Phenotypic Discovery of Neuroprotective Agents by Regulation of Tau Proteostasis via Stress-Responsive Activation of PERK Signaling

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Materials and Methods

Cell culture

BiFC-tau stable HEK293 human embryonic kidney cell line was kindly provided by Dr. Yun Kyung Kim, Korea Institute of Science and Technology (KIST), and was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) [Gibco] supplemented with 10% fetal bovine serum (FBS) [Gibco], 1% penicillin (100 units/mL)/streptomycin (100 μg/mL) [Gibco], Fungizone (0.25 μg/mL) [Gibco], and Geneticin (100 μg/mL) [Gibco]. SH-SY5Y human neuroblastoma cells (CRL-226, ATCC) were cultured in advanced DMEM/F12 [Gibco] supplemented with 10% FBS, 1% penicillin/streptomycin, and Fungizone. All cells were maintained at 5% CO₂ in a 37 ℃ incubator and confirmed to be mycoplasma negative using EZ-Mycoplasma detection kit [DoGenBio].

Stable cell line generation using viral production and transduction

Lentiviral- and retroviral-expression systems were used to stably express DsRed_IRES_Tau-EGFP and mCherry-GFP-Tau in HEK293 cell line, respectively. The mCherry-GFP-Tau expression vector was constructed by insertion of the MAPT gene into the retroviral expression vector pBABE-puro mCherry-GFP-LC3 (gifted from Dr. Heesun Cheong, Division of Chemical Biology, Research Institute, National Cancer Center, Korea). For virus production, HEK293 cells were seeded in 100-mm culture dishes and co-transfected with 3 μg of lentiviral transfer plasmid encoding pLenti-DsRed_IRES_MAPT:EGFP (Addgene, #92196), 3 μg of pCMV-VSV-G envelope plasmid (Addgene, #8454), and 3 μg of psPAX2 packaging plasmid (Addgene, #12260) using Lipofectamine [ThermoFisher Scientific]. For virus production of the retroviral system, HEK293 cells were co-transfected with 3 μg of retroviral transfer plasmid encoding pBabe-puro mCherry-GFP-MAPT, 3 μg of pCMV-VSV-G envelope plasmid (Addgene, #8454), and 3 μg of pUMVC packaging plasmid (Addgene, #8449) using Lipofectamine [ThermoFisher Scientific]. HEK293 cells were infected by the addition of viral stock in six-well cell culture dishes overnight. Following the infection, cells were subjected to selection by addition of Geneticin or puromycin for 7–10 days. Cells were then further sorted by a double-positive (DsRed and EGFP, or mCherry and GFP) gating using FACS Aria II [BD Bioscience]. HEK293-DsRed-EGFP-Tau stable cell lines were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, Fungizone (0.25 μg/mL), and Geneticin (100 μg/mL), and maintained at 5% CO₂ in a 37 ℃ incubator.

siRNA transfection

For PDIA3 and DNAJC3 knockdown experiments, short interfering RNA (siRNA) duplex against PDIA3 and DNAJC3 were purchased from BIONEER. siRNA oligonucleotides were transfected in BiFC-tau stably expressing HEK293 cells using Lipofectamine RNAiMAX
[ThermoFisher Scientific] and Opti-MEM [Gibco] on the basis of manufacturer's instructions. Oligonucleotide sequences for PDIA3 and DNAJC3 are as follows:

siPDIA3 #1 sense 5′- CGUCCUUCACACUCAGCUA -3′
sense
antisense 5′- UAGUGAGAUGAGAAGGACG -3′
sense
antisense 5′- GAAAUACCAGAGACAGUUU -3′
sense
antisense 5′- AAACUGGUCCUGGUAUUUC -3′
sense
antisense 5′- CUGCUAUCGCUUCCUUGA -3′
sense
antisense 5′- UCAAGGAAGCUAUGACG -3′
sense
antisense 5′- GUGAUGGCUUUUACCACU -3′
sense
antisense 5′- AGUAGGUAAAAGCCAUCA -3′

**Tau assembly monitoring and compound screening using BiFC-tau Venus HEK293 cell line**

Phenotype-based screening to monitor tau assembly was performed using HEK293 BiFC-tau Venus cells in a high-throughput manner. Cells were seeded into 384-well plates at a density of 2.8 × 10³ cells/well in 40 µL media for 24 h. Cells were treated with 80 nM thapsigargin [Sigma-Aldrich] in 10 µL media, then ~3,000-membered pDOS library compounds (10 µM) were treated using a 0.1 µL pinning tool. For the knockdown experiments, cells were transfected with 5 or 10 nM siRNAs using Lipofectamine RNAiMAX and incubated for 48 h, followed by thapsigargin treatment. After 24 h of incubation in a 37 °C, 5% CO₂ incubator, nuclei were stained by medium-diluted Hoechst 33342 (2 µg/mL) [ThermoFisher] for 20 min. Plates were scanned in an INCell Analyzer 2000 [GE Healthcare] at λ_ex/λ_em = 490/525 nm (for FITC channel) for Venus fluorescence and at λ_ex/λ_em = 350/455 nm (for DAPI channel) for nuclei. Bright-field images were also taken to check the cell morphologies and images were analyzed to quantify Venus intensity per cell using Developer software [GE Healthcare].

**Flow cytometry analysis using HEK293 DsRed–IRES–tau-EGFP cell line**

HEK293 DsRed-IRES-tau-EGFP cells were treated with each compound for the designated time or were transfected with siRNAs using Lipofectamine RNAiMAX. The cells were trypsinized, suspended in phosphate-buffered saline (PBS), and subjected to FACS analysis using Aria II. Data were analyzed to measure the fluorescence intensity ratio of EGFP to DsRed per cell using flowing software 2.5.1. Mean values of GFP and DsRed fluorescence intensity per cell with double-positive gating were used for the analysis.
Immunoblotting

Cells were collected and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 5 mM NaF, 2 mM Na3VO4, 1x Protease Inhibitor Cocktail [Roche]), followed by centrifugation at 200 g for 10 min at 4 °C for the whole-cell analysis and the determination of total protein concentration in each lysate using Pierce BCA Protein Assay Kit [ThermoFisher Scientific]. Equal amounts of each lysate were fractionated by PAGE, transferred to PVDF membrane [Bio-Rad], and blocked with 2% bovine serum albumin (BSA) [MP Biomedicals] in Tris Buffered Saline with Tween20 (TBS_T) [Sigma] for 1 h at room temperature. Membranes were probed with protein-specific antibodies overnight at 4 °C. The next day, membranes were washed three times in TBS_T and incubated for 1 h at room temperature with anti-rabbit or anti-mouse horseradish peroxidase secondary antibody [Cell Signaling Technology] in 2% BSA in TBS_T. After washing, membranes were exposed to detection reagent [GE Healthcare] and quantified using chemiluminescence [Bio-Rad].

The following antibodies were purchased as primary antibodies for immunoblot studies: Rabbit anti-ph(T982)-PERK polyclonal antibody (Abcam, ab192591); rabbit anti-PERK monoclonal antibody (Cell Signaling Technology, 3192); rabbit anti-ElF2S1 (phospho S51) polyclonal antibody (Abcam, Ab32157); rabbit anti-ph-elF2α (Ser51) monoclonal antibody (Cell Signaling Technology, 3597); mouse anti-elF2α monoclonal antibody (santa cruz, sc-133132); rabbit anti-ATF4 monoclonal antibody (Cell Signaling Technology, 11815); mouse anti-CHOP monoclonal antibody (Cell Signaling Technology, 2895); mouse anti-Tau-5 monoclonal antibody (Abcam, ab80579); mouse anti-Tau-5 monoclonal antibody (Invitrogen, AHB0042); rabbit anti-Tau (phospho S199) monoclonal antibody (Abcam, ab81268); rabbit anti-Tau (phospho T231) monoclonal antibody (Abcam, ab151559); rabbit anti-Tau (phospho S396) monoclonal antibody (Abcam, ab109390); rabbit anti-DNAJC3 monoclonal antibody (Cell Signaling Technology, 2940); rabbit anti-ERp57 polyclonal antibody (Abcam, ab10287); mouse anti-P4HB monoclonal antibody (Abcam, ab2792); mouse anti-GFP monoclonal antibody (Cell Signaling Technology, 2955); rabbit anti-SQSTM1/p62 polyclonal antibody (Cell Signaling Technology, 51145); rabbit anti-LC3B polyclonal antibody (Abcam, ab51520); rabbit anti-BDNF monoclonal antibody (Abcam, ab108319); rabbit anti-GAPDH monoclonal antibody (Cell Signaling Technology, 2118).

TS-FITGE

Procedures for TS-FITGE was previously described.[1] Briefly, HEK293 BiFC-tau cells or SH-SY5Y cells were treated with dimethylsulfoxide (DMSO, 0.1 %), SB1607 (10 μM), and SB1617 (10 μM), respectively, in the absence or presence of thapsigargin (200 nM) for 3 h. Application of SB1607, a significantly less active compound comparing to SB16xx compounds during the target identification process. The cell suspension was heated at a range of the designated temperatures for 3 min, followed by 25
°C for 3 min. The heated cell was washed with PBS and resuspended in lysis buffer A (0.4% NP-40 in PBS supplemented with Protease Inhibitor Cocktail). The cell suspension was freeze-thawed 3 times in liquid nitrogen for cell lysis. The cell lysate was clarified by centrifugation for 20 min at 20000 g, 4 °C. The protein concentration of the soluble fraction was quantified with Pierce BCA protein assay. 50 μg of the protein was precipitated with cold acetone followed by centrifugation for 7 min at 20000 g, 4 °C. The residual pellet was resuspended with 10 μl of conjugation buffer (30 mM Tris-HCl at pH 8.6, 2 M thiourea, 7 M urea, and 4% w/v CHAPS). 1 μl of 0.4 mM Cy2-N-hydroxysuccinimide (NHS) (for DMSO-treated group), Cy3-NHS (for SB1607-treated group), or Cy5-NHS (for SB1617-treated group) was mixed to the proteins and incubated at 4 °C for 45 min. The dye-conjugated proteomes were precipitated with cold acetone and resuspended with 50 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 40 mM DTT, and 1% IPG buffer). The same amount of DMSO-treated, SB1607-treated, and SB1617-treated samples were mixed, then a total of 150 μg (50 μg for Cy2-, Cy3-, and Cy5-labeled each) of proteomes was loaded on a 24-cm Immobiline Drystrip gel [GE Healthcare]. Isoelectric focusing was done by Ettan IPGphor 3 [GE Healthcare] followed by polyacrylamide gel electrophoresis (PAGE) with Ettan DALTsix system [GE Healthcare]. The gel was scanned with Typhoon Trio [GE Healthcare]. The protein spot location and fluorescence signals were analyzed by Melanie [GE Healthcare, Version 9.2.3]. The Cy2- (DMSO), Cy3- (SB1607), and Cy5- (SB1617) fluorescent intensity of each protein spot on TS-FITGE images were quantified and normalized by Melanie. Normalized Cy5/Cy2 and Cy5/Cy3 were used for a scatter plot to visualize thermal stabilization only by SB1617. Experimentally reproducible spots from the double-positive area on the scatter plot (Cy5/Cy2 and Cy5/Cy3) were analyzed by LC-MS/MS analysis.

In-gel digestion and mass spectrometry. The protein spots from the silver-stained gel were excised, destained, and digested with trypsin. The mixture was evaporated in SpeedVac and then dissolved in 10% acetonitrile with 0.1% formic acid. The resulting peptides were desalted in a trap column (180 μm × 20 mm, Symmetry C18) and separated on a C18 reversed-phase analytical column (75 μm × 200 mm, 1.7 μm, BEH130 C18) [Waters]. Mass spectrometry was done by nanoAcquity UPLC-ESI-QTOF/MS (SYNAPT G2-Si HDMS) [Waters] with an electrospray ionization PicoTip (±10 μm i.d., New Objective). The data were converted to .pkl files by Protein Lynx Global Server and searched by MASCOT engine with the SwissProt database.

