Supporting Information

Affinity-based selectivity profiling of an in-class selective competitive inhibitor of acyl protein thioesterase 2

Sang Joon Won[1], Joseph D. Eschweiler[2], Jaimeen D. Majmudar[2], Fei San Chong[2], Sin Ye Hwang[2], Brandon T. Ruotolo[2], and Brent R. Martin[1, 2, *]

[1]Program in Chemical Biology, [2]Department of Chemistry, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA

*Correspondence: brentrm@umich.edu

Contents

I. Supplementary Figures 2

Supplementary Figure 1. Competitive fluorescence polarization assay for ML349 binding. 2

Supplementary Figure 2. Native mass spectrometry analysis of ML349-biotin binding and stoichiometry. 2

II. Synthetic protocols and product characterization. 3

General Chemical Synthesis and Purification Procedures. 3

Synthetic Scheme 1. Synthesis of ML349-biotin. 4

Synthesis of ML349-biotin 4

Synthesis of *ortho*-ML349-biotin 7

III. Experimental Procedures 9

ML349-biotin pull-downs. 9

LC-MS proteomic analysis and data processing. 9

Recombinant protein expression and purification. 10

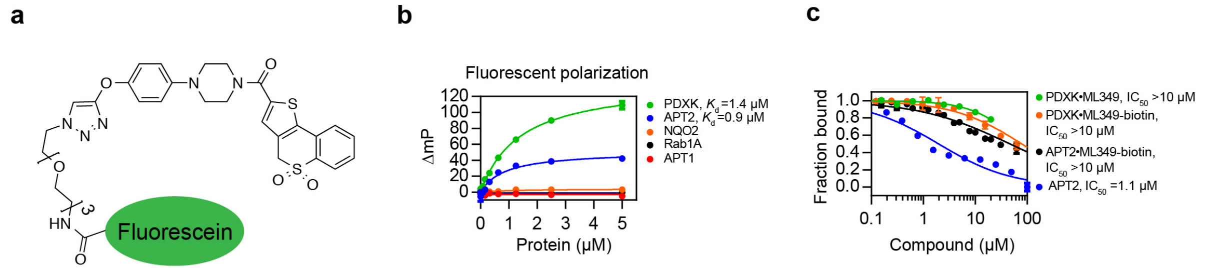
Steady-state kinetic analysis. 10

Native mass spectrometry analysis. 11

IV. References 11

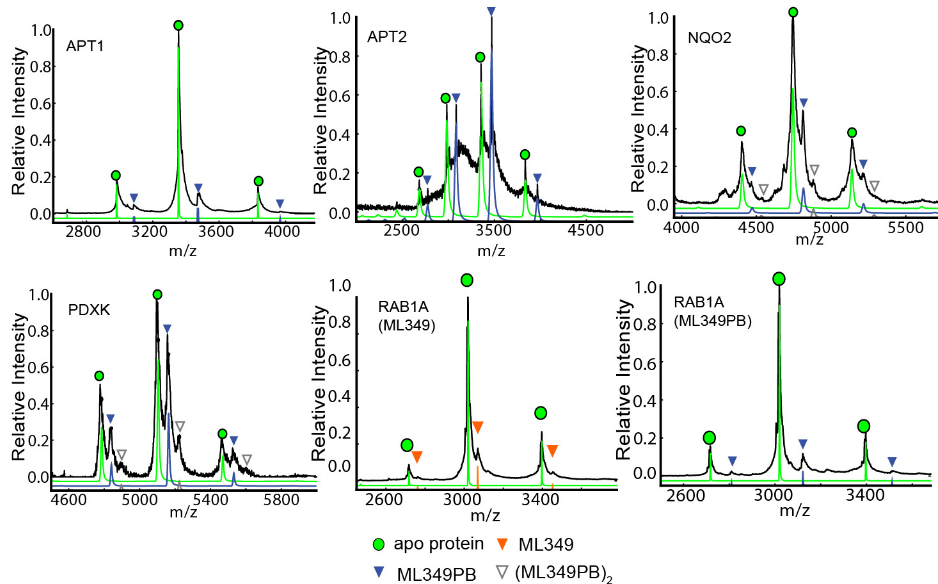
I. Supplementary Figures

Supplementary Figure 1. Competitive fluorescence polarization assay for ML349 binding. (a) Chemical structure of ML349-FL. (b) ML349-FL binding to candidate interacting proteins detected by fluorescence polarization. (c) Dose-dependent competition of ML349-FL binding to APT2 or PDXK by ML349 and ML349-biotin.



Supplementary Figure 2. Native mass spectrometry analysis of ML349-biotin binding and stoichiometry.

