Supporting information

Development of activity-based probes for cathepsin X

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General Methods. Unless otherwise noted, all resins and reagents were obtained from commercial suppliers and used without further purification. All solvents used were HPLC-grade and also purchased from commercial suppliers. All water sensitive reactions were performed in anhydrous solvents and under a positive pressure of argon. Reactions were analyzed by liquid chromatography-mass spectrometry (LC-MS) performed on an Agilent 1100 liquid chromatography system with an API 150EX single quadrupole mass spectrometer (Applied Biosystems). HPLC purifications were carried out on an ÄKTA explorer 100 (Amersham Pharmacia Biotech) with either C_4 or C_{18} reversed-phase columns (Waters Corp.). The mobile phase consisted of 95:4.9:0.1 water:acetonitrile:trifluoroacetic acid (TFA) and 99.9:0.1 acetonitrile:TFA. High-resolution mass spectrometry (HRMS) was performed using an LTQ-FTMS (Thermo Fisher Scientific).

Compound synthesis.

Synthesis of GB123 and Cy5DCG04.

GB123 was synthesized by following the previously reported procedure.[1] Cy5DCG04 was synthesized by following a previously reported solid-phase synthesis technique[2], and the Cy5 fluorophore was conjugated to the purified peptide at the final step. Briefly, the free amino version of DCG-04 (1.1 mg, 1.7 μmol, 1 eq) and Cy5-OSu (1.2 mg, 1.5 μmol, 0.9 eq) were dissolved in 150 μL of DMSO. Diisopropylethylamine (DIEA) was then added (2 μL, 11.4 μmol, 5 eq), and the compounds were allowed to react for 1 h at room temperature. The product was purified by HPLC using a gradient of 0-20% water:acetonitrile + 0.1% trifluoroacetic acid (TFA) for 5 column volumes, followed by a gradient of 20-70% water:acetonitrile + 0.1% TFA for 15 column volumes. Fractions containing the product were pooled, frozen, and evaporated to dryness to give 1.6 mg (1.2 μmol, 80%) of Cy5DCG04 as a blue solid.

Synthesis of MGP151.

The synthesis of MGP151 is similar to the synthesis of GB123 with slight modifications.[1] The chloromethylketone (CMK) of Fmoc-Leu-OH (Fmoc-Leu-CMK) and the carbazate linker on aminopolystyrene resin were prepared as previously described.[3] To synthesize MGP151, the carbazate resin was swelled in DMF for 15 min. A solution of Fmoc-Leu-CMK (3 eq) in DMF was added to the resin, and the resin was agitated for 3 h at 50 ^{o}C. The solution was drained, the resin was rinsed with DMF (3 X 3 mL), and 2,6-dimethylbenzoic acid (3 eq) and potassium fluoride (10 eq) in DMF were added to the resin. The resin was agitated for 14 h at room temperature. The solution was drained, and

the resin was rinsed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Note: To remove the excess potassium fluoride, float the resin in CH_2Cl_2 and transfer resin to a new tube (potassium fluoride sinks in CH_2Cl_2). The Fmoc group was then removed from the Leu residue using 5% diethylamine (DEA) in DMF for 15 min, followed by washing with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Fmoc-Tyr(Bu)-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the Tyr residue using 5% DEA in DMF for 15 min, followed by washing with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Fmoc-6-aminohexanoic acid (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the 6-aminohexanoic acid residue using 5% DEA in DMF for 15 min, followed by washing with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Fmoc-Lys(Boc)-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the Lys residue using 5% DEA in DMF for 15 min, followed by washing with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The resin was then acetylated using acetic anhydride (1.5 eq) and DIEA (2 eq) in CH_2Cl_2 for 1 h are room temperature. The peptide was cleaved

from the resin using a cleavage cocktail of 95% TFA, 2.5% TIS, and 2.5% water for 2 h at room temperature. The peptide was purified by HPLC using a gradient of 0-20% water:acetonitrile + 0.1% TFA for 2 column volumes, followed by a gradient of 20-80% water:acetonitrile + 0.1% TFA for 6 column volumes. Fractions containing the peptide product were pooled, frozen, and evaporated to dryness. This peptide was then coupled to the Cy5 fluorophore. Briefly, the peptide (1.0 mg, 1.4 μmol, 1 eq) and Cy5-OSu (1.5 mg, 1.9 μmol, 1.4 eq) were dissolved in 100 μL of DMSO. DIEA was then added (1.2 μL, 6.9 μmol, 5 eq), and the compounds were allowed to react for 1 h at room temperature. The product was purified by HPLC using a gradient of 0-20% water:acetonitrile + 0.1% TFA for 3 column volumes, followed by a gradient of 20-80% water:acetonitrile + 0.1% TFA for 10 column volumes. Fractions containing the product were pooled, frozen, and evaporated to dryness to give 1.8 mg (1.3 μmol, 95%) of MGP151 as a blue solid.

Synthesis of MGP140.

The synthesis of MGP140 is similar to the synthesis of Cy5DCG04 with slight modifications. Rink resin SS was swelled in DMF for 15 min, and the Fmoc group was removed by treatment with a 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with 3 X 3 mL of DMF and 3 X 3 mL of CH_2Cl_2. Fmoc-Phe-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the Phe residue, followed by washing with DMF and CH_2Cl_2 (as described above).

