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for

Straightforward Preparation and Assay of Aspartyl Protease Substrates with an Internal Thioester Linkage

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Experimental Section

General methods and Materials: Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. Side chain protected Fmoc and Boc amino acids, *N*-hydroxybenzotriazole (HOBt), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), Rink amide MBHA resin, 2-chlorotrityl chloride resin, and human liver cathepsin D were purchased from EMD Biosciences (San Diego, CA). MBHA resin was purchased from Advanced ChemTech (Louisville, KY). *N*-Hydroxyazabenzotriazole (HOAt) was purchased from PerSeptive Biosystems GmbH (Hamburg, Germany). *N*-Boc-Arg(Mts)-OH was purchased from Chem-Impex (Wood Dale, IL) as the cyclohexylamine salt. Di-*tert*-butyl dicarbonate (Boc_2O), 4,4'-dipyridyl disulfide, isobutyl chloroformate, 4-[4-(Dimethylamino)phenylazo]benzoic acid (Dabcyl), and 5-(2-Aminoethylamino)-1-naphthalenesulfonic acid (Edans) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). *N*,*N*'-Diisopropylcarbodiimide (DIC) was purchased from Acros Organics (Morris Plains, NJ). Diisopropylethylamine (*i*-Pr_2EtN) and dichloromethane (CH_2Cl_2) were distilled from CaH_2. Tetrahydrofuran was distilled from sodium benzophenone. In the synthesis of **5a** and **5b**, prior to preparation of the acidic media, the concentrated HBr (48% aq, Aldrich Chemical Company) was washed twice with 5% tributyl phosphate in $CHCl_3$ to remove contaminating Br_2.[1] Reaction progress was monitored with thin layer chromatography on Merck 60 F_{254} 0.25 μm silica plates. Fmoc quantitation was performed according to literature procedure.[2] The completion of peptide coupling reactions was verified by the Kaiser test.[3] High pressure liquid chromatography (HPLC) analysis of synthesized substrates was performed with a C18 reverse phase column (4.6 x 100 mm) [acetonitrile/H_2O-0.1% trifluoroacetic acid, 5 – 95% over 14 min, 0.4 mL/min, UV detection at 220, 254 and 280 nm for 22 min]. Purifications by HPLC were carried out on a preparatory reverse phase C18 column (24.1 x 250 mm) [acetonitrile/H_2O-0.1% trifluoroacetic acid, 5-95% over 50 min, 8 mL/min, 215, 254, 280 nm detection for 65 min]. Chemical shifts are reported in ppm relative to internal solvent peak and coupling

constants are reported in Hz. Elemental analyses were performed at the University of California at Berkeley elemental facility. Mass spectrometry data was collected with a Hewlett Packard Series 1100 LC-electrospray MS. All infrared (IR) spectra were recorded as thin films on a Fourier-Transform Infrared Spectrometer and only partial data are listed.

Preparation of Dabcyl-Lys-Pro-Ile-Val-Phe~NH~Phe-Arg-Leu-Edans (1a)

2-Chlorotrityl chloride resin (500 mg, 1.4 mmol/g resin) was swelled with CH_2Cl_2 (2.5 mL) for 1 h. The resin was then treated with a solution of *N*-Fmoc-Leu-OH (1.2 g, 3.5 mmol) and *i*-Pr_2EtN (0.96 mL, 7.0 mmol) in CH_2Cl_2 (3.5 mL), and the resulting slurry was stirred for 17 h on an orbital shaker. The resin was then filtered, washed (5 x 2.5 mL of NMP, 5 x 2.5 mL of CH_2Cl_2, 2 x 2.5 mL of diethyl ether), and dried under high vacuum. The yield for loading of the first amino acid was determined to be 77% by Fmoc quantitation. The resin was then swelled in CH_2Cl_2, and the Fmoc group was removed by treatment with 2.5 mL of a 20% piperidine solution in DMF, followed by filtration and washing. The second amino acid was coupled by treating the resin with a preformed solution containing *N*-Fmoc-Arg(Pbf)-OH (970 mg, 1.5 mmol), HOBt (200 mg, 1.5 mmol), and DIC (0.23 mL, 1.5 mmol) in NMP (2.5 mL) that had been pre-incubated for 10 min and shaken in an orbital shaker for 16 h. Filtration, washing, and removal of the Fmoc group was carried out according to the standard procedures described previously. The resin was then treated with a preformed solution containing *N*-Fmoc-Phe-OH (580 mg, 1.5 mmol), HOBt (200 mg, 1.5 mmol), and DIC (0.23 mL, 1.5 mmol) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no free amine was present (4 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Phe-OH (580 mg, 1.5 mmol), HOBt (200 mg, 1.5 mmol), and DIC (0.23 mL, 1.5 mmol) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no free amine was present (19 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Val-OH (1.0 g, 3.0