Representative mass / charge spectra for each recombinant protein are shown. 3 charge states were used for quantitation. (Green = apo protein, blue = ML349-biotin bound protein, orange = ML349 bound protein, white triangle = 2xML349-biotin bound protein).

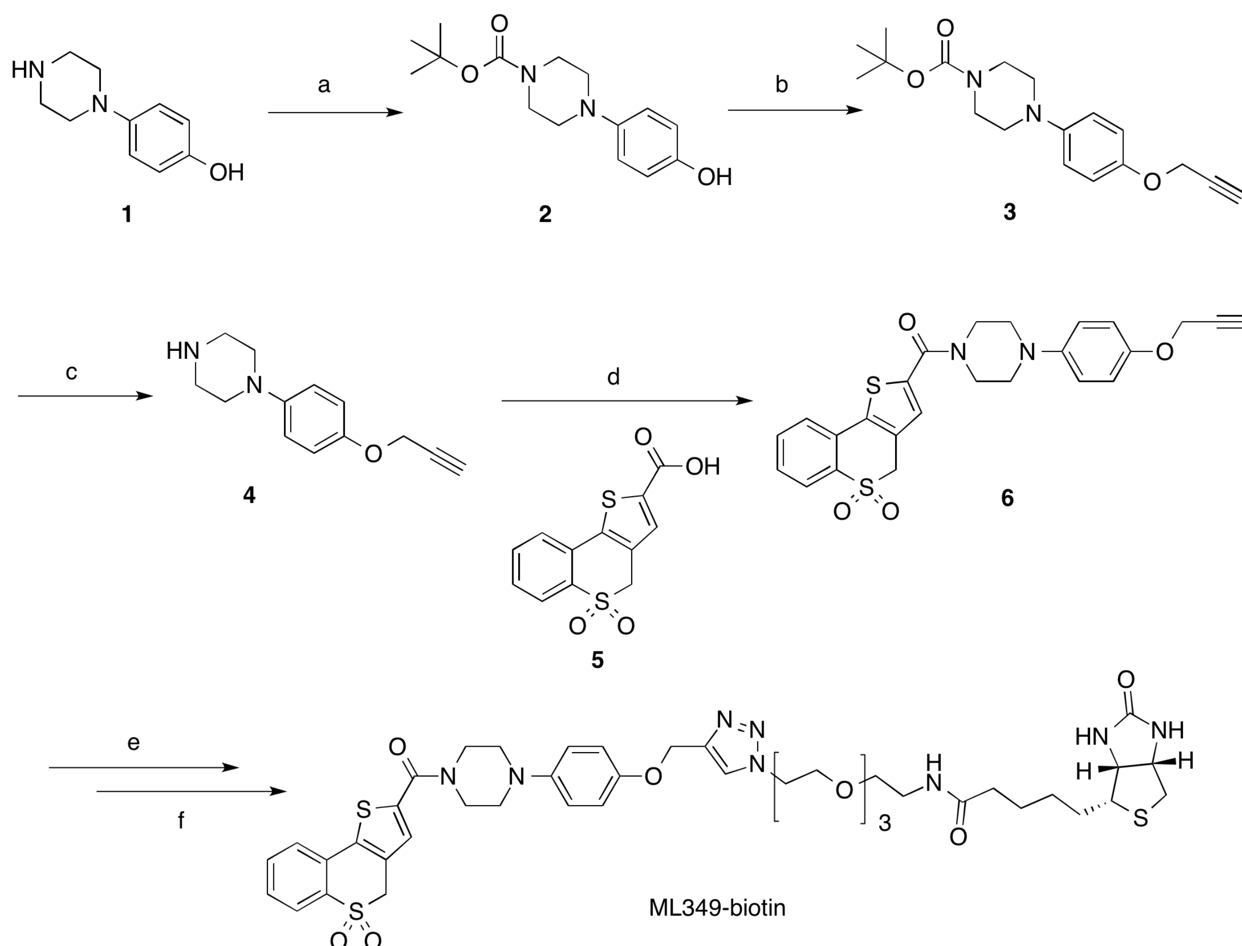


II. Synthetic protocols and product characterization.

General Chemical Synthesis and Purification Procedures.