Fmoc-Phe-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the Phe residue, followed by washing with DMF and CH_2Cl_2 (as described above). Fmoc-Lys(Boc)-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was then removed from the Lys residue, followed by washing with DMF and CH_2Cl_2 (as described above). Ethyl (2*S*, 3*S*)-oxirane-2,3-dicarboxylate (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 14 h at room temperature. The solution was drained, and the resin was rinsed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The peptide was cleaved from the resin using a cleavage cocktail of 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% water for 2 h at room temperature. The peptide was purified by HPLC using a linear gradient of 25-80% water:acetonitrile + 0.1% TFA. Fractions containing the peptide product were pooled, frozen, and evaporated to dryness. This peptide was then coupled to the Cy5 fluorophore. Briefly, the peptide (0.7 mg, 1.2 μmol, 1 eq) and Cy5-OSu (1.5 mg, 1.9 μmol, 1.5 eq) were dissolved in 100 μL of DMSO. DIEA was then added (1 μL, 5.7 μmol, 5 eq), and the compounds were allowed to react for 1 h at room temperature. The product was purified by HPLC using a gradient of 0-15% water:acetonitrile + 0.1% TFA for 5 column volumes, followed by a gradient of 15-40%

water:acetonitrile + 0.1% TFA for 10 column volumes. Fractions containing the product were pooled, frozen, and evaporated to dryness to give 1.4 mg (1.1 μmol, 93%) of MGP140 as a blue solid.

Synthesis of MGP302.

MGP302 was synthesized on 2-chlorotritylchloride resin. The resin was swelled in CH_2Cl_2 for 5 minutes. A solution of Fmoc-1,6-diaminohexane-HCl (1.2 eq) and DIEA (1.2 eq) in 4:1 CH_2Cl_2:DMF was added to the resin and the reaction was agitated for 5 min at room temperature, after which another 1.8 eq of DIEA was added. The resin was agitated for an additional 1 h at room temperature, and then MeOH was added to the reaction solution to cap the unreacted resin. The resin was agitated for an additional 30 min at room temperature, the solution was drained, and the resin was washed with CH_2Cl_2 (3 X 3 mL), DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), MeOH (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was removed from the diaminohexane residue using 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The non-natural amino acid Fmoc-1-Nal-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The Fmoc group was then removed from the 1-Nal residue, followed by washing with DMF and CH_2Cl_2 (as described above). The non-natural amino acid Fmoc-Phe(4-Me)-OH (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin

and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and DMF (3 X 3 mL). The Fmoc group was removed from the Phe(4Me) residue using 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Ethyl (2*S*, 3*S*)-oxirane-2,3-dicarboxylate (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 14 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The peptide was cleaved from the resin using a cleavage cocktail of 50% TFA, 3% TIS, and 47% CH_2Cl_2 for 2 h at room temperature. The peptide was purified by HPLC using a linear gradient of 20-80% water:acetonitrile + 0.1% TFA. Fractions containing the peptide product were pooled, frozen, and evaporated to dryness. This peptide was then coupled to the Cy5 fluorophore. Briefly, the peptide (0.9 mg, 1.5 μmol, 1 eq) and Cy5-OSu (1.5 mg, 1.9 μmol, 1.3 eq) were dissolved in 100 μL of DMSO. DIEA was then added (1.3 μL, 7.5 μmol, 5 eq), and the compounds were allowed to react for 1 h at room temperature. The product was purified by HPLC using a gradient of 10-20% water:acetonitrile + 0.1% TFA for 3 column volumes, followed by a gradient of 20-90% water:acetonitrile + 0.1% TFA for 15 column volumes. Fractions containing the product were pooled, frozen, and evaporated to dryness to give 1.2 mg (0.9 μmol, 63%) of MGP302 as a blue solid.

Synthesis of MGP310.

The synthesis of MGP310 is similar to the synthesis of AMS36 on BAL resin with slight modifications.[4] BAL resin was swelled in DMF for 15 min. 1-(aminomethyl)-napthalin (10 eq) was dissolved in DMF and added to the resin, and the reaction was agitated for 15 min at room temperature. $NaBH_3CN$ (10 eq) was added, and the resin was agitated for an additional 20 hours. The solution was drained, and the resin was washed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and MeCN (3 X 3 mL). Fmoc-Phe(4Me)-OH (10 eq) and HATU (9.5 eq) in DMF were added to the resin, followed by DIEA (20 eq). The reaction was agitated for 20 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The Fmoc group was removed from the Phe(4Me) residue using 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Ethyl (2*S*, 3*S*)-oxirane-2,3-dicarboxylate (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The resin was then treated with 1 M KOH in 3:1 THF:EtOH for 1 h at room temperature. The solution was drained, and the resin was washed with EtOH (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), DMF (3 X 3 mL), and CH_2Cl_2 (3 X 3 mL). Mono-trityl-1,6-diaminohexane (3 eq), PyBOP (3 eq), HOBt (3 eq), and DIEA (6 eq) were dissolved in DMF and added to the resin, and the resin was agitated for 20 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The peptide was cleaved from the resin using a cleavage cocktail of 95% TFA and 5% TIS for 1 h at room temperature. The

peptide was purified by HPLC using a linear gradient of 20-80% water:acetonitrile + 0.1% TFA. Fractions containing the peptide product were pooled, frozen, and evaporated to dryness. This peptide was then coupled to the Cy5 fluorophore. Briefly, the peptide (0.7 mg, 1.3 μmol, 1 eq) and Cy5-OSu (1.3 mg, 1.7 μmol, 1.3 eq) were dissolved in 100 μL of DMSO. DIEA was then added (1.2 μL, 6.9 μmol, 5 eq), and the compounds were allowed to react for 1 h at room temperature. The product was purified by HPLC using a gradient of 10-20% water:acetonitrile + 0.1% TFA for 3 column volumes, followed by a gradient of 20-90% water:acetonitrile + 0.1% TFA for 15 column volumes. Fractions containing the product were pooled, frozen, and evaporated to dryness to give 0.7 mg (0.6 μmol, 46%) of MGP310 as a blue solid.

Synthesis of MGP141.

