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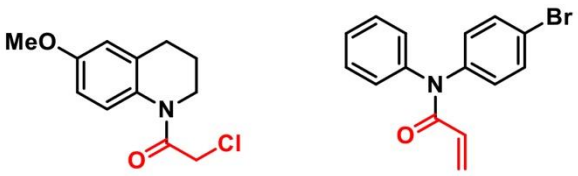
I. SUPPLEMENTARY FIGURES



KB02 KB05

Figure S1. Structures of broadly reactive scout fragments (KB02 and KB05).

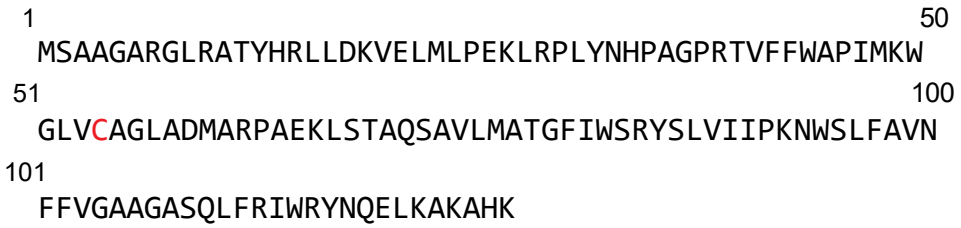


Figure S2. Peptide sequence of human MPC2 with C54 highlighted in red.

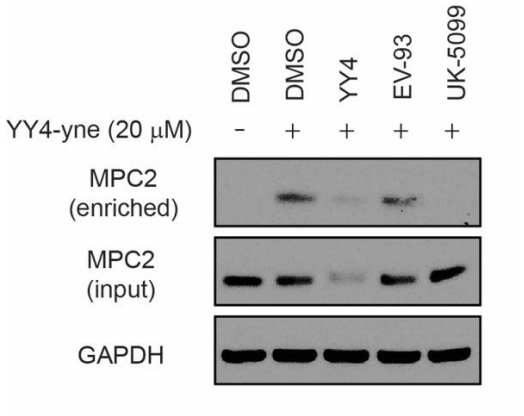


Figure S3. YY4-yne-coupled Western blotting of human T cell lysates treated *in vitro* with the indicated compounds (YY4, EV-93, and UK-5099; 20 μM, 1 h) or DMSO, followed by YY4-yne (20 μM, 1 h).

II. BIOLOGICAL METHODS

isoTOP-ABPP sample preparation

Expanded primary human T cells, prepared by a protocol from ref [1] were re-suspended in RPMI (2 x 10^6 cells/mL) containing 10% fetal bovine serum (FBS, Omega Scientific), penicillin (100 U/mL), streptomycin (100 µg/mL), and *L*-glutamine (2 mM). The cells were treated with DMSO or compounds for 3 h, pelleted (524 *g*, 5 min), washed with PBS, and lysed by sonication (2 x 8 pulses). Soluble and particulate proteomic fractions were separated by ultracentrifugation (100,000 *g*, 45 min), and protein concentration was normalized to 1.7 mg/mL using a standard DC protein assay (Bio-Rad). The resulting proteomes were analyzed by competitive isotopic Tandem Orthogonal Proteolysis Activity-Based Protein Profiling (isoTOP-ABPP) using a protocol described in the literature.[1]

Western blot analysis

Western blot analysis was performed on freshly isolated expanded T cells. For Western blot experiments, primary human T cells (2 x 10^7 cells/treatment) were re-suspended in RPMI media at 2 x 10^6 cells/mL and treated with the compounds or DMSO at 37 °C in a 5% CO_2 containing incubator for 3 h. Following this incubation period, the cells were pelleted (600 *g*, 5 min, 4 °C), washed with PBS (10 mL), transferred to 1.5 mL Eppendorf tubes, flash-frozen, and stored at –80 °C until further analysis. On the day of the analysis, the cell pellets were thawed on ice, re-suspended in cold PBS and lysed by sonication with probe sonicator (2 x 8 pulses). Protein concentrations for all the samples were adjusted to 1.5 mg/mL and 4x loading buffer was added (10 µL to 30 µL of proteome) and the samples were heated at 95 °C for 5 min. The proteins were resolved using SDS-PAGE (10% acrylamide gel) and transferred to 0.2 µM nitrocellulose membranes (GE Healthcare). The membrane was blocked with 5% milk in Tris-buffered saline (20 mM Tris-HCl 7.6, 150 mM NaCl) with 0.1% Tween 20 (TBST) buffer at rt for 1 h and incubated with primary antibodies [(MPC2; CST, 46141S, 1:500 dilution), (GAPDH; CST, 2118S, 1:5000 dilution)] in 5% milk in TBST at 4 °C overnight. Membrane was washed three times with TBST buffer and incubated with secondary antibody (CST, 7074S, 1:5000 dilution) in 5% milk in TBST at room temperature for 1 h. Membrane was washed three times with TBST buffer. The chemiluminescence signal in membrane was recorded after developing in ECL or ECL plus western blotting detection reagent using CL-XPosure film (Thermo Scientific). Relative band intensities were quantified using ImageJ.