All compounds were purchased from Sigma-Aldrich, unless otherwise noted and were of the highest purity available. NMR analysis was performed using a Varian 400 MHz NMR instrument. NMR integrations and coupling constants were computed using MestreNova. Small molecule high-resolution mass spectrometry was performed using an electrospray Agilent Q-TOF mass spectrometer (accuracy 1-5 ppm) and analyzed using the Agilent MassHunter software suite. Low-resolution mass spectrometry was performed using an electrospray Micromass LCT time-of-flight mass coupled to a HPLC pump with a rheodyne loop injector. Compounds were purified by normal phase flash silica-gel column chromatography or by semi-prep High-Performance Column Chromatography (HPLC). HPLC purifications were performed using a Waters semi-preparative 1525 binary pump system coupled to a photodiode array detector, an autosampler, and an automatic fraction collector. Separations were carried out on using the Waters Atlantis prep T3 C_{18} column (10 x 250 mm), in 95/5 water/acetonitrile 0.1% formic acid for 2 minutes, followed by a 40 min gradient increasing the mobile phase to 5/95 water/acetonitrile with 0.1% formic acid. Data were analyzed using the Waters Empower software. Resulting HPLC fractions were lyophilized using a Labconco FreeZone2.5-Plus freeze-drying system. In-gel fluorescence was imaged using the GE Typhoon scanner.

Synthetic Scheme 1. Synthesis of ML349-biotin.



(a) Di-*tert*-butyl dicarbonate, methanol, 12 hours, room temperature. 90-95% yield. (b) propargyl bromide, potassium carbonate, DMF, 16 hours, room temperature. 80-92% yield. (c) TFA, DCM, 5 hours, 60 °C. 60-90% yield. (d) Compound 5, HBTU, DIPEA, DCM, 16 hours, room temperature. 40-54% yield. (e) 11-Azido-3,6,9-trioxaunderdecan-1-amine, sodium ascorbate, $CuSO_4$, DMSO, 16 hours, room temperature. (f) NHS-Biotin, DMSO, DIPEA, room temperature, 3 hours. 52%- 60% yield.

Synthesis of ML349-biotin

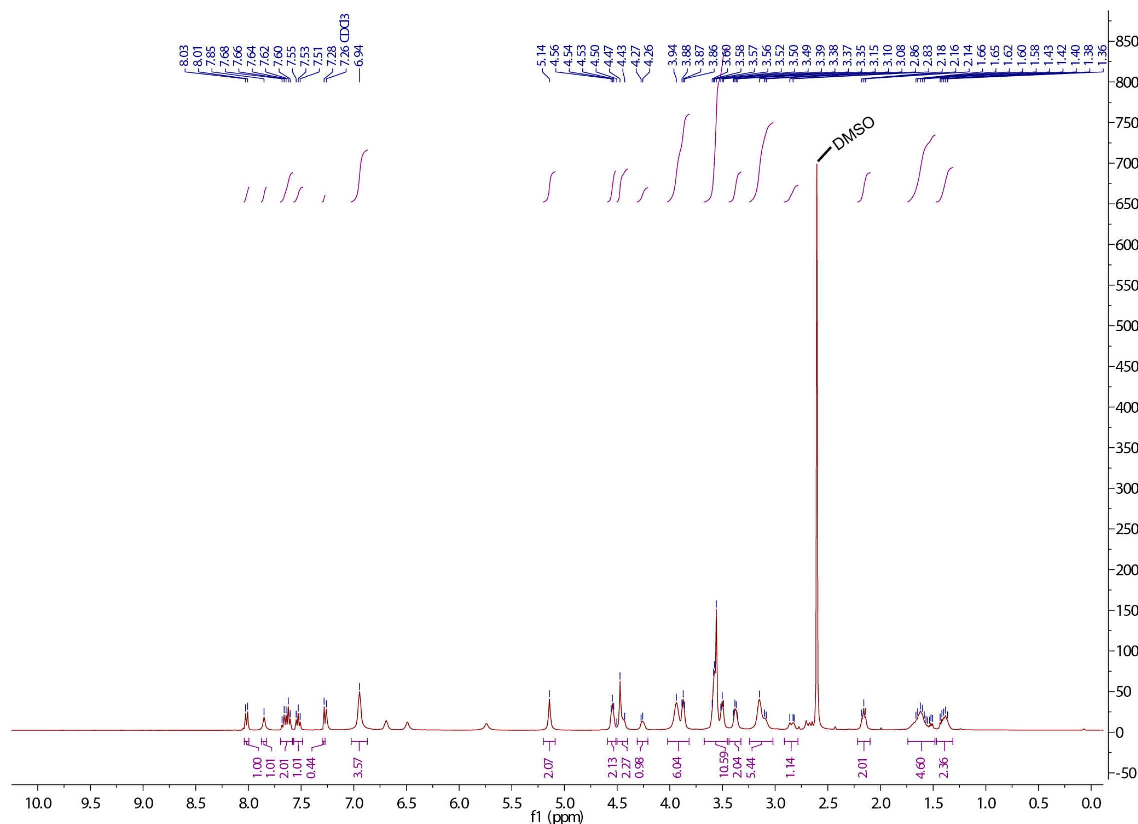
N-(2-(2-(2-(2-(4-((4-(4-(5,5-dioxido-4H-thieno[3,2-c]thiochromene-2-carbonyl)piperazin-1-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide. Compound 1 (Sigma, 900 mg, 1 eq) was treated with di-*tert*-butyl dicarbamate (1 eq.) in methanol for 12 hours. The contents were extracted with ethyl acetate, washed with brine, and then dried over sodium sulfate. The samples were concentrated under vacuum to yield compound 2, *tert*-butyl 4-(4-hydroxyphenyl)piperazine-1-