mmol), HOBt (410 mg, 3.0 mmol), and DIC (0.46 mL, 3.0 mmol) in NMP (5 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no amine was present (24 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Ile-OH (1.1 g, 3.0 mmol), HOBt (410 mg, 3.0 mmol), and DIC (0.46 mL, 3.0 mmol) in NMP (5 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no amine was present (20 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Pro-OH (1.0 g, 3.0 mmol), HOBt (410 mg, 3.0 mmol), and DIC (0.46 mL, 3.0 mmol) in NMP (5 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no amine was present (11 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Lys(Boc)-OH (1.4 g, 3.0 mmol), HOBt (410 mg, 3.0 mmol), and DIC (0.46 mL, 3.0 mmol) in NMP (5 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no amine was present (16 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing Dabcyl (510 mg, 1.8 mmol), HOAt (480 mg, 3.5 mmol), and DIC (0.33 mL, 2.1 mmol) in NMP (5 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed for 23 h, followed by filtration and washing, and then drying the resin under high vacuum. The side chain-protected peptide was then cleaved off support by treatment with 5 mL of a hexafluoroisopropanol/CH_2Cl_2 (1:4) solution for 1 h. The resin was filtered and washed (5 x 2 mL of CH_2Cl_2) and the combined filtrate was concentrated to dryness. The *N*-derivatized peptide (1.0 g, 0.7 mmol) was then dissolved in DMF (28 mL), and the carboxy terminus was activated by addition of HOBt (200 mg, 1.5 mmol) and EDC (290 mg, 1.5 mmol), followed by addition of Edans (370 mg, 1.4 mmol) to provide the side chain-protected FRET peptide. This peptide was purified by HPLC and lyophilized. Removal of the protecting groups was accomplished by treatment of the peptide with 5 mL of a trifluoroacetic acid/triisopropyl silane/water (95:2.5:2.5) solution for 2 h. The solution was

concentrated on a rotary evaporator, purified by HPLC and lyophilized to afford compound **1a** as a bright red solid. HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{79}H_{107}N_{17}O_{12}S$, 1518.8057; found, 1518.8084.

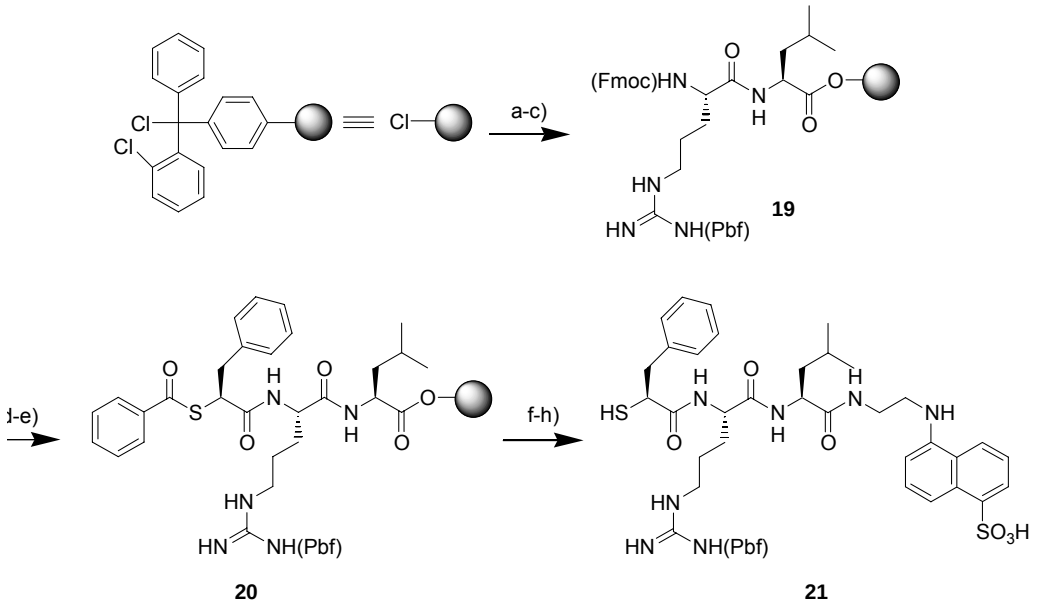
Preparation of (S)-2-(Benzoylthio)-3-phenylpropanoic acid (18)

Compound **18** was synthesized as reported in literature.[4]

