Supplementary Material

Experimental Methods

General. Rink Amide MBHA resin was purchased from Novabiochem. *O*-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Iris Biotech (Marktredwitz, Germany). Fmoc protected amino acids and all other reagents were of peptide synthesis grade and obtained from Auspep (Melbourne, Australia). Semi-preparative rp-HPLC purification of the linear peptides was performed using a Waters Delta 600 chromatography system fitted with a Waters 486 tunable absorbance detector with detection at 214 nm. Purification was performed by eluting with solvents A (0.1% TFA in water) and B (9:1 CH_3CN:H_2O, 0.1% TFA) on a Phenomenex Luna C_{18} 15m 250 x 21.2 mm steel jacketed column run at 20 mL/min. Analytical rp-HPLC analyses were performed using a Waters 600 chromatography system fitted with a Waters 996 photodiode array detector and processed using Waters Millenium software. Analytical analyses were performed using gradient elutions with solvents A and B on a Phenomenex Luna C_{18} 5m 250 x 4.6 mm column run at 1.0 mL/min. Mass spectra were obtained on a hybrid quadrupole TOF mass spectrometer (PE SCIEX API QSTAR Pulsar) equipped with an Ionspray (pneumatically assisted electrospray) operating at ambient temperature (ISMS). Calibration standard was ammonium adducts of polypropylene glycol (PPG1000).

Azide Irradiations. All alkyl azides were irradiated through clear soda-glass vials in which the compounds were enclosed. The azides were either irradiated using laboratory fluorescent lights or direct sunlight. The laboratory lighting was comprised of a series of 36W Osram Lumilux fluorescent tubes. This fluorescent lighting generally emits light of wavelength ranging from 280nm-700nm. The alkyl azide samples were routinely placed on a bench top about 3 metres away from these lights. For irradiation of solid azides the samples were simply placed in glass vials and left for the specified time either in laboratory light or sunlight. For irradiation of solutions of the azides, a 2mg/15ml solution was prepared in 60/40 acetonitrile/water.

Enzyme Assays

All enzymes were purchased from Merck Pty Ltd.

Assay Buffers

Caspase-1: 100mM NaCl, 50mM HEPES, 10mM DTT, 1mM EDTA, 10% Glycerol, 0.1% CHAPS, pH=7.4

Caspase-3: 100mM NaCl, 50mM HEPES, 10mM DTT, 1mM EDTA, 10% Glycerol, 0.1% CHAPS, pH=7.4

Cathepsin K: 100mM Na acetate, 10mM DTT, 120mM NaCl, pH=5.5

Enzymes Concentrations

Caspase-1: Recombinant human caspase-1 (3000U) was diluted with 700µL of buffer, divided into 70µL aliquots and stored at –80°C. The diluted solution (4.5µL) was used in each well for individual reactions.

Caspase-3: Recombinant human caspase-3 (5000U) was was diluted with 1ml of buffer, divided into 65µL aliquots and stored at –80°C. Each 65uL aliquot was subsequently diluted to 240uL and this solution used for each well (5µL).

Cathepsin K: Recombinant human cathepsin K (25mg) was purchased from Calbiochem. The enzyme solution was diluted with 600ml of buffer, divided into 65µL aliquots and stored at –80°C. Each 65uL aliquot was subsequently diluted to 240uL and this solution used for each well (5µL).

Substrates*:*

Caspase-1: A 2.8mM DMSO solution of Ac-YVAD-AMC (Merck) was diluted 200X in buffer to a final concentration of 14mM in each well (K_m=14nM).

Caspase-3: A 1.94mM DMSO solution of Ac-DEVD-AMC (Merck) was diluted 200X in buffer to a final concentration of 10mM in each well (K_m=9.7nM).

Cathepsin K: A 2.0mM DMSO solution of z-F-R-AMC (Merck) was diluted 200X in buffer to a final concentration of 10mM in each well (K_m=70mM).

Inhibitors

Each inhibitor that was tested for inhibitory potency was dissolved in DMSO to a concentration of 5mM and diluted out to the required concentration range. Seven-ten

different concentrations (2 fold dilutions) were used which spanned the IC_{50} value of the inhibitor and were each repeated in duplicate. 2μL of the DMSO solution was applied to each well.

