Supporting Information

Discovery of SARS-CoV-2 Main Protease Covalent Inhibitors from a DNA-Encoded Library Screening

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Table S1. The covalent library used in this study.

Library ID	BB1	BB2	BB3	BB4	Electrophile	Size
1	Amide formation with 191 BBs	Amide formation with 6 BBs	Suzuki reaction with 479 BBs	Reductive amination with 959 BBs		619.310 M
2	Amide formation with 191 BBs	Amide formation with 6 BBs	Suzuki reaction with 479 BBs	Reductive amination with 959 BBs		619.310 M
3	Amide formation with 191 BBs	Amide formation with 6 BBs	Suzuki reaction with 479 BBs	Reductive amination with 959 BBs		619.310 M
4	Amide formation with 191 BBs	Amide formation with 28 BBs	Amide formation with 191 BBs	/		1.069 M
5	Amide formation with 191 BBs	Amide formation with 28 BBs	Amide formation with 191 BBs	/		1.069 M
6	Amide formation with 191 BBs	Amide formation with 28 BBs	Amide formation with 191 BBs	/		1.069 M
7	Amide formation with 189 BBs	Amide formation with 37 BBs	Suzuki reaction with 383 BBs	/		2.772 M
8	Amide formation with 189 BBs	Amide formation with 37 BBs	Suzuki reaction with 383 BBs	/		2.772 M
9	Amide formation with 189 BBs	Amide formation with 37 BBs	Suzuki reaction with 383 BBs	/		2.772 M

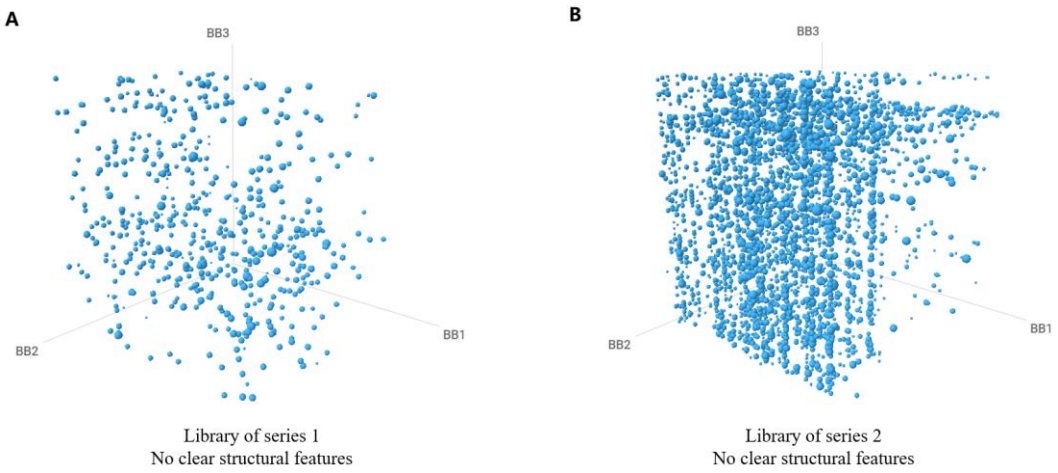


Figure S1. Cubic plot of libraries with preferred scaffolds from heat-on-beads elution methods. The three axes representing BB1 (cycle 1 building blocks), BB2 (cycle 2 building blocks), and BB3 (cycle 3 building blocks), respectively. Each blue dot represent a unique compound with different chemistry combination, and the size of dot proportional to the copy counts of each compound.

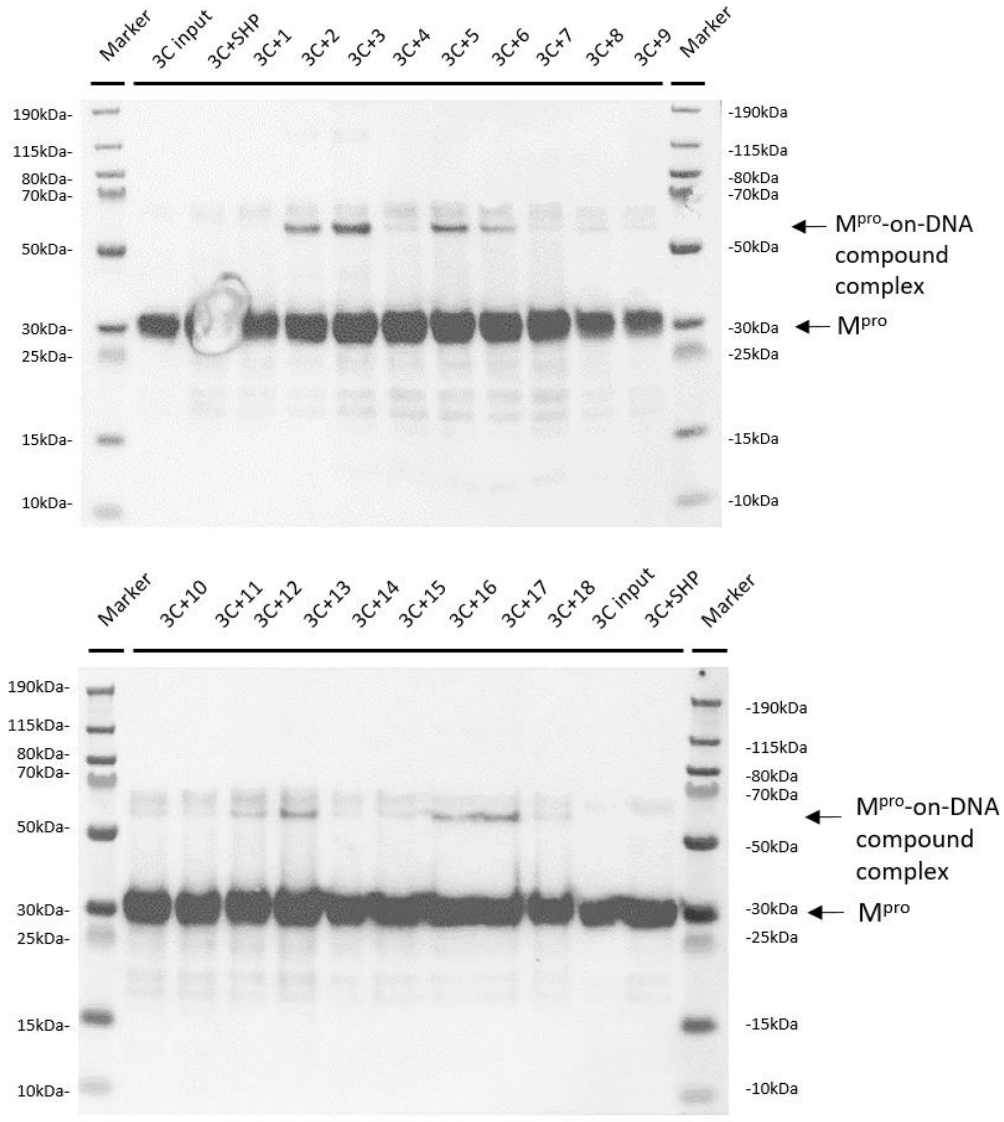


Figure S2. The gel shift of on-DNA version of hit compounds with SARS-CoV-2 M^{pro}. The protein band is detected by the western blot using anti-His antibody. SHP indicates the DNA tag only. The experiment had been repeated at least 3 times.

Table S2. Comparison of on-DNA gel shift results and covalent binding percentage as determined by off-DNA compounds.

On-DNA compound ID [a]	Covalent binding by On-DNA gel shift [b]	Off-DNA compound ID	Covalent binding percentage by LCMS [c]	Enzymatic activity SARS-CoV-2 [d]	Enrichment	Notes
1						
2	Y	**1a**	17.9%	>100	8.62	
3	Y	**1b**	29.3%	22.8	5.51	
4		**1c**	16.1%	61.6	7.57	
5	Y	**1d**	29.3%	>100	6.02	
6	Y	**1e**	48.8%	2.0	7.5	
7						
8						
9						
10						
11			7.9%	>100	6.02	
12	Y		NA	>100	5.51	
13	Y		NA	>100	6.02	
14			NA	>100	6.02	
15			11.5%	>100	7.50	
16	Y	**2a** and **2b**	12.1% and 9.4%	14.6	7.84	by-products
17	Y	**2c**	NA	>100	8.7	
18		**2d**	NA	36.51	7.26	

[a] The compound ID corresponding to the gel shift in Figure S2. [b] As determined by the visualized band shift in gel picture. [c] As determined by protein intact MS after 3h incubation. [b,c] The experiment had been repeated at least two times to confirm the on-DNA compounds and target interaction. [d] The experiment were calculated from ten data points with two independent determinations.

Experimental procedures

Reagents

Dimethylsulfoxide (DMSO, D2650) and sodium chloride (NaCl, S9888) were purchased from Sigma-Aldrich. His Pur™ Ni-NTA Magnetic Beads (88831), 1 M Tris-HCI, pH=7.5 (15567027), sheared salmon sperm DNA (10 mg/mL, AM9680), NuPAGE Bis-Tris 4%-12%, 15 wells (NP0336BOX) and ZebaSpin Desalting Plates, 7K MWCO (89808) were purchased from Thermo Fisher Scientific. The ultra-pure water (ddH_2O) was generated by Merck Milli-Q Direct. The other reagents were purchased from domestic vendors unless mentioned otherwise. MS reaction conversions were determined by UV absorption of LC/MS analysis. The centrifuge instruments were Allegra X-15R and Eppendorf-5424R. Real-time PCR (qPCR) and PCR were performed by Applied biosystems-Quantstudio 7 Flex and Biorad-C1000 Touch™ Thermal Cycler with Dual 48/48 Fast Reaction Module, respectively.

Covalent DEL selection

With a set of covalent libraries prepared, the covalent selection was performed with a competitive elution method. First, we incubated our covalent libraries with the purified *C*-terminal 6xHis tagged SARS-CoV-2 M^{pro} (36.6 kDa) in Tris-HCl (pH 7.5) buffer at room temperature for 1 hr. The reducing agents such as dithiothreitol (DTT) and tris(2-chloroethyl) phosphate (TCEP) were usually not included in the incubation buffer due to the potential reaction with electrophiles of covalent libraries. The amount of covalent library was 10 pmol for each condition and the amount of SARS-CoV-2 Mpro was ~5 μg.

Next, the solution mix was incubated with the His Pur™ Ni-NTA Magnetic Beads (Thermo Scientific™ 88831) for immobilization and the capturing capacity was pre-tested. After immobilization, the matrix was washed for more than 3 times, and the complexes of DEL molecules with target proteins were eluted from the matrix by higher concentration of imidazole (250 mM). The eluted samples were quantified by the quantitative PCR (qPCR) and amplified by the polymerase chain reaction (PCR). The amplified samples were further purified by gel and then subject to next-generation sequencing (NGS). After NGS, the raw data was processed and the tags were decoded to generate files including the structure information, copy numbers, and enrichment values for the subsequent data analysis. The competitive elution method avoided the background on the matrix by PCR amplification of eluted protein samples with covalently bound DEL molecules.

LC-MS analysis of covalent binding

The LC-MS was performed following previous reported protocols with slight modifications.[1] SARS-CoV-2 M^{pro} at 4 μM was reacted with inhibitors at 100 μM or 10 μM (2% (v/v) dimethylsulphoxide

(DMSO) final) in 20 mM Tris, pH 7.5 and 150 mM NaCl. After different incubation times, 10 μL aliquots were then assessed by electrospray mass spectrometry using a Waters Acquity UPLC/ESI-TQD with a 2.1350 mm Acquity UPLC BEH300 C4 column.

Gel shift assay

Gel shift assay was developed to quickly check the covalent binding of both on-DNA compounds and off-DNA compounds. It can be used as an alternative method for LCMS confirmation of covalent binding. For on-DNA compounds, the on-DNA compounds was incubated with the SARS-CoV-2 M^{pro} at room temperature for 60 min in the same buffer of covalent selection. The solution mixture was then subjected SDS-PAGE analysis and the SARS-CoV-2 M^{pro} was detected by the anti-His antibody following previous protocols.[2] The band shift of SARS-CoV-2 M^{pro} suggested the covalent binding of on-DNA compounds. For the off-DNA compounds, we synthesized the off-DNA compounds with a Cy5 tag. The SDS-PAGE analysis was performed similarly with the on-DNA compounds and the Cy5 signal was checked. The protein was then stained with coomassie brilliant blue. The overlap of Cy5 signal and coomassie brilliant blue band suggested the covalent binding of off-DNA compounds.

Enzymatic activity assay

To test for selectivity, seven human coronavirus M^{pro} (SARS-CoV-2, SARS, MERS, HKU1, OC43, 229E and NL63) were tested with the compound using a fluorometric assay. Human coronavirus Mpro (SARS-CoV-2, SARS, MERS, HKU1, OC43) were assayed with Dabcyl- KTSAVLQ⬇SGFRKM -(Edans) as substrates. Coronavirus Mpro (229E and NL63) were assayed with Dabcyl-YGSTLQ⬇AGLRKM -(Edans) and Dabcyl-YNSTLQ⬇SGLKKM -(Edans), respectively, as substrates. All assays were performed in 384-well white plates (Perkin elmer) in a total volume of 30 μL of the assay buffer containing 20 mM Tris-HCl (pH 7.3), 100 mM NaCl, 1mM EDTA, 0.1% BSA (v/v) in duplicate. A series of compound concentrations (0~100 μM final concentration at 2-fold serial dilutions) in 100% DMSO was prepared in a 384-well plate. Then 100× compound solutions were prepared in the assay buffer prior to assays. A total of 25 μL of each enzyme solution was distributed into wells, and 100× varying concentrations of compounds was added and incubated for 30 min. The enzyme reaction was initiated by adding 5 μL of the substrates, and fluorescence intensity was monitored at excitation/emission wavelengths of 340 nm/490 nm after 60 min incubation at 30 ℃. The relative fluorescence units (RFU) value was measured with an excitation wavelength of 360 nm and emission wavelength of 490 nm by using SpectraMax Paradigm Muti-Mode Detection Platform (Molecular Devices, USA). Experiments were performed in triplicate. Then the progress curve of peptide hydrolysis was plotted by GraphPad Prism 8.0.

In vitro inhibitory activity against SARS-CoV-2

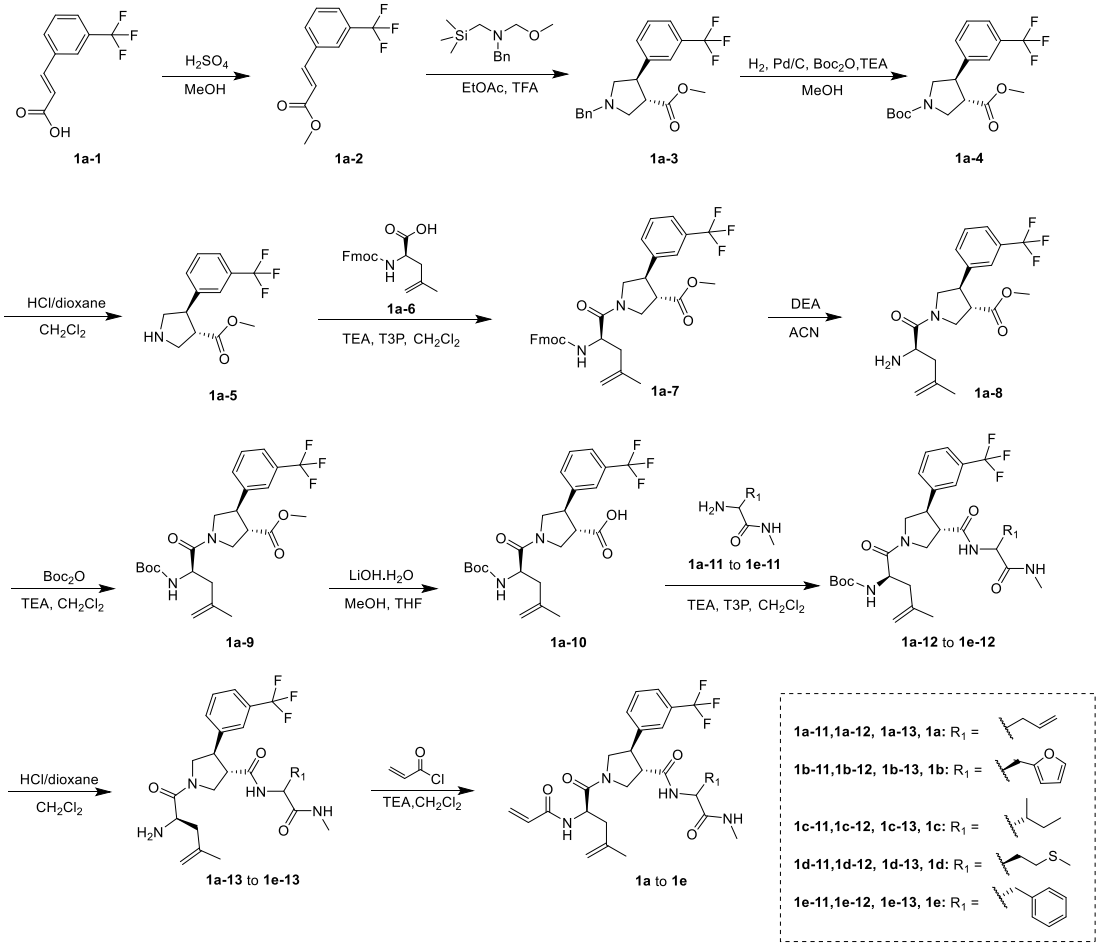
Ten-point dose-response curve (DRC) will be generated for each drug. Vero cells are seeded at 1.2 × 104 cells per well in black 384-well, μClear plates (Greiner Bio-One), 24 hrs prior to the experiment. Ten-point DRCs are generated with 3-fold dilutions, in duplicates or defined by the sponsor. Two μM pGP inhibitor (CP-100356) only group was used as a control for 0% inhibition when calculating the inhibition ratio of the tested compounds against SARS-CoV-2 infection. For viral infection, SARS-CoV-2 is added at a multiplicity of infection (MOI) of 0.0125. The cells are fixed at 24 hpi with 4% paraformaldehyde and then permeabilized with 0.25% TritonX-100. The virus is detected with Anti-SARS-CoV-2 protein antibody (1:3000 dilution in 5% normal goat serum in PBS) at 37 °C for 1 hr, and then stained with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:2000 in 5% normal goat serum in PBS) and 2.5μg/ml (1:4000 dilution) of Hoechst 33342. After each steps, the plates are washed with DPBS twice. Image are acquired by using Operetta high content imaging system (Equipment setting: 488/405 emission, 20X Objective, 5 images/well). The acquired images were analyzed using software to quantify cell numbers and infection ratios, and antiviral activity was normalized to positive (mock) and negative (2 μM of CP-100359 alone) controls in each assay plate. DRCs were fitted by sigmoidal dose-response models, with the following equation: Y = Bottom + (Top - Bottom)/(1 +(IC50/X)Hill slope), using XLfit 4 Software or Prism. EC50 and CC50 values were calculated from the normalized activity dataset-fitted curves.

Visualization and molecular modelling

The cubic scatter plots were generated with TIBCO Spotfire (version number: 7.11.2).[3] The docking poses of representative compounds were obtained using the covalent docking feature in MOE.[4] The starting protein structure for docking was prepared from the crystal structure of SARS-CoV-2 M^{pro} covalently bound with an aldehyde-based inhibitor (PDB code: 6M0K)[5], by breaking the C-S bond between Cys145 and the ligand, and correcting pronated states of protein residues using MOE. Cys145 was indicated as the reactive site for our selected electrophiles, and Michael 1-4 addition was established as the reaction template. The possibilities of compounds bound with cysteines other than Cys145 near the catalytic site were also evaluated, yet no plausible binding modes were identified.

Synthetic procedures for off-DNA compounds and its intermediates

The general procedure for the synthesis of compounds 1a to 1e is showed in scheme S1.



Scheme S1. General procedures for the synthesis of compounds **1a** to **1e**.

Synthesis of (E)-methyl 3-(3-(trifluoromethyl) phenyl) acrylate (1a-2):

To a mixture of (*E*)-3-[3-(trifluoromethyl) phenyl] prop-2-enoic acid (**1a-1**, 10.0 g, 46.2 mmol) and H_2SO_4 (4.54 g, 46.2 mmol) in MeOH (100 mL) was added in one portion at 25 °C under N_2. The mixture was stirred at 60 °C for 5 hrs. TLC (PE/EtOA = 1/1, product R_f = 0.77) showed one new spot. LCMS showed **1a-1** was consumed completely. The residue was poured into water (50.0 mL) and stirred for 20 min. The aqueous phase was extracted with CH_2Cl_2 (30.0 mL x 3). The combined organic phase was washed with brine (30.0 mL x 3), dried with anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography (SiO_2, PE/EtOA = 1/1), to get the white solid of **1a-2** (9.92 g, 41.5 mmol, 89.7% yield, 96.30% purity).

^{1}H NMR (400MHz, CD_3OD): δ 7.75-7.77 (m, 1H), 7.69-7.73 (m, 1H), 7.67-7.69 (m, 1H), 7.61-7.66 (m, 1H), 7.48-7.58 (m, 1H), 6.44-6.55 (m, 1H), 3.57-4.04 (m, 3H);
LCMS: m/z = 231.1 $(M+H)^+$, Rt = 1.665 min.

Synthesis of methyl (3S,4R)-1-benzyl-4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylate (1a-3):
To a mixture of **1a-2** (3.00 g, 13.0 mmol) and *N*-(methoxymethyl)-1-phenyl-*N*-(trimethylsilylmethyl) methanamine (5.57 g, 23.4 mmol) in EtOAc (80.0 mL) was added TFA (297 mg, 2.61 mmol, 193 uL) in one portion at 25 °C under N_2. The mixture was stirred at 60 °C for 2 hrs. TLC (PE/EtOA = 5/1, product R_f =0.63) showed one new spot. LCMS showed **1a-2** was consumed completely. The residue was poured into water (20.0 mL) and stirred for 10 min. The aqueous phase was extracted with EtOA (30.0 mL x 3). The combined organic phase was washed with brine (30.0 mL x 3), dried with anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography (SiO_2, PE/EtOA = 5/1), to get the white oil of **1a-3** (4.00 g, 11.0 mmol, 84.40% yield).
LCMS: m/z = 364.4 $(M+H)^+$, Rt = 0.477 min.

Synthesis of (3S,4R)-1-tert-butyl 3-methyl 4-(3-(trifluoromethyl) phenyl) pyrrolidine-1, 3-dicarboxylate (1a-4)
To a mixture of **1a-3** (4.0 g, 11.0 mmol) in MeOH (20 mL), then added Boc_2O (12.0 g, 55.0 mmol, 12.6 mL) and TEA (3.34 g, 33.0 mmol, 4.60 mL) and Pd/C (0.50 g, 10% purity) under N_2 atmosphere. The suspension was degassed and purged with H_2 for 3 times. The mixture was stirred under H_2 (30 psi) at 25 °C for 12 hrs. LCMS and TLC (PE/EtOA = 3/1, product R_f = 0.5) showed **1a-3** was consumed completely. The reaction mixture was filtered through a gelite pad, the filtrate was concentrated afford the crude. The residue was purified by flash silica gel chromatography (ISCO®; 12 g SepaFlash® Silica Flash Column, Eluent of 0~100% EtOA/PE gradient @ 40 mL/min). Obtained **1a-4** (2.50 g, 6.49 mmol, 59.0% yield, 97.0% purity) as a colorless oil.
^{1}H NMR (400MHz, CD_3OD): δ 7.59-7.64 (m, 2H), 7.51-7.59 (m, 2H), 3.81-3.92 (m, 2H), 3.66-3.78 (m, 1H), 3.61 (s, 3H), 3.53-3.60 (m, 1H), 3.36-3.45 (m, 2H), 1.45-1.51 (m, 9H);
LCMS: m/z = 318.2 $(M+H-56)^+$, Rt = 0.616 min.

Synthesis of methyl (3S,4R)-4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylate (1a-5):
To a solution of **1a-4** (2.50 g, 6.70 mmol) in CH_2Cl_2 (20.0 mL), was added HCl/dioxane (4 M, 1.67 mL). The mixture was stirred at 25 °C for 30 min. LCMS showed **1a-4** was consumed completely. The mixture was concentrated under reduced pressure to give a residue. Obtained **1a-5** (2.28 g, crude) as a yellow solid.

LCMS: m/z = 274.2 $(M+H)^+$, Rt = 0.838 min.

Synthesis of methyl (3S,4R)-1-[(2R)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-4-methyl-pent-4-enoyl]-4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylate (1a-7)

To a solution of (2*R*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-4-methyl-pent-4-enoic acid (**1a-6**, 1.27 g, 3.61 mmol) in CH_2Cl_2 (10.0 mL), was added TEA (914 mg, 9.04 mmol, 1.26 mL) and **1a-5** (1.19 g, 4.34 mmol). After addition, the mixture was added T3P (3.45 g, 5.42 mmol, 3.22 mL, 50% purity) at 0 °C, the mixture was stirred at 25 °C for 2 hrs. TLC (PE/EtOA = 3/1, product R_f = 0.46) showed the **1a-6** was consumed completely. The reaction mixture was filtered through a gelite pad, and the filrate was concentrated afford the crude. The residue was diluted with H_2O 60.0 mL and extracted with CH_2Cl_2 (20.0 mL x 3). The combined organic layers were washed with aqueous NaCl (20.0 mL x 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 20 g SepaFlash® Silica Flash Column, Eluent of 0~100% EtOA/PE gradient @ 40 mL/min). Obtained **1a-7** (1.90 g, 3.10 mmol, 85.7% yield, 99.81% purity) as a yellow oil.

LCMS: m/z = 607.4 $(M+H)^+$, Rt = 0.637 min.

Synthesis of methyl (3S,4R)-1-[(2R)-2-amino-4-methyl-pent-4-enoyl]-4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylate (1a-8)

To a solution of **1a-7** (1.88 g, 3.10 mmol) in ACN (16 mL) was added DEA (1.16 g, 3.10 mmol, 4 mL). Then the mixture was stirred at 25 °C for 1 hr. LCMS showed **1a-7** was consumed completely. The mixture was concentrated under reduced pressure to give a residue. Obtained **1a-8** (1.80 g, crude) as a yellow solid.

LCMS: m/z = 385.1 $(M+H)^+$, Rt = 0.628 min

Synthesis of methyl (3S, 4R)-1-[(2R)-2-(tert-butoxycarbonylamino) -4-methyl-pent-4-enoyl] -4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylate (1a-9)

To a solution of **1a-8** (1.80 g, 4.68 mmol) in CH_2Cl_2 (10.0 mL), was added TEA (1.42 g, 14.0 mmol, 1.96 mL) and Boc_2O (4.09 g, 18.7 mmol, 4.3 mL). Then the mixture was stirred at 25 °C for 3 hrs. LCMS and TLC (PE/EtOA = 3/1, product R_f = 0.32) showed **1a-8** was consumed completely. The residue was diluted with CH_2Cl_2 30 mL and extracted with H_2O (10.0 mL x 3). The combined organic layers were washed with aqueous NaCl (10.0 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 20 g SepaFlash® Silica Flash Column, Eluent of 0~100% EtOA/PE gradient @ 40 mL/min).

Obtained **1a-9** (1.38 g, 2.42 mmol, 51.7% yield, 85.00% purity) as a colorless oil.

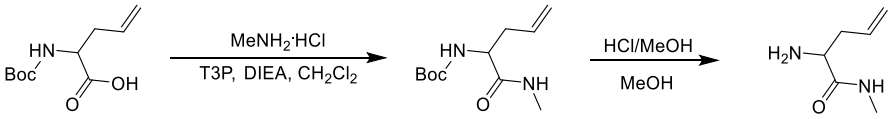
LCMS: m/z = 485.3 $(M+H)^+$, Rt = 0.582 min

Synthesis of (3S, 4R)-1-[(2R)-2-(tert-butoxycarbonylamino)-4-methyl-pent-4-enoyl]-4-[3-(trifluoromethyl) phenyl] pyrrolidine-3-carboxylic acid (1a-10)

To a solution of **1a-9** (1.38 g, 2.85 mmol) in THF (6.0 mL) and MeOH (6.0 mL), was added a solution of $LiOH{\cdot}H_2O$ (358 mg, 8.54 mmol) in H_2O (2.0 mL), the mixture was stirred at 25 °C for 2 hrs. TLC (PE/EtOA = 3/1, product R_f = 0.08) showed **1a-9** was consumed completely. The residue was diluted with H_2O 30.0 mL and citric acid to make pH = 4~5. Then the mixture was washed with EtOA (10.0 mL x 3). The combined organic layers were washed with aqueous NaCl (10.0 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. Obtained **1a-10** (1.26 g, 2.62 mmol, 91.9% yield, 97.81% purity) as a colorless oil.

LCMS: m/z = 471.2 $(M+H)^+$, Rt = 0.563 min

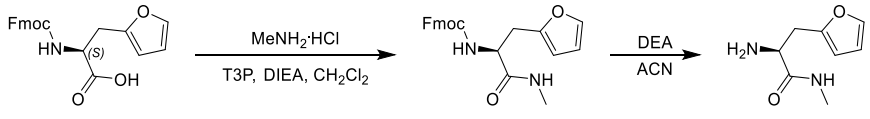
Synthesis preparation of compound 2-amino-N-methylpent-4-enamide (1a-11)



1a-14 **1a-15** **1a-11**

Step 1: To a solution of 2-(*tert*-butoxycarbonylamino)pent-4-enoic acid (**1a-14**, 900 mg, 4.18 mmol) in CH_2Cl_2 (10.0 mL) were added T3P (3.99 g, 6.27 mmol, 3.7 mL), DIEA (3.78 g, 29.3 mmol, 5.0 mL) and methanamine hydrochloride (1.41 g, 20.9 mmol, 5.0 *equiv*) at 0 °C. The mixture was stirred at 25 °C for 2 hrs. TLC (CH_2Cl_2/MeOH = 10/1, R_f = 0.08) indicated **1a-14** was consumed completely. The reaction mixture was diluted with CH_2Cl_2 100 mL and washed with the saturated solution of $NaHCO_3$ (200 mL x 1). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give **1a-15** (900 mg, 3.94 mmol, 94.3% yield) as a white solid. *Step 2:* To a solution of **1a-15** (100 mg, 438 umol) in MeOH (2.0 mL) was added HCl/MeOH (4 M, 1.1 mL). The mixture was stirred at 25 °C for 1 hr. TLC (DCM/MeOH = 10/1, R_f = 0.08) indicated **1a-15** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue. Compound **1a-11** (50.0 mg, 303 umol, 69.3% yield, HCl) was obtained as white solid.

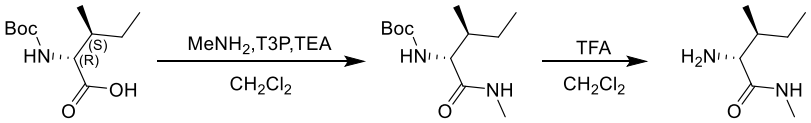
Synthesis preparation of compound (2S)-2-amino-3-(2-furyl)-N-methyl-propanamide (1b-11)



1b-14 **1b-15** **1b-11**

Step 1: To a mixture of (2*S*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-3-(2-furyl) propanoic acid (**1b-14**, 500 mg, 1.32 mmol, 1.0 *equiv*) in CH_2Cl_2 (5.0 mL) was added T3P (1.26 g, 1.98 mmol, 1.18 mL, 50% purity,) and methanamine hydrochloride (89.1 mg, 1.32 mmol) and DIEA (1.19 g, 9.24 mmol, 1.61 mL) in one portion at 0 °C under N_2. The mixture was stirred at 0 °C for 5 min, then heated to 25 °C and stirred for 1 hr. LCMS showed **1b-14** was consumed completely. The reaction mixture was partitioned between CH_2Cl_2 10.0 mL and sat $NaHCO_3$ 10.0 mL. The organic phase was separated, washed with brine (10.0 mL x 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. Compound 9H-fluoren-9-ylmethyl *N*-[(1*S*)-1-(2-furylmethyl)-2-(methylamino)-2-oxo-ethyl] carbamate (425 mg, crude) was obtained as a white solid. *Step 2:* To a mixture of 9H-fluoren-9-ylmethyl *N*-[(1*S*)-1-(2-furylmethyl)-2-(methylamino)-2-oxo-ethyl] carbamate (425 mg, 1.09 mmol) in DEA (1.0 mL) and ACN (6.0 mL) was added in one portion at 25 °C under N_2. The mixture was stirred at 25 °C for 1 hr. Concentrated under reduced pressure to give a residue. Compound **1b-11** (425 mg, crude) was obtained as a yellow solid.

Synthesis preparation of (2R,3S)-2-amino-N, 3-dimethyl-pentanamide (1c-11)

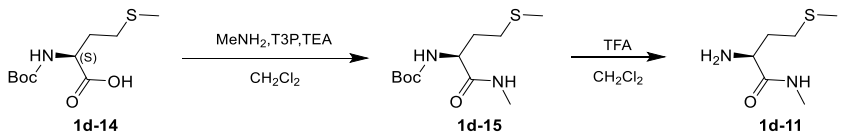


1c-14 **1c-15** **1c-11**

Step 1: To a solution of (2*R*, 3*S*)-2-(*tert*-butoxycarbonylamino)-3-methyl-pentanoic acid (**1c-14**, 1 g, 4.32 mmol) in CH_2Cl_2 (10.0 mL) was added TEA (1.75 g, 17.2 mmol, 2.41 mL) and methanamine hydrochloride (729 mg, 10.8 mmol). Then the mixture was added T3P (5.50 g, 8.65 mmol, 5.14 mL, 50% purity) at 0 °C. After addition, the mixture was stirred at 25 °C for 2 hrs. LCMS showed **1c-14** was consumed completely. The mixture was diluted with CH_2Cl_2 10.0 mL and extracted with H_2O (10.0 mL x 3). The combined organic layers were washed with aqueous NaCl (10.0 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. Obtained **1c-15** (970 mg, crude) as a white solid. *Step 2:* To a solution of **1c-15** (500 mg, 2.05 mmol) in CH_2Cl_2 (6.0 mL), was added TFA (3.08 g, 27.0 mmol, 2 mL), the mixture was stirred at 25 °C for 30 min. LCMS showed **1c-15** was consumed completely. The mixture was concentrated under reduced pressure to give a residue. Without purification, obtained **1c-11** (910 mg, crude) as a yellow oil.

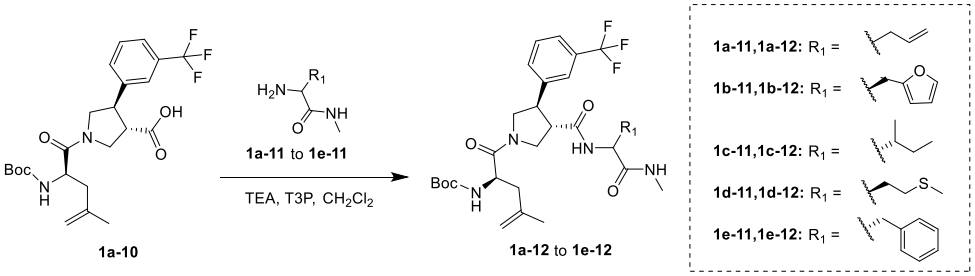
LCMS: m/z = 145.3 $(M+H)^+$, Rt = 0.162, 0.225 min

Synthesis preparation of (2S)-2-amino-N-methyl-4-methylsulfanyl-butanamide (1d-11)



Step 1: To a solution of (2*S*)-2-(*tert*-butoxycarbonylamino)-4-methylsulfanyl-butanoic acid (**1d-14**, 1.0 g, 4.01 mmol) in CH_2Cl_2 (10.0 mL), was added TEA (1.62 g, 16.0 mmol, 2.23 mL) and methanamine; hydrochloride (677 mg, 10.0 mmol). Then the mixture was added T3P (5.10 g, 8.02 mmol, 4.77 mL, 50% purity) at 0 °C, the mixture was stirred at 25 °C for 2 hrs. LCMS showed (**1d-14** was consumed completely. The mixture was diluted with CH_2Cl_2 10.0 mL and extracted with H_2O (10.0 mL x 3). The combined organic layers were washed with aqueous NaCl (10.0 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. Without purification. Obtained *tert*-butyl *N*-[(1*S*)-1-(methylcarbamoyl)-3-methylsulfanyl-propyl] carbamate (**1d-15**, 1.10 g, crude) as a yellow solid.
Step 2: To a solution of **1d-15** (500 mg, 1.91 mmol) in CH_2Cl_2 (6.0 mL) was added TFA (3.08 g, 27.0 mmol, 2.0 mL). The mixture was stirred at 25 °C for 30 min. LCMS showed the **1d-15** was consumed completely. The mixture was concentrated under reduced pressure to give **1d-11** (986.5 mg, crude) as a yellow oil.
LCMS: m/z = 163.3 $(M+H)^+$, Rt = 0.145 min.

Synthesis of compound 1a-12 to 1e-12:



To a mixture of **1a-10** and 2-amino-2-R_1 group-*N*-methylacetamide (**1a-11**, **1b-11**, **1c-11**, **1d-11** or **1e-11**, 2.0 *equiv*) in THF was added DIEA (1.1 *equiv*) and HATU (2.0 *equiv*) in one portion at 25°C under N_2. The mixture was stirred at 25 °C for 1 hr. The reaction mixture was partitioned between EtOAc and H_2O. The organic phase was separated, washed with brine 2 times, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography **(**ISCO®; 4 g SepaFlash® Silica Flash Column, Eluent of 0~100% EtOA/ PE gradient @ 6 mL/min), to give compound **1a-12**, **1b-12**, **1c-12**, **1d-12** or **1e-12** crude, which was used next step without further purification.

- Compound *tert*-butyl *N*-[(1*R*)-1-[(3*S*,4*R*)-3-[[1-benzyl-2-(methylamino)-2-oxo-ethyl] carbamoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-1-carbonyl]-3-methyl-but-3-enyl] carbamate (**1a-12**):

yellow solid;

LCMS: m/z = 581.1 $(M+H)^+$, Rt = 0.949 min.

- Compound *N*-[(1*R*)-1-[(3*S*,4*R)*-3-[[(1*S*)-1-(2-furylmethyl)-2-(methylamino)-2-oxo-ethyl] carbamoyl]-4-[3-(trifluoromethyl)phenyl] pyrrolidine-1-carbonyl]-3-methyl-but-3-enyl] carbamate (**1b-12**): white solid;

  LCMS: m/z = 621.2 $(M+H)^+$, Rt = 0.962 min.

- Compound *tert*-butyl *N*-[(1*R*)-3-methyl-1-[(3*S*,4*R*)-3-[[(1*R*,2*S*)-2-methyl-1 (methylcarbamoyl) butyl]carbamoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-1-carbonyl] but-3-enyl] carbamate (**1c-12**): yellow oil;

  ^{1}H NMR (400MHz, $CDCl_3$): δ 7.39-7.62 (m, 4H), 5.96-6.13 (m, 1H), 5.42-5.77 (m, 1H), 5.03-5.26 (m, 1H), 4.76-4.95 (m, 2H), 4.44-4.61 (m, 1H), 4.15-4.36 (m, 2H), 3.85-4.08 (m, 2H), 2.94-3.15 (m, 1H), 2.69-2.89 (m, 3H), 2.24-2.47 (m, 2H), 1.76-1.83 (m, 3H), 1.38-1.48 (m, 9H), 0.68-0.98 (m, 8H);

  LCMS: m/z = 597.4 $(M+H)^+$, Rt = 1.793 min.

- Compound *tert*-butyl *N*-[(1*R*)-3-methyl-1-[(3*S*,4*R*)-3-[[(1*S*)-1-(methylcarbamoyl)-3-methylsulfanyl-propyl] carbamoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-1-carbonyl]but-3-enyl] carbamate (**1d-12**): yellow oil;

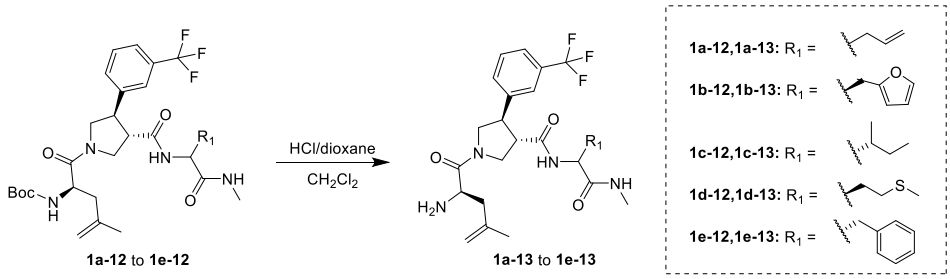
  ^{1}H NMR (400MHz, $CDCl_3$): δ 7.41-7.59 (m, 4H), 6.31-6.58 (m, 1H), 6.15 (br d, J = 15.6 Hz, 1H), 5.17 (br d, J = 8.4 Hz, 1H), 4.76-4.96 (m, 2H), 4.43-4.70 (m, 2H), 3.49-4.42 (m, 6H), 2.77-2.88 (m, 2H), 2.70 (t, J = 5.6 Hz, 1H), 2.22-2.66 (m, 4H), 1.88-2.15 (m, 5H), 1.75-1.82 (m, 3H), 1.38-1.46 (m, 9H);

  LCMS: m/z = 615.4 $(M+H)^+$, Rt = 0.515 min.

- Compound *tert*-butyl *N*-[(1*R*)-1-[(3*S*,4*R*)-3-[[1-benzyl-2-(methylamino)-2-oxo-ethyl] carbamoyl]-4-[3-(trifluoromethyl)phenyl] pyrrolidine-1-carbonyl]-3-methyl-but-3-enyl] carbamate (**1e-12**): white solid;

  LCMS: m/z = 631.3 $(M+H)^+$, Rt = 0.982 min.

Synthesis of compound 1a-13 to 1e-13:



To a mixture of **1a-12** (**1b-12**, **1c-12**, **1d-12** or **1e-12**) in CH_2Cl_2 was added TFA (1.0 *equiv*) in one portion at 25 °C under N_2. The mixture was stirred at 25 °C for 40 min. LCMS showed reactant was consumed completely. Concentrated under reduced pressure to give a residue, to get **1a-13** (**1b-13**, **1c-13**, **1d-13** or **1e-13**) crude, which was used next step without further purification.

- Compound (3*S*,4*R*)-1-[*(2R)*-2-amino-4-methyl-pent-4-enoyl]-*N*-[1-benzyl-2-(methylamino)-2-oxo-ethyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carboxamide (**1a-13**): brown solid;
  LCMS: m/z = 481.2 $(M+H)^+$, Rt = 0.776 min.

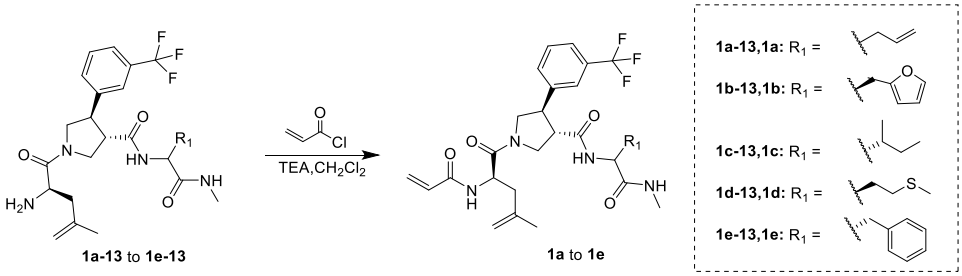
- Compound (3*S*,4*R*)-1-[*(2R)*-2-amino-4-methyl-pent-4-enoyl]-*N*-[(1*S*)-1-(2-furylmethyl)-2-(methylamino)-2-oxo-ethyl]-4-[3-(trifluoro methyl) phenyl] pyrrolidine-3-carboxamide (**1b-13**): brown solid;
  LCMS: m/z = 521.3 $(M+H)^+$, Rt = 0.776 min.

- Compound (3*S*,4*R*)-1-((*R*)-2-amino-4-methylpent-4-enoyl)-*N*-((2*R*,3*S*)-3-methyl-1-(methylamino)-1-oxopentan-2-yl)-4-(3-(trifluoromethyl) phenyl)pyrrolidine-3-carboxamide (**1c-13**): white solid;
  ^{1}H NMR (400 MHz, CD_3OD): δ 7.46-7.74 (m, 4H), 4.95-5.07 (m, 2H), 3.33-4.37 (m, 8H), 2.56-2.76 (m, 4H), 2.39-2.56 (m, 1H), 1.79-1.89 (m, 3H), 1.60-1.74 (m, 1H), 1.07-1.38 (m, 1H), 0.78-1.00 (m, 3.6H), 0.64-0.74 (m, 2H), 0.48-0.56 (m, 1.4H);
  LCMS: m/z = 497.3 $(M+H)^+$, Rt = 1.073, 1.202 min.

- Compound (3*S*,4*R*)-1-((*R*)-2-amino-4-methylpent-4-enoyl)-*N*-((*S*)-1-(methylamino)-4-(methylthio)-1-oxobutan-2-yl)-4-(3-(trifluoromethyl)phenyl)pyrrolidine-3-carboxamide (**1d-13**): white solid;
  ^{1}H NMR (400 MHz, CD_3OD): δ 7.52-7.71 (m, 4H), 4.93-5.07 (m, 3H), 3.32-4.43 (m, 7.8H), 3.20-3.30 (m, 0.2H), 2.57-2.75 (m, 4H), 2.35-2.52 (m, 2H), 2.05 (d, J = 1.6 Hz, 3H), 1.89-1.93 (m, 1.5H), 1.79-1.89 (m, 3.5H), 1.58-1.75 (m, 1H);
  LCMS: m/z = 515.3 $(M+H)^+$, Rt = 0.978, 1.073 min.

- Compound (3*S*,4*R*)-1-[(2*R*)-2-amino-4-methyl-pent-4-enoyl]-*N*-[1-benzyl-2-(methylamino)-2-oxo-ethyl]-4-[3-(trifluoromethyl)phenyl] pyrrolidine-3-carboxamide (**1e-13**): yellow liquid;
  LCMS: m/z = 531.2 $(M+H)^+$, Rt = 0.793 min, 0.817 min.

Synthesis of compound 1a to 1e



To a mixture of **1a-13** (**1b-13**, **1c-13**, **1d-13** or **1e-13**) and TEA (3.0 *equiv*) in CH_2Cl_2 was added prop-2-enoyl chloride (1.1 *equiv*) in one portion at -78 °C under N_2. The mixture was stirred at -78 °C for 5 min. The reaction mixture was partitioned between H_2O and CH_2Cl_2. The organic phase was separated, washed with brine 2 times, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by prep-HPLC (column: Phenomenex Luna C18 100*30mm*3um; mobile phase: [water (0.225%FA)-ACN]; B%: 10%-80%, 8min), to get compound **1a**, **1b**, **1c**, **1d** or **1e** (10%~70% yield).

- Compound (3*S*,4*R*)-1-((*R*)-2-acrylamido-4-methylpent-4-enoyl)-*N*-(1-(methylamino)-1-oxopent-4-en-2-yl)-4-(3-(trifluoromethyl)phenyl)pyrrolidine-3-carboxamide (**1a**): white solid;
  ^{1}H NMR (400 MHz, CD_3OD): δ 7.37-7.61 (m, 4H), 6.08-6.30 (m, 2H), 5.16-5.71 (m, 2H), 5.04-5.15 (m, 1H), 4.71-4.77 (m, 2H), 4.60-4.77 (m, 1H), 3.22-4.54 (m, 7H), 3.17-3.17 (m, 1H), 2.61-2.72 (m, 3H), 2.02-2.60 (m, 4H), 1.79 (d, *J* = 12.8 Hz, 3H);
  LCMS: m/z = 535.4 $(M+H)^+$, Rt = 1.440,1490 min;
  HPLC: 99.5% purity (220 nm).

- Compound (3*S*,4*R*)-1-((*R*)-2-acrylamido-4-methylpent-4-enoyl)-*N*-((*S*)-3-(furan-2-yl)-1-(methylamino)-1-oxopropan-2-yl)-4-(3-(trifluoromethyl)phenyl)pyrrolidine-3-carboxamide (**1b**): white solid;
  ^{1}H NMR (400 MHz, CD_3OD): δ 7.53-7.68 (m, 4H), 7.23 (s, 1H), 6.21-6.37 (m, 2H), 6.13-6.14 (m, 1H), 5.65-5.80 (m, 2H), 4.81-4.87 (m, 3H), 4.56 (dd, *J* = 5.6, 9.2 Hz, 1H), 3.90-4.12 (m, 3H), 3.62-3.80 (m, 1H), 3.42-3.58 (m, 1H), 3.17-3.26 (m, 1H), 2.91-2.99 (m, 1H), 2.78-2.86 (m, 1H), 2.68-

2.73 (m, 3H), 2.52-2.53 (m, 1H), 2.39-2.46 (m, 1H), 1.76-1.86 (m, 3H);
LCMS: m/z = 575.1 $(M+H)^+$, Rt =1.567 min;
HPLC: 99.8% purity (220 nm).

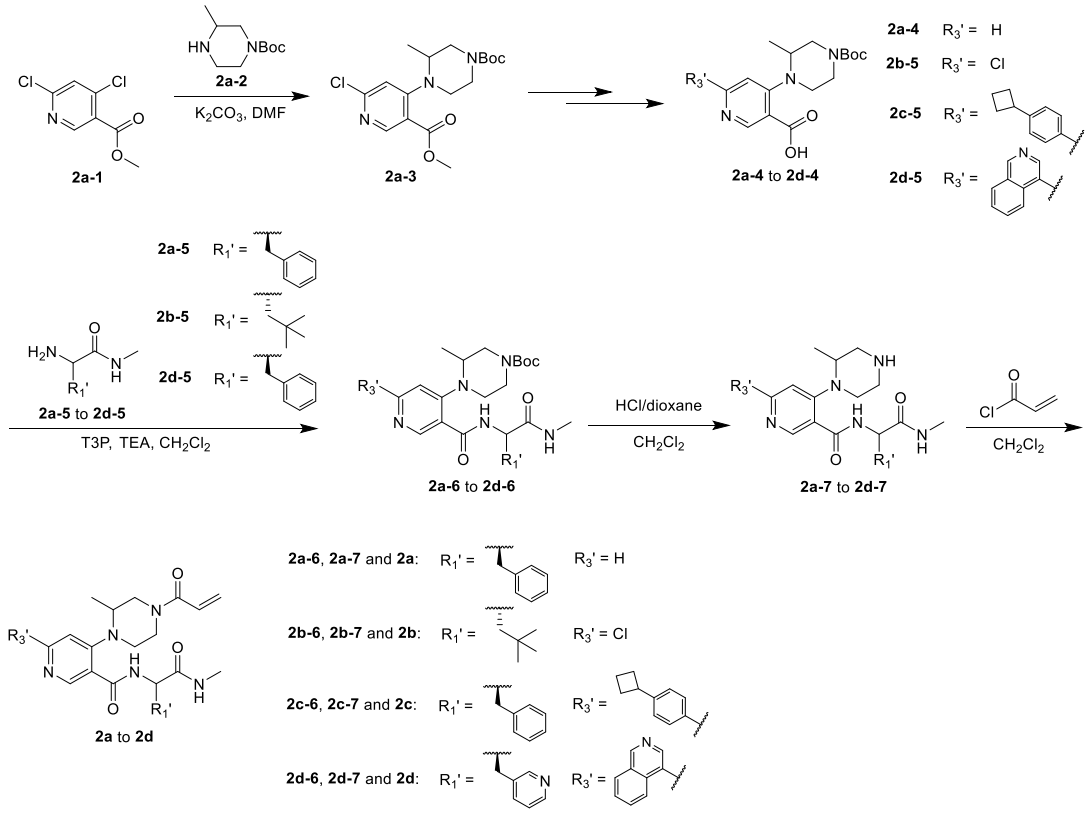
- Compound (3*S*,4*R*)-1-((*R*)-2-acrylamido-4-methylpent-4-enoyl)-*N*-((2*R*,3*S*)-3-methyl-1-(methylamino)-1-oxopentan-2-yl)-4-(3-(trifluoromethyl) phenyl) pyrrolidine-3-carboxamide (**1c**): white solid;
^{1}H NMR (400 MHz, CD_3OD): δ 7.46-7.75 (m, 4H), 6.10-6.46 (m, 2H), 5.64-5.77 (m, 1H), 4.90-4.96 (m, 1H), 4.84 (br d, J = 5.6 Hz, 2H), 4.40-4.52 (m, 0.5H), 4.21-4.29 (m, 1H), 4.10-4.20 (m, 0.5H), 3.86-4.10 (m, 2H), 3.71-3.86 (m, 1H), 3.54-3.70 (m, 1H), 3.45-3.54 (m, 1H), 3.32-3.44 (m, 1H), 2.59-2.74 (m, 3H), 2.49-2.57 (m, 1H), 2.36-2.45 (m, 1H), 1.77-1.85 (m, 3H), 1.60-1.76 (m, 1H), 1.27-1.38 (m, 0.6H), 1.05-1.18 (m, 0.4H), 0.84-0.93 (m, 3H), 0.67 (br d, J = 4.4 Hz, 1.6H), 0.51 (dd, J = 6.8, 5.2 Hz, 1.4H);
LCMS: m/z = 551.3 $(M+H)^+$, Rt = 1.526, 1.596 min;
HPLC: 100% purity (220 nm).

- Compound (3*S*,4*R*)-1-((*R*)-2-acrylamido-4-methylpent-4-enoyl)-*N*-((*S*)-1-(methylamino)-4-(methylthio)-1-oxobutan-2-yl)-4-(3-(trifluoromethyl) phenyl) pyrrolidine-3-carboxamide (**1d**): white solid;
^{1}H NMR (400 MHz, CD_3OD): δ 7.53-7.74 (m, 4H), 6.21-6.40 (m, 2H), 5.67-5.75 (m, 1H), 4.91-4.97 (m, 0.5H), 4.81-4.87 (m, 2H), 4.38-4.54 (m, 1H), 4.34 (dd, J = 9.6, 4.1 Hz, 0.5H), 4.04-4.21 (m, 1H), 3.94-4.04 (m, 1H), 3.83-3.93 (m, 0.5H), 3.71-3.82 (m, 1H), 3.42-3.70 (m, 1.5H), 3.35-3.41 (m, 0.4H), 3.19-3.31 (m, 0.6H), 2.73 (d, J = 4.0 Hz, 1.5H), 2.61-2.67 (m, 1.5H), 2.37-2.59 (m, 3.1H), 1.93-2.10 (m, 3H), 1.87-1.93 (m, 2H), 1.59-1.85 (m, 4H);
LCMS: m/z = 569.3 $(M+H)^+$, Rt = 1.434, 1.495 min;
HPLC: 99.82% purity (220 nm).

- Compound (3*S*,4*R*)-*N*-[1-benzyl-2-(methylamino)-2-oxo-ethyl]-1-[(2*R*)-4-methyl-2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carboxamide (**1e**): white solid;
^{1}H NMR (400 MHz, CD_3OD): δ 7.48-7.70 (m, 4H), 6.93-7.33 (m, 5H), 6.11-6.42 (m, 2H), 5.64-5.95 (m, 1H), 4.71-4.88 (m, 3H), 4.24-4.65 (m, 2H), 3.40-4.64 (m, 4H), 2.99-3.39 (m, 2H), 2.70-2.97 (m, 1H), 2.56-2.69 (m, 3H), 2.35-2.56 (m, 2H), 1.73-1.88 (m, 3H);
LCMS: m/z = 585.5 $(M+H)^+$, Rt = 1.591, 1.666 min;

HPLC: 99.7% purity (220 nm).

The general procedure for the synthesis of compounds 2a to 2d is showed in scheme S2.



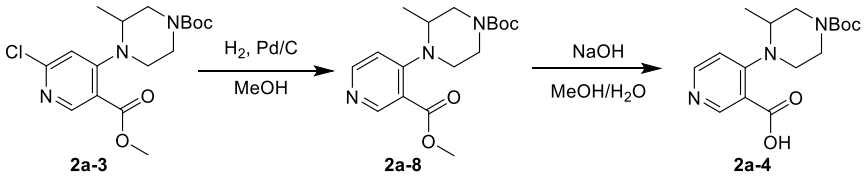
Scheme S2. General procedures for the synthesis of compounds **2d** to **2e**.

Synthesis of compound tert-butyl 4-(2-chloro-5-methoxycarbonyl-4-pyridyl)-3-methyl-piperazine-1-carboxylate (2a-3):

To a solution of methyl 4,6-dichloronicotinate (**2a-1**, 4.00 g, 19.4 mmol) in DMF (20.0 mL) was added K_2CO_3 (8.05 g, 58.3 mmol) and *tert*-butyl 3-methylpiperazine-1-carboxylate (**2a-2**, 5.83 g, 29.1 mmol). The mixture was stirred at 55 °C for 12 hrs. LCMS showed 38.5% of methyl 4,6-dichloronicotinate remained. Several new peaks were shown on LCMS and 54.5% of desired compound was detected. The reaction mixture was quenched by addition H_2O (20.0 mL) at 25 °C, and then diluted with CH_2Cl_2 (20.0 mL x 3). The combined organic layers were washed with brine (50.0 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, PE/EtOA = 1/0 to 3/1, product R_f = 0.3). Compound **2a-3** (2.44 g, 6.44 mmol, 33.2% yield) was obtained as white oil.

LCMS: m/z = 369.8 $(M+H)^+$, Rt = 0.568 min.

Synthesis of compound 4-(4-tert-butoxycarbonyl-2-methyl-piperazin-1-yl)pyridine-3-carboxylic acid (2a-4):



Step 1: To a solution of **2a-3** (2.44 g, 6.60 mmol) in MeOH (15.0 mL) and THF (5.0 mL) was added Pd/C (10%, 200 mg) under N_2 atmosphere. The suspension was degassed and purged with H_2 for 3 times. The mixture was stirred under H_2 (15 Psi) at 25°C for 3 hrs. LCMS showed **2a-3** was consumed completely. The reaction mixture was filtered through a gelite pad, and the filrate was concentrated to afford *tert*-butyl 4-(3-methoxycarbonyl-4-pyridyl)-3-methyl-piperazine-1-carboxylate (**2a-8**, 2.13 g, 5.97 mmol, 90.5% yield, 94.0% purity) as yellow oil.

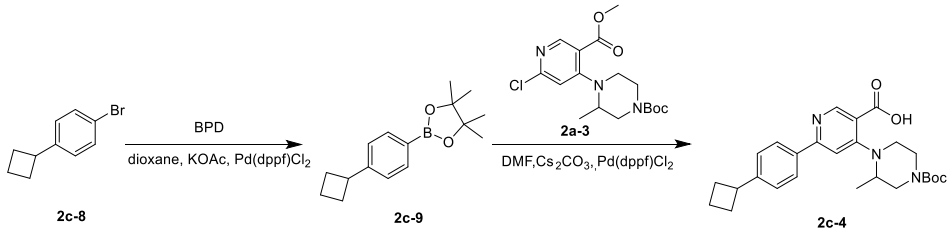
LCMS: m/z = 336.2 $(M+H)^+$, Rt = 0.435 min.

Step 2: To a solution of **2a-8** (2.13 g, 6.35 mmol) in THF (10.0 mL) and MeOH (10.0 mL) was added a solution of NaOH (635 mg, 15.9 mmol) in H_2O (5.0 mL), the mixture was stirred at 60 °C for 4 hrs. LCMS showed **2a-8** was consumed completely. The mixture was concentrated under reduced pressure to give a residue. The residue was added MeOH (20.0 mL) and was filtered through a gelite pad, and the filtrate was concentrated afford the crude. Without purification, obtained **2a-4** (1.95 g, 5.95 mmol, 93.6% yield) as a yellow solid.

^{1}H NMR (400 MHz, MeOD): δ 8.29-8.36 (m, 1H), 8.13 (d, J = 6.0 Hz, 1H), 6.83 (d, J = 6.0 Hz, 1H), 4.10 (br s, 2H), 3.81-3.90 (m, 1H), 3.25-3.40 (m, 3H), 3.03-3.22 (m, 1H), 1.44-1.53 (m, 9H), 1.11 (d, J = 6.8 Hz, 3H);

LC-MS: m/z = 322.2 $(M+H)^+$, Rt = 0.406 min.

Synthesis of compound 4-(4-(tert-butoxycarbonyl)-2-methylpiperazin-1-yl)-6-(4-cyclobutylphenyl) nicotinic acid (2c-4):



Step 1: To a solution of 1-bromo-4-cyclobutyl-benzene (**2c-8**, 50.0 mg, 237 umol) in dioxane (2.0 mL) were added BPD (72.2 mg, 284 umol), KOAc (46.5 mg, 474 umol) and $Pd(dppf)Cl_2$ (17.3 mg, 23.7 umol) under N_2 atmosphere. The mixture was stirred at 80 °C for 3 hrs. TLC (PE/EtOA = 10/1, product R_f = 0.5) showed **2c-8** was consumed completely. The reaction mixture was diluted with H_2O (20.0 mL) and extracted with EtOA 60 mL (30.0 mL x 2). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, PE/EtOA = 1/0 to 10/1). The first batch of compound 2-(4-cyclobutylphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**2c-9**, 50.0 mg, 81.8%yield) was obtained as white solid. (The second batch of **2c-9** (500 mg, 81.77% yield) was re-prepared and obtained as white solid.)

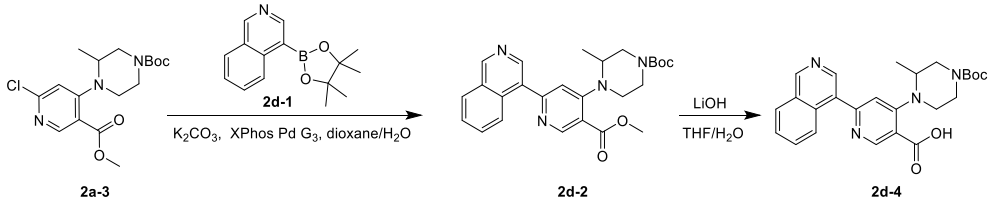
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 8.0 Hz, 2H), 7.23 (d, *J* = 7.8 Hz, 2H), 2.3-2.4 (m, 2H), 2.1-2.2 (m, 2H), 2.0-2.1 (m, 1H), 1.8-1.9 (m, 2H), 1.3-1.4 (m, 12H).

Step 2: To a solution of **2a-3** (200 mg, 541 umol) and **2c-9** (140 mg, 541 umol) in DMF (2 mL) and H_2O (0.2 mL) were added cyclopentyl (diphenyl) phosphane dichloropalladium iron (39.6 mg, 54.1 umol) and Cs_2CO_3 (529 mg, 1.62 mmol). The mixture was stirred at 100 °C for 3 hrs. TLC (PE/ EtOA = 10/1, product R_f = 0.49) indicated **2a-3** was consumed completely. The reaction mixture was diluted with H_2O 100 mL and extracted with EtOA (50.0 mL x 2). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, PE/EtOA = 1/0 to 10/1). **2c-4** (200 mg, 71.7% yield) was obtained as yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.49 (s, 1H), 7.98 (d, *J* = 8.4 Hz, 2H), 7.65 (s, 1H), 7.37 (d, *J* = 8.4 Hz, 2H), 4.18-4.23 (m, 2H), 3.57-3.71 (m, 1H), 2.82-3.37 (m, 5H), 2.41 (qt, *J* = 8.4, 2.5 Hz, 2H), 2.20 (td, *J* = 9.2, 2.6 Hz, 3H), 2.04-2.12 (m, 1H), 1.52 (s, 9H), 1.00 (d, *J* = 6.4 Hz, 3H);

LCMS: m/z = 452.2 $(M+H)^+$, Rt = 0.886 min.

Synthesis of compound 4-(4-tert-butoxycarbonyl-2-methyl-piperazin-1-yl)-6-(4-isoquinolyl) pyridine-3-carboxylic acid (2d-4):



Step 1: To a solution of *tert*-butyl 4-(2-chloro-5-methoxycarbonyl-4-pyridyl)-3-methyl-piperazine-1-carboxylate (**2a-3**, 300 mg, 811μmol) in dioxane (8.0 mL) and H_2O (2.0 mL) was added K_2CO_3 (336

mg, 2.43 mmol) and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoquinoline (**2d-1**, 310 mg, 1.22 mmol). After addition XPhos Pd G3 (137 mg, 162 μmol) and the mixture was stirred at 110 °C for 12 hrs under N_2 atmosphere. LCMS showed desired mass. TLC (CH_2Cl_2/MeOH = 10/1, product R_f = 0.4) showed new spot. The reaction mixture was partitioned between EtOA (10 mL) and H_2O (20.0 mL). The organic phase was separated, washed with brine (10 mL x 3), dried over Na_2SO_4, filtered and concentrated under reduced pressure to get *tert*-butyl 4-[2-(4-isoquinolyl)-5-methoxycarbonyl-4-pyridyl]-3-methyl-piperazine-1-carboxylate (**2d-2**, 360 mg, 778μmol) as a white solid.

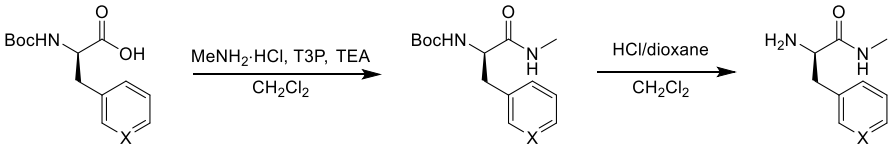
^{1}H NMR (400 MHz, CD_3OD): δ 9.32-9.35 (m, 1H), 8.76-8.79 (m, 1H), 8.54-8.57 (m, 1H), 8.19-8.25 (m, 1H), 8.03-8.08 (m, 1H), 7.83-7.87 (m, 1H), 7.78 (s, 1H), 7.24-7.28 (m, 1H), 3.96-3.98 (m, 3H), 3.84-3.92 (m, 1H), 3.34-3.50 (m, 2H), 3.14-3.29 (m, 2H), 1.52-1.95 (m, 2H), 1.47-1.49 (m, 9H), 1.20-1.20 (m, 3H);

LCMS: m/z = 463.4 $(M+H)^+$, Rt = 0.483 min.

Step 2: To a solution of **2d-2** (360 mg, 778 μmol) in THF (4.0 mL) was added NaOH (93.4 mg, 2.33 mmol) H_2O (1.0 mL) and MeOH (1.0 mL). LCMS showed **2d-2** was consumed completely. Then adjust pH = 6~7 by 1M HCl. The reaction mixture was partitioned between EtOA (50.0 mL) and H_2O (10.0 mL). The organic phase was separated, washed with aqueous NaCl (10.0 mL x 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue, to get compound **2d-4** (200 mg, 57.3% yield) as a white solid.

LCMS: m/z = 449.3 $(M+H)^+$, Rt = 0.434 min.

Synthesis of compound 2a-5 and 2d-5:



2a-9, X = C
2d-9, X = N

2a-10, X = C
2d-10, X = N

2a-5, X = C
2d-5, X = N

Step 1: To a solution of (2*R*)-2-(*tert*-butoxycarbonylamino)-3-phenyl-propanoic acid (**2a-9**) or *(2R)*-2-(tert-butoxycarbonylamino)-3-(3-pyridyl)propanoic acid (**2d-9**) in CH_2Cl_2 was added TEA (4 *equiv*), methanamine hydrochloride (2.5 *equiv*) and T3P (2.0 *equiv*, 50% purity). The mixture was stirred at 25 °C for 2 hrs. LCMS showed reactant was consumed completely. The residue was poured into water and stirred for 5 min. The aqueous phase was extracted with CH_2Cl_2 3 times. The combined organic phase was washed with brine 3 times, dried with anhydrous Na_2SO_4, filtered and concentrated in vacuum, to get compound **2a-10** (84.8% yield) or **2d-10** (81.0% yield).

- Compound *tert*-butyl *N*-[*(1R)*-1-benzyl-2-(methylamino)-2-oxo-ethyl] carbamate (**2a-10**): white

solid;

^{1}H NMR (400MHz, $CDCl_3$): δ 7.27 (s, 5H), 7.10-7.10 (m, 1H), 5.72-5.82 (m, 1H), 4.95-5.22 (m, 1H), 3.02-3.11 (m, 2H), 2.69-2.78 (m, 3H), 1.42 (s, 9H);

LCMS: m/z = 223.1 $(M+H)^+$, Rt = 0.508 min.

- Compound *tert*-butyl *N*-[(1*R*)-2-(methylamino)-2-oxo-1-(3-pyridylmethyl)ethyl]carbamate (**2d-10**): white solid;

  LCMS: m/z = 280.1 $(M+H)^+$, Rt =0.385 min.

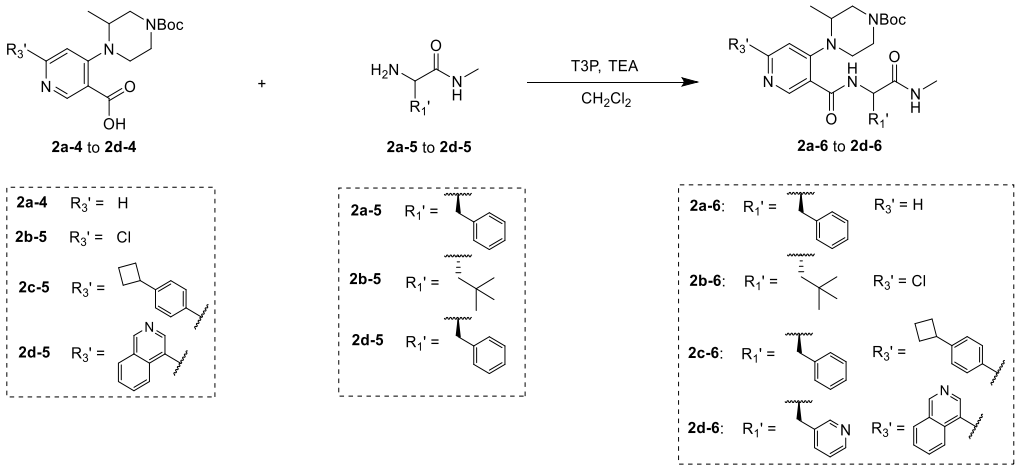
Step 2: To a mixture of **2a-10** or **2a-d** in CH_2Cl_2 was added HCl/dioxane (4 M) at 25 °C under N_2. The mixture was stirred at 25 °C for 1 hr. LCMS showed reactant was consumed completely. The residue was filtered and concentrated in vacuum, to get compound **2a-11** (91.1% yield, HCl) or **2d-11** (99.9% yield, HCl).

- Compound (2*R*)-2-amino-*N*-methyl-3-phenyl-propanamide (**2a-11**): white solid;

  LCMS: m/z = 179.2 $(M+H)^+$, Rt = 0.478 min.
- Compound (2*R*)-2-amino-*N*-methyl-3-(3-pyridyl)propanamide (**2d-11**): white solid;

  LCMS: m/z = 180.1 $(M+H)^+$, Rt =0.145 min

Synthesis of compound 2a-6 to 2d-6



To a mixture of **2a-4** (**2b-4**, **2c-4** or **2d-4**) in CH_2Cl_2 was added TEA (3.0 *equiv*) and **2a-5** (**2b-5** or **2d-5** (1.7 *equiv*) and T3P (1.5 *equiv*, 50 % purity). The mixture was stirred at 20 °C for 1 hr. TLC (CH_2Cl_2/MeOH = 10/1, product R_f = 0.4) showed **2a-4** (**2b-4**, **2c-4** or **2d-4**) was consumed completely and LCMS showed desired mass was detected. The reaction mixture was quenched by addition H_2O (15.0 mL) at 25 °C, and then diluted with CH_2Cl_2 (40.0 mL x 2) and NH_4Cl (20.0 mL). The combined

organic layers were washed with brine (30.0 mL x 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by prep-TLC (SiO_2, CH_2Cl_2/ MeOH = 10/1), to get compound **2a-6**, **2b-6**, **2c-6** or **2d-6** (15%~75% yield).

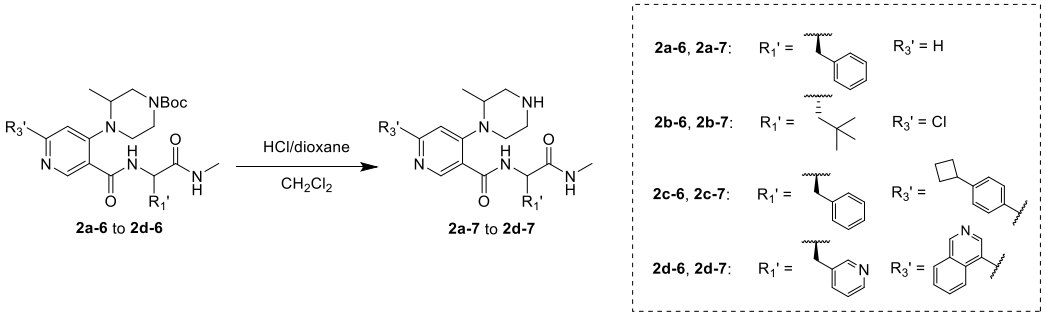
- Compound *tert*-butyl 4-[3-[[(1*R*)-1-benzyl-2-(methylamino)-2-oxo-ethyl]carbamoyl]-4-pyridyl]-3-methyl-piperazine-1-carboxylate (**2a-6**): yellow oil;
  LCMS: m/z = 482.3 $(M+H)^+$, Rt = 0.444 min

- Compound *tert*-butyl 4-[2-chloro-5-[[(1*S*)-3,3-dimethyl-1-(methylcarbamoyl) butyl]carbamoyl]-4-pyridyl]-3-methyl-piperazine-1-carboxylate (**2b-6**): white solid;
  LCMS: m/z = 496.3 $(M+H)^+$, Rt = 0.558 min.

- Compound *tert*-butyl 4-[5-[[(1*R*)-1-benzyl-2- (methylamino)-2-oxo-ethyl]carbamoyl]-2-(4-cyclobutylphenyl)-4-pyridyl]-3-methyl-piperazine-1-carboxylate (**2c-6**): colorless oil;
  LCMS: m/z = 612.5 $(M+H)^+$, Rt = 0.892 min.

- Compound *tert*-butyl 4-[2-(4-isoquinolyl)-5-[[*(*1*R*)-2-(methylamino)-2-oxo-1-(3-pyridylmethyl) ethyl]carbamoyl]-4-pyridyl]-3-methyl-piperazine-1-carboxylate (**2d-6**): yellow solid;
  LCMS: m/z = 610.3 $(M+H)^+$, Rt = 0.438 min.

Synthesis of compound 2a-7 to 2d-7



To a solution of **2a-6** (**2b-6**, **2c-6** or **2d-6**) in CH_2Cl_2 was added TFA (100 *equiv*). The mixture was stirred at 25 °C for 30 min. LCMS showed reactant was consumed completely and desired mass was detected. The reaction mixture was concentrated under reduced pressure to give a residue, to get crude product **2a-7**, **2b-7**, **2c-7** or **2d-7**.

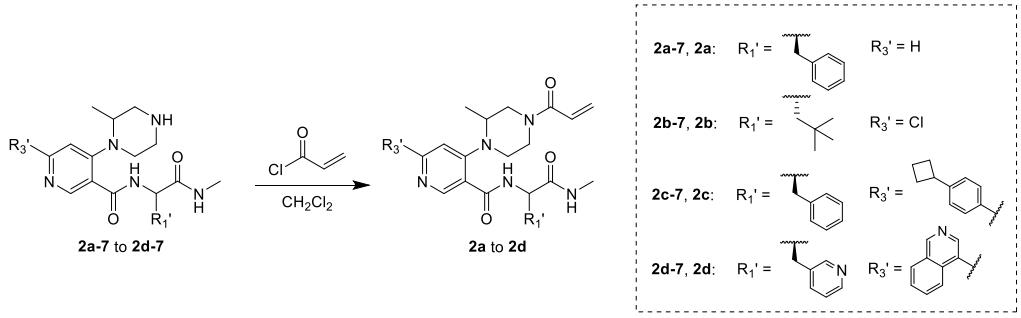
- Compound *N*-[(1*R*)-1-benzyl-2-(methylamino)-2-oxo-ethyl]-4-(2-methylpiperazin-1-yl) pyridine-3-carboxamide (**2a-7**): yellow solid;
  LCMS: m/z = 382.2 $(M+H)^+$, Rt = 0.361 min.

- Compound 6-chloro-*N*-[(1*S*)-3,3-dimethyl-1-(methylcarbamoyl)butyl]-4-(2-methylpiperazin-1-yl) pyridine-3-carboxamide (**2b-7**): yellow oil;
  LCMS: m/z = 396.1 $(M+H)^+$, Rt = 0.425 min.

- Compound The *N*-[(1*R*)-1-benzyl-2-(methylamino)-2-oxo-ethyl]-6-(4-cyclobutylphenyl)-4-(2-methylpiperazin-1-yl) pyridine-3-carboxamide (**2c-7**): white solid;
  LCMS: m/z = 512.3 $(M+H)^+$, Rt = 2.377 min.

- Compound 6-(4-isoquinolyl)-*N*-[(1*R*)-2-(methylamino)-2-oxo-1-(3-pyridylmethyl)ethyl]-4-(2-methylpiperazin-1-yl) pyridine-3-carboxamide (**2d-7**): yellow oil;
  LCMS: m/z = 510.4 $(M+H)^+$, Rt = 0.329 min.

Synthesis of compound 2a to 2d



To a solution of **2a-7** (**2b-7**, **2c-7** or **2d-7**) in CH_2Cl_2 was added TEA (1.0 *equiv*) and acryloyl chloride (1.0 *equiv*). The mixture was stirred at -78 °C for 5 min. LCMS showed reactant was consumed completely and desired mass was detected. The reaction mixture was quenched by addition H_2O at 25°C, and then diluted with CH_2Cl_2 2 times. The combined organic layers were washed with H_2O 2 times, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by prep-HPLC (column: Phenomenex Luna C18 100*30mm*3um; mobile phase: [water (0.225%FA)-ACN]; B%: 0%-40%, 8min), to get compound **2a**, **2b**, **2c** or **2d** (5%~30% yield).

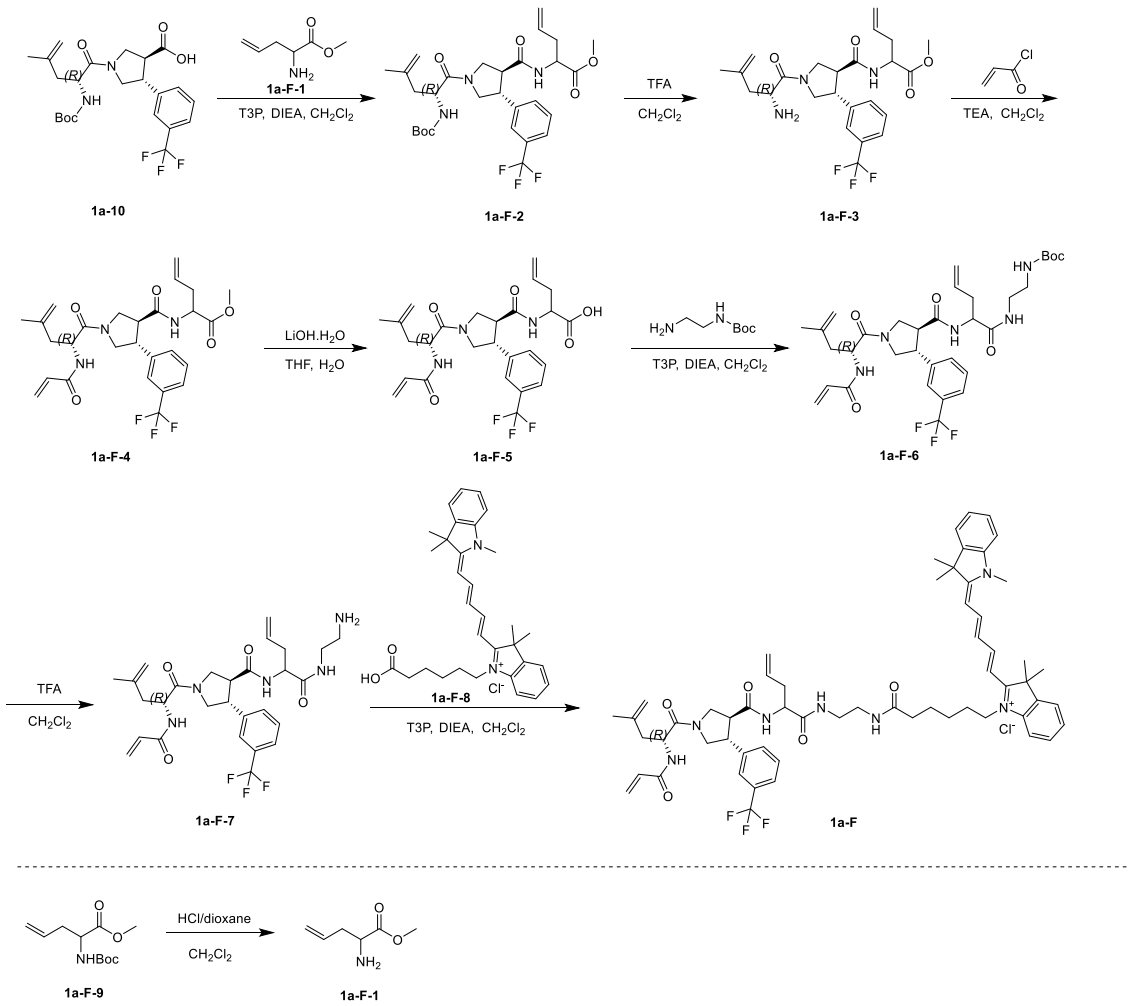
- Compound *N*-[(1*R*)-1-benzyl-2-(methylamino)-2-oxo-ethyl]-4-(2-methyl-4-prop-2-enoyl-piperazin-1-yl)pyridine-3-carboxamide (**2a**): white solid;
  ^1H NMR (400 MHz, CD_3OD): δ 8.18-8.38 (m, 2H), 7.24-7.37 (m, 5H), 7.04-7.13 (m, 1H), 6.68-6.84 (m, 1H), 6.21-6.32 (m, 1H), 5.77-5.86 (m, 1H), 4.81 (br s, 2H), 3.86-3.99 (m, 1H), 3.35-3.75 (m, 3H), 3.14-3.25 (m, 2H), 2.97-3.08 (m, 2H), 2.76 (d, *J* = 4.4 Hz, 3H), 0.91-1.01 (m, 3H);
  LC-MS: m/z = 436.4 $(M+H)^+$, Rt = 1.443 min;
  HPLC: 94.32% (220 nm).