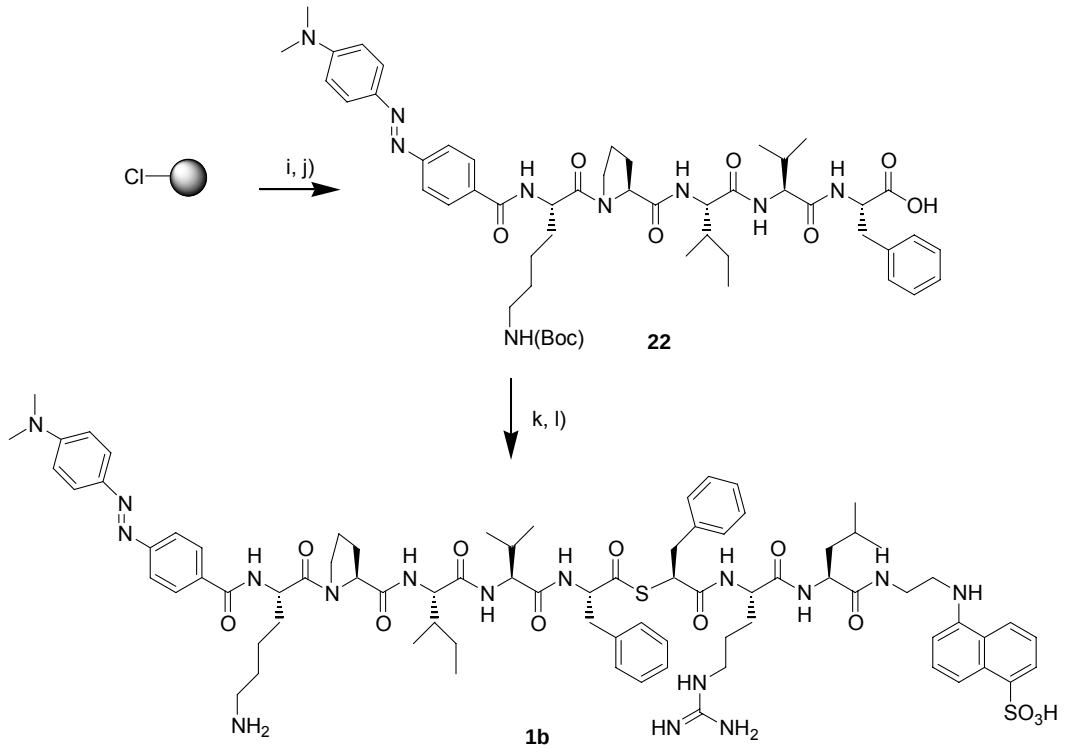
Preparation of Dabcyl-Lys-Pro-Ile-Val-Phe~S~Phe-Arg-Leu-Edans (1b)

Scheme 1. Preparation of **1b**.

(A)



(B)



Reaction conditions: a) Fmoc-Leu-OH, *i*-PrEt$_2$N; b) piperidine/DMF/CH_2Cl_2 (1:2:2); c) Fmoc-Arg(Pbf)-OH, DIC, HOBt, DMF; d) piperidine/DMF/CH_2Cl_2 (1:2:2); e) **18**, DIC, HOBt, DMF; f) hexafluoroisopropanol/CH_2Cl_2 (1:4); g) Edans, DIC, HOBt, DMF; h) 5N NH_3 (aq), mercaptoethanol; i) Fmoc-based SPPS; j) Dabcyl, DIC, HOAt, DMF; k) PyBOP, HOBt, *i*-PrEt$_2$N, then **21**; l) trifluoroacetic acid

Part A: 2-Chlorotrityl chloride resin (2 g, 1.4 mmol/g resin) was swelled with CH_2Cl_2 (10 mL) for 1 h. The resin was then treated with a solution of *N*-Fmoc-Leu-OH (4.9 g, 14 mmol) and *i*-Pr$_2$EtN (3.8 mL, 28 mmol) in CH_2Cl_2 (14 mL), and the resulting slurry was stirred for 16 h on an orbital shaker. The resin was then filtered, washed (3 x 10 mL of CH_2Cl_2/CH_3OH/*i*-Pr$_2$EtN (17:2:1), 3 x 10 mL of CH_2Cl_2, 2 x 10 mL of NMP, 2 x 10 mL of CH_2Cl_2, 2 x 10 mL of diethyl ether), and dried under high vacuum. The yield for loading of the first amino acid was determined to be 76% by Fmoc quantitation. The resin was then swelled in CH_2Cl_2, and the Fmoc group was removed by treatment with 10 mL of a 20% piperidine solution in DMF, followed by filtration and washing (5 x 10 mL of DMF, 5 x 10 mL of CH_2Cl_2). The second amino acid was coupled by treating the resin with a preformed solution containing *N*-Fmoc-Arg(Pbf)-OH (6.8 g, 10.5 mmol),