carboxylate (1.3 g, 95 % yield) . ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (ddd, *J* = 36.0, 9.8, 3.0 Hz, 4H), 3.74 – 3.48 (m, 4H), 3.07 – 2.95 (m, 4H), 1.48 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.0, 151.0, 119.4, 116.1, 80.3, 77.5, 77.2, 76.9, 51.4, 28.6. Compound 2 (125 mg) was treated with propargyl bromide (2 eq.) and potassium carbonate (2 eq.) in dimethylformamide (DMF). The mixture was stirred for 16 hours at room temperature. The crude mixture was extracted with ethyl acetate several times before washing with brine and drying over sodium sulfate. The sample was further purified by flash column chromatography (1:1 ethyl acetate/hexanes) to yield compound 3, *tert*-butyl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate (130 mg, 92 % yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.83 – 6.73 (m, 4H), 4.50 (d, *J* = 2.5 Hz, 2H), 3.50 – 3.42 (m, 4H), 2.93 – 2.85 (m, 4H), 2.47 (t, *J* = 2.4 Hz, 1H), 1.39 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.2, 151.7, 146.0, 118.2, 115.4, 79.3, 56.0, 50.2, 28.1. The compound 3 (1 eq.) was treated with trifluoroacetic acid (TFA, 2.5 eq.) in dichloromethane (DCM) for 5 hours at 60 ºC reflux. The residual TFA was boiled off and the sample was dried at high-vacuum to yield compound 4 (87 mg, 90 % yield) and continued without further purification. Synthesis of compound 5 was previously described[1], and an aliquot of the characterized product was used here. Compound 4 (50 mg, 1 eq.) was coupled with compound 5 (1 eq.) by using HBTU (Sigma, 3 eq.), DIPEA (Sigma, 2 eq.), and DMF as solvent. The reaction mixture was stirred for 16 hours at room temperature and the crude mixture was extracted with DCM several times, followed by a brine wash and sodium sulfate treatment to remove excess polar materials. The sample was further purified by flash column chromatography (40 % ethyl acetate: 60 % hexanes) to yield compound 6, (5,5-dioxido-4*H*-thieno[3,2-*c*]thiochromen-2-yl)(4-(4-(prop-2-yn-1-yloxy)phenyl)piperazin-1-yl)methanone (46 mg, 54 % yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.8 Hz, 1H), 7.72 – 7.49 (m, 3H), 7.23 (s, 1H), 6.97 (t, *J* = 10.9 Hz, 4H), 4.66 (d, *J* = 2.4 Hz, 2H), 4.45 (s, 2H), 3.99 (s, 4H), 3.19 (s, 4H), 2.52 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.2, 134.0, 133.6, 130.0, 129.3, 127.9, 125.8, 124.2, 115.9, 56.3, 51.2. The alkynyl portion of compound 6 (30 mg, 1 eq.) was conjugated with 11-Azido-3,6,9-trioxaundecan-1-amine (Sigma, 1 eq.) via Copper(I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) with $CuSO_4$ (catalytic amount) and sodium ascorbate (0.2 eq.) in DMSO for 16 hours at 50 °C. The crude mixture was purified by HPLC and then lyophilized to yield the intermediate product with free amine at the end of the linker, (4-(4-((1-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)phenyl)piperazin-1-yl)(5,5-dioxido-4*H*-thieno[3,2-*c*]thiochromen-2-yl)methanone, (31 mg, 70% yield) ^{1}H NMR (401 MHz, Methanol-d_4) δ 8.08 (s, 1H), 8.01 (d, *J* = 7.8 Hz, 1H), 7.80 – 7.73 (m, 2H), 7.62 (ddd, *J* = 8.3, 6.3, 2.4 Hz, 1H), 7.47 (s, 1H), 7.04 – 6.92 (m, 4H), 5.12 (s, 2H), 4.67 (d, *J* = 7.7 Hz, 2H), 4.60 (t, *J* = 5.0 Hz, 2H), 3.92 (dt, *J* = 13.3, 4.9 Hz, 5H), 3.71 – 3.54 (m,