YY4-yne-coupled Western blotting assay

Western blot analysis was performed on expanded T cells. For YY4-yne-coupled Western blotting experiments, primary human T cells (5 x 10^7 cells/treatment) were re-suspended in RPMI media at 2 x 10^6 cells/mL and pre-treated with compounds or DMSO at 37 °C in a 5% CO_2 containing incubator for 3 h (or otherwise indicated times)

before further treating with YY4-yne for 2 h (In time course studies, T cells were directly treated with YY4-yne for indicated times). Following this incubation period, the cells were pelleted (600 *g*, 5 min, 4 °C), washed with PBS (10 mL), transferred to 1.5 mL Eppendorf tubes, flash-frozen, and stored at –80 °C until further analysis. On the day of the analysis, the cell pellets were thawed on ice, re-suspended in cold PBS and lysed by sonication with probe sonicator (2 x 8 pulses). Protein concentrations for all the samples were adjusted to 1.5 mg/mL. For input samples, 4x loading buffer was added (10 µL to 30 µL of proteome) and the samples were heated at 95 °C for 5 min, and stored at –20 °C. For enrichment samples, whole cell lysate (500 µL of 1.5 mg/mL) was subjected to click reaction by adding 55 µL of a freshly prepared CuAAC reagent mixture to conjugate biotin to probe-labeled proteins. CuAAC reaction mixture consisted of biotin-PEG3-azide (10 µL of 10 mM stocks in DMSO, final concentration = 100 µM), tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 20 µL of fresh 50 mM stock in water, final concentration = 1 mM), tris(benzyltriazolylmethyl)amine ligand (TBTA; 60 µL of 1.7 mM stock in DMSO:*t*-butanol 1:4, final concentration = 100 µM), $CuSO_4$ (20 µL of 50 mM stock in water, final concentration = 2 mM). Upon addition of the CuAAC reaction mixture, each reaction was immediately mixed by vortexing and then allowed to react at ambient temperature for 1 h. The mixtures were transferred to 15 mL Falcon tubes and 2 mL cold methanol was added. The resulting cloudy mixtures were centrifuged (5,000 *g*, 10 min, 4 °C) to pellet the proteins. After removing the supernatant, the protein pellets were washed with 1 mL cold 1:1 MeOH:$CHCl_3$ and were resuspended in cold 4:1 MeOH:$CHCl_3$ (2.5 mL) by sonication. The cloudy mixtures were centrifuged (5,000 *g*, 10 min, 4 °C) to pellet the proteins. The pellets were resuspended in 1 mL of 1.2% SDS/PBS with sonication (2 x 8 pulses) and heated at 95 °C for 5 min. Once solubilized, the samples were diluted with 4 mL PBS and streptavidin-agarose beads were added for the enrichment (final SDS concentration: 0.2% in PBS). The beads (100 µL of a 50% slurry per sample) were washed with PBS (2 x 10 mL) and resuspended in 1 mL of PBS per sample prior to addition. The final mixture was rotated for 3 h at room temperature. Following this enrichment step, the beads were pelleted by centrifugation (2,000 *g*, 2 min) and washed to remove non-specifically binding proteins (4 x 10 mL, 0.2% SDS in PBS). The beads were transferred to 1.5 mL Eppendorf tubes with 1 mL of 0.2% SDS/PBS and pelleted by centrifugation (5,000 *g*, 2 min). After removal of the supernatant, 50 µL of 2x SDS loading buffer was added to each sample. After boiling for 10 min, the beades were pelleted by centrifugation (5,000 *g*, 1 min) and samples were transferred to new 1.5 mL Eppendorf tubes and stored at –20 °C before further use. Western blotting experiments were performed by described above. Relative band intensities were quantified using ImageJ.

