Supporting information

Discovery of *h*ALPL Agonists for Osteogenesis from A Selenium-containing DNA-encoded Natural Product Library

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1. General information

All commercially available organic compounds were purchased from Sigma-Aldrich, bidepharm, adamas-beta and Shanghai GeorGene Biotech Co., Ltd. in China. Unless otherwise noted, all commercial reagents and solvents were used without additional purification. The substrates BSEAs, carboxyl-containing BSEAs and terminal alkyne-bearing natural products are all available in our recently research[1]. Water was purified with a Millipore Milli-Q system. NMR spectra were recorded on Bruker AM-400 or AM-500 instruments. Chemical shifts were reported as δ (ppm) and coupling constants were reported as J (hertz) referenced to Tetramethylsilane (TMS) as an internal standard for ^{1}H NMR ($CDCl_3$, δ 7.26; DMSO-d_6, δ 2.50) and ^{13}C NMR ($CDCl_3$, δ 77.0; DMSO-d_6, δ 39.5). The following abbreviations are used to designate multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, td = triplet of doublets, dt = doublet of triplets. High-resolution mass spectra were obtained on an Agilent Technologies 6230 Accurate Mass TOF LC/MS instrument or an AB Sciex 4600 QTOF MS instrument.

DNA headpiece (HP-NH_2, 5'- /5phos/GAGTCA/iSp9/iUniAmM/iSp9/TGACTCCC-3') were obtained from commercial sources unless otherwise noted and used as received. Oligonucleotides were purchased from GenScript in desalted form as lyophilized material. Ligation buffer, DNA-ligase and high-fidelity Phusion DNA polymerase were purchased from New England Biolabs. PCR purification and gel extraction kits were purchased from Qiagen. All gel images were captured by a Bio-Rad Chemidoc image system.

2. Abbreviations

α-MEM: Minimum Essential Medium-α
Abs: Absorbance
ALP: Alkaline Phosphatase
BB: Building Block
BMSCs: Bone Marrow-derived Mesenchymal Stromal Cells
BSEAs: Benzoselenazolones
BV/TV: Bone Tissue Volume/Total Volume
DCM: Dichloromethane
DCE: 1,2-Dichloroethane
DEL: DNA-encoded chemical library
DMAP: 4-Dimethylaminopyridine
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic acid
DPF: Days Post-fracture
EDCI: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA: Ethylene Diamine Tetraacetic Acid
EC_{50}: Concentration for 50% of Maximal Effect

ECM: Extracellular Matrix
ESI-MS: Electrospray Ionization Mass Spectrometry
FBS: Fetal Bovine Serum
Fmoc: 9-Fluorenylmethyloxycarbonyl
*h*ALPL: Human Recombinant Alkaline Phosphatase, Tissue-nonspecific
*h*BMSCs: Human Bone Marrow-Derived mesenchymal stem cells
HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic Acid
HP-DNA: Headpiece DNA
HPLC: High Performance Liquid Chromatography
H & E: Hematoxylin and Eosin
HPP: Hypophosphatasia
K_a: association constant
K_d: dissociation constant
K_D: Dissociation Constants
*n*DEL: DNA-encoded Natural Product Library
NMR: Nuclear Magnetic Resonance
NGS: Next Generation Sequencing
PBS: Phosphate Buffer Saline
PBST: Phosphate Buffered Saline with Tween-20
pNPP: p-Nitrophenyl Phosphate
PPi: inorganic Pyrophosphate
PCR: Polymerase Chain Reaction
Pre-OBs: Pre-osteoblasts
qRT-PCR: quantitative Real-time PCR
RIPA: Radio-immunoprecipitation Assay
RNA: Ribonucleic Acid
RT: Room temperature
rpm: revolutions per minute
SAR: Structure-activity Relationship
SDS-PAGE: SDS-polyacrylamide Gel Electrophoresis
Se-HAP: Selenium-hydroxyapatite
Se-*n*DEL: Selenium-containing DNA-encoded Natural Product Library
SeNEx: Selenium-nitrogen Exchange
sNHS: N-hydroxysulfosuccinimide Sodium Salt
SO/FG: Safranin O/Fast Green
SPR: Surface Plasmon Resonance
TBE: Tris-Borate Electrophoresis Buffer
Tb.N: Trabecular Number
Tb.SP: Trabecular Separation
Tb.Th: Trabecular Thickness
TMS: Tetramethylsilane
TNAP: Tissue-nonspecific Alkaline Phosphatase
TRAP: Tartrate-Resistant Acid Phosphatase
μCT: micro-computed Tomography

UPLC/MS: Ultra-performance Liquid Chromatography−Mass Spectrometry
WT: Wild Type

3. Method

3.1. Cells culture

HEK293T cells (NCACC, SCSP-502) were adherent cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, 11965092) with 10% fetal bovine serum (FBS; Hyclone, SH30070.03) at 37˚C with 5 % CO_2. Mouse pre-osteoblasts (Pre-OBs) were isolated from calvarial bones. Bones were digested with 0.3% collagenase (Sigma, C6885) for 2 h, and terminated by Minimum Essential Medium-α (α-MEM; Gibco,12561056) supplemented with 10% FBS. Then, the cells were resuspended and cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine and 1% Penicillin-Streptomycin (Gibco, 15140122) at 37˚C with 5 % CO_2. The adherent cells were cultured for 3–4 days and passaged at 90% confluence. Early passage (P3-P5) of pre-o at 37˚C with 5 % CO_2. Steoblasts were used for subsequent study. Human Bone Marrow-Derived mesenchymal stem cells (*h*BMSCs) were obtained from 3 female patients (age 50–61 years) undergoing routine total hip replacement surgery at the Ninth People's Hospital (Shanghai, China). All patients gave written, informed consent to donate biological material for research purposes. The *h*BMSCs were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% Penicillin-Streptomycin at 37˚C with 5 % CO_2. Early passage (P3-P5) of *h*MSCs were induced of osteogenic differentiation. The ethics for harvesting human mesenchymal stem cells was also approved by the Ethics Committee of the Ninth People's Hospital, Shanghai Jiaotong University School of Medicine (Ethics Number: SH9H-2022-TK36-1).

*3.2. h*ALPL protein purification

The *h*ALPL protein was purified according to the previous operations[2]**.** For the overexpression in the mammalian cells, the human wild-type ALPL gene (amino acid 18–500) was similarly cloned into a modified pcDNA3.4 vector (Invitrogen, USA) with a Pre-Scission Protease recognition sequence followed by a Flag tag and 10× polyhistidine tag at C-terminal, as well as an HA signaling peptide at N-terminal. Expi293F cells (Thermo Fisher Scientific, A14527) were cultured in a chemically defined Union-293 medium (Union-Biotech, UM293-01) at 37 °C, 120 rpm, supplied with 5% CO_2. When cell density reached 2.5×10^6 cells/milliliter, the cells were transiently transfected with the pcDNA3.4-based expression plasmids using PEI MAX (Polysciences, 24765). The cells were harvested 96 h after transfection for protein purification. The supernatants of the cell cultures were collected by centrifugation (Eppendorf 5424R) and then subjected to the affinity chromatography with Nickel-chelating resin (Smart-Lifesciences, China), rinsed with a washing buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10% Glycerol) and 50 mM imidazole). After eluted with an elution buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol and 300 mM imidazole), the elution fractions containing the desired protein were combined, dialyzed to remove imidazole, concentrated by ultrafiltration, aliquoted, and examined with SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.3. SDS-PAGE

Briefly, 5 μg of the purified *h*ALPL was transferred to another 1.5 mL tube, 4 μL NuPAGE LDS sample buffer (4 ×, catalog number: 1874129) was added. The solution was heated at 95 °C for 10 min., samples were loaded to GenScript SurePAGE, Bis-Tris, 10 cm × 8 cm gels (catalog number: M00653) and analyzed by electrophoresis, 130 V for 1 h. The buffering system employed was 1 × MOPS SDS Running Buffer (ABCONE, catalog number: M22785). The intensities of fluorescence were analyzed by ChemiDoc MP Imaging system. Then coomassie (0.5%) was added and the gel was read after washing 2−3 times.

3.4. *h*ALPL enzymatic assays

The assay used p-nitrophenyl phosphate (pNPP; Sangon Biotechnology, China) as the phosphatase substrate which could be enzymatically hydrolyzed into a yellow-colored product (maximal absorbance (Abs) at 405 nm). The reactions were started by adding 50 μl substrate solution comprising of 10 mM pNPP in lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.5) to each well which was pre-loaded with 50 μl aliquot of *h*ALPL diluted 1:100 in the same buffer. The absorbance of 405 nm was measured at 2-min intervals at 37 °C by using a microplate reader (Enspire, PerkinElmer).

3.5. EC_{50} assay for *h*ALPL activation by compounds

Experimental reactions were set up in triplicate by pre-incubation of the *h*ALPL with test compounds two-fold diluted in a final concentration ranging from 100 μM to 1.56 μM in lysis buffer with 5% DMSO for 30 min at room temperature. Subsequently addition of 50 μl pNPP substrate into each well and absorbance at 405 nm was measured on a microplate reader after 30 min. Raw absorbance data were subjected to normalization using the formula:

$$\text{Normalized intensity} = \frac{\text{A sample} - \text{A blank}}{\text{A control} - \text{A blank}}$$

Where A sample A blank, A control represent the absorbance of test samples, blank (medium only), and untreated control (DMSO only), respectively. EC_{50} was calculated by non-linear regression fit using GraphPad Prism v9.0.

3.6. Surface Plasmon Resonance (SPR) binding assay

The SPR binding assays were performed on a Biacore 8K instrument (GE Healthcare). The running buffer (PBS-T80+DMSO) contained 20 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, 0.05% (v/v) surfactant Tween-80, and 5% DMSO. Purified His-labelled *h*ALPL was dissolved at 30 μg/mL in an acetate buffer (pH 4.5), and captured on the Sensor chip CM5 (GE Healthcare, 29-1049-88) using the standard Amine Coupling Kit (GE Healthcare, BR100050). Chemicals in a series of two-fold dilutions were flowed through the sensor chip at a flow rate of 30 μL/min for 90 s for the association step followed by dissociation for an additional 90 s using the Low Weight Molecular multi-cycle kinetics/affinity method provided by GE Healthcare. Solvent correction was carried out before and after each analysis with eight

different concentrations of DMSO solution per cycle. The binding affinities (K_D) of oridonin were determined by fitting the data to a 1:1 binding model using Biacore 8K Evaluation software (GE Healthcare). Binding kinetics of F5, E7 and F8 were analyzed with Biacore evaluation software (Cytiva) to calculate the k_a and k_d values. The K_D values were calculated as the ratio of ligand dissociation rate to association rate (k_d/k_a).

3.7. Rescue experiment

Pre-incubate purified *h*ALPL (0.1 mg/mL,10 μL) with tetramisole (Aladdin, T335508-25g) or SBI-425 (MedChemexpress, HY-124756-5mg) at the final inhibitor concentration of 200 μM or 200 nM respectively in 20 μL lysis buffer with 5% DMSO at 25°C for 30 min, followed by rescue treatment through supplementation with either F5 or E7 or F8 (two-fold diluted in a final concentration ranging from 100 μM to 1.56 μM in lysis buffer with 5% DMSO) at 25°C for 30 min. The enzymatic activity was performed according to the previously described methodology (Methods section 3.4), data were analyzed from three independent experiments (triplicates each) using non-linear regression fit using GraphPad Prism v9.0.

3.8. Cell lysate-based enzymatic assays

Hek293T cells were seeded at a standardized density of 1×10^5 cells/well in 24-well tissue culture plates (CCP-Nunc, 144530) to achieve 70-80% confluency at the time of transfection. Following 24-hour adherence in complete DMEM supplemented with 10% FBS at 37°C with 5% CO_2, cells were transfected using PEI MAX according to the manufacturer's protocol. Plasmid DNA (1 μg/well) of various ALPL mutations and transfection reagent (2 μL/well) were maintained at constant ratios across all experimental conditions to ensure transfection consistency. At 24 hours post-transfection, transfection efficiency was assessed using fluorescence microscopy (Nikon Eclipse Ti) for GFP-tagged constructs. For protein extraction, culture medium was aspirated and cells were washed twice with ice-cold PBS (pH 7.4). Cellular lysis was performed using RIPA buffer (Absin, abs9231-100ml) supplemented with 1× protease inhibitor cocktail (Sigma-Aldrich, P8215-1ML). Following 30-minute incubation on ice with periodic vortexing, lysates were clarified by centrifugation at 13,000 × g for 10 minutes at 4°C. Supernatants were aliquoted and stored at -80°C or processed immediately for enzymatic assays. The enzymatic activity were performed according to the previously described methodology (Methods section 3.4), cell lysates were dispensed in triplicate (10 μL/well) into 96-well flat-bottom plates (Sigma-Aldrich, M0661-1CS), followed by treatment with either F5 or E7 or F8 at a final concentration of 10 μM in lysis buffer with 5% DMSO at 25°C for 30 min. Data were analyzed from three independent experiments (triplicates each) using non-linear regression fit using GraphPad Prism v9.0.

3.9. Alkaline Phosphatase (ALP) Staining

Bone Marrow-derived Mesenchymal Stromal Cells (BMSCs) were seeded in 48-well plates (CCP-Nunc, 150787) at a density of 1×10^4 cells/well. The control group was maintained in complete growth medium (α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin (MedChemexpress, HY-K0014-10mL) while experimental groups were cultured in osteogenic induction medium (complete medium supplemented with 50 μg/mL ascorbic acid,

10 mM β-glycerophosphate, and 100 nM dexamethasone) (Sigma, SCM121) containing varying concentrations (0, 0.8, 1.6, or 3.2 μM) of test compounds F5, E7, or F8. Medium was replaced with fresh one every third day. After 7 days of culture, cells were washed twice with PBS and fixed with 4% paraformaldehyde (Alfa Aesar, J61899.AK) for 15 min at room temperature. ALP activity was assessed using a BCIP/NBT chromogenic kit (Beyotime, C3206) according to the manufacturer's protocol, with incubation at 37°C for 30 min protected from light. Following three PBS washes, plates were air-dried and imaged using a Cytation 5 cell imaging multimode reader (BioTek Instruments, USA). Staining intensity was quantified via densitometric analysis using ImageJ software (NIH, version 1.53).

3.10. Total RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was isolated from E7-treated pre-osteoblast cultures using TRIzol Reagent (Thermo Fisher Scientific, 15596018) following established chaotropic lysis protocols. Subsequent cDNA synthesis was performed with the PrimeScript RT Master Mix (Takara Bio, Otsu, Japan) under optimized reverse transcription conditions (37°C for 15 min, 85°C for 5 sec). Quantitative Real-time PCR (qPCR) was performed using the Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (Yeasen, 11202ES08). Osteogenic differentiation markers (RUNX2, OSTERIX, OCN, OPN, COL1A1) were analyzed following 72-hour exposure to 2 μM F5 compound, with primer sequences validated through NCBI Primer-BLAST specificity checks. Normalization against β-actin reference gene was performed using the comparative threshold cycle (2-ΔΔCT) method.

Table S1. Primers for the RT-qPCR

Gene	Forward	Reverse
Col1a1	TCCAGGATGTTAGGAACTGTG	AGGCCTGAGTTCATGTTGCT
Runx2	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
Alp	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Ocn	ATGGCTTGAAGACCGCCTAC	GACAGGGAGGATCAAGTCCC
Opn	CTTTCACTCCAATCGTCCCTAC	GCTCTCTTTGGAATGCTCAAGT
Osterix	AGGCACAAAGAAGCCATAC	AATGAGTGAGGGAAGGGT
β-actin	CAGCAAGCAGGAGTACGATGA	AAAACGCAGCTCAGTAACAGTC

3.11. Mice

All animal experiments were approved by the Institutional Animal Care and Ethics Committee of Ninth People's Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China, Ethics Number: SH9H-2022-A037-SB). Animals used in this experiment were 8-wk-old male mice purchased from Weitonglihua Corporation (Beijing, China). All mice were maintained on C57BL/6 background, all mice were housed in pathogen-free conditions with constant ambient temperature (22 ± 2 ℃) and humidity (55 ± 10%), with an alternating 12-h light/dark cycle. Animals were fed standard rodent chow and had access to fresh water ad libitum.

3.12. Mouse Femoral Fracture

Under isoflurane inhalation anesthesia, the skin over the femoral region was incised with a scalpel blade. Muscles were bluntly dissected to expose the femur, followed by lateral dislocation of the patella to reveal the femoral condyle. The medullary cavity was reamed using a 25G needle (BD) prior to insertion of an isodiameter intramedullary pin. A mid-shaft femoral osteotomy was performed using a dental drill. The pin was retained in situ to stabilize the fracture and permit postoperative ambulation. Muscular layers were re-approximated and the skin was closed with 4/0 nylon sutures.

3.13. Pharmaceutical intervention

Fracture model mice were randomly assigned to three treatment groups (n = 3 per group). Intramuscular injections (200 μL per dose) were administered at postoperative days 3, 7, 10, and 14: Group 1 (vehicle control) received phosphate-buffered saline (PBS), Group 2 received 10 μM E7, and Group 3 received 20 μM E7. Evaluate fracture healing using the aforementioned X-ray and gait analysis methods.

3.14. X-Ray Assessment

Femoral fracture progression was monitored using a digital radiography system (MultiFocus 10x15, Faxitron Bioptics, LLC.). Mice were anesthetized with inhaled isoflurane and positioned in a prone orientation on the scanning platform, with the affected limb abducted and the knee joint maintained at 90° flexion. High-resolution digital images were acquired. Longitudinal imaging was performed at two-day intervals for a duration of three weeks.

3.15. micro-CT scanning

Fresh bone samples were fixed in 4% paraformaldehyde for 24–48 hours at 4°C to preserve microstructure. After fixation, samples were transferred to PBS containing 0.01% sodium azide to prevent bacterial growth. Samples were secured in a cylindrical holder with foam padding to minimize movement during scanning. Then micro-CT scanning was performed using a high-resolution micro-CT scanner (μCT-100, SCANCO Medical AG, Wangen-Brütti-sellen, Switzerland). Raw projection data were reconstructed using SCANCO micro-CT software (v6.5–3) with a modified Feldkamp algorithm. Three-dimensional reconstructions of the CT scanning and subchondral bone parameters were analyzed using the μCT-100 software (v6.5–3, SCANCO Medical AG) according to the standard protocol.

3.16. Histology

The Fractured femu were harvested from the 8-week-old male C57BL6/J mice, fixed with 4% paraformaldehyde (prepared in 0.1 M phosphate-buffered saline, pH 7.4) for 1 hours, Sequential dehydration was performed through a graded ethanol series (70%, 80%, 95%, and 100% v/v; 1 hour per concentration) to minimize tissue shrinkage artifacts. For osseous components, specimens underwent decalcification in 0.5 M EDTA (pH 7.4) with daily solution, decalcified tissues were cleared in xylene and embedded in paraffin (melting point 56–58°C) under standardized anatomical orientation. Serial sections (5 μm thickness) were obtained using a rotary microtome (Leica, RM2235), mounted on poly-L-lysine-coated slides, and dried at 45°C for 24 hours to ensure adhesion. Histological analyses were conducted through multiplex

staining protocols: 1) Hematoxylin and Eosin (H & E)—Standard protocol for paraffin tissue sections. 2) Safranin O/Fast Green (SO/FG) — Differential staining of sulfated glycosaminoglycans (red) versus collagen (green) in cartilage matrices. 3) Tartrate-Resistant Acid Phosphatase (TRAP)—Enzymatic activity visualization using naphthol AS-BI phosphate substrate to identify osteoclastic resorption zones. All reagents (Servicebio, Wuhan, China) were applied following manufacturer-validated protocols. The samples were coated with 10 nm of gold using a gold sprayer (Leica, ACE600a) before SEM scans (Carl Zeiss, Gemini300,). SEM images were captured in conventional high-vacuum mode at 5 kV and a working distance of 5.6 mm. Staining intensity was quantified via densitometric analysis using ImageJ software (NIH, version 1.53).