HOBt (1.4 g, 10.5 mmol), and DIC (1.6 mL, 10.5 mmol) in DMF (10.5 mL) that had been pre-incubated for 10 min and the resulting slurry was shaken in an orbital shaker for 19 h to obtain dipeptide **19**. The resin was then filtered and washed, and the Fmoc group was removed according to the standard procedure described previously. The resin was then treated with a preformed solution containing **18** (2.3 g, 8.0 mmol), HOBt (1.0 g, 8.0 mmol), and DIC (1.3 mL, 8.0 mmol) in 16 mL of DMF that had been pre-incubated for 10 min. The reaction was allowed to proceed overnight, and then the resin was filtered, washed, and dried under high vacuum to obtain support-bound **20**. The side chain-protected peptide was then cleaved off solid support by treatment with 10 mL of a hexafluoroisopropanol/CH_2Cl_2 (1:4) solution. The filtrate was collected, and the resin was washed with 1 x 10 mL cleavage solution, 5 x 5 mL CH_2Cl_2, and the combined filtrate was concentrated by rotary evaporation. The solid was then purified by HPLC and lyophilized to afford the side chain-protected peptide. The carboxy terminus was activated by treatment with DIC (0.28 mL, 1.8 mmol) and HOBt (240 mg, 1.8 mmol) in DMF (1.8 mL) for 10 min, followed by addition of Edans. The reaction was allowed to proceed overnight, and the crude solution was purified by HPLC to afford peptide **19** as a white solid. Prior to the coupling reaction leading to the FRET thioester substrate, intermediate **19** was treated with a 5 N aqueous ammonia solution that contained a few drops of 2-mercaptoethanol under an Argon atmosphere. The reaction was allowed to proceed for 4 h, and after concentration by rotary evaporation, the free thiol-containing peptide was purified by HPLC and lyophilized to obtain intermediate **21**.

Part B: 2-Chlorotrityl chloride resin (2 g, 1.4 mmol/g resin) was swelled with CH_2Cl_2 (10 mL) for 1 h. The resin was then treated with a solution of *N*-Fmoc-Phe-OH (5.4 g, 14 mmol) and *i*-Pr_2EtN (3.8 mL, 28 mmol) in CH_2Cl_2 (14 mL) and the resulting slurry stirred for 16 h on an orbital shaker. The resin was then filtered, washed, and dried under high vacuum. The yield for loading of the first amino acid was determined to be 73% by Fmoc quantitation. The resin was then

swelled in CH_2Cl_2, and the Fmoc group was removed by treatment with 10 mL of a 20% piperidine solution in DMF, followed by filtration and washing. The second amino acid was coupled by treating the resin with a preformed solution containing *N*-Fmoc-Val-OH (3.4 g, 10 mmol), HOBt (1.4 g, 10 mmol), and DIC (1.6 mL, 10 mmol) in DMF (10 mL) that had been pre-incubated for 10 min and the resulting slurry was shaken in an orbital shaker for 19 h. The resin was then filtered, washed, and the Fmoc group was removed. The resin was then treated with a preformed solution containing *N*-Fmoc-Ile-OH (3.5 g, 10 mmol), HOBt (1.4 g, 10 mmol), and DIC (1.6 mL, 10 mmol) in DMF (10 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no free amine was present (18 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Pro-OH (3.4 g, 10 mmol), HOBt (1.4 g, 10 mmol), and DIC (1.6 mL, 10 mmol) in DMF (10 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no free amine was present (3 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing *N*-Fmoc-Lys(Boc)-OH (4.7 g, 10 mmol), HOBt (1.4 g, 10 mmol), and DIC (1.6 mL, 10 mmol) in DMF (10 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed until no free amine was present (13 h), followed by filtration, washing, and removal of the Fmoc group. The resin was then treated with a preformed solution containing Dabcyl (1.7 g, 6.0 mmol), HOAt (820 mg, 6.0 mmol), and DIC (0.93 mL, 6.0 mmol) in DMF (20 mL) that had been pre-incubated for 10 min. The reaction was allowed to proceed for 48 h, followed by filtration and washing, and dried under high vacuum. The side chain-protected peptide was then cleaved off support by treatment with 5 mL of a hexafluoroisopropanol/CH_2Cl_2 (1:4) solution for 1 h. The filtrate was collected, and the resin was washed (5 x 2 mL CH_2Cl_2). The combined filtrate was concentrated by rotary evaporation, purified by HPLC, and lyophilized to afford **22** as a bright red solid. Peptide **22** was then dissolved in DMF (480 μL) and then was activated by addition of PyBOP (25 mg, 48 μmol), HOBt (6.5 mg, 48 μmol), and *i*-Pr_2EtN. The activated ester of **22** was then reacted with **21** to obtain a red

solid that was then purified by HPLC and lyophilized to afford the side chain-protected FRET peptide. Removal of the side chain protecting groups was accomplished by treatment with trifluoroacetic acid for 1 h. After reaction completion, 3 volumes of toluene were added to the reaction mixture, which was then concentrated to dryness by rotary evaporation. The crude product was purified by HPLC and lyophilized to obtain **1b** as a red solid. HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{79}H_{106}N_{16}O_{12}S_2$, 1535.7632; found, 1535.7696.

Synthesis of (R)-2-bromo-3-phenylpropanoic acid (5, R = Bn)

A 500-mL 3-neck round-bottom flask with an overhead stirrer was charged with KBr (18.7 g, 157 mmol) and 249 mL of 0.75 M aq HBr (21.3 mL of 48% HBr (aq) in 228 mL of water). After cooling the solution to -7 ^oC, sodium nitrite (5.6 g, 81 mmol) was added over 1 h followed by the addition of D-phenylalanine (7.00 g, 42.4 mmol) while maintaining the temperature of the reaction solution between -4 ^oC and -7 ^oC. The reaction mixture was vigorously stirred for 1.5 h and then was extracted with EtOAc that had been precooled to 0 ^oC (3 x 200 mL). The combined organic layers were washed with brine (2 x 150 mL), dried over $MgSO_4$, and concentrated in vacuo. Purification by column chromatography over silica gel (60:40:1 hexanes/ethyl acetate/acetic acid) gave 5.12 g (22.4 mmol, 52.7%**)** of **6** as a colorless viscous oil. Only 2% racemization was observed by LC. ^1H NMR (400 MHz, $CDCl_3$): δ 3.40 (dd, 1H, *J* = 14.2, 7.3), 3.62 (dd, 1H, *J* = 14.2, 6.1), 4.61 (t, 1H, *J* = 7.6), 7.37-7.51 (m, 5H), 12.24 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.07, 136.49, 129.38, 128.98, 127.70, 45.16, 40.79). Anal. calcd for $C_9H_9BrO_2$: C, 47.19; H, 3.96. Found: C, 47.29; H, 3.74. ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_9H_{10}BrO_2$, 228.98; 230.98. Found: 228.9, 230.9. IR (cm^{-1}): 3031 (br), 2664, 2574, 1717. The analytical data corresponded exactly according to that described in the literature.[5]

Synthesis of (S) 2-bromo-3-phenylpropanoic acid (ent-5, R = Bn)

Compound **(ent-5, R = Bn)** was prepared in the same manner as **5** (R = Bn), starting from L-phenylalanine.

Synthesis of 7 (R = Bn)

A 100-mL round-bottom flask was charged with a solution of *N*-Boc-L-phenylalanine (13 mmol, 3.5 g) and THF (44 mL). The resulting solution was stirred under a N_2 atmosphere and cooled to 0 ^oC and then *i*-Pr_2EtN (9.0 mL, 52 mmol) was added. Isobutyl chloroformate (3.5 mL, 52 mmol) was then added, and the resulting mixture was stirred for 1 h at 0 ^oC. Then H_2S was bubbled through the mixture for 2 h, after which the H_2S flow was stopped, and the reaction was allowed to proceed overnight. The reaction mixture was then concentrated in vacuo, dissolved in CH_2Cl_2, washed with citric acid (10% w/w in water) (2 x 200 mL) and then brine (2 x 200 mL), dried over $MgSO_4$, and concentrated in vacuo to afford 3.5 g of a yellow viscous oil, which contained **7a** (6.6 mmol, 51%, based on ^1H NMR data) and the unreacted starting material. This oil was used in subsequent reaction steps without further purification. ^1H NMR (400 MHz, $CDCl_3$): δ7.18-7.34 (m, 5H), 4.52-4.60 (m, 1H), 3.16 (dd, 1H, *J* = 13.8, 8.8), 3.09 (dd, 1H, *J* = 21.0, 13.9), 1.41 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 129.28, 128.74, 128.50, 127.22, 77.34, 77.02, 76.70, 28.25, 27.99). HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{20}NO_3S$, 282.1164; found, 282.1164. IR (cm^{-1}): 3337, 3025, 3088, 3058, 2980, 2924, 2552, 1703.

Preparation of Thioester Peptide Library of Format Ac-Lys-Pro-Ile-X-Phe~S~Phe-Arg-Leu-NH_2 (12a-12q)

MBHA resin (3.0 g, 0.70 mmol/g resin) was treated with a 10% solution of *i*-Pr_2EtN in CH_2Cl_2 (5 mL/g resin) for 45 min. After displaying a positive Kaiser test, the resin was filtered and washed with CH_2Cl_2 (5 x 15 mL). A preformed solution of *N*-Boc-Leu-OH (2.6 g, 11 mmol), PyBOP (5.5 g, 11 mmol), and HOBt (1.4 g, 11 mmol) in CH_2Cl_2/DMF (1:1, 10.5 mL) was poured onto the resin, and the resulting slurry was stirred for 4 min on an orbital shaker, followed by addition of *i*-Pr_2EtN (2.9 mL, 21 mmol). The resulting slurry was shaken in a wrist-action shaker for 14 h. The resin was then filtered, washed, and dried under high vacuum. After swelling the resin in CH_2Cl_2 for 2 h, the *N*-Boc protecting group was removed by treatment with 50% TFA in CH_2Cl_2 (20 mL) for 15 min, followed

by filtration, and washing (5 x 15 mL of NMP, 5 x 15 mL of CH_2Cl_2, 2 x 15 mL of diethyl ether). The resin was then swollen in a minimum amount of CH_2Cl_2/DMF (1:1) and treated with a solution of *N*-Boc-Arg(Mts)-OH (2.9 g, 6.3 mmol), PyBOP (3.3 g, 6.3 mmol), and HOBt (0.85 g, 6.3 mmol) in CH_2Cl_2/DMF (1:1, 6.3 mL). The resulting slurry was shaken for 4 min, before the addition of *i*-Pr_2EtN (1.7 mL, 13 mmol). After no unreacted amine was present (within 14 h), the resin was filtered, washed, and dried overnight under high vacuum. After removal of the *N*-Boc protecting group, the resin was swollen and treated with a preformed solution of the anhydride of **5 (R = Bn)** (10.5 mmol) in CH_2Cl_2 (21 mL). This solution was obtained by stirring **5 (R = Bn)** (21 mmol, 4.8 g) and DIC (10.5 mmol, 1.64 mL) in CH_2Cl_2 (21 mL) in a frit syringe fitted with a filter. After 30 min, the solution was filtered directly onto the resin. The solid remaining in the syringe was washed with DMF (21 mL) thus bringing the total concentration to 0.5 M. The resulting slurry was then stirred for 2 h, after which no unreacted amine was present. The resin was filtered and washed. For the S_N2 displacement of bromide by **7 (R = Bn)**, a preformed solution of **7 (R = Bn)** (1.8 g, 6.3 mmol)**,** *i*-Pr_2EtN (1.7 mL, 13 mmol), and CH_2Cl_2 (6.3 mL) was added to the resin. The slurry was flushed under N_2, followed by agitation in a wrist-action shaker for 16 h. The resin was then filtered, washed, dried under high vacuum, split into 17 portions of approximately 200 mg, and transferred to 10 mL syringes equipped with a fritted filter. Each porton of resin was then swollen in of CH_2Cl_2 (2 mL), followed by removal of the *N*-Boc protecting group, and then treatment with one of the following *N*-Boc-protected amino acids, [*N*-Boc-Ala-OH (200 mg, 1 mmol), *N*-Boc-Asn(Xan)-OH (400 mg, 1 mmol), *N*-Boc-Glu(OBzl)-OH (300 mg, 1 mmol), *N*-Boc-Gln-OH (200 mg, 1 mmol), *N*-Boc-Asp(OBzl)-OH (300 mg, 1 mmol), *N*-Boc-Gly-OH (200 mg, 1 mmol), *N*-Boc-His(Bom)-OH (400 mg, 1 mmol), *N*-Boc-Ile-OH (200 mg, 1 mmol), *N*-Boc-Leu-OH (300 mg, 1 mmol), *N*-Boc-Lys(2-Cl-Z)-OH (400 mg, 1 mmol), *N*-Boc-Phe-OH (300 mg, 1 mmol), *N*-Boc-Pro-OH (200 mg, 1 mmol), *N*-Boc-Ser(Bzl)-OH (300 mg, 1 mmol), or *N*-Boc-Thr(Bzl)-OH (300 mg, 1 mmol)], as well as PyBOP (500 mg, 1 mmol) and HOBt (100 mg, 1 mmol) in CH_2Cl_2/NMP (1:1, 1 mL). The resulting slurry was shaken for 4 min, before the