- Compound 6-chloro-*N*-[(1*S*)-3,3–dimethyl-1-(methylcarbamoyl)butyl]-4-(2-methyl-4-prop-2-enoyl-piperazin-1-yl) pyridine-3-carboxamide (**2b**): white solid;
  ^{1}H NMR: (400 MHz, $CDCl_3$): δ 9.36-9.64 (m, 1H), 8.98 (br s, 1H), 6.89-7.12 (m, 1H), 6.49-6.68 (m, 1H), 6.28-6.46 (m, 1H), 5.88-6.10 (m, 1H), 5.70-5.85 (m, 1H), 4.48-4.71 (m, 1H), 4.15-4.42 (m, 1H), 3.49-4.00 (m, 3H), 3.09-3.41 (m, 2H), 2.72-3.03 (m, 4H), 1.84-2.02 (m, 1H), 1.61-1.67 (m, 1H), 0.86-1.09 (m, 12H);
  LC-MS: m/z = 450.4 $(M+H)^+$, Rt = 1.107 min;
  HPLC: 100% purity (220 nm).

- Compound *N*-[(1*R*)-1-benzyl-2-(methylamino)-2-oxo-ethyl]-6-(4-cyclobutylphenyl)-4-(2-methyl-4-prop-2-enoyl- piperazin-1-yl) pyridine-3-carboxamide (**2c**): white solid.
  ^{1}H NMR (400 MHz, CD_3OD): δ 8.80 (d, *J* = 4.8 Hz, 0.5H), 8.66 (d, *J* = 10.4 Hz, 0.5H), 7.91 (dd, *J* = 8.0, 5.6 Hz, 2H), 7.43-7.56 (m, 1H), 7.22-7.42 (m, 7H), 6.78 (dd, *J* = 16.8, 10.4 Hz, 1H), 6.28 (br d, *J* = 16.4 Hz, 1H), 5.82 (br dd, *J* = 10.4, 1.6 Hz, 1H), 4.79-4.87 (m, 2H), 4.62 (s, 1H), 3.50-3.82 (m, 5H), 2.99-3.23 (m, 3H), 2.77-2.79 (m, 3H), 2.35-2.45 (m, 2H), 2.16-2.25 (m, 3H), 1.87-1.97 (m, 1H), 0.89-1.01 (m, 3H);
  LCMS: m/z = 566.4 $(M+H)^+$, Rt = 2.40 min;
  HPLC: 98.20% purity (220 nm).

- Compound 6-(4-isoquinolyl)-*N*-[(1*R*)-2-(methylamino)-2-oxo-1-(3-pyridylmethyl)ethyl]-4-(2-methyl-4-prop-2-enoyl-piperazin-1-yl)pyridine-3-carboxamide (**2d**): white solid;
  ^{1}H NMR (400 MHz, CD_3OD): δ 9.27-9.39 (m, 1H), 8.57-8.72 (m, 1H), 8.52-8.57 (m, 1H), 8.39-8.52 (m, 2H), 8.18-8.25 (m, 1H), 8.00-8.11 (m, 1H), 7.79-7.87 (m, 2H), 7.72-7.79 (m, 1H), 7.40-7.49 (m, 1H), 7.30-7.39 (m, 1H), 6.68-6.86 (m, 1H), 6.17-6.32 (m, 1H), 5.75-5.82 (m, 1H), 4.80-4.87 (m, 2H), 3.87-4.06 (m, 1H), 3.67-3.86 (m, 2H), 3.33-3.67 (m, 2H), 3.21-3.28 (m, 1H), 3.10-3.21 (m, 2H), 2.72-2.82 (m, 3H), 0.93-1.09 (m, 3H);
  LC-MS: EB2938-27-P1A8, m/z = 564.4 $(M+H)^+$, Rt =1.226 min;
  HPLC: 96.04% purity (220 nm).

The general procedure for the synthesis of compounds 1a-F is showed in scheme S3.



Scheme S3. General procedures for the synthesis of **1a-F**

Synthesis of compound methyl 2-aminopent-4-enoate (1a-F-1):

To a solution of methyl 2-*(tert*-butoxycarbonylamino)pent-4-enoate (**1a-F-9**, 450 mg, 1.96 mmol) in CH_2Cl_2 (6 mL) was added HCl/dioxane (4 M, 2.0 mL) .The mixture was stirred at 25 °C for 2 hrs. TLC (CH_2Cl_2/MeOH = 10/1, product R_f = 0.2) indicated **1a-F-9** was consumed completely. The reaction mixture was concentrated under reduced pressure to give **1a-F-1** (225 mg, crude) as a yellow oil, which was used for next step directly.

^{1}HNMR (400 MHz, CD_3OD): δ 5.75-5.81 (m, 1H), 5.27-5.32 (m, 2H), 4.14-4.17 (m, 1H), 3.85 (s, 3H), 2.63-2.75 (m, 2H).

Synthesis of methyl 2-[[(3S,4R)-1-[(2R)-2-(tert-butoxycarbonylamino)-4-methyl-pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carbonyl]amino]pent-4-enoate (1a-F-2):

To a solution of **1a-10** (270 mg, 574 umol) in CH_2Cl_2 (3 mL) was added T3P (548 mg, 861 umol, 512 uL), DIEA (223 mg, 1.72 mmol, 300 uL) and ***1a-F-1*** (95.0 mg, 574 umol, HCl). The mixture was stirred at 25 °C for 4 hrs. The reaction mixture was quenched by addition H_2O (5 mL) at 25°C, and extracted with CH_2Cl_2 (10.0 mL x 3). The combined organic layers were dried over Na_2SO_4 filtered and concentrated under reduced pressure to give a residue, which was purified by column chromatography on silica gel eluted with CH_2Cl_2/MeOH (1/0 to 10/1) to give **1a-F-2** (230 mg, 66.2% yield) as a yellow solid.

LCMS: m/z = 582.4 $(M+H)^+$, Rt = 0.577 min.

Synthesis of methyl 2-[[(3S,4R)-1-[(2R)-2-amino-4-methyl-pent-4-enoyl]-4-[3-(trifluoromethyl) phenyl]pyrrolidine-3-carbonyl]amino]pent-4-enoate (1a-F-3):

To a solution of **1a-F-2** (230 mg, 395 umol) in CH_2Cl_2 (3 mL) was added TFA (1.0 mL). The mixture was stirred at 25 °C for 2 hrs. The reaction mixture was concentrated under reduced pressure to give **1a-F-3** (190 mg, crude) as a yellow oil, which was used for next step directly.

LCMS: m/z = 482.1 $(M+H)^+$, Rt = 0.817 min.

Synthesis of methyl 2-[[(3S,4R)-1-[(2R)-4-methyl-2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carbonyl]amino]pent-4-enoate (1a-F-4):

To a solution of **1a-F-3** (180 mg, 374 umol) in CH_2Cl_2 (2.0 mL) was added TEA (893 mg, 8.83 mmol, 1.23 mL). Then prop-2-enoyl chloride (33.8 mg, 374 umol, 30.5 uL) was added. The mixture was stirred at -75 °C for 0.5 hr. The reaction mixture was quenched by addition H_2O (3.0 mL) at 25 °C, and extracted with CH_2Cl_2 (5.0 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **1a-F-4** (160 mg, 79.9% yield) as a yellow oil, which was used for next step directly.

LCMS: m/z = 536.3 $(M+H)^+$, Rt = 0.545 min.

Synthesis of 2-[[(3S,4R)-1-[(2R)-4-methyl-2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carbonyl]amino]pent-4-enoic acid (1a-F-5):

To a solution of **1a-F-4** in THF (1.0 mL) and H_2O (1.0 mL) was added $LiOH.H_2O$ (18.8 mg, 448 umol). The mixture was stirred at 25 °C for 1 hr. The pH of reaction mixture was adjust to 3 by addition HCl (1 M) and extracted with EtOAc (5.0 mL x 3). The combined organic layers were dried over Na_2SO_4 filtered and concentrated under reduced pressure to give **1a-F-5** (150 mg, crude) as a yellow solid, which was used for next step directly.

LCMS: m/z = 522.1 $(M+H)^+$, Rt = 0.889 min.

Synthesis of tert-butyl N-[2-[2-[[(3S,4R)-1-[(2R)-4-methyl-2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carbonyl]amino]pent-4-enoylamino]ethyl]carbamate (1a-F-6)：

To a solution of **1a-F-5** (135 mg, 259 umol) in CH_2Cl_2 (2.0 mL) was added T3P (247 mg, 388 umol, 231 uL) and DIEA (100 mg, 776 umol, 135 uL). Then *tert*-butyl *N*-(2-aminoethyl)carbamate (62.2 mg, 388 umol, 61.0 uL) was added. The mixture was stirred at 25 ℃ for 3 hrs. The reaction mixture was quenched by addition H_2O (3.0 mL) at 25℃, and extracted with CH_2Cl_2 (5.0 mL x 3). The combined organic layers were dried over Na_2SO_4 filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography on silica gel eluted with CH_2Cl_2/MeOH (1/0 to 10/1) to give **1a-F-6** (90.0 mg, 44.5% yield) as a yellow solid.

^{1}HNMR (400 MHz, CD_3OD): δ 7.48-7.70 (m, 4H), 6.18-6.39 (m, 2H), 5.65-5.71 (m, 1H), 5.01-5.35 (m, 1H), 4.70-4.87 (m, 4H), 4.10-4.50 (m, 2H), 3.85-4.08 (m, 2H), 3.34-3.80 (m, 3H), 3.03-3.27 (m, 5H), 2.14-2.55 (m, 4H), 1.75-1.83 (m, 3H), 1.42 (s, 9H).

LCMS: m/z = 664.1 $(M+H)^+$, Rt = 0.938 min.

Synthesis of (3S,4R)-N-[1-(2-aminoethylcarbamoyl)but-3-enyl]-1-[(2R)-4-methyl-2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carboxamide (1a-F-7)：

To a solution of **1a-F-6** (80.0 mg, 121 umol) in CH_2Cl_2 (1.0 mL) was added TFA (0.3 mL). The mixture was stirred at 25 ℃ for 16 hrs. The reaction mixture was concentrated under reduced pressure to give **1a-F-7** (65.0 mg, crude) as a yellow solid, which was used for next step directly.

LCMS: m/z = 564.4 $(M+H)^+$, Rt = 0.449 min.

Synthesis of (3S,4R)-N-[1-[2-[6-[3,3-dimethyl-2-[(1E,3E,5Z)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dienyl]indol-1-ium-1-yl]hexanoylamino]ethylcarbamoyl]but-3-enyl]-1-[(2R)-4-methyl -2-(prop-2-enoylamino)pent-4-enoyl]-4-[3-(trifluoromethyl)phenyl]pyrrolidine-3-carboxamide (1a-F)：

To a solution of **1a-F-7** (60.0 mg, 106 umol) in CH_2Cl_2 (1.0 mL) was added T3P (101mg, 160 umol, 95.0 uL) and DIEA (41.3 mg, 319 umol, 55.6 uL). Then 6-[3,3-dimethyl-2-[(1*E*,3*E*,5*Z*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dienyl]indol-1-ium-1-yl]hexanoic acid (**1a-F-8**, 51.5 mg, 106 umol) was added. The mixture was stirred at 25 ℃ for 2 hrs. The reaction mixture was quenched by addition H_2O (2.0 mL) at 25℃, and extracted with EtOAc (5.0 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue, which was purified by prep-HPLC (Column: Phenomenex Gemini-NX 80 * 30 mm * 3 um. phase: [water(0.05%

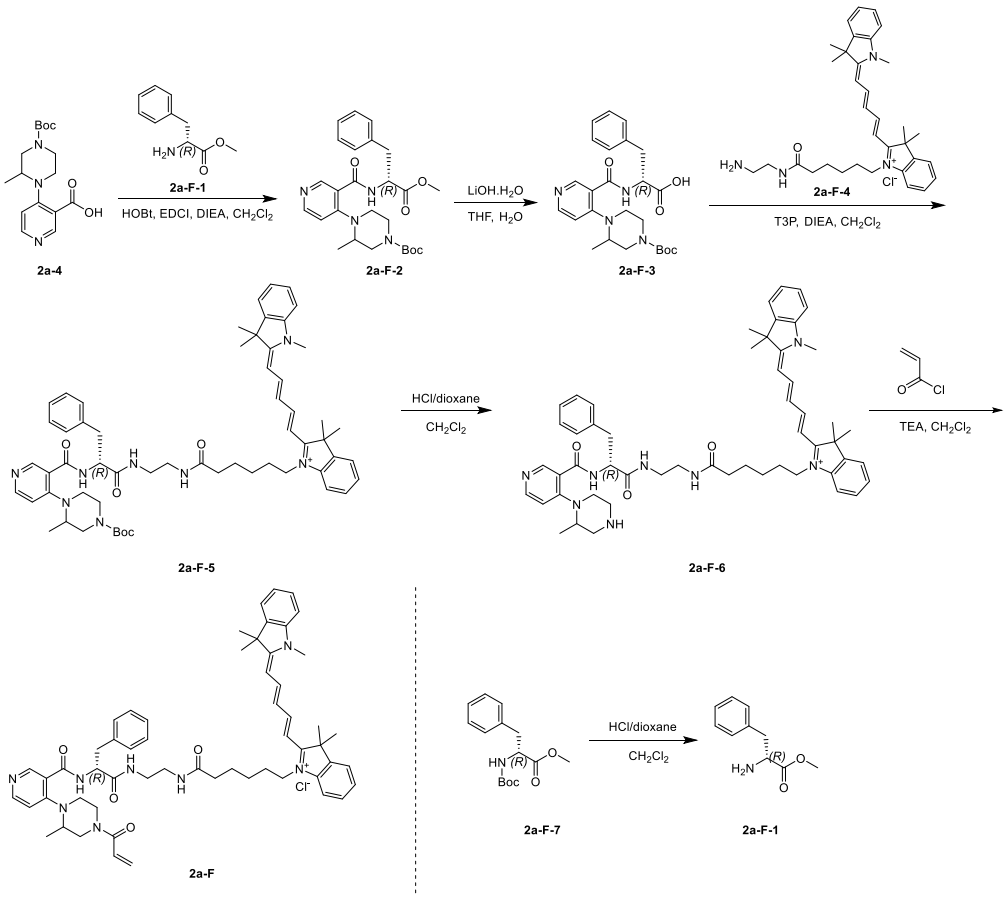
HCl)-ACN]; B%: 32%-62%, 8 min) to give **1a-F** (7.12 mg, 6.30% yield) as a blue solid.

^{1}HNMR (400 MHz, CD_3OD): δ 8.22-8.28 (m, 2H), 7.51-7.69 (m, 4H), 7.49 (d, J = 7.6 Hz, 2H), 7.37-7.45 (m, 2H), 7.22-7.33 (m, 4H), 6.62 (t, J = 12.4 Hz, 1H), 6.14-6.40 (m, 4H), 5.62-5.72 (m, 1H), 5.23-5.38 (m, 1H), 4.90-5.15 (m, 1H), 4.79-4.86 (m, 2H), 4.71-4.77 (m, 1H), 4.01-4.54 (m, 5H), 3.64-4.00 (m, 3H), 3.62 (s, 3H), 3.32-3.60 (m, 2H), 3.16-3.29 (m, 4H), 2.37-2.59 (m, 2H), 2.18-2.22 (m, 4H), 1.80-1.86 (m, 2H), 1.70-1.80 (m, 15H), 1.63-1.70 (m, 2H), 1.40-1.54 (m, 2H);

LCMS: m/z = 515.3 $(M+H)^+$, Rt = 2.091 min;

HPLC: 97.56% purity (220 nm).

The general procedure for the synthesis of compounds 2a-F is showed in scheme S4.



Scheme S4. General procedures for the synthesis of **2a-F**

Synthesis of compound (2R)-2-amino-3-phenyl-propanoate (2a-F-1):

Referenced to synthesis procedure of **1a-F-1**, to give **2a-F-1** (1.56 g, 91.2% yield) as a white solid.

^{1}H NMR (400 MHz, CD_3OD): δ 7.30-7.40 (m, 3H), 7.26-7.27 (m, 2H), 4.31-4.35 (m, 1H), 3.80 (s, 3H), 3.24-3.30 (m, 1H), 3.16-3.21 (m, 1H);

LCMS: m/z = 180.1 $(M+H)^+$, Rt = 0.366 min.

