Supporting Information

Development of near-infrared fluorophore (NIRF)-labeled activity-based probes for in vivo imaging of legumain

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General methods for solid phase peptide synthesis.

AOMK inhibitor (LI-0) and probe (LP-0) were synthesized as described previously (*1*). All aza-peptidyl epoxide inhibitor (LI-1) and probes (LP-1, tat LP-1, r8 LP-1, penetratin LP-1 and cholesterol LP-1) were synthesized by following the previously reported procedures (*2, 3*) with slight modifications. Fmoc protecting groups from Rink SS resin (0.75 mmol/g) were removed by treatment with 20% piperidine in DMF for 15 min, followed by three washes with DMF. A 1.2 M solution of bromoacetic acid (10 eq) in NMP and DIC (10 eq) were added to the resin. The resin was shaken 1.5 hrs and washed three times. A solution of Mono-Fmoc protected hydrazide (3 eq) in NMP was added and shaken overnight. Resin loading was determined by Fmoc-quantification (0.2-0.3 mmol/g). A 0.5M solution of *N*-Fmoc-protected amino acid (3 eq.) and HOBt (3 eq.) in DMF and DIC (3 eq.) were added to the resin. The resin was shaken 1.5-2hrs. For each of the following steps, Fmoc-deprotection and coupling reactions were repeated as described above. Capping of *N*-terminal amine was achieved by shaking the resin with a

0.5 M solution of acetic anhydride (5 eq.) and DIEA (5 eq.) in DMF for 5 min.

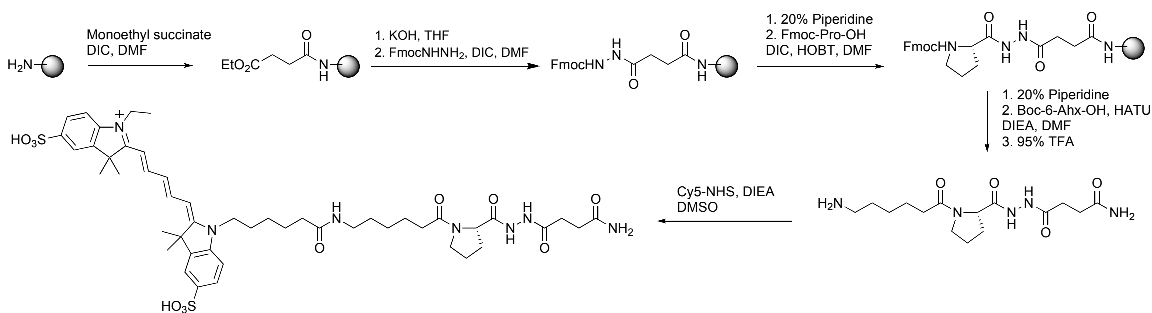
General method of cleavage from the resin and purification of peptides.

The 3-way nylon stopcock was replaced with polypropylene Needle Valve (Waters). A solution of 95% TFA/2.5% TIS/2.5% H_2O was added to the resin. After standing for 1h, the cleavage mixture was collected, and the resin was washed with the fresh cleavage solution and 1:1 mixture of water and acetonitrile. The combined fractions were evaporated and the crude residue was purified by HPLC. Fractions containing product were collected and lyophilized to dryness.

Synthesis of LP-1 ctrl

LP-1 ctrl was synthesized by following Scheme S1. Rink SS resin was deprotected and loaded with monoethyl succinate (5 eq) and DIC (5 eq) in DMF for 2 hrs. The rest of the amino acids were added successively and the final product was purified by following the same methods described above.

