

Supporting Information

“Activity-Based High-Throughput Profiling of Metalloprotease Inhibitors Using Small Molecule Microarrays”

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1. General Information:

Starting materials and reagents were purchased commercially and used without further purification, unless otherwise stated. All moisture-sensitive reactions were performed under a positive pressure of nitrogen. ¹HNMR spectra were recorded on a 300 MHz Bruker ACF300 or DPX300 NMR spectrometer. Chemical shifts are reported in parts per million referenced to internal standard ((CH₃)₄Si = 0.00 ppm). ESI mass spectra were acquired in the positive mode using a Finnigan/Mat TSQ7000 spectrometer. Analytical RP-HPLC separations were performed on Phenomex C18 (150 x 3.0 mm) column, using a Shimadzu Prominence HPLC system equipped with a Shimadzu SPD-20A detector. Eluents A (0.1 % TFA/acetonitrile) and B (0.1 % TFA/water) were used as the mobile phases. Thermolysin (E.C. 3.4.24.27) and GM6001 were purchased from Calbiochem (USA) and Bacterial collagenase (E.C. 3.4.24.3) was purchased from Sigma-Aldrich (Milwaukee, USA) at the highest grade available and used without further purification.

2. Design Consideration of the Library

We designed and synthesized a library of metalloprotease inhibitors with the scaffold HONH-Suc(2-*i*Bu)-P₂'-P₃'-Gly-Gly-Lys(biotin)-CONH₂ (Figure S1a) that comprised hydroxamic acid “warhead” (a potent zinc binding group) to target these compounds to the active site of metalloproteases. The P₁' position was maintained as isobutyl and the biotin linker was incorporated as an inert and versatile tag for alternative and future applications of the library. Our design was based on known molecular templates of Marimastat, Batimastat, and GM6001, three broad-spectrum hydroxamate inhibitors of matrix metalloproteases (Figure S1b).⁸

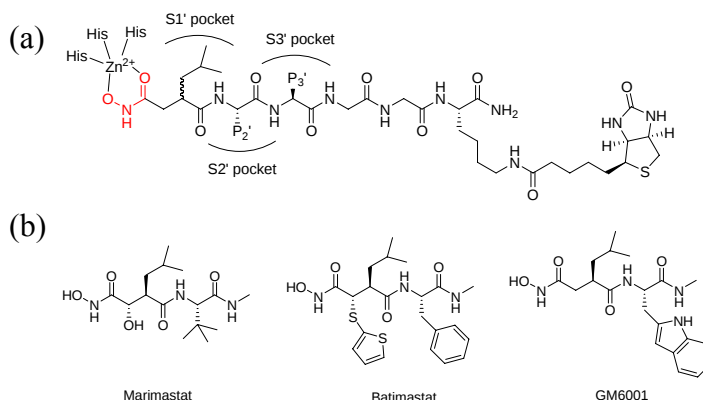


Figure S1. (a) General structure of the succinyl hydroxamate-based inhibitor library. The P₁', P₂' and P₃' substitutions of the inhibitor project into the enzyme's S₁', S₂' and S₃' pockets respectively, hydroxamic acid chelates with the zinc ion in the metalloprotease's active site. (b) Structures of broad-spectrum metalloprotease inhibitors

3. Chemical Synthesis of the 400 Hydroxamates

3.1. Synthesis of succinyl hydroxamate warhead.

Synthesis of mono-substituted succinyl hydroxamate involved five steps (Figure S2). First the 4-methylpentanoic acid was condensed with 2-oxazolidone in the presence of DCC/DMAP. This step was quite efficient and gave 93% high yield. Subsequently, reaction of the oxazolidinone **2** with sodium bis(trimethylsilyl)amide and then *tert*-butyl bromoacetate produced the racemic alkylated product **3** in 83% yield. Deprotection of the *tert*-butyl ester followed by coupling with trityl hydroxylamine generated the succinyl hydroxamate **5** in 70% yield over two steps. Removal of the oxazolidone protection group with lithium hydroxide/hydrogen peroxide produced the desired racemic monosubstituted succinyl hydroxamic acid **6** in 80% yield.

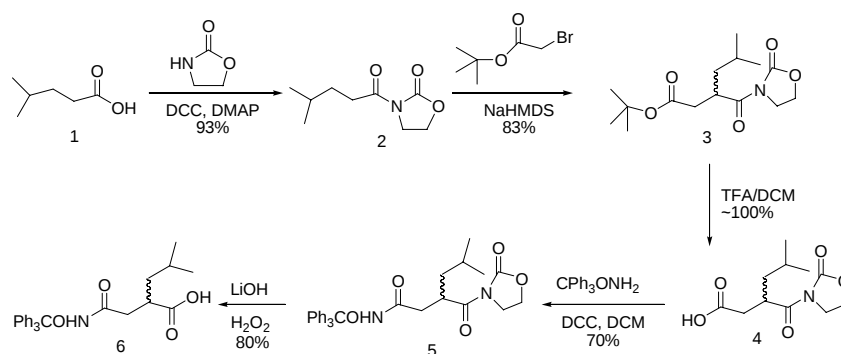


Figure S2. Synthesis route towards the hydroxamate warhead

3-(4-methylpentanoyl)oxazolidin-2-one (**2**).¹

To a suspension of 2-oxazolidinone (6.97g, 80.0mmol), DMAP (1.17g, 9.6mmol) and 4-methylpentanoic acid (12.1ml, 96.0 mmol) in CH₂Cl₂ (150 mL) at 0 °C, under a nitrogen atmosphere, was added DCC in one portion (19.81g, 96.0mmol). After 10 min the temperature was raised to RT and stirring was continued overnight. The dicyclohexylurea formed was filtered and the precipitate washed with CH₂Cl₂ (20 ml). The filtrate was washed with sat. NaHCO₃ (100 ml), dried with Na₂SO₄ and concentrated at reduced pressure to furnish the crude product, which was purified by silica gel chromatography (20%-30% EtOAc in hexanes) to afford **2** as a colorless oil (13.76g, 74.4mmol, 93% yield). ¹H-NMR (300 MHz, CDCl₃) δ 4.26 (t, *J* = 16.1 Hz, 2H), 3.85 (t, *J* = 16.1 Hz, 2H), 2.73 (t, *J* = 15.2 Hz, 2H), 1.50-1.33 (m, 3H), 0.77 (q, 6H); ESI-MS calcd for C₉H₁₅NO₃Na [M+Na]⁺ 208.2, found 208.0.

tert-butyl 5-methyl-3-(2-oxooxazolidine-3-carbonyl)hexanoate (**3**).²

To a flame-dried 500 ml three-necked RBF was added 12.03 g (65.0mmol) of oxazoline **2** in 150 ml of dry THF. This mixture was cooled to -78 °C, and 1.0 M sodium bis(trimethylsilyl)amide in THF (85.0ml, 85.0mmol) was added dropwise. The internal reaction temperature was kept below -71 °C. The solution was then stirred at -78 °C for 1 hour, then neat *tert*-butyl bromoacetate (14.4ml, 97.5mmol) was added via a syringe over a 10-minute period. The mixture was stirred at -78 °C for 2 hours then warmed to 0 °C and quenched with Et₂O and aqueous 2 M NH₄Cl. The aqueous layer was separated and extracted with diethyl ether. The combined organic layers were washed with brine, dried, filtered, and concentrated to give brown oil. The oil was purified by silica gel chromatography (15%-20% EA in hexane) to isolate 16.13 g (53.9mmol, 83% yield) of the desired product **3** as colorless oil. ¹H-NMR (300 MHz, CDCl₃) δ 4.37 (t, *J* = 17.2 Hz, 2H), 4.20-4.10 (m, 1H), 3.97 (t, *J* = 17.2 Hz, 2H), 2.63 (q, 1H), 2.35 (q, 1H), 1.52-1.48 (m, 2H); 1.35 (s, 9H), 1.17-1.24 (m, 1H), 0.84 (q, 4H). ESI-MS calcd for C₁₅H₂₅NO₅Na [M+Na]⁺ 322.3, found 322.1.

5-methyl-3-(2-oxooxazolidine-3-carbonyl)hexanoic acid (4).

Compound **3** (14.95g, 50.0mmol) was dissolved in 300 ml of DCM, then TFA (38.5ml, 500 mmol) was added, the mixture was vigorously stirred at room temperature for 4 hours. After removal of the solvent, the product was diluted with ethyl acetate (100 ml), which was then washed with water and brine, followed by drying in Na₂SO₄. The solution was concentrated to give the corresponding carboxylic acid **5** as a brownish oil, which was dried *in vacuo* for 4 hours to completely remove TFA (99% yield). ¹H-NMR (300 MHz, CDCl₃) δ 4.44-4.37 (m, 2H), 4.28-4.12 (m, 1H), 4.05-3.95 (m, 2H), 2.81 (q, 1H), 2.52 (q, 1H); 1.57-1.51 (m, 2H), 1.35-1.23 (m, 1H), 0.91(q, 4H); ESI-MS calcd for C₁₁H₁₇NO₅Na [M+H]⁺ 244.2, found 244.0.

5-methyl-3-(2-oxooxazolidine-3-carbonyl)-N-(trityloxy)hexanamide (5).

Carboxylic acid **4** (12.20g, 50.0mmol) in DCM (150 ml) was cooled down to 0 °C before addition of DCC (12.38g, 60.0mmol). Ten minutes later, the trityl-protected hydroxyamine (16.52g, 60.0 mmol) was subsequently added to the reaction mixture, and the mixture was allowed to warm up to room temperature and stirred for 3 hours. The dicyclohexylurea formed was filtered and the filtrate was washed with saturated NaHCO₃, brine, dried over Na₂SO₄, then concentrated to give a brown oil, which was purified by silica gel chromatography (20%-30% EA in hexane) to isolate 17.50g (35 mmol, 70% yield) of the desired product **5** as white solid. ¹H-NMR (300 MHz, CDCl₃) δ 7.66 (br s, 1H), 7.35 (s, 15H), 4.35 (t, *J* = 16.4Hz, 2H), 4.13-3.89 (m, 3H), 2.33 (q, 1H); 1.62-1.45 (m, 1H), 1.35-1.22 (m, 2H), 1.17-0.98(m, 1H), 0.82(m, 6H); ESI-MS calcd for C₃₀H₃₂N₂O₅Na [M+Na]⁺ 523.2, found 523.1.

4-methyl-2-(2-oxo-2-(trityloxyamino)ethyl)pentanoic acid (6).

To imide **5** (17.5 g, 35 mmol) in 4/1 THF/H₂O (600 ml) was added 30% aqueous H₂O₂ (14.3 ml, 136 mmol) at 0 °C over 5 minutes. The internal temperature was kept at or below 5 °C. Lithium hydroxide (2.8 g, 116.7 mmol) in 20 ml of water was then added over 5 minutes to ensure the internal temperature was kept below 4 °C. After addition, the mixture was allowed to warm to room temperature. After two hours, the reaction mixture was cooled to 0 °C and sodium nitrite (7.2 g, 103.7 mmol.) was added. The mixture was allowed to warm to room temperature and concentrated under reduced pressure. The resulting liquid was extracted with CH₂Cl₂. The combined organic layers were washed with aqueous 1 M NaOH. The combined aqueous layers were acidified with aqueous 1 M HCl to pH = 6, then extracted with ethyl acetate. The combined organic layers were dried, filtered, and concentrated to give 12.1 g (28 mmol, 80% yield) of **6** as an off-white solid. ¹H-NMR (300 MHz, CDCl₃) δ 7.46 (br s, 1H), 7.28 (s, 15H), 2.83-2.79(m, 1H), 2.40-2.22 (m, 1H), 1.80-1.72 (m, 2H), 1.36-1.29 (m, 2H); 0.95(q, 6H); ESI-MS calcd for C₂₇H₂₉NO₄Na [M+Na]⁺ 454.2, found 454.0.

3.2. Construction of the 400-member library

The construction of the 400-members MMP inhibitors library was achieved by standard Fmoc solid phase peptide synthesis, IRORI split-and-pool directed sorting technology (Figure S3).³ The synthesis involved the use of only 20 reaction bottles in three rounds of synthesis and sorting. Final products were released from the solid phase by standard TFA cleavage protocol. The average concentration of individual inhibitors in DMSO stock solution was adjusted to a uniform 230 μM (estimated using ACC dye conjugation as 1% additive in the final coupling step).

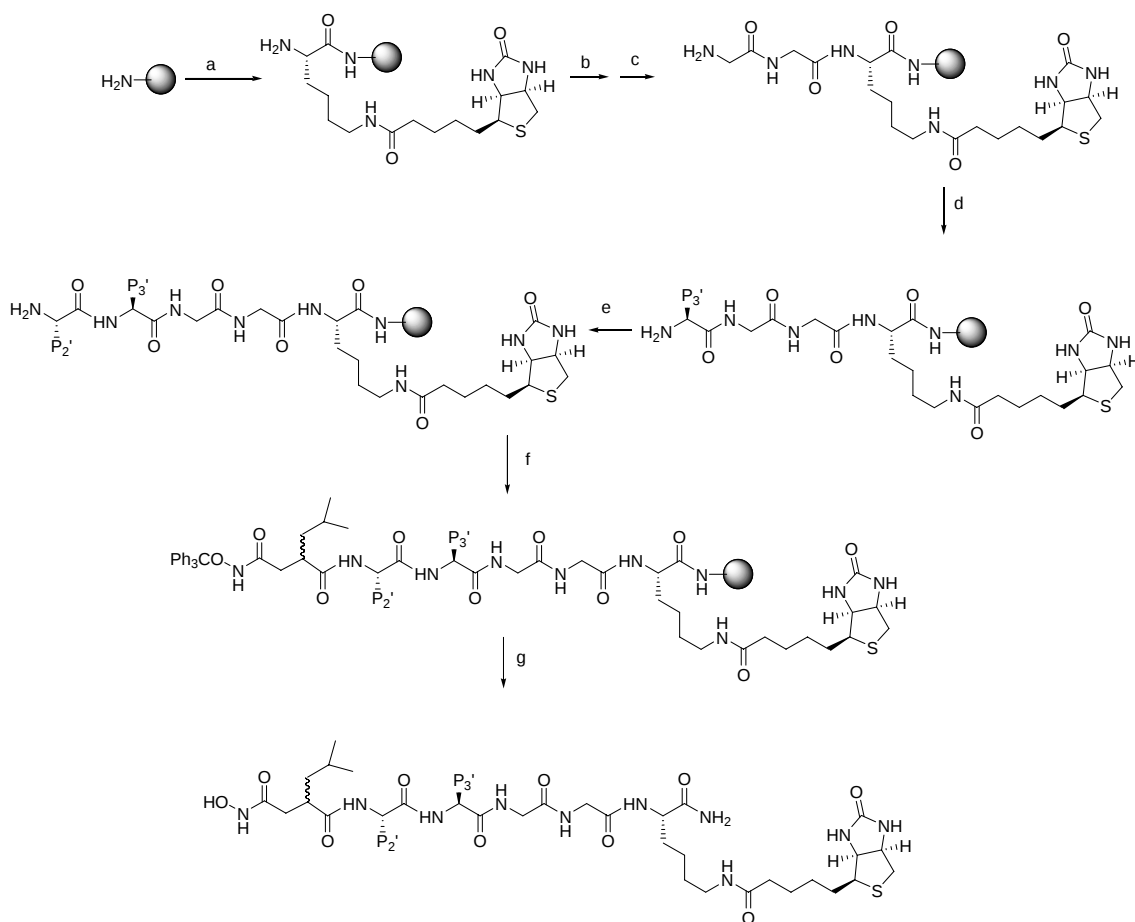
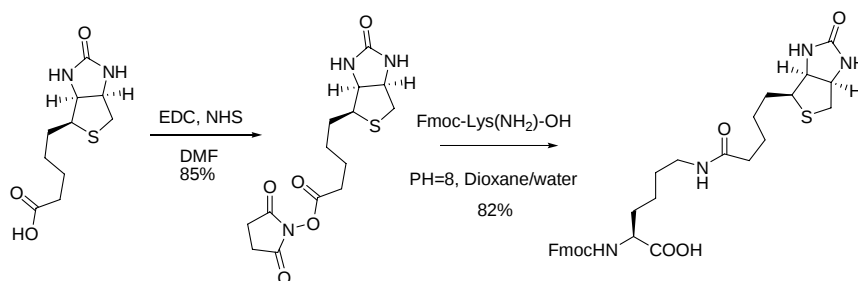