3.17. Gait analysis

Gait parameters were assessed using the CatWalk XT system. (Noldus Information Technology, Wageningen, Netherlands) according to the manufacturer's instructions. Briefly, mice were individually placed on an illuminated glass walkway and allowed to ambulate freely to traverse from one end to the other. A high-speed color camera (100 Hz) positioned beneath the platform recorded the dynamic illuminated contact areas between paws and glass surface. Signal intensity (0-255 arbitrary units) correlated with the contact area and increased proportionally to the applied paw pressure. Only uninterrupted runs meeting predefined velocity criteria (5-25 cm/sec) were collected for analysis. Spatial-temporal parameters, including paw print area, pressure distribution, and stride kinematics, were automatically quantified using the CatWalk Software v10.6 with paw recognition algorithms.

3.18. Statistical Analysis

Experiments were repeated at least three times, and the results were expressed as means ± standard deviation (S.D.) unless otherwise indicated. Sample numbers are indicated in figure legends. Data analysis was performed with GraphPad Prism v9.0. Significance was assumed at a p-value <0.05 by using a two-tailed Student's t-test or one-way ANOVA for comparisons of two groups or more than two groups, respectively, in the software for normally distributed data sets with equal variances, (*$p < 0.05$, very significant for **$p < 0.01$, and the most significant for ***$p < 0.001$).

4. Library synthesis, characterization and purification

4.1. General strategies for oligonucleotides conjugation

4.1.1. Synthesis of HP-AOP

Fmoc-NH-PEG5-CH2CH2COOH (AOP, 200mM in DMSO, 3.6 mL), S-NHS (150 mM in 2:1 DMSO / H_2O, 3.6 mL) and EDC (200 mM in DMSO, 3.6 mL) in DMSO (15 mL) were mixed and the mixture was shaken at room temperature for 30 minutes. Subsequently, the HP-DNA (1 mmol) was dissolved borate buffer (250 mM, pH 9.5, 15 mL). Activated compound was combined with the DNA solution and the resulting mixture was shaken at 45°C for 12 hours. After the reaction was completed as monitored by UPLC-MS, a 10% v/v aqueous piperidine

solution was added to the DNA solution containing the -NFmoc group, and the reaction was allowed to proceed at room temperature for 30 min. The reaction mixture was added 5 M NaCl solution (10 % by volume) and cold ethanol (2.5 folds by volume, ethanol stored at -20°C). The mixture was stored at -80 °C for more than 30 minutes. After that, the mixture was centrifuged for 15 minutes at 4 °C in a microcentrifuge at 12000 rpm. The supernatant was removed and the pellet was dissolved in water to the final concentration of 1 mM and used directly for the next step of click coupling reaction without further purification.

4.1.2. On-DNA amide bond formation of Fmoc-protected amino acids

In a 96-well plate to Fmoc-protected amino acids (BB1, 200mM in DMSO, 10 μL), S-NHS (150 mM in 2:1 DMSO / H_2O, 10 μL) and EDC (200 mM in DMSO, 10 μL) in DMSO (42 μL) were added and the mixture was shaken at room temperature for 30 minutes. Subsequently, the HP-AOP (1 mM in borate buffer (250 mM, pH 9.5), 42 μL) was combined with the activated solution and the resulting mixture was shaken at 45°C for 12 hours. After the reaction was completed as monitored by UPLC-MS, a 10% v/v aqueous piperidine solution was added to the DNA solution containing the -NFmoc group, and the reaction was allowed to proceed at room temperature for 30 min. The reaction mixture was added 5 M NaCl solution (10 % by volume) and cold ethanol (2.5 folds by volume, ethanol stored at -20°C). The mixture was stored at -80 °C for more than 30 minutes. After that, the mixture was centrifuged for 15 minutes at 4 °C in a microcentrifuge at 12000 rpm. The supernatant was removed and the pellet was dissolved in water to the final concentration of 1 mM and used directly for the next step of click coupling reaction without further purification.

4.1.3. On-DNA amide bond formation of carboxyl-containing benzoselenazolones (BSEAs)

In a 96-well plate to carboxyl-containing BSEAs (BB2, 200mM in DMSO, 20 μL), S-NHS (150 mM in 2:1 DMSO/H_2O, 20 μL) and EDC (200 mM in DMSO, 20 μL) in DMSO (83 μL) were added and the mixture was shaken at room temperature for 30 minutes. Subsequently, the HP-AOP-BB1 (1 mM in borate buffer (250 mM, pH 9.5), 83 μL) was combined with the activated solution and the resulting mixture was shaken at 45°C for 12 hours. The reaction mixture was added 5 M NaCl solution (10 % by volume) and cold ethanol (2.5 folds by volume, ethanol stored at -20°C). The mixture was stored at -80 °C for more than 30 minutes. After that, the mixture was centrifuged for 15 minutes at 4 °C in a microcentrifuge at 12000 rpm. The supernatant was removed and the pellet was dissolved in water to the final concentration of 1 mM and used directly for the next step of click coupling reaction without further purification.

4.1.4. On-DNA SeNEx click reaction

In a 96-well plate to DNA-AOP-BB1-BB2 (1 mM, 15 μL), DMSO (143 μL), $AgNO_3$ (10 mM in ddH_2O, 15 μL) NH_3•H_2O (50 mM in ddH_2O, 15 μL), alkyne-bearing natural products (BB3, 20 mM in DMSO, 37.5 μL) were added. The resulting mixture was vortexed and stood at room temperature for 12 hours. The reaction mixture was added 5 M NaCl solution (10 % by volume) and cold ethanol (2.5 folds by volume, ethanol stored at -20°C). The mixture was stored at -80 °C for more than 30 minutes. After that, the mixture was centrifuged for 15 minutes at 4 °C in a microcentrifuge at 12000 rpm. The supernatant was removed and the pellet was dissolved in water to the final concentration of 1 mM.

4.2. Library step

4.2.1. Oligo DNA Annealing

The two complementary single-stranded Oligo DNA strands (purchased from GenScript, each at 2.0 mM in aqueous solution) were mixed in equal volumes. The mixture was heated at 95°C for 3 min and then allowed to cool gradually to room temperature, yielding double-stranded Oligo DNA at a final concentration of 1 mM.

4.2.2. DNA Purification/Handling

To the post-reaction mixture (containing chemically/enzymatically modified Oligo DNA), 10% (v/v) of 5 M NaCl aqueous solution was added and mixed by pipetting. Subsequently, 2.5–3 volumes of ice-cold absolute ethanol (pre-chilled at −20°C) were added with thorough mixing until a white turbidity appeared. The solution was then incubated at −80°C for at least 30 min. After chilling, the mixture was centrifuged under the following conditions: 1) 1.5 mL microcentrifuge tubes were centrifuged in an Eppendorf Centrifuge FRESCO21 with 12,000 × g for 15 min; 2) 96-well plates or 15/50 mL conical tubes were centrifuged in an Eppendorf Centrifuge 5810R with 3,900 rpm for 30 min. The supernatant was carefully discarded, and the pellet was air-dried to remove trace ethanol. The precipitate was then resuspended in an appropriate volume of *dd*H_2O and quantified by Nanodrop 2000 from Thermo Scientific via absorption at 260 nm to adjust the final concentration to 1 mM.

4.2.3. Agarose Gel Electrophoresis Analysis

From each well of the post-ligation reaction mixture, 1 μL was diluted with an appropriate volume of *dd*H_2O, and 10 μL of the diluted solution (containing ~100 ng of DNA) was mixed with 2 μL of 6× loading buffer (Yeasen Biotechnology, 10102ES76). The samples were loaded onto a 4% agarose gel (50-well format), with DNA ladder (Sangon Biotech, B500005-0001) loaded in the first lane of each row as a reference. Electrophoresis was performed in 0.5× TBE buffer (Sangon Biotech, B548105-0005) at 150 V for 28 min. After separation, the gel was visualized under UV illumination using a gel imaging system.

4.2.4 Synthesis of Se-*n*DEL

i) Round 1

A total of 2.6 mL of HP-AOP solution was evenly aliquoted into 259 wells (10 μL/well). To each well, 10 μL of Oligo0 (1 mM) and 10 μL of OligoA1-259 (code1, 1 mM) were added, followed by 10% (v/v) T4 DNA ligase and 10× T4 ligation buffer. The mixture was thoroughly mixed and incubated at room temperature for 12 h. Agarose gel electrophoresis (Figure S1A) confirmed successful ligation, with a product band of ~30 bp. DNA from each well was isolated by ethanol precipitation. After centrifugation, the pellet was resuspended in 10 μL of borate buffer (250mM, pH 9.5). HP-DNA and 259 Fmoc-protected amino acids (BB1) (Figure S2) conjugates were prepared as described in Section 4.1.2 subsequently pooled, followed by Fmoc deprotection, and resuspended in *dd*H_2O to a final concentration of 1 mM.

ii) Round 2

200 μL of the Round 1 product was aliquoted into 10 wells (20 μL/well), each well received 10 μL of OligoB1-10 (code2, 1 mM), 10% (v/v) T4 ligase, and 10× ligation buffer, followed by incubation at room temperature for 12 h. Gel electrophoresis (Figure S1B) revealed a ~40

bp product. DNA from each well was isolated by ethanol precipitation, after centrifugation the pellet was resuspended in 20 µL of borate buffer (250mM, pH 9.5), and reacted with carboxyl-containing BSEAs (Figure S3) as described in Section 4.1.3. Pooled DNA was reprecipitated and adjusted to a final concentration of 1 mM in *dd*H_2O.

iii) Round 3

150 µL of the Round 2 product was aliquoted into 10 wells (15 µL/well), each well received 15 µL of OligoC1-10 (code3, 1 mM) and 15 µL of OligoD (closing tag, 1 mM), followed by 10% (v/v) T4 DNA ligase and 10× T4 ligation buffer. The mixture was thoroughly mixed and incubated at room temperature for 12 h. Gel analysis (Figure S1C) showed a dominant ~60 bp band, with minor incomplete ligation products. DNA from each well was isolated by ethanol precipitation, after centrifugation the pellet was resuspended in 15 µL of borate buffer (250mM, pH 9.5), and reacted with alkyne-bearing natural products (Figure S4) as described in Section 4.1.4. Pooled DNA was reprecipitated and adjusted to a final concentration of 1 mM in *dd*H_2O. The final Se-*n*DEL library comprised 2.59×10^4 unique members (259 × 10 × 10).

5. Se-*n*DEL selection against *h*ALPL

5.1 Selection of hits from the Se-*n*DEL

The purified *h*ALPL was used to bind to Ni-charged Magbeads (GenScript, L00295). The Magbeads were washed twice using PBST in 5 minute intervals. The beads were separated using a magnetic rack to separate from the supernatant. For each screening, 10 µg His-tagged *h*ALPL was immobilized on 12 µL Magbeads with 200 µg/mL Salmon sperm DNA (Thermo Fisher Scientific, 15632-011) as the blocking buffer, by incubation at room temperature for 1 hour in a total volume of 50 µL. Negative control was set with *h*ALPL replaced by PBS. After two washes of the immobilized target and the negative control with PBST, Se-*n*DEL was added to them. The final volume of the selection system is 100 µL, with concentration of each DNA-encoded chemicals around 0.05 pM. The selection system was incubated at room temperature with rotation for 1 hour. Following five washes with PBST, *h*ALPL bound chemical-DNA conjugates were eluted from the target by heating at 95 °C for 10 min in 90 µL PBST. The resulting final elution was collected for sequencing analyses.

5.2 Next generation Sequencing and selection data Analysis

The Se-*n*DEL library contains a total of 25900 chemical structures, each of which was encoded with a unique DNA sequence. Deep sequencing of Se-*n*DEL was carried out using Illumina method. The Illumina adaptor sequences around the DNA coding sequences were trimmed by CLC genomics workbench version 12 (Qiagen). The resulting DNA sequences were 30 base pairs in length corresponding to the DNA sequences of building blocks in 3 rounds of "split-pool" iterations. For each testing sample, the DNA coding sequences were mapped to the reference DEL library. No mismatch was allowed in the mapping. The mapped coding sequences were counted for all compounds across different samples. DEL decoding and data analysis was performed as previously described[3]. In brief, DNA tags were amplified by PCR using the following primers:

Forward: 5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG
Reverse: 5’-CAAGCAGAAGACGGCATACGAGATGTCGTGATGTGACTGGAGTTC.

6. SeNEx chemistry-based in situ nanomole-scale parallel synthesis and enzymatic activity screening

6.1 Nanomole-scale Parallel Synthesis of in situ Se-Natural Product Library

20 μL of BSEAs (10 mM in DMSO), 10 μL of alkyne-bearing oridonin (20 mM in DMSO), and 1 μL of $AgNO_3$ (20 mM in ddH2O) and 1 μL of $NH_3 \cdot H_2O$ (0.3 M) and 18 μL of DMSO were added into each well of 96 well microplate, the plate was sealed, and the reaction mixture were agitated (12000 rpm) at room temperature for 6 hours. The resulting solutions were directly utilized for subsequent biological activity evaluation procedures without further purification, with the compound concentration in each well being approximately 4 mM.

6.2 In situ Enzymatic Activity Screening against *h*ALPL

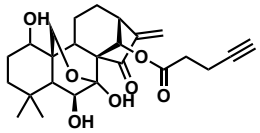
Experimental reactions were set up in triplicate by pre-incubation of the *h*ALPL with test compounds at a final concentration of 10 μM in lysis buffer with 5% DMSO for 30 min at room temperature. Subsequently addition of 50 μl pNPP substrate into each well and absorbance at 405 nm was measured on a microplate reader after 30 min. Raw absorbance data were subjected to normalization using the formula:

$$\text{Normalized intensity} = \frac{\text{A sample} - \text{A blank}}{\text{A control} - \text{A blank}}$$

Where A sample A blank, A control represent the absorbance of test samples, blank (medium only), and untreated control (DMSO only), respectively.

7. Chemical synthesis

(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7-oxododecahy-dro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl pent-4-ynoate (S1)

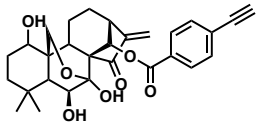


S1

To a solution of Oridonin (364 mg, 1 mmol) and 4-epentynoic acid (147 mg, 1.5 mmol) in 10 mL DCM was added EDCI (282mg, 2 mmol) and DMAP (147 mg, 1.2 mmol) successively, then the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (PE: EA = 2:3) to afford the title compound **S1** as a pale yellow solid in 60% yield (266 mg). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.88 – 7.78 (m, 2H), 7.58 (d, J = 8.1 Hz, 2H), 6.07 (d, J =

3.7 Hz, 3H), 5.94 (d, *J* = 10.2 Hz, 1H), 5.64 (s, 1H), 4.45 (d, *J* = 10.0 Hz, 2H), 4.15 (d, *J* = 10.2 Hz, 1H), 3.94 – 3.82 (m, 1H), 3.53 (dd, *J* = 10.3, 6.8 Hz, 1H), 3.38 – 3.32 (m, 1H), 3.14 (d, *J* = 9.7 Hz, 1H), 2.60 – 2.53 (m, 1H), 2.17 (qd, *J* = 13.4, 8.0 Hz, 1H), 1.91 (dd, *J* = 13.0, 5.8 Hz, 1H), 1.76 (dt, *J* = 13.6, 6.3 Hz, 1H), 1.50 (dddt, *J* = 30.2, 23.9, 13.8, 7.7 Hz, 3H), 1.32 (dt, *J* = 13.3, 3.4 Hz, 1H), 1.26 – 1.14 (m, 2H), 1.00 (d, *J* = 2.8 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 207.57, 170.44, 151.18, 119.99, 96.16, 83.51, 74.54, 73.97, 72.04, 71.97, 62.97, 62.31, 59.51, 54.30, 41.80, 40.91, 38.82, 33.80, 33.78, 33.20, 30.74, 29.79, 22.14, 20.19, 13.99.
HRMS (ESI) calculated for $[M+H]^+$ $[C_{25}H_{33}O_7]^+$ 445.2221, found 445.2227.

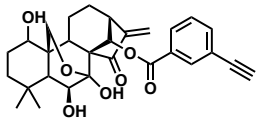
(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7oxododecahydro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl 4-ethynylbenzoate (S2)



S2

Following the general procedure[1], **S2** was obtained as a white solid (354mg, 72% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.88 – 7.78 (m, 2H), 7.58 (d, *J* = 8.1 Hz, 2H), 6.07 (d, *J* = 3.7 Hz, 3H), 5.94 (d, *J* = 10.2 Hz, 1H), 5.64 (s, 1H), 4.45 (d, *J* = 10.0 Hz, 2H), 4.15 (d, *J* = 10.2 Hz, 1H), 3.94 – 3.82 (m, 1H), 3.53 (dd, *J* = 10.3, 6.8 Hz, 1H), 3.38 – 3.32 (m, 1H), 3.14 (d, *J* = 9.7 Hz, 1H), 2.60 – 2.53 (m, 1H), 2.17 (qd, *J* = 13.4, 8.0 Hz, 1H), 1.91 (dd, *J* = 13.0, 5.8 Hz, 1H), 1.76 (dt, *J* = 13.6, 6.3 Hz, 1H), 1.50 (dddt, *J* = 30.2, 23.9, 13.8, 7.7 Hz, 3H), 1.32 (dt, *J* = 13.3, 3.4 Hz, 1H), 1.26 – 1.14 (m, 2H), 1.00 (d, *J* = 2.8 Hz, 6H). HRMS (ESI) calculated for $[M+H]^+$ $[C_{29}H_{33}O_7]^+$ 493.2221, found 493.2227.

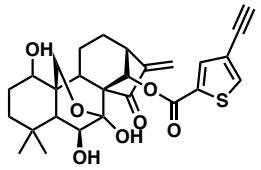
(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7-oxododecahyd-ro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl 3-ethynylbenzoate (S3)



S3

To a solution of Oridonin (364 mg, 1 mmol) and 3-ethynyl-benzoic acid (219 mg, 1.5 mmol) in 10 mL DCM was added EDCI (282mg, 2 mmol) and DMAP (147 mg, 1.2 mmol) successively, then the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (PE: EA = 2:3) to afford the title compound **S3** as a pale yellow solid in 70% yield (345 mg). ^{1}H NMR (500 MHz, DMSO) δ 7.88 (dd, *J* = 7.7, 1.5 Hz, 2H), 7.71 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.51 (t, *J* = 7.7 Hz, 1H), 6.12 (s, 1H), 6.09 – 6.05 (m, 2H), 5.94 (d, *J* = 10.3 Hz, 1H), 5.65 (s, 1H), 4.45 (d, *J* = 5.1 Hz, 1H), 4.30 (s, 1H), 4.15 (d, *J* = 10.2 Hz, 1H), 3.90 – 3.84 (m, 1H), 3.54 (dd, *J* = 10.3, 6.8 Hz, 1H), 3.41 – 3.37 (m, 1H), 3.16 (d, *J* = 9.7 Hz, 1H), 2.56 (dt, *J* = 13.7, 8.9 Hz, 1H), 2.17 (qd, *J* = 13.4, 8.0 Hz, 1H), 1.92 (dd, *J* = 13.0, 5.8 Hz, 1H), 1.76 (dt, *J* = 13.6, 6.4 Hz, 1H), 1.58 – 1.42 (m, 3H), 1.32 (dt, *J* = 13.4, 3.4 Hz, 1H), 1.24 (dd, *J* = 13.7, 3.4 Hz, 1H), 1.18 (t, *J* = 7.2 Hz, 1H), 1.01 (d, *J* = 2.0 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 207.71, 164.35, 151.18, 136.37, 132.80, 131.43, 130.25, 129.54, 122.42, 120.04, 96.33, 82.97, 82.30, 75.10, 74.46, 72.06, 63.08, 62.49, 59.69, 54.33, 41.84, 40.96, 38.82, 33.82, 33.18, 30.78, 29.82, 22.09, 20.22. HRMS (ESI) calculated for $[M+H]^+$ $[C_{29}H_{33}O_7]^+$ 493.2221, found 493.2225.