Enzyme Inhibition Assays.

In each well of a 96 well plate was placed 1μL of substrate, 2μL of inhibitor and 4.5-5ml of the enzyme made up to 200μL in buffer. The enzyme was incubated with inhibitor for 5 minutes prior to the addition of substrate and the reaction monitored on a fluorostar spectrophotometer (BMG Technologies) in a 96-well plate format, using an excitation wavelength of 380nm and emission of 460nm to detect free AMC. The IC_{50} value for each inhibitor was determined using non-linear regression by fitting the data to a sigmoidal dose-response curve plotting log [I] vs Vi/Vo. The assays for caspases-1, -3 were conducted at room temperature, the assay for cathepsin K was conducted at 30°C.

Synthesis of Aldehyde 2.

Boc-Leucinyl-Norleucinol (2g, 6.1mmol)[1] was dissolved in neat TFA and stirred at room temperature for 30 minutes. The TFA was removed *in vacuo* and the residue dissolved in DMF (20ml) and to this solution was added HBTU (2.27g, 6.0mmol), 2-naphthoic acid (1.05g, 6.0 mmol) and finally DIPEA (4 ml, 24mmol). This solution was stirred for 1 hour, after which it was acidified by the addition of 10% citric acid solution (100mL). The mixture was extracted with ethyl acetate (2 x 50ml) and the aqueous layer discarded. The combined ethyl acetate layer was washed with citric acid solution (2 x 100ml), water (1x 100ml), sat. $NaHCO_3$ (2 x 100mml), water (5x100ml) and dried and evaporated to give 2-naphthoyl-leucinyl-norleucinol (1.8g, 4.68mmol) in 78% yield. This compound was pure enough to be used without further purification.

MS (M+H) = 385 Da.

^{1}Hnmr (600MHz, d_6-DMSO) δ 8.301 (s, 1H, 2-Nap-H); 7.93-7.82 (m, 4H, 2-Nap-H), 7.58-7.53 (m, 2H, 2-Nap-H), 6.79 (d, 1H, J=8.1Hz, Leu-NH), 6.48 (d, 1H, J=7.8Hz, Nle-NH), 4.73 (m, 1H, Leu-α-CH), 3.93 (m, 1H, Nle-α-CH), 3.71(dd, 1H, J=11.03, 3.52Hz), Nle-

CH_2-OH), 3.61(dd, 1H, J=11.03, 5.90Hz, Nle-CH_2-OH), 1,87-1.72 (m, 3H, Leu-βH,-γH), 1.55 (m, 1H, Nle-βH), 1.48 (m, 1H, Nle-βH), 1.28 (m, 4H, Nle-γH,δH), 1.02 (d, 3H, J=4.96Hz, Leu-δ–CH_3), 1.01 (d, 3H, J=4.84Hz, Leu-δ–CH_3), 0.80 (t, 3H, J= 6.93Hz, Nle-ε-CH_3).

2-naphthoyl-leucinyl-norleucinol (50mg, 0.13mmol) was dissolved in dichloromethane (5ml) and to the solution was added Dess-Martin periodinane (100mg, 0.24mmol). The mixture was stirred at room temperature for two hours after which a solution of 10% sodium thiosulphate in saturated Na HCO_3 (10ml) was added and solution stirred for a further hour. The solution was extracted with dichloromethane (2x10ml) and the combined dichloromethane layers dried and evaporated. The resultant residue was dissolved in 60/40 acetonitrile/water solution (10ml) and purified by preparative HPLC. The title compound (2-naphthoyl-leucinyl-norleucinal) was lyophilized to a white powder (16mg, 0.041mmol) in 32% yield.