Preparation of tandem mass tag (TMT) labeled samples for multiplexed MS-based analysis

YY4-yne-coupled Western blotting samples were prepared as described above with the following modifications. After the click reaction and proteome precipitation, the pellets were solubilized in proteomics-grade urea (500 µL, 6 M in DPBS) containing 10 µL of 10% SDS by sonication. 50 µL of 1:1 mixture of TCEP (200 mM in DPBS) and K_2CO_3

(600 mM in DPBS) were added to each sample and the mixture was incubated at 37 °C for 30 min to reduce the disulfides. Reduced thiols were then alkylated by incubating with 70 μL iodoacetamide (400 mM in DPBS) at room temperature protected from light for 30 min. To each solution, 130 μL of 10% SDS (in DPBS) was added and diluted to ~0.2% SDS with DPBS (5.5 mL) and incubated with pre-equilibrated streptavidin agarose beads (Thermo Fisher Scientific, Cat # 20347, 100 μL 1:1 slurry) for 1.5 h at room temperature on a rotator. The beads were centrifuged (1,400 g, 2 min) and washed sequentially with 0.2% SDS in DPBS (1 x 5 mL), DPBS (2 x 5 mL) and 200 mM EPPS Sigma Aldrich, E9502, pH 8, 1 x 5 mL) to remove unbound proteins, excess detergent and reagents. The beads were transferred to a low-binding 1.5 mL microfuge tube (Axygen, CNT-1.5FL) and the enriched proteins were on-bead digested overnight at 37 °C in ~200 μL of 2 M urea in 200 mM EPPS containing 2 μg sequencing grade porcine trypsin (Promega, V5111) and $CaCl_2$ (1 mM). The samples were centrifuged to separate the beads and supernatant, then anhydrous acetonitrile was added to the supernatant to 30% final volume. 6 μL (20 μg/μL) of respective 10-plex TMT tag (Thermo Scientific, cat # 90110) was added and the reaction was incubated at room temperature for 1 h with occasional vortexing before quenching with 6 μL of 5% hydroxylamine for 15 min followed by the addition of 4 μL formic acid. All 10-plex samples were evaporated to dryness using SpeedVac vacuum concentrator, reconstituted in ~400 μL Buffer A (95% H_2O, 5% acetonitrile, 0.1% formic acid) with sonication, and combined. The samples were subjected to high pH fractionation as previously described[1] and the resulting 7 combined fractions were re-suspended in buffer A (10 μL) and analyzed on the Orbitrap Fusion mass-spectrometer (5 μL injection volume).[1] The resulting data was analyzed as described previously.[1]

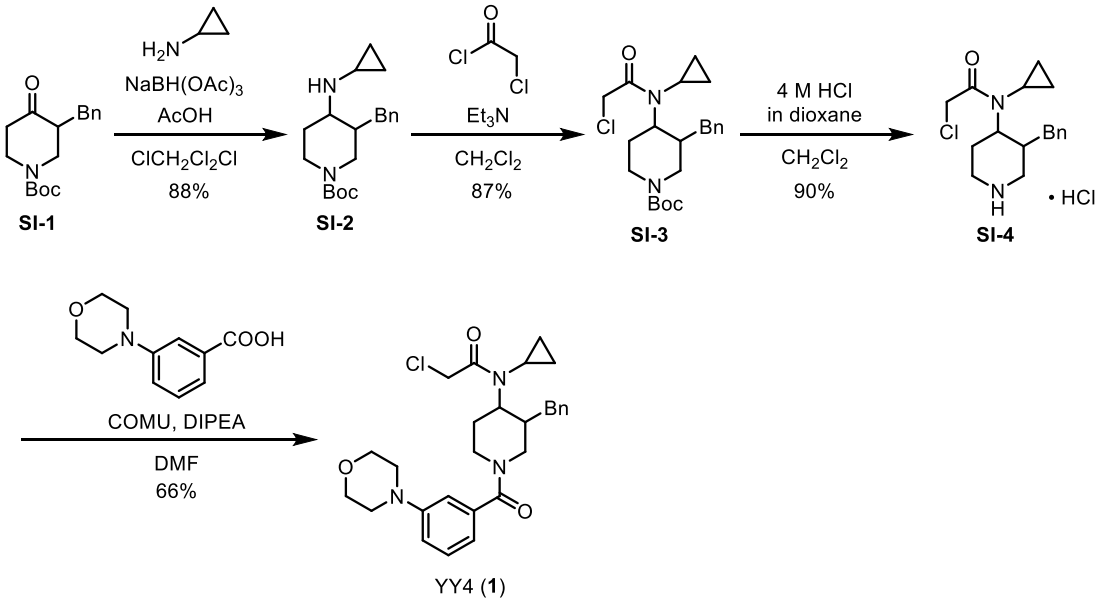
III. SYNTHETIC METHODS AND COMPOUNDS CHARACTERIZATION