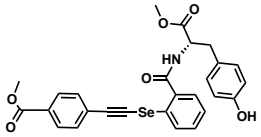
(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7-oxododecahydro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl 4-ethynylthiophene-2-carboxylate (S4)



S4

To a solution of Oridonin (364 mg, 1 mmol) and 4-ethynylthiophene-2-carboxylic acid (228 mg, 1.5 mmol) in 10 mL DCM was added EDCI (282mg, 2 mmol) and DMAP (147 mg, 1.2 mmol) successively, then the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (PE: EA = 2:3) to afford the title compound **S4** as a pale yellow solid in 50% yield (249 mg). ^{1}H NMR (500 MHz, DMSO) δ 8.15 – 8.06 (m, 1H), 7.58 (d, *J* = 1.5 Hz, 1H), 6.05 (d, *J* = 7.1 Hz, 2H), 6.01 (s, 1H), 5.90 (d, *J* = 10.3 Hz, 1H), 5.65 (s, 1H), 4.44 (d, *J* = 5.1 Hz, 1H), 4.21 (s, 1H), 4.13 (d, *J* = 10.2 Hz, 1H), 3.86 (d, *J* = 10.2 Hz, 1H), 3.52 (dd, *J* = 10.3, 6.8 Hz, 1H), 3.36 (d, *J* = 5.6 Hz, 1H), 3.15 (d, *J* = 9.7 Hz, 1H), 2.58 – 2.52 (m, 1H), 2.12 (tt, *J* = 13.1, 6.6 Hz, 1H), 1.90 (dd, *J* = 13.0, 5.7 Hz, 1H), 1.75 (dt, *J* = 13.9, 6.3 Hz, 1H), 1.58 – 1.40 (m, 3H), 1.32 (dt, *J* = 13.3, 3.4 Hz, 1H), 1.21 (td, *J* = 13.6, 3.5 Hz, 1H), 1.15 (d, *J* = 6.9 Hz, 1H), 1.00 (d, *J* = 3.5 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 207.49, 160.05, 151.10, 137.76, 135.66, 134.76, 121.47, 119.97, 96.25, 81.45, 78.22, 75.26, 74.47, 72.04, 63.04, 62.51, 59.64, 54.33, 41.82, 40.93, 38.81, 33.81, 33.18, 30.75, 29.80, 22.10, 20.20. HRMS (ESI) calculated for $[M+H]^+$ $[C_{27}H_{31}O_7S]^+$ 499.1785, found 499.1792.

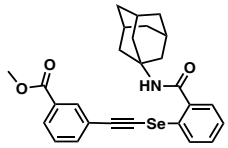
methyl (S)-4-(((2-((3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2yl)carbamoyl)phenyl)se-lanyl)ethynyl)benzoate (A1)



A1

To a stirring solution of BTSA (**Se-16**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**ak2**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5 equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **A1**. This compound was obtained in 92% yield (96 mg) as a white foamy solid. ^{1}H NMR (400 MHz,$CDCl_3$) δ 8.21 (d, *J* = 7.6 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 2H), 7.57 (d, *J* = 8.4 Hz, 2H), 7.53 – 7.47 (m, 2H), 7.33 – 7.24 (m, 1H), 6.96 (d, *J* = 8.4 Hz, 2H), 6.79 (d, *J* = 7.6 Hz, 1H), 6.74 (d, *J* = 8.4 Hz, 2H), 5.71 (s, 1H), 5.04 (dt, *J* = 7.2, 5.4 Hz, 1H), 3.93 (s, 3H), 3.79 (s, 3H), 3.27 – 3.12 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.00, 166.77, 166.62, 155.09, 134.34, 132.53, 131.30, 130.46, 130.31, 129.75, 129.53, 129.38, 128.01, 127.19, 126.89, 126.32, 115.61, 103.71, 78.35, 53.80, 52.62, 52.26, 36.95. HRMS (ESI) calculated for $C_{27}H_{24}NO_6Se^+$ ($[M+H]^+$): 538.0763; found: 538.0770.

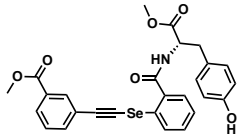
methyl 3-(((2-(((3s,5s,7s)-adamantan-1yl)carbamoyl)phenyl)selanyl)ethynyl)benzoate (A2)



A2

To a stirring solution of BTSA (**Se-5**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**ak3**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5 equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **A2**. This compound was obtained in 94% yield (93 mg) as a white foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 – 8.17 (m, 2H), 7.99 (dt, *J* = 8.0, 1.6 Hz, 1H), 7.69 (dt, *J* = 7.6, 1.6 Hz, 1H), 7.52 – 7.42 (m, 2H), 7.46 – 7.38 (m, 1H), 7.27 (td, *J* = 7.6, 0.8 Hz, 1H), 5.92 (s, 1H), 3.93 (s, 3H), 2.13 (s, 9H), 1.78 – 1.67 (m, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.69, 166.39, 135.73, 133.72, 132.69, 131.99, 131.75, 130.40, 130.34, 129.18, 128.45, 126.31, 125.98, 123.96, 102.72, 76.26, 52.82, 52.26, 41.60, 36.26, 29.46. HRMS (ESI) calculated for $C_{27}H_{28}NO_3Se^+$ ($[M+H]^+$): 494.1229; found: 494.1235.

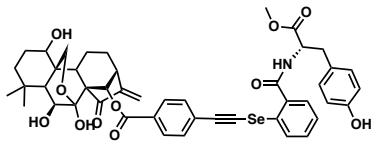
methyl (S)-3-(((2-((3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)phenyl)selanyl)ethynyl)benzoate (A3)



A3

To a stirring solution of BTSA (**Se-16**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**ak3**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5 equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **A3**. This compound was obtained in 92% yield (100 mg) as a white foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (dd, *J* = 8.4, 1.2 Hz, 1H), 8.20 (t, *J* = 1.6 Hz, 1H), 8.00 (dt, *J* = 8.0, 1.2 Hz, 1H), 7.70 (dt, *J* = 7.6, 1.2 Hz, 1H), 7.54 – 7.46 (m, 2H), 7.43 (t, *J* = 7.6 Hz, 1H), 7.33 – 7.25 (m, 1H), 6.96 (d, *J* = 8.4 Hz, 2H), 6.77 (d, *J* = 7.6 Hz, 1H), 6.74 (d, *J* = 8.4 Hz, 2H), 5.51 (s, 1H), 5.04 (dt, *J* = 7.2, 5.4 Hz, 1H), 3.93 (s, 3H), 3.79 (s, 3H), 3.29 – 3.12 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.96, 166.78, 166.47, 155.01, 135.78, 134.50, 132.74, 132.53, 130.50, 130.42, 130.38, 129.79, 129.33, 128.52, 127.32, 126.85, 126.25, 123.83, 115.60, 103.22, 75.58, 53.80, 52.61, 52.32, 36.95. HRMS (ESI) calculated for $C_{27}H_{24}NO_6Se^+$ ($[M+H]^+$): 538.0763; found: 538.0767.

(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7oxododecahydro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl4-(((2(((S)-3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)phenyl)selanyl)ethyn-yl)benzoate (F5)

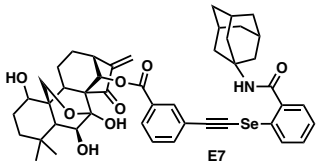


F5

To a stirring solution of BTSA (**Se-16**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**S2**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5

equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **F5**. This compound was obtained in 89% yield (154 mg) as a white foamy solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.27 – 9.16 (m, 2H), 8.11 (d, J = 8.1 Hz, 1H), 8.05 (dd, J = 7.8, 1.4 Hz, 1H), 7.86 (d, J = 8.2 Hz, 2H), 7.64 (dd, J = 11.7, 7.6 Hz, 3H), 7.47 (t, J = 7.5 Hz, 1H), 7.13 – 7.04 (m, 2H), 6.69 – 6.63 (m, 2H), 6.10 – 6.03 (m, 3H), 5.93 (d, J = 10.2 Hz, 1H), 5.64 (s, 1H), 4.61 (ddd, J = 10.1, 7.7, 5.2 Hz, 1H), 4.43 (d, J = 4.9 Hz, 1H), 4.15 (d, J = 10.2 Hz, 1H), 3.87 (d, J = 10.2 Hz, 1H), 3.66 (s, 3H), 3.54 (dd, J = 10.3, 6.8 Hz, 1H), 3.39 (s, 0H), 3.15 (d, J = 9.7 Hz, 1H), 3.09 (dd, J = 13.9, 5.2 Hz, 1H), 3.01 (dd, J = 13.9, 10.1 Hz, 1H), 2.54 (s, 1H), 2.17 (tq, J = 13.2, 7.9, 7.4 Hz, 1H), 1.92 (dd, J = 12.9, 5.8 Hz, 1H), 1.76 (dt, J = 13.8, 6.2 Hz, 1H), 1.51 (dtt, J = 23.4, 15.3, 8.5 Hz, 3H), 1.35 – 1.21 (m, 2H), 1.17 (d, J = 6.8 Hz, 1H), 1.00 (d, J = 2.9 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 207.69, 172.32, 167.43, 164.52, 156.44, 151.23, 133.36, 133.29, 131.63, 130.45, 130.10, 129.76, 129.62, 129.01, 127.87, 127.31, 127.01, 119.99, 115.56, 103.91, 96.34, 79.58, 74.95, 74.51, 72.05, 63.08, 62.51, 59.69, 55.30, 54.33, 52.55, 41.89, 40.96, 38.82, 35.84, 33.82, 33.18, 30.77, 29.82, 22.10, 20.23. HRMS (ESI) calculated for $C_{46}H_{48}NO_{11}Se^+$ ([M+H]$^+$): 870.2387; found: 870.2395.

(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7oxododecahydro-1H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl 3-(((2-(((3s,5s,7s)-adamantan-1-yl)carbamoyl)phenyl)selanyl)ethynyl)benzoate (E7)

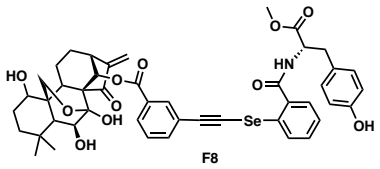


E7

To a stirring solution of BTSA (**Se-5**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**S3**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5 equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **E7**. This compound was obtained in 92% yield (155 mg) as a white foamy solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.07 (dd, J = 8.1, 1.1 Hz, 1H), 8.03 – 7.96 (m, 2H), 7.93 (d, J = 1.8 Hz, 1H), 7.86 (dt, J = 7.8, 1.5 Hz, 1H), 7.78 (dt, J = 7.6, 1.5 Hz, 1H), 7.60 (td, J = 7.7, 1.4 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.40 (td, J = 7.5, 1.1 Hz, 1H), 6.12 (s, 1H), 6.09 – 6.04 (m, 2H), 5.94 (d, J = 10.3 Hz, 1H), 5.65 (s, 1H), 4.43 (d, J = 5.1 Hz, 1H), 4.18 – 4.11 (m, 1H), 3.87 (dd, J = 10.2, 1.6 Hz, 1H), 3.54 (dd, J = 10.3, 6.8 Hz, 1H), 3.38 (dd, J = 11.0, 5.4 Hz, 1H), 3.16 (d, J = 9.7 Hz, 1H), 2.60 – 2.52 (m, 1H), 2.23 – 2.13 (m, 1H), 2.09 (d, J = 2.6 Hz, 9H), 1.93 (dd, J = 12.9, 5.8 Hz, 1H), 1.81 – 1.72 (m, 1H), 1.67 (s, 6H), 1.57 – 1.44 (m, 3H), 1.32 (dt, J = 13.3, 3.4 Hz, 1H), 1.24 (dt, J = 13.7, 3.9 Hz, 1H), 1.17 (d, J = 6.8 Hz, 1H), 1.00 (d, J = 2.2 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 207.71, 167.05, 164.43, 151.21, 135.94, 132.58, 132.36, 132.05, 131.49, 129.92, 129.60, 129.38, 129.20, 126.67, 123.31, 120.03, 102.89, 96.34, 77.67, 75.11, 74.47, 72.04, 63.09, 62.51, 59.68, 54.32, 52.66, 41.85, 41.27, 41.15, 40.96, 40.58, 38.81, 36.47, 33.82, 33.19, 30.79, 29.82, 29.35, 22.10, 20.22. HRMS (ESI) calculated for $C_{46}H_{52}NO_8Se^+$ ([M+H]$^+$): 826.2853; found: 826.2859.

(5S,6aS,9R,11bR,14S)-1,5,6-trihydroxy-4,4-dimethyl-8-methylene-7-oxododecahydro-1H-6,

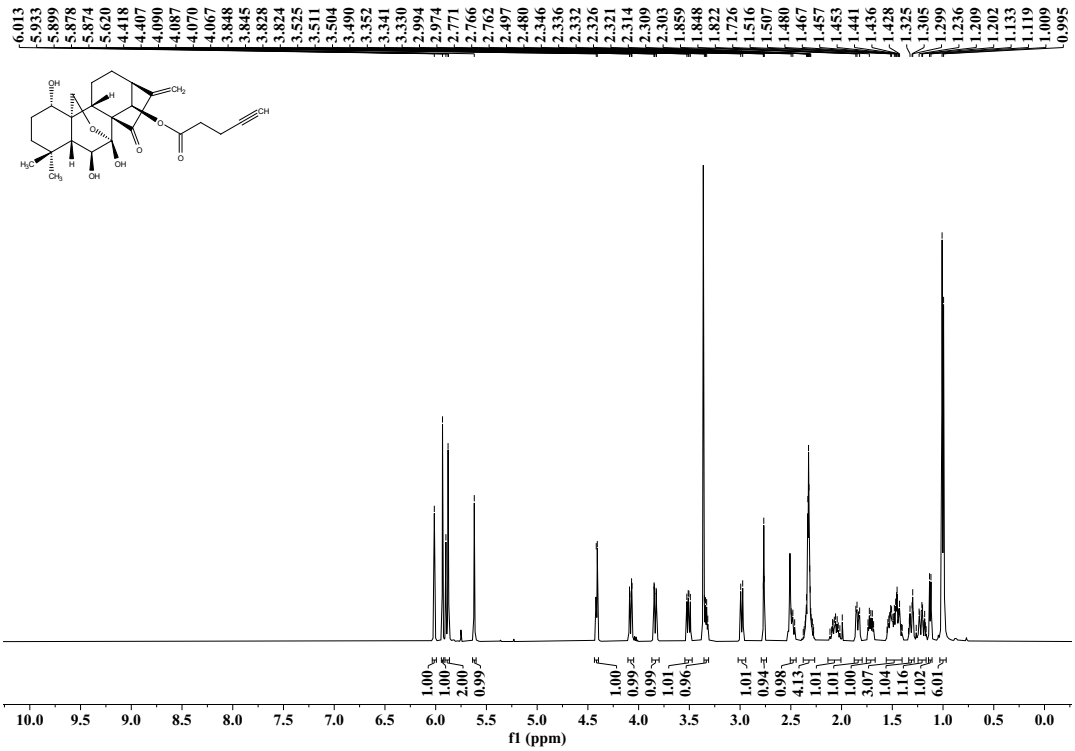
11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalen-14-yl 3-(((2-(((S)-3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)phenyl)selanyl)ethynyl)benzoate (F8)