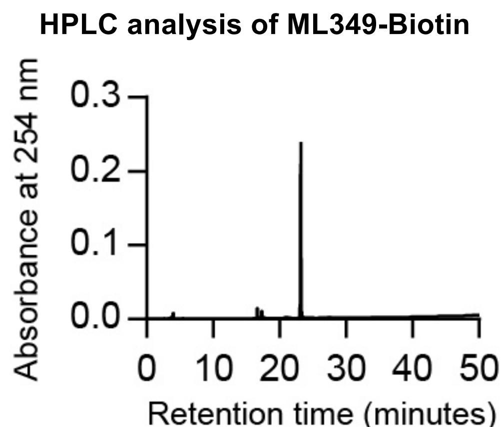
11H), 3.12 (dt, J = 24.8, 5.1 Hz, 6H). This product was treated with Biotin-NHS ester (15 mg, Click Chemistry Tools), DIPEA (2 eq.) in DMSO for 16 hours at room temperature. The resulting crude mixture was purified by HPLC to yield ML349-biotin (22 mg, 52 % yield).

Final Product: ^{1}H NMR (400 MHz, Chloroform-d) δ 8.02 (d, J = 7.8 Hz, 1H), 7.85 (s, 1H), 7.64 (dt, J = 15.7, 7.6 Hz, 2H), 7.53 (t, J = 7.5 Hz, 1H), 7.28 (s, 1H), 6.94 (s, 4H), 5.14 (s, 2H), 4.54 (t, J = 4.9 Hz, 2H), 4.47 (s, 2H), 4.26 (d, J = 6.1 Hz, 1H), 4.02 – 3.82 (m, 6H), 3.67 – 3.45 (m, 11H), 3.37 (q, J = 5.2 Hz, 2H), 3.12 (d, J = 20.1 Hz, 5H), 2.91 – 2.78 (m, 1H), 2.16 (t, J = 7.5 Hz, 2H), 1.58 (ddt, J = 31.9, 11.7, 6.5 Hz, 5H), 1.40 (dt, J = 15.0, 7.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 164.1, 162.2, 136.3, 134.0, 133.6, 130.1, 130.1, 130.1, 129.3, 128.1, 125.9, 124.2, 115.7, 77.2, 70.5, 70.4, 70.1, 69.9, 69.4, 62.4, 61.8, 60.2, 55.6, 51.2, 50.4, 40.6, 39.1, 35.9, 28.3, 28.1, 25.6. $C_{43}H_{54}N_8O_9S_3$, HRMS (ESI positive), [M+H] predicted m/z: 923.3249, found 923.3232. HPLC purity: 99% (shown below).