Synthetic

General Methods

All chemicals, including anhydrous solvents, were obtained from commercial suppliers and used without further purification. Merck silica gel TLC plates (0.25 mm, 60 F_{254}) were used to monitor reaction progress and were visualized under UV light (254 nm) or by staining with potassium permanganate ($KMnO_4$). Flash chromatography was performed using SiliaFlash® F60 silica gel (SiO_2, 40-60 µM, 60 Å), loaded with dichloromethane unless otherwise noted. NMR spectra were recorded at ambient temperature on Bruker DRX-500 or Bruker AV-600 (5 mm DCH cryoprobe) instruments. Chemical shifts are recorded in ppm relative to tetramethylsilane (TMS, ^1H, 0 ppm) or solvent signals: $CDCl_3$ (^1H, 7.26), D_2O (^1H, 4.80). Peaks are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) (Hz). High-resolution mass spectra (HRMS) were obtained on an Agilent LC/MSD TOF mass spectrometer by electrospray ionization-time-of-flight (ESI-TOF).

Supplementary Scheme 1. Synthetic route to generate YY4 (**1**).



Amine **SI-2**

Cyclopropylamine (1.68 mL, 24.1 mmol) and acetic acid (1.38 mL, 24.1 mmol) were added to a solution of ketone **SI-1**[1] (4.65 g, 16.1 mmol) in 1,2-dichloroethane (35 mL) at room temperature. After stirring for 1 h, $NaBH(OAc)_3$ (4.09 g, 19.3 mmol) was added to the mixture. After stirring for 13 h, the reaction was quenched by slowly adding

saturated aqueous $NaHCO_3$ at 0 °C and the mixture was extracted with CH_2Cl_2 (x3). The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by column chromatography (hexane/EtOAc = 1/1) to afford **SI-2** (4.67 g, 88%) as a white solid.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 7.25 – 7.15 (m, 3H), 4.15 – 3.45 (m, 2H), 3.30 – 2.60 (m, 4H), 2.45 (t, *J* = 12.0 Hz, 1H), 2.30 – 1.96 (m, 2H), 1.80–1.55 (m, 2H), 1.43 (s, 9H), 0.60 – 0.25 (m, 4H).
HRMS ESI-TOF (*m/z*): $[M+H]^+$ for $C_{20}H_{30}N_2O_2$ 331.2380, found 331.2388.

Chloroacetamide **SI-3**

Et_3N (4.9 mL, 35.3 mmol) and chloroacetyl chloride (1.69 mL, 21.2 mmol) were added to a solution of amine **SI-2** (4.67 g, 14.1 mmol) in CH_2Cl_2 (70 mL) at 0 °C. After stirring for 30 min at room temperature, the reaction was quenched by adding saturated aqueous NH_4Cl and the mixture was extracted with CH_2Cl_2 (x3). The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by column chromatography (hexane/EtOAc =1/1) to afford **SI-3** (4.99 g, 87%) as a white amorphous solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.22 (m, 2H), 7.22 – 7.09 (m, 3H), 4.51 – 4.04 (m, 4H), 4.05 – 3.66 (s, 1H), 2.90 – 2.25 (m, 7H), 1.81 (d, *J* = 12.8 Hz, 1H), 1.43 (s, 9H), 1.10 – 0.90 (m, 3H), 0.90 – 0.76 (m, 1H).
HRMS ESI-TOF (*m/z*): $[M+Na]^+$ for $C_{22}H_{31}ClN_2O_3$ 429.1915, found 429.1919.