The synthesis of MGP141 is identical to the synthesis of MGP140 with one simple modification. Instead of coupling the growing peptide to ethyl (2*S*, 3*S*)-oxirane-2,3-dicarboxylate, the peptide was coupled to monoethyl succinate (3 eq) using DIC (3 eq)/HOBt (3 eq) in DMF. Subsequent coupling steps, cleavage from the resin, purification of the peptide, and coupling of the peptide to Cy5 follow the same protocols as described for the synthesis of MGP140. MGP141 (2.2 mg, 1.8 μmol, 95%) was obtained as a blue solid.

Synthesis of bMGP310.

The synthesis of bMGP310 is similar to the synthesis of MGP310. BAL resin was swelled in DMF for 15 min. 1-(aminomethyl)-napthalin (10 eq) was dissolved in DMF

and added to the resin, and the reaction was agitated for 15 min at room temperature. $NaBH_3CN$ (10 eq) was added, and the resin was agitated for an additional 20 hours. The solution was drained, and the resin was washed with DMF (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), and MeCN (3 X 3 mL). Fmoc-Phe(4Me)-OH (10 eq) and HATU (9.5 eq) in DMF were added to the resin, followed by DIEA (20 eq). The reaction was agitated for 20 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The Fmoc group was removed from the Phe(4Me) residue using 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). Ethyl (2*S*, 3*S*)-oxirane-2,3-dicarboxylate (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 2 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The resin was then treated with 1 M KOH in 3:1 THF:EtOH for 1 h at room temperature. The solution was drained, and the resin was washed with EtOH (3 X 3 mL), CH_2Cl_2 (3 X 3 mL), DMF (3 X 3 mL), and CH_2Cl_2 (3 X 3 mL). Mono-Fmoc-1,6-diaminohexane (3 eq), PyBOP (3 eq), HOBt (3 eq), and DIEA (6 eq) were dissolved in DMF and added to the resin, and the resin was agitated for 20 h at room temperature. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The Fmoc group was removed from the 1,6-diaminohexane linker using 20% piperidine/DMF (v/v) for 15 min. The solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). D-(+)-biotin (3 eq) and HOBt (3 eq) were dissolved in DMF and added to the resin. DIC (3 eq) was then added to the resin and solution, and the resin was agitated for 20 h at room temperature. The

solution was drained, and the resin was washed with DMF (3 X 3 mL) and CH_2Cl_2 (3 X 3 mL). The peptide was cleaved from the resin using a cleavage cocktail of 95% TFA and 5% TIS for 1 h at room temperature. The peptide was purified by HPLC using a linear gradient of 20-90% water:acetonitrile + 0.1% TFA. Fractions containing the peptide product were pooled, frozen, and evaporated to dryness. bMGP310 (6.0 mg, 7.9 μmol, 24%) was obtained as a white solid.

Characterization of all compounds.

All final compounds used for biological studies were purified by HPLC and characterized by HRMS.

Cy5DCG04 $[M+H]^+$ calcd. for $C_{66}H_{91}N_8O_{16}S_2^+$, 1315.5989, found 1315.5957.

MGP151 $[M+H]^+$ calcd. for $C_{72}H_{96}N_7O_{15}S_2^+$, 1362.6400, found 1362.6375.

MGP140 $[M+H]^+$ calcd. for $C_{63}H_{78}N_7O_{14}S_2^+$, 1220.5043, found 1220.5018.

MGP302 $[M+H]^+$ calcd. for $C_{68}H_{83}N_6O_{13}S_2^+$, 1255.5454, found 1255.5411.

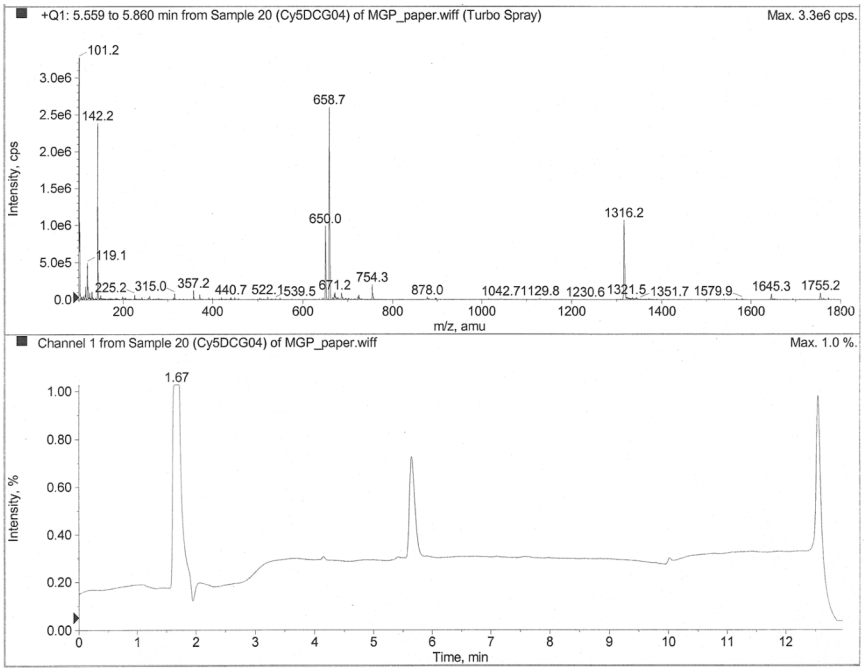
MGP310 $[M+H]^+$ calcd. for $C_{66}H_{77}N_6O_{11}S_2^+$, 1169.5086, found 1169.5055.

MGP141 $[M+H]^+$ calcd. for $C_{63}H_{80}N_7O_{13}S_2^+$, 1206.5250, found 1206.5208.

