ELECTRONIC SUPPLEMENTARY INFORMATION FOR:

Potent and selective inhibitors of human peptidylglycine α -amidating monooxygenase

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<u>General</u>

Melting points were established using a Stanford Research Systems MPA 100 OptiMelt automated melting point system with digital image processing technology. ¹H and ¹³C NMR spectra were recorded on Varian Mercury 300 and Inova 500 spectrometers. Chemical shifts are reported as δ in parts per million and coupling constants are reported as *J* values in Hz. ESI mass spectra were recorded using a VG Quattro II triple quadrupole mass spectrometer. EI mass spectra were recorded on a VG AutoSpec M series sector (EBE) mass spectrometer. Microanalyses were carried out on a Carlo Erba 1106 automatic analyser by the Research School of Chemistry Microanalytical Service at the Australian National University.

Recombinant solutions of *Xenopus laevis* (frog) PAM were purchased from Wako Pure Chemical Industries. H889 and DMS53 small cell lung carcinoma, PC3 prostate cancer and SW1783 brain tumor cells were obtained from the American Type Culture Collection (ATCC). The H889, DMS53 and PC3 strains were grown in GIBCO[®] RPMI-1640 medium supplemented with 10% fetal bovine serum obtained from Invitrogen, whereas the SW1783 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, MEM nonessential amino acids and MEM sodium pyruvate solution obtained from Invitrogen. The tripeptide substrate (*R*)-tyrosyl-(*S*)-valylglycine-OH used in inhibition assays was obtained from Bachem AG. Glycine-extended calcitonin (procalcitonin) was purchased from GL Biochem (Shanghai) Ltd. Flurorescamine was purchased from Sigma Aldrich Chemical Co. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. PAM assays were run at least in duplicate with $K_{M,app}$ and IC₅₀ data plotted and calculated using SciDAVis 0.2.4.

Cell Culture Maintenance and PAM Extraction Procedure

PAM extraction from cells was performed using a modified literature procedure.¹ The desired cancer cell lines were grown to a high density under a moist 5% $CO_2/95\%$ air atmosphere at

37 °C, in medium refreshed every two days. The cells were passaged weekly. Once a large number of cells were available, growth medium was collected and concentrated with a Millipore Amicon[®] Ultra YM-10 filter (Cell secreted PAM), and cells were harvested and counted using a hemocytometer. Adherent cell lines (DMS53, PC3, SW1783) were washed twice with Dulbecco's phosphate-buffered saline (DPBS), lifted with 0.05% trypsin-EDTA and the suspension diluted with medium. The cells were pelleted by centrifugation before being washed twice more with DPBS and re-pelleted by centrifugation (1200 g, 5 min, 4 °C). Suspension cells (H889) were collected by centrifugation (1200 g, 5 min, 4 °C) after counting. Around 50 million cells were collected for each extraction. Extraction buffer (20 mM TES/10 mM mannitol, pH 7.4) containing leupeptine (2 μg/ml), benzamidine (16 μg/mL), PMSF (30 μg/mL) and Cu₂SO₄ (1 μM) was added to the cell pellet and the mixture was homogenised. The homogenate was centrifuged (1800 g, 5 min, 4 °C), and the supernatant was separately centrifuged at 30,000 g for 30 min (4 °C). The supernatant was collected (S30 extract containing PAM) and stored at -80 °C to maintain PAM activity. The pellet from the 30,000 g centrifugation was resuspended in extraction buffer containing 1% Triton X-100 and the mixture was centrifuged at 100,000 g for 1 h (4 °C). The supernatant was collected (S100 extract containing PAM) and stored at -80 °C. Table 2 of the manuscript refers to the S30 extract for H889 and the S100 extract for DMS53 and PC3.

PAM Assay Procedure (Human Cell Extracts and Media)

Assays for PAM inhibition were performed with 1.25 mM ascorbic acid, 10 μ M copper sulfate, 150 μ g/mL bovine liver catalase, 1% ethanol, 1% DMSO and 0.05 mM substrate [(*R*)-Tyr-(*S*)-ValGly, and varying concentrations of inhibitors in 150 mM MES buffer at pH 5.8. Assays were initiated through the addition of the appropriate amount of enzyme to give a final volume of 200 μ L, and then the mixtures were incubated at 37 °C for 2 h (1 h for amide substrates). The assays were quenched through the addition of 50 μ L of 1 M NaOH, and the mixtures were neutralised with 50

 μ L of 1 M HCl. The solutions were passed through Amicon[®] Ultra YM-3 filters and the filtrates were analysed directly using HPLC.