Chloroacetamide **SI-4**

4 M HCl in dioxane (12.3 mL, 12.3 mmol) was added to a solution of **SI-3** in CH_2Cl_2 (60 mL) at 0 °C. After stirring for 24 h, the reaction mixture was concentrated in vacuo and the crude product was filtered and washed with Et_2O to afford **SI-4** (3.77 g, 90%) as a white solid.

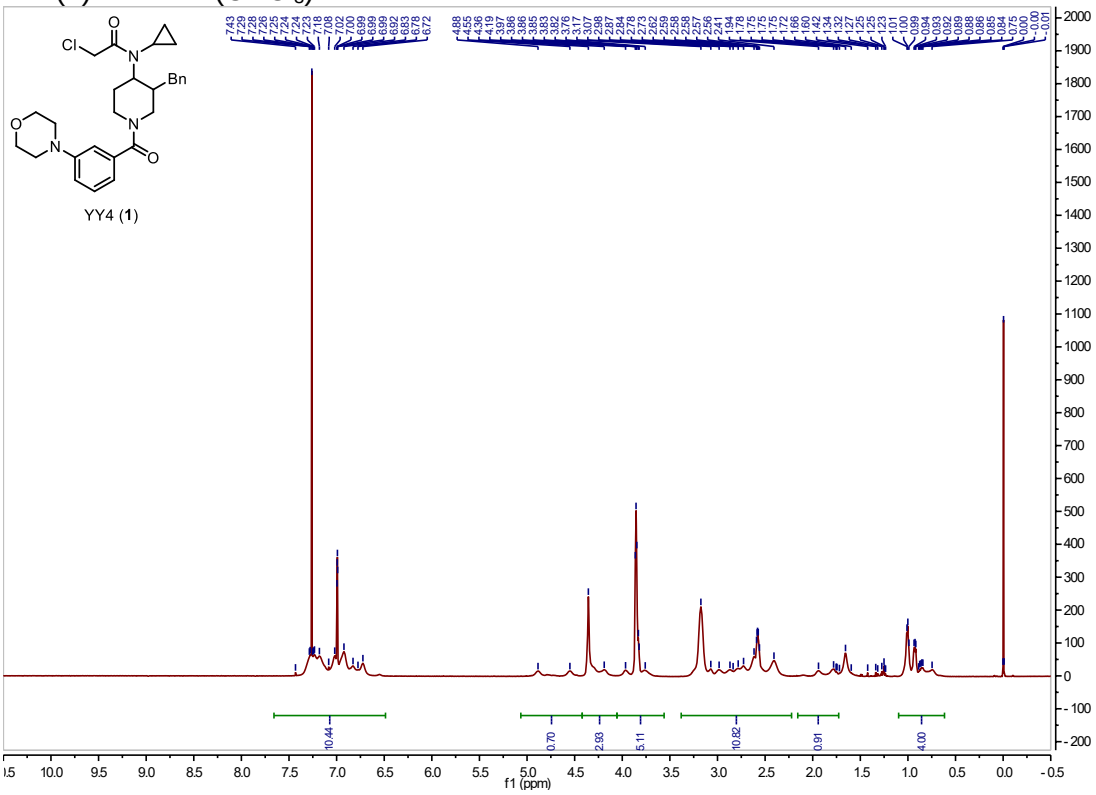
^{1}H NMR (600 MHz, D_2O) δ 7.47 – 7.21 (m, 5H), 4.66 – 4.47 (m, 2H), 4.28 – 4.14 (m, 1H), 3.65 – 3.42 (m, 1H), 3.30 (dd, *J* = 13.4, 4.2 Hz, 1H), 3.21 – 3.07 (m, 2H), 2.93 – 2.59 (m, 5H), 2.26 – 2.15 (m, 1H), 1.13 – 0.91 (m, 3H), 0.87 – 0.76 (m, 1H).
HRMS ESI-TOF (*m/z*): $[M+H]^+$ for $C_{17}H_{23}ClN_2O$ 307.1572, found 307.1582.