Scheme S1. Synthesis of LP-1 ctrl



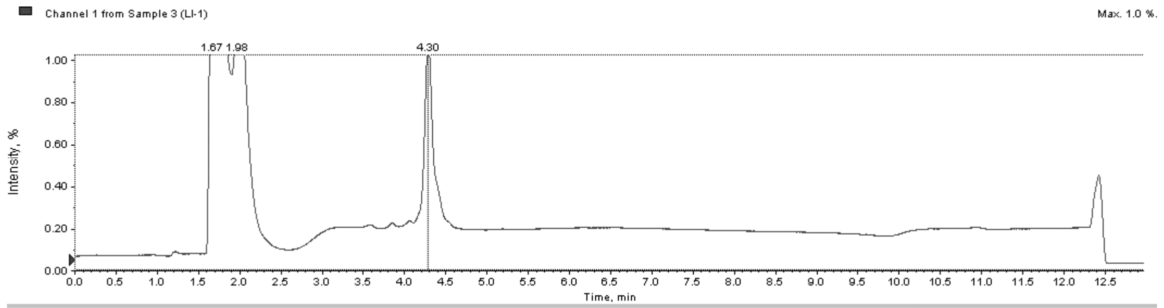
Characterization of all compounds.

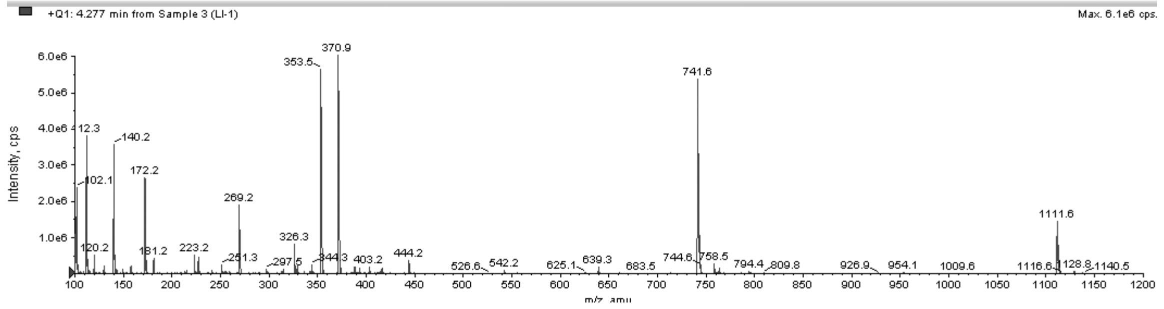
All final compounds used for biological studies were purified by HPLC and characterized by either [a] high-resolution mass spectrometry (HRMS) or [b] MALDI-TOF.

LI-1 $[M+H]^+$ calcd. for $C_{15}H_{23}N_4O_7$, 371.1567; found 371.1568[a]
LI-0 $[M+H]^+$ calcd. for $C_{21}H_{27}N_2O_7$,419.1818; found 419.1816[a]
LP-1 $[M+H]^+$ calcd. for $C_{52}H_{70}N_7O_{14}S_2$, 1080.4422; found 1080.4692[a]
LP-0 $[M+H]^+$ calcd. for $C_{58}H_{74}N_5O_{14}S_2$, 1128.4674; found 1128.4711[a]
LP-1 Ctrl $[M+H]^+$ calcd. for $C_{48}H_{66}N_7O_{11}S_2$, 980.4262; found 980.4435[a]
tat LP-1 $[M+H]^+$ calcd. for $C_{123}H_{203}N_{42}O_{29}S_3$, 2828.4863; found 2828.61[b]
r8 LP-1 $[M+H]^+$ calcd. for $C_{118}H_{193}N_{44}O_{28}S_3$, 2770.4188; found 2770.42[b]
Penetratin LP-1 $[M]^+$ calcd. for $C_{174}H_{261}N_{45}O_{40}S_4$, 3748.8655; found 3748.9038[a]
Cholesterol LP-1 $[M+H]^+$ calcd. for $C_{92}H_{133}N_{10}O_{18}S_3$, 1761.8956; found 1762.12[b]