CETSA

HEK293 BiFC-tau cells were treated with DMSO (0.1%), SB1607 (10 μM), or SB1617 (10 μM), respectively, in the presence of thapsigargin (200 nM) for 3 h. The cell suspension was heated at a range of the designated temperatures for 3 min, followed by 25 °C for 3 min. The heated cell was washed with PBS and resuspended in lysis buffer A. The cell suspension
was freeze-thawed 3 times in liquid nitrogen for cell lysis. The cell lysate was clarified by centrifugation for 20 min at 20000 g, 4 °C. The same volume of clarified cell lysate was combined with SDS buffer, then subjected to immunoblotting.

**Pulldown assay**

SH-SY5Y cells were seeded on 6-well plates, then treated with 1 µM thapsigargin and 5 µM SB1624 (probe compound) in the presence or absence of 40 µM SB1617 for 2.5 h. Cells were exposed to 356 nm UV irradiation for 30 min on ice. After washing with cold PBS, cells were lysed in RIPA buffer and cleared by centrifugation at 20000 g for 15 min, 4 °C. The protein concentration of supernatants was determined by Pierce BCA Protein Assay Kit and the protein concentration was adjusted to 1 mg/mL. Click reaction was pursued on the proteome with biotin-azide (50 µM, Sigma Aldrich), TBTA (100 µM, Sigma Aldrich), CuSO₄ (1 mM, Sigma Aldrich), TCEP (1 mM, Sigma Aldrich), and iBuOH (5%) for 1 h. Cold acetone was added to the mixture for 20 min at −20 °C for the protein precipitation. After the centrifugation at 15000 g for 10 min, 4 °C, the pellet was dissolved in PBS containing 1.2% SDS by sonication and diluted to 0.2% sodium dodecyl sulfate (SDS) by adding PBS. The samples were incubated with 20 µL streptavidin agarose beads [Sigma Aldrich] by rotating for 3 h at room temperature. The beads were washed with PBS containing 0.2% SDS four times. The proteins were eluted by boiling with a 3× SDS sample buffer and analyzed by SDS-PAGE and western blot.

**Surface plasmon resonance (SPR) assay**

SPR experiments were performed using a Biacore T100 instrument [GE Healthcare]. Recombinant PDIA3 or DNAJC3 proteins were immobilized through amide bonds to a CM5 sensor chip [GE Healthcare] by activating the carboxyl group on the CM5 chip with a 1:1 mixture of N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide. The protein immobilization reactions were carried out in PBS with 0.005% Tween 20 at pH 4.7 and pH 5.0 for PDIA3 and DNAJC3, respectively. Bindings between the compounds and proteins were monitored by flowing various concentrations of compounds in PBS (pH 7.3) containing 3% DMSO and 0.005% Tween 20 at 25 °C. Data were analyzed to calculate kinetic parameters by fitting sensorgrams with the 1:1 binding model using Biacore T100 Evaluation software [GE Healthcare].

**PEG maleimide modification assay**

Cysteine modification assay using PEG maleimide was carried out by following a paper. After compound treatment, BiFC-tau HEK293 cells were washed with cold PBS and incubated with 20 mM N-ethylmaleimide (NEM, Sigma Aldrich) in PBS for 20 min on ice to alkylate the reduced form of cysteines. After washing with cold PBS, the cells were lysed in RIPA buffer. For the whole-cell analysis, lysates were centrifuged with 200 g for 5 min at 4 °C.
to remove insoluble matters. The protein concentration of supernatants was determined by Pierce BCA Protein Assay Kit. Equal amounts of each lysate were treated with 12 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma Aldrich) for 20 min at room temperature to reduce the oxidized forms of PDI. Lysates were incubated with 15 mM of methoxy polyethylene glycol 5000 maleimide (mPEG-mal5000, Sigma Aldrich) for 1 h at room temperature. After adding with the SDS sample buffer to the lysates and brief vortex, SDS samples were directly used for SDS-PAGE and western blot.

RNA extraction and quantitative real-time PCR

To analyze the levels of autophagy-related genes, SH-SY5Y cells were treated with 1 µM thapsigargin in the presence or absence of 5 µM SB1617 for 8 h. Total RNA was extracted using the RNeasy kit [Qiagen] according to the manufacturer’s instructions. RNA was quantified using NanoVue [GE Healthcare] and cDNAs were prepared with AccuPower CycleScript RT PreMix dT20 [Bioneer] according to the manufacturer’s instructions. Quantitative RT-polymerase chain reaction (qRT-PCR) experiments were conducted with KAPA SYBR FAST ABI Prism qPCR Master Mix [KAPA Biosystems]. The data were analyzed by the comparative Ct method and normalized against housekeeping genes.

Live-cell imaging for mCherry-GFP-Tau fluorescence

DeltaVision Elite imaging system [GE Healthcare] was used for imaging of HEK293T cells stably expressing mCherry-GFP-Tau. For live-cell imaging, the chamber was maintained with 37 °C, 5% CO2 condition. Cells were treated with 100 nM thapsigargin and 40 µM SB1617 and monitored by real-time imaging. Images were obtained with a 60× scale, using mCherry/mCherry (excitation: 575/25 nm, emission: 625/45 nm), GFP/GFP (excitation: 475/28 nm, emission: 525/48 nm) filter sets. Images were analyzed and merged with SoftWorks deconvolution software.

Animals and ethics statement

CD-1 (ICR) male mice (2–3 months old, weight 25–35 g; DBL, Korea) were used for the TBI study. Mice were kept in an adjusted environment (22 ± 2 °C, 55 ± 5% humidity, 12-h light/dark cycle with lights on at 8:00 am) and received a standard diet by Purina, Korea. All rats were given ad libitum access to water and food. To avert and minimize stress related to transportation, experiments were initiated after the animals were allowed 1 week of acclimatization to environmental conditions. The animal care protocol and experimental procedure were approved by the Committee on Animal Use for Research and Education at Hallym University (protocol # Hallym 2018-82), following the National Institutes of Health guidelines. This manuscript was also written in compliance with the Animal Research: Reporting In Vivo Experiments guidelines.[3]
Pharmacokinetic study

CD-1 (ICR) male mice (7 weeks old, KOATECH, Korea) were administrated with SB1617 (5% DMSO/35% PEG-400/65% DW) intravenously (5 mg/kg, 3.3 mL/kg) or intraperitoneally (5 mg/kg, 3.3 mL/kg), and blood was collected at the designated time points from orbital venous plexus. The concentration of SB1617 was investigated by LC-MS/MS analysis (Agilent 1200, 4000 Qtrap) from the plasma samples. Pharmacokinetic parameters were obtained from the plasma concentration–time plot using WinNonlin software [Pharsight, USA].

Blood-brain barrier penetration study

CD-1 (ICR) male mice (7 weeks old, KOATECH, Korea) were intraperitoneally (5 mg/kg, 3.3 mL/kg) administrated with SB1617 (5% DMSO/50% PEG-400/45% DW). Blood samples were collected in heparin-treated tubes at the designated time points (0.5 h and 3 h) from orbital venous plexus and the mice were sacrificed followed by the brain tissue harvest. Plasma and brain homogenate samples were analyzed by LC-MS/MS (Agilent 1260, Agilent 6460). The ratio of SB1617 in the brain to that in the plasma were calculated.

Experimental controlled cortical impact model for traumatic brain injury (TBI)

A controlled cortical impact (CCI) model of experimental TBI was performed as previously described.[4] Briefly, mice were deeply anesthetized with 3% inhaled isoflurane in a 70:30 mixture of nitrous oxide and oxygen using an isoflurane vaporizer [VetEquip], positioned in a stereotaxic apparatus [David Kopf Instruments], and maintained on 1–1.5% isoflurane. A craniotomy was made approximately 4 mm over the right hemisphere using a portable drill (2 mm lateral to the midline and 1 mm posterior to the bregma). Using a controlled cortical impact device (Leica Impact One, Leica Biosystems), a 2 mm flat-tip impactor was accelerated down to a 1.4 mm depth at a velocity of 5 m/sec. All rats were maintained at a core temperature of 36–37.5 °C with a homeothermic blanket control unit [Harvard Bioscience] during and after surgery, until ambulatory. Animals were assigned randomly to traumatic brain injury according to an online randomization tool (randomizer.org). Sham-operated groups only received craniotomy.

SB1617 administration for TBI experiments

To investigate the neuroprotective effects of SB1617 against brain injury after TBI, SB1617 was intraperitoneally administered twice per day at a dose of 5 mg/kg (5% DMSO/50% PEG-400/45% DW). Control group mice were treated intraperitoneally with equal volumes of vehicle.
**Tissue preparation**

Mice were deeply anesthetized with urethane (1.5 g/kg, intraperitoneal) in saline (0.9% NaCl) at a volume of 0.01 mL/g body weight. A toe pinch was used to evaluate the effectiveness of anesthesia. Then, mice were intracardially perfused with saline, followed by 4% paraformaldehyde in PBS. The brains were post-fixed with 4% paraformaldehyde for 1 h and immersed in 30% sucrose for cryoprotection. Thereafter, the entire brain was frozen and coronally sectioned on a cryostat microtome (CM1850, Leica) at 30 μm thickness.

**Immunohistochemistry**

Sections were immersed in 1.2% H₂O₂ for 20 min at room temperature to inhibit endogenous peroxidase activity. After washing in PBS, the sections were incubated with a mouse monoclonal anti-NeuN antibody (diluted 1:500; Millipore) in PBS containing 0.3% Triton X-100 at 4 °C overnight to evaluate neuronal loss after TBI. After washing in PBS, the sections were incubated in biotinylated anti-mouse IgG (diluted 1:250; Vector) to detect NeuN antibody and biotinylated anti-rat IgG (diluted 1:250; Vector) to investigate the degree of leakage of endogenous immunoglobulin G after TBI for 2 h at room temperature. Thereafter, sections were immersed in the avidin-biotin-peroxidase complex [Vector] for 2 h at room temperature. Between incubations, the sections were washed with PBS. The immune reaction was visualized with 3,3’-diaminobenzidine [Sigma-Aldrich] in 0.01 M PBS containing 0.015% H₂O₂, and the sections were mounted on gelatin-coated slides. The immunoreactions were observed under an Olympus IX70 inverted microscope.

**Immunofluorescence analysis**

Immunofluorescence labeling was performed from routine immunostaining protocols as previously reported. Sections were immersed in 1.2% hydrogen peroxide for 15 min at room temperature to inhibit endogenous peroxidase activity. After washing in PBS, the sections were incubated with each specific type of polyclonal or monoclonal primary antibody in PBS containing 0.3% Triton X-100 at 4 °C overnight. Primary antibodies used in this study were as follows: mouse monoclonal anti-4-hydroxynonenal (4-HNE; diluted 1:500; Alpha Diagnostic International), mouse monoclonal anti-Tau (Tau5; diluted 1:500; Abcam), mouse monoclonal anti-phospho Tau (Ser202, Thr205; AT8; diluted 1:200; Invitrogen), and mouse monoclonal anti-PDI (diluted 1:100; Abcam). After washing in PBS, sections were applied with fluorescent-conjugated secondary antibodies (diluted 1:250; Invitrogen). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; diluted 1:1000; Invitrogen). Fluorescence-stained sections were mounted on gelatin-coated slides and coverslipped with DPX [Sigma-Aldrich]. The sections were photographed using a confocal microscope (LSM 710; Carl Zeiss).
Quantification of IHC/IF data

To count the NeuN-positive cells, every 6th sections were collected at an interval of 180 μm from 1.2 to 2.1 mm, posterior to the bregma. Five coronal sections were analyzed from each mouse using the microscope with a 20× objective. These sections were then coded and given to a blinded experimenter who counted the total number of NeuN-positive cells in the hippocampal CA1 and cortex from the ipsilateral hemisphere. Data were expressed as the average number of NeuN-positive cells per each region. To quantify PDI-, Tau5-, AT8-, 4HNE-immunofluorescence intensity, five coronal sections were evaluated by a blinded experimenter using ImageJ [National Institute of Health]. Briefly, the image was loaded into ImageJ and changed into 8-bit via the menu option (Image/Color/Split Channels). The image was binarized, and the menu option (Analyze/Measure) was selected, and then each immunofluorescence signal was expressed as the mean gray value.

Assessment of neurological deficits

To assess whether SB1617 administration attenuated TBI-induced neurological deficits, neurological function was evaluated by using a neurological severity score (NSS).