YY4 (**1**)

COMU (225 mg, 0.524 mmol) and DIPEA (228 μL, 1.13 mmol) were added to a solution of 3-morpholinobenzoic acid (109 mg, 0.524 mmol) and **SI-4** (150 mg, 0.437 mmol) in DMF (3 mL) at 0 °C. After stirring for 15 min at 0 °C, the reaction mixture was quenched by adding saturated aqueous NH_4Cl and water. The mixture was extracted with EtOAc (x3) and the combined organic extracts were washed with water (x2), brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by preparative TLC (EtOAc) to afford YY4 (**1**) as a white amorphous solid (142 mg, 66%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 – 6.49 (m, 9H), 5.06 – 3.56 (m, 9H), 3.38 – 2.22 (m, 11H), 2.16 – 1.73 (m, 1H), 1.10 – 0.62 (m, 4H).
HRMS ESI-TOF (*m/z*): $[M+H]^+$ for $C_{26}H_{34}ClN_3O_3$ 496.2361, found 496.2373.

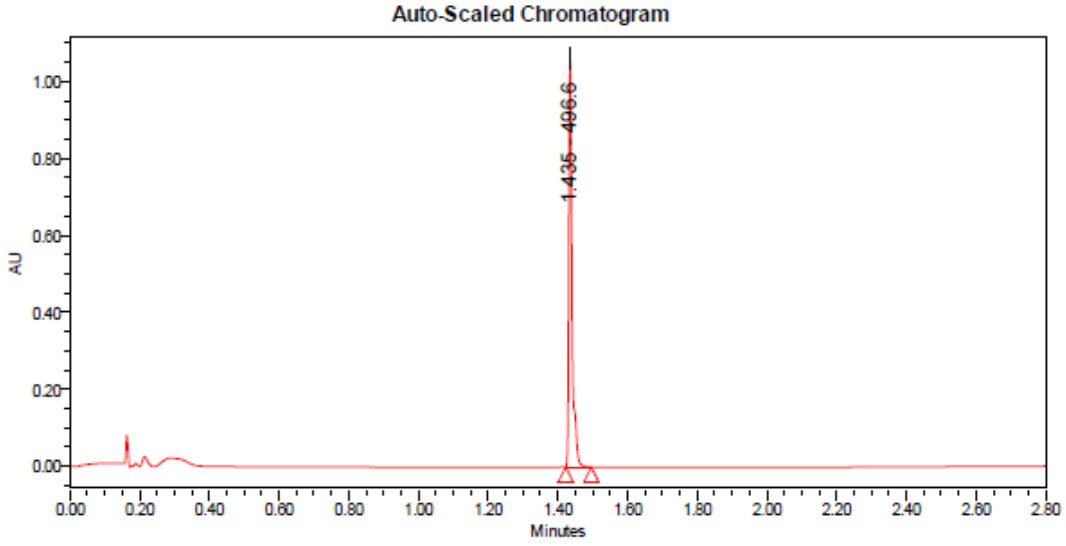
YY4 (**1**) ^{1}H NMR ($CDCl_3$)



Confirmation of purity of YY4 (**1**) by HPLC-MS.

SAMPLE INFORMATION

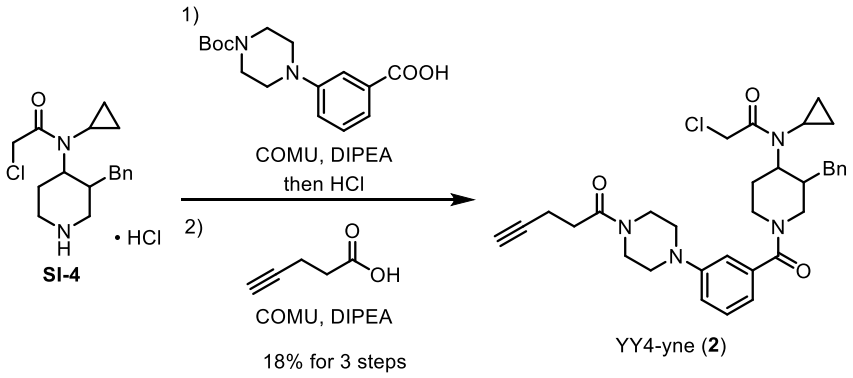
Sample Name:	YY4	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Generic_LC
Vial:	1:C,8	Acq. Method Set:	A_Grad_10_99
Injection #:	1	Processing Method	Default_UV
Injection Volume:	0.50 ul	Channel Name:	MaxPlot
Run Time:	2.8 Minutes	Proc. Chnl. Descr.:	PDA Spectrum PDA MaxPlot
Date Acquired:	10/18/2019 5:32:37 PM PDT		
Date Processed:	10/18/2019 5:35:38 PM PDT		