Synthesis of compound tert-butyl 4-[3-[[(1R)-1-benzyl-2-methoxy-2-oxo-ethyl]carbamoyl]-4-pyridyl]-3-methyl-piperazine-1-carboxylate (2a-F-2):

Referenced to synthesis procedure of **1a-F-2**, to give **2a-F-2** (249 mg, 53.7% yield) as a yellow oil.

LCMS: m/z = 483.7 $(M+H)^+$, Rt = 0.489 min.

Synthesis of compound (2R)-2-[[4-(4-tert-butoxycarbonyl-2-methyl-piperazin-1-yl)pyridine-3-carbonyl]amino]-3-phenyl-propanoic acid (2a-F-3):.

To a mixture of **2a-F-2** (130 mg, 269 umol) in THF (2.0 mL) and H_2O (2.0 mL) was added $LiOH.H_2O$ (33.9 mg, 808 umol) at 25 °C and stirred for 16 hrs. The reaction mixture was adjust pH to 5 with HCl (1 M, 3.0 mL), then concentrated in vacuum to give a residue. The residue was purified by prep-HPLC (Phenomenex Gemini-NX 80 * 30 mm * 3 um; mobile phase: [water (0.05% FA)-ACN]; B%: 15%-47%, 8 min) to give **2a-F-3** (90.5 mg, 71.4% yield) as a white solid.

^1H NMR(400 MHz, CD_3OD): δ 8.24-8.38 (m, 2H), 7.26-7.31 (m, 4H), 7.24-7.25 (m, 1H), 7.10-7.23 (m, 1H), 4.78-4.81 (m, 1H), 3.67-3.84 (m, 2H), 3.39-3.54 (m, 2H), 2.99-3.20 (m, 5H), 1.49 (s, 9H), 0.94-1.02 (m, 3H);

LCMS: m/z = 469.2 $(M+H)^+$, Rt = 0.473 min.

Synthesis of compound tert-butyl 4-[3-[[(1R)-1-benzyl-2-[2-[6-[3,3-dimethyl-2-[(1E,3E,5Z)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dienyl]indol-1-ium-1-yl]hexanoylamino] ethylamino]-2-oxo-ethyl]carbamoyl]-4-pyridyl]-3-methyl-piperazine-1-carboxylate (2a-F-5):

To a solution of **2a-F-3** (43.1 mg, 91.9 umol) and **2a-F-4** (50.0 mg, 83.5 umol) in CH_2Cl_2 (0.5 mL) was added DIEA (54.0 mg, 418 umol, 72.7 uL) and T3P (79.7 mg, 125 umol, 74.5 uL). The blue mixture was stirred at 25 °C for 16 hrs. TLC (CH_2Cl_2/MeOH = 10/1, product R_f = 0.3, UV) showed the reaction was completed. The reaction mixture was quenched by addition of H_2O (10.0 mL) and extracted with CH_2Cl_2 (10.0 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue as a blue oil. The residue was purified by column chromatography on silica gel eluted with CH_2Cl_2/MeOH (1/0 to 9/1) to give **2a-F-5** (34.0 mg, 37.9% yield) as a blue oil.

LCMS: m/ z = 975.3 $(M+H)^+$, Rt = 0.898 min.

Synthesis of N-[(1R)-1-benzyl-2-[2-[6-[3,3-dimethyl-2-[(1E,3E,5Z)-5-(1,3,3-trimethylindolin-2-

ylidene)penta-1,3-dienyl]indol-1-ium-1-yl]hexanoylamino]ethylamino]-2-oxo-ethyl]-4-(2-methylpiperazin-1-yl)pyridine-3-carboxamide (2a-F-6):

To a solution of **2a-F-5** (30.0 mg, 30.7 umol) in CH_2Cl_2 (0.5 mL) was added HCl/dioxane (4 M, 0.1 mL). The blue mixture was stirred at 25 °C for 0.5 hr. The reaction mixture was quenched by addition of $NaHCO_3$ (10.0 mL) at 25 °C and extracted with CH_2Cl_2 (10.0 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **2a-F-6** (26.0 mg, 96.6% yield) as blue oil.

LCMS: m/ z = 875.5 $(M+H)^+$, Rt = 0.811 min.

Synthesis of N-[(1R)-1-benzyl-2-[2-[6-[3,3-dimethyl-2-[(1E,3E,5Z)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dienyl]indol-1-ium-1-yl]hexanoylamino]ethylamino]-2-oxo-ethyl]-4-(2-methyl-4-prop-2-enoyl-piperazin-1-yl)pyridine-3-carboxamide (2a-F):

To a solution of **2a-F-6** in CH_2Cl_2 (0.5 mL) was added TEA (15.9 mg, 157 umol, 21.9 uL) and prop-2-enoyl chloride (7.13 mg, 78.8 umol, 6.42 uL). The blue mixture was stirred at -78 °C for 0.5 hr. The reaction mixture was quenched by addition of H_2O (10.0 mL) and extracted with CH_2Cl_2 (10.0 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue as a blue oil. The residue was purified by prep-HPLC (Phenomenex Gemini-NX 80 * 30mm * 3um; mobile phase: [water (0.05% HCl)-ACN]; B%: 17%-47%, 8 min) to give **2a-F** (6.10 mg, 24.5% yield) as a blue solid.

^{1}H NMR (400 MHz, CD_3OD): δ 8.11-8.31 (m, 4H), 7.49 (d, J = 7.6 Hz, 2H), 7.26-7.43 (m, 13H), 6.61-6.77 (m, 2H), 6.25-6.30 (m, 2H), 5.81-5.86 (m, 1H), 4.72-4.77 (m, 1H), 4.44-4.48 (m, 1H), 4.20-4.21 (m, 2H), 4.08 (t, J = 7.2 Hz, 2H), 3.68-3.89 (m, 2H), 3.63 (s, 3H), 3.34-3.38 (m, 2H), 3.24-3.29 (m, 4H), 2.92-3.04 (m, 2H), 2.23 (t, J = 6.8 Hz, 2H), 1.78-1.84 (m, 2H), 1.71-1.72 (m, 12H), 1.65-1.68 (m, 2H), 1.44-1.50 (m, 2H), 1.15-1.18 (m, 3H);

LCMS: m/z = 929.8 $(M+H)^+$, Rt = 1.62 min;

HPLC: 97.90% purity (220 nm).

QC results for off-DNA compounds

QC results for compound 1a:

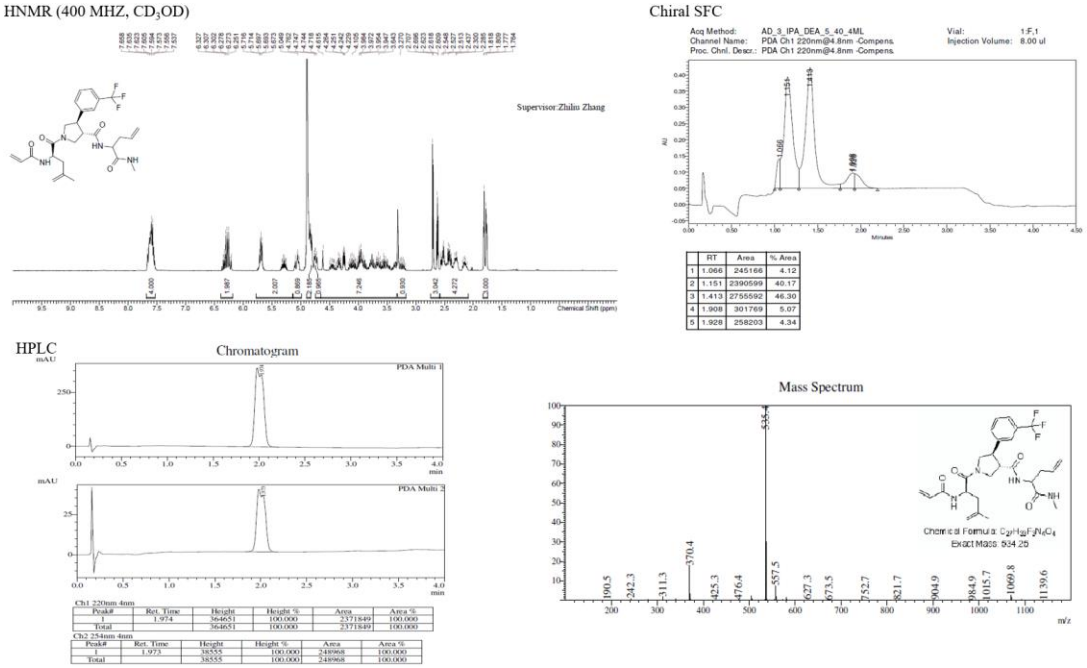


Figure S3. HNMR, HPLC, MS and Chiral SFC results of compound **1a**.

QC results for compound 1b:

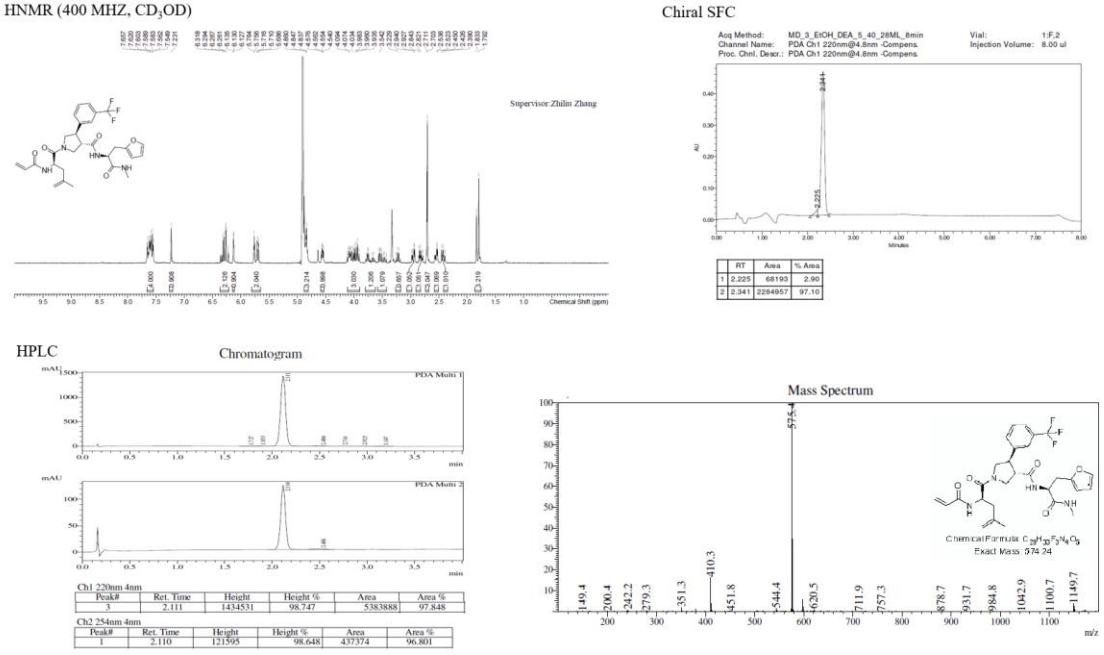


Figure S4. HNMR, HPLC, MS and Chiral SFC results of compound **1b**.

QC results for compound 1c:

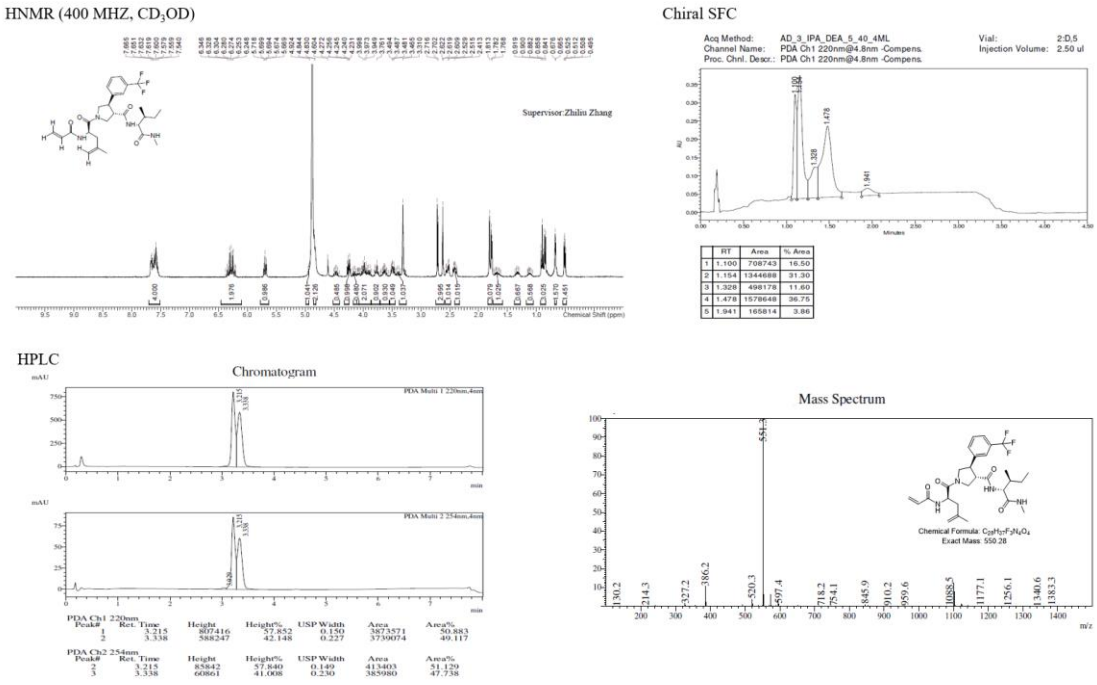


Figure S5. HNMR, HPLC, MS and Chiral SFC results of compound **1c**.

QC results for compound 1d:

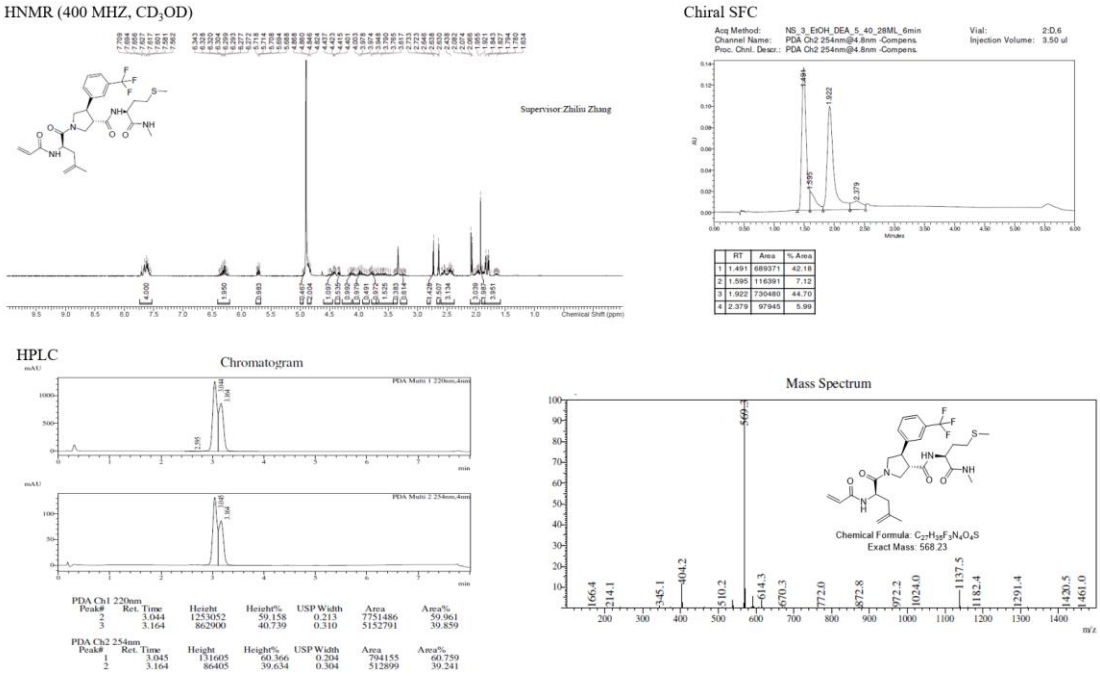


Figure S6. HNMR, HPLC, MS and Chiral SFC results of compound **1d**.