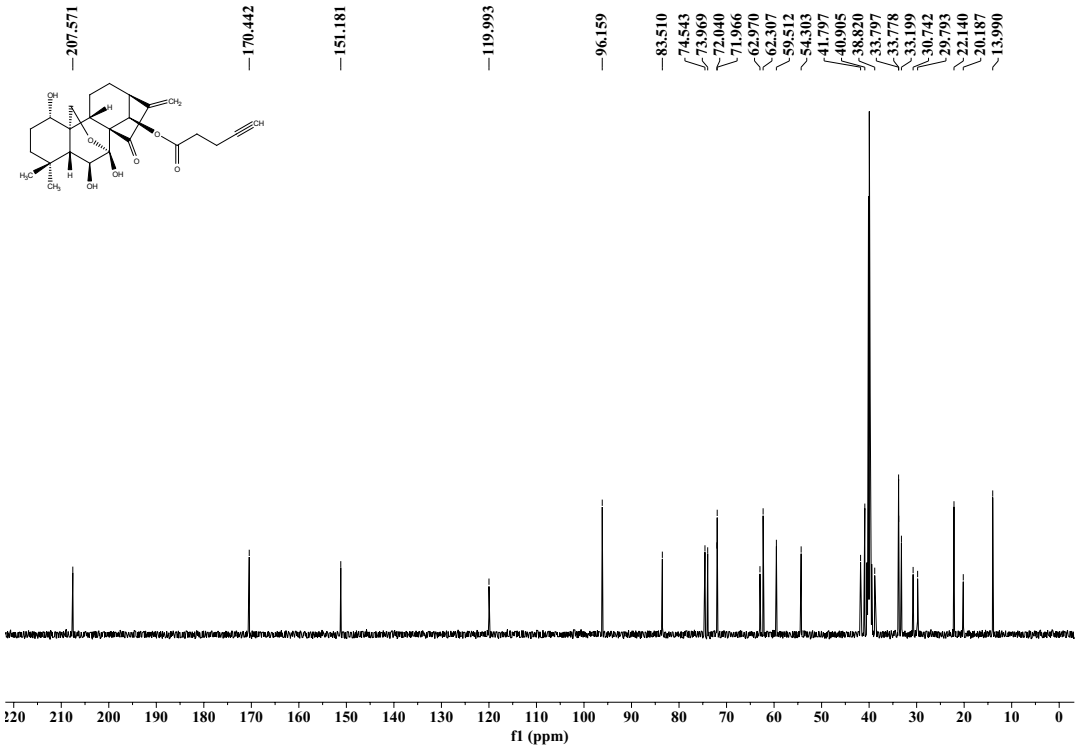


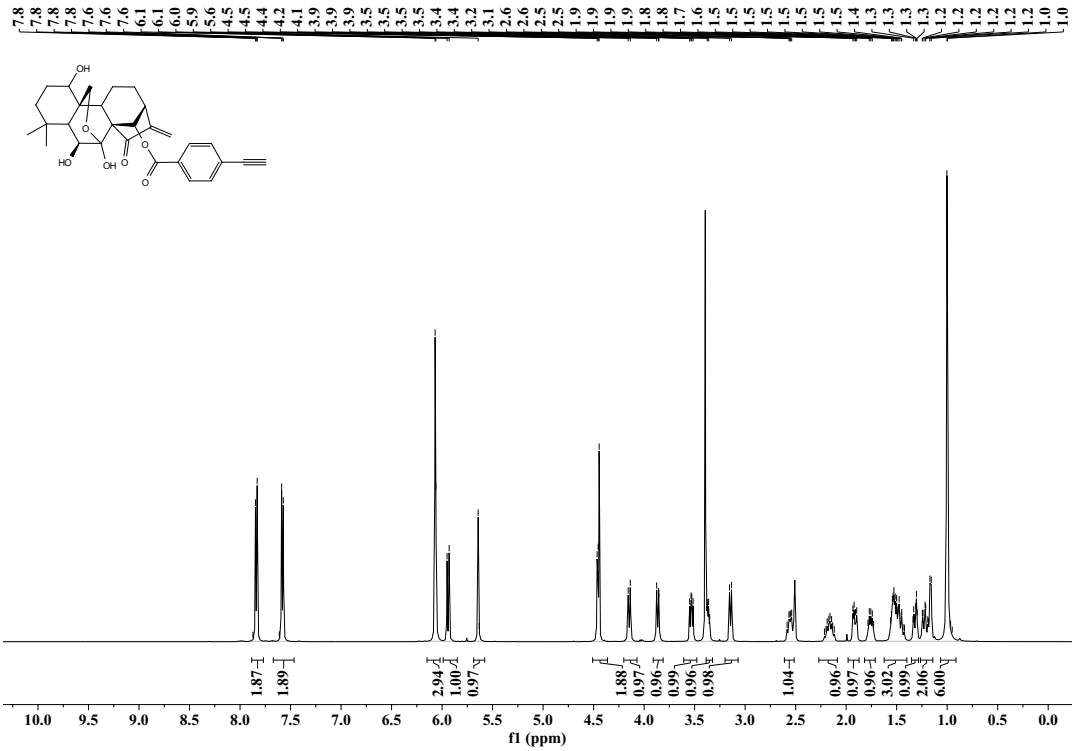
F8

To a stirring solution of BTSA (**Se-16**) (0.2 mmol, 1 equiv) and $AgNO_3$ (0.01 mmol, 0.05 equiv) in DCE (2 mL) was added alkyne (**S3**) (0.24 mmol, 1.2 equiv) and $NH_3 \cdot H_2O$ (0.3 mmol, 1.5 equiv), then the reaction mixture was stirred at room temperature for 2 h. After that, the solvent was removed and the residue was purified by flash chromatography on silica gel to give the title compounds **F8**. This compound was obtained in 91% yield (154 mg) as a white foamy solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.20 (d, J = 7.8 Hz, 2H), 8.09 (d, J = 8.1 Hz, 1H), 8.05 (d, J = 7.8 Hz, 1H), 7.94 (d, J = 1.8 Hz, 1H), 7.87 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.66 (t, J = 7.6 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.47 (t, J = 7.5 Hz, 1H), 7.09 (d, J = 8.2 Hz, 2H), 6.67 (d, J = 8.1 Hz, 2H), 6.13 (s, 1H), 6.07 (t, J = 1.8 Hz, 2H), 5.95 (d, J = 10.2 Hz, 1H), 5.65 (s, 1H), 4.61 (ddd, J = 10.3, 7.7, 5.2 Hz, 1H), 4.44 (s, 1H), 4.15 (d, J = 10.2 Hz, 1H), 3.87 (d, J = 10.2 Hz, 1H), 3.67 (s, 3H), 3.55 (dd, J = 10.3, 6.8 Hz, 1H), 3.16 (d, J = 9.7 Hz, 1H), 3.10 (dd, J = 13.9, 5.2 Hz, 1H), 3.01 (dd, J = 13.9, 10.1 Hz, 1H), 2.56 (dt, J = 13.7, 8.9 Hz, 1H), 2.17 (ddd, J = 21.1, 13.5, 6.9 Hz, 1H), 1.93 (dd, J = 12.9, 5.8 Hz, 1H), 1.76 (dt, J = 15.5, 6.3 Hz, 1H), 1.61 – 1.11 (m, 7H), 1.00 (s, 6H).^{13}C NMR (126 MHz, DMSO) δ 207.73, 172.35, 167.44, 164.43, 156.46, 151.20, 135.97, 133.45, 133.30, 132.38, 131.50, 130.46, 129.99, 129.81, 129.60, 129.01, 127.88, 126.95, 123.21, 120.05, 115.57, 103.41, 96.34, 77.08, 75.13, 74.47, 72.05, 63.10, 62.51, 59.69, 55.30, 54.32, 52.55, 41.85, 40.96, 38.81, 35.85, 33.82, 33.19, 30.78, 29.83, 22.10, 20.22. HRMS (ESI) calculated for $C_{46}H_{48}NO_{11}Se^+$ ([M+H]$^+$): 870.2387; found: 870.2390.