Peak Results

	Name	RT	Area	Height	Amount	Units	Base Peak (m/z)
1		1.435	674704	1064029			496.57

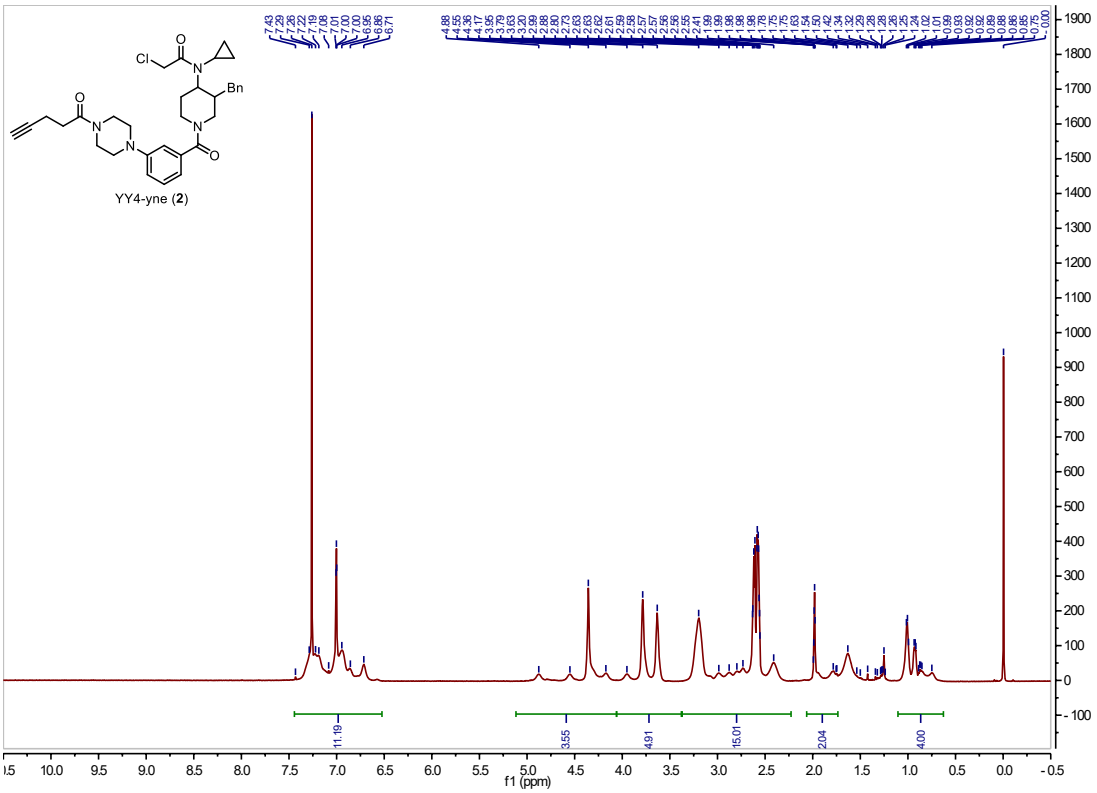
Supplementary Scheme 2. Synthetic route to generate YY4-yne (**2**).