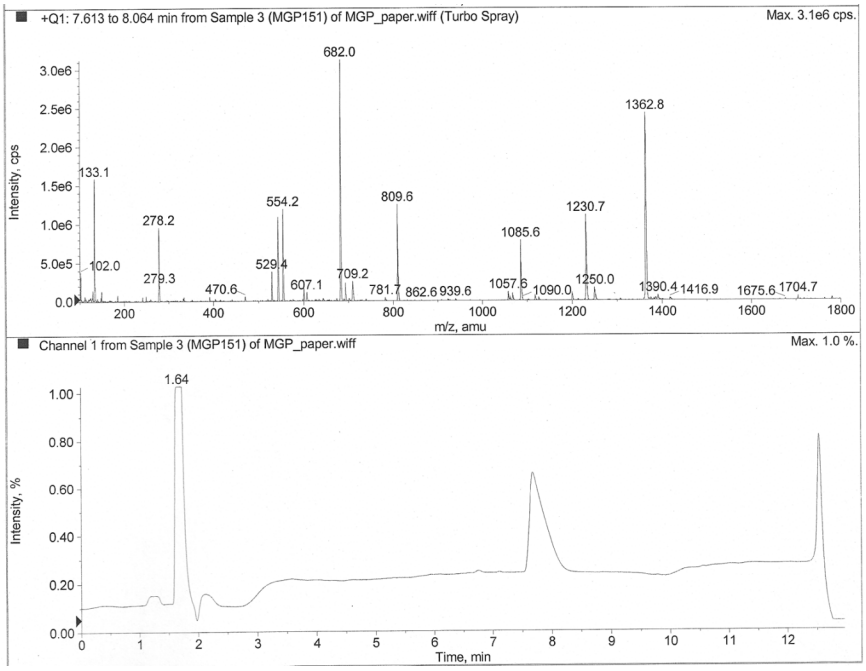
bMGP310 $[M+H]^+$ calcd. for $C_{41}H_{53}N_6O_6S^+$, 757.3747 found 757.3747.

LC-MS spectra of purified compounds (top: observed mass of the major peak from the UV trace, bottom: UV trace at 215 nm). All of the peptides were characterized by HPLC and characterized by HRMS. All of the compounds are ≥ 97% pure.