HPLC separation and quantification of the tripeptide substrate ((R)-Tyr-(S)-ValGly-OH) and the amidated product ((R)-Tyr-(S)-Val-NH₂) from assays of PAM in cancer cell extracts used a Waters Alliance 2695 separation module and a Waters 600E Pump connected to a two position, six port switching valve (Switch 1), a Waters Reagent Manager (Switch 2) containing fluorescamine (30 mg/100 mL acetonitrile) and a Waters 2475 Fluorescence Detector (**Figure S1**).



Figure S1. HPLC setup for post-column fluorescent labelling of (*R*)-Tyr-(*S*)-ValGly and (*R*)-Tyr-(*S*)-Val-NH₂.

Injected samples underwent an online solid phase extraction on a C-18 (SPE) YMC ODS-AQ cartridge column (4.0 x 23 mm) with the switching valve open to waste (Switch 1 OFF). After 5 minutes, switch 1 and switch 2 were turned ON, allowing separation of (R)-Tyr-(S)-ValGly-OH and (R)-Tyr-(S)-Val-NH₂ with a C-18 YMC ODS-AQ column (4.6 x 100 mm) and a Phenomenex Phenosphere Cation-Exchange (SCX) column (4.6 x 250 mm) over 30 minutes. The *N*-terminus of substrate and product were fluorescently labelled by reaction with fluorescamine in a post-column Waters 1000 RXN coil at 80 °C and detected with a Waters 2475 Fluorescence Detector (Ex. 390 nm, Em. 470 nm). The solvent and gradient system for the separation are shown in **Table S1**. Data were collected and processed with Empower Pro - Empower 2 software using an IBM data station. Between each sample injection a cleaning routine was applied (30 minutes total) using solvents delivered from both the Waters 2695 separations module (Table S2) and the Waters 600E pump

(Table S3).

Table S1. Solvent and gradient system for Waters 2695 separations module used for separation of the tripeptide substrate and the amide product over 30 mins. Events: 5 minutes switch 1 and switch 2 were turned ON.

Time (min)	Flow (mL/min)	Acetonitrile (%)	Buffer A (%) [*]	Gradient Curve
0.00	1.00	0.0	100.0	0
3.00	1.00	0.0	100.0	6
8.00	1.00	10.0	90.0	6

Table S2. Solvent and gradient system for Waters 2695 separations module used for cleaning routine. Events: 0.2 minutes switch 1 is OFF; 11.0 minutes switch 1 is ON; 25.0 minutes switch 1 is OFF.

Time (min)	Flow	Acetonitrile (%)	Buffer A (%) [*]	Buffer B $(\%)^*$	Gradient
	(mL/min)				Curve
0.00	1.00	10.0	90.0	0.0	0
0.50	1.00	10.0	90.0	0.0	6
1.00	1.00	50.0	50.0	0.0	6
5.00	1.00	50.0	50.0	0.0	6
5.10	0.10	50.0	50.0	0.0	6
11.00	0.10	50.0	50.0	0.0	6
11.50	0.50	50.0	50.0	0.0	6
12.00	0.50	50.0	50.0	0.0	6
13.00	1.00	50.0	50.0	0.0	6
18.00	1.00	50.0	50.0	0.0	6
20.00	1.00	10.0	90.0	0.0	6
25.00	1.00	10.0	90.0	0.0	6
25.10	1.20	0.0	0.0	100.0	6

Table S3. Solvent and gradient system for Waters 600E pump used for cleaning routine. Events: 0.2 minutes switch 1 is OFF; 11.0 minutes switch 1 is ON; 25.0 minutes switch 1 is OFF.

Time	Flow	Sodium Phosphate	$W_{ator}(0/)$	Urea 5M, pH 7	Gradient
(min)	(mL/min)	500 mM, pH 7 (%)	water (%)	(%)	Curve
0.00	1.00	0.0	100.0	0.0	0
0.50	1.00	0.0	100.0	0.0	6
1.00	1.00	0.0	00.0	100.0	6
2.00	1.00	0.0	00.0	100.0	6
4.00	1.00	0.0	100.0	0.0	6
5.00	1.00	100.0	0.0	0.0	6
7.00	1.00	100.0	0.0	0.0	6
7.10	1.00	0.0	100.0	0.0	6
11.10	1.00	0.0	100.0	0.0	6
12.00	1.00	0.0	100.0	0.0	6
20.00	1.00	0.0	100.0	0.0	6
21.00	0.00	0.0	100.0	0.0	6

^{*}Buffer A is the Waters AccQ.Tag[™] Eluent A (100 mL in 1000 mL water); Buffer B is the Waters AccQ.Tag[™] Eluent A (25 mL in 1000 mL water).