YY4-yne (**2**)

COMU (274 mg, 0.641 mmol) and DIPEA (304 μL, 1.75 mmol) were added to a solution of 3-(4-Boc-piperazin-1-yl)benzoic acid (196.4 mg, 0.641 mmol) and **SI-4** (200 mg, 0.583 mmol) in DMF (3 mL) at 0 °C. After stirring for 15 min at 0 °C, the reaction mixture was quenched by adding aq. 1 M HCl. The mixture was extracted with EtOAc (x3) and the combined organic extracts were washed with water (x2), brine, dried (Na_2SO_4), and concentrated in vacuo. 4 M HCl in dioxane (1 mL) was added to a solution of crude mixture in CH_2Cl_2 (3 mL) at 0 °C. After stirring for 24 h at room temperature, the reaction mixture was concentrated in vacuo and the crude product was used in the next step without purification. COMU (158 mg, 0.369 mmol) and DIPEA (161 μL, 0.923 mmol) were added to a solution of the crude mixture and 4-pentynoic acid (36.2 mg, 0.369 mmol) in DMF (3 mL) at 0 °C. After stirring for 15 min at 0 °C, the reaction mixture was quenched by adding saturated aqueous NH_4Cl and water. The mixture was extracted with EtOAc (x3) and the combined organic extracts were washed with water (x2), brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by preparative TLC (EtOAc) to afford YY4-yne (**2**) as a white amorphous solid (61 mg, 18% for 3 steps).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 – 6.44 (m, 9H), 5.08 – 3.46 (m, 9H), 3.42 – 2.25 (m, 15H), 2.15 – 1.73 (m, 2H), 1.14 – 0.45 (m, 4H).
HRMS ESI-TOF (*m/z*): $[M+H]^+$ for $C_{33}H_{39}ClN_4O_3$ 575.2783, found 575.2782.


YY4-yne (2)
f1 (ppm)

Confirmation of purity of YY4-yne (**2**) by HPLC-MS.

SAMPLE INFORMATION

Sample Name:	YY4-yne	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Generic_LC
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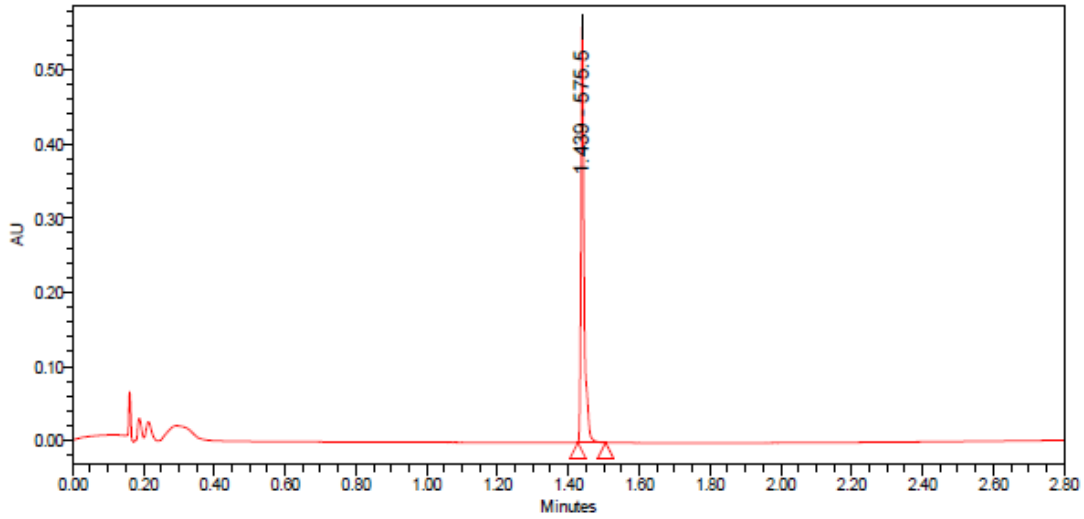
Injection #:	1	Processing Method	Default_UV
Injection Volume:	0.50 ul	Channel Name:	MaxPlot
Run Time:	2.8 Minutes	Proc. Chnl. Descr.:	PDA Spectrum PDA MaxPlot
Date Acquired:	10/18/2019 5:36:04 PM PDT		
Date Processed:	10/18/2019 5:39:05 PM PDT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units	Base Peak (m/z)
1		1.439	333565	561726			575.54

[1] E. V. Vinogradova, D. C. Lazar, R. M. Suciu, Y. Wang, G. Bianco, Y. Yamashita, V. M. Crowley, D. Remillard, K. M. Lum, G. M. Simon, E. K. Kemper, M. R. Lazear, S. Yin, M. M. Blewett, M. M. Dix, N. Nguyen, M. N. Shokhirev, E. Chin, L. Lairson, S. Forli, J. R. Teijaro, B. F. Cravatt, *bioRxiv* **2019**, 808113.