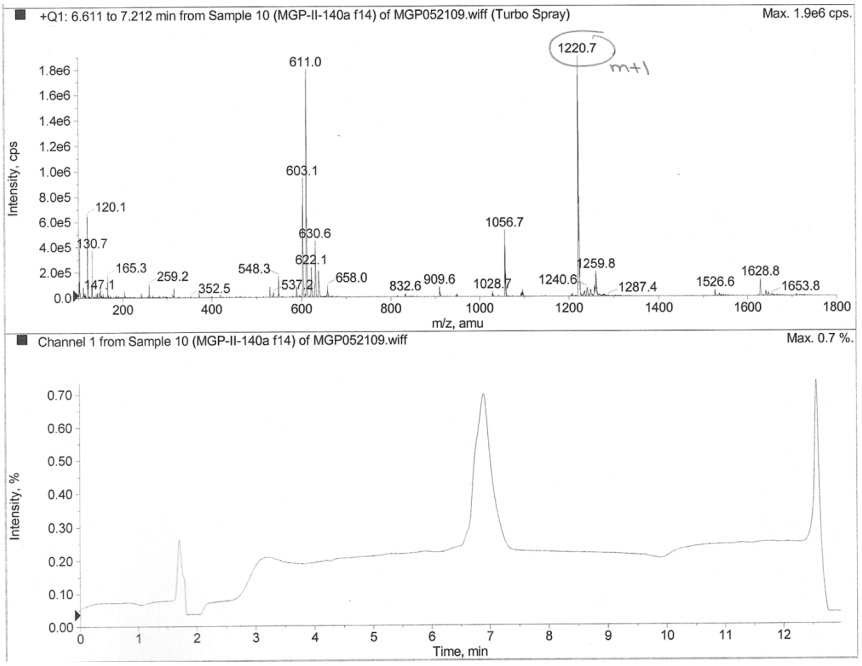
Cy5DCG04



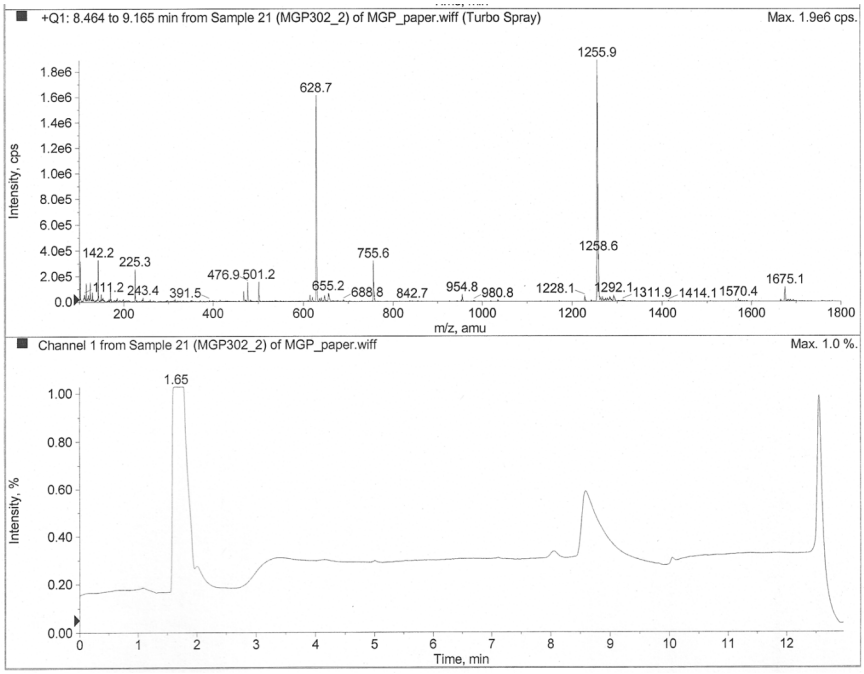
MGP151



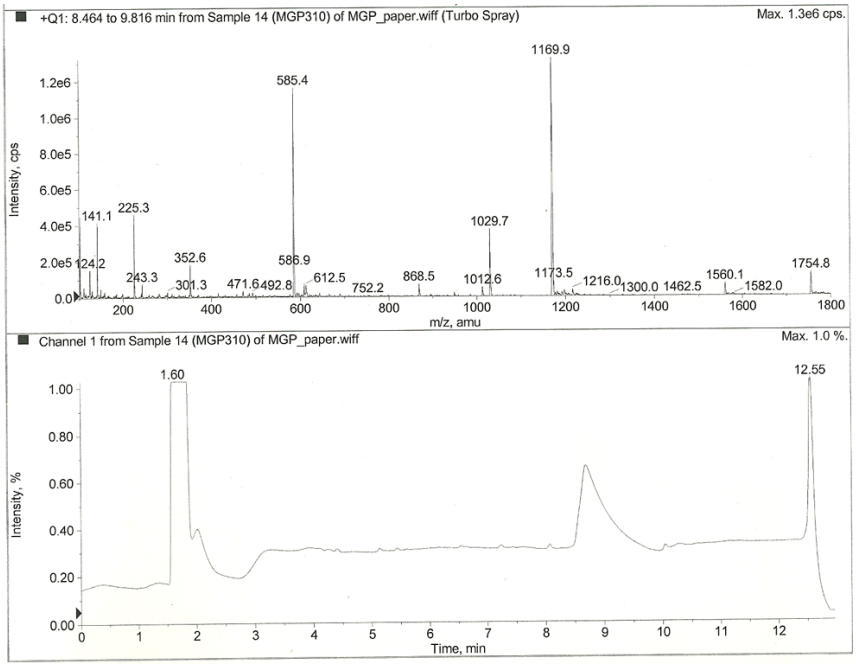
MGP140



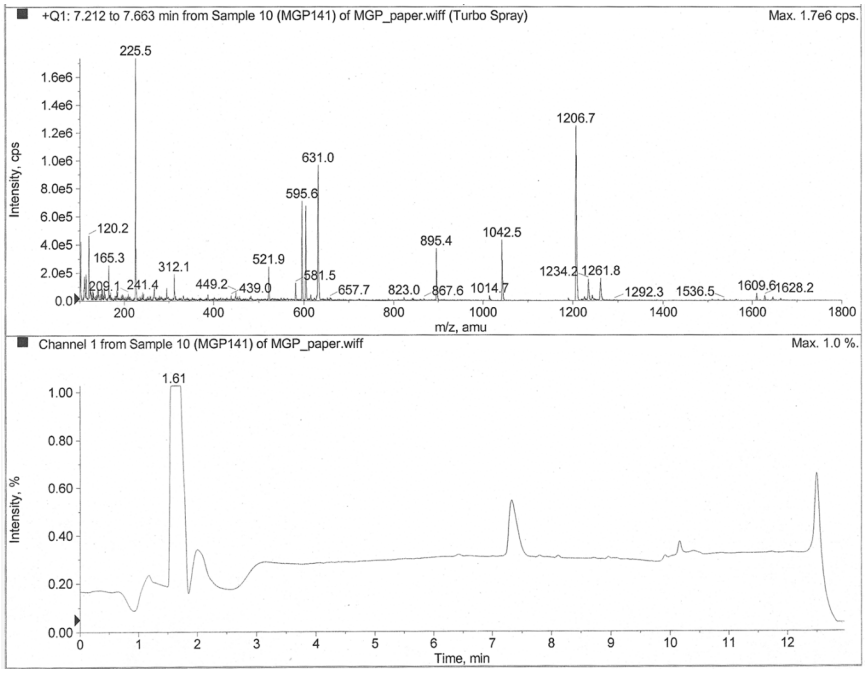
MGP302



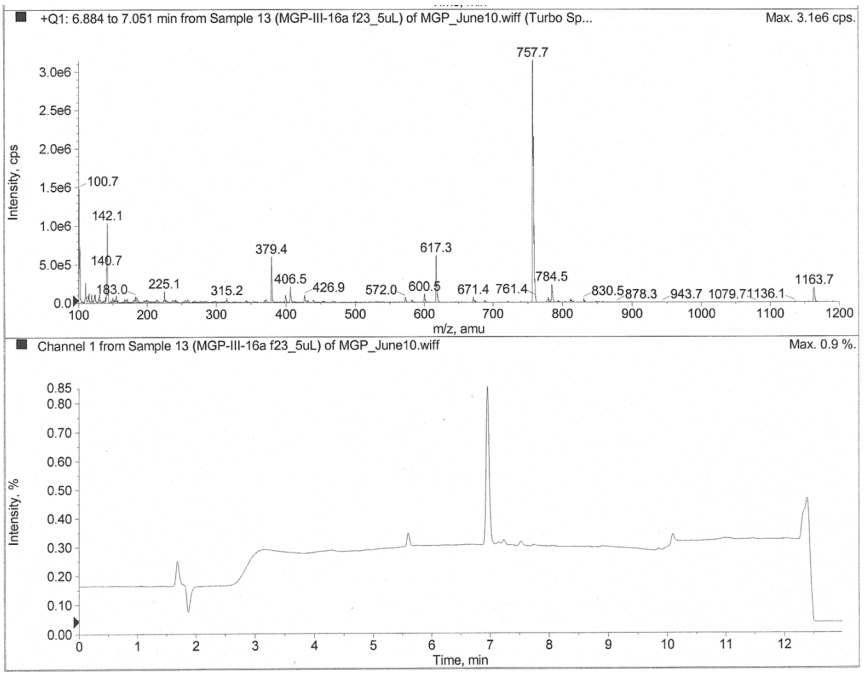
MGP310



MGP141



bMGP310



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(2) Greenbaum, D.; Medzihradszky, K. F.; Burlingame, A.; Bogyo, M. *Chem Biol* **2000**, *7*, 569-81.