addition of *i*-Pr_2EtN (0.3 mL, 2 mmol). The slurry was then shaken in a wrist-action shaker for 16 h. The resin was then filtered, washed, and the *N*-Boc protecting group was removed. After deprotection the resin was swollen and treated with a solution of *N*-Boc-Ile-OH (200 mg, 1 mmol), PyBOP (500 mg, 1 mmol), and HOBt (100 mg, 1 mmol) in CH_2Cl_2/NMP (1:1, 1 mL). The resulting slurry was shaken for 4 min, before the addition of *i*-Pr_2EtN (0.3 mL, 2 mmol). The slurry was then shaken in a wrist-action shaker for 18 h. The resin was then filtered, washed, and the *N*-Boc protecting group was removed followed by treatment with a solution of *N*-Boc-Pro-OH (200 mg, 1 mmol), PyBOP (500 mg, 1 mmol), and HOBt (100 mg, 1 mmol) in CH_2Cl_2/NMP (1:1, 1 mL). The resulting slurry was shaken for 4 min, before the addition of *i*-Pr_2EtN (0.3 mL, 2 mmol). The slurry was then shaken in a wrist-action shaker for 19 h. The resin was then filtered and washed. After removal of the *N*-Boc protecting group the resin was treated with a solution of *N*-Boc-Lys(2-Cl-Z)-OH (400 mg, 1 mmol), PyBOP (500 mg, 1 mmol), and HOBt (100 mg, 1 mmol) in CH_2Cl_2/NMP (1:1, 1 mL). The resulting slurry was shaken for 4 min, before the addition of *i*-Pr_2EtN (0.3 mL, 2 mmol). The slurry was then shaken in a wrist-action shaker for 16 h. The resin was then filtered, washed, and the *N*-Boc protecting group was removed followed by capping of the *N*-terminal amine with acetic anhydride by treatment of resin with 1 mL of a dichloromethane solution containing acetic anhydride (0.5 M) and pyridine (1.0 M). After no free amine was present the resin was filtered, washed, and dried overnight under high vacuum. Cleavage of substrates off the resin and removal of side chain-protecting groups was achieved by treatment with 2 mL of a 10% solution of trifluoromethanesulfonic acid in TFA for 4 h. The resin was then filtered and washed (5 x 1 mL of TFA, 5 x 1 mL of CH_2Cl_2, 2 x 1 mL of CH_3OH). The filtrates were collected, concentrated in vacuo, purified by preparative HPLC, and lyophilized to afford **12a** (31.6 mg, 24%), **12b** (14.7 mg, 11%), **12c** (16.8 mg, 12%), **12d** (27.7 mg, 20%), **12e** (12.6 mg, 9%), **12f** (22.4 mg, 18%), **12g** (32.3 mg, 25%), **12h** (14.4 mg, 10%), **12i** (20.1, 14%), **12j** (49.7 mg, 32%), **12k** (22.7 mg, 15%), **12l** (10.8 mg, 8%), **12m** (7.7 mg, 6%), **12n** (15.7 mg, 10%), **12o** (14.4 mg, 10%), **12p** (27.4 mg, 21%), **12q** (11.4 mg, 8%). HRMS-

FAB (*m/z*): **12a**: $[M+H]^+$ calcd for $C_{52}H_{80}N_{12}O_9S$, 1049.5970; found, 1049.5957. HRMS-FAB (*m/z*): **12b**: $[M+H]^+$ calcd for $C_{53}H_{81}N_{13}O_{10}S$, 1092.6028; found, 1092.6058. HRMS-FAB (*m/z*): **12c**: $[M+H]^+$ calcd for $C_{54}H_{82}N_{12}O_{11}S$, 1107.6025; found, 1107.6023. HRMS-FAB (*m/z*): **12d**: $[M+H]^+$ calcd for $C_{54}H_{83}N_{13}O_{10}S$, 1106.6185; found, 1106.6191. HRMS-FAB (*m/z*): **12e**: $[M+H]^+$ calcd for $C_{53}H_{80}N_{12}O_{11}S$, 1093.5868; found, 1093.5879. HRMS-FAB (*m/z*): **12f**: $[M+H]^+$ calcd for $C_{51}H_{78}N_{12}O_9S$, 1035.5814; found, 1035.5807. HRMS-FAB (*m/z*): **12g**: $[M+H]^+$ calcd for $C_{55}H_{86}N_{12}O_9S$, 1091.6440; found, 1091.6439. HRMS-FAB (*m/z*): **12h**: $[M+H]^+$ calcd for $C_{54}H_{84}N_{12}O_9S$, 1077.6263; found, 1077.6283. HRMS-FAB (*m/z*): **12i**: $[M+H]^+$ calcd for $C_{55}H_{87}N_{13}O_9S$, 1106.6549; found, 1106.6552. HRMS-FAB (*m/z*): **12j**: $[M+H]^+$ calcd for $C_{55}H_{86}N_{12}O_9S$, 1091.6440; found, 1091.6439. HRMS-FAB (*m/z*): **12k**: $[M+H]^+$ calcd for $C_{58}H_{84}N_{12}O_9S$, 1125.6283; found, 1125.6277. HRMS-FAB(*m/z*): **12l**: $[M+H]^+$ calcd for $C_{52}H_{80}N_{12}O_{10}S$, 1065.5919; found, 1065.5910. HRMS-FAB (*m/z*): **12m**: $[M+H]^+$ calcd for $C_{53}H_{82}N_{12}O_{10}S$, 1079.6076; found, 1079.6075. HRMS-FAB (*m/z*): **12n**: $[M+H]^+$ calcd for $C_{58}H_{84}N_{12}O_{10}S$, 1141.6232; found, 1141.6243. HRMS-FAB (*m/z*): **12o**: $[M+H]^+$ calcd for $C_{55}H_{82}N_{14}O_9S$, 1115.6188; found, 1115.6202. HRMS-FAB (*m/z*): **12p**: $[M+H]^+$ calcd for $C_{54}H_{82}N_{12}O_9S$, 1075.6127; found, 1075.6123. HRMS-FAB (*m/z*): **12q**: $[M+H]^+$ calcd for $C_{55}H_{86}N_{12}O_9S$, 1091.6440; found, 1091.6428.