These tests were performed at 1, 12, 24, 48, 72 h, and 7 days after TBI or Sham surgery. The NSS assesses the functional neurological status of mice based on the presence of reflexes and the ability to perform motor (muscle status, abnormal movement) and behavioral tasks such as beam walking, beam balance, and spontaneous locomotion. This is graded from 0 to 10 (0 = normal function: 10 = maximal deficit). One point is awarded for failing to perform a particular task or for the lack of a tested reflex. Thus, a higher score implies a more severe injury.

Pole climbing test

Pole climbing test was performed to analyze the motor coordination of the mice. Mice were placed head upward on the tip of the vertical pole (60 cm high with a rough surface). Thereafter, both time taken to orientate the body completely downwards (time to turn) and to reach the bottom with all four paws (time to finish) were recorded. The maximal testing time was 60 s. Each mouse was tested three times at the time points given followed by a calculation of means that were used for statistical analysis.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (GraphPad, San Diego, CA, USA). For Figure 2E, statistical relevance was assessed using the one-way analysis of variance (ANOVA) test with Bonferroni’s post hoc analysis. For Figure 2G, the statistical relevance of siRNA-treated groups compared to the scRNA-treated control was assessed using the one-way ANOVA test with Dunnett’s post hoc analysis. For Figure 3F, statistical relevance was assessed using the two-way ANOVA test with Bonferroni’s post hoc
analysis. For Figure 3G, multiple comparisons were performed within sets for each gene using the one-way ANOVA test with Bonferroni’s post hoc analysis. For the in vivo results (Figure 4), comparisons were conducted using the repeated measures ANOVA test with Student-Newman-Keuls post hoc analysis. All data are presented per group as the mean ± standard error of the mean (SEM). A P value < 0.05 was considered significant.
**Supplementary Figures**

**Figure S1.** BiFC-tau screening system validation ($Z'$ factor > 0.5) and screening data. (A) The robustness of the high-throughput BiFC-tau screening system was validated by obtaining $Z'$-factor as higher than 0.5. Fold chances of fluorescent intensity in BiFC-tau Venus HEK293 cells were plotted and $Z'$- factor was calculated by applying the equation shown in (A). (B) Screening data using HEK293 BiFC-tau Venus cells. Hit compounds were selected below 50% of fluorescence intensity induced by thapsigargin treatment with >90% viability during the screening.

|        | avg | stdv | $Z'$ |
|--------|-----|------|------|
| DMSO   | 1.0 | 0.1  |      |
| thapsigargin | 4.9 | 0.4  | 0.6  |

\[ Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \]

mean ($\mu$), standard deviation ($\sigma$), positive controls ($\mu_p, \sigma_p$), negative controls ($\mu_n, \sigma_n$)
Figure S2. Structure-activity relationship study data. (A) Schematic representation of chemical structures of the pyrimidodiazepine analogues investigated for structure-activity relationship study. (B–I) Dose-dependency data of the compounds shown in (A) and Table S1 for the inhibition of BiFC-tau-Venus assembly. The varying concentrations of the compounds were co-treated with 80 nM thapsigargin to HEK293-BiFC-tau-Venus cells for 24 h. Data are shown as the mean ± SEM. IC50 values are listed in Table S1.
Figure S3. SB1617 treatment without thapsigargin did not alter BiFC-tau Venus-fluorescence intensity. The varying concentrations of SB1617 were treated to HEK293 BiFC-tau-Venus cells without thapsigargin for 24 h. Data are shown as the mean ± SEM.
Figure S4. Quantification data of Figure 1C for total tau (Tau5) and phospho-tau (S199, T231, S396) levels (A), and for the ratio of p-tau to total tau (B) by western blot analysis upon treatment of 80 nM thapsigargin (TG) with either 5 μM of SB1617 or SB1607 for 20 h in BiFC-tau Venus HEK293 cells. TG treatment increased the levels of total tau as well as p-tau, whereas SB1617 treatment reduced the levels of both tau and p-tau increased by TG. However, there was no significant change in the ratio of p-tau to total-tau upon SB1617 treatment. Data are shown as the mean ± SEM. Multiple group comparisons were performed by one-way ANOVA with Bonferroni’s multiple comparison analysis, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
Figure S5. Post-sorted cell populations within HEK293 DsRed-IRES-EGFP-Tau stable cells were used for tau analysis. The stable cells showed more than 50-times population of double-positive cells (FITC- and PE-positive) compared to that of the negative cells (HEK293, not transfected). FITC channel: EGFP-tau; PE channel: DsRed.

### HEK293 DsRed-IRES-EGFP-Tau stable cells

R-1 (red color, live cells), H-2 (FITC-positive), H-3 (PE-positive)  
**Green color**: live and double-positive (FITC and PE)

|     | events | % total | % Live |
|-----|--------|---------|--------|
| **Negative** |        |         |        |
| All events    | 10000  | 100     |        |
| R-1 (Live)    | 7623   | 76.23   | 100    |
| R-1 and H-2 and H-3 | 116  | 1.16    | 1.52   |

|     | Events | % total | % Live |
|-----|--------|---------|--------|
| **Stable** |        |         |        |
| All events    | 16879  | 100     |        |
| R-1           | 11637  | 68.94   | 100    |
| R-1 and H-2 and H-3 | 8757 | 51.94   | 75.34  |
Figure S6. Gating strategy used for the investigation of tau proteostasis alterations in DsRed-IRES-EGFP-tau HEK293 cells. FITC-positive and PE-positive areas were determined as higher than $2 \times 10^3$ based on the signals from the negative cells (See Figure S4). We matched the total number of double-positive cells in each condition for fair comparison in FACS histogram, of which x-axis indicates FITC/PE intensity per cell (FITC channel: EGFP-tau; PE channel: DsRed). Representative data comparing thapsigargin-treated versus thapsigargin & SB1617-treated (A) or thapsigargin & SB1607-treated (B) cells are shown as overlaid histograms. Mean values of FITC/PE for each condition were converted to percentage values, then plotted as bar-graphs for statistical analysis.
Figure S7. (A) Quantitative data of flow cytometry data in Figure 1G. DsRed-IRES-EGFP-tau HEK293 cells were treated with 100 nM TG together with either 5 µM of SB1617 or SB1607 for 20 h (n ≥ 4). Multiple group comparisons were performed using one-way analysis of variance (ANOVA) with Bonferroni’s post hoc analysis, ***P ≤ 0.001. (B) Dose-dependency data of SB1617 in DsRed-IRES-GFP-tau HEK293 cells. Cells were treated with varying concentrations of SB1617 in the presence of 100 nM thapsigargin (TG) for 20 h. The fluorescence ratio of GFP to DsRed per cell was measured by FACS. Statistical significance for TG & SB1617-treated groups compared to the TG-treated group was assessed by one-way ANOVA with Dunnett’s multiple comparison analysis., ns. P > 0.05, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Data in (A) and (B) are shown as the mean ± SEM.
Figure S8. Full-range images of TS-FITGE 2D gels shown in Figure 2A. Merged gel images of Cy2- (blue, DMSO), Cy3- (green, SB1607), and Cy5- (red, SB1617) channels at the designated temperature. Reproducible red spots are marked with numbers (from 1 to 11). The identity of proteins from those spots are listed in Table S2.
Figure S9. Quantification data of TS-FITGE in Figure S8. The Cy2- (DMSO, vehicle), Cy3- (SB1607, weakly active compound), and Cy5- (SB1617, active compound) fluorescent intensity of each protein spot on TS-FITGE images were quantified and normalized by Melanie (GE Healthcare, Version 9.2.3). Normalized Cy5/Cy2 and Cy5/Cy3 signals were used for the scatter plots to visualize the thermal stabilization only by SB1617. The red box in each scatter plot is the quadrant with protein spots with significant thermal stabilization. Red dots are the protein spots quantitatively selected from 2D gels at the dedicated temperature by Melanie software and confirmed their protein identity by LC-MS/MS analysis.
**Figure S10.** Target validation for candidate proteins identified from TS-FITGE results shown in Table S2. (A) BiFC-tau Venus HEK293 cells were transfected with siRNAs of each target candidate protein for 48 h then were treated with 80 nM thapsigargin for 20 h. Compared to the siPDIA3 (si1-PDIA3)-treated condition, other gene knockdown conditions did not reduce the BiFC-tau-Venus fluorescence intensity. Statistical significance of siRNA-treated groups compared to the scRNA-treated control was assessed by one-way ANOVA with Dunnett’s multiple comparison analysis. ns. P > 0.05, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Data are shown as the mean ± SEM. (B) The protein knockdown efficiency was demonstrated by qRT-PCR data. Cells were transfected with siRNAs for 48 h. Data are shown as the mean ± SEM.

**Figure S11.** Representative images of cellular thermal stability shift assay (CETSA) data of SB1607- and SB1617-treated BiFC-tau Venus HEK293 cells toward DNAJC3, PDIA3, and GAPDH, visualized by immunoblotting. Quantified data are shown on the right side with T_m values. Both DNAJC3 and PDIA3 are thermally stabilized upon SB1617 treatment compared to the conditions with a vehicle or SB1607 treatment.
Figure S12. The biophysical kinetic studies of SB1617 toward the interactions with PDIA3 or DNAJC using surface plasmon resonance (SPR) spectroscopy. SPR data were used to investigate biophysical kinetics for the association ($k_a$), the dissociation ($k_d$), and the binding affinity ($K_D$) of SB1617 toward immobilized PDIA3 (A) and DNAJC3 (B) ($n = 3$). The overlaid SPR sensorgrams are from 0.125, 0.25, 0.5, 1.5, 3, 4, 6 µM of SB1617 in (A) and 0.625, 1.25, 2.5, 5, 10 µM of SB1617 in (B) in an order of increasing responses.
**Figure S13.** PEG-maleimide modification assay to monitor the oxidation status of PDI upon siRNA-based PDIA3 depletion. (A) Immunoblot data for PDI and GAPDH after PEG-maleimide modification upon siRNA treatment. Cells were treated with 10 mM 1,4-dithiothreitol (DTT) and 5 mM tetramethylazodicarboxamide (DA) for 15 min to obtain the reduced and oxidized forms of PDI, respectively. Oxidized forms of PDI were detected at the higher molecular weight than reduced PDI shown at 57 kDa. (B) Knockdown efficiency for PDIA3 was observed by anti-PDIA3 antibody. The data were taken from the cells depleted with PDIA3 using siRNAs but were not processed for the PEG-maleimide modification steps. Data shown in (A) and (B) were obtained from parallel experiments.
Figure S14. Quantification data of Figure 3A for total tau (Tau5) and phospho-tau (S199, T231, S396) levels (A), and for the ratio of p-tau to total tau (B) by western blot analysis. Depletion of either PDIA3 or DNAJC3 suppressed total-tau and p-tau levels in tau-overexpressing cells, whereas the depletion of either PDIA3 or DNAJC3 induced no significant change in a ratio of p-tau to total-tau. Data are shown as the mean ± SEM. Multiple group comparisons were performed by one-way ANOVA with Bonferroni’s multiple comparison analysis, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
**Figure S15.** PDIA3 and DNAJC3 double knockdown effects on tau assembly inhibition in BiFC-tau Venus HEK293 cells. BiFC-tau cells were transfected with si1-PDIA3 and/or si1-DNAJC3 for 48 h, then treated with 80 nM thapsigargin for 20 h. For double knockdown experiments, a half concentration of siRNAs (5 nM) was used compared to that used in Figure 2E. Statistical significance of siRNA-treated groups compared to the scRNA-treated control was assessed by one-way ANOVA with Dunnett’s multiple comparison analysis., * P ≤ 0.05, ** P ≤ 0.01. Data are shown as the mean ± SEM.

**Figure S16.** PDIA3 and DNAJC3 double knockdown effects on tau clearance in DsRed-IRES-EGFP-tau HEK293 cells. Cells were transfected with si2-PDIA3 and/or si1-DNAJC3 for 48 h, then treated with 100 nM thapsigargin for 20 h. The ratio between GFP/DsRed fluorescence intensity per cell was measured by FACS analysis. For double knockdown experiments, a half concentration of siRNAs (5 nM) was used compared to that used in Figure 2G. Statistical significance of siRNA-treated groups compared to the scRNA-treated control was assessed by one-way ANOVA with Dunnett’s multiple comparison analysis., * P ≤ 0.05, ** P ≤ 0.01. Data are shown as the mean ± SEM.
Figure S17. PDIA3 and DNAJC3 double knockdown effects on PERK signaling activation in BiFC-tau Venus HEK293 cells. BiFC-tau cells were transfected with si2-PDIA3 and/or si1-DNAJC3 for 48 h. PERK activation upon gene knockdown was confirmed by increased p-eIF2α (A) and enhanced ATF4 (B) levels in BiFC-tau cells.