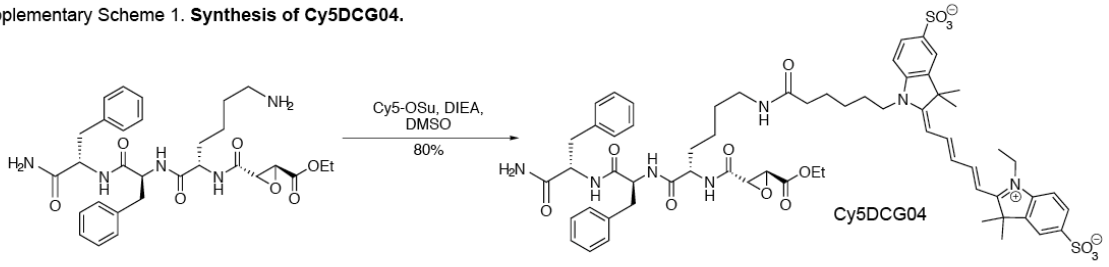
(3) Kato, D.; Boatright, K. M.; Berger, A. B.; Nazif, T.; Blum, G.; Ryan, C.; Chehade, K. A.; Salvesen, G. S.; Bogyo, M. *Nat Chem Biol* **2005**, *1*, 33-8.

(4) Sadaghiani, A. M.; Verhelst, S. H.; Bogyo, M. *J Comb Chem* **2006**, *8*, 802-4.

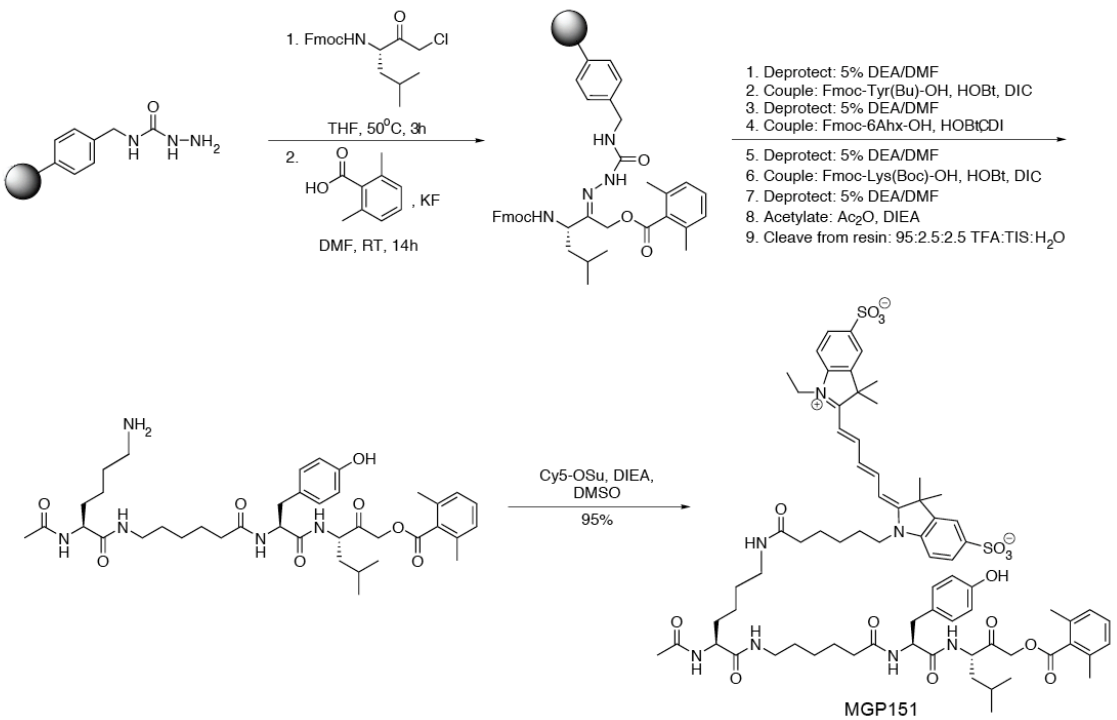
Supplementary Table 1. Quantifiaction of mean fluorescence units for cells shown in figure 5, panel c. Values were obtained from confocal images using ImageJ. Values for multiple cells were determined and averaged.

	MGP140	**GB111-NH_2/ MGP140**	**JPM-OEt/ MGP140**
Average MFI of cells	97.7 ± 0.6	91.0 ± 0.3	85.7 ± 0.2
Background fluorescence (MFI)	95.9	90.4	85.4
MFI-background	1.7 ± 0.6	0.6 ± 0.3	0.3 ± 0.2
Percent inhibition	n/a	65%	81%

