Supporting Information
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MATERIALS AND METHODS

General Experimental details.

Flash column chromatography was carried out with silica gel 40-63 μm (VWR). Analytical TLC was performed on Merck Silica gel 60 F254 aluminium sheets (0.25 mm layer coating) and was visualised with short wavelength (366 nm) ultraviolet light. TLC Staining was performed with a solution of anisaldehyde.

Proton NMR (1H NMR) were recorded at 300 MHz or 600 MHz on Bruker AMX300 and AMX600 instruments respectively. 13C NMR experiments were run on the same instruments at 150 MHz or 75 MHz respectively. All NMR experiments were conducted at room temperature. LC-MS was conducted on a Waters UPLC/SQD-LC mass spectrometer with a linear gradient from 95% of solvent A (water with 0.1% formic acid) to 95% solvent B (acetonitrile with 0.1% formic acid) over 4 minutes at a flow rate of 0.6 mL/min. Preparative (RP)HPLC was carried out using a Phenomenex Jupiter 4 μm Proteo 90 Å, LC Column (diameter = 250 mm × 21.2 mm): flow rate 10.0 mL/min, detection at 230, 254 and 280 nm using a gradient of 95% water (0.1% TFA)/5% acetonitrile (0.1% TFA), to 40% water (0.1% TFA)/60% acetonitrile (0.1% TFA), over 45 min, using a Dionex Ultimate 3000 equipped with a fraction collector. Analytical (RP)HPLC was carried out using a Phenomenex SphereClone™ 5 μm ODS(2) 80 Å, LC Column (diameter = 250 mm × 4.6 mm): flow rate 1.0 mL/min, detection at 230, 254 and 280 nm using a gradient of 95% water (0.1% TFA)/5% acetonitrile (0.1% TFA), to 5% water (0.1% TFA)/95% acetonitrile (0.1% TFA), over 45 min, using a Dionex Ultimate 3000 HPLC system.

Standard protected amino acids and resins for Fmoc peptide synthesis were purchased from Merck Biosciences or Cambridge Reagents Ltd and were used as supplied. 2-fluoro-β-Alanine and other routine chemicals were purchased from Sigma while 2,2-difluoro-β-alanine was prepared according to the method reported by Cheguillaume et al., which is based on that originally described by Katritzky. Briefly N,N-(dibenzyl)-1H-benzotriazolyl-1-methylamine is employed to alkylate the zinc enolate derived from ethyl bromodifluoroacetate in a Reformatsky-type reaction to afford the N,N-dibenzyl-2,2-difluoro-β-alanine ethyl ester. Debenzylation with concomitant ethyl ester hydrolysis was achieved by catalytic hydrogenation over Pd(OH)2 in aqueous HCl. 2,2-difluoro-β-alanine hydrochloride was then isolated as a white solid. 1H NMR (300 MHz, D2O) δ 3.63 (2H, t, J = 15.2, CH2), 19F NMR δ -110.1 (s, CF2), 13C NMR (75 MHz, D2O) δ 167.2 (C=O, t) 113.6 (t, CF2), 42.3 (t, CH2) ppm. ESI+ MS (m/z) calculated for C3H5F2NO2 125.0288 found [MH]+ 126.1 Da.

General peptide synthesis. For manual solid phase syntheses, steps were performed in sintered 12 ml ISOLUTE SPE syringe tubes (Biotage). For a 0.05 mmol scale synthesis, couplings were performed using 10 equivalents of the amino acid dissolved in DMF (1.1 mL), 10 equivalents of HBTU/HOBt (1.1 mL from a 0.45 M stock in DMF) and 17.5 equivalents of DIPEA (150 μL) for at least 4 hours with agitation. Fmoc deprotection was carried out with a minimal volume of 20% piperidine in DMF (v/v) for 15 minutes. Washings between each step were carried out with DMF (3 x 5.0 mL) and DCM (2 x 5.0 mL). Automated peptide synthesis was carried out on an ABI 433A synthesiser (Applied Biosystems) using the FastMoc protocol.

Peptides were cleaved from the resin using TFA/water/EDT, 95.5:2.5:2.5, v/v/v) for 4-5 hours. After filtration of the resin, peptides were precipitated in cold diethyl ether (5 volumes). Crude peptides were obtained by centrifugation at 3000 RPM for 15 minutes at 4 °C. The ethereal layer was decanted and the crude peptide was resuspended in fresh diethyl ether before being
pelleted again by centrifugation. Crude peptide pellets were typically resuspended in the minimum volume of water with up to 35% acetonitrile before purification by (RP)HPLC.

**General procedure for thioester/Hydrazide formation**

Model peptides were dissolved in 0.1 M Na phosphate buffer (0.9 mL, final concentration of approximately 1 mg/mL, 0.9 mM) and TCEP.HCl (<5 mg)* was added, followed by sodium 2-mercaptoethanesulfonate (MESNa, 0.1 g). The final reaction pH was nearer pH 4.5. The reaction mixture was shaken (700 rpm) in an Eppendorf thermomixer at the desired temperature (40-60 °C) for 24-48 h. 50 µL aliquots of the reaction mixture were removed and analysed by HPLC and LC-MS.

To form the C-terminal hydrazide the reaction was carried out exactly as above except hydrazinium acetate was added to a final concentration of 5% w/v from a 50% w/v stock solution (prepared by dissolving e.g. 250 mg hydrazinium acetate in 250 µL distilled water). In these reactions the final pH was nearer pH 7.0.

In peptide cyclisation reactions the procedure for forming thioesters was followed, using pre-neutralised TCEP.HCl each time.

*TCEP can also be added from a pre-neutralized 1 M stock solution

**Preparative HPLC Purification MS-characterization of model peptides:** Peptide 5a: \( t_R = 25.0 \) min. ESI+ MS (m/z) calc. 1059.2, found [MH]+ 1059.6 Da. 6a: \( t_R = 25.7 \) min. ESI+ MS (m/z) calc. 1073.3, found [MH]+ 1074.1 Da. 7a, \( t_R = 25.7 \) min. ESI+ MS (m/z) calc. 1087.3, found [MH]+ 1087.5 Da. 8a: \( t_R = 25.3 \) (epimer1), 27.2 (epimer2). ESI+ MS (m/z) calc. 1091.2, found [MH]+ 1091.7 Da. 9a: \( t_R = 27.1 \) min. ESI+ MS (m/z) calc. 1109.2 found [MH]+ 1109.7 Da.

**Agardhipeptin A analogues:** Peptide 10a: \( t_R = 33.9 \) min. ESI+ MS (m/z) calc. 944.3, found [MH]+ 945.8 Da. 10b: \( t_R = 25.7 \) min. ESI+ MS (m/z) calc. 823.3, found [MH]+ 824.8 Da. 11a, \( t_R = 34.6 \) min. ESI+ MS (m/z) calc. 976.3, found [MH]+ 977.8 Da. 11b, \( t_R = 25.7 \) min. ESI+ MS (m/z) calc. 855.3, found [MH]+ 856.6 Da.

**Microcin J25 fragments:** linear 17 \( t_R = 26.5 \) min. ESI+ MS (m/z) calc. 1033.4 found [MH]+ 1034.6 Da. linear 18 \( t_R = 27.1 \) min, ESI+ MS (m/z) calc. 1047.4 found [MH]+ 1048.2 Da. Cyclic 19 \( t_R = 26.5 \) min, ESI+ MS (m/z) calc. 912.4 found [MH]+ 913.2 Da.
References:
