Supporting Information

The discovery of macrocyclic XIAP antagonists from a DNA-programmed chemistry library, and their optimization to give lead compounds with *in vivo* antitumor activity

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X-ray Co-crystal Structures. BIR2 (156-231) was mixed with a 10-fold molar excess of compounds **5, 7** and **8** (1 mM final concentration). Crystals were grown at 20 °C by vapor diffusion in the presence of 0.98M Ammonium Sulfate and 0.1M Ammonium Formate. They would nucleate within 1-3 days after being streak seeded. Crystals were cryoprotected in a solution consisting of 50% mother liquor plus 50% glycerol. The crystals diffracted to: compound **5** 1.97 Å, compound **7** 2.09 Å and compound **8** 2.02 Å. The coordinates have been deposited with PDB; deposition numbers 4WVT (compound **5**), 4WVS (compound **7**), and 4WVU (compound **8**).

XIAP-BIR3 SMAC Peptide Fluorescence Polarization Assay (FPA). Assays were performed in black, flat-bottom, 384-well plates. The final assay volume was 50 μL prepared from additions of N-His-Tb-BIR3(241-356, XIAP), fluoresceinated modified SMAC peptide (prepared by the method described in: Nikolovska-Coleska. Z.; Wang, R.; Fang, X.; Pan, H.; Tomita, Y.; L,i P.; Roller, P. P.; Krajewski, K.; Saito, N. G.; Stuckey, J. A.; Wang, S. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal. Biochem.* **2004**, *332,* 261-273) and test compounds in assay buffer consisting of 20 mM Sodium Phosphate, 1 mM EDTA, 50 mM NaCl, and 0.05% Pluronic F68. The reaction was incubated at room temperature for 60 minutes and fluorescence polarization of the reaction was detected on the LJL Analyst Plate Reader (LJL Biosystems). Inhibition data were calculated from mP values generated by the no protein control reactions for 100% inhibition and vehicle-only reactions for 0% inhibition. The final concentration of reagents in the assay was 130 nM N-His-Tb-BIR3(241-356, XIAP), 1.4 nM fluoresceinated modified SMAC peptide, and 1% DMSO. Dose response curves were generated to determine the concentration required for inhibiting 50% of polarization activity (IC_{50}). Compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) and evaluated at eleven concentrations. IC_{50} values were derived by non-linear regression analysis.

All the following assays performed by the same FPA method as above

cIAP-1 His-BIR3 SMAC Peptide Fluorescence Polarization Assay (FPA). Construct was N-His-Tb-BIR3(262-352); final protein concentration in assay was 36.1 nM; peptide tracer - same source and concentration as XIAP-BIR3 Smac assay.

cIAP-1 His-BIR2-3 SMAC Peptide Fluorescence Polarization Assay (FPA). Construct was N-His-Tb-BIR2-3(154-352); final protein concentration in assay was 5.9 nM; tracer was a proprietary fluoresceinated dimeric SMAC peptide at 3.0 nM final concentration.

XIAP His-BIR2-3 SMAC Peptide Fluorescence Polarization Assay (FPA). Construct was N-His-Tb-BIR2-3(125-356)-C202A-C213G; final protein concentration in assay was 8.7 nM; tracer was a proprietary fluoresceinated dimeric SMAC peptide at 4.5 nM final concentration.

XIAP-BIR2 SMAC Peptide AlphaScreen Assay. Assays were performed in white, flat-bottom, 384-well ProxiPlates (Perkin Elmer). The final assay volume was 10 μL prepared from additions of His-BIR2 (124-240/C202A/C213G), Biotinylated SMAC peptide (sequence AVPIAQK biotinylated at the *C*-terminus), and test compounds in assay buffer consisting of 25 mM Hepes, 100 mM NaCl, 0.1% BSA, and 5 mM $CaCl_2$. The reaction was incubated at room temperature for 60 minutes. After 60 minutes, 2.5 μL of Alphascreen detection reagent (Perkin Elmer) was added to the reaction mixture and incubated at room temperature in the dark for 120 minutes. The Alphascreen signal generated by the reaction was detected on the Envision Plate Reader. Inhibition data were calculated from an Alphascreen signal generated by the no protein control reactions for 100% inhibition and vehicle-only reactions for 0% inhibition. The final concentration of reagents in the assay was 50 nM His-BIR2 (124-240/C202A/C213G), 50 nM Biotinylated SMAC peptide, 4 μg/mL Alphascreen detection reagents, and 0.5% DMSO. Dose response curves were generated to determine the concentration required for inhibiting 50% of the activity (IC_{50}). Compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) and evaluated at eleven concentrations. IC_{50} values were derived by non-linear regression analysis.

Caspase-3 Rescue Assay. THP-1 cells (ATCC catalog #TIB202) were used as a source of caspase-3 for the assay. Briefly cells were harvested, washed two times with PBS (Life Technologies catalog #14190-144) and disrupted with 5 volumes of ice cold lysis buffer LB according to previously described protocol.[29] Following 20 minute incubation on ice the cells were subjected to a single freeze thaw cycle using liquid nitrogen. Cell debris were pelleted by centrifugation (Beckman GS-6KR) at 1000 x G, 5 min, 4 ^{o}C. The lysates (100 μl aliquots) were transferred to new chilled tubes, frozen in liquid nitrogen and stored at -80 ^{o}C until needed. On the day of the assay the lysates were activated by making 1 mM ATP (Sigma catalog #A7699) & 5 μM cytochrome C (Calbiochem cat #250600) and incubated for 3 hrs at 30 ^{o}C. Assays were performed in black, flat-clear bottom, 96-well Optilux assay plates (BD Falcon catalog #353948). The final assay volume was 100 μL prepared from additions of N-His-Tb-BIR2-3(124-356)-C202A-C213G protein, XIAP, fluoresceinated caspase-3 substrate (Peptides International catalog # PI3171), and test compounds in assay buffer consisting of 30 mM Hepes pH 7.5**,** 0**.**1 M NaCl**,** 5% Sucrose**,** 0**.**1% CHAPS, 20 mM beta-mercaptoethanol. The reaction was started by adding caspase-3 substrate, incubating at 37 ^{o}C and determining the reaction rate by measuring fluorescence every 3 minutes (90 minutes total) using a FluoroScan Ascent (Labsystems) set at 390ex/485em wavelengths. Inhibition data were calculated from average reaction rate values generated by using a proprietary XIAP BIR2-3 antagonist at 20 μM for 100% inhibition and vehicle-only reactions for 0% inhibition. The final concentration of reagents in the assay were 80 nM N-His-Tb-BIR2-3(124-356)-C202A-C213G), XIAP), 100 μM caspase-3 substrate and 3% DMSO. Dose response curves were generated to determine the concentration required for rescuing 50% of caspase-3 reaction rate (IC_{50}). Compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) and evaluated at eight concentrations. IC_{50} values were derived by non-linear regression analysis.

Cellular Proliferation Assay. Tumor cells were grown in 96 well plates (Falcon catalog #353936) in RPMI (Life Technologies catalog #11875-085) containing 10% FBS (Summit catalog #FP-200-05) at a density that maintains logrithemic growth over 96 hrs and incubated overnight in a humidified atmosphere at 37 ºC, 5% CO_2. Next day 20 µl of MTS (Promega catalog numbers G109X and G110X) working reagent was added to a row of wells containing cells and incubate for 3 hrs at 37 ºC. The optical density, determined by reading in a spectramax 250 platereader (Molecular Devices) at 490 nm wavelength, was used as time = 0. To a second plate compound titrated in DMSO was added to wells containing 2 ng/ml rhTNF (R&D Systems catalog #210-TA). Following an additional 72 hrs incubation the optical density was again determined at 490 nm wavelength. The optical density at time 0 was subtracted from all values obtained from treated wells. The percent inhibition was determined by the following formula; %Inhibition = $(1\text{-}OD_{avg\ treated}/OD_{avg\ untreated})x100\%$. Compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) and evaluated at eight concentrations. IC_{50} values were derived by non-linear regression analysis.

Pharmacokinetic Parameters Obtained in Mice. A single dose of compounds **17-19** (1 mg/kg for **17** & **18** and 0.2 mg/kg for **19**) in 100% phosphate buffer solution were administered by tail vein injection (iv bolus, 5 mL/kg) to fed male Balb/C mice (*n = 3 per compound*). Blood samples (~0.2 mL) were obtained by retro-orbital bleeding at 0.05, 0.25, 0.5, 1, 3, 5 and 7 hours post dose. Within each group, three mice were used to bleed at two to three time points, resulting in a composite PK profile. Blood samples were allowed to clot on ice and then centrifuged to collect serum. Serum samples were stored at -20 °C until analysis by LC-MS/MS.

***In Vivo* Antitumor Activity in the Subcutaneously Implanted A875 and MDA-MB-231 Xenograft Models in Nude Mice.** Female Balb/C athymic (nu+/nu+) mice, 6–8 weeks old, were obtained from Sprague-Dawley Co. (Indianapolis, IN). Animals were provided with food and water *ad libitum* and housed five per cage. Mice were maintained in accordance with Bristol-Myers Squibb's Institutional Animal Care and Use Committee in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) guidelines for the humane treatment and care of laboratory mice. A875 or MDA-MB-231 tumor fragments maintained by serial passage *in vivo* were implanted subcutaneously in the hind flank using an 18 g trocar. Approximately two weeks post implant, when tumor sizes reached 100–150 mm^3, intravenous dosing was initiated using gavage needles with either compound at the indicated concentrations or vehicle (7.5% 1M NaCl, 12% hydroxypropyl beta-cyclodextrin 80.5% water) in the control group. Tumor growth was assessed twice weekly by vernier caliper measurement. Group sizes were *n* = 8 or 9. Treatments resulting in greater than 20% lethality and/or 20% body weight loss were considered toxic. Antitumor activity was determined by calculating the maximum percent tumor growth inhibition (TGI) of treated animals at the indicated time points using the formula:

$\%TGI = \{(C_t\text{-}T_t)/(C_t\text{-}C_0)\} \times 100$, where C_t = the median tumor volume (mm^3) of vehicle treated control (C) mice at time t. T_t = median tumor volume of treated (T) mice at time t. C_0 is the

median tumor volume of control mice at time 0. Activity is defined as a continuous %TGI >50% for at least one tumor volume doubling time after the start of drug treatment.

NMR Analysis of Key Macrocycles

Compound 5: ^{1}H NMR Major conformer ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.75 (1H, bs s), 8.88 (1H, d, *J* = 7.6 Hz), 8.8 (2H, br s), 8.12 (1H, d, *J* = 7.6 Hz), 8.04 (1H, s), 7.84 (1H, d, *J* = 7.6 Hz), 728-7.00 (9H, m), 6.83 (2H, d, *J* = 8.8 Hz), 6.63 (2H, d, *J* = 8.4 Hz), 5.13 (1H, d, *J* = 4 Hz), 4.62-4.23 (6H, m), 3.92-3.57 (5H, m), 3.42-3.35 (1H, m), 3.2-2.5 (6H, m), 2.39 (3H, t, *J* = 4.8 Hz), 2.33-2.13 (2H, m), 1.93-1.56 (3H, m), 1.3 (3H, dd, *J* = 6.8 Hz).

Minor conformer ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.75 (1H, bs s), 8.8 (2H, br s), 8.74 (1H, d, *J* = 6.8 Hz), 8.28 (1H, d, *J* = 8.4 Hz), 8.2 (1H, s), 8.02 (1H, d, *J* = 8.4 Hz), 7.30-6.98 (11H, m), 6.66 (2H, d, *J* = 8.8 Hz), 5.13 (1H, d, *J* = 4 Hz), 4.62-4.23 (6H, m), 3.92-3.57 (5H, m), 3.42-3.35 (1H, m), 3.2-2.5 (6H, m), 2.44 (3H, t, *J* = 5.2 Hz), 2.33-2.13 (2H, m), 1.93-1.56 (3H, m), 1.3 (3H, dd, *J* = 6.8 Hz).

Compound 8: ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.76 (1H, br s), 9.65 (1H, s), 8.78 (2H, br s), 8.73 (1H, d, *J* = 8.4 Hz), 8.45 (1H, d, *J* = 9.2 Hz), 8.22 (1H, d, *J* = 8.4 Hz), 7.77 (1H, s), 7.27-7.17 (7H, n), 7.11 (2H, d, *J* = 8.4 Hz), 6.52 (1H, s), 5.15-2.06 (1H, m), 4.63-4.56 (3H, m), 4.49-4.36 (4H, m), 3.88 (2H, d, *J* = 5.2 Hz), 3.81 (1H, t, *J* = 10.0 Hz), 3.02 (1H, dd, *J* = 11.6 Hz), 2.98 (2H, dd, *J* = 3.2, 6.8 Hz), 2.88 (1H, dd, *J* = 6.0, 14.0 Hz), 2.81 (1H, dd, *J* = 7.2, 14.4 Hz), 2.74-2.63 (2H, m), 2.08 (1H, dd, *J* = 6.8, 13.2 Hz), 1.37 (1H, dd, *J* = 9.6, 11.2 Hz), 1.34 (3H, d, *J* = 6.8 Hz), 0.95 (3H, d, *J* = 6.8 Hz), 0.91 (3H, d, *J* = 6.8 Hz).

Compound 10: ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.85 (1H, br s), 8.76 (2H, br s), 8.66 (1H, d, *J* = 8.0 Hz), 8.55 (1H, d, *J* = 9.2 Hz), 8.28 (1H, s), 8.22 (1H, d, *J* = 8.8 Hz), 7.33 (2H, d, *J* = 7.2 Hz), 7.25 (2H, t, *J* = 7.6 Hz), 7.19 (1H, t, *J* = 7.2 Hz), 7.17 (2H, d, *J* = 8.4 Hz), 6.82 (2H, d, *J* = 8.8 Hz), 5.54-5.50 (1H, m), 5.33 (1H, d, *J* = 14.0 Hz), 5.16 (1H, d, *J* = 14.4 Hz), 5.59 (1H, dt, *J* = 2.8, 10.4 Hz), 4.47 (1H, dd, *J* = 3.2, 10.8 Hz), 4.32-4.25 (4H, m), 3.89 (1H, dd, *J* = 3.2 Hz), 3.84 (1H, dd, *J* = 6.0, 12.4 Hz), 3.01-2.63 (6H, m), 1.96-1.87 (1H, m), 1.53 (1H, d, *J* = 15.2 Hz), 1.31 (3H, d, *J* = 7.2 Hz), 0.89 (3H, d, *J* = 6.8 Hz), 0.86 (3H, d, *J* = 6.0 Hz).

Compound 16: ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.87 (2H, br s), 8.80 (4H, br s), 8.71 (2H, d, *J* = 8.0 Hz), 8.48 (2H, d, *J* = 8.0 Hz), 8.39 (2H, s), 8.22 (2H, d, *J* = 8.0 Hz), 7.86-7.83 (8H, m), 7.76 (2H, d, *J* = 8.4 Hz), 7.48-7.47 (2H, m), 7.46 (2H, d, *J* = 3.2 Hz), 7.44 (2H, d, *J* = 3.6 Hz), 7.15 (4H, d, *J* = 8.4 Hz), 6.8 (4H, d, *J* = 8.4 Hz), 5.34-5.19 (2H, m), 4.82 (4H, dd, *J* = 12.0, 14.8 Hz), 4.72 (2H, dd, *J* = 7.6, 14.0 Hz), 4.59-4.51 (4H, m), 4.47 (2H, t, *J* = 7.6 Hz), 4.43-4.38 (2H, m), 3.9 (2H, dd, *J* = 6.0, 12.4 Hz), 3.8 (2H, dd, *J* = 9.6, 15.2 Hz), 3.62-3.58 (2H, m), 3.19 (2H, dd, *J* = 5.6, 14.0 Hz), 3.04-3.01 (4H, m), 2.82-2.77 (4H, m), 2.25 (2H, dd, *J* = 10.0, 21.2 Hz), 2.06 (2H, ddd, *J* = 6.8, 13.6, 20.0 Hz), 1.36 (6H, d, *J* = 6.8 Hz), 0.94 (6H, d, *J* = 6.8 Hz), 0.9 (6H, d, *J* = 6.8 Hz).

Compound 17: **^{1}H NMR** (C_2D_6SO, 400 MHz) δ 12.86 (2H, br s), 8.78 (4H, br s), 8.69 (2H, d, *J* = 7.6 Hz), 8.47 (2H, d, *J* = 8.0 Hz), 8.417 (2H, s), 8.24 (2H, d, *J* = 8.0 Hz), 7.87-7.82 (6H, m), 7.77 (2H, d, *J* = 8.8 Hz), 7.50-7.44 (6H, m), 7.16 (4H, d, *J* = 8.4 Hz), 6.8 (4H, d, *J* = 8.4 Hz), 5.35-5.26 (2H, m), 8.85 (1H, d, *J* = 12.0 Hz), 4.8 (1H, d, *J* = 11.6 Hz), 4.56-4.52 (4H, m), 4.46-4.37 (4H, m), 3.87 (2H, dd, *J* = 5.6 Hz), 3.79 (2H, t, *J* = 5.2 Hz), 3.51-3.32 (12H, m), 3.18 (2H, dd, *J* = 3.2, 15.2 Hz), 3.02 (4H, dd, *J* = 4.8, 12.4 Hz), 2.79 (4H, dd, *J* = 10.0, 12.8 Hz), 1.88-1.56 (10H, m), 1.35 (6H, d, *J* = 7.2 Hz), 1.18-0.97 (10H, m).

Compound 18: **^{1}H NMR** (C_2D_6SO, 400 MHz) δ 12.88 (2H, br s), 8.80 (4H, br s), 8.58 (2H, d, *J* = 8.0 Hz), 8.50 (2H, d, *J* = 8.0 Hz), 8.40 (2H, s), 8.21 (2H, d, *J* = 9.2 Hz), 7.87-7.82 (6H, m), 7.75 (2H, d, *J* = 8.4 Hz), 7.47-7.43 (6H, m), 7.16 (4H, d, *J* = 8.8 Hz), 6.81 (2H, d, *J* = 8.4 Hz), 5.25-5.16 (2H, m), 4.85 (2H, d, *J* = 11.6 Hz), 4.81 (2H, d, *J* = 11.6 Hz), 4.75 (2H, dd, *J* = 6.4, 13.2 Hz), 4.58-4.37 (10H, m), 4.47-4.37 (4H, m), 3.94 (2H, q, *J* = 6.0 Hz), 3.78 (2H, t, *J* = 5.6 Hz), 3.22-3.17 (2H, m), 3.06-3.03 (4H, m), 2.82-2.76 (4H, m), 2.32-2.21 (2H, m), 2.88 (6H, d, *J* = 6.8 Hz), 1.01 (18H, s).

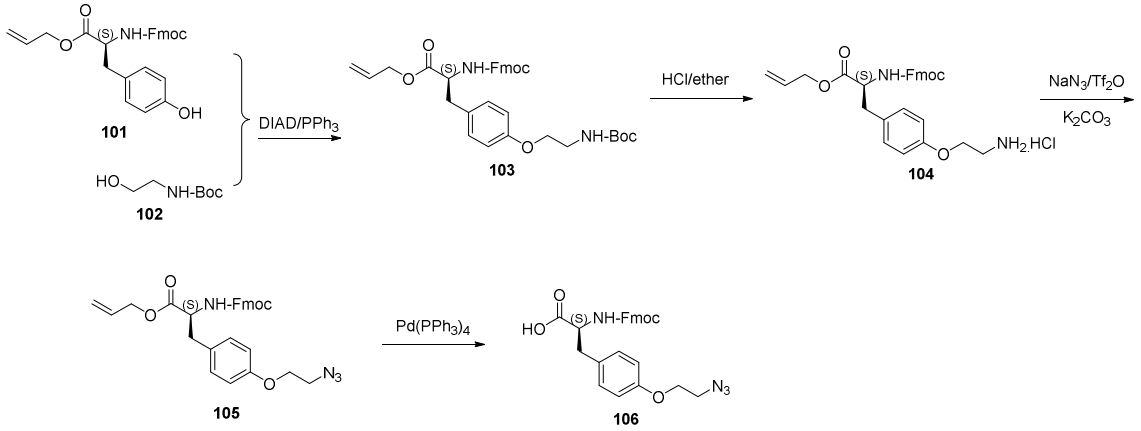
Compound 19: **^{1}H NMR** (C_2D_6SO, 400 MHz) δ 12.96 (2H, br s), 8.77 (4H, br s), 8.65 (2H, d, *J* = 7.6 Hz), 8.34 (2H, d, *J* = 7.6 Hz), 7.99 (2H, br s), 7.83-7.65 (5H, m), 7.65 (2H, d, *J* = 8.0 Hz), 7.44-7.39 (5H, m), 5.22-5.12 (2H, m), 4.72-1.49 (8H, m), 4.38 (2H, dd, *J* = 7.6, 8.0 Hz), 3.86 (2H, dd, *J* = 5.6, 12.0 Hz), 3.76 (2H, br s), 3.25-3.05 (22H, m), 1.34 (6H, d, *J* = 6.8 Hz), 0.81 (12H, dd, *J* = 6.8 Hz).

Mass Spectrometry Data:

Compound #	Molecular Formula	Expected m/z	Observed m/z
1	$C_{34}H_{42}N_8O_7$	675.3249	675.3265
2	$C_{40}H_{53}N_9O_8$	788.4095	788.7100
3	$C_{35}H_{51}N_9O_7$	710.3984	710.3991
4	$C_{35}H_{52}N_{10}O_7$	725.4093	725.4095
5	$C_{42}H_{50}N_8O_8$	795.3824	795.3740
6	$C_{41}H_{48}N_8O_8$	781.3668	781.3669
8	$C_{37}H_{47}N_9O_7$	730.3671	730.3673
9	$C_{37}H_{47}N_9O_7$	730.3671	730.3674
10	$C_{35}H_{44}N_8O_7$	689.3411	689.45
11	$C_{42}H_{50}N_8O_7$	779.3875	779.3875
12	$C_{40}H_{48}N_8O_7$	753.3719	753.3720
13	$C_{74}H_{94}N_{18}O_{14}$	730.3677	730.39*
14	$C_{70}H_{88}N_{16}O_{14}$	1377.6739	1377.6758
15	$C_{78}H_{92}N_{16}O_{14}$	1477.7052	1477.7062
16	$C_{78}H_{92}N_{16}O_{14}$	1477.7052	1477.7076
17	$C_{84}H_{100}N_{16}O_{14}$	1557.7678	1557.7696
18	$C_{80}H_{96}N_{16}O_{14}$	1505.7365	1505.7387
19	$C_{64}H_{80}N_{16}O_{12}$	1265.6214	1265.61
* Compounds were observed in the second charge state.			

Preparation of Intermediate Building Blocks:

Preparation of 106:



Compound 104: DIAD (9.3 g, 37 mmol) was added dropwise into the reaction mixture of compound **101** (11 g, 24 mmol), compound **102** (6 g, 37 mmol) and PPh_3 (9.6 g, 37 mmol) in THF (150 mL) at 60 ℃. Then the reaction mixture was stirred at 60 ℃ for additional 30 minutes. The mixture was cooled to ambient temperature, and then 2 N HCl/ether (100 mL) was added to the reaction mixture and stirred through overnight to form a precipitate. The solid was washed with ethyl acetate to give compound **104**, which was taken onto the next step without further purification (13 g, 68%).

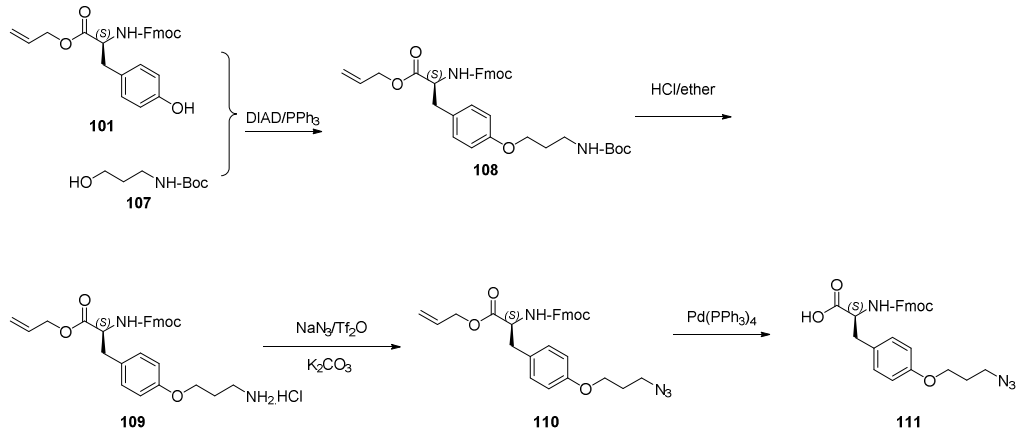
Compound 105: Tf_2O (11 g, 41 mmol) was added dropwise into a reaction mixture of NaN_3 (3.0 g, 42 mmol) in acetonitrile (40 mL) at 0 ℃. The reaction mixture was stirred at 0 ℃ for additional 30 minutes. The solid was washed with acetonitrile (20 mL), and the solution was used directly in the next step.

TfN_3 in acetonitrile (60 ml) was added dropwise into a reaction mixture of compound **104** (13 g, 24 mmol) and K_2CO_3 (7.0 g, 49 mmol) in acetonitrile (70 mL) and water (70 mL) at 0 ℃. Then the reaction mixture was stirred at 0 ℃ for additional hour. The mixture was extracted with ethyl acetate and water. The organic layer was separated and dried with Na_2SO_4. The solvent was removed to give a crude product which was purified with a silica gel column to give compound **105** (8 g, 65%).

Compound 106: A solution of compound **105** (8.0 g, 15 mmol), $Pd(PPh_3)_4$ (0.1 g), and morpholine (2.4 g, 30 mmol) in CH_2Cl_2 (50 mL) was stirred at ambient temperature for 2 hours. The solvent was removed to give a crude product which was dissolved in a solution of K_2CO_3 (2 g) in water (50 mL). The aqueous solution was extracted with diethyl ether (200 ml). The water phase was acidified with 1N HCl and extracted with ethyl acetate. The organic layer was separated and dried with Na_2SO_4. The crude product was further precipitated by ether to give the compound **106** (4.4 g, 60%). ^{1}H NMR (400 MHz, C_2D_6SO) δ 8.85 (s, 1H), 7.88 (d, J = 7.6 Hz, 2H), 7.65 (t, J = 7.2 Hz, 2H), 7.41 (dt, J = 7.2, 2.0 Hz, 2H), 7.34-7.27 (m, 2H), 7.20 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 7.6 Hz, 2H), 4.21-4.01 (m, 6H), 3.61 (t, J = 4.4 Hz, 2H), 3.05-3.01 (m, 1H),

2.82 (dd, J = 13.2, 10.4 Hz, 1H); MS (m/z): $[M+Na]^+$ calcd. for $C_{26}H_{24}N_4NaO_5$, 495.1644; found, 495.2.

Preparation of compound 111:



Compound 109: DIAD (6.8 g, 27 mmol) was added dropwise into the reaction mixture of compound **101** (8.0 g, 18 mmol), compound **107** (4.7 g, 27 mmol) and PPh_3 (7.0 g, 27 mmol) in THF (150 mL) at 60 ℃. Then the reaction mixture was stirred at 60 ℃ for additional 30 minutes. The mixture was cooled to ambient temperature, then 2 N HCl/ether (100 ml) was added and the reaction mixture was stirred overnight. The resulting precipitate was filtered and washed with ethyl acetate to give compound **109**, which was taken onto the next step without further purification (5.4 g, 57%).

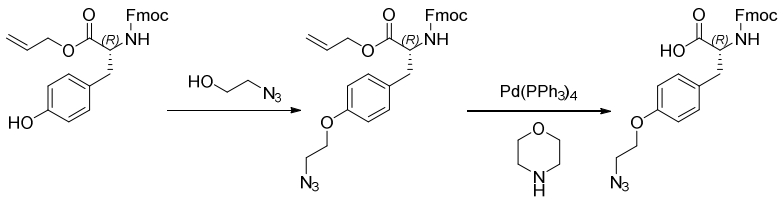
Compound 110: Tf_2O (5.9 g, 21 mmol) was added dropwise into the solution of NaN_3 (1.4 g, 21 mmol) in acetonitrile (20 mL) at 0 ℃. Then the reaction mixture was stirred at 0 ℃ for additional 30 minutes. The solid was filtered and washed with acetonitrile (10 ml), and the resulting solution was used directly in the next step.

TfN_3 in acetonitrile (30 ml) was added dropwise into a mixture of compound **109** (5.4 g, 11 mmol) and K_2CO_3 (3.0 g, 21 mmol) in acetonitrile (50 mL) and water (50 mL) at 0 ℃. Then the reaction mixture was stirred at 0 ℃ for additional 1 hour. The mixture was extracted with water and ethyl acetate. The organic layer was separated and dried over Na_2SO_4. The solvent was removed to give a crude product which was purified by silica gel chromatography to yield **110** (4 g, 75%).

Compound 111: A solution of compound **110** (4.0 g, 7.6 mol), $Pd(PPh_3)_4$ (0.1 g), and morpholine (1.3 g, 15 mmol) in CH_2Cl_2 (50 mL) was stirred at ambient temperature for 2 hours. The solvent was removed to give a residue which was dissolved in a solution K_2CO_3 (2 g) in water (50 mL) and the impurity was extracted away by ether (about 200 mL). The water phase was acidified with 1 N HCl and extracted with ethyl acetate. The organic layer was separated and dried with Na_2SO_4. The crude product was further precipitated by the addition of ether to yield **111** (3.1 g 81%). ^1H NMR (400 MHz ,C_2D_6SO) δ 12.73 (br s, 1H), 7.87 (d, J = 7.6 Hz, 2H), 7.67 (d, J = 8.4 Hz, 1H), 7.63 (t, J = 7.6 Hz, 2H), 7.4 (dt, J = 7.6, 2.0 Hz, 2H), 7.32-7.25 (m, 2H), 7.17

(d, J = 8.8 Hz, 2H), 6.81 (d, J = 8.4 Hz, 2H), 4.19-4.09 (m, 4H), 3.95 (t, J = 6.0 Hz, 2H), 3.46 (t, J = 6.4, 2H); MS (*m/z*): $[M+Na]^+$ calcd. for $C_{27}H_{26}N_4NaO_5$, 509.1801; found, 509.2.

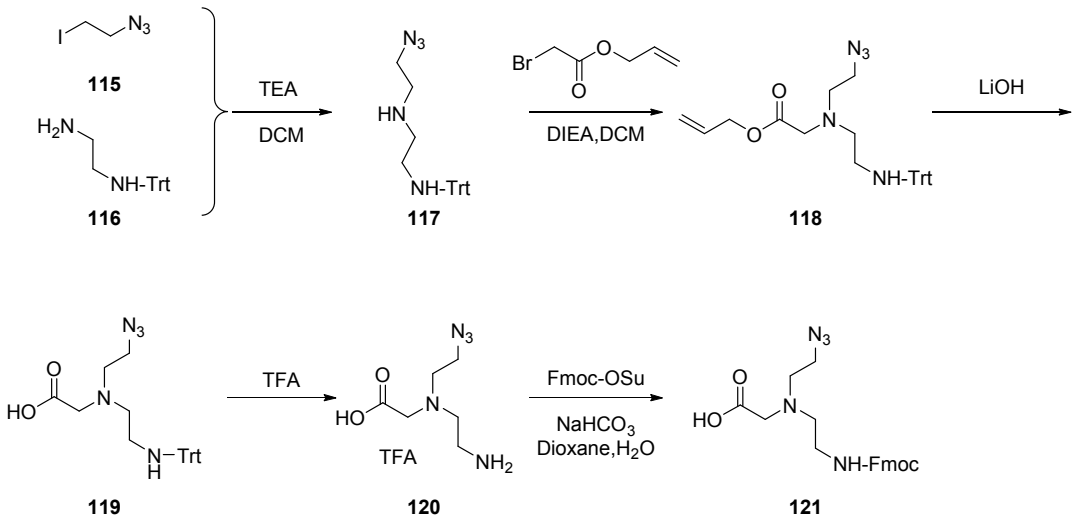
Preparation of (R)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(4-(2-azidoethoxy)phenyl)propanoic acid. (Compound 114)



Compound 113: To a solution of **112** (26.0 g, 58.7 mmol), PPh_3 (23 g, 88 mmol) and 2-azidoethanol (10.2 g, 117 mmol) in THF (200 mL) was added DIAD (23.7 g, 117 mmol) at 60 ^oC. The mixture was stirred at 60 ^oC for additional 30 minutes. The solvent was removed to give a crude product which was purified by a silica gel column (eluting with 10-30% ethyl acetate/petroleum ether) to provide **113** (15 g, yield 50 %). ^1H NMR (300 MHz, $CDCl_3$) δ 7.8 (d, 3H), 7.7-7.6 (d, 2H), 7.5-7.3 (m, 4H), 7.1-7.0 (m, 3H), 6.9-6.7 (m, 2H), 6.0-5.8 (m, 1H), 5.4-5.2 (m, 2H), 4.6 (m, 2H), 4.5-4.1 (m, 4H), 3.5-3.4 (m, 1H), 3.2-2.9 (m, 2H), 2.6 (s, 1H).

Compound 114: To a solution of **113** (15 g, 29 mmol) and morpholine (2.4 g, 28 mmol) in CH_2Cl_2 (60 mL) was added $Pd(PPh_3)_4$ (0.1 g). The mixture was stirred for 2 hours. The organic layers were concentrated, extracted with ethyl acetate and washed with 1 N HCl (aq), saturated NaCl, dried over Na_2SO_4, and filtered. The filtrate was concentrated and purified by a silica gel column (eluting with 20-50% ethyl acetate/petroleum ether) to provide **114** (7.1 g, 51%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.9 (d , 2H), 7.8-7.6 (m, 3H), 7.5 (m, 2H), 7.1-7.0 (m, 3H),7.4 (m, 2H), 7.3 (m, 2H), 7.0-6.8 (m, 2H), 4.3-4.1 (m, 6H), 3.7-3.6 (m, 2H), 3.1-3.0 (m, 1H), 2.9-2.8 (m, 1H); MS (*m/z*): $[M+Na]^+$ calcd. for $C_{26}H_{24}N_4NaO_5$, 495.1644; found, 495.2.

Preparation of 2-((2-(((9H-fluoren-9-yl)methoxy)carbonylamino)ethyl)(2-azidoethyl)amino)acetic acid. (Compound 121)



Procedure:

Compound 117: To a solution of **116** (10 g, 33 mmol) and in CH_2Cl_2 (100 mL) was added triethyl amine (7 mL, 50 mmol), then a solution of **115** (10 g, 50 mmol) in CH_2Cl_2 (10 mL) was added dropwise at 0 ℃. The reaction mixture was stirred at ambient temperature overnight. The residue was treated with water and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated to give **117** (10 g, 82 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.2-2.3 (t, 2H), 2.7-2.8 (m, 4H), 3.3-3.4 (t, 2H), 7.1-7.2 (m, 3H), 7.2-7.3 (t, 6H), 7.5-7.6 (d, 6H).

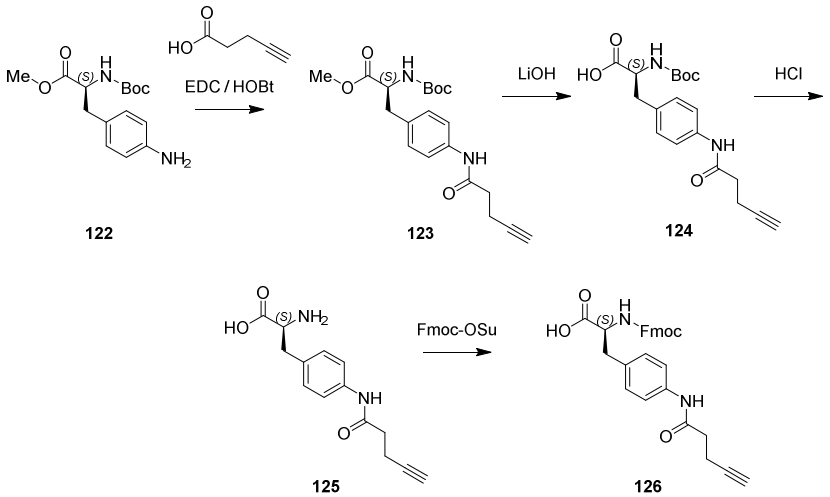
Compound 118: To a solution of **117** (10. g, 27 mmol) in CH_2Cl_2 (100 mL) was added DIPEA (6.6 mL, 40. mmol), then allyl 2-bromoacetate (5.8 g, 32 mmol) was added dropwise at 0 ℃. The reaction mixture was stirred at ambient temperature overnight. The residue was treated with water and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated to give a crude oil, which was purified by column (Al_2O_3) to afford **118** (9.0 g, 71 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.2-2.3 (t, 2H), 2.7-2.9 (m, 4H), 3.2 (t, 2H), 3.3 (s, 2H), 4.5-4.6 (d, 2H), 5.2-5.4 (m, 2H), 5.9-6.0 (m, 1H), 7.1-7.2 (m, 3H), 7.2-7.3 (t, 6H), 7.5-7.6 (d, 6H).

Compound 119: To a solution of **118** (9.0 g, 19 mmol) in THF/H_2O (100 mL, 1:1) was added LiOH (2.3 g, 57 mmol). The reaction mixture was stirred at ambient temperature for 4 hours. TLC indicated reaction completion. The residue was treated with water and extracted with ethyl acetate. The aqueous layer was adjusted to pH 5 with 1 N HCl solution. The precipitate was filtered washed with petroleum ether and dried *in vacuo* to afford **119** (6.0 g, 73 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.3-2.4 (t, 2H), 2.6-2.7 (t, 2H), 2.8-2.9 (t, 2H), 3.1-3.2 (s, 2H), 3.3-3.4 (t, 2H), 7.1-7.2 (m, 3H), 7.2-7.3 (t, 6H), 7.5-7.6 (d, 6H).

Compound 120: To a solution of **119** (6 g, 14 mmol) in CH_2Cl_2 (40 mL) was added TFA (20 mL) dropwise at 0 ℃ . The reaction mixture was stirred at ambient temperture overnight. TLC indicated reaction completion. The residue was concentrated to afford **120** (2.9 g, 69%).

Compound 121: To a solution of **120** (2.9 g, 9.6 mmol) in dioxane/H_2O (50 mL, 1:1) was added $NaHCO_3$ (2.0 g, 24 mmol), then Fmoc-OSu (3.9 g, 11 mmol) was added portion wise at 0 ℃. The reaction mixture was stirred at ambient temperature overnight. TLC indicated reaction completion. The residue was treated with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated to give a crude product, which was purified by recrystallization to afford **121** (2.5 g, 64 %). ^{1}H NMR (C_2D_6SO, 400 MHz) δ 12.17 (br s, 1H), 7.89 (d, *J* = 7.2 Hz, 2H), 7.68 (d, *J* = 7.2 Hz, 2H), 7.42 (t, *J* = 7.2 Hz, 2H), 7.33 (t, *J* = 7.6 Hz, 2H), 7.14 (br s, 1H), 4.29 (s, *J* = 6.4 Hz, 2H), 4.21 (t, *J* = 6.4 Hz, 1H), 3.39 (s, 2H), 3.30 (t, *J* = 5.6 Hz, 2H), 3.07 (d, *J* = 5.6 Hz, 2H), 2.85 (t, *J* = 5.6 Hz, 2H), 2.71 (t, *J* = 6.4 Hz, 2H). ^{13}C NMR (125 MHz, C_2D_6SO) δ 172.7, 156.1, 143.9, 140.7, 128.9, 127.6, 127.3, 127.0, 125.1, 121.3, 120.1, 120.0, 65.3, 54.3, 53.3, 53.0, 48.8, 46.7); MS (m/z): $[M]^+$ calcd. for $C_{21}H_{24}N_5O_4$, 410.1828; found, 410.2.

Preparation of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(4-pent-4-ynamidophenyl)propanoic acid. (126)



Compound 123: To a solution of **122** (20.5 g, 69.7 mmol), pent-4-ynoic acid (13.7 g, 139.2 mmol) and hydroxybenzotriazole (11.3 g, 83.6 mmol) in CH_2Cl_2 (150 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (16 g, 84 mmol) at ambient temperature. The reaction mixture was stirred at ambient temperature overnight. The mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated and purified by a silica gel column (eluting with 10-40% ethyl acetate / petroleum ether) to provide **123** (15.6 g, yield 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5-7.4 (d, 2H), 7.2-7.0 (d, 2H), 5.0-4.9 (m, 1H), 4.6 (m, 1H), 3.8-3.7 (s, 3H), 3.2-3.0 (m, 2H), 2.7-2.6 (m, 4H), 2.1-2.0 (m, 1H), 1.5-1.4 (s, 9H).

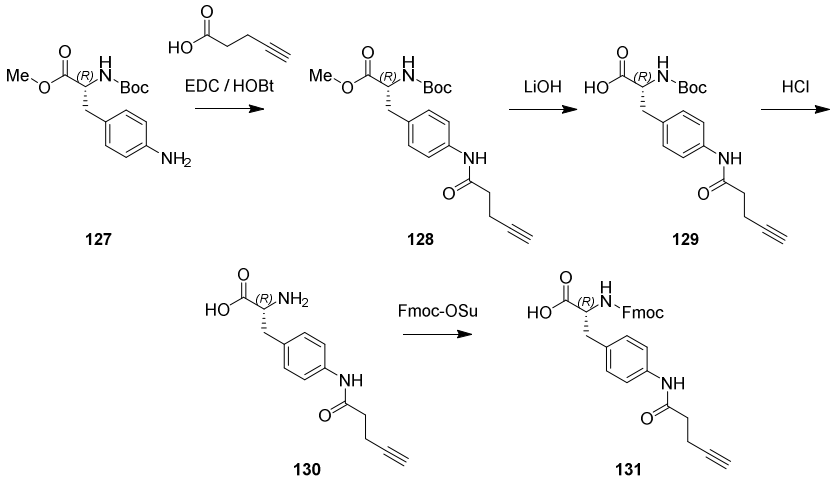
Compound 124: To a solution of **123** (15.6 g, 41.7 mmol) in THF (50 mL) and H_2O (50 mL)

was added LiOH (3.5 g, 83 mmol). The mixture was stirred at ambient temperature overnight. The solvent was removed *in vacuo*, and the resulting residue was extracted with Et_2O. At an ice bath, a 2 N aqueous solution of HCl was added to adjust the pH of the reaction mixture to 5. The mixture was then extracted with ethyl acetate, and the combined organic layers were wash with brine, dried and concentrated to give **124** (12 g, yield 80 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.0-7.9 (m, 1H), 7.5-7.4 (d, 2H), 7.1-7.0 (d, 2H), 5.0 (m, 1H), 4.6 (m, 1H), 3.2-3.0 (m, 2H), 2.7-2.6 (m, 4H), 2.1-2.0 (m, 1H), 1.5-1.4 (s, 9H).

Compound 125: To a solution of **124** (12 g, 33 mmol) in CH_2Cl_2 (60 ml) was added Et_2O/HCl (25 mL, 5.1 mol/L) dropwise at an ice bath for 30 minutes. The reaction was stirred at ambient temperature overnight. The solvent was removed *in vacuo* yielding **125** (9.5 g, 96.3 %).

Compound 126: To a solution of **125** (9.5 g, 32 mmol) and K_2CO_3 (4.4 g, 32 mmol) in acetonitrile (40 mL) and water (40 mL) was added Fmoc-OSu (8.7 g, 25 mmol) in acetonitrile (40 mL) over 10 minutes under an ice bath. The mixture was stirred for 30 minutes. The mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated and recrystallized to give **126** (5.9 g, 40. %). ^{1}H NMR (400 MHz, C_2D_6SO) δ 12.29 (br s, 1H), 9.93 (s, 1H), 7.88 (d, J = 7.2 Hz, 2H), 7.70-7.64 (m, 3H), 7.52 (d, J = 8.0 Hz, 2H), 7.42-7.39 (m, 2H), 7.34-7.28 (m, 2H), 7.2 (d, J = 8.4 Hz, 2H), 4.23-4.15 (m, 4H), 3.04 (dd, J = 13.6, 3.6 Hz, 1H), 2.87-2.79 (m, 2H), 2.51-2.43 (m, 4H)); MS (m/z): $[M+Na]^+$ calcd. for $C_{29}H_{26}N_2NaO_5$, 505.1739; found, 505.2.

Preparation of (R)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(4-pent-4-ynamidophenyl)propanoic acid. (131)



Compound 128: To a solution of **127** (9.5 g, 39 mmol), pent-4-ynoic acid (4.75 g, 48.5 mmol) and hydroxybenzotriazole (5.2 g, 39 mmol) in CH_2Cl_2 (70 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (7.4 g, 39 mmol) at ambient temperature. Then the reaction mixture was stirred at room temperature for overnight. The mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated and purified by a silica gel column (eluting with 10-40 % ethyl

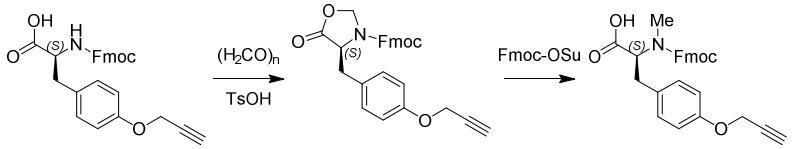
acetate / petroleum ether) to provide **128** (4 g, yield 30 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5-7.4 (d, 2H), 7.2-7.0 (d, 2H), 5.0-4.9 (m, 1H), 4.6 (m, 1H), 3.8-3.7 (s, 3H), 3.2-3.0 (m, 2H), 2.7-2.6 (m, 4H), 2.1-2.0 (m, 1H), 1.5-1.4 (s, 9H).

Compound 129: To a solution of **128** (4.0 g, 11 mmol) in THF (15 mL) and H_2O (15 mL) was added LiOH (0.90 g, 21 mmol). The mixture was stirred at ambient temperature overnight. The volatile organics were removed *in vacuo*. The crude reaction mixture was diluted with Et_2O. HCl (2 N aq) was added to to the crude mixture at 0 ^{o}C adjust the pH of the reaction mixture to 5, then extracted with ethyl acetate, washed by brine, dried, and concentrated to give **129** (3.5 g, 91 %).

Compound 130: To a solution of **129** (3.5 g, 9.7 mmol) in CH_2Cl_2 (20 mL) was added Et_2O/HCl (10 mL, 5.4 mol/L) dropwise in an ice bath for 30 minutes. The reaction was stirred at ambient temperature overnight. Then the resulting mixture was evaporated at to give **130** (2.0 g, 69 %).

Compound 131: To a solution of **130** (2.0 g, 6.8 mmol) and K_2CO_3 (0.90 g, 6.8 mmol) in acetonitrile (10 mL) and water (10 mL) was added Fmoc-OSu (1.8 g, 5.4 mmol) in acetonitrile (10 mL) over 10 minutes in an ice bath. The mixture was stirred for 30 minutes. The reaction mixture was extracted with ethyl acetate. The combined organic layers were washed with saturated NaCl, dried over Na_2SO_4, and filtered. The filtrate was concentrated and recrystallized to provide **131** (1.0 g, yield 39 %). ^{1}H NMR (400 MHz, C_2D_6SO) δ 12.71 (br s, 1H), 9.93 (s, 1H), 7.88 (d, 2H), 7.71 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 8 Hz, 2H), 7.51 (d, 2H), 7.43-7.39 (m, 2H), 7.39-7.27 (m, 2H), 7.19 (d, J = 8.4 Hz, 2H), 4.23-4.14 (m, 4H), 3.03 (dd, J = 13.6, 3.6 Hz, 1H), 2.85-2.79 (m, 2H), 2.51-2.46 (m, 4H, m); MS (*m/z*): $[M+Na]^+$ calcd. for $C_{29}H_{26}N_2NaO_5$, 505.1739; found, 505.2.

Preparation of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)-3-(4-(prop-2-ynyloxy)phenyl)propanoic acid. (Compound 134)



132 133 134

Compound 133: To a solution of **132** (6.0 g, 14 mmol) and paraformaldehyde (6 g) in toluene (5 mL) was added TsOH (0.23 g, 1.4 mmol). The mixture was stirred at refluxing for overnight. The mixture was concentrated and purified by a silica gel chromatography (eluting with 20-50% ethyl acetate/petroleum ether, 2% acetic acid) to provide **133** (4.1 g, yield 66.6 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.9-7.7 (d, 2H), 7.7-7.3 (m, 6H), 7.2-7.1 (d, 1H), 7.0-6.8 (d, 2H), 4.9 (m, 1H), 4.7-4.6 (s, 3H), 4.5-4.3 (m, 2H), 4.3-4.1 (m, 1H), 3.4-3.3 (m, 1H), 3.2-3.0 (m,1H), 2.6-2.4 (m, 1H).

Compound 134: To a solution of **133** (4.1 g, 9.1 mmol) and triethylsilane (4.7 g, 41 mmol) in

DCE (25 mL) was added TFA (25 mL) over 5 minutes under ice bath. The mixture was stirred for overnight. The mixture was extracted with DCE. The combined organic layers were washed with saturated NaCl, dried over Na_2SO_4, and filtered. The filtrate was concentrated and purified by a silica gel column (eluting with 20-50 % ethyl acetate/petroleum ether, 2% acetic acid) to provide **134** (2.1 g, yield 51 %). ^{1}H NMR (400 MHz, C_2D_6SO) δ 12.88 (br s, 1H), 7.88 (d, *J* = 7.6 Hz, 2H), 7.59-7.49 (m, 2H), 7.41 (t, *J* = 7.2 Hz, 2H), 7.36-7.29 (m, 2H), 7.14 (d, *J* = 8.4 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.86 (t, *J* = 5.2 Hz, 2H), 4.76-4.59 (m, 3H), 4.35-4.18 (m, 3H), 3.56-3.51 (m, 1H), 3.06-2.95 (m, 1H), 2.70-2.67 (m, 3H); MS (*m/z*): $[M+Na]^+$ calcd. for $C_{28}H_{25}NNaO_5$, 478.1630; found, 478.2.