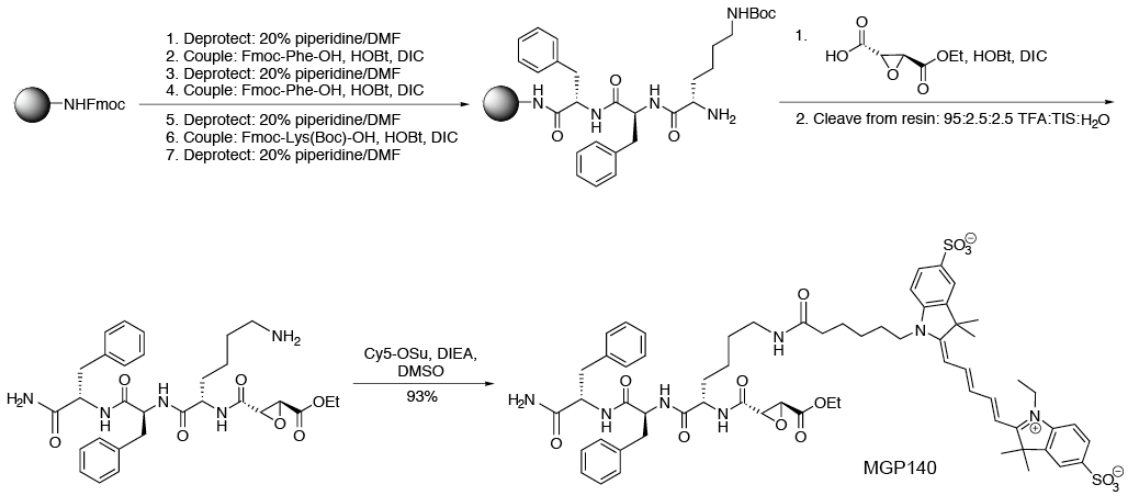
Supplementary Scheme 1. **Synthesis of Cy5DCG04.**



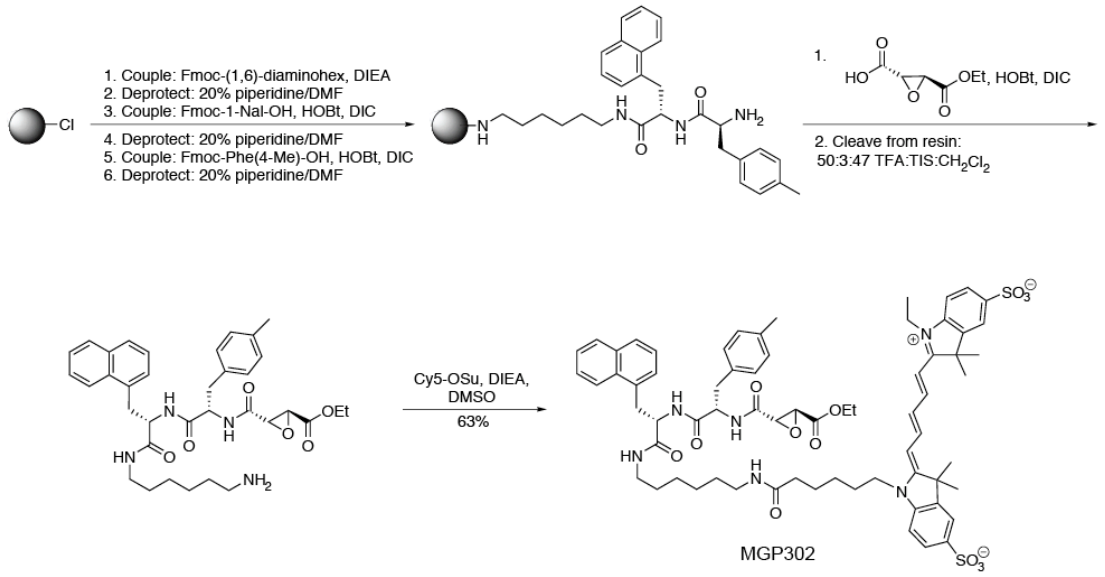
Supplementary Scheme 2. **Synthesis of MGP151.**



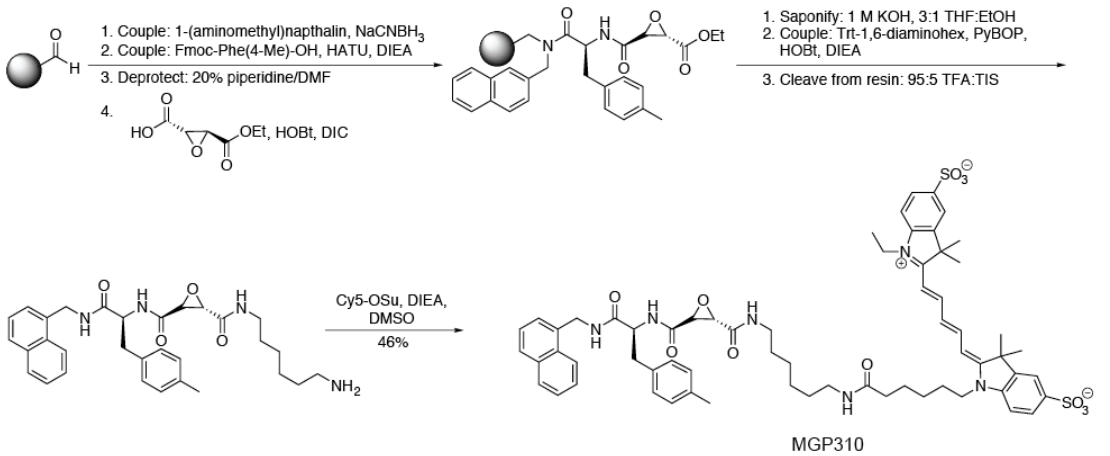
Supplementary Scheme 3. **Synthesis of MGP140.**



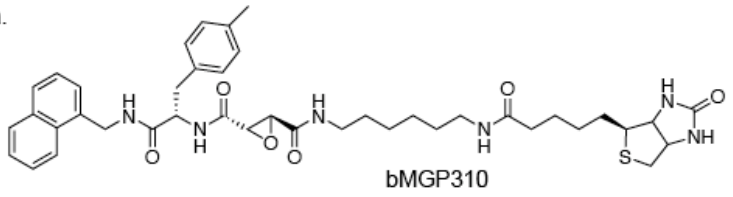
Supplementary Scheme 4. **Synthesis of MGP302.**