Figure S18. Investigation of the PERK signaling pathway by western blot analyses. PERK signaling pathway was investigated by western blot analyses upon treatment with thapsigargin (TG) with or without SB1617 up to 24 h. (A) PERK signaling activation by monitoring phospho-PERK and phospho-eIF2α up to 24 h. Human neuroblastoma SH-SY5Y cells were treated with 1 μM TG with or without 10 μM SB1617. (B) PERK activation (p-PERK, p-eIF2α, and ATF4) was promoted using a lower dosage of SB1617. SH-SY5Y cells were treated with 1 μM TG with or without 5 μM SB1617.
**Figure S19.** Conditional activation of the PERK signaling pathway by SB1617 treatment monitored using western blot analyses in BiFC-tau Venus HEK293 cells. (A) To mimic the ER stress condition, BiFC-tau cells were treated with 200 nM thapsigargin (TG) without or with 10 µM SB1617. (B) BiFC-tau cells were treated with 200 nM TG only or 10 µM SB1617 only.

**Figure S20.** Upregulation of PDIA3 and DNAJC3 mRNA levels upon ER stress. mRNA levels of PDIA3 and DNAJC3 were assessed by RT-qPCR. SH-SY5Y human neuroblastoma cells were treated with 1 µM thapsigargin (TG) with or without 5 µM SB1617 for 4 h (A) and 8 h (B). All mRNA levels were normalized to GAPDH. Data are shown as the mean ± SEM. Multiple group comparisons were performed by one-way ANOVA with Bonferroni’s multiple comparison analysis, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
Figure S21. Quantification data of western blot analysis in Figure 3H for the ratio of LC3B-II to LC3B-I (A) and the level of p62 (B) upon treatment of 5 µM SB1617 in the absence and presence of 500 nM TG for 6–8 h in HEK293-BiFC-tau cells. Data are shown as the mean ± SEM.

Figure S22. Representative data (A) and the quantitative data (B) of immunoblot analysis of total tau upon treatment for 18 h with SB1617 (5 µM) and TG (100 nM) in the absence and presence of the autophagy inhibitors, 3-methyladenine (3-MA, 5 mM) and bafilomycin A1 (Baf, 50 nM), in HEK293 BiFC-tau cells. Results are representative of at least three independent experiments. Data are shown as the mean ± SEM. Multiple group comparisons were performed by two-way ANOVA with Bonferroni’s post hoc analysis, ns, P > 0.05*, P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
Figure S23. *In vivo* pharmacokinetic characterization of SB1617 using male ICR mice. (A) The concentration of SB1617 in plasma up to 24 h. Intraperitoneally (IP) injected data are shown in red dots and intravenously (IV) injected data are shown in blue dots. (B) Pharmacokinetic parameters. Data are shown as the mean ± SEM (male ICR mice, n = 3).
Figure S24. Evaluation of *in vivo* blood-brain barrier permeability of SB1617. SB1617 concentration in plasma and brain tissue at the indicated times. Male ICR mice were intraperitoneally injected with SB1617 as 5 mg/kg. Data are shown as the mean ± SEM (n = 3).

| Time (h) | Concentration (ng/mL) | Brain/Plasma ratio |
|----------|-----------------------|--------------------|
|          | Plasma | Brain tissue       |                    |
| 0.5      | 885 ± 187 | 421 ± 58.2 | 0.50 ± 0.14 |
| 3        | 92.4 ± 29.8 | 55.9 ± 5.00 | 0.66 ± 0.21 |

Figure S25. Timeline showing the *in vivo* experimental design with a TBI mouse model. Initial time points for vehicle/SB1617 injections are shown by red arrows. The first compound/vehicle injection is right after the TBI/sham surgery, and the second compound/vehicle injection is 12 h post-TBI/sham surgery for the investigation of 24 h vehicle/SB1617 treated condition (vehicle/SB1617 treated twice in total).
Figure S26. (A) Representative immunofluorescence images showing expression of the ER stress marker PDI in the ipsilateral hippocampal CA1 and cortex sections at 24 h after sham surgery or TBI. Scale bar, 20 µm. (B) Quantification of the expression of PDI. Data are represented as means ± SEM.; n = 3–6 from each group; * P < 0.05 versus vehicle-treated TBI group.

Figure S27. Representative immunofluorescence images showing expression of the oxidative stress marker 4HNE in the ipsilateral hippocampal CA1 (A) and cortex (C). Scale bar, 20 µm. Quantification of the intensity of 4HNE from ipsilateral hippocampal CA1 (B) and cortex (D). Data are mean ± SEM; n = 3–6 from each group, * P < 0.05 versus vehicle-treated TBI group.
Figure S28. Quantitative data of Figures 5A–C. Ipsilateral hippocampal CA1 and cortex sections from vehicle- and SB1617-treated mice at 24 h (A, B) 72 h (C) after sham surgery or TBI were stained for total tau (A), phsopho-tau (B), and live neuronal cells (C) with anti-Tau5, anti-AT8, and NeuN, respectively. Data are represented as means ± SEM; n = 3–6 from each group; * P < 0.05 versus vehicle-treated group.
## Supplementary Tables

### Table S1. Description for the small molecule screening.

| Category       | Parameter                  | Description                                                                 |
|----------------|----------------------------|------------------------------------------------------------------------------|
| Assay          | Type of assay              | Cell-based                                                                  |
|                | Target                     | Phenotype-based, HTS/HCS format                                             |
|                | Primary measurement        | Fluorescence intensity per cell from BiFC-tau-Venus                         |
|                | Key reagents               | Thapsigargin                                                                |
|                | Assay protocol             | ‘Tau screening’ section in methods                                          |
|                | Additional comments        | The assay was adapted from ref [8].                                          |
| Library        | Library size               | 3,000 compounds                                                             |
|                | Library composition        | Small-molecules                                                             |
|                | Source                     | In house synthesized library                                                |
|                | Additional comments        | Details about parts of library design can be found in ref[9].                |
| Screen         | Format                     | 384-well plates                                                             |
|                | Concentration(s) tested    | 10 µM compound, 0.1 % DMSO                                                  |
|                | Plate controls             | In each plate, positive (thapsigargin and DMSO) and negative (DMSO), control active compound (thapsigargin and P52F08 as 70% inhibition for the BiFC-Venus fluorescence intensity, the compound is an unpublished molecule) |
|                | Reagent/ compound dispensing system | Pin tool 0.1 µL for screening compound treatment, multichannel pipette for thapsigargin treatment |
|                | Detection instrument and software | INCell Analyzer 2000 (GE Healthcare), Developer software (GE Healthcare)     |
|                | Assay validation/QC        | Z’ score, 0.6 (Figure S1B)                                                  |
|                | Normalization              | DMSO control as 0 % and thapsigargin control as 100 %                       |
| Post-HTS analysis | Hit criteria              | > 50 % inhibition, > 90 % viability, solubility                             |
|                | Hit rate                   | 4 %                                                                         |
|                | Additional assay(s)        | Investigation of Tau and phospho-Tau by Western blot                        |
|                | Confirmation of hit purity and structure | Compounds were resynthesized.                                               |
**Table S2.** Protein list obtained from the red spots of TS-FITGE images shown in fig. S4 by mass spectroscopy analyses. Column Etc. \(^a\) Proteins selected for further target validation. Proteins known to be non-specific binders to aliphatic chains e.g. linkers.\(^{10}\) Proteins known to be non-specific binders from unpublished data.

| Spot # | Match to     | MW    | Mascot Score | Queries matched | Sequence Coverage (%) | Protein                                                                 | Etc  |
|-------|--------------|-------|--------------|-----------------|-----------------------|------------------------------------------------------------------------|------|
| 1     | HSP74_HUMAN  | 94240 | 347          | 20              | 30                    | Heat shock 70 kDa protein 4                                             | c    |
| 2     | C1TC_HUMAN   | 101495| 721          | 82              | 38                    | C-1-tetrahydrofolate synthase, cytoplasmic                               | a    |
|       | SIAS_HUMAN   | 40281 | 159          | 7               | 19                    | Sialic acid synthase                                                     | b    |
| 3     | ACAD8_HUMAN  | 45040 | 95           | 2               | 5                     | Acyl-CoA dehydrogenase family member 8, mitochondrial precursor          | c    |
| 4     | PGAM1_HUMAN  | 28786 | 151          | 10              | 37                    | Phosphoglycerate mutase 1                                               | b    |
| 5     | ECHM_HUMAN   | 31367 | 182          | 15              | 39                    | Enoyl-CoA hydratase, mitochondrial precursor                              | c    |
|       | PRDX6_HUMAN  | 25019 | 139          | 18              | 59                    | Peroxiredoxin-6                                                         | a    |
| 6     | GSTP1_HUMAN  | 23341 | 305          | 23              | 56                    | Glutathione S-transferase P                                             | a    |
| 7     | NAMPT_HUMAN  | 55487 | 307          | 28              | 47                    | Nicotinamide phosphoribosyltransferase                                   | a    |
|       | ATPA_HUMAN   | 59714 | 167          | 13              | 21                    | ATP synthase subunit alpha, mitochondrial precursor                      | b    |
| 8     | TXND5_HUMAN  | 47599 | 472          | 34              | 43                    | Thioredoxin domain-containing protein 5 precursor                        | c    |
|       | ACTB_HUMAN   | 41710 | 395          | 25              | 38                    | Actin, cytoplasmic 1                                                     | b    |
|       | ATPB_HUMAN   | 56525 | 258          | 13              | 28                    | ATP synthase subunit beta, mitochondrial precursor                       | l    |
| 9     | PDIA3_HUMAN  | 56747 | 817          | 47              | 49                    | Protein disulfide-isomerase A3 precursor                                 | a    |
|       | VATB2_HUMAN  | 56465 | 177          | 14              | 31                    | Vacuolar ATP synthase subunit B, brain isoform                           | c    |
|       | ALBU_HUMAN   | 69321 | 155          | 9               | 9                     | Serum albumin precursor                                                  | c    |
| 10    | DNJC3_HUMAN  | 57544 | 67           | 8               | 13                    | DNAJ homolog subfamily C member 3                                       | a    |
| 11    | GSTO1_HUMAN  | 27548 | 171          | 19              | 48                    | Glutathione omega-1 transferase                                          | c    |
Table S3. Description of the Neurological severity score (NSS) for mice.

| Task               | Description                                                                 | Points |
|--------------------|-----------------------------------------------------------------------------|--------|
| Exit circle        | Ability and initiative to exit a circle of 30 cm diameter (time limit: 3 minutes) | o      |
| Mono-/Hemiparesis  | Paresis of upper and/or lower limb of the contralateral side                  | o      |
| Straight walk      | Alertness, initiative, and motor ability to walk straight, once the mouse is put on the floor | o      |
| Startle reflex     | Innate reflex; the mouse will bounce in response to a loud hand clap         | o      |
| Seeking behavior   | Physiological behavior as a sign of “interest” in the environment           | o      |
| Beam balancing     | Ability to balance on a beam of 7 mm width for at least 10 seconds           | o      |
| Round stick balancing | Ability to balance on a round stick of 5 mm diameter for at least 10 seconds | o      |
| Beam walk: 3 cm    | Ability to cross a 30 cm long beam of 3 cm width                            | o      |
| Beam walk: 2 cm    | Same task, increased difficulty on a 2 cm wide beam                          | o      |
| Beam walk: 1 cm    | Idem, increased difficulty on a 1 cm wide beam                              | o      |

Maximal score 10

*Mice are awarded one point for each failure to perform a task.
Table S4. DNA sequences of the primers used for Real-Time quantitative PCR.