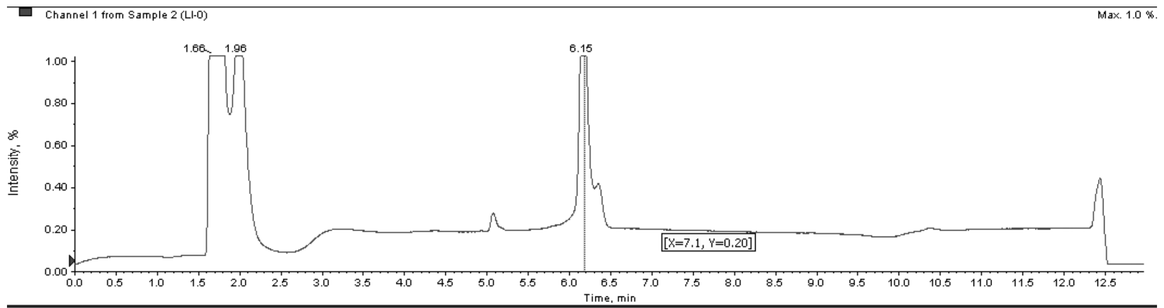
LC-MS spectra of the purified compounds (top: UV trace at 215nm, bottom: observed mass of the major peak from the UV trace). All of the peptides were purified by HPLC and characterized by HR-MS, All of the compounds are over 97% pure. However, some of the compounds (LI-0, LP-1, LP-0, LP-1 ctrl) showed a minor shoulder in each LC-MS trace. All of these peaks exhibit identical mass and fragmentation pattern compared to their parent peaks, therefore we believe that these peaks are racemized byproducts that occur when using standard DIC/HOBT coupling methods. We also believe that based on the area of each peak the amounts of these impurities are minimal (3~7%) and the nature of these impurities did not affect the outcome of the overall experiments

