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Supporting Information for Proteomics

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Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays

Supplementary Material

Materials. Unless otherwise noted, chemicals were obtained from commercial suppliers and used without further purification. Rink amide AM polystyrene resin and Fmoc-amino acids were purchased from Novabiochem (San Diego, CA). Diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), AcOH, trifluoroacetic acid (TFA), collidine, and triisopropylsilane (TIS) were purchased from Aldrich. Anhydrous, low amine content *N, N'*-dimethylformamide (DMF) was purchased from EM Science (Cincinnati, OH). 7-(9-fluorenylmethylcarbonyl)amino-4-carbamoylmethylcoumarin (Fmoc-ACC-OH) was prepared as reported [1]. Library synthesis was performed in 96-well plates by using the MultiChem synthesis apparatus from Robbins Scientific (Sunnyvale, CA).

Fmoc-Arg(Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-Cl. To a flame-dried three-neck 100 mL round bottom flask under nitrogen fitted with a stir bar was added 20 mL of freshly distilled ether and 929 μL of DMF (12 mmol, 1 equiv). The flask was cooled to 0 ºC in an ice bath and 1.4 mL $(COCl)_2$ (16 mmol, 1.2 equiv) was added. Once the gas has ceased evolving, the ether was evaporated under a stream of nitrogen while the flask was maintained at 0 ºC. 7.8 g of Fmoc – Arg(Pbf)-OH (7.8 g, 12 mmol, 1 equiv) in 30 mL of freshly distilled methylene chloride was added and allowed to stir for 5 minutes at 0 ºC. The ice bath was removed, and the solution was stirred for 10 minutes at rt. Diisopropylethylamine (2.1 mL, 12 mmol, 1 equiv) was added to the solution, and the preparation was added immediately to the resin.

Fmoc-Lys(Boc, *tert*-butoxycarbonyl)-F. Fmoc-Lys(Boc)-F was prepared from the corresponding protected amino acid according to published procedures [2]. To a 100 mL round bottom flask under nitrogen fitted with a stir bar was added Fmoc-Lys(Boc)-OH (30 g, 64 mmol), 320 mL of methylene chloride, pyridine (5.2 mL, 64 mmol), and cyanuric fluoride (11 mL, 128 mmol). The reaction mixture was stirred for 3 h after which 680 mL of methylene chloride was added. The organic layer was extracted with 1 M $NaHCO_3$ (3 x 300 mL) and 1M $NaHSO_4$ (3 x 300 mL). The organic layer was dried over Na_2SO_4 and filtered. Evaporation of the solvent provided 29.1 g (97%) of the product as a white solid, which was used without further purification.

P_1 = Arg library synthesis. For the preparation of the P_1 = Arg sublibrary, 4.0 g (2.8 mmol) of Rink amide AM polystyrene resin (loading 0.69 mmol/g) was added to a fritted syringe and washed with DMF (3 x 40 mL). The Fmoc group was removed (treatment with 20% piperidine in DMF (40 mL) for 20 min), and the resin was filtered and washed with DMF (5 x 40 mL). The 7-(9-fluorenylmethylcarbonyl)amino-4-carbamoylmethylcoumarin (Fmoc-ACC-OH)[1] (2.4 g, 5.5 mmol), 1-hydroxybenzotriazole (HOBt) (0.85 g, 5.5 mmol), and DMF (430 mL) were added to the resin. The

resin was agitated for 20 min and diisopropylcarbodiimide (DIC) (0.86 ml, 5.5 mmol) was added. The resin was agitated for 15 h, filtered, washed with DMF (3 x 60 mL), and subjected a second time to the acylation conditions. Any unacylated linker was then capped by adding a preactivated solution of AcOH (0.92 mL, 16 mmol), HOBt (2.5 g, 16 mmol), and DIC (2.5 mL, 16 mmol) in DMF (40 mL) to the resin and agitating the reaction mixture for 4 h. The resin was washed with DMF (5 x 40 mL), the Fmoc group was removed, and the resin was filtered and washed with DMF (5 x 40 mL) and CH_2Cl_2 (2 x 40 mL). To load the P_1 residue, a 0.4 M solution of diisopropylethylamine and crude Fmoc-Arg(Pbf)-Cl in CH_2Cl_2 (*vida supra*) was added to the resin. The resin was agitated for 1 h, filtered, washed with CH_2Cl_2 (2 x 40 mL) and the acylation repeated. The resin was washed with CH_2Cl_2 (2 x 40 mL), DMF (2 x 40 mL), THF (2 x 40 mL), and DMF (2 x 40 mL). The unacylated ACC was capped by adding a preactivated solution of AcOH (1.6 mL, 28 mmol), 3-nitro-1,2,4-triazole (3.2 g, 28 mmol), and DIC (4.4 ml, 28 mmol) in DMF (25 mL) to the resin and agitating the reaction mixture for 21 h. The resin was filtered, washed with DMF (3 x 60 mL), THF (3 x 60 mL), MeOH (3 x 60 mL), and dried under vacuum. The resin was then distributed into 361 wells of 96-well Robbins blocks (Flexchem System; Robbins Scientific, Sunnyvale, CA) (approximately 4.0 μmol (10 mg) per well) and solvated with DMF (2 x 200 μL/well). The Fmoc group was removed (3 x 200 μL/well), and the resin was filtered and washed with DMF (3 x 1 mL/well). To load the P_2 residue, the resin in each well was subjected to 200 μL of a 0.4 M solution of an Fmoc amino acid (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt, trityl)-OH, Fmoc-Asp(O-*t*-Bu, *t*-butyl)-OH, Fmoc-Glu(O-*t*-Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O-*t*-Bu)-OH, Fmoc-Thr(O-*t*-Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(O-*t*-Bu)-OH, or Fmoc-Val-OH) (0.08 mmol/well), preactivated with DIC (0.08 mmol per well) and HOBt (0.08 mmol per well), in DMF. After agitating the resin for 4 h, the resin in each fritted syringe was washed with DMF (3 x 1 mL/well), the Fmoc group was removed, and the resin was filtered and washed with DMF (3 x 1 mL). To load the P_3 residue, 200 μL of a 0.4 M solution of each Fmoc amino acid, preactivated with HOBt and DIC, in DMF, was added to the appropriate wells (0.1 mmol/well). The resin was agitated for 4 h and washed with DMF (3 x 1 mL). The Fmoc group was removed, and the resins washed with DMF (3 x 1 mL) and filtered. To load the P_4 alanine, 0.2 mL of a 0.4 M Fmoc-Ala-OH solution, preactivated with HOBT and DIC, in DMF was added to each of the wells (0.08 mmol/well). The resin was agitated for 4 h, filtered, and washed with DMF (3 x 1 mL). The Fmoc group was removed, and the resin in each well was filtered and washed with DMF (3 x 1 mL). To acetylate the substrates, 0.2 mL of a 0.4 M AcOH solution, preactivated with HOBt and DIC, in DMF was added to each of the wells (0.08 mmol/well). The resin was agitated for 3 h, and the resin in each well was thoroughly washed with DMF (3 x 500 μL), 1:9 DIEA/DMF (2 x 500 μL), DMF (3 x 500 μl), THF (2 x 500 μL), MeOH (2 x 500 μL), THF (2 x 500 μL), and CH_2Cl_2 (3 x 500 μL). The reaction blocks were placed on 96-deep-well microtiter plates, and the substrates were cleaved from the resin by repeated subjections to a

solution of 95:2.5:2.5 TFA/TIS/H_2O (6 x 200 μL per well) over 1 h. The collection plate was concentrated. The compounds were diluted with 1:1 CH_3CN/H_2O (0.5 mL/well), and the microtiter plates were concentrated again. A representative number of the library members were analyzed by HPLC-MS to demonstrate good purity (HPLC conditions: CH_3CN/H_2O-0.1% TFA, 5-45% for 14 min, 0.4 mL/min, detection at 220/230/254nm for 22 min).

Substrate	***m/z* calcd**	***m/z* found**	**t_R**
Ac-Ala-Ala-Ala-Arg-ACC-NH_2	629.3	630.4	6.0
Ac-Ala-Glu-Glu-Arg-ACC-NH_2	745.3	746.3	5.9
Ac-Ala-Gly-Ile-Arg-ACC-NH_2	657.3	658.5	6.8
Ac-Ala-His-Glu-Arg-ACC-NH_2	753.3	754.5	5.8
Ac-Ala-Ile-Leu-Arg-ACC-NH_2	713.4	714.5	8.0
Ac-Ala-Lys-Pro-Arg-ACC-NH_2	712.4	713.5	5.8
Ac-Ala-Asp-Ser-Arg-ACC-NH_2	689.3	690.3	5.8
Ac-Ala-Glu-Tyr-Arg-ACC-NH_2	779.3	780.5	6.6
Ac-Ala-Phe-Met-Arg-ACC-NH_2	765.3	766.5	8.1
Ac-Ala-Val-His-Arg-ACC-NH_2	723.4	724.5	5.9
Ac-Ala-Leu-Asp-Arg-ACC-NH_2	715.3	716.5	6.9
Ac-Ala-Met-Gly-Arg-ACC-NH_2	675.3	676.4	6.5
Ac-Ala-Pro-Lys-Arg-ACC-NH_2	712.4	713.5	5.8
Ac-Ala-Arg-Gln-Arg-ACC-NH_2	757.4	758.5	5.7
Ac-Ala-Thr-Trp-Arg-ACC-NH_2	774.3	775.5	7.5
Ac-Ala-Trp-Met-Arg-ACC-NH_2	804.3	805.5	8.5
Ac-Ala-Trp-Tyr-Arg-ACC-NH_2	836.4	837.5	8.3
Ac-Ala-Val-Leu-Arg-ACC-NH_2	699.4	700.5	8.0
Ac-Ala-Tyr-Arg-Arg-ACC-NH_2	806.4	807.5	6.4
Ac-Ala-Met-Gln-Arg-ACC-NH_2	746.3	747.3	8.4
Ac-Ala-Pro-Arg-Arg-ACC-NH_2	740.4	741.5	7.5
Ac-Ala-Asn-Ser-Arg-ACC-NH_2	688.3	689.3	6.6
Ac-Ala-Gln-Thr-Arg-ACC-NH_2	716.3	717.5	6.0
Ac-Ala-Ser-Thr-Arg-ACC-NH_2	675.3	676.4	7.4
Ac-Ala-Thr-Val-Arg-ACC-NH_2	687.3	688.3	8.2
Ac-Ala-Arg-Met-Arg-ACC-NH_2	774.4	775.5	8.2
Ac-Ala-Leu-Tyr-Arg-ACC-NH_2	763.4	764.5	7.6

P_1= Lys sublibrary synthesis. The P_1 = Lys sublibrary was prepared analogously to the P_1 = Arg sublibrary, but the P_1 Lys was loaded by subjecting the resin to a 0.4 M solution of Fmoc-Lys(Boc)-F (*vida supra*) and collidine in CH_2Cl_2 overnight. A representative number (~10%) of the library members were analyzed by HPLC-MS to demonstrate good purity (HPLC conditions: CH_3CN/H_2O-0.1% TFA, 5-45% for 14 min, 0.4 mL/min, detection at 220/230/254nm for 22 min).

Substrate	***m/z* calcd**	***m/z* found**	**t_R**
Ac-Ala-Ala-Ala-Lys-ACC-NH_2	601.3	602.3	7.1
Ac-Ala-Ala-Met-Lys-ACC-NH_2	661.3	662.3	8.4
Ac-Ala-Asp-Ala-Lys-ACC-NH_2	645.3	646.3	7.2
Ac-Ala-Asp-Asn-Lys-ACC-NH_2	688.3	689.3	6.6
Ac-Ala-Glu-Asp-Lys-ACC-NH_2	703.3	704.3	7.0
Ac-Ala-Glu-Pro-Lys-ACC-NH_2	685.3	686.3	7.4
Ac-Ala-Phe-Gly-Lys-ACC-NH_2	735.3	736.3	9.4
Ac-Ala-Phe-Gln-Lys-ACC-NH_2	734.3	664.2	9.3
Ac-Ala-Gly-Phe-Lys-ACC-NH_2	649.3	735.3	8.7
Ac-Ala-Gly-Arg-Lys-ACC-NH_2	672.3	673.3	6.6
Ac-Ala-His-Asp-Lys-ACC-NH_2	711.3	712.2	6.7
Ac-Ala-His-Pro-Lys-ACC-NH_2	693.3	694.3	7.1
Ac-Ala-Ile-Lys-Lys-ACC-NH_2	700.4	701.3	7.7
Ac-Ala-Ile-Ser-Lys-ACC-NH_2	659.3	660.5	8.2
Ac-Ala-Lys-Lys-Lys-ACC-NH_2	700.3	701.4	8.5
Ac-Ala-Lys-Ser-Lys-ACC-NH_2	674.3	675.3	6.5
Ac-Ala-Lys-Trp-Lys-ACC-NH_2	773.4	774.3	9.1
Ac-Ala-Leu-Lys-Lys-ACC-NH_2	700.4	701.4	8.0
Ac-Ala-Leu-Tyr-Lys-ACC-NH_2	735.4	736.3	9.6
Ac-Ala-Thr-Phe-Lys-ACC-NH_2	707.3	708.4	8.9
Ac-Ala-Met-Asn-Lys-ACC-NH_2	704.3	705.3	7.8
Ac-Ala-Met-Trp-Lys-ACC-NH_2	776.3	777.3	10.8
Ac-Ala-Asn-Glu-Lys-ACC-NH_2	702.3	703.3	6.7
Ac-Ala-Asn-Gln-Lys-ACC-NH_2	701.3	702.3	6.5
Ac-Ala-Pro-Phe-Lys-ACC-NH_2	703.3	704.3	9.7
Ac-Ala-Pro-Arg-Lys-ACC-NH_2	712.4	713.3	6.9
Ac-Ala-Gln-Gly-Lys-ACC-NH_2	644.3	645.3	6.5
Ac-Ala-Gln-Ser-Lys-ACC-NH_2	674.3	675.2	6.1
Ac-Ala-Gln-Trp-Lys-ACC-NH_2	773.4	774.3	9.2