Absorbance-based Assay of Aspartyl Protease Cathepsin D

Release of 4-thiopyridone after proteolytic cleavage of the thioester peptide substrates was monitored using a Spectra Max 190 96-well microtiter plate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 324 nm. Substrate samples were stored at -20 ^oC as 80 mM stock solutions in DMSO. A 0.1 M stock solution of 4,4'-dipyridyl disulfide was prepared in DMSO and stored at -20 ^oC prior to use. Standard assay conditions were 0.1 M citrate buffer, pH 4, 5% DMSO, 25 nM cathepsin D, 2.5 mM thiopeptide substrate. Hydrolysis reactions were carried out in triplicate and monitored for up to 15 minutes. Relative rates of hydrolysis were determined over the linear region after subtracting background hydrolysis without enzyme which was negligible.

Fluorescence assays

Cleavage of the FRET substrates (**1a** and **1b**) was monitored using a Spectra Max Gemini XS (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 360 nm and an emission wavelength of 490 nm. A stock solution of the substrate sample was prepared in DMSO and diluted with assay buffer to a final concentration varying from 0.1 μM to 10 μM**.** Standard assay conditions were 0.1 M citrate buffer, 0.1% Tween-20, pH 4, 5% DMSO, 2 nM cathepsin D. Relative rates of hydrolysis were determined over the linear region after subtracting background hydrolysis without enzyme which was negligible. HPLC analysis of proteolytis of compound **1b** is provided in Figure 1.

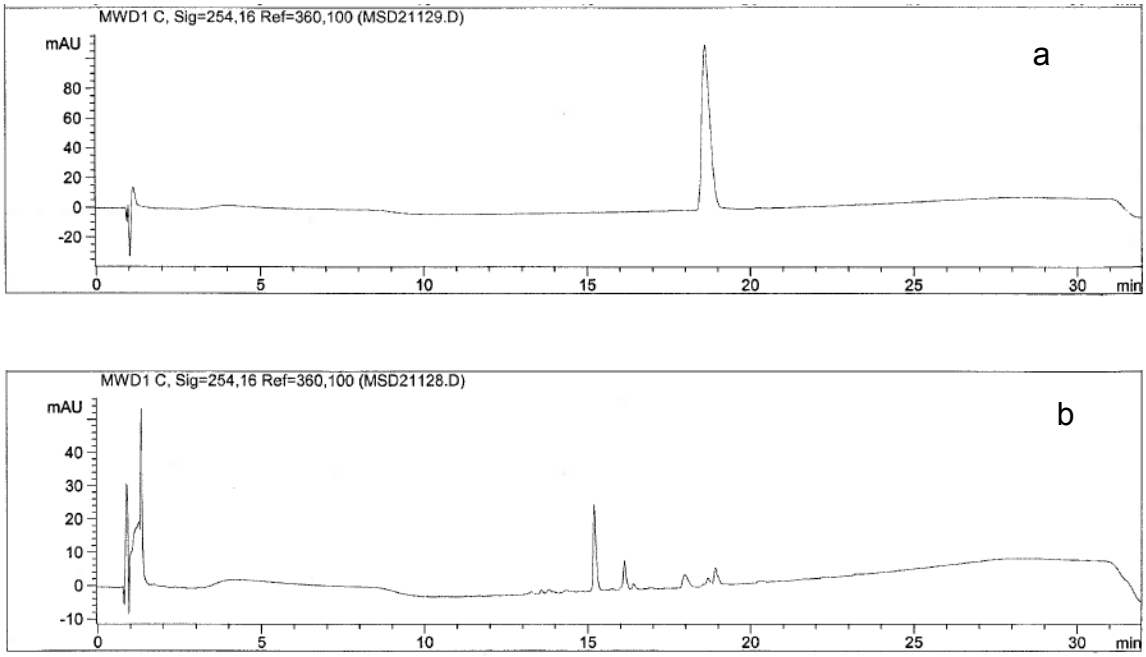


Figure 1. HPLC analysis of proteolytic cleavage of compound **1b** with cathepsin D.

a) **1a** before treatment with cathepsin D. R_T(min): 18.597; MS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{79}H_{106}N_{16}O_{12}S_2$, 1535.77; found, 1536.2.

b) **1b** after treatment with cathepsin D. Peak 1 (major diastereomer of C-terminal cleavage fragment): R_T(min): 15.190; Area: 162.058; MS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{33}H_{45}N_7O_6S_2$, 700.30; found, 700.8. Peak 2 R_T(min): 16.114; Area: 71.255;

MS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{33}H_{45}N_7O_6S_2$, 700.30; found, 700.0. Peak 3 (N-terminal cleavage fragment): R_T(min): 17.974; Area: 40.841; MS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{46}H_{63}N_9O_7$, 854.50; found, 855.0. Peak 4 (unreacted **1b**): R_T(min): 18.910; Area: 31.160; MS-ESI (*m*/*z*): $[M+2H]/2^+$ calcd for $C_{79}H_{106}N_{16}O_{12}S_2$, 768.39; found, 769.0.

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