MS (M+H) = 383

^{1}Hnmr (600MHz, $CDCl_3$) δ 9.60 (s, 1H, CHO), 8.31 (s, 1H, 2-Nap-H); 7.94-7.82 (m, 4H, 2-Nap-H), 7.60-7.54 (m, 2H, 2-Nap-H), 6.71 (m, 2H, Leu-NH, Nle-NH), 4.80 (m, 1H, Leu-α-CH), 4.51 (m, 1H, Nle-α-CH), 1.96-1.72 (m, 4H, Leu-βH,-γH, Nle-βH), 1.64 (m, 1H, Nle-βH), 1.30 (m, 4H, Nle-γH,δH), 1.03 (d, 3H, J=6.41Hz, Leu-δ–CH_3), 1.02 (d, 3H, J=6.35Hz, Leu-δ–CH_3), 0.83 (t, 3H, J= 7.01Hz, Nle-ε-CH_3).

Synthesis of monoacyl aminal 3

The primary amide 2-Nap-Leu-Nle-NH_2 was synthesised by standard solid phase peptide synthesis. Rink amide MBHA resin (SV=0.67 meq/g, 0.746g) was deprotected with 50/50 DMF/piperidine solution (2x1min). Norleucine, Leucine and 2-naphthoic acid were coupled onto the resin via the procedure described below. Four equivalents of Fmoc-protected amino acid (norleucine, leucine and 2-naphthoic acid), 4 equivalents of HBTU and two equivalents of DIPEA were employed in each coupling. Fmoc-deprotections and resin neutralization was achieved by 2 x 1 minute treatments with excess 1:1 piperidine:DMF. Coupling yields were monitored by quantitative ninhydrin assay. The peptide was cleaved from resin by treatment with a solution containing 95% trifluoroacetic acid (TFA):2.5% H_2O: 2.5% triisopropylsilane (TIPS) (25mL solution per 1gm of peptide-resin) for 2h at room temperature. The TFA solution was filtered, concentrated *in vacuo,* diluted with 50:50

water/acetonitrile solution (10ml), purified by semi-preparative rp-HPLC and lyophilized to a white powder (120mg, 0.3mmol) in 60% yield. This was used directly for the next reaction.

ESI-MS (M+H) = 398

^{1}Hnmr (600MHz, d_6-DMSO) δ 8.67 (d, 1H, J=7.98Hz, LeuNH), 8.51 (s, 1H, 2-Nap-H); 8.04-7.96 (m, 4H, 2-Nap-H), 7.85 (d, 1H, J=8.18Hz, Nle-NH), 7.63-7.58 (m, 2H, 2-Nap-H), 7.30 (s,1H, -NH_2) 7.01 (s, 1H, -NH_2), 4.56 (m, 1H, Leu-α-CH), 4.20 (m, 1H, Nle-α-CH), 1,77-1.74 (m, 2H, Leu-βH), 1.67 (m, 1H, Nle-βH), 1.59 (m, 1H, Leu γ-CH), 1.53 (m, 1H, Nle-βH), 1.24 (m, 4H, Nle-γH,δH), 0.94 (d, 3H, J=6.22Hz, Leu-δ–CH_3), 0.90 (d, 3H, J=6.30Hz, Leu-δ–CH_3), 0.81 (t, 3H, J= 6.93Hz, Nle-ε-CH_3).

2-Nap-Leucinyl-Norleucinyl amide (100mg, 0.25mmol) was dissolved 50/50 water/acetonitrile (15ml) and to this solution was added [bis(trifluoroacetoxy)iodo]benzene (0.108g, 0.25mmol). The mixture was stirred at room temperature for 3hrs after which a further 50mg of the iodobenzene reagent was added and reaction mixture stirred for a further 3hrs. This solution was then filtered and injected directly onto a preparative HPLC column and purified. The monoacyl aminal **3** was obtained as a white solid (63mg, 0.17mmol) in 68% yield.