QC results for compound 1e:

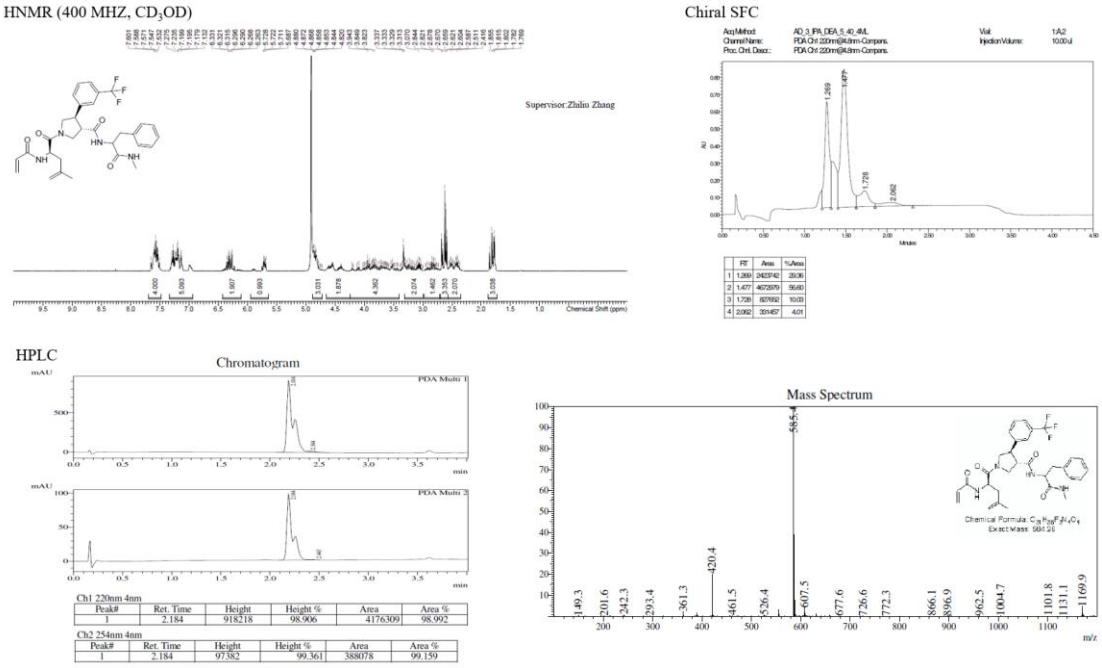


Figure S7. HNMR, HPLC, MS and Chiral SFC results of compound **1e**.

QC results for compound 2a:

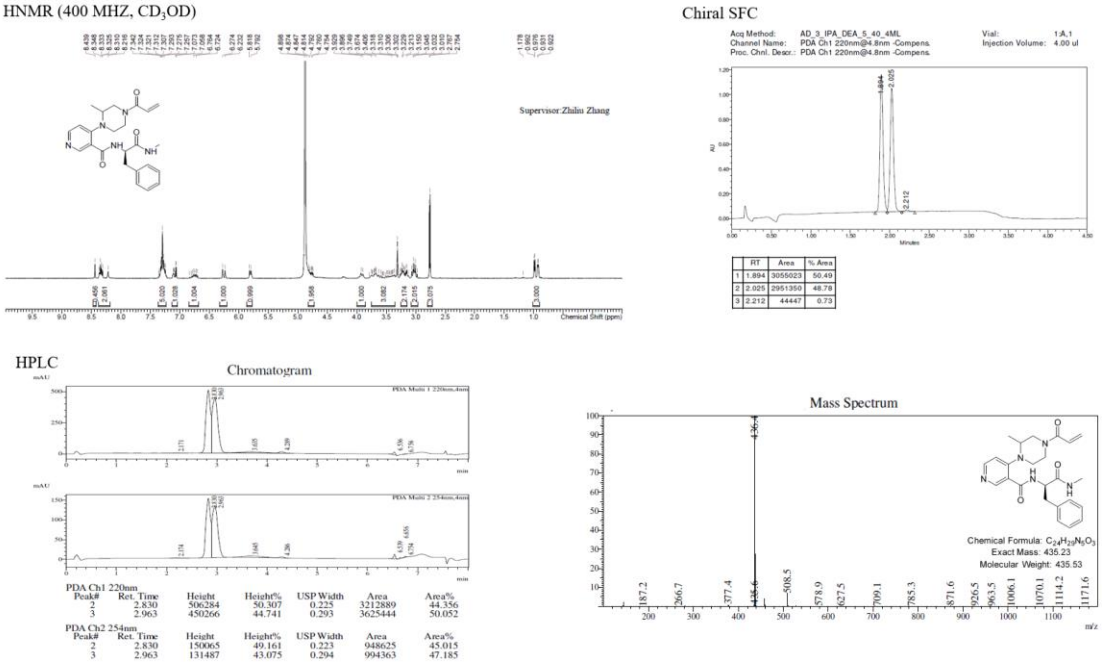


Figure S8. HNMR, HPLC, MS and Chiral SFC results of compound **2a**.

QC results for compound 2b:

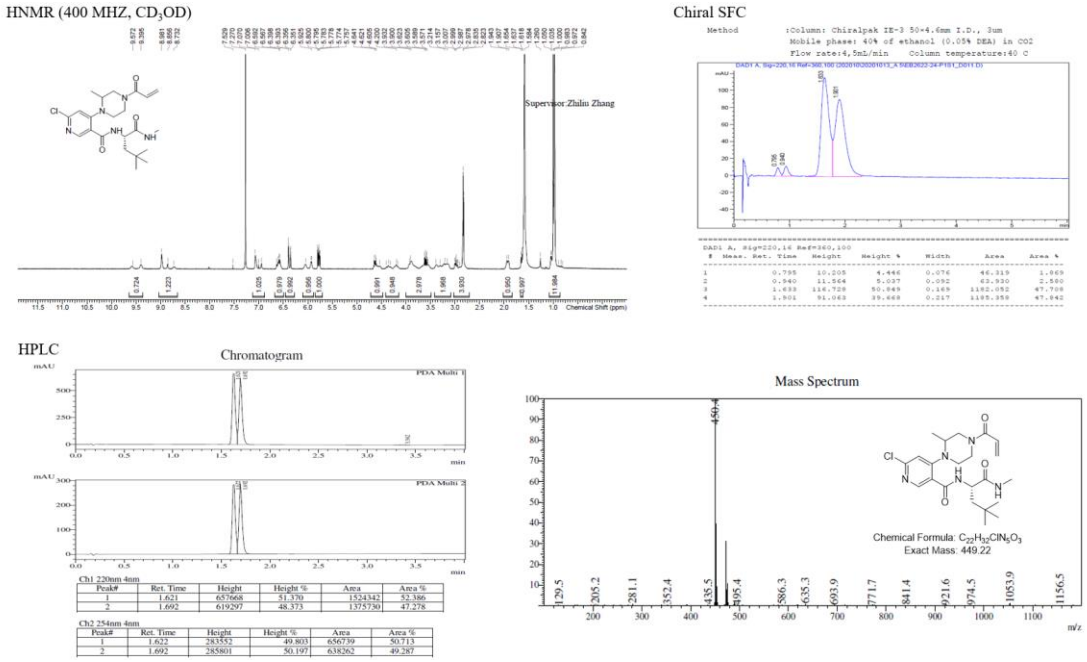


Figure S9. HNMR, HPLC, MS and Chiral SFC results of compound **2b**.

QC results for compound 2c:

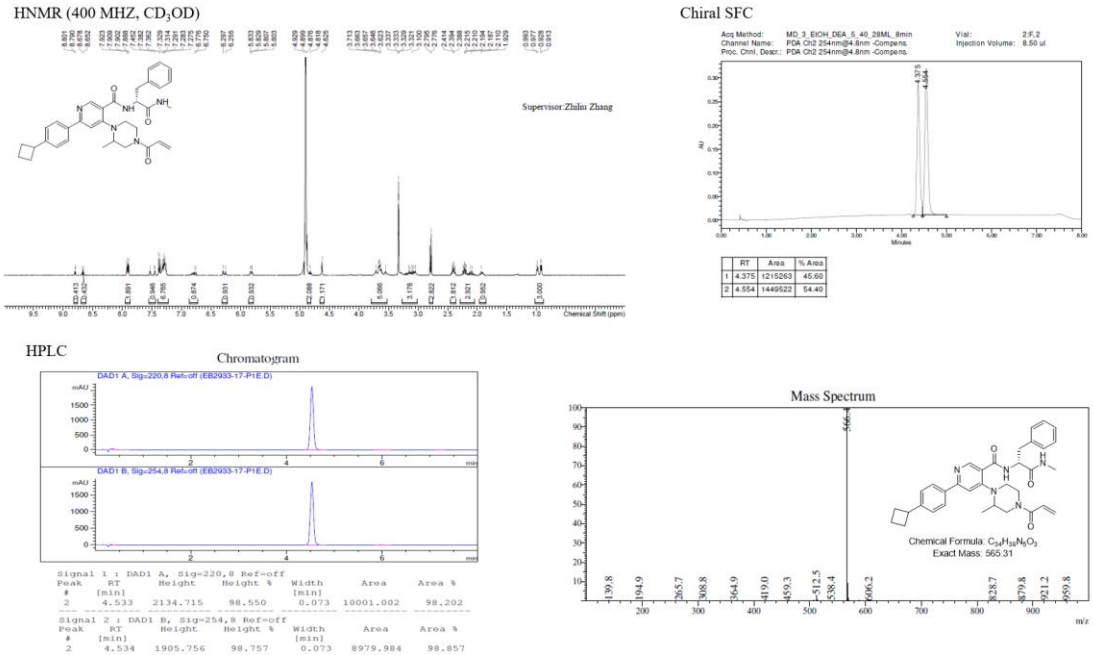


Figure S10. HNMR, HPLC, MS and Chiral SFC results of compound **2c**.

QC results for compound 2d:

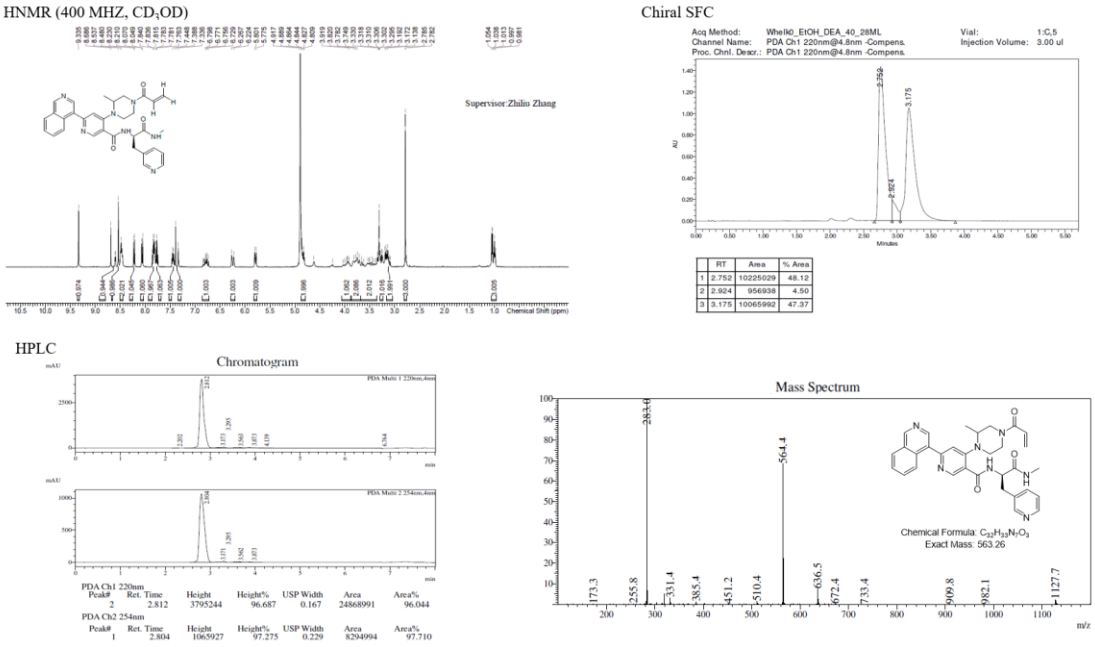


Figure S11. HNMR, HPLC, MS and Chiral SFC results of compound **2d**.

QC results for compound 1a-F:

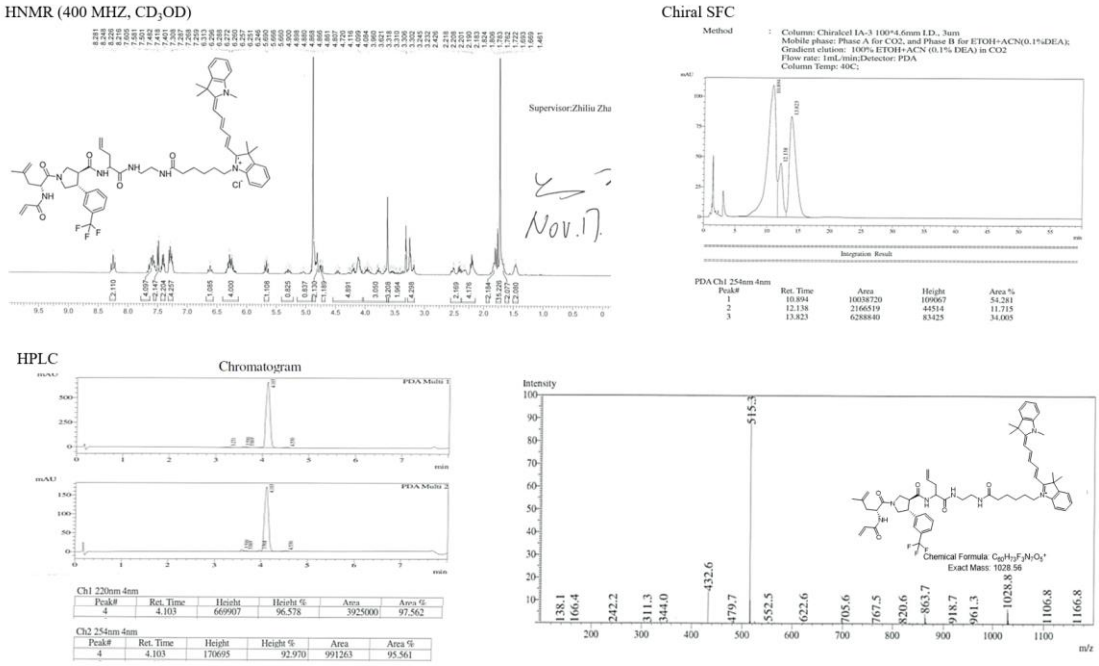


Figure S12. HNMR, HPLC, MS and Chiral SFC results of compound **1a-F**.

QC results for compound 2a-F:

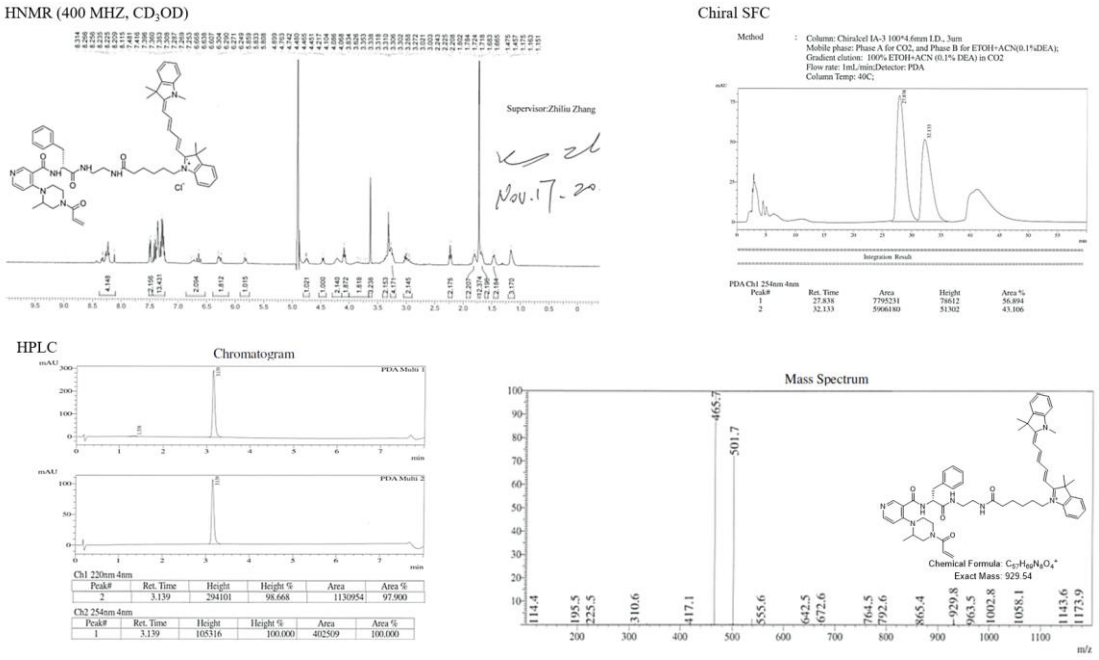


Figure S13. HNMR, HPLC, MS and Chiral SFC results of compound **2a-F**.

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