| Gene symbol | Forward | Reverse |
|-------------|---------|---------|
| ATF4        | GTTCTCCAGCGACAAGGCTA | ATCCCTCTTGTCTGTGTTGG |
| ATG3        | CCAACATGGAATGGGCTAC | ACCGCCAGCATCAGTGTG |
| ATG5        | AGTATCAGACACGGTATGG | TGCAAAGGCCCCTGACACTG |
| ATG12       | GAGACCAGCCTGTTAGCAA | CTGAACCCTCAGTGCAAAC |
| NBR1        | GGAAGCAGAAAGAGACCTGAG | CCAGAGTCTGTAGGAGTGTG |
| p62/SQSTM11 | GTGTAGGAACCCGCTACAA | GCGATCTTCCTCATCTGTC |
| GABARAP     | ACATGCCTACAGTGACGA | TTTCACTCCCTTCACAATC |
| GABARAPL2   | CGTGGAGTCCGGCGGATC | AGGCATGAGGACACATGCA |
| GAPDH       | ACCAGGCCCCAGCAAGCACAAG | TTCAAGGGGCTTACATGGCAACTG |
| GSTP1       | GACTACAACCTGCTGGACTT | ATTGATGGGAGGTTCACGTA |
| PRDX6       | ACAATTGTAAGAGCCCACAG | ACCAAAAACAAACACACCACAG |
| C1TC        | AAACCGAAGCCTTGTGTAAG | CCAGTCACCACAGCTATTC |
| NAMPT       | AGGGTTACAAAGGTTGCTGCCACC | CTCCAGGAAACCAGAGGAAT |
| DNAJC3      | AGATCGAGCAGAGGCCTATTT | TCTCGTTTTGCGACCTT |
| PDIA3       | CTGAACCTATCCAGAGCAGCA | ATACTTGCGCTCCAGGT |

S37
Synthetic Procedures and Compound Characterization

General Information on Synthetic Protocols

NMR spectra were obtained on an Agilent 400-MR DD2 Magnetic Resonance System (400 MHz, Agilent, USA), Varian/Oxford As-500 (500 MHz, Varian Assoc., Palo Alto, USA), or Bruker Avance 600 MHz Cryo-NMR Spectrometer (600 MHz, Bruker, Germany). Chemical shift values were recorded as parts per million (δ), referenced to tetramethylsilane (TMS) as the internal standard, or to the residual solvent peak (CDCl₃, ¹H: 7.26, ¹³C: 77.16, DMSO-d₆, ¹H: 2.50, ¹³C: 39.52). Multiplicities were indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); td (triplet of doublets); br s (broad singlet), br d (broad doublet) and so on. Coupling constants were reported in hertz (Hz). IR spectra were measured on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. High-resolution mass spectra were analyzed at the Mass Spectrometry Laboratory of National Instrumentation Center for Environmental Management (NICEM) at Seoul National University on a LCQ LC/MS (Thermo) using the electrospray ionization (ESI) method or by Ultra High-Resolution ESI Q-TOF mass spectrometer (Bruker). Commercially available reagents were obtained from Sigma-Aldrich, TCI, Acros, or Alfa Aesar, and used without further purification unless noted otherwise. All solvents were obtained by passing them through activated alumina columns of solvent purification systems from Glass Contour. Analytical thin-layer chromatography (TLC) was performed using Merck Kieselgel 60 F₂₅₄ plates, and the components were visualized by observation under UV light (254 and 365 nm) or by treating the plates with ninhydrin followed by thermal visualization. Flash column chromatography was performed on Merck Kieselgel 60 (230–400 mesh).
General procedure for the preparation of 1–9

The synthesis of the 1–9 was previously reported [see: Kim, J. et al. Diversity-Oriented Synthetic Strategy for Developing Chemical Modulator of Protein-Protein Interaction. Nat. Commun. 7, 13196 (2016)].[11]

Compound 1. The synthesis and characterization of compound 1 were identical to the previous report.[11]

Compound 2. A light yellow solid; $R_t = 0.15$ (DCM/MeOH = 20:1); 62 % overall yield; $^1$H NMR (400 MHz, CDCl3) $\delta$ 8.04 (br d, $J = 2.0$ Hz, 1H), 7.98 (s, 1H), 6.68 (br s, 1H), 4.16 (dd, $J = 9.8$, 4.7 Hz, 1H), 3.83 (m, 2H), 3.64 (t, $J = 10.0$ Hz, 1H), 3.28 (ddd, $J = 12.8$, 6.9, 2.2 Hz, 1H), 3.09 (S, 6H), 1.10 (m, 21H); $^{13}$C NMR (100 MHz, CDCl3) $\delta$ 167.4, 162.2, 157.7, 157.1, 92.4, 65.2, 64.5, 47.4, 42.0, 18.1, 12.0; HRMS(ESI+): Calcd for C19H36N5OSi $[M+H]^+$ 378.2684, found 378.2682, $\Delta$ppm –0.53; mp: 76–78 °C.

Compound 3. A yellow oil; $R_t = 0.36$ (DCM/MeOH = 20:1); 61 % overall yield; $^1$H NMR (400 MHz, CDCl3) $\delta$ 8.01 (s, 1H), 7.96 (s, 1H), 7.27 (m, 2H), 7.20 (m, 3H), 6.74 (br s, 1H), 4.18 (dd, $J = 9.8$, 4.3 Hz, 1H), 3.89 (m, 3H), 3.67 (t, $J = 10.2$ Hz, 1H), 3.60 (m, 1H), 3.30 (dd, $J = 11.0$, 7.4 Hz, 1H), 3.09 (s, 3H), 2.96 (m, 2H), 1.13 (m, 21H); $^{13}$C NMR (100 MHz, CDCl3) $\delta$ 167.1, 162.1, 157.8, 157.0, 139.0, 128.8, 128.6, 126.5, 92.7, 65.2, 64.4, 54.8, 47.5, 40.8, 34.1, 18.1, 12.0; HRMS(ESI+): Calcd for C26H42N5OSi $[M+H]^+$ 468.3153, found 468.3151, $\Delta$ppm –0.43;
Compound 4. A yellow oil; $R_f = 0.42$ (DCM/MeOH = 20:1); 57 % overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.24 (d, $J = 2.0$ Hz, 1H), 8.00 (s, 1H), 7.27–7.15 (m, 5H), 6.40 (br s, 1H), 4.85 (d, $J = 15.3$ Hz, 1H), 4.38 (d, $J = 15.3$ Hz, 1H), 4.19 (dd, $J = 9.6, 4.5$ Hz, 1H), 4.04 (m, 1H), 3.78 (m, 2H), 3.65 (t, $J = 9.8$ Hz, 1H), 3.25 (ddd, $J = 12.9, 7.0, 2.3$ Hz, 1H), 1.37 (d, $J = 6.3$ Hz, 3H), 1.24 (d, $J = 6.7$ Hz, 3H), 1.12 (m, 21H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 168.1, 162.3, 157.2, 157.1, 139.8, 128.3, 127.6, 126.7, 96.4, 65.2, 64.8, 56.7, 47.2, 45.9, 21.2, 20.7, 18.1, 12.0; HRMS(ESI+): Calcd for C$_{27}$H$_{44}$N$_5$O$_2$Si$^+$ [M+H]$^+$ 482.3310, found 482.3313, $\Delta$ppm +0.62;

Compound 5. The synthesis and characterization of 5 were identical to the previous report.$^{[11]}$

Compound 6. A yellow oil; $R_f = 0.5$ (DCM/MeOH = 20:1); 61% overall yield; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.52 (s, 1H), 8.27 (s, 1H), 7.58 (d, $J = 8.3$ Hz, 2H), 7.35 (br d, $J = 5.4$ Hz, 1H), 7.01 (d, $J = 8.3$ Hz, 2H), 4.32 (dd, $J = 9.5, 4.6$ Hz, 1H), 4.01 (dd, $J = 12.7, 6.4$ Hz, 1H), 3.95 (br s, 1H), 3.88 (s, 3H), 3.76 (t, $J = 10.3$ Hz, 1H), 3.28 (dd, $J = 12.7, 7.3$ Hz, 1H), 1.17 (m, 21H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 168.9, 161.3, 161.1, 159.0, 158.1, 131.9, 129.7, 113.9, 107.2, 64.94, 64.92, 55.5, 47.9, 18.08, 18.07, 12.0; HRMS(ESI+): Calcd for C$_{24}$H$_{37}$N$_4$O$_2$Si$^+$ [M+H]$^+$ 441.2680, found 441.2679, $\Delta$ppm –0.22.
Compound 7. A light yellow oil; $R_f = 0.39$ (DCM/MeOH = 20:1); 73% overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.15 (s, 1H), 8.05 (s, 1H), 7.30 (d, $J = 8.0$ Hz, 2H), 7.23 (d, $J = 8.0$ Hz, 2H), 6.86 (br s, 1H), 4.75, 4.68 (ABq, $J_{AB} = 15.6$ Hz, 2H), 4.18 (dd, $J = 9.8$, 4.3 Hz, 1H), 3.87 (m, 2H), 3.66 (t, $J = 10.0$ Hz, 1H), 3.33 (dd, $J = 11.0$, 7.4 Hz, 1H), 3.02 (s, 3H), 1.12 (m, 21H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 167.3, 162.5, 157.2, 157.1, 136.0, 133.3, 129.2, 128.9, 92.8, 65.2, 64.6, 55.8, 47.3, 40.4, 18.1, 12.0; HRMS(ESI+): Calcd for C$_{25}$H$_{39}$ClN$_5$OSi$^+$ [M+H]$^+$ 488.2607, found 488.2608, $\Delta$ppm +0.20.

Compound 8. A yellow oil; $R_f = 0.35$ (DCM/MeOH = 20:1); 76% overall yield; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.12 (d, $J = 2.4$ Hz, 1H), 8.03 (s, 1H), 7.23 (m, 2H), 6.99 (m, 2H), 6.86 (d, $J = 2.9$ Hz, 1H), 4.73, 4.66 (ABq, $J_{AB} = 12.2$ Hz, 2H), 4.16 (dd, $J = 9.8$, 4.9 Hz, 1H), 3.86 (m, 2H), 3.64 (t, $J = 10.0$ Hz, 1H), 3.31 (ddd, $J = 13.0$, 7.1, 2.9 Hz, 1H), 2.99 (s, 3H), 1.09 (m, 21H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 167.4, 162.5, 162.3 (d, $^{1}$J$_{CF}$ = 244.4 Hz), 157.19, 157.17, 133.1 (d, $^{3}$J$_{CF}$ = 3.0 Hz), 129.5 (d, $^{3}$J$_{CF}$ = 7.6 Hz), 115.6 (d, $^{2}$J$_{CF}$ = 21.2 Hz), 92.8, 65.2, 64.6, 55.7, 47.3, 40.2, 18.1, 12.0; HRMS(ESI+): Calcd for C$_{25}$H$_{39}$FN$_5$OSi$^+$ [M+H]$^+$ 472.2902, found 472.2904, $\Delta$ppm +0.42.

Compound 9a. A yellow oil; $R_f = 0.35$ (DCM/MeOH = 20:1); 67% overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.14 (s, 1H), 8.05 (s, 1H), 7.27 (d, $J = 8.2$ Hz, 2H), 6.99 (d, $J = 8.2$ Hz, 2H), 6.80 (br s, 1H), 4.76, 4.68 (ABq, $J_{AB} = 15.4$ Hz, 2H), 4.18 (dd, $J = 9.8$, 4.3 Hz, 1H), 3.87 (m, 2H), 3.66 (t, $J = 10.0$ Hz, 1H), 3.33 (m, 1H), 3.02 (s, 3H), 1.16 (m, 21H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 167.3, 162.4, 157.2, 157.1, 139.3, 134.2, 129.3, 119.3, 92.8, 65.1, 64.5, 55.9, 47.3, 40.2, 18.1, 12.0; HRMS(ESI+): Calcd for C$_{25}$H$_{39}$N$_8$OSi$^+$ [M+H]$^+$ 495.3011, found 495.3011.
Synthetic procedure for the preparation of 1

To a solution of 1 (90.0 mg, 0.198 mmol) in tetrahydrofuran (THF, 4 mL) was added tetra-n-butylammonium fluoride solution (TBAF, 1.0 M solution in THF, 0.257 mL, 0.257 mmol) and the mixture was stirred at room temperature (r.t.). After completion of the reaction as indicated by TLC, the reaction mixture was quenched with saturated NaHCO₃(aq). The resultant was extracted twice with DCM, dried with anhydrous Na₂SO₄(s), filtered, and concentrated in vacuo. To a solution of the crude product in dichloromethane (DCM, 4 mL) was added p-toluenesulfonyl chloride (p-TsCl, 49.1 mg, 0.257 mmol) and then, the mixture was stirred at r.t. After completion of the reaction as indicated by TLC, solvent was removed under reduced pressure and the residue was purified by silica-gel flash column chromatography to obtain desired product SB1601 (59.9 mg, 67% overall yield) as a white solid. Rᵣ = 0.39 (EtOAc); ¹H NMR (500 MHz, CDCl₃) δ 8.12 (s, 1H), 7.44–7.32 (m, 7H), 7.18 (d, J = 7.8 Hz, 2H), 6.62 (s, 1H), 5.71 (br d, J = 4.9 Hz, 1H), 4.73, 4.69 (ABq, Jₐₕ = 16.5 Hz, 2H), 4.49 (br s, 1H), 3.75 (d, J = 6.8 Hz, 1H), 3.56 (d, J = 13.2 Hz, 1H), 3.40 (dt, J = 13.2, 4.9 Hz, 1H), 3.00 (s, 3H), 2.95 (t, J = 6.8 Hz, 1H), 2.39 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 166.3, 163.5, 156.0, 144.7, 138.2, 134.9, 130.0, 128.8, 127.8, 127.7, 127.3, 103.5, 87.7, 66.8, 58.6, 56.4, 48.8, 40.0, 21.7; IR (neat) νₘₐₓ: 3249, 1737, 1559, 1336, 1166, 678 cm⁻¹; HRMS(EI+): Calcd for C₂₃H₂₆N₅O₃S⁺ [M+H]⁺ 452.1751, found 452.1753, Δppm +0.44; mp: 150–152 °C.