Substrate (cont'd)	*m/z* calcd	*m/z* found	t_R
Ac-Ala-Arg-His-Lys-ACC-NH_2	752.4	753.3	6.6
Ac-Ala- Arg-Thr-Lys-ACC-NH_2	716.4	717.4	6.5
Ac-Ala-Ser-Ile-Lys-ACC-NH_2	659.3	660.3	8..2
Ac-Ala-Ser-Pro-Lys-ACC-NH_2	643.3	644.3	7.2
Ac-Ala-Ser-Arg-Lys-ACC-NH_2	702.3	703.3	6.6
Ac-Ala-Ser-Val-Lys-ACC-NH_2	645.3	646.3	7.9
Ac-Ala-Val-Gly-Lys-ACC-NH_2	615.3	616.3	7.6
Ac-Ala-Val-Ser-Lys-ACC-NH_2	645.3	646.3	7.4
Ac-Ala-Trp-Ile-Lys-ACC-NH_2	758.4	759.3	11.4
Ac-Ala-Trp-Thr-Lys-ACC-NH_2	746.3	747.3	9.9
Ac-Ala-Tyr-Ile-Lys-ACC-NH_2	735.4	736.4	9.8
Ac-Ala-Tyr-Val-Lys-ACC-NH_2	721.3	722.3	9.0

Determination of K_ms. Individual kinetic constants were determined for selected good substrates for each enzyme. Substrate stock solutions were prepared in DMSO. The substrates were diluted in assay buffer (25 mM Tris pH 7.2, 150 mM NaCl, 5 mM $CaCl_2$, and 0.01% tween) to a final concentration ranging from 12 μM to 1200 μM. The concentration of DMSO in the assays was less than 6%. The enzyme concentrations varied from 0.8 nM to 375 nM, depending on the enzyme. The enzyme concentration was always held at least 50-fold lower than the lowest substrate concentration in order to satisfy steady-state assumptions. Hydrolysis of ACC substrates was monitored fluorometrically with an excitation wavelength of 380 nm and emission wavelength of 460 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). Rates were measured at rt and fit to the Michaelis-Menten equation for steady state kinetics in order to determine the K_ms. All individual rates were measured in duplicate.

Enzyme	Substrate	K_m (μM)
Human thrombin	Ac-AMPK-ACC-NH_2	140 ± 20
tPA	Ac-AFGK-ACC-NH_2	590 ± 56
tPA	Ac-AYTK-ACC-NH_2	220 ± 21
uPA	Ac-ASGK-ACC-NH_2	530 ± 33
uPA	Ac-ATSK-ACC-NH_2	610 ± 46
Factor Xa	Ac-AFGK-ACC-NH_2	250 ± 20
Factor Xa	Ac-AGFK-ACC-NH_2	220 ± 8.9
Factor Xa	Ac-ALFK-ACC-NH_2	190 ± 41
plasmin	Ac-ARFK-ACC-NH_2	170 ± 12

[1] Maly, D. J., Leonetti, F., Backes, B. J., Dauber, D. S., *et al.*, *J .Org. Chem.* 2002, *67*, 910-915.
[2] Olah, G. A., Nojima, M., Kerekes, I., *Synthesis* 1973, 487-488.