PAM Assay Procedure (Frog)²

Assays for PAM inhibition were performed with 3 mM ascorbic acid, 2 μ M copper sulfate, 100 μ g/mL bovine liver catalase, 0.01% Tween 20, 1% ethanol and 0.1 mM substrate [(*R*)-Tyr-(*S*)-ValGly], and varying concentrations of inhibitors in 100 mM MES buffer at pH 6.6. Assays were initiated through the addition of the appropriate amount of enzyme to give a final volume of 60 μ L, and then the mixtures were incubated at 37 °C for 1 h. The assays were quenched through the addition of 30 μ L of 1 M NaOH, then the mixtures were neutralized with 30 μ L of 1 M HCl. Two 20 μ L aliquots were then taken and diluted to 110 μ L with water. The duplicate diluted solutions were then analyzed directly using HPLC, and the ratio of the substrate [(*R*)-Tyr-(*S*)-ValGly] to the product [(*R*)-Tyr-(*S*)-Val-NH₂] determined by integration, to give the amount processed. HPLC separation and quantification of the tripeptide substrate and the amidated product from frog PAM assays used a Waters Alliance 2695 separations module with a Waters Symmetry C-18 5 μ m column (250 mm x 4.6 mm) and a Waters 2996 photodiode array detector. Data were collected and processed with Empower Pro - Empower 2 software using an IBM data station. Alternatively, HPLC separation and quantification of the tripeptide substrate and the amidated product was carried out using the HPLC system described for the assays of human PAM in extracts and media.

Synthesis and Analytical Data for O-Acylglycolic Acid Inhibitors

General Procedure 1: Synthesis of O^{*}-acylglycolic acid benzyl esters

To a solution of the fatty acid (4.01 mmol) in acetone (80 mL) was added potassium carbonate (8.07 mmol) and benzyl bromoacetate (4.01 mmol). The mixture was heated at reflux for 23 h then filtered and evaporated under reduced pressure. The crude oil was partitioned between diethyl ether (50 mL) and water (50 mL). The organic fraction was dried over magnesium sulfate, filtered and evaporated under reduced pressure to give the benzyl ester.

General Procedure 2: Synthesis of O^a-acylglycolic acids

To a solution of the benzyl ester (1.40 mmol) in methanol (25 mL) was added 5% palladium on charcoal (0.15 g). The mixture was stirred under a hydrogen atmosphere at room temperature for 19 h, filtered through celite and evaporated under reduced pressure. The residue was subjected to silica gel flash column chromatography (ethyl acetate/hexanes/acetic acid) to afford the fatty acid glycolate.

Benzyl O⁻⁻decanoylglycolate

Following general procedure 1, the crude reaction mixture was filtered and evaporated under reduced pressure to afford a colourless oil which was subjected to flash silica gel column chromatography (ethyl acetate/hexanes) to give the title compound (52%) as a colourless oil, spectroscopically identical to that previously reported.² ¹**H** NMR (300 MHz, CDCl₃): δ 7.35 (m, 5H), 5.19 (s, 2H), 4.65 (s, 2H), 2.42 (t, *J* 7.5 Hz, 2H), 1.65 (m, 2H), 1.28 (m, 12H), 0.90 (t, *J* 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.3, 168.0, 135.3, 128.9, 128.8, 128.6, 67.4, 60.9, 34.2, 32.3, 29.8, 29.7, 29.5, 25.2, 23.1, 14.6; **HRMS** (EI) calcd. for C₁₉H₂₈O₄ (M⁺) *m/z* 320.1988, found *m/z* 320.1989.

O[•]-Decanoylglycolic acid (1a)

Following general procedure 2, the suspension of the crude product was filtered through celite, and the filtrate was concentrated under reduced pressure, to give a colourless solid. The crude residue was recrystallised from ethyl acetate/hexanes to give the title compound **1a** (80%) as a colourless solid, spectroscopically identical to that previously reported.² **mp.** 54 °C; ¹**H NMR** (300 MHz, CDCl₃): δ 4.62 (s, 2H), 2.40 (t, *J* 7.5 Hz, 2H), 1.60-1.67 (m, 2H), 1.26 (m, 12H), 0.88 (t, *J* 6.9 Hz, 3H); ¹³C NMR (100 MHz, CD₃OD): δ 174.9, 171.9, 61.7, 34.6, 33.0, 30.6, 30.4, 30.1, 25.9, 23.7, 14.4; **MS** (ESI, +ve) *m/z* 253.4 (M+Na⁺, 100%); **Elemental Analysis:** C, 62.22; H, 9.90 (C₁₂H₂₂O₄ requires C, 62.58; H, 9.63%); **HRMS** (ESI, +ve) calcd. for C₁₂H₂₂O₄Na (M+Na⁺) *m/z* 253.1416, found *m/z* 253.1415.