Supplementary Scheme 5. **Synthesis of MGP310**

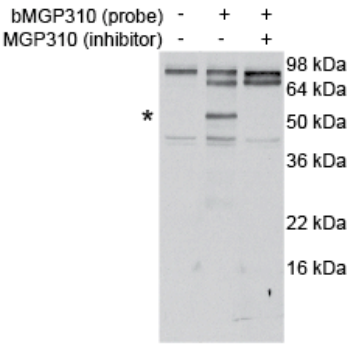


a.



bMGP310

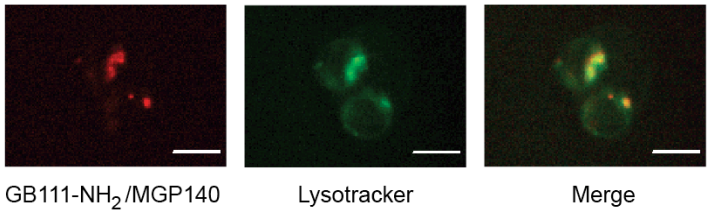
b.



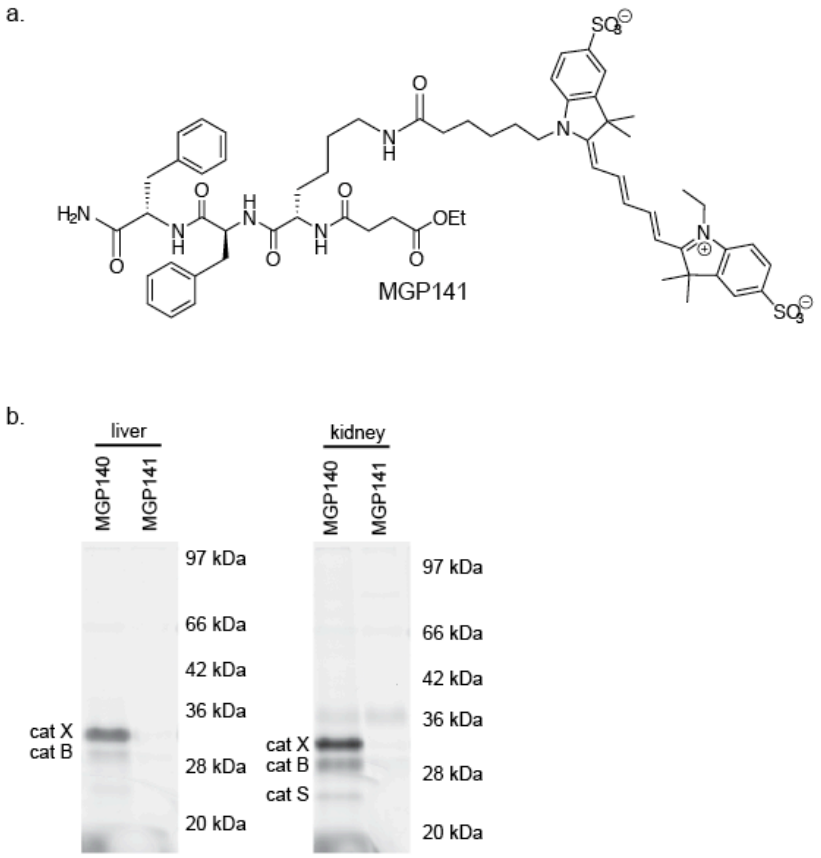
c.

	protein	accession number	MW	peptide fragments bMGP310	peptide fragments MGP310/bMGP310
*	bile acid-CoA:amino acid N-acyltransferase	IPI00207010	46 kDa	31	3
	pyruvate carboxylase, mitochondrial	IPI00210435	130 kDa	8	8
	keratin, type II cytoskeletal 1	IPI00421857	65 kDa	5	5
	propionyl-coenzyme A carboxylase	IPI 00765682	80 kDa	3	7
	serum albumin	IPI00191737	69 kDa	3	7

Supplementary Figure 1. **Determination of bMGP310 probe target in rat liver lysates.** a) Structure of bMGP310. b) Western blot of rat liver lysates treated with 1 μM bMGP310. Where indicated, lysates were pretreated with MGP310 before probe labeling to verify that protein target labeling by bMGP310 was activity-based. Activity-based labeling was visualized by SDS-PAGE analysis, followed by biotin blotting using streptavidin-HRP. c) Mass spectrometric identification of proteins labeled by bMGP310. After bMGP310 labeling, labeled proteins were enriched on immobilized streptavidin and analyzed by LC-MS/MS. All identified proteins are listed with their accession number, theoretical molecular weight, number of identified peptide fragments in the bMGP310-treated sample, and number of identified peptide fragments in the sample pretreated with MGP310 before labeling with bMGP310.



Supplementary Figure 2. **GB111-NH_2/MGP140 labeling is found in the lysosomal compartments of live KG-1 cells.** Live KG-1 cells were treated with 10 µM GB111-NH_2 for 1 h at 37 °C, followed by labeling with 5 µM MGP140 for 30 min at 37 °C. The cells were then washed with fresh media containing 10 µM GB111-NH_2 for 3 h at 37 °C. The cells were then treated with 50 nM LysoTracker Green DND-26. Images were taken with a 40X objective. Red is Cy5 fluorescence, green is LysoTracker fluorescence, and yellow is the overlap of the green and red signals. Scale bar is 10 µm.



Supplementary Figure 3. **Structure and *in vivo* labeling of MGP141.** a) Structure of MGP141, a control probe for MGP140 that lacks the epoxide warhead. b) Labeling of cathepsins *in vivo* with MGP141. Balb/c mice were injected with MGP140 or MGP141. The probes were allowed to circulate for 2h, after which the mice were sacrificed. Tissue lysates from liver and kidney were analyzed by SDS-PAGE, and labeling of the cathepsins is indicated. Labeling of the cathepsins is not observed in tissue from mice treated with MGP141.