8. Supplementary NMR spectra



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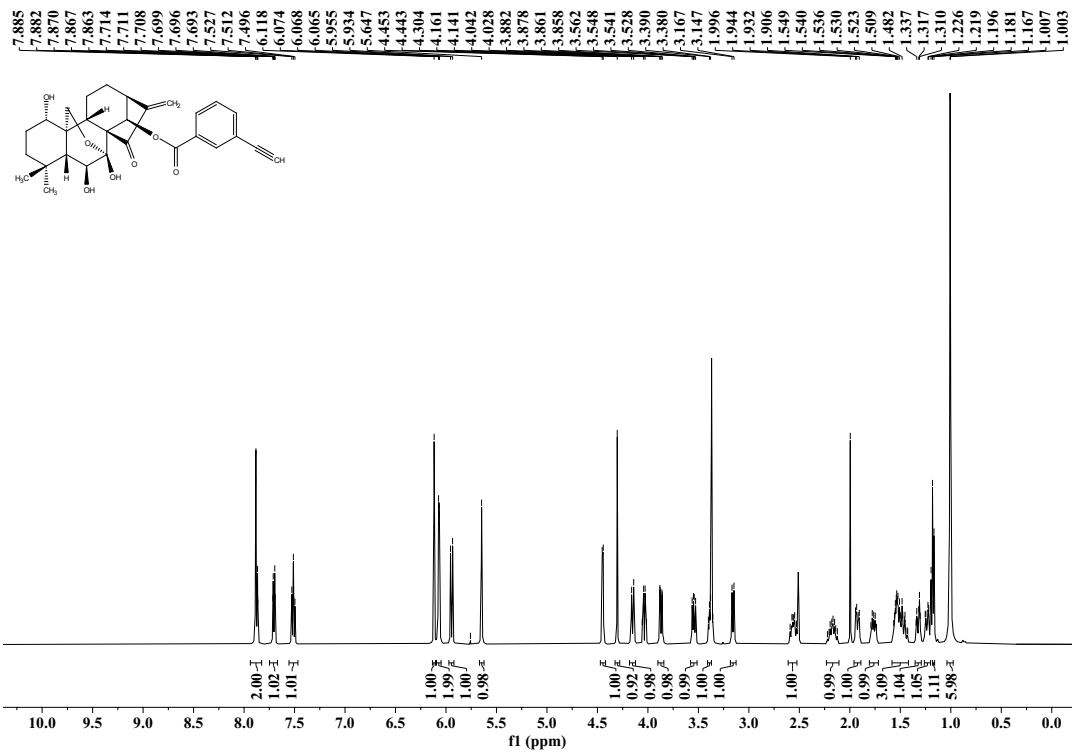
1.87 1.89 2.94 1.00 0.97 1.88 0.97 0.96 0.99 0.96 0.98 1.04 0.96 0.97 0.96 3.02 0.99 2.06 6.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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CH3
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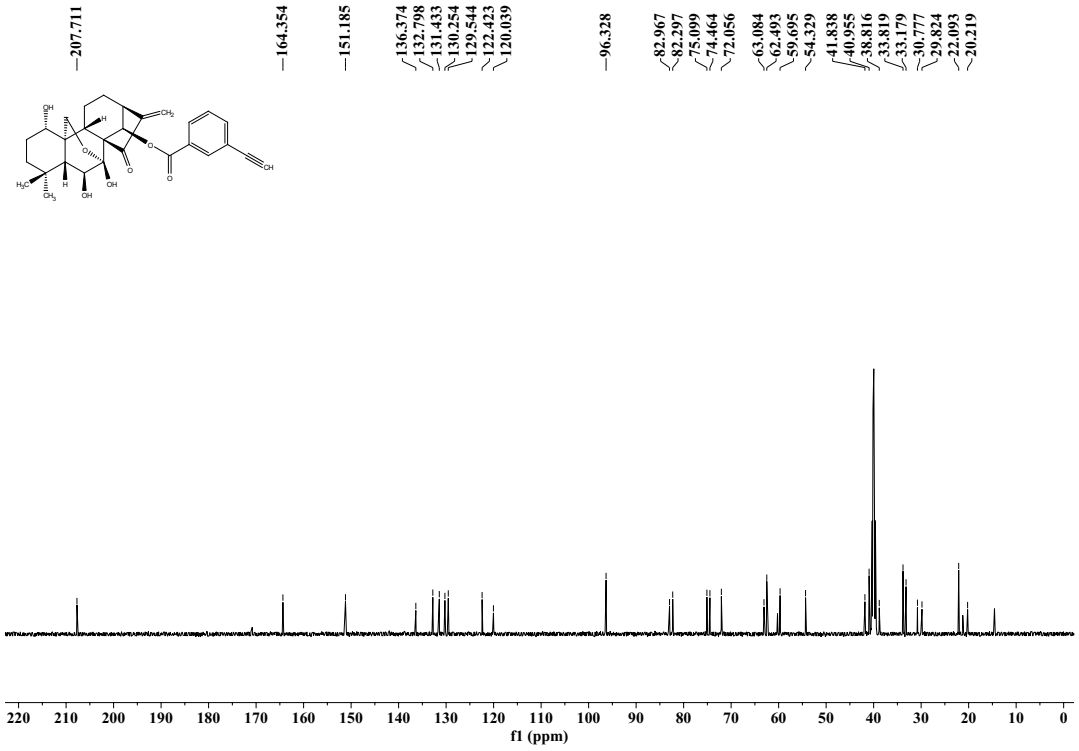
2.00 1.02 1.01 1.00 1.99 1.00 0.98 1.00 0.92 0.98 0.98 0.99 1.00 1.00 1.00 0.99 1.00 0.99 3.09 1.04 1.05 1.11 5.98
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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62.493
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38.816
33.819
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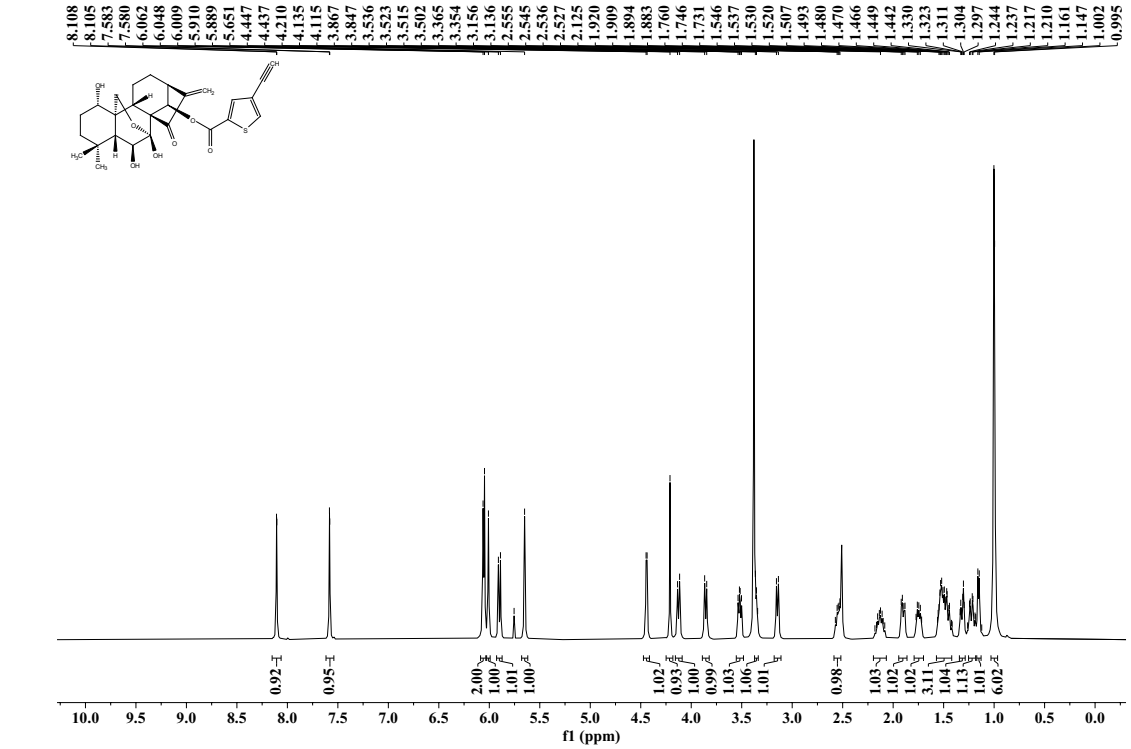
f1 (ppm)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1.00
1.02
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0.99
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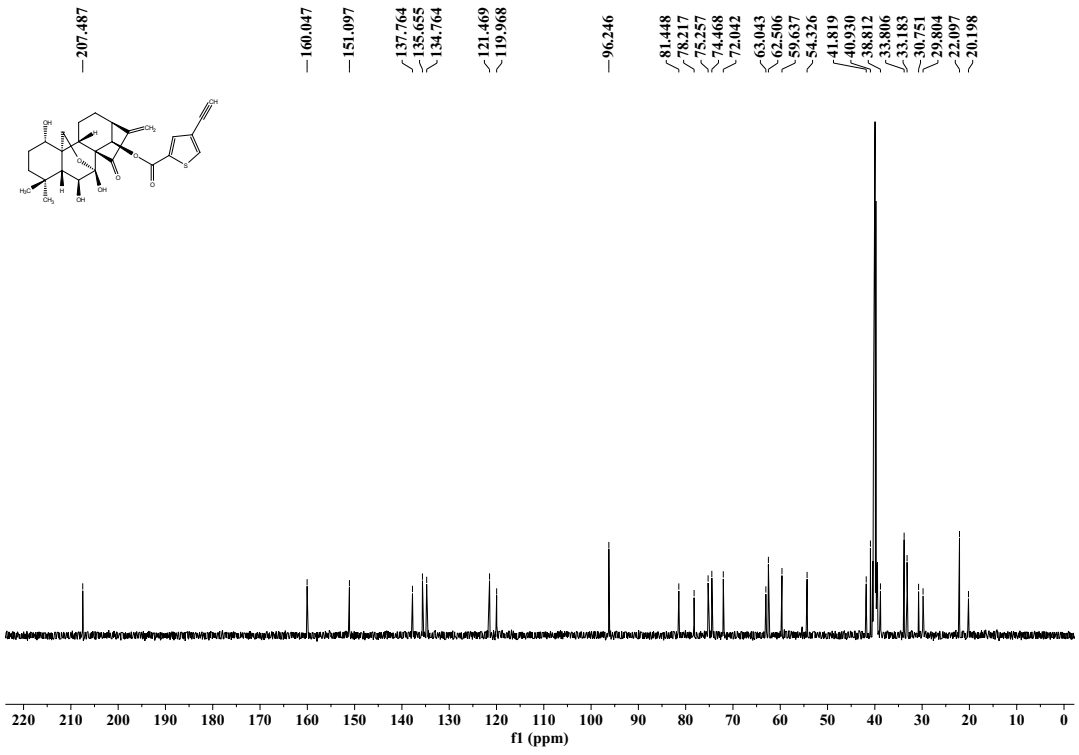
f1 (ppm)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72.042
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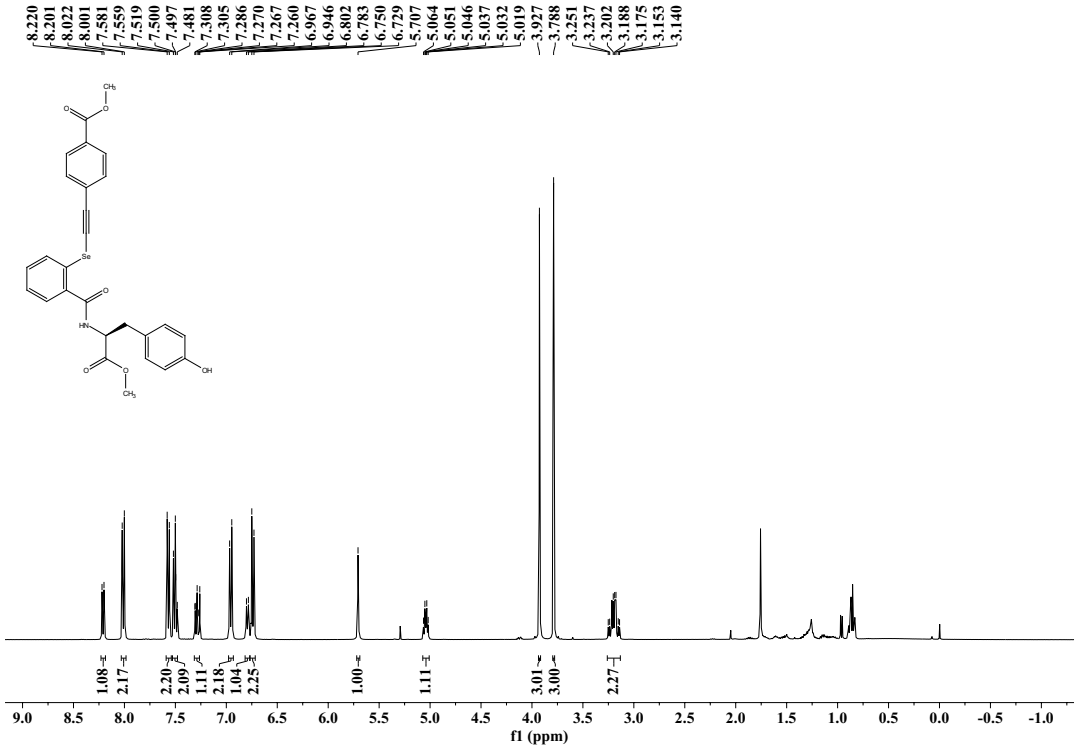
f1 (ppm)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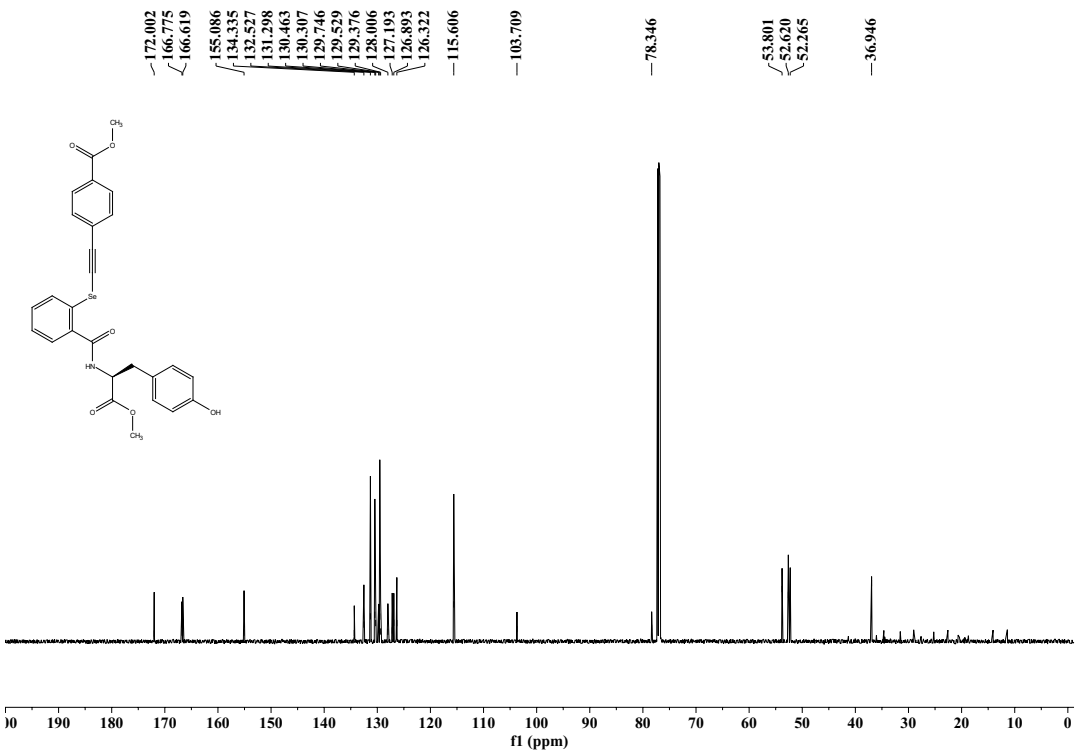
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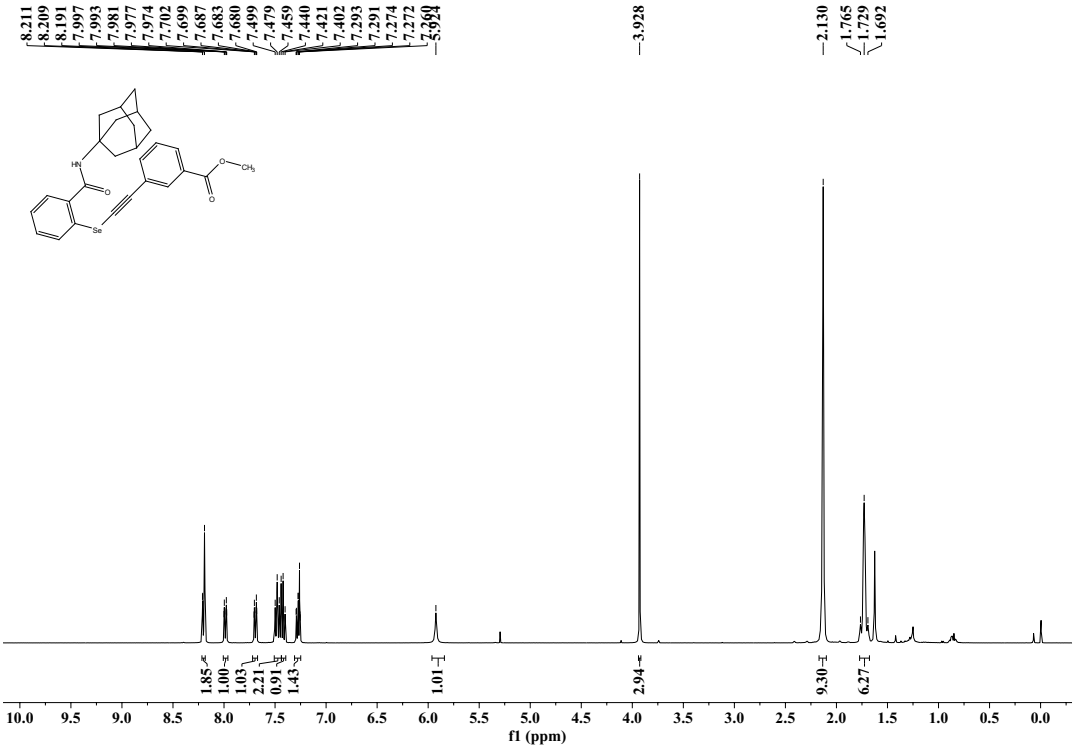
f1 (ppm)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7.680
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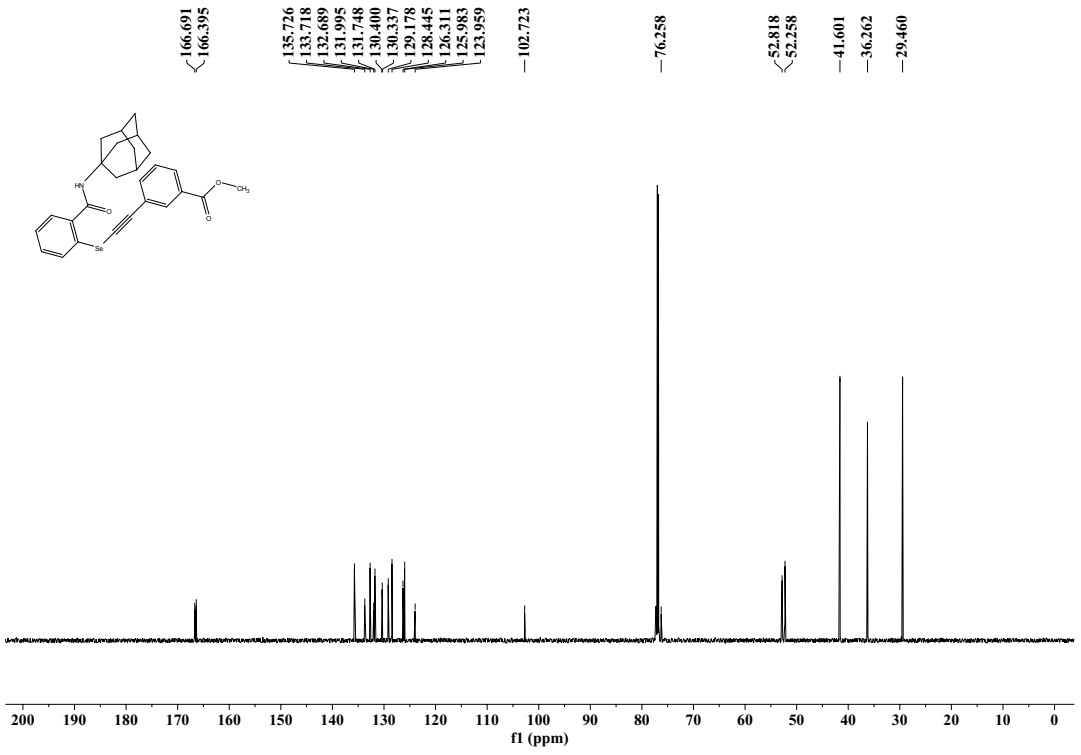
f1 (ppm)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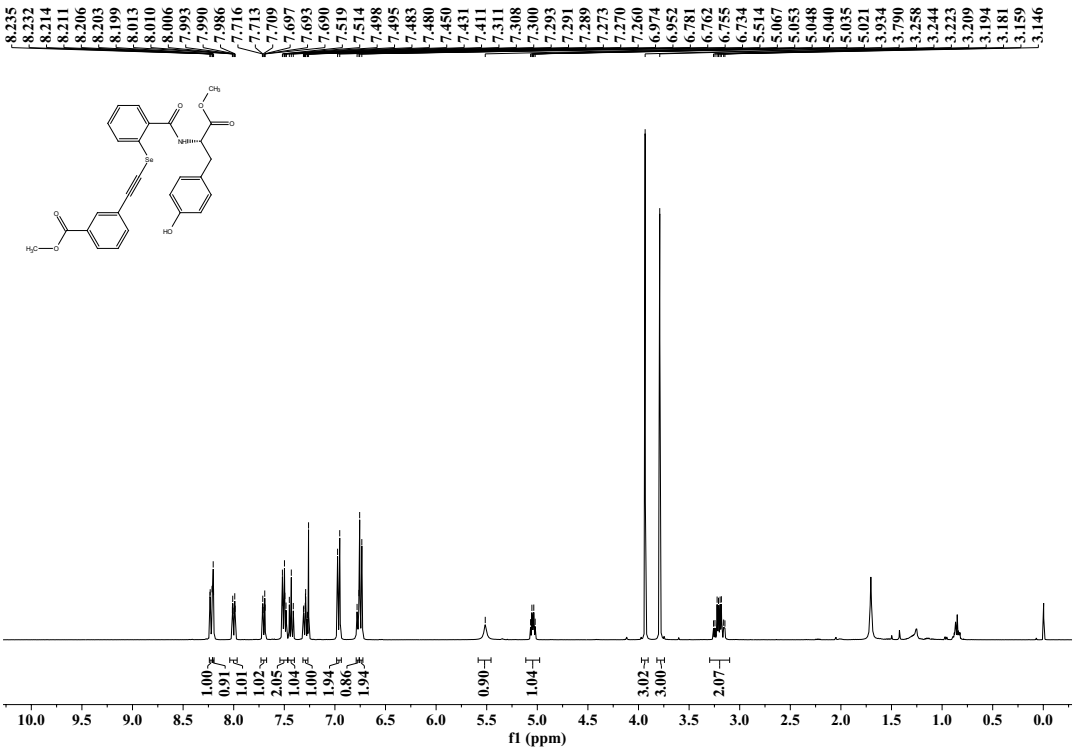
f1 (ppm)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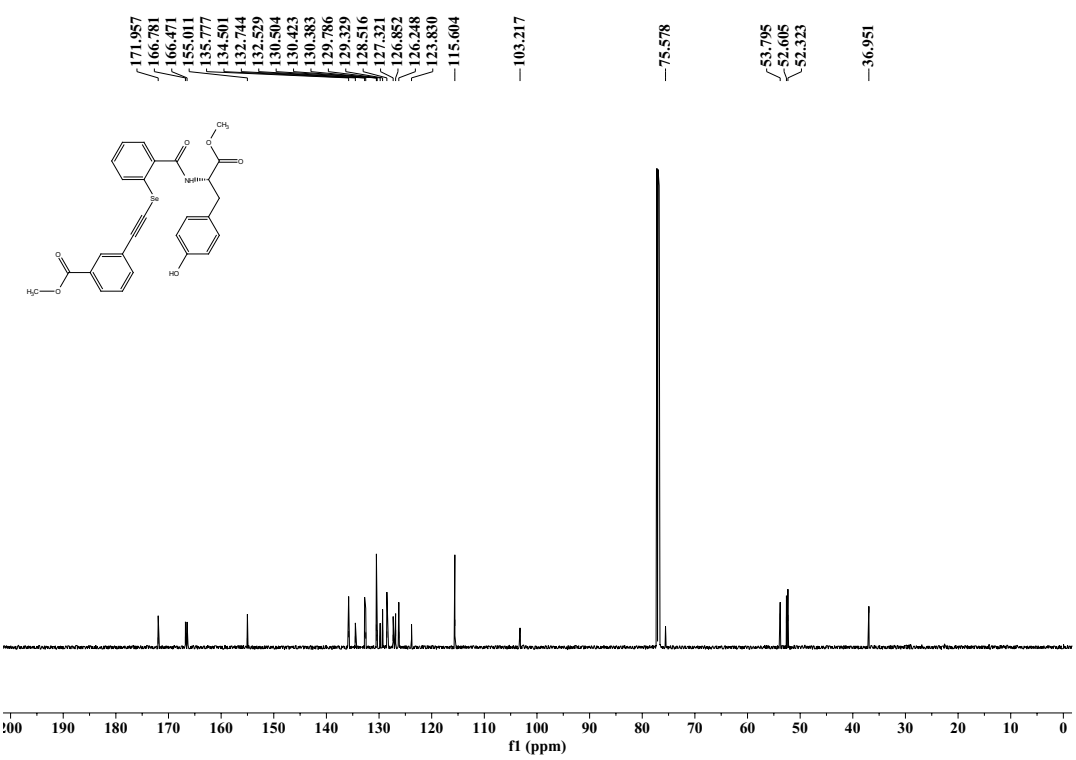
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200
190
180
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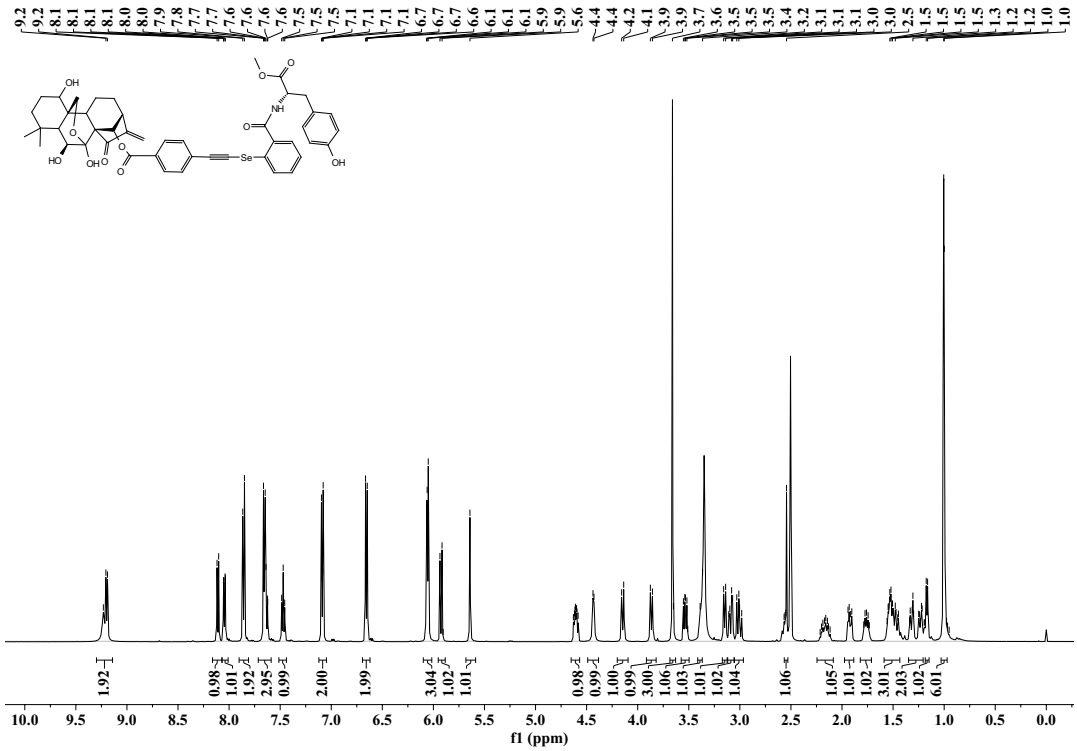
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O
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NH
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Se
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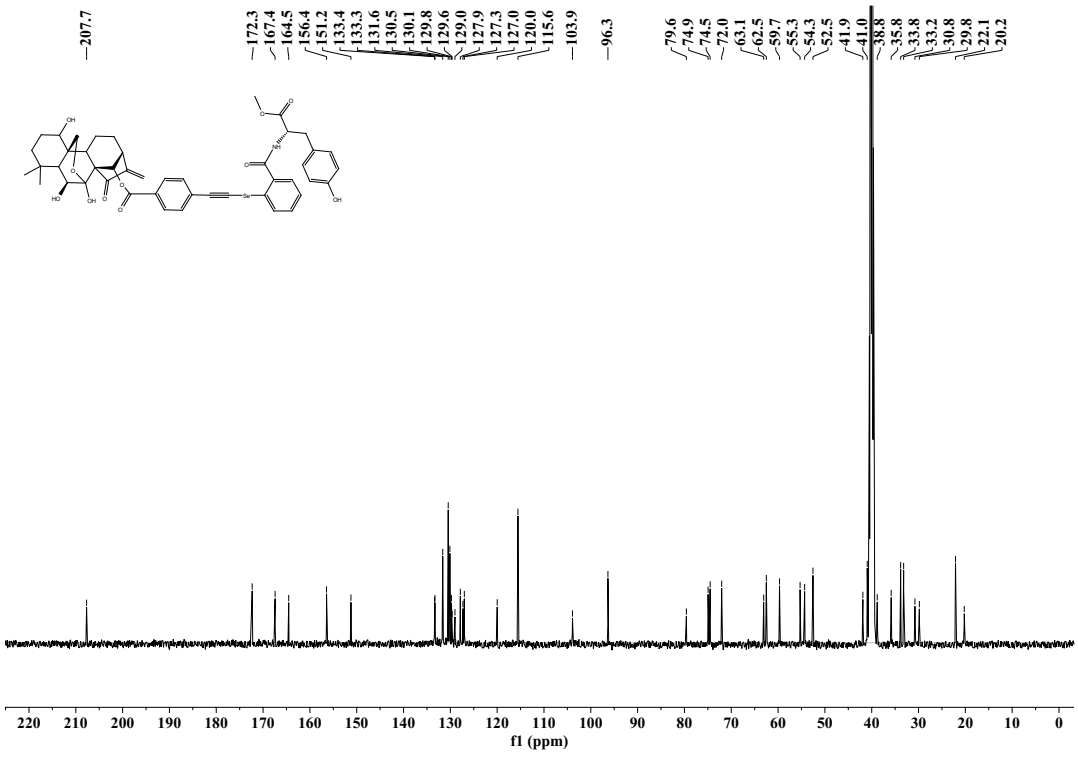
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79.6 74.9 74.5 72.0 63.1 62.5 59.7 55.3 54.3 52.5 41.9 41.0 38.8 35.8 33.8 33.2 30.8 29.8 22.1 20.2
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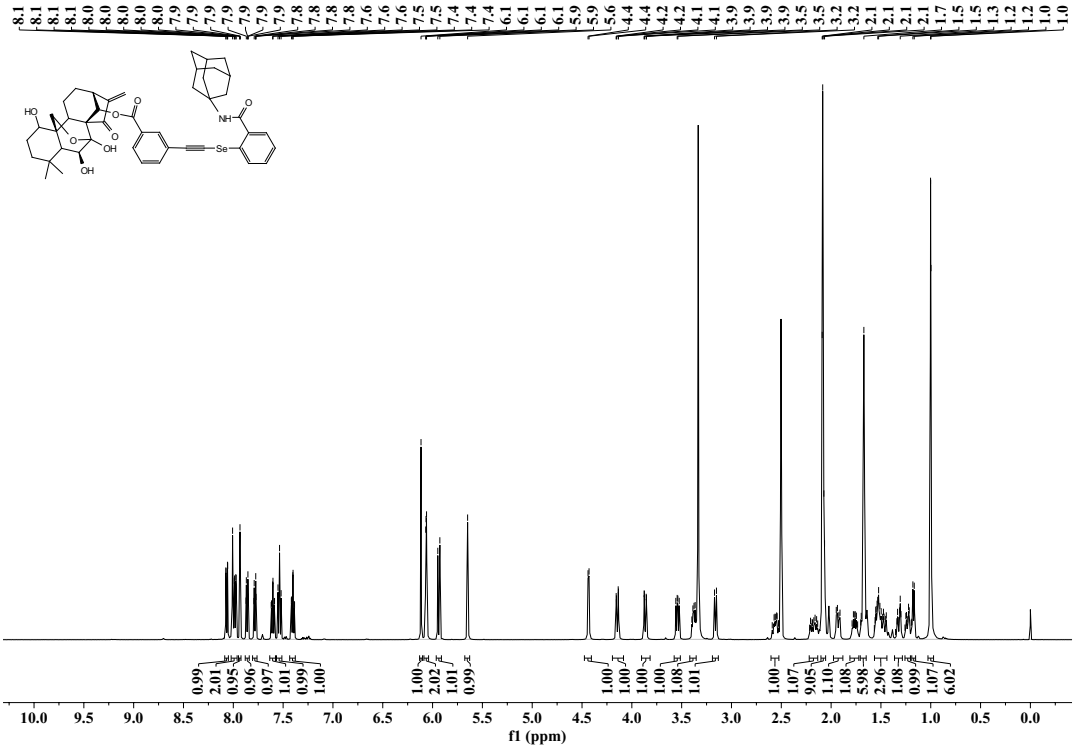
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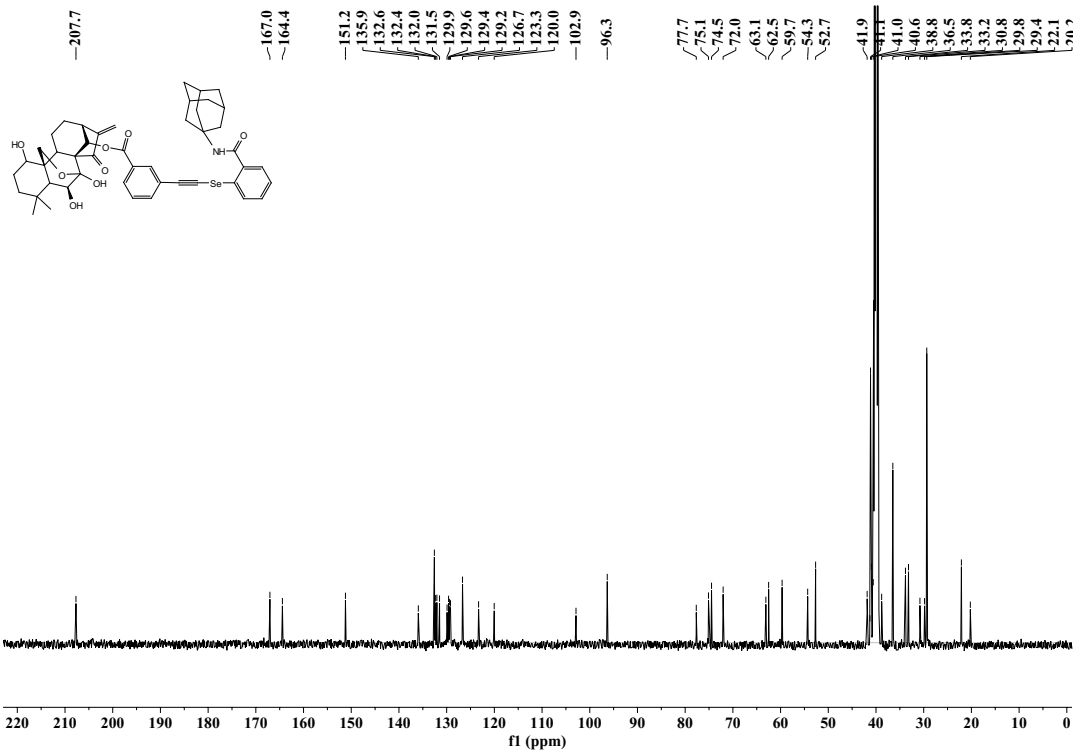
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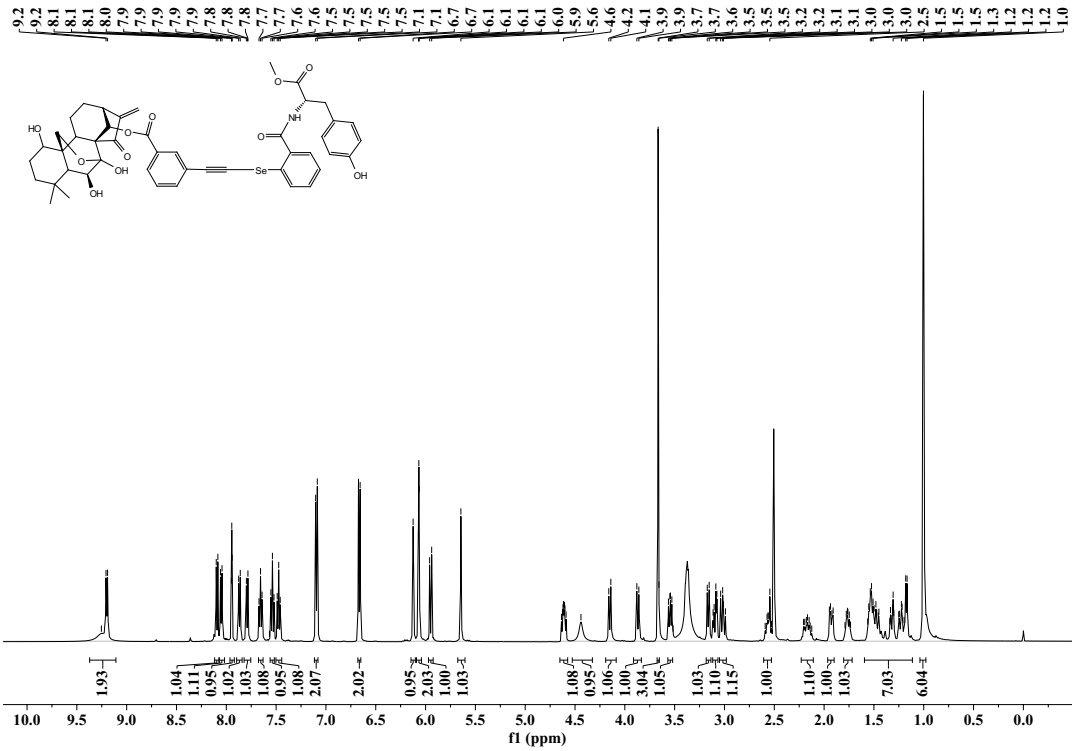
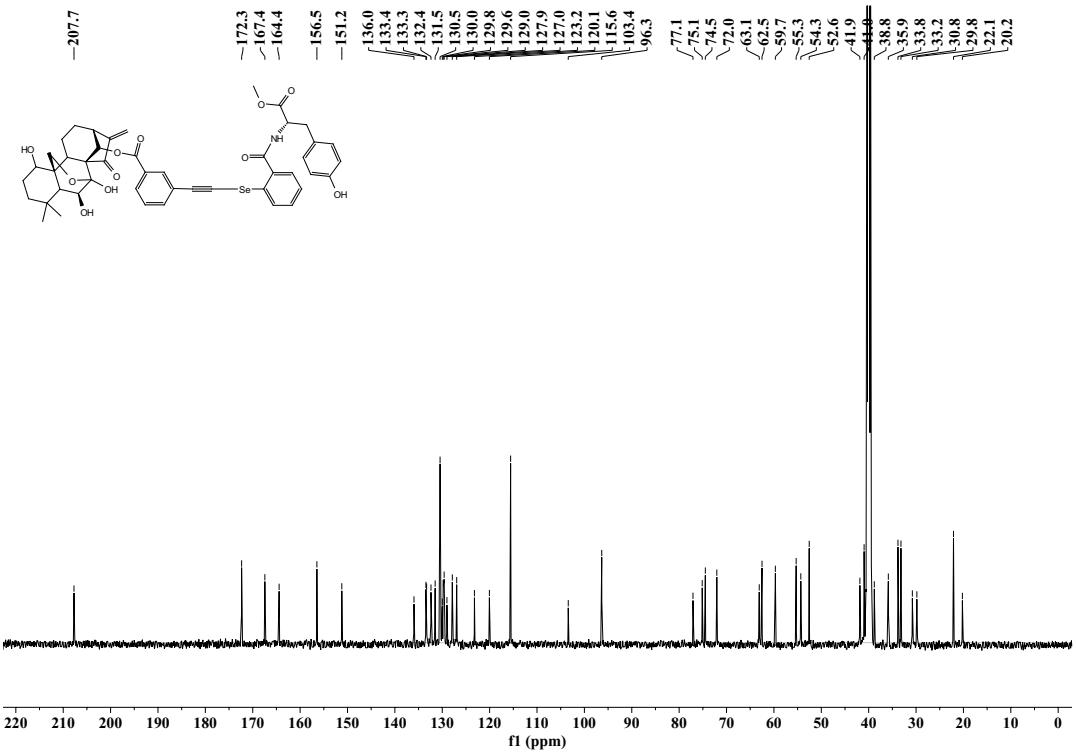
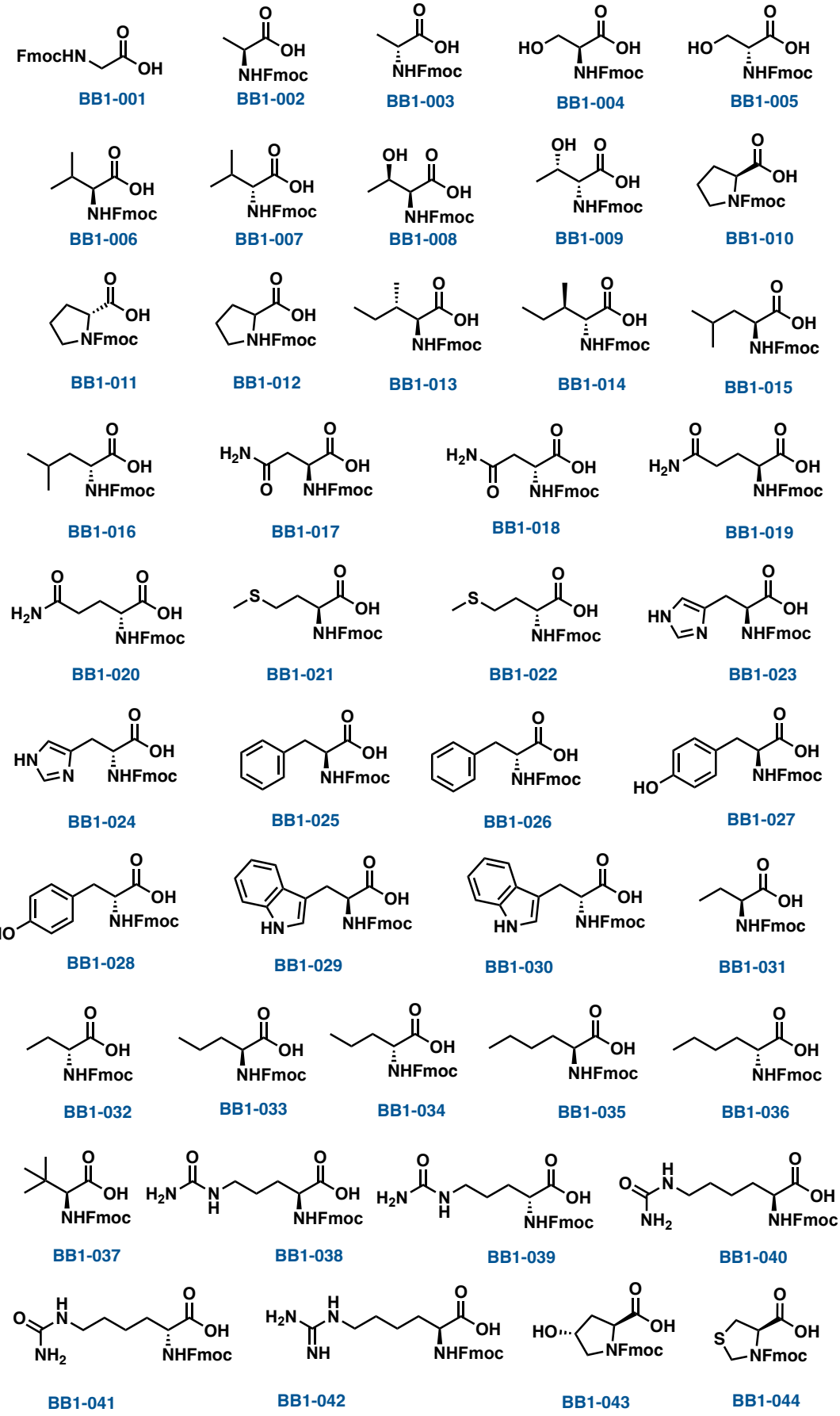