Figure S3. Procedure for the synthesis of 400-member MMP inhibitors on solid-phase. Reagents and conditions: (a) i: Fmoc-Lys(Biotin)-OH, HOBt, DIC, DIEA, 12 hrs; ii: 20% piperidine/DMF, 2 hrs (b) i: Fmoc-Gly-OH, HOBt, DIC, DIEA, 6 hrs; ii: 20% piperidine/DMF, 2 hrs (c) i: Fmoc-Gly-OH, HOBt, DIC, DIEA, 6 hrs; ii: 20% piperidine/DMF, 2 hrs (d) i: Fmoc-AA₃-OH, HOBt, DIC, DIEA, 8 hrs; ii: 20% piperidine/DMF, 2 hrs (e) i: Fmoc-AA₂-OH, HOBt, DIC, DIEA, 8 hrs; ii: 20% piperidine/DMF, 2 hrs (f) i: CPh₃ONH-Suc(2-iBu)-OH, HOBt, HBTU, DIEA, 12 hrs; ii: 20% piperidine/DMF, 2 hrs (g) 95% TFA/ 5% TIS, 2 hrs

3.2.1. Synthesis of Fmoc-Lys(Biotin)-OH



Biotin-

NHS. To a solution of D-biotin (24.43g, 100 mmol) in DMF was added N-hydroxysuccinimide (13.81g, 120 mmol) and EDC (23 g, 120 mmol). The reaction was allowed to proceed overnight. The resulting mixture was dried *in vacuo* to remove DMF. The gel-like residue was recrystallized from EtOH/Acetic acid/H₂O(95:1:4) to afford biotin-NHS as a white solid (29 g; 85% yield): ¹H-NMR (300 MHz, CD₃OD) δ 6.40 (br s, 1H), 6.35 (br s, 1H), 4.33-4.29 (m, 1H), 4.17-4.13 (m, 1H), 3.12-3.08 (m, 1H), 2.86-2.81 (m, 5H) including 2.81 (s, 4H), 2.67 (t, *J* = 7.2 Hz, 2H),

2.61-2.59 (m, 1H), 1.70-1.24 (m, 6H); ESI-MS calcd for $C_{14}H_{20}N_3O_5S$ $[M+H]^+$ 342.1, found 342.1.

Fmoc-Lys(Biotin)-OH. To a solution of Fmoc-Lys(Boc)-OH (39.8 g, 85 mmol) in DCM (80.0 ml) was added TFA (80 ml). The reaction was stirred at room temperature for 1 hour and concentrated *in vacuo*. The oil residue was dissolved in 280 ml of 1:1 Dioxane/water co-solvent, and the pH of the solution was adjusted to 8~8.5 by using a 4 M NaOH solution at 0 °C. Biotin-NHS (29 g, 85 mmol) and was subsequently added, and the reaction mixture was stirred at room temperature overnight. The resulting gelatinous solid formed was added ether, stirred for 5 minutes. The supernatant ether was decanted followed by addition of acetone and adjusting the pH of the resulting solution to 3 using 2 M HCl at 0 °C. Finally, the solid was filtered, washed several times with MeOH to afford the pure Fmoc-K(Biotin)-OH as a white solid (41.5 g, 82%): 1H -NMR (300 MHz, CD_3OD) δ 7.89 (d, J = 7.6 Hz, 2H), 7.72 (d, J = 7.7 Hz, 2H), 7.42 (t, J = 7.3 Hz, 2H), 7.33 (t, J = 7.3 Hz, 2H), 6.41 (br s, 1H), 6.35 (br s, 1H), 4.31-4.22 (m, 4H), 4.13-4.09 (m, 1H), 3.91-3.84 (m, 1H), 3.10-3.00 (m, 3H), 2.80 (dd, J = 4.9 Hz, J = 12.2 Hz, 1H), 2.56 (d, J = 12.5 Hz, 1H), 2.03 (t, J = 7.3 Hz, 2H), 1.61-1.29 (m, 12H); ESI-MS calcd for $C_{31}H_{39}N_4O_6S$ $[M+H]^+$ 595.3, found 595.2.

3.2.2. Experimental details of the solid phase synthesis

Synthesis of Fmoc-Lys (biotin)-OH resin. To a 1-litre glass bottle was added a solution of 20% piperidine in DMF and 12 g of Rink Amide AM resin (loading: 0.5 mmol/g). The mixture was shaken for 2 hours. The resin was filtered and washed with DMF (3 x), DCM (3 x) and DMF (3 x). The preactivated solution containing Fmoc-Lys(biotin)-OH (4.0 eq), HOBt (4.0 eq), DIC (4.0 eq), DIEA (8.0 eq) in DMF (400 ml) was added. The reaction was shaken overnight at room temperature. The resin was then washed with DMF (3 x), DCM (3 x) and DMF (3 x). Subsequently, Fmoc was removed following 20% piperidine treatment as described below.

Loading GG linker. To a 1-litre bottle was added biotin-linked resin and a preactivated solution of Fmoc-Gly-OH (4.0 eq), HOBt (4.0 eq), DIC (4.0 eq) and DIEA (8.0 eq) in 500 ml DMF. The reaction mixture was shaken for 6 hours and the resin was then washed with DMF, DCM, and DMF. Subsequently, Fmoc was deprotected by 20% piperidine in DMF for 2 hours. The procedures were repeated to load the second Fmoc-Gly-OH coupling.

Loading of P_3' , P_2' residues. To a 1-litre glass bottle was added a solution of 20% piperidine in DMF and 400 microreactors (each containing 30 mg of Lysin(biotin)-GG-Fmoc resin and an Rf tag). The mixture was shaken for 2 hours and then the microreactors were washed with DMF (3 x), DCM (3 x) and DMF (3 x). The microreactors were then distributed into twenty bottles and each bottle was subjected to a solution of a unique Fmoc-protected amino acid (4.0 eq), and HOBt (4.0 eq), DIC (4.0 eq) and DIEA (8.0 eq) in DMF (50 ml). After shaken for 8 hours, the microreactors were washed with DMF (3 x 400 ml), DCM (3 x 400 ml) and DMF (3 x 400 ml). The Fmoc group was then deprotected by 20% piperidine, and the microreactors were washed with DMF. To load the residue of P_2' , repeat the above procedure.

Loading P_1' residue: succinic acid derivative (w/ 1% Fmoc-ACC). The 400 microreactors were added a preactivated solution of succinic acid derivative (4.0 eq), Fmoc-ACC (0.04 eq), HOBt (4.0 eq), HBTU (4.0 eq) and DIEA (8.0 eq) in DMF. The reaction mixture was shaken overnight and then the microreactors were washed with DMF, DCM and DMF. The Fmoc group was removed and the microreactors were washed with DMF, MeOH and dried under vacuum.

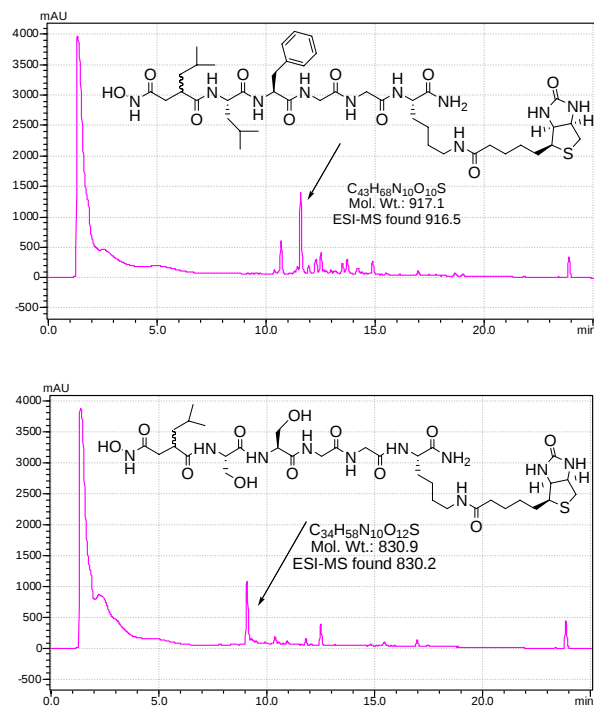
Cleavage from the solid support. Each dried microreactor was treated with an 1-ml solution containing TFA (95%), TIS (5%) and the mixture was shaken for 4 hours at rt. The cleavage solution was then transferred to 96-well plates and then concentrated *in vacuo*.

Precipitation. To each vessel was added ether and the plate was kept in a -20 °C freezer overnight. Removal of the ether layer after centrifugation for 10 minutes gave 400 peptide products. Then each peptide was dissolved in DMSO (0.5 ml).

Quantification. The final coupling step incorporated 1% coumarin to each of the small molecule inhibitors in order to facilitate a fluorescence-based approach for concentration determination. 10 μ l of the peptide solutions were diluted into PBS buffer (pH 7.4) to a final volume of 200 μ l. The solutions were transfer to 96-well microtitre plates scanned for fluorescence under the coumarin channel $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 455$ nm using a SpectraMAXTM Gemini XS fluorescence plate reader (Molecular Devices, USA). A linear concentration gradient was also using unconjugated aminomethylcarboxycoumarin (ACC) and this standard was used to estimate concentrations of each library member.

4. Library characterization

The purity of the final products was determined by Shimadzu automated rp-HPLC, with single major peaks obtained in most cases (214 nm) (see Figure S5 for profiles of selected library members). ESI-MS spectrometry was taken for selected samples to further confirm successful synthesis (Table S1).



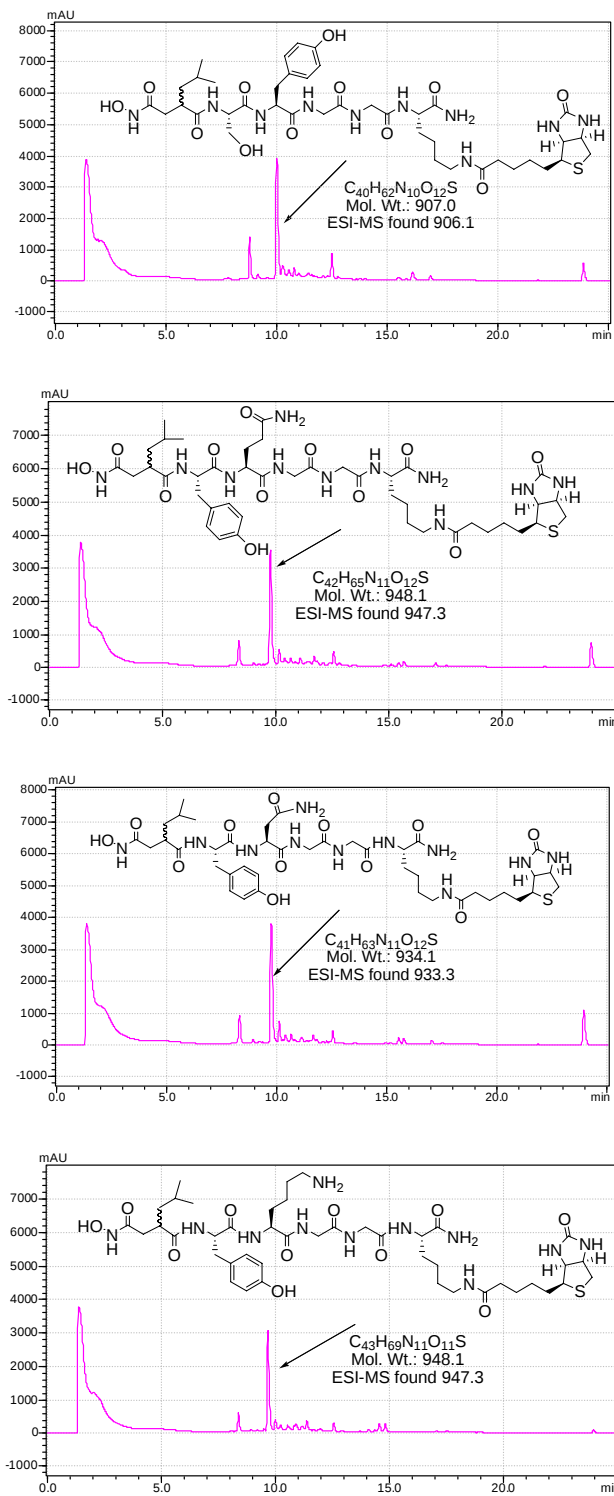


Figure S5. Six representative library members: HPLC profiles and ESI-MS results.

5. Nanodroplet SMM Profiling

5.1. SMM profiling procedure.

6 μ l of the original working bodipy-casein stock from the EnzChekTM Protease Assay Kit (Molecular Probes, USA) was diluted into 25 μ l of PBS (pH 7.4) and applied under coverslip to N-hydroxysuccinimide derivatized slides, prepared as previously described.^{4,5} After a 3 h incubation, the amine reactive slides were quenched with PBS containing 0.5 M glycine. The resulting casein-coated slides were stored at 4 °C in the dark until ready for use. The slides were stable for up to a month without appreciable drop in activity, but were typically used within a week of preparation.

The enzyme (e.g. thermolysin or collagenase) were prepared to a 0.5 mg/ml concentration and mixed with approximately 2 μ M of inhibitor. Enzymes were kept on ice until spotting with an ESI SMATM arrayer (Ontario, Canada). SMM spotting was done in duplicate with a spot spacing of 750 μ m using SMP15B spotting pins (TeleChem International Inc, USA) (The pins produced 9.4 nl spots with a diameter of 550 μ m). The pins were rinsed between samples using two cycles of wash (for 10 s) and sonication (for 10 s) in reservoirs containing 70% ethanol followed by drying under reduced pressure (for 10 s). After spotting, slides were incubated in a dark, humid chamber for 2 hours with saturation above ~ 85% to minimize evaporation of the droplets followed by analysis on an ArrayWoRxTM microarray scanner (Applied Precision, USA) equipped with the relevant filters for bodipy ($\lambda_{ex/em}$ = 490/528nm). Under these conditions spots remained visibly hydrated for periods beyond 24 hours though the fluorescence intensities of spots did not increase greatly beyond 4 hours, indicating the reaction was complete.⁴ A typical image generated was shown in Figure 2b in the maintext.

5.2. Data processing and analysis.

Mean spot intensities were background subtracted and averaged. Inhibition potency was calculated using normalization against equivalent concentrations of enzymes spotted without inhibitor. (1-Averaged Spot Intensity with Inhibitor X/Average Spot Intensity with Thermolysin) x 100%. The results were visualized using TreeView (<http://rana.lbl.gov/EisenSoftware.htm>) and cluster analysis was performed using average-linkage clustering with Gene Cluster (<http://rana.lbl.gov/EisenSoftware.htm>).⁶

6. Complementary Profiling Using Microplates

Microplate experiments were carried out using the EnzChekTM Protease Assay Kit (Molecular Probes, USA) comprising bodipy cross-linked casein. The reaction was performed in black flat-bottom polypropylene 384-well plates (Nunc, USA). Briefly a 1 mg/ml stock solution of the Bodipy FL casein was prepared according to the vendors' protocol. 0.1 μ l of the solution was used per assay and resuspended in 28 μ l of PBS (pH 7.4). To this mixture, 2 μ l of an enzyme-inhibitor mix was added (containing 0.5 mg/ml of thermolysin and 2 μ M of each specific inhibitor) thereby initiating the reaction. The plate was allowed to incubate at room temperature for 1 hour before being interrogated for end-point fluorescence at λ_{ex} = 485 nm and λ_{em} = 530 nm using a SpectraMAXTM Gemini XS fluorescence plate reader (Molecular Devices, USA). Inhibition potency was calculated using normalization against equivalent concentrations of enzymes spotted without inhibitor (1-Averaged Intensity with Inhibitor X/Average Intensity with Thermolysin) x 100%.

6.1. IC₅₀ Measurements

The IC₅₀ values were obtained using dose-dependent reactions by varying the concentrations of the inhibitor, under the same enzyme-concentration. Briefly, a two-fold dilution series from approximately

800 nM to 6.25 nM (final concentration) was prepared for each inhibitor to be tested in black flat-bottom polypropylene 384-well plates (Nunc, USA). 400 ng of enzyme (thermolysin/collagenase) was added to each of these dilutions and the reaction was initiated by introducing 0.5 μ g of Bodipy FL casein to each well, bringing the final volume to 50 μ l. The plate was allowed to incubate at room temperature for 1 hour before being interrogated for end-point fluorescence at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 530$ nm using a SpectraMAXTM Gemini XS fluorescence plate reader (Molecular Devices, USA). The IC₅₀ was calculated by fitting the fluorescent outputs obtained using the Graphpad Prism software v.4.03 (GraphPad, San Diego). Results are shown in Figure S6 and Table S1.

6.2. K_i Measurements

The IC₅₀ values were obtained using dose-dependent reactions by varying both the concentrations of the substrate and inhibitor, whilst maintaining the same enzyme concentration. Briefly, a two-fold dilution series from approximately 80 nM to 5 nM (final concentration) was prepared for each inhibitor to be tested in black flat-bottom polypropylene 384-well plates (Nunc, USA). 400 ng of Thermolysin was added to each of these dilutions and the reaction was initiated by introducing 1 μ g, 0.5 μ g and 0.3 μ g of Bodipy FL casein, bringing the final volume to 30 μ l. The plate was scanned immediately under the kinetic mode at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 530$ nm at a 40 s intervals for a period of 8 minutes using a SpectraMAXTM Gemini XS fluorescence plate reader (Molecular Devices, USA). Kinetic data obtained were analyzed using the Dixon plot (1/ initial velocity plotted against inhibitor concentration); affording three different linear graphs (corresponding to each of the substrate concentrations used) that converged on the horizontal axis (as is expected of classical non-competitive inhibitors) to give direct readouts of the K_i values. Results are shown in Figure S7 and Table S1.

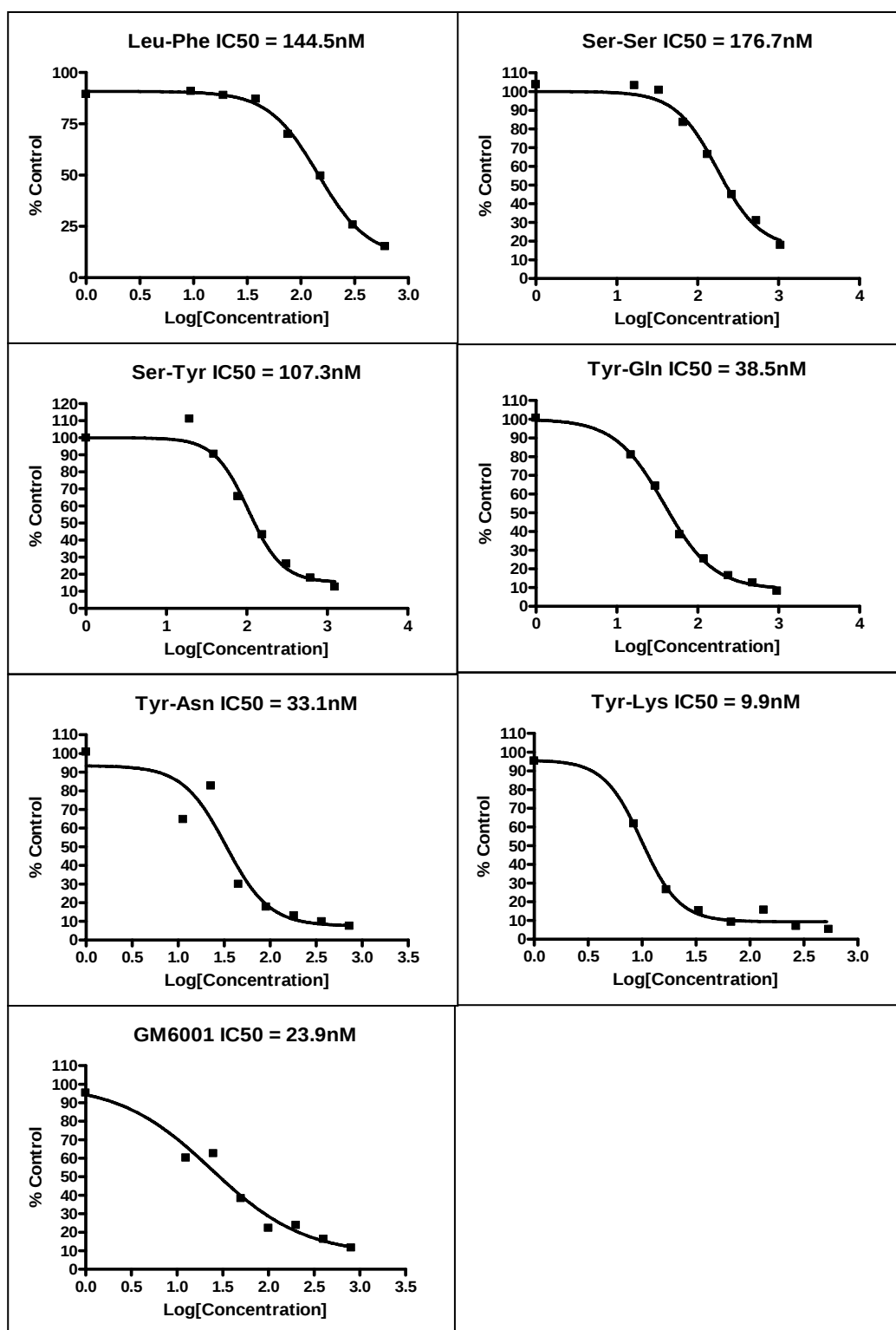


Figure S6. Graphs for determining IC₅₀ values of selected samples for thermolysin inhibition.