ESI-MS (M+H) = 370

^{1}Hnmr (600MHz, d_6-DMSO) δ 8.71 (d, 2H, J=7.94Hz, Leu-NH and Nle-NH), 8.51 (s, 1H, 2-Nap-H); 8.07 (br. s, 3H, -NH_3), 8.04-7.96 (m, 4H, 2-Nap-H), 7.64-7.59 (m, 2H, 2-Nap-H), 4.88 (m, 1H, Nle-α-CH), 4.60 (m, 1H, Leu-α-CH), 1,82-1.74 (m, 2H, Leu-βH), 1.74-1.68 (m, 1H, Nle-βH), 1.58-1.53 (m, 1H, Leu γ-CH), 1.39-1.33 (m, 1H, Nle-βH), 1.32-1.26 (m, 4H, Nle-γH,δH), 0.96 (d, 3H, J=6.38Hz, Leu-δ–CH_3), 0.92 (d, 3H, J=6.43Hz, Leu-δ–CH_3), 0.87 (t, 3H, J= 6.89Hz, Nle-ε-CH_3).

Irradiation of 2-Nap-Leu-Nle-N_3 1 in the presence $NaBH_4$

Under Laboratory Lights

A pure sample of 2-Nap-Leu-Nle-N_3 (2mg) and $NaBH_4$ (20mg) were dissolved in 60/40 acetonitrile/water mixture (15ml). The solution was divided into two portions in two identical soda-glass vials. One vial was covered with aluminium foil and the other exposed to laboratory lighting. Both solutions were analysed by HPLC every 24hrs. At the end of 72

hours the irradiated solution was comprised of a mixture of 25% amine (either **10** or both **10** and **11**), 8% alcohol **6** and starting material 67%.

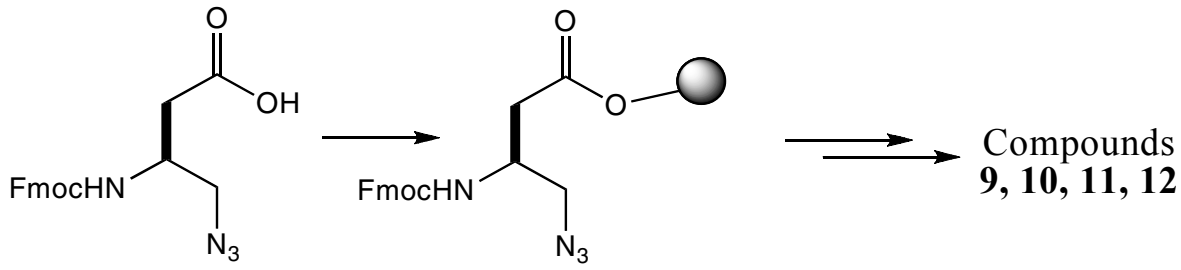
Large Scale in Sunlight

A pure sample of 2-Nap-Leu-Nle-N_3 (60mg, 0.15mmol) was dissolved in 20ml of 60/40 acetonitrile water mixture together with $NaBH_4$ (20eq.). The solution was placed in a soda-glass vial and left in direct sunlight for 3 hrs. The solution was subsequently filtered and purified by preparative HPLC. The major product that was isolated was amine **10** (10mg, 14%) which was lyophilized as a white powder. The rest of the mixture mainly consisting of starting material.

MS (M+H) = 384

^{1}Hnmr (600MHz, d_6-DMSO) δ 8.70 (d, 1H, J=7.76Hz, Leu-NH), 8.50 (s, 1H, 2-Nap-H); 8.01-7.95 (m, 4H, 2-Nap-H), 7.82 (d, 1H, J=8.63Hz, Nle-NH), 7.74 (br.s, 2H, -NH_2), 7.64-7.59 (m, 2H, 2-Nap-H), 4.53 (m, 1H, Leu-α-CH), 3.94 (m, 1H, Nle-α-CH), 2.89 (m, 1H, CH-NH_2), 2.80 (m, 1H, CH-NH_2), 1.79 (m, 1H, Leu-βH), 1.72 (m, 1H, Leu-γH), 1.61 (m, 1H, Leu-βH), 1.49 (m, 1H, Nle-βH), 1.39 (m, 1H, Nle-βH), 1.32-1.17 (m, 4H, Nle-γH, -δH), 0.95 (d, 3H, J=6.55Hz, Leu-δ–CH_3), 0.92 (d, 3H, J=6.51Hz, Leu-δ–CH_3), 0.79 (t, 3H, J= 6.75Hz, Nle-ε-CH_3).

Synthesis of compound 9 -12

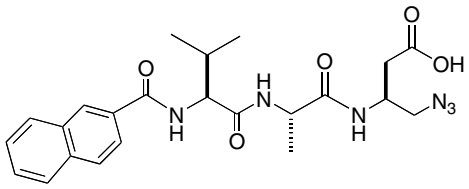


Scheme 1: Preparation of inhibitors **9-12**

The azidomethylene derivative of L-Fmoc-aspartic acid was prepared by deprotection of the corresponding tert-butyl ester[2] and loaded onto TCP resin (substitution 0.72 mmol.g^{-1}, 0.25 mmol scale syntheses, 350 mg resin). Peptides were synthesized manually by standard solid phase methods using HCTU/DIPEA activation for Fmoc chemistry on TCP resin. Four equivalents of Fmoc-protected amino acids, 4 equivalents of HCTU and two equivalents of DIPEA were employed in each coupling. Fmoc-deprotections and resin neutralization was

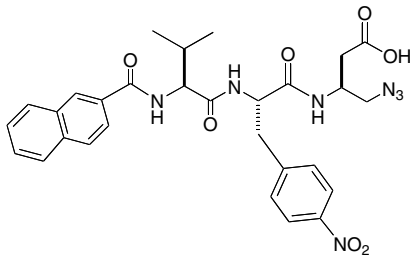
achieved by 2 x 2 min. treatments with excess 1:1 piperidine:DMF. Coupling yields were monitored by quantitative ninhydrin assay[3] and double couplings were employed for yields below 99.8%. N-terminal acetylation was achieved by treating the fully assembled and protected peptide-resins (after removal of the N-terminal Fmoc group) with HCTU-activated acetic acid as per normal coupling. The peptide was cleaved from resin and protecting groups simultaneously removed by treatment for 2h at room temperature with a solution containing 95% trifluoroacetic acid (TFA):2.5% H_2O: 2.5% triisopropylsilane (TIPS) (25mL solution per 1gm of peptide-resin). The TFA solutions were filtered, concentrated *in vacuo,* diluted with 50% A:50% B, lyophilized, and subsequently purified by semi-preparative rp-HPLC. All experimental procedures were performed with minimum exposure of the resin or the cleaved peptide to fluorescent lighting especially after final purification by preparative HPLC and lyophilization.

4-Azido-3-(S)-(2-(S)-{3-methyl-2-(S)-[(naphthalene-2-carbonyl)-amino]-butyrylamino}-propionylamino)-butyric acid. 2-Nap-Val-Ala-Asp-N_3. 9



^{1}Hnmr (600MHz, d_6DMSO) δ (ppm) 8.49 (s, 1H), 8.41 (d, J = 8.64 Hz, 1H), 8.12 (d, J = 7.44 Hz, 1H), 8.04 (m, 2H), 7.97 (m, 2H), 7.94 (m, 1H), 7.59 (m, 2H), 4.37 (dd, J = 8.22, 8.04 Hz, 1H), 4.27 (m, 1H), 4.14 (m, 1H), 3.39 (dd, J = 12.48, 4.44 Hz, 1H), 3.34 (dd, J = 12.60, 6.78 Hz, 1H), 2.41 (m, 2H), 2.16 (m, 1H), 1.22 (d, J = 7.02 Hz, 3H), 0.95 (m, 6H).
HRMS (M+H) calc. for $C_{23}H_{29}N_6O_5$ 469.2199, Found 469.2213.

(*S*)-4-azido-3-((*S*)-2-((*S*)-3-methyl-2-(naphthalene-2-carboxamido)butanamido)-3-(4-nitrophenyl)propanamido)butanoic acid 10

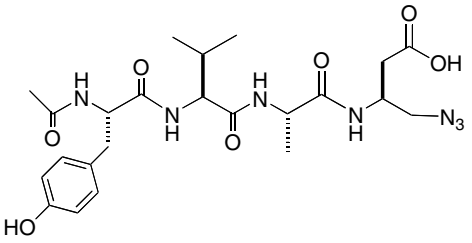


^{1}Hnmr (600MHz, d_6-DMSO) δ 8.43 (s, 1H), 8.34 (d, 1H, J=8.62Hz), 8.22 (m, 2H), 8.01 (d, J=8.75, 2H), 7.97 (d, J=8.58, 2H), 7.90 (m, 1H), 7.60 (m, 2H), 7.49 (d, J=8.75Hz, 2H), 4.62