9. Supporting Figures

9.1 Figure S1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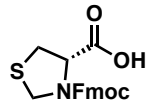

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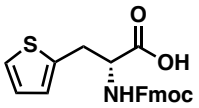

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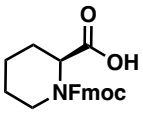

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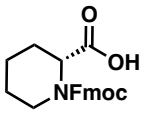

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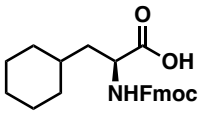

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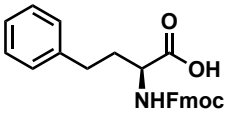

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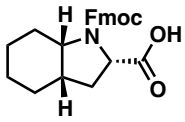

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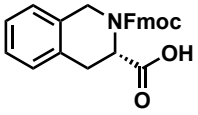

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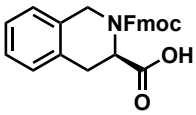

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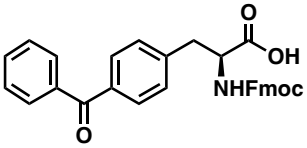

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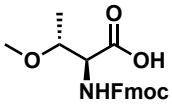

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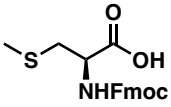

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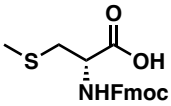

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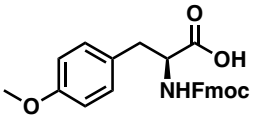

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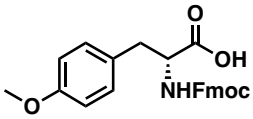

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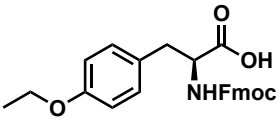

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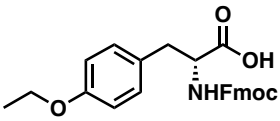

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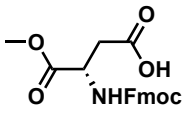

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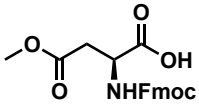

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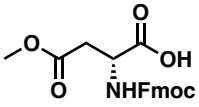

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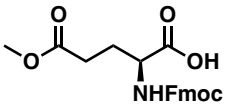

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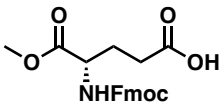

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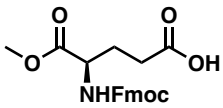

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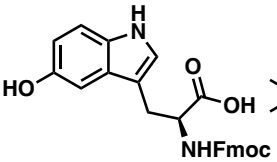

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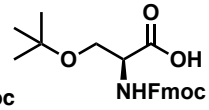

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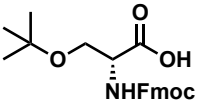

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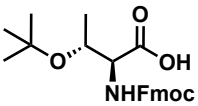

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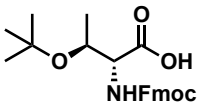

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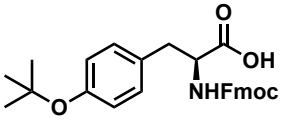

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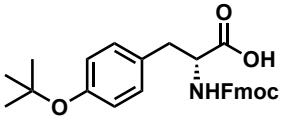

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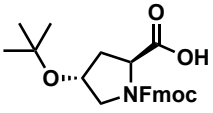

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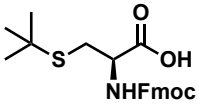

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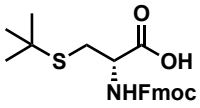

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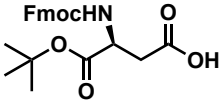

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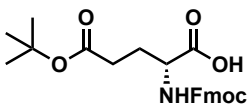

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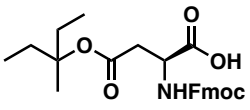

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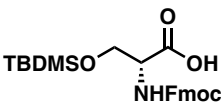

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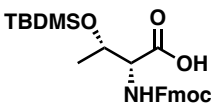

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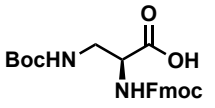

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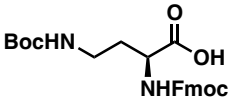

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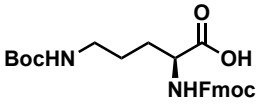

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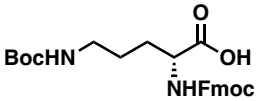

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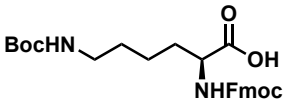

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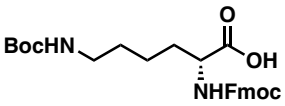

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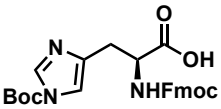

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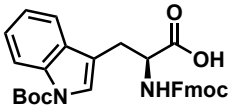

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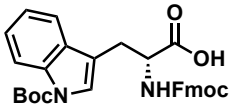

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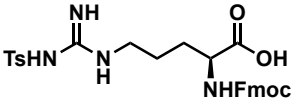

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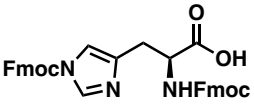

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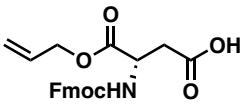

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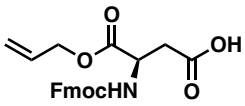

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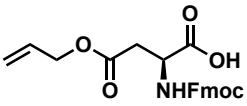

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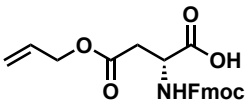

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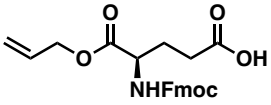

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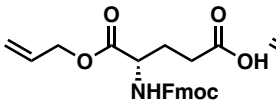

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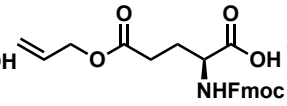

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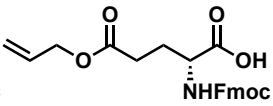

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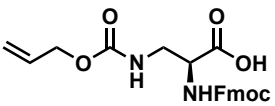

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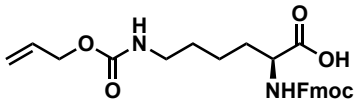

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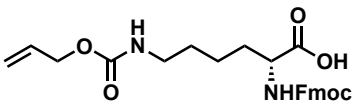

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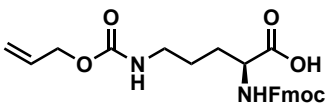

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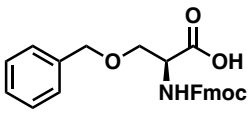

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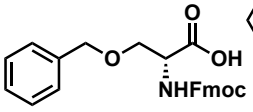

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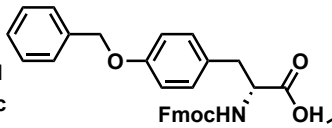

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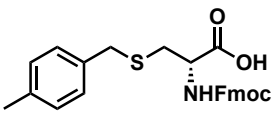

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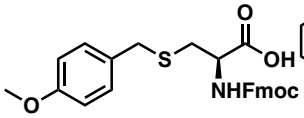

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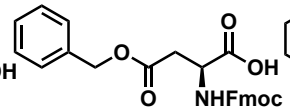
BB1-123



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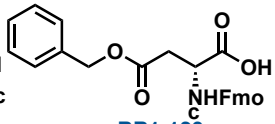

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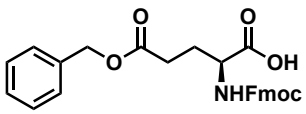
BB1-126



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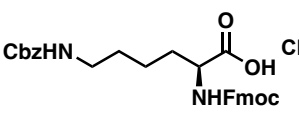

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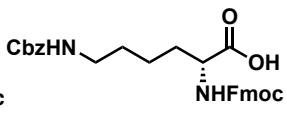

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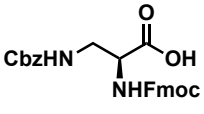

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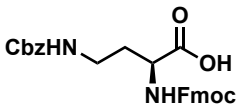

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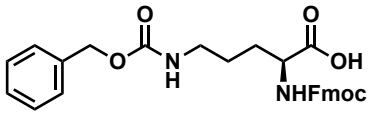

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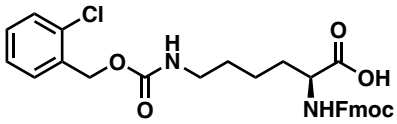

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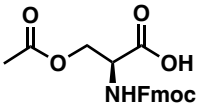

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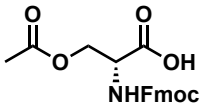

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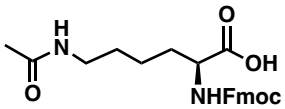

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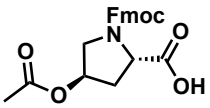

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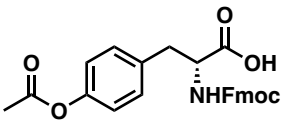

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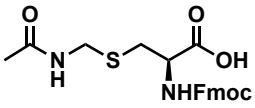

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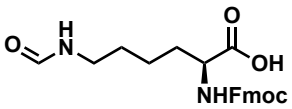

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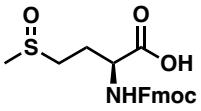

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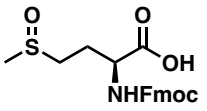

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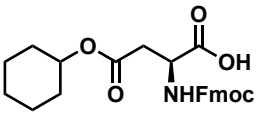

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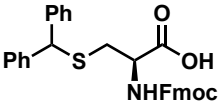

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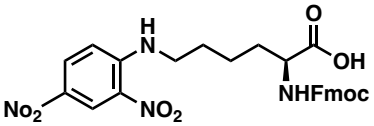

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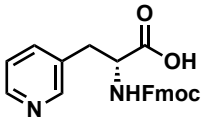

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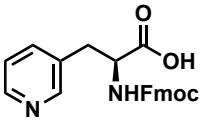

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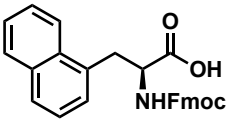
BB1-148



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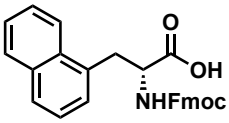

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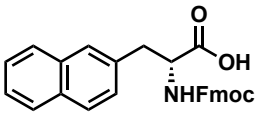

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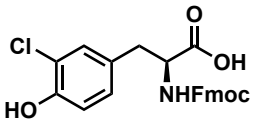

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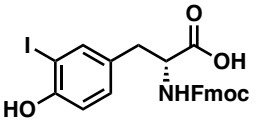

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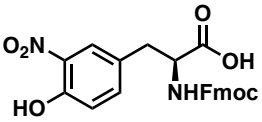

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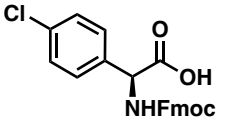

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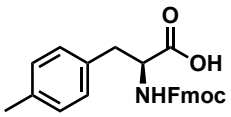

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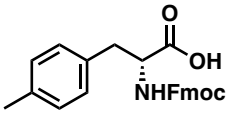