SB1602. A white solid; Rᵣ = 0.30 (EtOAc); 65.7 mg, 71 % overall yield; ¹H NMR (500 MHz, CDCl₃) δ 8.11 (s, 1H), 7.43–7.37 (m, 6H), 7.32 (m, 1H), 6.83 (m, 2H), 6.61 (s, 1H), 5.93(br d, J = 5.4 Hz, 1H), 4.73, 4.67 (ABq, Jₐₕ = 16.3 Hz, 2H), 4.46 (br t, J = 4.9 Hz, 1H), 3.82 (s, 3H),
SB1603. A yellow solid; \( R_f = 0.30 \) (EtOAc); 55.4 mg, 58 % overall yield; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.09 (s, 1H), 7.62 (m, 1H), 7.52 (d, \( J = 8.2 \) Hz, 2H), 7.45 (m, 1H), 7.37 (m, 2H), 7.31 (m, 3H), 6.74 (s, 1H), 6.06 (br s, 1H), 4.88 (br s, 1H), 4.60 (s, 2H), 3.99 (d, \( J = 7.4 \) Hz, 1H), 3.58–3.45 (m, 3H), 2.91 (s, 3H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.4, 163.9, 156.1, 148.2, 137.9, 134.5, 132.0, 131.7, 130.7, 128.7, 127.8, 127.4, 124.2, 102.0, 87.9, 68.3, 58.1, 56.7, 48.1, 40.0; IR (neat) \( \nu_{\text{max}} \): 2912, 1540, 1353, 1169, 738, 699 cm\(^{-1}\); HRMS(ESI+): Calcd for C\(_{22}\)H\(_{23}\)N\(_6\)O\(_5\)S\(^+\) [M+H]\(^+\) 483.1445, found 483.1443, \( \Delta \text{ppm} \) -0.41; mp: 80–82 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and 2-nitrobenzenesulfonyl chloride.

SB1604. The synthesis, characterization and X-ray crystallographic analysis (CCDC number 1500586) of SB1604 was previously reported.\(^{[11]}\)
SB1605. A pale yellow solid; \( R_f = 0.3 \) (EtOAc); 53.5 mg, 59% yield; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.16–8.14 (m, 3H), 7.51–7.40 (m, 7H), 6.54 (s, 1H), 5.65 (d, \( J = 3.9 \) Hz, 1H), 4.71 (s, 2H), 4.53 (br s, 1H), 3.82 (dd, \( J = 7.4, 2.0 \) Hz, 1H), 3.64 (d, \( J = 13.3 \) Hz, 1H), 3.45 (m, 1H), 3.05–3.01 (m, 4H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.4, 163.3, 156.2, 150.5, 143.6, 138.2, 129.1, 129.0, 127.5, 127.4, 124.5, 102.5, 87.5, 58.9, 56.6, 48.6, 39.5; IR (neat) \( \nu_{\text{max}} \): 2970, 1739, 1558, 1530, 1400, 1351, 1169, 738 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{22}\)H\(_{23}\)N\(_5\)O\(_5\)S\(^+\) [M+H]\(^+\) 483.1445, found 483.1439, \( \Delta \text{ppm} \) –1.24; mp: 97–99 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and 4-nitrobenzenesulfonyl chloride.

SB1606. A white solid; \( R_f = 0.4 \) (EtOAc); 56.8 mg, 63% overall yield; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.12 (s, 1H), 7.45–7.39 (m, 6H), 7.34 (m, 1H), 7.03 (m, 2H), 6.57 (s, 1H), 5.97 (br s, 1H), 4.72, 4.69 (ABq, \( J_{\text{AB}} = 16.5 \) Hz, 2H), 4.47 (br s, 1H), 3.77 (dd, \( J = 7.6, 2.2 \) Hz, 1H), 3.58 (d, \( J = 13.2 \) Hz, 1H), 3.40 (dt, \( J = 13.2, 4.9 \) Hz, 1H), 2.99 (s, 3H), 2.97 (d, \( J = 7.5 \) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.3, 165.6 (d, \( J_{\text{CF}} = 255.1 \) Hz), 163.4, 156.0, 138.2, 133.9 (d, \( J_{\text{CF}} = 3.0 \) Hz), 130.5 (d, \( J_{\text{CF}} = 9.9 \) Hz), 128.8, 127.5, 127.3, 116.7 (d, \( J_{\text{CF}} = 22.0 \) Hz), 103.0, 87.6, 58.7, 56.4, 48.7, 39.7; IR (neat) \( \nu_{\text{max}} \): 3245, 2921, 1571, 1351, 1169, 1155, 679 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{22}\)H\(_{23}\)F\(_3\)N\(_5\)O\(_3\)S\(^+\) [M+H]\(^+\) 456.1500, found 456.1501, \( \Delta \)ppm +0.22; mp: 105–107 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and 4-fluorobenzenesulfonyl chloride.
**SB1607.** A white solid; \( R_t = 0.25 \) (EtOAc); 55.8 mg, 75 % overall yield; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.14 (s, 1H), 7.37–7.26 (m, 5H), 6.58 (s, 1H), 5.92 (br d, \( J = 4.3 \) Hz, 1H), 4.73 (d, \( J = 16.0 \) Hz, 1H), 4.64 (br s, 1H), 4.55 (d, \( J = 16.0 \) Hz, 1H), 4.10 (dd, \( J = 7.6, 1.8 \) Hz, 1H), 3.92 (t, \( J = 7.4 \) Hz, 1H), 2.96 (s, 3H), 2.73 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.6, 163.5, 156.1, 138.0, 128.7, 127.6, 127.3, 102.5, 87.3, 67.1, 58.2, 56.6, 48.7, 40.4, 38.5; IR (neat) \( \nu_{\text{max}} \): 2912, 2109, 1571, 1538, 1350, 1168, 738 cm\(^{-1}\); HRMS(ESI+): Calcd for \( \text{C}_{17}\text{H}_{22}\text{N}_5\text{O}_3\text{S}^+ \) [M+H]\(^+\) 376.1438, found 376.1437, \( \Delta \text{ppm} \) –0.27; mp: 136–138 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and methanesulfonyl chloride.

**SB1608.** A white solid; \( R_t = 0.38 \) (EtOAc); 67.6 mg, 78% overall yield; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.12 (s, 1H), 7.56 (m, 1H), 7.56 (m, 2H), 7.44–7.37 (m, 6H), 7.33 (m, 1H), 5.95 (br d, \( J = 4.9 \) Hz, 1H), 4.73, 4.69 (ABq, \( J_{\text{AB}} = 16.3 \) Hz, 2H), 4.50 (br t, \( J = 4.9 \) Hz, 1H), 3.75 (dd, \( J = 7.3, 1.5 \) Hz, 1H), 3.56 (d, \( J = 12.7 \) Hz, 1H), 3.40 (dt, \( J = 13.2, 5.0 \) Hz, 1H), 3.00 (s, 3H), 2.93 (t, \( J = 6.8 \) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.3, 163.5, 155.9, 138.2, 137.9, 133.6, 129.4, 128.8, 127.7, 127.6, 127.2, 103.2, 87.7, 66.8, 58.6, 56.3, 48.7, 40.0; IR (neat) \( \nu_{\text{max}} \): 3380, 1739, 1536, 1352, 1170, 974, 723, 696 cm\(^{-1}\); HRMS(ESI+): Calcd for \( \text{C}_{22}\text{H}_{24}\text{N}_5\text{O}_3\text{S}^+ \) [M+H]\(^+\) 438.1594, found 438.1595, \( \Delta \text{ppm} \) +0.23; mp: 120–122 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and benzenesulfonyl chloride.
SB1609. A white solid; $R_t = 0.32$ (EtOAc); 54.5 mg, 61% overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.11 (s, 1H), 7.39–7.35 (m, 2H), 7.30–7.21 (m, 8H), 6.60 (s, 1H), 5.76 (br s, 1H), 4.66, 4.50 (ABq, $J_{AB} = 15.6$ Hz, 2H), 4.32 (br s, 1H), 4.09 (s, 2H), 3.97 (d, $J = 7.4$ Hz, 1H), 3.72 (t, $J = 6.8$ Hz, 1H), 3.28 (m, 2H), 2.81 (s, 3H); $^13$C NMR (100 MHz, CDCl$_3$) $\delta$ 166.4, 163.6, 156.0, 138.0, 131.0, 129.1, 128.8, 127.9, 127.7, 127.3, 102.2, 87.0, 68.0, 59.4, 57.7, 57.2, 48.1, 40.1; IR (neat) $\nu_{\text{max}}$: 2970, 1741, 1558, 1403, 1349, 1161, 1049, 969, 696 cm$^{-1}$; HRMS(ESI+): Calcd for C$_{23}$H$_{26}$N$_5$O$_3$S $[M+H]^+$ 452.1751, found 452.1747, $\Delta$ppm –0.88; mp: 78–80 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and benzylsulfonyl chloride.

\begin{center}
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\end{center}

SB1610. A white solid; $R_t = 0.15$ (EtOAc); 53.8 mg, 62% overall yield; $^1$H NMR (600 MHz, DMSO-$d_6$, 77 °C) $\delta$ 8.93 (s, 1H), 8.86 (d, $J = 4.0$ Hz, 1H), 8.05 (m, 2H), 7.56 (dd, $J = 7.3$, 5.1 Hz, 1H), 7.38 (m, 2H), 7.30 (m, 3H), 6.97 (br s, 1H), 6.66 (s, 1H), 4.80 (br s, 1H), 4.66 (d, $J = 15.6$ Hz, 1H), 4.66 (d, $J = 15.6$ Hz, 1H), 3.71 (br d, $J = 6.6$ Hz, 1H), 3.52 (m, 1H), 3.33 (d, $J = 13.9$ Hz, 1H), 3.04 (br s, 1H), 2.90 (s, 3H); $^13$C NMR (150 MHz, DMSO-$d_6$, 77 °C) $\delta$ 165.5, 162.9, 155.3, 153.8, 147.2, 137.9, 134.9, 134.2, 128.0, 127.1, 126.6, 124.0, 101.7, 86.7, 66.4, 57.0, 55.6, 47.6, 39.8; IR (neat) $\nu_{\text{max}}$: 3313, 1732, 1555, 1353, 1171, 980, 957, 690 cm$^{-1}$; HRMS(ESI+): Calcd for C$_{21}$H$_{23}$N$_6$O$_3$S $[M+H]^+$ 439.1547, found 439.1553; $\Delta$ppm +1.37, mp: 185–187 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and pyridine-3-sulfonyl chloride.

\begin{center}
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SB1611. A white solid; $R_t = 0.33$ (EtOAc); 51.8 mg, 59% overall yield; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.10 (dd, $J = 4.9$, 1.5 Hz, 1H), 8.03 (s, 1H), 7.57 (dd, $J = 3.7$, 1.2 Hz, 1H), 7.39–7.36 (m, 2H), 7.30–7.28 (m, 3H), 7.25 (d, $J = 4.9$ Hz, 1H), 7.22 (dd, $J = 4.9$, 3.9 Hz, 1H), 6.60

\begin{center}
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(s, 1H), 4.75 (br s, 1H), 4.66 (d, J = 15.7 Hz, 1H), 4.49 (d, J = 15.7 Hz, 1H), 3.63 (dd, J = 7.3, 1.5 Hz, 1H), 3.52 (dt, J = 13.9, 5.0 Hz, 1H), 3.28 (d, J = 13.2 Hz, 1H), 2.93 (t, J = 7.1 Hz, 1H), 2.86 (s, 3H);

$^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 165.6, 163.3, 155.7, 138.1, 136.9, 135.5, 134.1, 128.5, 127.5, 127.0, 102.0, 87.1, 66.6, 57.4, 56.1, 47.9, 40.2; IR (neat) $\nu_{\text{max}}$: 3241, 1739, 1577, 1542, 1355, 1166, 1026, 970, 674 cm$^{-1}$; HRMS(ESI$^+$): Calcd for C$_{20}$H$_{22}$N$_5$O$_3$S$_2$ $[M+H]^+$ 444.1159, found 444.1159; mp: 112–114 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 1 and 2-thiophenesulfonyl chloride.