^{1}H NMR of ML349-biotin



HPLC analysis of ML349-Biotin



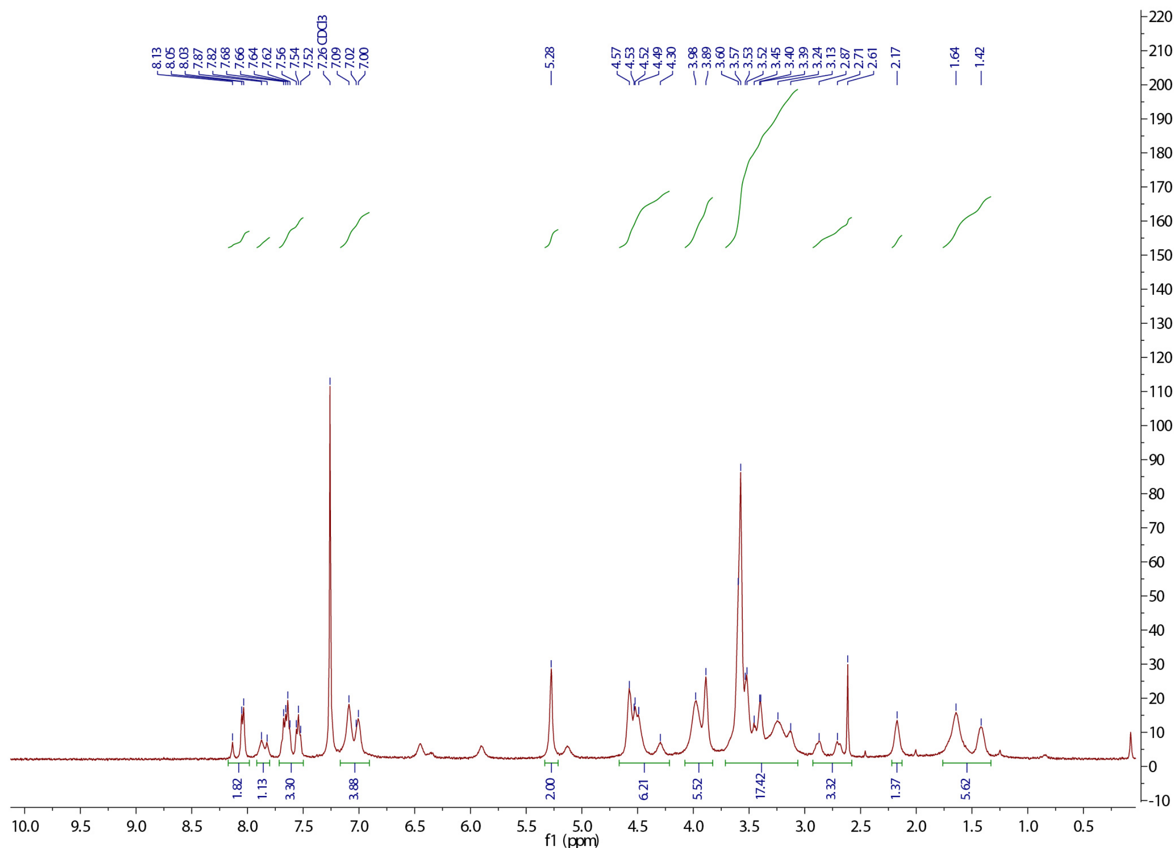
Synthesis of *ortho*-ML349-biotin

N-(2-(2-(2-(2-(4-(2-(4-(5,5-dioxido-4H-thieno[3,2-c]thiochromene-2-carbonyl)piperazin-1-yl)phenoxy)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide. Synthesized as described above but used 2-(piperazin-1-yl)phenol (50 mg) during step (a). Intermediate compound 2, *tert*-butyl 4-(2-hydroxyphenyl)piperazine-1-carboxylate, (60 mg, 90% yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 – 6.96 (m, 2H), 6.89 (dd, *J* = 8.0, 1.5 Hz, 1H), 6.78 (td, *J* = 7.6, 1.5 Hz, 1H), 3.52 (t, *J* = 5.0 Hz, 4H), 2.75 (t, *J* = 5.0 Hz, 4H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.6, 151.2, 138.7, 126.3, 121.1, 120.0, 114.4, 79.8, 52.2, 44.3, 28.4. Intermediate compound 3, *tert*-butyl 4-(2-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate, (20 mg, 60% yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 – 6.87 (m, 4H), 4.72 (d, *J* = 2.4 Hz, 2H), 3.57 (t, *J* = 5.0 Hz, 4H), 2.98 (t, *J* = 5.0 Hz, 4H), 2.50 (t, *J* = 2.4 Hz, 1H), 1.46 (s, 9H). Intermediate compound 6, (5,5-dioxido-4*H*-thieno[3,2-*c*]thiochromen-2-yl)(4-(2-(prop-2-yn-1-yloxy)phenyl)piperazin-1-yl)methanone,(13 mg, 40% yield)^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (dt, *J* = 8.2, 2.4 Hz, 1H), 7.65 (dtd, *J* = 15.0, 7.8, 1.3 Hz, 2H), 7.54 (td, *J* = 7.6, 1.5 Hz, 1H), 7.23 (s, 1H), 7.15 – 6.97 (m, 4H), 4.79 (d, *J* = 2.4 Hz, 2H), 4.44 (d, *J* = 5.2 Hz, 2H), 4.14 – 3.91 (m, 4H), 3.25 (d, *J* = 23.1 Hz, 4H), 2.54 (t, *J* = 2.4 Hz, 1H). Intermediate compound before coupling with NHS-biotin, (4-(2-((1-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)phenyl)piperazin-1-yl)(5,5-dioxido-4*H*-thieno[3,2-*c*]thiochromen-2-yl)methanone, (12 mg, 65% yield) ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.03 (t, *J* = 8.0 Hz, 1H), 7.94 (s, 1H), 7.70 – 7.59 (m, 2H), 7.52 (t, *J* = 7.2 Hz, 1H), 7.31 (s, 1H), 7.09 – 6.88 (m, 4H), 5.26 (d, *J* = 3.7 Hz, 2H), 4.69 – 4.43 (m, 4H), 3.91 (p, *J* = 5.0 Hz, 6H), 3.79 – 3.46 (m, 9H), 3.37 (p, *J* = 5.1 Hz, 2H), 3.14 (d, *J* = 5.8 Hz, 5H).