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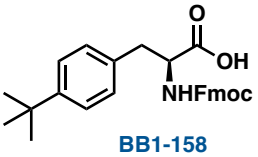

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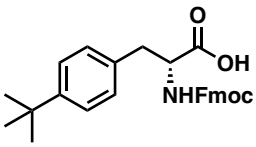

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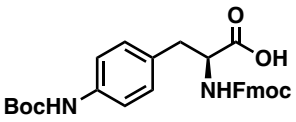

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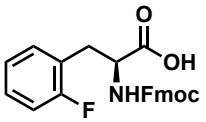

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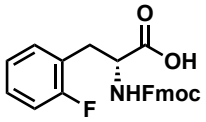

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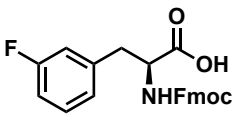

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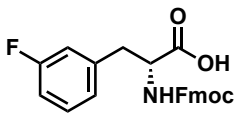

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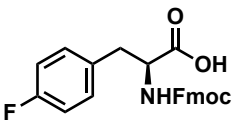

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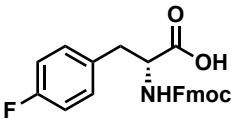

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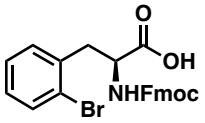

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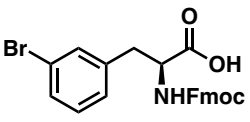

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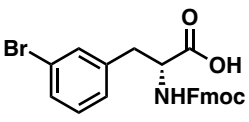

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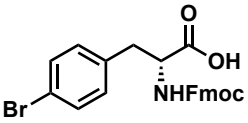

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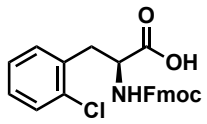

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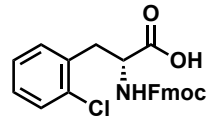

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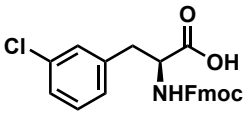

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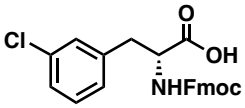

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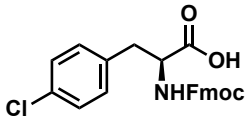

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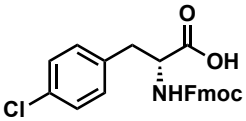

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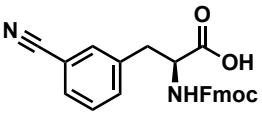

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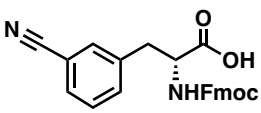

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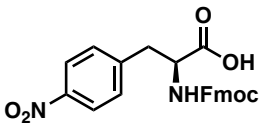

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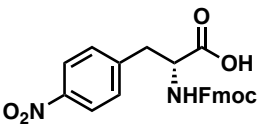

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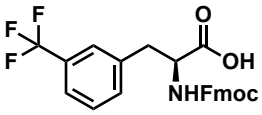

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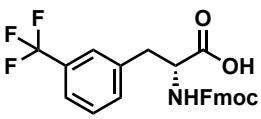

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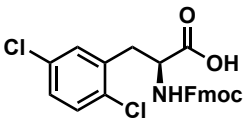

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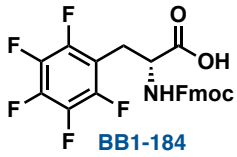

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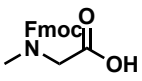

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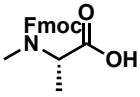

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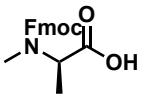

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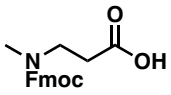

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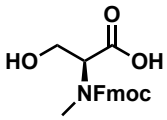

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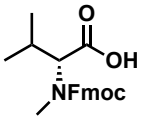

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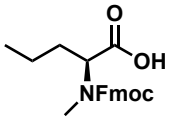

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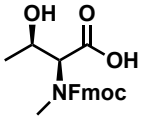

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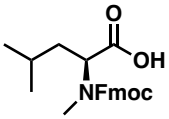

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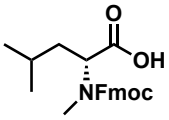

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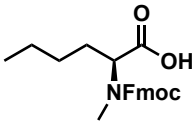

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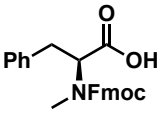

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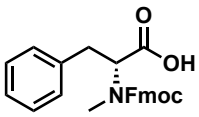

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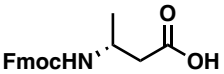

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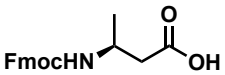

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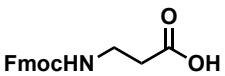

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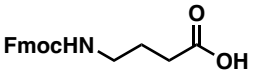

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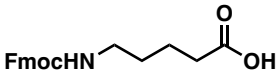

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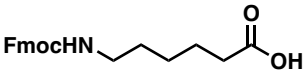

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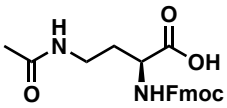

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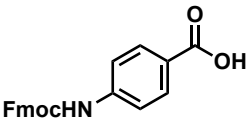

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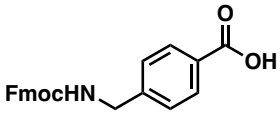

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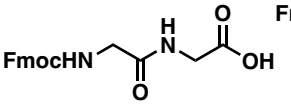

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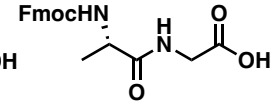

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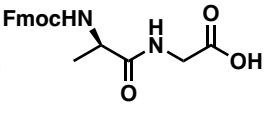

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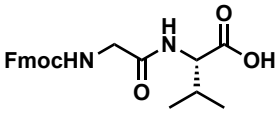

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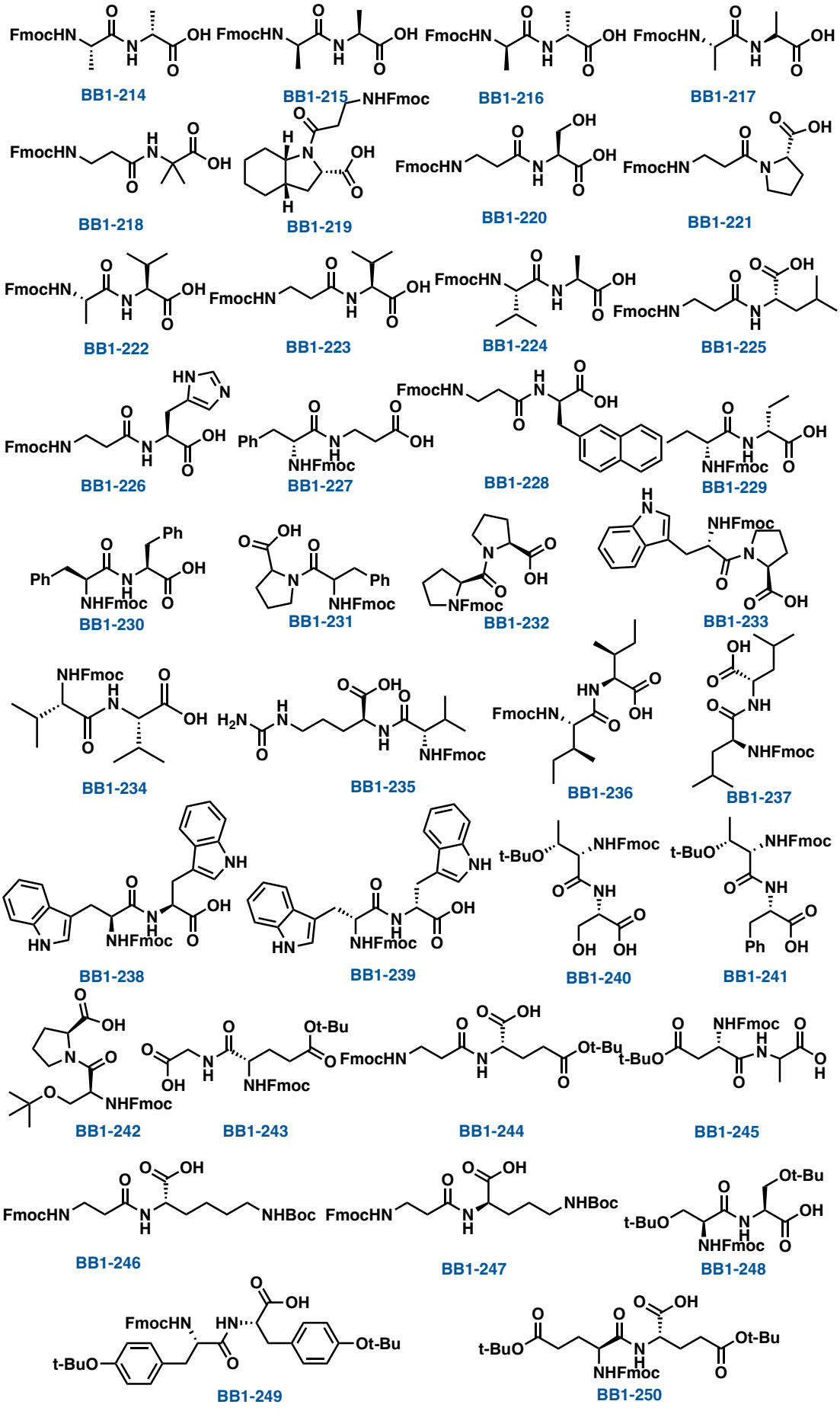
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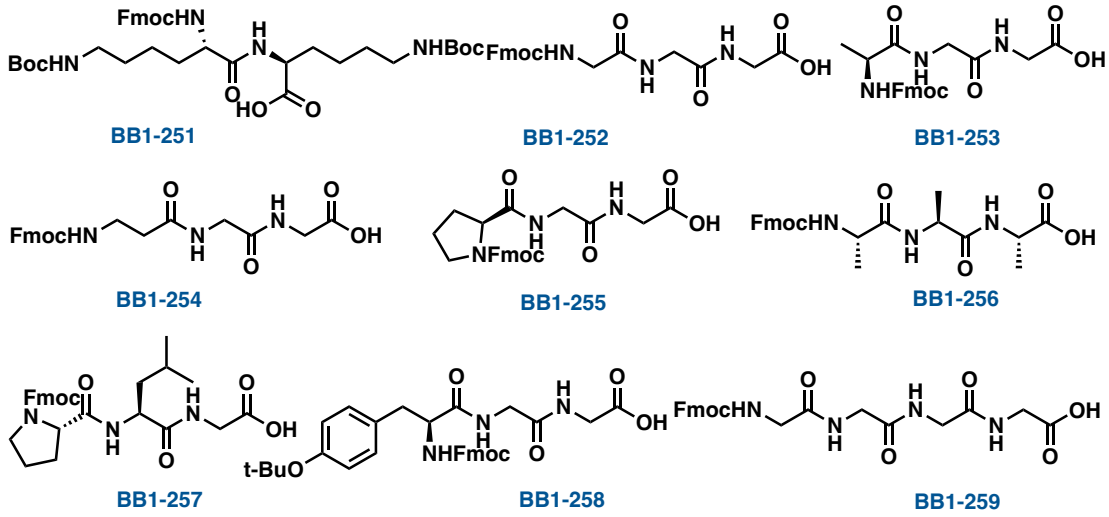

Figure S1. Structure of Fmoc-protected amino acids (BB1) employed in this work.

9.2 Figure S2

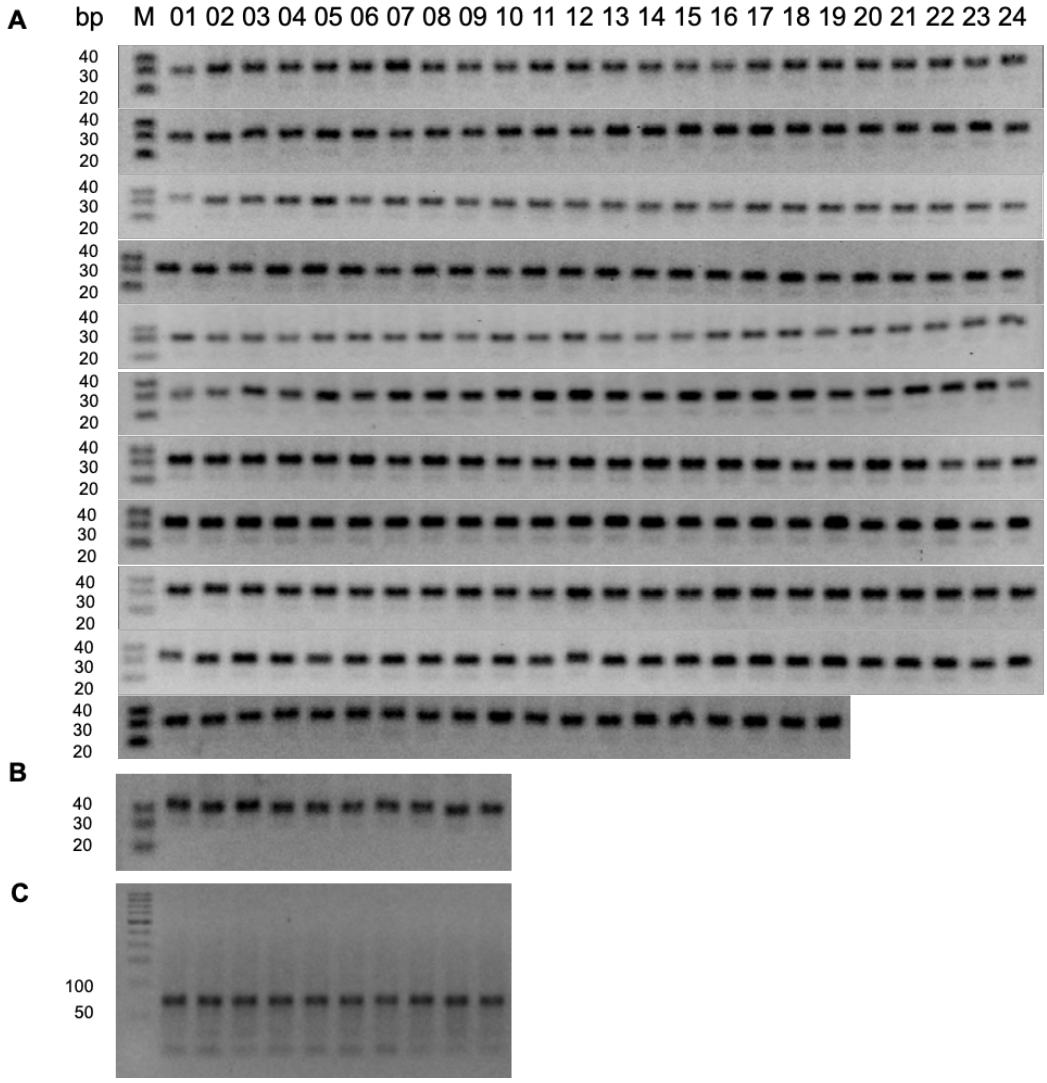

Figure S2. A) Agarose gel verification of HP-DNA ligation to Oligo0 and Code1 1-259. The ligated band was observed at approximately 30 bp. B) Agarose gel verification round1 mixtures

ligation to Code2 1-10. The ligated band was observed at approximately 40 bp. C) Agarose gel verification of Round2 ligation to Code3 1-10 and closing tag. The ligated band was observed at approximately 60 bp. The first lane represents the marker, while the remaining lanes correspond to the tested samples.

9.3 Figure S3

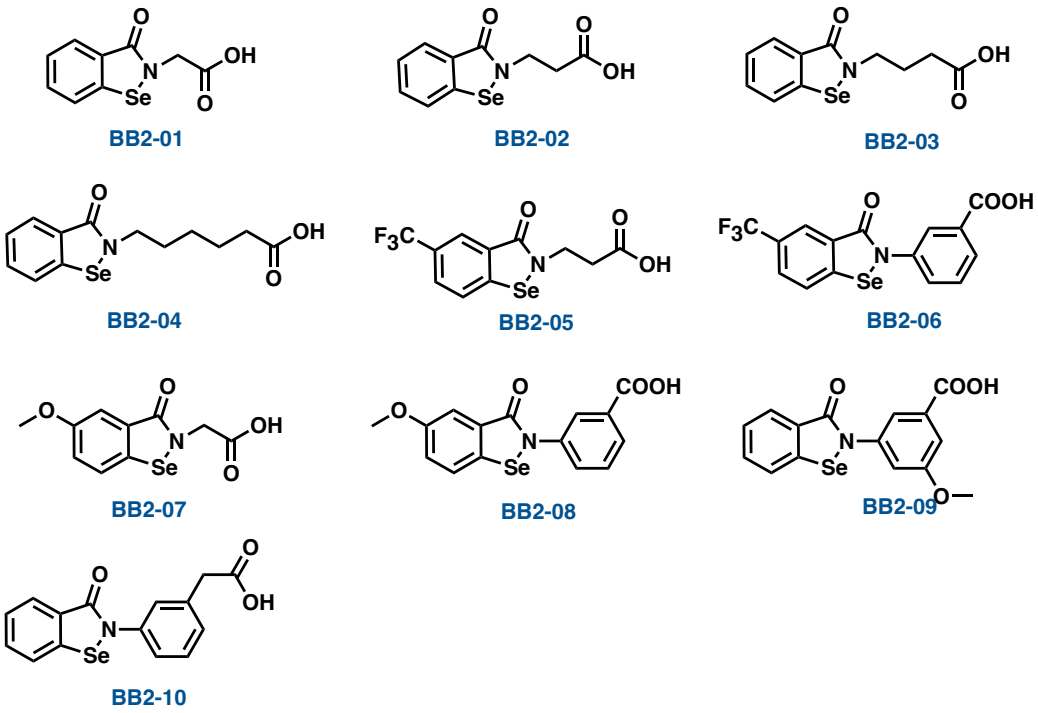

Figure S3. Structure of carboxyl-containing benzoselenazolones (BB2) employed in this work.

9.4 Figure S4

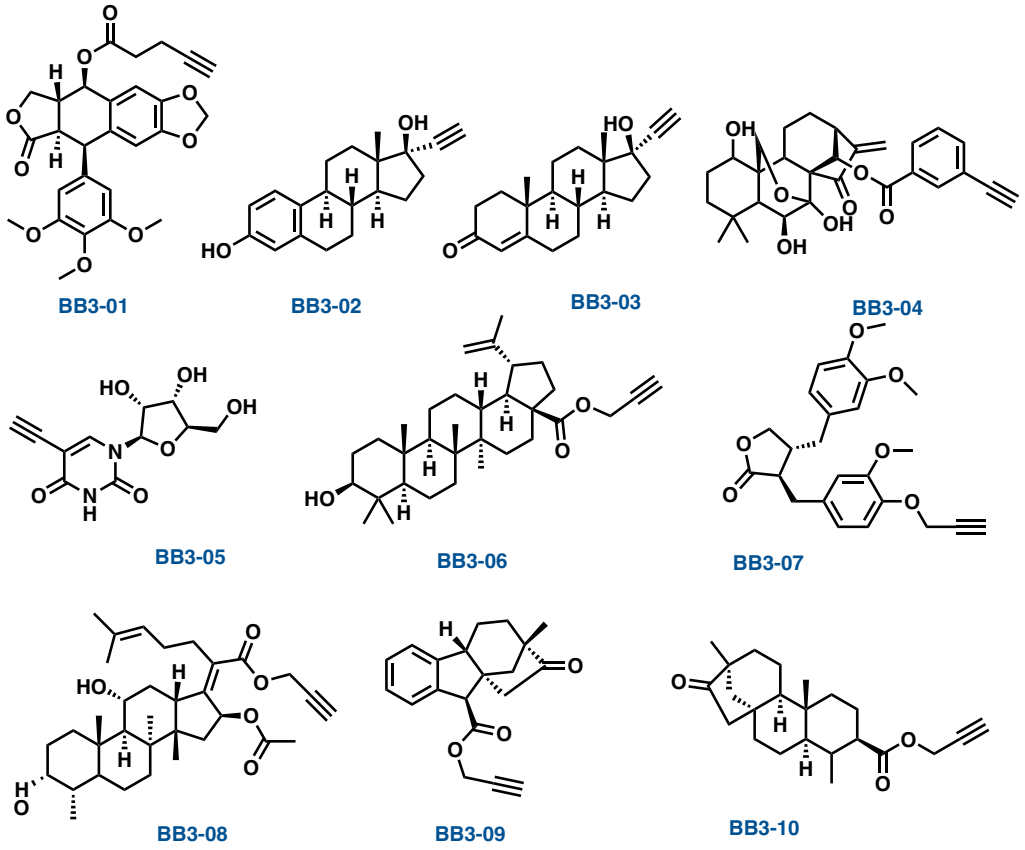

Figure S4. Structure of terminal alkyne-bearing natural products (BB3) employed in this work.

