu, G.-M. Tan, M.-Q. Dong, P. Xu, P.-H. Zhang and S.-M. He, *Nat. Biotechnol*., 2018, **36**, 1059–1061.

2 G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw and K. I. Goldberg, *Organometallics*, 2010, **29**, 2176–2179.

3 E. A. Althoff and N. W. Cornish, United States Patent, US 2005/0221402A1, Oct. 6, 2005.

4 M. Mutz, K. J. Barr and J. Gestwichi, PCT Patent, WO2010077317A2, July 8, 2010.

5 T. Joshi, G. J. Barbante, P. S. Francis, C. F. Hogan, A. M. Bond and L. Spiccia, *Inorg. Chem*., 2011, **50**, 12172−12183.

6 J.-H. Kim, D. A. Heller, H. Jin, P. W. Barone, C. Song, J. Zhang, L. J. Trudel, G. N. Wogan, S. R. Tannenbaum and M. S. Strano, *Nat. Chem*., 2009, **1**, 473–481.

7 M. K. Parai, D. J. Huggins, H. Cao, M. N. L. Nalam, A. Ali, C. A. Schiffer, B. Tidor and T. M. Rana, *J. Med. Chem*., 2012, **55**, 6328−6341.

8 K. Susumu, H. T. Uyeda, I. L. Medintz, T; Pons, J. B. Delehanty and H. Mattoussi, *J. Am. Chem. Soc*., 2007, **129**, 13987–13996.

9 F. Li and Y. Wang, PCT Patent, WO 2012/103279 A2, Jan 26, 2012.

10 F. P. Jørgensen and M. Bols, *J. Org. Chem*., 2018, **83**, 6050–6055.

11 F. P. Jørgensen, D. Madsen, M. Meldal, J. V. Olsen, M. Petersen, J. Granhøj and M. Bols, *J. Med. Chem*., 2019, **62**, 5191−5216.

12 T. Tamura, T. Ueda, T. Goto, T. Tsukidate, Y. Shapira, Y. Nishikawa, A. Fujisawa and I. Hamachi, *Nat. Commun*., 2018, **9**, 1870.

13 F. Friscourt, C. J. Fahrni and G.-J. Boons, *J. Am. Chem. Soc*., 2012, **134**, 18809–18815.

14 A. Banerjee, S. Maschauer, H. Hübner, P. Gmeiner and O. Prante, *Bioorg. Med. Chem. Lett*., 2013, **23**, 6079–6082.

15 H. S. Gill, J. N. Tinianow, A. Ogasawara, J. E. Flores, A. N. Vanderbilt, H. Raab, J. M. Scheer, R. Vandlen, S.-P. Williams and J. Marik, *J. Med. Chem*., 2009, **52**, 5816–5825.

16 M. A. Brun, K.-T. Tan, R. Griss, A. Kielkowska, L. Reymond and K. Johnsson, *J. Am. Chem. Soc*., 2012, **134**, 7676–7678.

17 S. Fujishima, R. Yasui, T. Miki, A. Ojida and I. Hamachi, *J. Am. Chem. Soc*., 2012, **134**, 3961–3964.

18 M. Tsukagoshi, PCT Patent, WO2018097239A1, May 31, 2018.

19 A. M. Gazzali, L. Colombeau, P. Arnoux, H. A. Wahab, C. Frochot, R. Vanderesse and S. Acherar, *Tetrahedron*, 2017, **73**, 532–541.

20 L. Laraia, A. Friese, D. P. Corkery, G. Konstantinidis, N. Erwin, W. Hofer, H. Karatas, L. Klewer, A. Brockmeyer, M. Metz, B. Schölermann, M. Dwivedi, L. Li, P. Rios-Munoz, M. Köhn, R. Winter, I. R. Vetter, S. Ziegler, P. Janning, Y.-W. Wu and H. Waldmann, *Nat.Chem. Biol*., 2019, **15**, 710–720.