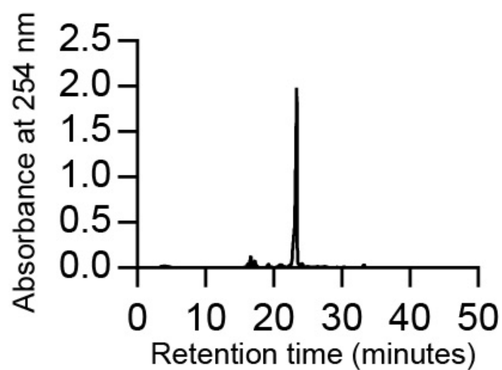
Final product: (9 mg, 60% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.18 – 7.98 (m, 2H), 7.85 (m, *J* = 20.2 Hz, 1H), 7.72 – 7.51 (m, 3H), 7.05 (m, *J* = 34.3 Hz, 4H), 5.28 (s, 2H), 4.67 – 4.21

(m, 6H), 3.93 (m, *J* = 36.5 Hz, 6H), 3.71 – 3.06 (m, 17H), 2.93 – 2.58 (m, 3H), 2.17 (s, 1H), 1.53 (d, *J* = 90.0 Hz, 6H). $C_{43}H_{54}N_8O_9S_3$, HRMS (ESI positive), [M+H] predicted m/z: 923.3249, found 923.3242. HPLC purity: 99% (shown below).

^{1}H NMR of *ortho*-ML349-biotin



HPLC analysis of *o*-ML349-Biotin



III. Experimental Procedures

ML349-biotin pull-downs.

Human HEK-293T cells were resuspended in phosphate buffered saline supplemented with 0.01% (v / v) triton-X (PBST) and sonicated briefly. Streptavidin-Agarose beads (EMD Millipore) (100 μL slurry) were incubated with 50 μM of ML349-biotin, *o*-ML349-biotin, or azide-PEG$_3$-biotin (Click Chemistry Tools) in PBST for 30 minutes at 4 °C, and washed three times. Next, 1 mg of cell lysate was added for 1 hour at 4 °C, centrifuged at 1000 x g for 1 minute, and then quickly washed 3 times with PBS. The enriched beads were then resuspended in 50 μL of PBS supplemented with 10 μM of ML349 and incubated for 1 hour at 4 °C. After centrifugation at 1000x g for 1 minute, the supernatant was transferred to another tube and treated with either 1 μM of FP-TAMRA for in-gel fluorescence or further processed for mass spectrometry analysis, diluting the sample in 6 M urea (4-fold) for 20 minutes at room temperature. Next, the sample was reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 20 minutes at 37 °C, and alkylated with 20 mM iodoacetamide for 1 hour at 37 °C in the dark. After 3-fold dilution in PBS, 2.5 μg of mass spectrometry-grade trypsin (Promega) was added and incubated overnight at 37 °C. Samples were desalted using Oasis HLB μElution plates (Waters), lyophilized using the Savant SPD1010 SpeedVac (Thermo Scientific) and reconstituted in LC-MS buffer (3% acetonitrile in LC-MS grade water, 0.1% formic acid, and 10 fmol/μL *Saccharomyces cerevisiae* alcohol dehydrogenase (P00330) digest, Waters).

LC-MS proteomic analysis and data processing.

Tryptic peptides were injected onto a 1D Waters NanoAquity UPLC system UPLC system equipped with a 5 μM Symmetry C18 (180 μm x 20 mm) trap column and a 1.8 μm High Strength Silica (HSS-T3) analytical column (75 μm x 150 mm). Initial loading of tryptic peptides was performed over 3 minutes by loading onto a trap column, followed by analytical separation over a 90-minute gradient (7% to 35% acetonitrile) coupled to a Waters Synapt G2S HDMS time-of-flight mass spectrometer with ion mobility separation using data independent acquisition methods (Waters Corp.). The nanoLC flow rate was 500 nl / min. Lock mass (Glu-fibronogen peptide, m/z = 785.8426) was collected every 30 seconds for internal reference mass calibration. The capillary voltage was set at 3.00 kV. The quadruple mass analyzer was manually set for mass 500, 600 and 700 and the sampling cone was set to operate at 32 eV. The nano flow gas was set to flow at 0.2 bar with the purge gas set to flow at 50 L / h and the source temperature was set at 70 °C. All measurements were collected with the mass spectrometer operating in resolution mode (resolving power of at least 20,000 FWHM (full-width at half maximum) in

positive-mode ESI for 400 m/z). For Ion Mobility Separation (IMS) wave height was set as 40 V and IMS wave velocity as 600 m/s, with the spectral acquisition time each mode being 0.5 s. Collision energy (CE) ramp from 16 eV to 60 eV during each 0.5 s-integration was used as standard setting for the elevated energy MS scan. Data was analyzed with Progenesis QI for proteomics software (Nonlinear dynamics). The false discovery rate (FDR) was set to 1% with 1 maximum missed cleavage. The minimum ion matching requirements were set to 2 fragments / peptide, 5 fragments / protein and 2 peptides / protein. The Top3 (3 most intense peptides) method was used for quantitative analysis. Briefly, the 3 most-intense peptides from each protein were used to quantify the average abundance of a protein[2]. Proteins with a minimum of 5-fold enrichment, a *p*-value less than 0.05, and at least 3 quantified peptides were assigned as putative ML349-biotin binding proteins.