9.5 Figure S5

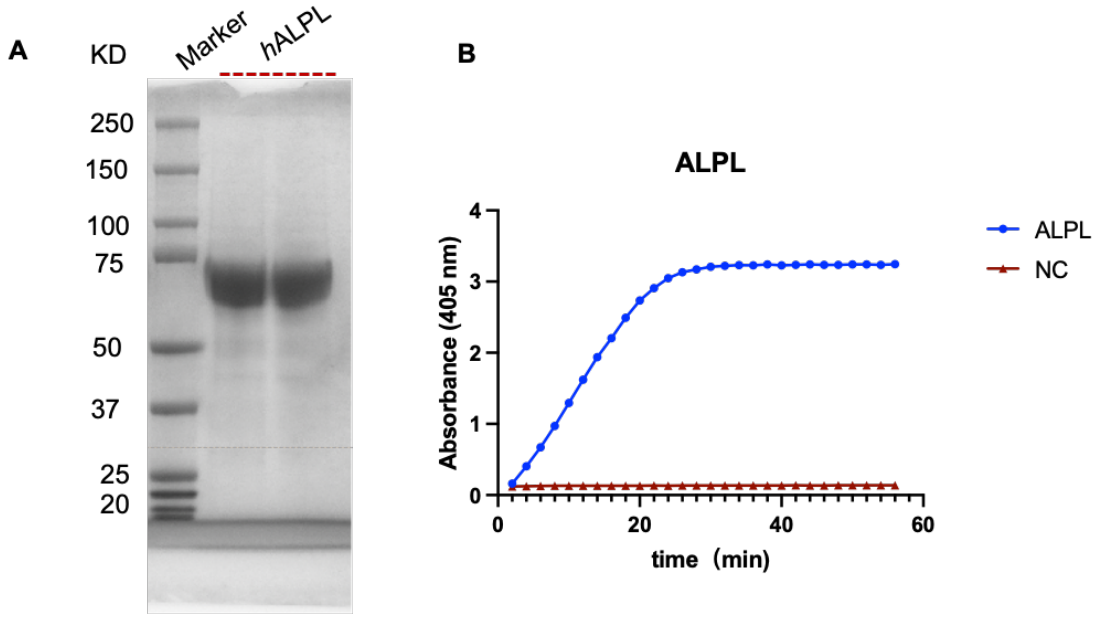

Figure S5. A) SDS-PAGE analysis of the Ni-NTA affinity purified *h*ALPL. The MW of his-tagged *h*ALPL is ~62 KD. B) Enzyme activity of purified *h*ALPL and No-protein negative control.

9.6 Figure S6

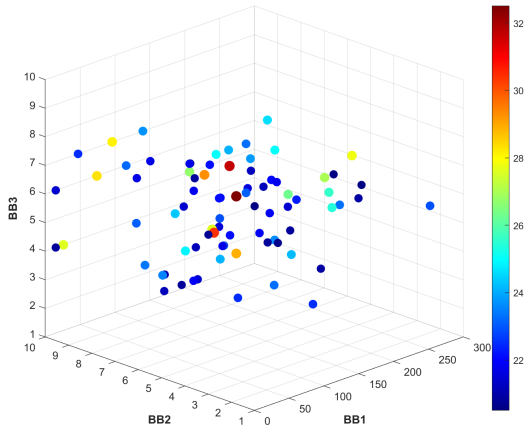

Figure S6. Affinity-based selection against the protein-free control.

9.7 Figure S7

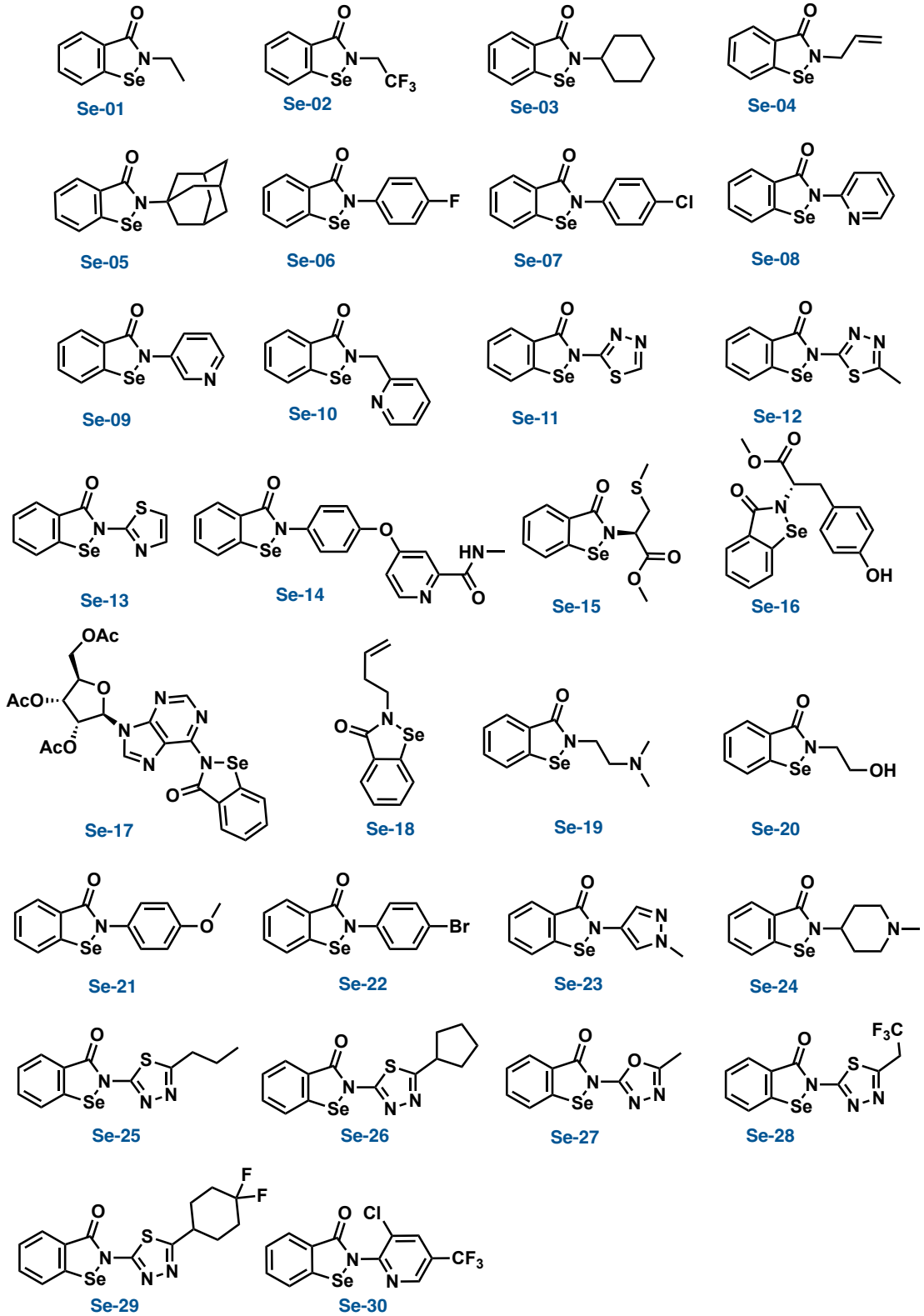

Figure S7. Benzoselenazolones (BSEAs) used in parallel selenium-containing Oridonin library synthesis.

9.8 Figure S8

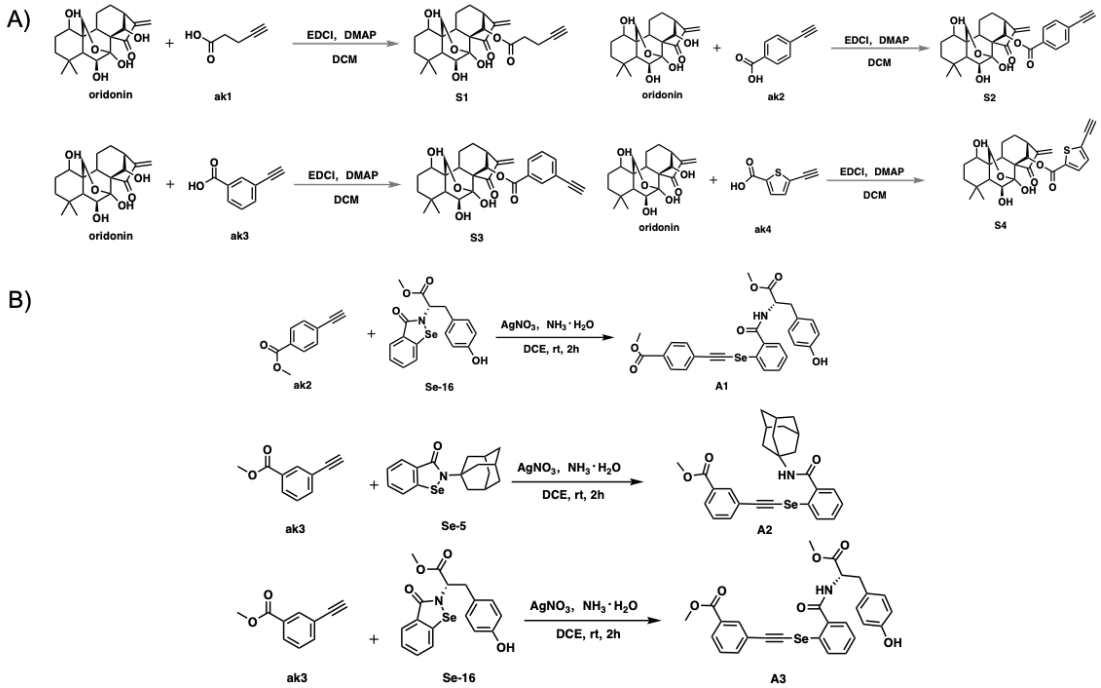

Figure S8. Synthesis of terminal alkyne-bearing oridonin S1-S4 and key intermediates A1-A3. A) Oridonin (1 mmol), acid (1.5 mmol), EDCI (2 mmol), DMAP (1.2 mmol) in DCM (10 mL), rt, 3h. B) BSEAs (0.2 mmol), alkynes (0.24 mmol), $NH_3 \cdot H_2O$ (0.3 mmol), and $AgNO_3$ (5 mol%) in DCE (2 mL), rt, 2 h (without air exclusion).

9.9 Figure S9

A

Entry	K_a (1/Ms)	k_d (1/s)	K_D (μM)	EC_{50} (μM)
Oridonin	NR	NR	63.1	NR
F5	1.15×10^{3}	1.54×10^{-2}	13.4	3.74
E7	3.57×10^{3}	3.75×10^{-2}	10.5	5.85
F8	8.69×10^{2}	3.41×10^{-2}	39.3	3.83
S2	NA	NA	NA	54.52
S3	NA	NA	NA	53.88

B

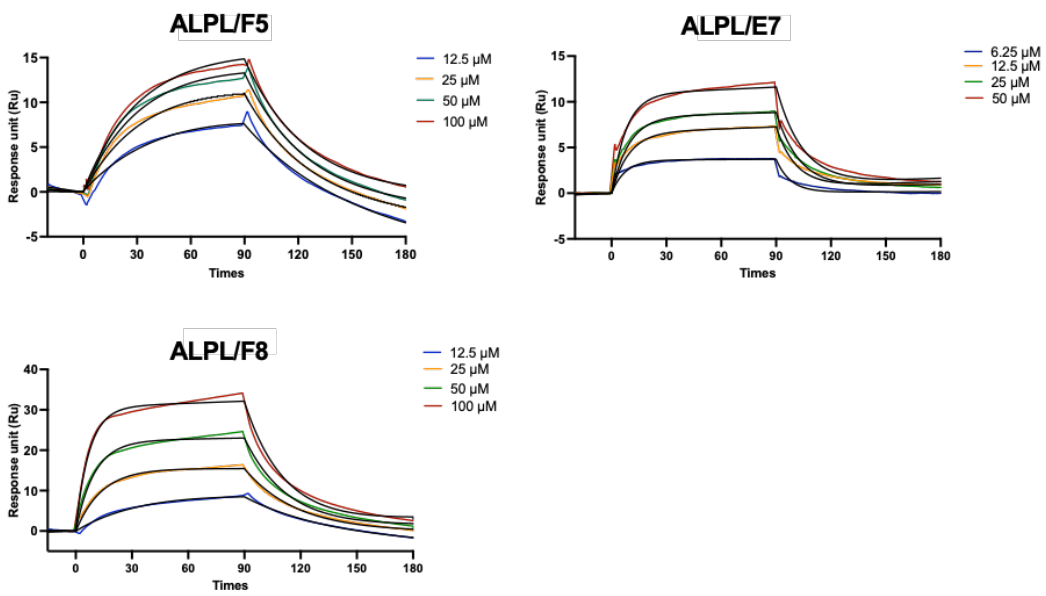

Figure S9. A) EC_{50} and K_D of the compounds further elucidate the structure-activity Relationship. B) Surface plasmon resonance sensorgram from a 2-fold dilution series of F5, E7, and F8 demonstrates the association between *h*ALPL and the compounds mentioned above. Binding kinetics of F5, E7 and F8 were analyzed based on the SPR association and dissociation curves. The K_D values were calculated as the ratio of ligand dissociation rate to association rate (k_d/k_a).

9.10 Figure S10

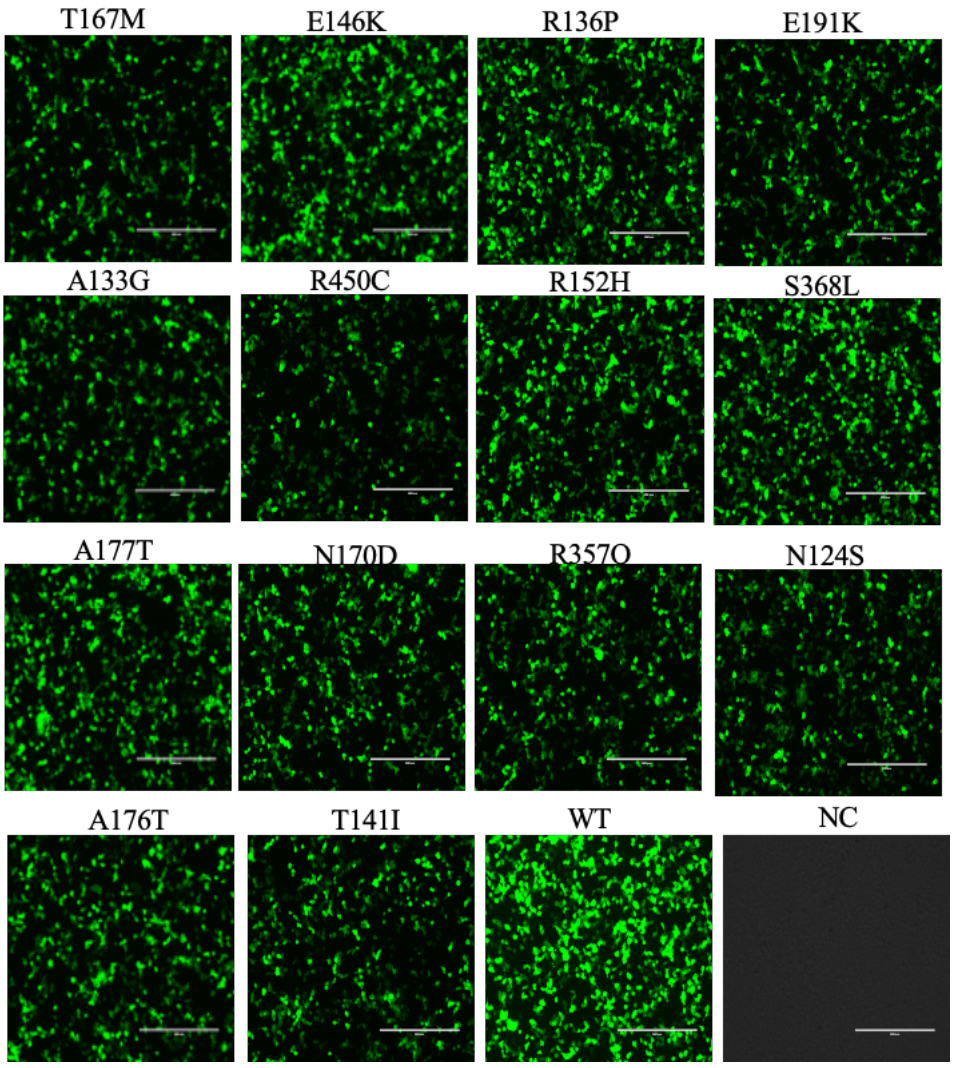

Figure S10. Imaging of cell Hek293T cells overexpressing different GFP-tagged *h*ALPL mutations.

9.11 Figure S11

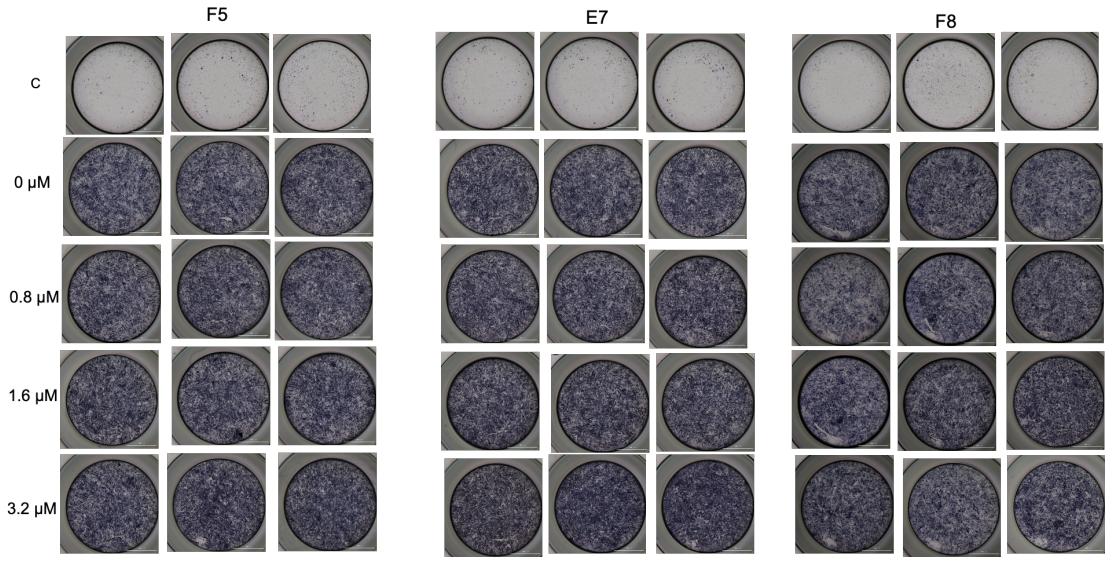

Figure S11. Alkaline phosphatasestaining of the bone marrow-derived mesenchymal stem cells (BMSC) after 7-day treatment with PBS or F5, E7, and F8 at different concentrations. Group C was treated with complete medium. Other groups were treated with complete medium supplemented with an induction cocktail. Induction cocktail contains β-glycerophosphate, vitamin C, and dexamethasone. Each group was performed in triplicate.

10. Reference

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