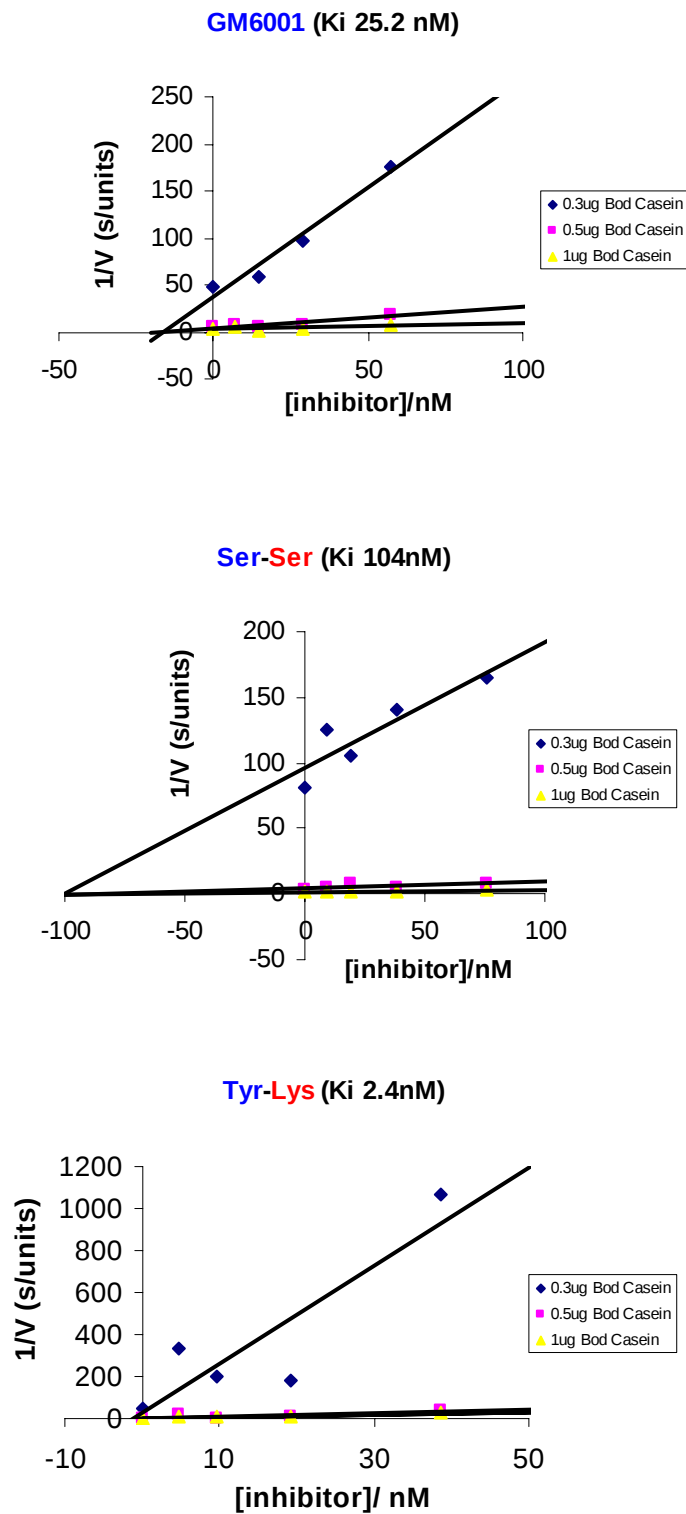


Figure S7. Graphs for determining K_i values of 2 representative inhibitors and GM6001 against thermolysin.

Table S1. K_i/IC_{50} values of 6 selected inhibitors from the library together with commercial inhibitor GM6001 is tabulated together with results obtained from large-scale microarray and microplate screens. ESI-MS results are also displayed.

P2' - P3'	IC₅₀ (nM)	K_i (nM)	ESI-MS found (calculated)
Leu-Phe	144.5	ND^a	916.5 (917.1)
Ser-Ser	176.7	104	830.2 (830.9)
Ser-Tyr	107.3	ND	906.2 (907.0)
Tyr-Gln	38.5	ND	947.3 (948.1)
Tyr-Asn	33.1	ND	933.3 (934.1)
Tyr-Lys	9.9	2.4	947.3 (948.1)
GM6001	23.9	25.2	-

^aND = Not determined

6.3. Comparison of SMM vs microplate data

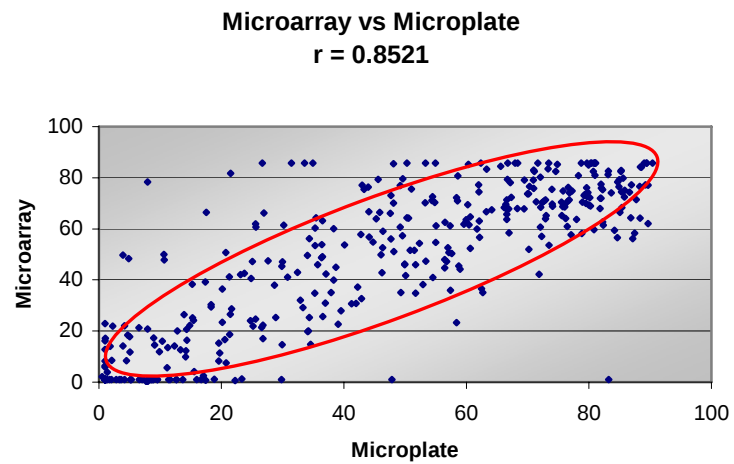


Figure S8. C) Normalized microarray data across all 400 samples were plotted against data obtained using the microplate method. Correlation analysis yielded a Pearson correlation coefficient of 0.852 using Graphpad InStat 3.0 (GraphPad, San Diego).

7. X-Ray Structure of Thermolysin

The crystal structure of thermolysin has been published,⁷ but no structural information is available for thermolysin containing a bound hydroxamic acid inhibitor. Nevertheless the structure of thermolysin complexed with an alternative class of phosphorus containing inhibitors – phosphoramidon (Figure 1B)⁸ ($K_i = 28$ nM)⁹ has been solved. Inspection of this structure (Figure S9) leads to a reasonable understanding of the preference of hydrophobic leu in the S_1' pocket, which constitutes a deep hydrophobic cavity particularly well suited for this side chain.¹⁰ The stereoimages (Figure S9b & c) also demonstrate that the tryptophan side chain at the P_2' position forms a hydrogen bond with Asn111. This could account for a similar preference that is also seen for Tyr at the P_2' position. The S_2' and S_3' pockets are open and accessible to the solvent and are not very well defined. It is perhaps for this reason there have been limited reports detailing the P_3' preference of thermolysin,¹¹ and our findings provide, to our knowledge, the first detailed evidence of the P_3' inhibitor selectivity of thermolysin. It would be interesting in further extensions to analyze the structures of thermolysin together with the inhibitors discovered herein to better understand the docking position of these inhibitors and their interactions with the S_3' site. By taking advantage of the P_3' selectivity, class specific inhibitors may be further designed with strong selectivity and narrow spectrums towards selected metalloproteases.