(m, 1H), 4.28 (t, J=8.48Hz, 1H), 4.14 (m, 1H), 3.37 (m, 2H), 3.11 (dd, J= 4.89, 13.46, 1H), 2.92 (dd, J= 9.55, 13.54, 1H), 2.43 (m, 2H), 2.05 (m, 1H), 0.88 (d, J=6.71, 3H), 0.82 (d, J=6.74, 3H).

MS (M+H) = 590

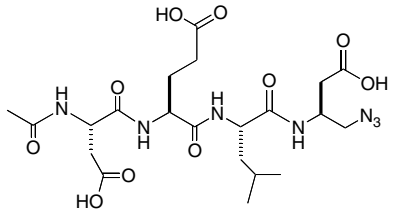
4-Azido-3-(S)-(2-(S)-{2-(S)-[2-(S)-Acetylamino-3-(4-hydroxy-phenyl)propionylamino]-3-methyl-butyrylamino}-propionylamino)-butyric acid. Ac-Tyr-Val-Ala-Asp-N_3. 11



^{1}Hnmr (600MHz, d_6DMSO) δ (ppm) 9.11 (br, 1H), 8.00 (m, 3H), 7.78 (d, J = 8.82 Hz, 1H), 7.01 (d, J = 8.40 Hz, 2H), 6.61 (d, J = 8.40 Hz, 1H), 4.45 (m, 1H), 4.23 (m, 1H), 4.14 (m, 2H), 3.39 (dd, J = 12.48, 4.38 Hz, 1H), 3.34 (dd, J = 12.54, 6.72 Hz, 1H), 2.86 (dd, J = 13.98, 3.96 Hz, 1H), 2.59 (dd, J =13.98, 10.2 Hz, 1H), 2.41(m, 2H), 1.96 (m, 1H), 1.73 (s, 2H), 1.19 (d, J = 7.08 Hz, 3H), 0.84 (d, J = 6.78 Hz, 3H), 0.81 (d, J = 6.78 Hz, 3H).

HRMS (M+H) calc. for $C_{23}H_{34}N_7O_7$ 520.2520, Found 520.2508.

4-Azido-3-(S)-{2-(S)-[2-(S)-(2-(S)-acetylamino-3-carboxy-propionylamino)-4-carboxy-butyrylamino]-4-methyl-pentanoylamino}-butyric acid. Ac DEVD-N_3. 12



^{1}Hnmr (600MHz, d_6-DMSO) δ 8.20 (d, J=7.59, 1H), 7.99 (d, J=7.59, 1H), 7.97 (d, J=8.05, 1H), 7.82 (d, J=7.53, 1H), 4.52 (m, 1H), 4.22 (m, 2H), 4.13 (m, 1H), 2.67 (dd, J= 5.90, 16.60, 1H), 2.46 (dd, J= 7.94, 16.57, 1H), 2.42 (d, J=6.92, 2H), 2.22 (m, 2H), 1.92 (m, 1H), 1.84 (s, 3H), 1.74 (m, 1H), 1.58 (m, 1H), 1.45 (m, 2H), 0.87 (d, J=6.61, 3H), 0.83 (d, J=6.54, 3H).

HRMS (M+H) calc. for $C_{21}H_{34}N_7O_{10}$ 544.2367, Found 544.2386.

Reference

1. Higuchi, N., Masayuki, S., Iwasawa, N., Sumida, M., Shibata, H., US patent 5081284, 1992.
2. Boeijen, A; van Ameijde, J; Liskamp R. M. J. *J. Org. Chem.* **2001**, 6, 8454-8462
3. Sarin, V.; Kent, S. B. H. Tan, J. P.; Merrifield, R. B. *Anal. Biochemistry* **1981**, *117*, 147.