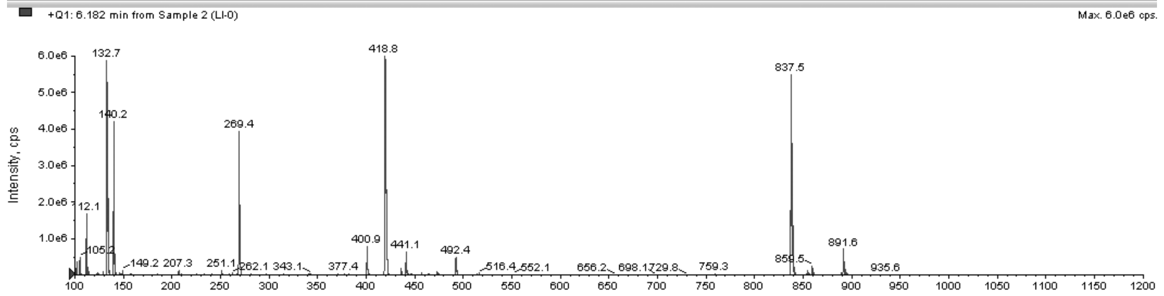
LI-1



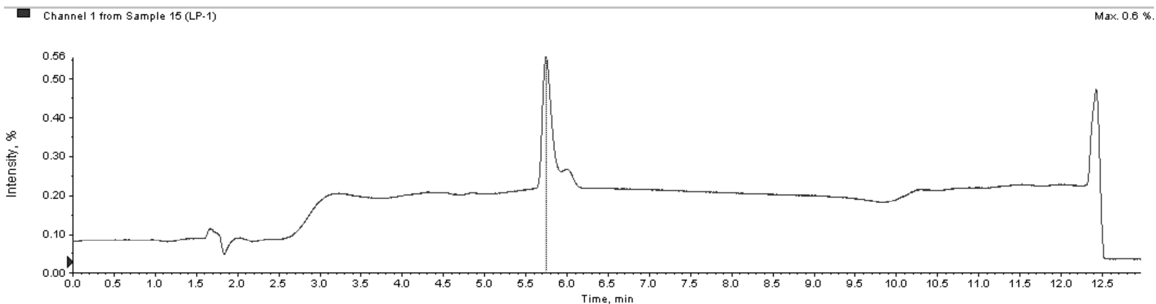


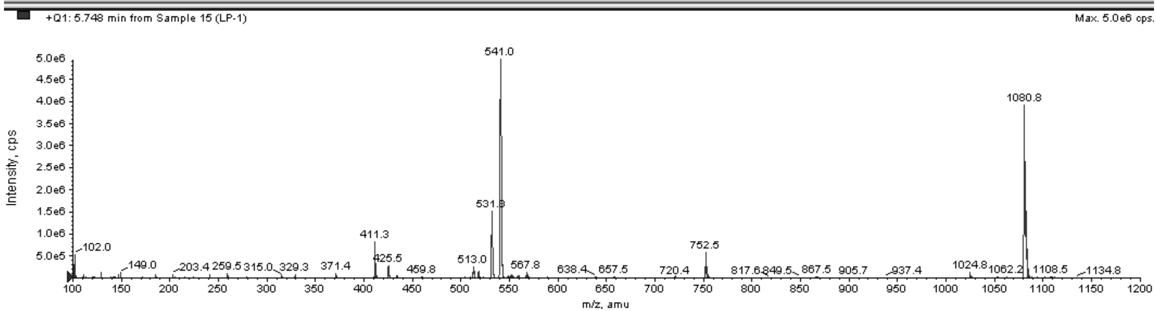
LI-0



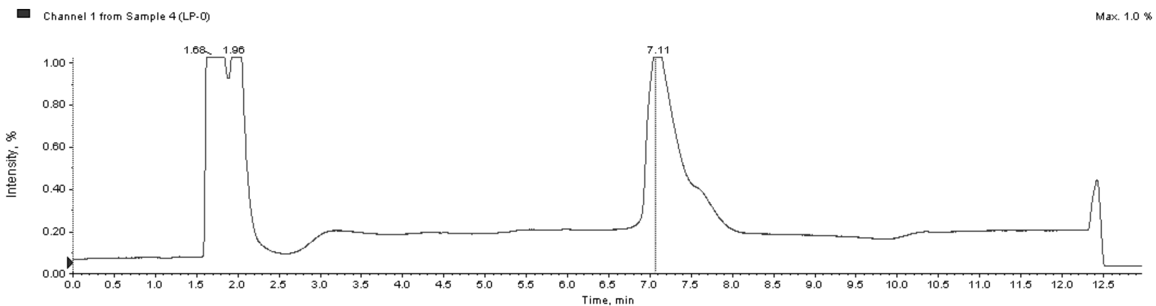


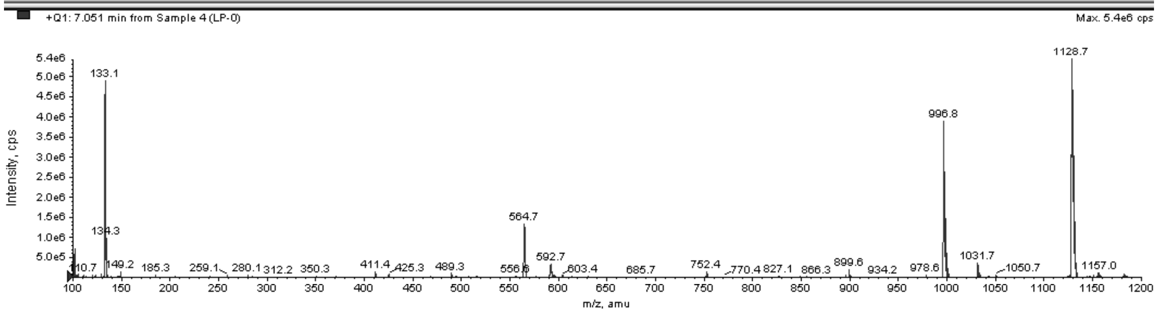
LP-1



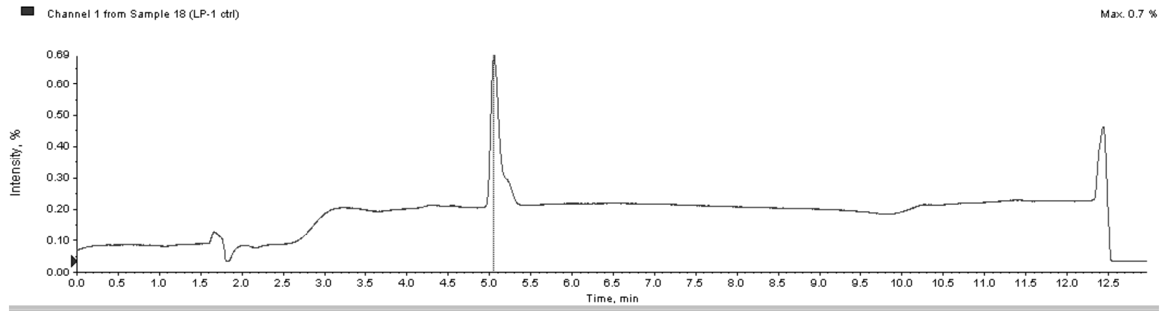


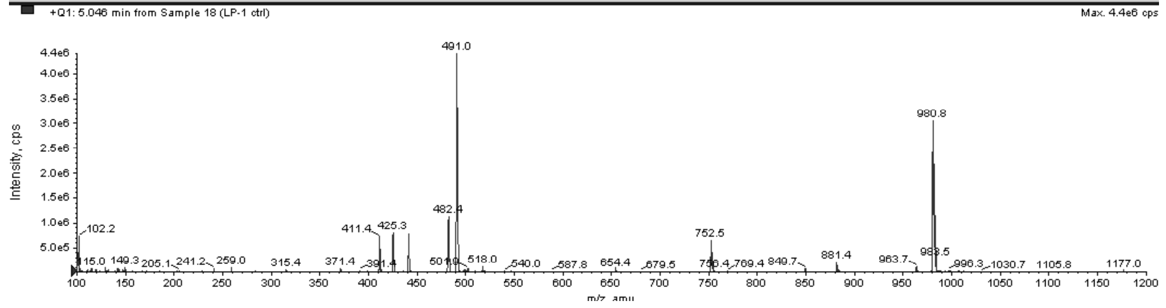
LP-0



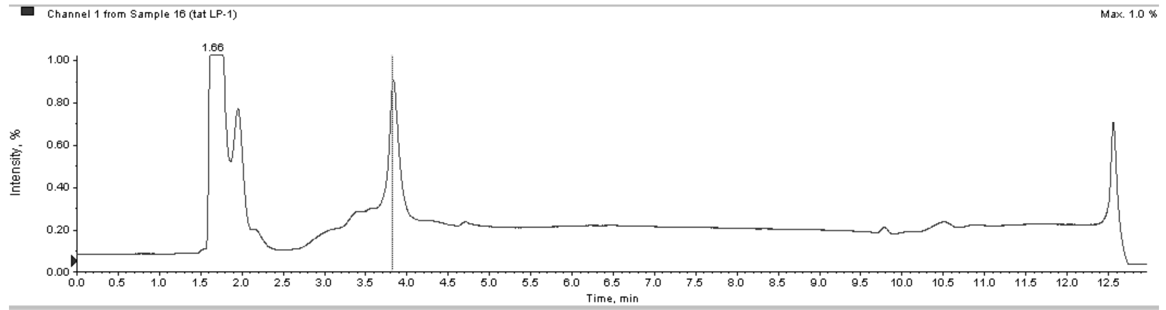


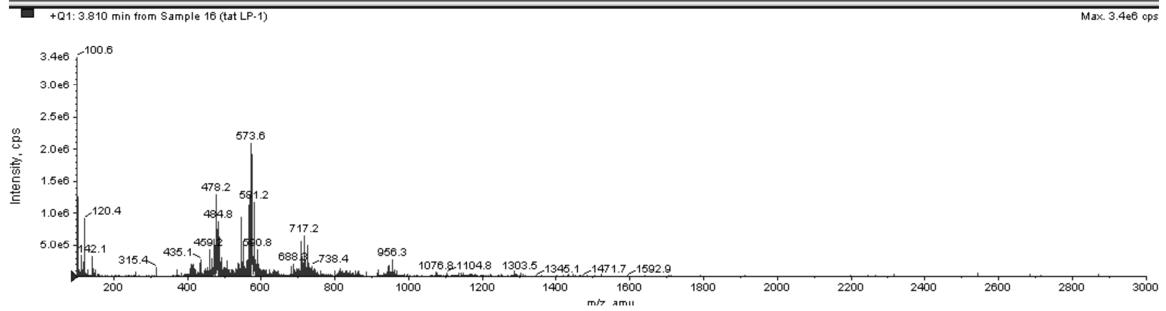
LP-1 Ctrl



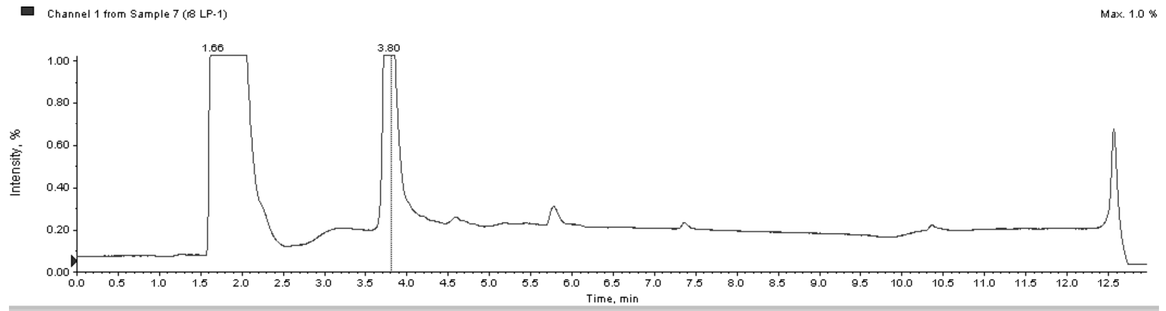


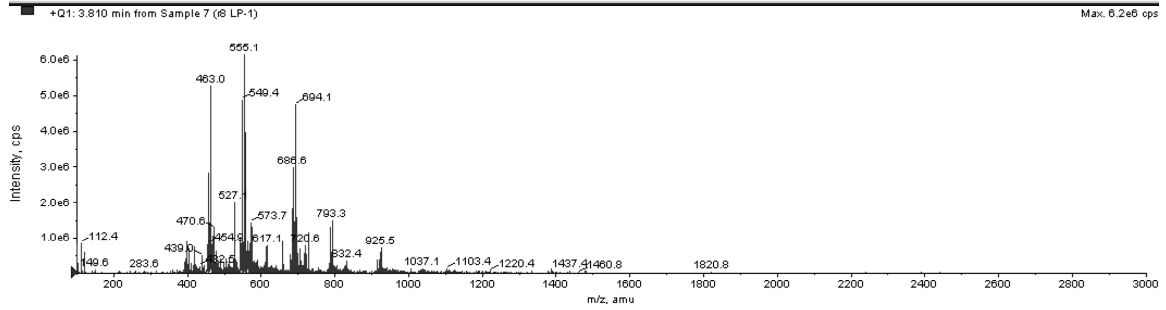
tLP-1



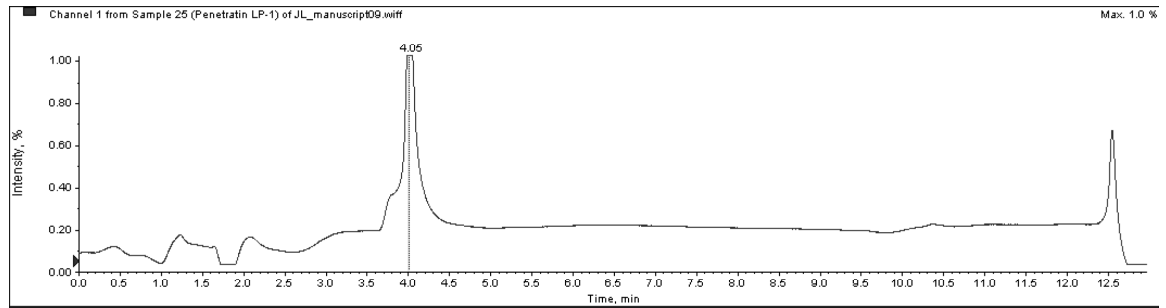


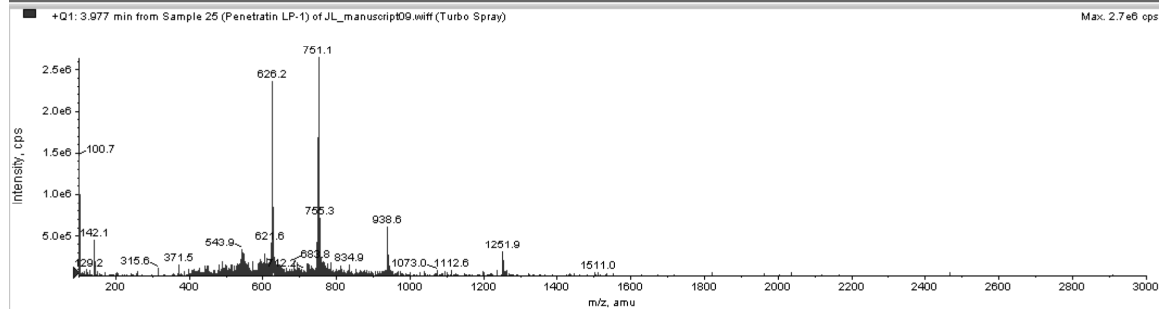
r8 LP-1