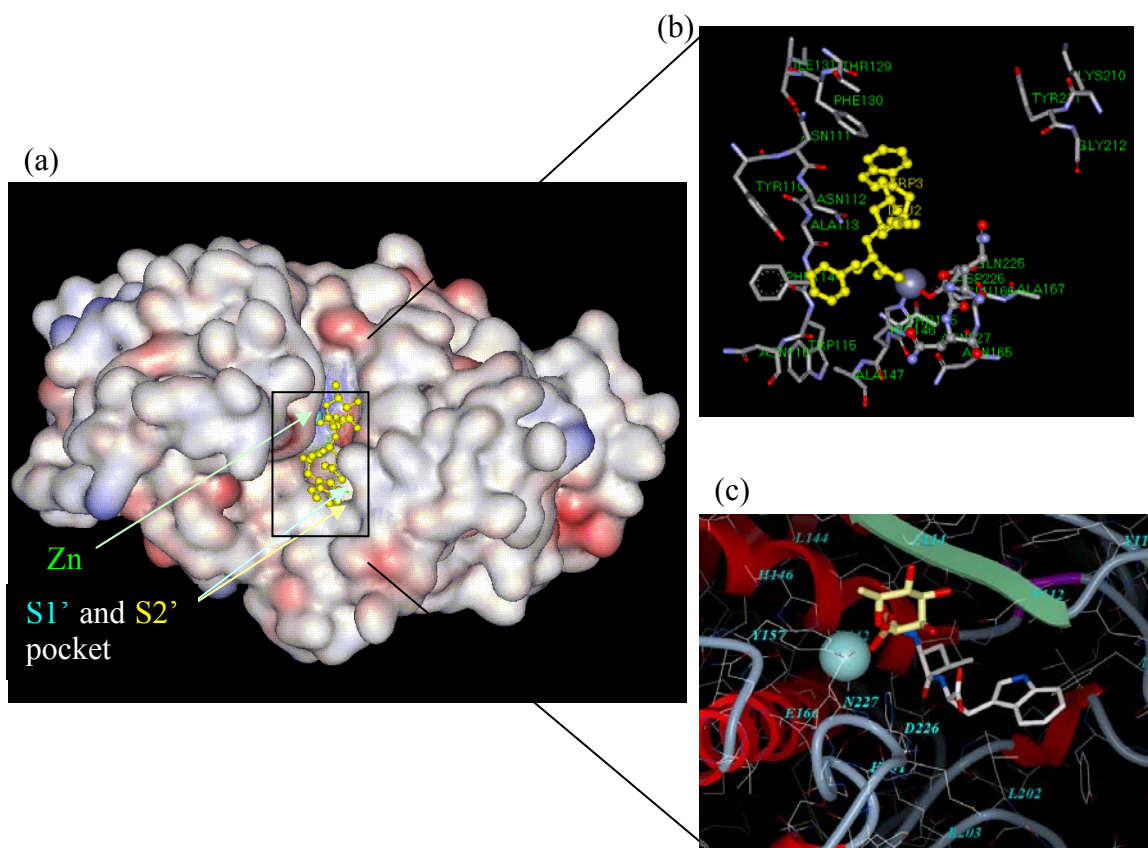


Figure S9. The crystal structure of thermolysin complexed with phosphoramidon (in yellow), a known inhibitor containing P_1' -Leu, P_2' -Trp (PDB accession: 1TLP)⁸ with a K_i of 28 nM against thermolysin at pH 7.⁹ (a) Electrostatic potential surface of thermolysin. (b) The side chains corresponding to residues proximate to phosphoramidon are shown together with the zinc atom (blue sphere). Images were generated using WebLab ViewerLite (Accelrys, San Diego, USA). (c) Ribbon diagram highlighting the substrate-binding pocket of thermolysin bound with phosphoramidon. Image was generated using ICM Browser 3.3 (Molsoft, La Jolla, USA)

8. Collagenase Experiments

Similar to thermolysin, we profiled the *Clostridium histolyticum* collagenase (ChC) against all 400 inhibitors using the nanodroplet profiling strategy (Figure S10 & S11), and confirmed by microplate experiments (Figure S12 & Table S2). As with thermolysin, a single microarray surface was able to accommodate the entire 400-member library (with relevant controls) spotted in duplicate. The results from duplicated experiments are presented in Figure S10.

We went on to perform a more detailed evaluation of selected inhibitors using IC₅₀ measurements (Figure S12 & Table S2). The predictions from the large-scale nanodrop screens were consistent with the IC₅₀ values obtained (Table S2). All inhibitors tested gave IC₅₀ values in the nanomolar range. Inspection of the clustering data (Figure S11) demonstrated that aromatic residues (Tyr, Trp, Phe) and hydrophobic residues (Leu, Ile) contribute positively to potency at the P₂' position. The tryptophan residue at the P₃' position also renders high inhibition potency. This agrees well with reported inhibitors for ChC that prefer glycine or leucine in the P₁' position and aromatic or hydrophobic functionalities in the P₂' and P₃' positions.

Despite documented attempts by Supuran *et al.*,¹² the ChC structure has yet to be successfully solved through crystallography (though the collagen binding domain of this protein has recently been elucidated¹³). Nevertheless, it is predicted that proline and Xaa within the collagenase repeating unit (Gly-Pro-Xaa-I-Gly-Pro-Xaa, Xaa – any amino acid, I-scissile site) occupy the P₁' and P₂' site for ChC. Also there have been a number of notable developments in recent years of hydroxamate inhibitors targeted against this protein. Specifically sulfonylated L-alanine hydroxamates with 2-chlorophenyl in the P₂' position and pentafluorophenyl in the P₃' position gave a K_i of 5 nM against ChC.¹² We also characterized two inhibitors HONH-Suc(2-iBu)-Tyr-Lys-Gly-Gly-Lys(Biotin)-CONH₂ and HONH-Suc(2-iBu)-Tyr-Asn-Gly-Gly-Lys(Biotin)-CONH₂ that gave relatively potent IC₅₀ values in the low nanomolar range (Table S2).

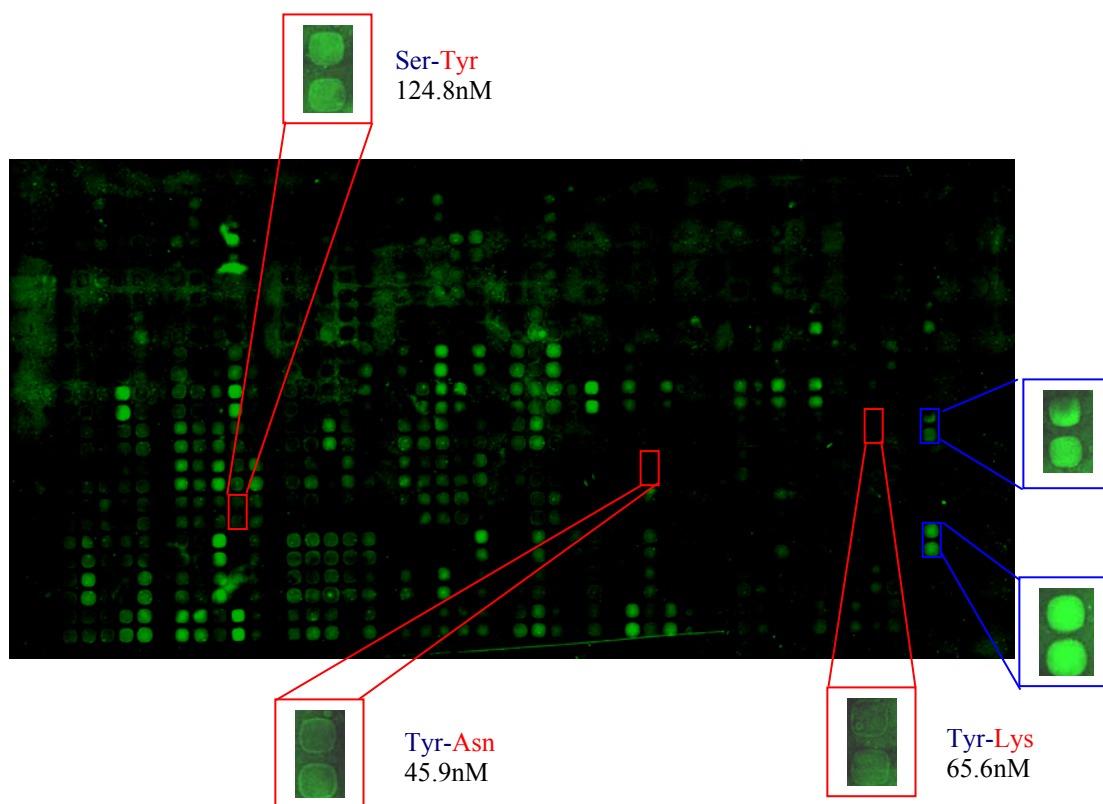


Figure S10. Representative microarray image with all 400 members of the library screened against Collagenase. Samples were spotted in duplicate and slides were scanned under the fluorescein channel to reveal potent inhibitors (benchmarked against equivalent enzyme concentrations spotted in absence of any inhibitor). Thermolysin samples printed without any inhibitor are boxed in blue, and those with selected inhibitors in red.