**SB1612.** A yellow solid; $R_t = 0.27$ (EtOAc); 41.8 mg, 52 % overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.37 (d, J = 8.6 Hz, 2H), 8.12 (d, J = 9.0 Hz, 2H), 8.06 (s, 1H), 6.69 (s, 1H), 5.93 (br s, 1H), 4.54 (br s, 1H), 3.85 (dd, J = 7.6, 1.4 Hz, 1H), 3.46 (m, 2H), 3.07 (s, 6H), 3.01 (t, J = 7.0 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 166.8, 163.4, 156.1, 150.7, 144.2, 129.3, 124.6, 102.1, 88.3, 67.1, 56.5, 48.5, 43.2; IR (neat) $\nu_{\text{max}}$: 3409, 2924, 1579, 1524, 1351, 1171, 738, 685 cm$^{-1}$; HRMS(ESI$^+$): Calcd for C$_{16}$H$_{19}$N$_6$O$_5$S $[M+H]^+$ 407.1132, found 407.1130, Δppm –0.49; mp: 118–120 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 2 and 4-nitrobenzenesulfonyl chloride.

**SB1613.** A pale yellow solid; $R_t = 0.45$ (EtOAc); 61.0 mg, 62 % overall yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.33 (d, J = 8.6 Hz, 2H), 8.08 (s, 1H), 7.99 (d, J = 8.6 Hz, 2H), 7.30–7.21 (m, 5H), 6.55 (s, 1H), 6.08 (br s, 1H), 4.54 (br s, 1H), 3.97 (m, 1H), 3.84 (d, J = 7.0 Hz, 1H), 3.46 (m, 3H), 3.09 (s, 3H), 3.00 (m, 2H), 2.89 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 166.5, 163.5, 156.0, 150.6, 144.1, 139.6, 129.2, 128.9, 128.5, 126.2, 124.5, 102.6, 88.1, 67.0, 56.5, 54.5, 48.4, 42.5, 33.8; IR (neat) $\nu_{\text{max}}$: 3411, 1739, 1580, 1524, 1354, 1169, 738 cm$^{-1}$; HRMS(ESI$^+$): Calcd for C$_{23}$H$_{25}$N$_6$O$_5$S $[M+H]^+$ 497.1602, found 497.1603, Δppm +0.20; mp: 108–110 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 3 and 4-nitrobenzenesulfonyl chloride.
SB1614. A light yellow solid; \( R_t = 0.5 \) (EtOAc); 51.6 mg, 51% overall yield; \( ^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.28 (d, \( J = 8.2 \) Hz, 2H), 8.05 (s, 1H), 7.92 (d, \( J = 7.8 \) Hz, 2H), 7.37 (d, \( J = 7.4 \) Hz, 2H), 7.28 (m, 2H), 7.18 (m, 1H), 6.85 (s, 1H), 5.82 (br s, 1H), 4.59 (m, 2H), 4.49 (d, \( J = 14.8 \) Hz, 1H), 3.96 (m, 1H), 3.85 (d, \( J = 7.8 \) Hz, 1H), 3.55 (d, \( J = 13.3 \) Hz, 1H), 3.38 (m, 1H), 3.27 (t, \( J = 7.2 \) Hz, 1H), 1.34 (d, \( J = 6.3 \) Hz, 3H), 1.29 (d, \( J = 6.3 \) Hz, 3H); \( ^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 167.0, 163.4, 155.9, 150.5, 144.2, 140.3, 128.9, 128.3, 127.9, 126.6, 124.6, 106.9, 87.5, 67.2, 56.7, 56.6, 48.3, 46.3, 20.7, 20.0; IR (neat) \( \nu \) max: 2970, 1738, 1568, 1531, 1348, 1169, 1061, 737 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{24}\)H\(_{27}\)N\(_6\)O\(_5\)S\(_2\) \([M+H]^+\) 511.1758, found 511.1761, Δppm +0.59; mp: 85–87 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 4 and 4-nitrobenzenesulfonyl chloride.

SB1615. A pale yellow solid; \( R_t = 0.35 \) (EtOAc); 44.7 mg, 56% overall yield; \( ^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.46 (s, 1H), 8.41 (d, \( J = 8.6 \) Hz, 2H), 8.09 (d, \( J = 9.0 \) Hz, 2H), 6.85 (s, 1H), 5.74 (br d, \( J = 3.9 \) Hz, 1H), 5.56 (s, 1H), 5.17 (s, 1H), 4.63 (br s, 1H), 3.91 (dd, \( J = 7.6, 1.8 \) Hz, 1H), 3.66 (d, \( J = 12.5 \) Hz, 1H), 3.50 (m, 1H), 3.25 (t, \( J = 7.2 \) Hz, 1H), 2.17 (s, 3H); \( ^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 167.4, 162.8, 157.0, 150.8, 143.8, 142.1, 129.1, 124.9, 119.5, 114.5, 87.9, 67.0, 56.2, 48.9, 22.9; IR (neat) \( \nu \) max: 3290, 2977, 1740, 1557, 1529, 1346, 1170, 907, 737 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{17}\)H\(_{18}\)N\(_2\)O\(_5\)S\(_2\) \([M+H]^+\) 404.1023, found 404.1023; mp: 200–202 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 5 and 4-nitrobenzenesulfonyl chloride.
**SB1616.** A white solid; $R_f = 0.35$ (EtOAc); 41.8 mg, 45 % overall yield; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.41 (m, 3H), 8.03 (d, $J = 8.3$ Hz, 2H), 7.65 (br d, $J = 4.4$ Hz, 1H), 7.52 (d, $J = 8.3$ Hz, 2H), 7.09 (d, $J = 8.3$ Hz, 2H), 6.47 (s, 1H), 4.89 (br s, 1H), 3.87 (s, 3H), 3.70 (d, $J = 7.3$ Hz, 1H), 3.55 (m, 1H), 3.39 (d, $J = 14.0$ Hz, 1H), 3.08 (t, $J = 7.3$ Hz, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 163.3, 162.5, 160.2, 156.6, 150.6, 142.5, 131.2, 129.6, 129.1, 125.1, 113.7, 113.5, 87.5, 66.9, 55.7, 55.3, 48.1; IR (neat) $\nu_{max}$: 3244, 3109, 3005, 1739, 1570, 1528, 1352, 1168, 740 cm$^{-1}$; HRMS(ESI$^+$): Calcd for C$_{21}$H$_{20}$N$_5$O$_6$S$^+$ [M+H]$^+$ 470.1129, found 470.1127, Δppm –0.43; mp: 250–252 °C. The product was synthesized according to the synthetic procedure for the preparation of **SB1601** from 6 and 4-nitrobenzenesulfonyl chloride.

**SB1617.** (58.3 mg, 57% overall yield) as a light yellow solid. $R_f = 0.40$ (EtOAc); $^1$H NMR (400 MHz, CDCl$_3$) δ 8.24 (d, $J = 8.6$ Hz, 2H), 8.13 (s, 1H), 7.63 (d, $J = 8.6$ Hz, 2H), 7.42, 7.35 (ABq, $J_{AB} = 8.2$ Hz, 4H), 6.56 (s, 1H), 5.73 (d, $J = 3.9$ Hz, 1H), 4.67, 4.62 (ABq, $J_{AB} = 17.0$ Hz, 2H), 4.54 (br s, 1H), 3.84 (d, $J = 7.4$ Hz, 1H), 3.61 (d, $J = 13.3$ Hz, 1H), 3.45 (dt, $J = 13.3$, 4.7 Hz, 1H), 3.07 (t, $J = 7.0$ Hz, 1H), 2.99 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.4, 163.4, 156.3, 150.6, 143.7, 136.8, 133.2, 129.1, 129.03, 128.95, 124.6, 102.8, 87.5, 67.0, 58.0, 56.5, 48.6, 40.1; IR (neat) $\nu_{max}$: 3248, 3016, 1739, 1565, 1529, 1350, 1169, 738 cm$^{-1}$; HRMS(ESI$^+$): Calcd for C$_{22}$H$_{22}$ClN$_6$O$_5$S$^+$ [M+H]$^+$ 517.1055, found 517.1053, Δppm –0.39; mp: 110–112 °C. The product was synthesized according to the synthetic procedure for the preparation of **SB1601** from 7 and 4-nitrobenzenesulfonyl chloride.
SB1618. A light yellow solid; \( R_f = 0.39 \) (EtOAc); 54.5 mg, 55 % overall yield; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.24 (d, \( J = 8.8 \) Hz, 2H), 8.12 (s, 1H), 7.69 (d, \( J = 8.8 \) Hz, 2H), 7.36 (dd, \( J = 8.1, 5.6 \) Hz, 2H), 7.12 (t, \( J = 8.6 \) Hz, 2H), 6.61 (s, 1H), 5.76 (br d, \( J = 4.4 \) Hz, 1H), 4.64 (s, 2H), 4.54 (br s, 1H), 3.84 (dd, \( J = 7.8, 1.5 \) Hz, 1H), 3.59 (d, \( J = 13.2 \) Hz, 1H), 3.45 (dt, \( J = 13.7, 4.9 \) Hz, 1H), 3.07 (t, \( J = 7.1 \) Hz, 1H), 2.98 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.4, 163.4, 162.3 (d, \( J_{CF} = 244.4 \) Hz), 156.3, 150.6, 143.8, 133.8 (d, \( J_{CF} = 3.8 \) Hz), 129.3 (d, \( J_{CF} = 7.6 \) Hz), 129.0, 124.5, 115.7 (d, \( J_{CF} = 21.3 \) Hz), 102.8, 87.6, 67.0, 57.9, 56.5, 48.6, 40.0; IR (neat) \( \nu_{\text{max}} \): 3246, 3018, 1739, 1567, 1533, 1498, 1349, 1170, 739 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{22}\)H\(_{22}\)FN\(_9\)O\(_5\)S\(_2\)\([M+H]\)^+ 501.1351, found 501.1353, \( \Delta \)ppm +0.40; mp: 102–104 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 8 and 4-nitrobenzenesulfonyl chloride.