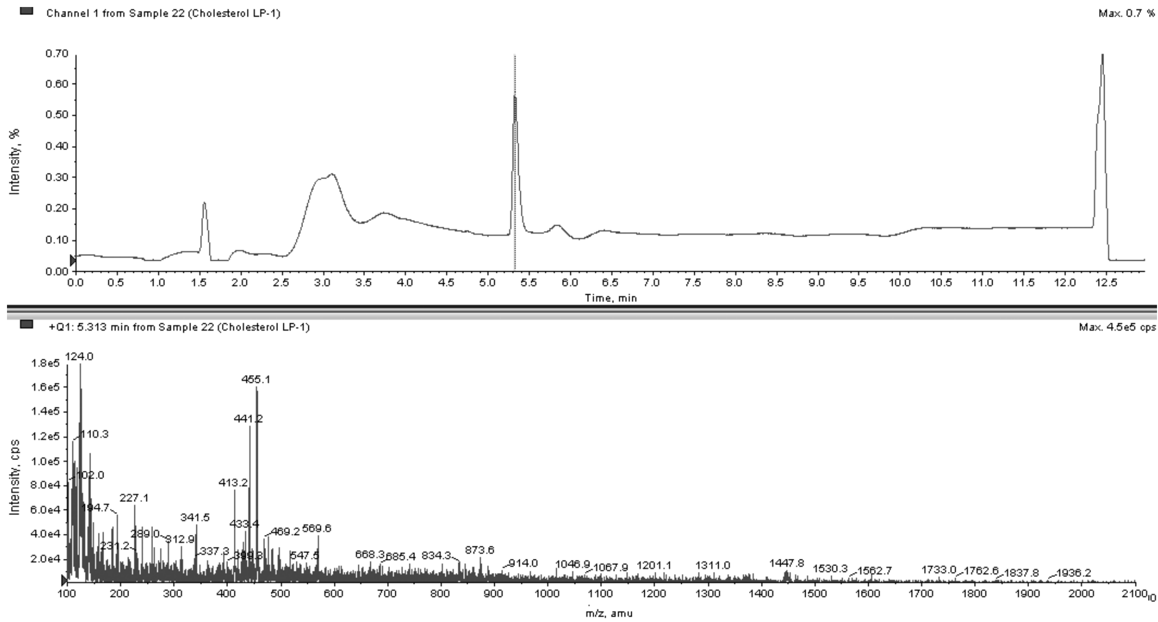


Penetratin LP-1



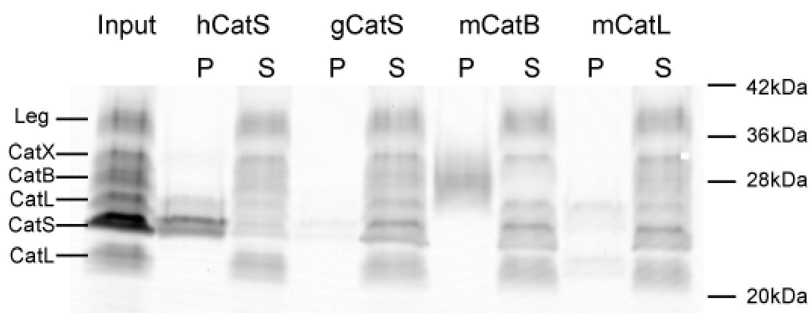


Cholesterol LP-1



Cathepsin immunoprecipitation

Figure S1. Immunoprecipitation of cathepsins in intact raw 264.7 cells. Cells were pretreated with 100 μM of LI-1 for 1 hr and labeled by addition of LP-1 (25 μM). High concentration of LP-1 was used to bring out all the off-target labeling in intact cells.



RAW 264.7 macrophages (600,000 cells/well) were seeded in a 6 well plate 24 hrs prior to labeling. Cells were pretreated with 100 μM of LI-1 for 1 hr and labeled by addition of LP-1 (25 μM). High concentration of LP-1 was used to