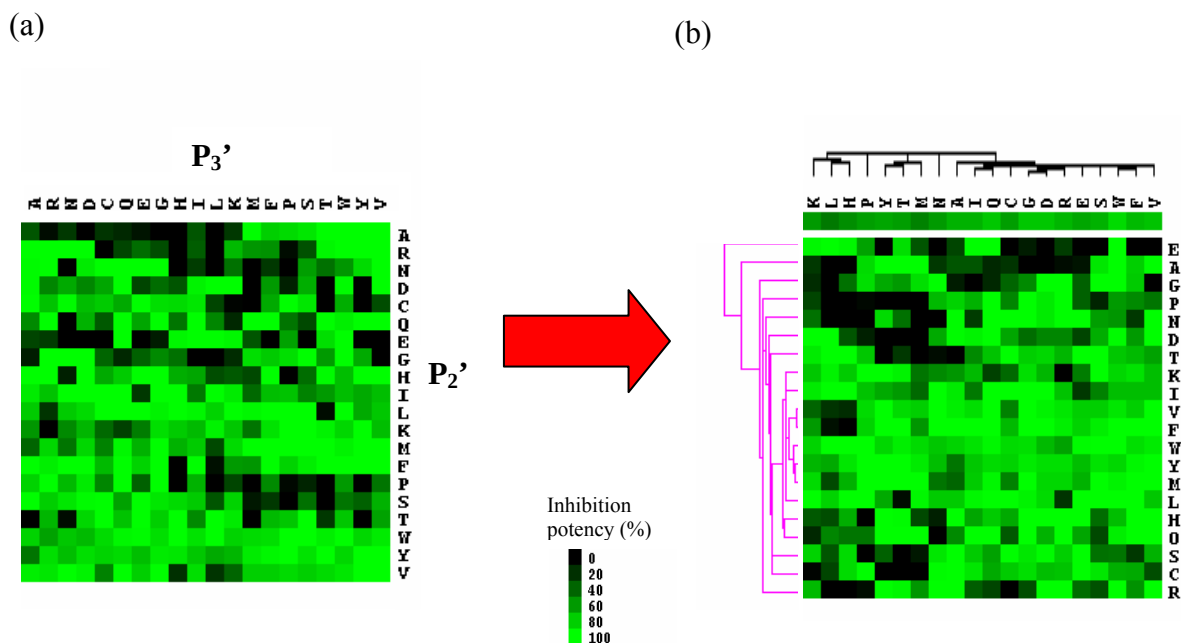


Figure S11. Microarray results with all 400 members of the library screened against Collagenase. (a) Samples were spotted in duplicate and results were averaged from two microarray experiments followed by visualization in TreeView. The intensity scale shown inset highlights the most potent inhibitors in bright green with the corresponding scale shown inset. (b) Results across all 400 members of the library were clustered and analyzed to compare importance of various amino acid substituents at both the P₂' and P₃' positions. Data points were clustered using Gene Cluster and visualized in TreeView. The intensity scale shown inset highlights the most potent inhibitors in bright green. (See Materials and Methods sections for calculation of inhibition potency)

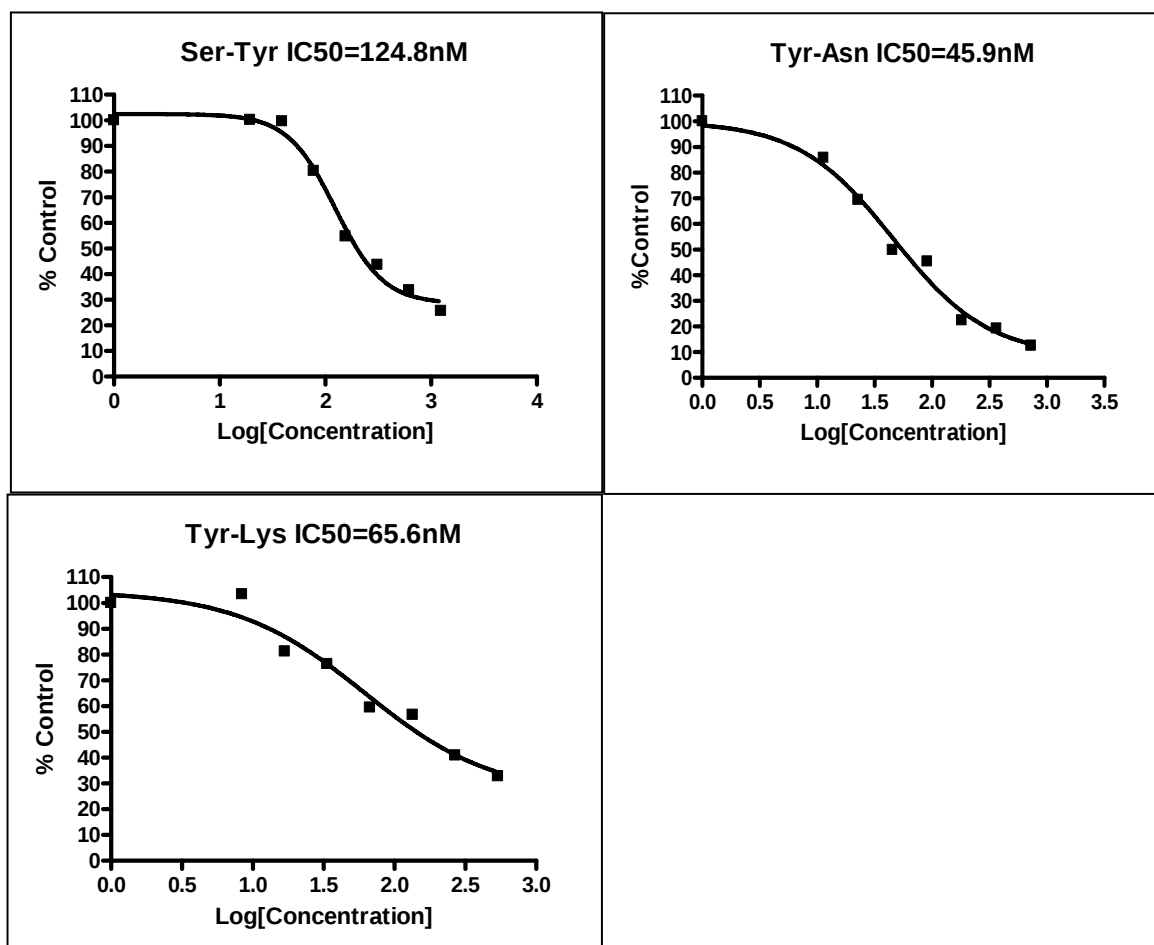


Figure S12. Graphs for determining IC₅₀ values of selected samples for Collagenase inhibition.

Table S2. IC₅₀ values of 3 selected inhibitors obtained from large-scale microarray screens. ESI-MS results are also displayed.

P2' - P3'	IC₅₀ (nM)	ESI-MS found (calculated)
Ser-Tyr	124.8	906.2 (907.0)
Tyr-Asn	45.9	933.3 (934.1)
Tyr-Lys	65.6	947.3 (948.1)

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