Recombinant protein expression and purification.

Human *LYPLA1* (*APT1*), *LYPLA2* (*APT2*), *Rab1A, NQO2*, and *PDXK* genes were amplified from HEK-239T cell cDNA and sub-cloned into the pTrcHis2A bacterial expression vector (Sigma), transformed into BL21(DE3) cells, and induced with 0.5 mM IPTG for 16 hours at 25 º C. Cell pellets were lysed in 50 mM HEPES pH 7.8, 300 mM NaCl, and 10 % glycerol by sonication and cleared by centrifugation at 35000 x g for 30 minutes. The supernatant was incubated with Talon cobalt affinity beads for 1 hour (Invitrogen), washed, and eluted with imidazole. The eluted samples were dialyzed overnight in 50 mM HEPES pH 7.8, 150 mM NaCl and supplemented with 20 % glycerol before for storage at -80 º C.

Steady-state kinetic analysis.

Resorufin acetate (Sigma) hydrolysis assays were performed to characterize APT2 inhibition, subtracting any spontaneous hydrolysis from the catalytic-dead enzyme (APT2-S122A)[1]. Steady-state inhibitors IC_{50} values were calculated by pre-incubating 10 nM protein with varying inhibitor concentrations for 30 minutes at room temperature. A fixed concentration of substrate (50 μM final) was aliquoted into each well and the reaction was initiated by the addition of the enzyme-inhibitor mixture and initial rates were measured. The data containing 8 replicates per inhibitor concentration was imported to Graphpad Prism 6 and a standard non-logarithmic sigmoidal dose response curve was fitted to each enzyme experiment. Each K_i value was calculated using Cheng-Prusoff equation, referencing previously reported K_m values. Fluorescence polarization assays were performed as previously described.[1]

Native mass spectrometry analysis.

Native MS experiments were performed using a Synapt G2 ion mobility-mass spectrometer (Waters Corp.). Samples were buffer exchanged into 200 mM ammonium acetate using a Micro Biospin column (Bio-Rad, Inc. Hercules, California). Each protein was incubated at a final concentration of 10 μM protein : 10 μM ligand (ML349 or ML349-biotin) in 5% DMSO for 5 min. Samples were electrosprayed from in-house gold-coated borosilicate capillaries. Instrument parameters were optimized for high transmission of protein-ligand ions with minimal gas-phase dissociation. The backing pressure was set to ~8 mBar and the capillary potential was set to 1.6 kV, with a sampling cone potential of 30 V. For monomeric species, the trap collision energy was set to 20 V for efficient transmission of ions with no observable unfolding or ligand dissociation. For dimeric species, the trap collision energy was set to 50 V for more efficient desolvation, with no apparent ligand dissociation. Spectra were acquired from 1000 to 10000 m/z for 1 minute. Other instrument parameters were set to those found described as optimal in previous literature[3]. The resulting datasets were processed and deconvolved using Unidec[4] to yield relative abundances of apo and bound proteins. Relative abundance ratios were also validated manually using background subtraction and integration tools mMass[5]. Ratios of manually integrated peak areas were compared with the relative intensities derived from Unidec and found to be within 5% error in all cases.

11(12):3374-3382

IV. References

[1] Won, S. J., Davda, D., Labby, K. J., Hwang, S. Y., Pricer, R. E., Majmudar, J. D., Armacost, K. A., Rodriguez, L. A., Rodriguez, C. L., Chong, F. S., Torossian, K. A., Palakurthi, J., Hur, E. S., Meagher, J. L., Brooks, C. L., Stuckey, J. A., and Martin, B. R. (2016) Molecular mechanism for isoform-selective inhibition of acyl protein thioesterases 1 and 2 (APT1 and APT2), *ACS Chem. Biol.* 11(12):3374-3382.

[2] Silva, J. C., Gorenstein, M. V., Li, G.-Z., Vissers, J. P. C., and Geromanos, S. J. (2006) Absolute Quantification of Proteins by LCMSE : A Virtue of Parallel ms Acquisition, *Mol. Cell. Proteomics 5*, 144-156.

[3] Zhong, Y., Hyung, S. J., and Ruotolo, B. T. (2011) Characterizing the resolution and accuracy of a second-generation traveling-wave ion mobility separator for biomolecular ions, *Analyst 136*, 3534-3541.

[4] Marty, M. T., Baldwin, A. J., Marklund, E. G., Hochberg, G. K., Benesch, J. L., and Robinson, C. V. (2015) Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles, *Anal. Chem. 87*, 4370-4376.
[5] Strohalm, M., Kavan, D., Novak, P., Volny, M., and Havlicek, V. (2010) mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data, *Anal. Chem. 82*, 4648-4651.