SB1619. A pale yellow solid; \( R_f = 0.40 \) (EtOAc); 61.2 mg, 59 % overall yield; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.24 (d, \( J = 8.6 \) Hz, 2H), 8.11 (s, 1H), 7.72 (d, \( J = 8.6 \) Hz, 2H), 7.37 (d, \( J = 8.2 \) Hz, 2H), 7.09 (d, \( J = 8.2 \) Hz, 2H), 6.61 (s, 1H), 5.86 (br d, \( J = 4.3 \) Hz, 1H), 4.64 (s, 2H), 4.54 (br s, 1H), 3.84 (d, \( J = 7.8 \) Hz, 1H), 3.59 (d, \( J = 13.2 \) Hz, 1H), 3.45 (dt, \( J = 13.4, 4.8 \) Hz, 1H), 3.08 (t, \( J = 7.0 \) Hz, 1H), 2.98 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 166.4, 163.5, 156.2, 150.6, 143.8, 139.3, 134.9, 129.2, 129.0, 124.5, 119.4, 102.8, 87.6, 67.0, 58.0, 56.5, 48.6, 40.1; IR (neat) \( \nu_{\text{max}} \): 2900, 2109, 1572, 1530, 1349, 1168, 738 cm\(^{-1}\); HRMS(ESI\(^+\)): Calcd for C\(_{22}\)H\(_{22}\)N\(_9\)O\(_5\)S\(_2\)\([M+H]\)^+ 524.1459, found 524.1460, \( \Delta \)ppm +0.19; mp: 88–90 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 9a and 4-nitrobenzenesulfonyl chloride.
SB1620. A white solid; Rf = 0.40 (EtOAc); 57.2 mg, 59 % overall yield; 1H NMR (400 MHz, CDCl3) δ 8.11 (s, 1H), 7.50 (dd, J = 8.6, 4.7 Hz, 2H), 7.39, 7.33 (ABq, JAB = 8.4 Hz, 4H), 7.10 (t, J = 8.4 Hz, 2H), 6.56 (s, 1H), 5.92 (br d, J = 4.7 Hz, 1H), 4.65 (s, 2H), 4.48 (br s, 1H), 3.78 (d, J = 7.4 Hz, 1H), 3.56 (d, J = 13.2 Hz, 1H), 3.41 (dt, J = 13.5, 4.8 Hz, 1H), 2.99 (m, 4H); 13C NMR (100 MHz, CDCl3) δ 166.2, 165.7 (d, JCF = 255.8 Hz), 163.5, 156.1, 136.8, 133.0, 130.5 (d, JCF = 9.9 Hz), 129.1, 129.0, 116.8 (d, JCF = 22.8 Hz), 103.3, 87.6, 66.8, 57.9, 56.3, 48.7, 40.2; IR (neat) νmax: 3015, 1739, 1570, 1491, 1351, 1231, 1171, 1157, 678 cm–1; HRMS(ESI+): Calcd for C22H22ClF5N5O3S+ [M+H]+ 490.1110, found 490.1112, Δppm +0.41; mp: 150–152 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 7 and 4-fluorobenzenesulfonyl chloride.

SB1621. A white solid; Rf = 0.35 (EtOAc); 61.6 mg, 62 % overall yield; 1H NMR (400 MHz, CDCl3) δ 8.10 (s, 1H), 7.45 (d, J = 8.6 Hz, 2H), 7.37, 7.32 (ABq, JAB = 8.2 Hz, 4H), 6.88 (d, J = 8.6 Hz, 2H), 6.57 (s, 1H), 5.85 (br s, 1H), 4.65 (s, 2H), 4.47 (br s, 1H), 3.85 (s, 3H), 3.75 (d, J = 7.4 Hz, 1H), 3.54 (d, J = 13.3 Hz, 1H), 3.40 (dt, J = 13.2, 4.9 Hz, 1H), 2.99 (m, 4H); 13C NMR (100 MHz, CDCl3) δ 166.1, 163.7, 163.5, 156.0, 136.9, 132.9, 129.9, 129.3, 129.2, 128.9, 114.6, 103.7, 87.7 (C11, d, J = 2.3 Hz), 66.9, 57.9, 56.3, 55.8 (C25, d, J = 2.3 Hz), 48.8, 40.3; IR (neat) νmax: 2970, 1739, 1574, 1496, 1349, 1161, 680 cm–1; HRMS(ESI+): Calcd for C23H25ClF5N5O3S+ [M+H]+ 502.1310, found 502.1310; mp: 156–158 °C. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 7 and 4-methoxybenzenesulfonyl chloride.
Synthetic procedure for the preparation of SB1622

To a solution of SB1617 (58.3 mg, 0.113 mmol) in DCM (5 mL) were sequentially added Et$_3$N (0.032 mL, 0.226 mmol) and acetyl chloride (0.010 mL, 0.147 mmol) at 0 °C. The resulting mixture was left to stir and allowed to warm to r.t. After completion of the reaction as indicated by TLC, the resultant was quenched with saturated NaHCO$_3$(aq) and extracted twice with DCM. The combined organic layer was dried over anhydrous Na$_2$SO$_4$(s) and the filtrate was condensed under reduced pressure, followed by silica-gel flash column chromatography to afford the desired product SB1622 (51.2 mg, 81% yield) as a white solid.

**SB1622.** $R_f = 0.2$ (Hexane/EtOAc = 1:1); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.50 (s, 1H), 8.24 (d, $J = 9.0$ Hz, 2H), 7.59 (d, $J = 9.0$ Hz, 2H), 7.45, 7.39 (ABq, $J_{AB} = 8.2$ Hz, 4H), 6.47 (s, 1H), 4.81–4.65 (m, 3H), 4.45 (br s, 1H), 3.80 (d, $J = 8.2$ Hz, 1H), 3.38 (br s, 1H), 3.14 (s, 3H), 2.96 (dd, $J = 7.8, 5.5$ Hz, 1H), 2.06 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.1, 165.8, 161.0, 156.1, 150.6, 144.0, 135.7, 133.7, 129.3, 128.9, 128.6, 124.8, 86.3, 69.1, 58.2, 57.6, 46.1, 40.2, 23.6; IR (neat) $\nu_{max}$: 2970, 1739, 1674, 1559, 1530, 1350, 1229, 1169, 738 cm$^{-1}$; HRMS(ESI+): Calcd for C$_{24}$H$_{24}$ClN$_6$O$_6$S$^+$ [M+H]$^+$ 559.1161, found 559.1175, ∆ppm +2.50, mp: 105–107 °C.

**SB1623.** A light yellow solid; $R_f = 0.35$ (Hexane/EtOAc = 1:1); 56.2 mg, 85 % yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.53 (s, 1H), 8.23 (d, $J = 8.6$ Hz, 2H), 7.59 (d, $J = 9.0$ Hz, 2H), 7.45, 7.40 (ABq, $J_{AB} = 8.2$ Hz, 4H), 6.51 (s, 1H), 4.74 (m, 3H), 4.45 (br s, 1H), 3.75 (d, $J = 7.8$ Hz, 1H), 3.40 (br d, $J = 8.6$ Hz, 1H), 3.14 (s, 3H), 2.94 (m, 1H), 1.63 (m, 1H), 1.05 (m, 2H), 0.81 (m, 1H), 0.70 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 174.8, 165.9, 161.0, 156.1 (C2, d, $J = 3.8$ Hz), 150.6, 144.0, 135.7, 133.7, 129.3, 128.9, 128.7, 124.8, 86.4, 69.0, 58.2, 57.6, 46.5, 40.2
(C25, d, $J = 3.7$ Hz), 14.0; IR (neat) $\nu_{\text{max}}$: 2970, 1739, 1665, 1558, 1398, 1350, 1168, 738 cm$^{-1}$; HRMS(ESI+): Calcd for C$_{25}$H$_{26}$ClN$_6$O$_6$S$^+$ [M+H]$^+$ 585.1318, $\Delta$ppm +1.03; found 585.1324; mp: 124–126 oC. The product was synthesized according to the synthetic procedure for the preparation of SB1622 from SB1617 and cyclopropanecarbonyl chloride.

**Compound 9b.** A pale yellow solid; $R_f = 0.45$ (EtOAc); 73.0 mg, 61 % overall yield; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.12 (s, 1H), 7.79 (d, $J = 8.3$ Hz, 2H), 7.36 (d, $J = 8.3$ Hz, 2H), 7.28 (d, $J = 8.3$ Hz, 2H), 7.07 (d, $J = 8.3$ Hz, 2H), 6.62 (s, 1H), 5.90 (br d, $J = 3.9$ Hz, 1H), 4.66, 4.62 (ABq, $J_{AB} = 16.8$ Hz, 2H), 4.49 (br s, 1H), 3.81 (d, $J = 6.8$ Hz, 1H), 3.56 (d, $J = 13.0$ Hz, 1H), 3.43 (dt, $J = 13.6$, 5.0 Hz, 1H), 3.06 (t, $J = 6.8$ Hz, 1H), 2.97 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 166.3, 163.5, 156.2, 139.2, 138.7, 137.7, 135.0, 129.2, 129.0, 119.4, 103.4, 101.6, 87.7, 66.9, 58.0, 56.4, 48.7, 40.3; IR (neat) $\nu_{\text{max}}$: 2969, 2107, 1736, 1567, 1351, 1166, 733 cm$^{-1}$; HRMS(ESI+): Calcd for C$_{22}$H$_{22}$I$_2$N$_8$O$_3$S$^+$ [M+H]$^+$ 605.0575, found 605.0577, $\Delta$ppm +0.33; mp: 98–100 oC. The product was synthesized according to the synthetic procedure for the preparation of SB1601 from 9a and 4-iodobenzenesulfonyl chloride.
Synthetic procedure for the preparation of SB1624

To a solution of 9b (73.0 mg, 0.121 mmol) in dimethylformamide (DMF, 6 mL) were added trimethylsilylacetylene (0.033 mL, 0.242 mmol), Pd(PPh\(_3\))\(_2\)Cl\(_2\) (8.50 mg, 0.012 mmol, 10 mol %), copper(I) iodide (CuI, 11.6 mg, 0.061 mmol) and triethylamine (TEA, 0.051 mL, 0.363 mmol). Then the resulting mixture was left to stir at r.t. After completion of the reaction indicated by TLC, the resultant was quenched with saturated NH\(_4\)Cl(aq) and extracted twice with ethyl acetate. The combined organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and filtered. The filtrate was condensed under reduced pressure, followed by silica-gel flash column chromatography to afford intermediate B (54.0 mg, 81% yield). To a solution of intermediate B (54.0 mg, 0.098 mmol) in THF (2 mL) was added TBAF (1.0 M solution in THF, 0.127 mL, 0.127 mmol). The reaction mixture was then left to stir at r.t. After completion of the reaction as indicated by TLC, the solvent was removed under reduced pressure and the residue was purified through the silica-gel flash column chromatography to obtain product SB1624 (25.1 mg, 51% yield, 41% overall yield) as a light yellow solid. 

\[ R_f = 0.45 \text{ (EtOAc)} \]

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.14 (s, 1H), 7.53 (s, 4H), 7.37 (d, \(J = 8.0\) Hz, 2H), 7.08 (d, \(J = 8.0\) Hz, 2H), 6.63 (s, 1H), 5.57 (br s, 1H), 4.66 (s, 2H), 4.49 (br s, 1H), 3.81 (d, \(J = 8.0\) Hz, 1H), 3.57 (d, \(J = 12.0\) Hz, 1H), 3.43 (m, 1H), 3.31 (s, 1H), 3.02 (m, 4H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 166.3, 163.3, 155.9, 139.2, 137.9, 135.0, 133.0, 129.3, 128.0, 127.7, 119.4, 103.4, 87.7, 81.80, 81.78, 66.9, 58.0, 56.4, 48.8, 40.3; IR (neat) \(\nu_{\text{max}}\): 3373, 3215, 2955, 2102, 1737, 1574, 1353, 1166, 973 cm\(^{-1}\); HRMS(ESI+): Calcd for C\(_{24}\)H\(_{23}\)N\(_8\)O\(_3\)S\(^+\) [M+H]\(^+\) 503.1608, found 503.1607, \(\Delta\)ppm −0.20; mp: 139–141 °C.
$^1$H and $^{13}$C NMR Spectra
Sample Name: 2E56-Vt-free_1
Data Collection date: 11/05/2013
Archive Directory: /home/pa/petri/ventura/data
Sample Directory: L07_4.01_20131105_20131105_01
Profile: S0408
Pulse Sequence: PROTON (2p9a)
Instrument: 500L
Data collected on: Nov 20 2013

[Graph showing NMR spectra with peaks labeled]

Sample #7, Operator: sbjpack
Delay, Delay 1: 1.000 sec
Pulse 45.0 Degrees
Acq. Time 1.111 sec
Width 10000.3 Hz
16 repetitions
OFF-RES 25, 100.51568 Mhz
Sweep 3.56
Power 53.40
Continuously auto-clocked
DATA PROCEDURE
Line Broadening 3.3 Hz
PT size 65536
Total time 10 sec

[Graph showing NMR spectra with peaks labeled]

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