bring out all the off-target labeling in intact cells. Cells were washed with PBS buffer and lysed by addition of citrate-phosphate lysis buffer (50mM citrate-phosphate, 1% CHAPS, 0.5% Triton, 5mM DTT, pH 4.5). 20 μL of crude lysates were diluted to 500 ml in RIPA buffer (phosphate-buffered saline, 0.5% NP-40, 1mM EDTA, pH 7.4). Anti-mouse cathepsin B (E Weber

Halle), anti-mouse cathepsin L (R&D Systems), anti-goat cathepsin S (Abchem) and anti-human cathepsin S (R&D Systems) were added and mixed for 15 min at 0 °C. 40 μL of Protein A/G Plus Agarose beads (Santa Cruz Biotechnology) were added and mixed overnight at 4 °C. Samples were spun down and supernatant and beads were separated. Beads were washed with RIPA buffer and boiled after addition of sample buffer 2X (20% glycerol, 100 mM Tris HCl, pH 6.8, 6% SDS, and 10% b-mercaptoethanol). Acetone was added to the supernatant and kept at –80 °C for 2 h. Precipitated proteins were collected by centrifugation in the cold for 30 min, dried, and dissolved by boiling in sample buffer. Samples without immunoprecipitation (input), pellets (P) and supernatant (S) were separated on a 12.5% SDS-PAGE and visualized by scanning of the gel with a Typhoon flatbed laser scanner (excitation/emission 633/680 nm).

1. Kato, D., Boatright, K. M., Berger, A. B., Nazif, T., Blum, G., Ryan, C., Chehade, K. A., Salvesen, G. S., and Bogyo, M. (2005) Activity-based probes that target diverse cysteine protease families, *Nat Chem Biol 1*, 33-38.
2. Kato, D., Verhelst, S. H., Sexton, K. B., and Bogyo, M. (2005) A general solid phase method for the preparation of diverse azapeptide probes directed against cysteine proteases, *Org Lett 7*, 5649-5652.
3. Edgington, L. E., Berger, A. B., Blum, G., Albrow, V. E., Paulick, M. G., Lineberry, N., and Bogyo, M. (2009) Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes, *Nat Med 15*, 967-973.