Benzyl *O*^a-hexanoylglycolate

Following general procedure 1, the crude reaction mixture was filtered and evaporated under reduced pressure to give a colourless oil which was subjected to silica gel flash column chromatography (ethyl acetate/hexanes), providing the title benzyl ester as a colourless oil (91%). ¹H NMR (300 MHz, CDCl₃): δ 7.37 (m, 5H), 5.20 (s, 2H), 4.65 (s, 2H), 2.41 (t, *J* 7.5 Hz, 2H), 1.66 (quintet, *J* 7.5 Hz, 2H), 1.32 (m, 4H), 0.89 (t, *J* 7.1 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.1, 167.8, 135.0, 128.6, 128.5, 128.3, 67.0, 60.5, 33.7, 31.1, 24.4, 22.2, 13.8; MS (ESI, +ve) *m/z* 264 (M⁺, 2%), 148 (94), 120 (57), 107 (71), 99 (100), 91 (95); HRMS (ESI, +ve) calcd. for C₁₅H₂₀O₄ (M⁺) *m/z* 264.1362, found *m/z* 264.1362.

O[•]-Hexanoylglycolic acid (5a)

Following general procedure 2, the crude reaction mixture was filtered through celite and evaporated under reduced pressure to give a yellow oil. The oil was dissolved in ethyl acetate, silica gel (5 g) was added and the mixture was shaken then filtered. The filtrate was evaporated under reduced pressure to afford the fatty acid glycolate **5a** as a yellow oil (98%), spectroscopically

identical to that reported in the literature.³ ¹**H** NMR (300 MHz, CDCl₃): δ 4.67 (s, 2H), 2.43 (t, *J* 7.5 Hz, 2H), 1.68 (quintet, *J* 7.5 Hz, 2H), 1.29 (m, 4H), 0.91 (t, *J* 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.3 (x 2), 60.0, 33.6, 31.1, 24.4, 22.2, 13.8; **Elemental Analysis:** C, 55.10; H, 8.20 (C₈H₁₄O₄ requires C, 55.16; H, 8.10%); **MS** (ESI, -ve) *m/z* 173.2 ([M-H⁺], 10%), 115 (75), 75 (100); **HRMS** (ESI, -ve) calcd. for C₈H₁₃O₄ (M-H⁺) *m/z* 173.0814, found *m/z* 173.0815.

Benzyl O-octanoylglycolate

Following general procedure 1, the crude reaction mixture was filtered and evaporated under reduced pressure to afford the title benzyl ester as a colourless oil (100%). ¹H NMR (300 MHz, CDCl₃): δ 7.36 (m, 5H), 5.20 (s, 2H), 4.65 (s, 2H), 2.40 (t, *J* 7.5 Hz, 2H), 1.65 (quintet, *J* 7.5 Hz, 2H), 1.28 (m, 8H), 0.87 (t, *J* 6.8 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.1, 167.8, 135.0, 128.6, 128.5, 128.3, 67.0, 60.5, 33.7, 31.6, 28.9, 28.8, 24.7, 22.5, 14.0; MS (EI, +ve) *m/z* 292 (M⁺, 2%), 148 (78), 127 (87), 120 (48), 107 (57), 91 (100); HRMS (EI, +ve) calcd. for C₁₇H₂₄O₄ (M⁺) *m/z* 292.1675, found *m/z* 292.1678.

O^a-Octanoylglycolic acid (5b)

Following general procedure 2, the crude reaction mixture was filtered through celite and evaporated under reduced pressure to give green crystals. These were subjected to silica gel flash column chromatography (ethyl acetate/hexanes/acetic acid) to give the glycolic acid **5b** as colourless crystals (83%), spectroscopically identical to those reported in the literature.³ **mp.** 50.3-50.8 °C; ¹**H NMR** (300 MHz, CDCl₃): δ 4.67 (s, 2H), 2.43 (t, *J* 7.5 Hz, 2H), 1.67 (quintet, *J* 7.5 Hz, 2H), 1.30 (m, 8H), 0.89 (t, *J* 6.9 Hz, 3H); ¹³**C NMR** (75 MHz, CDCl₃): δ 173.9, 173.2, 60.0, 33.7, 31.6, 28.9, 28.8, 24.7, 22.6, 14.0; **Elemental Analysis:** C, 59.34; H, 8.85 (C₁₀H₁₈O₄ requires C, 59.39; H, 8.97%); **MS** (ESI, -ve) *m/z* 201.1 ([M-H⁺], 30%), 143 (50), 75 (100); **HRMS** (ESI, -ve) calcd. for C₁₀H₁₇O₄ (M-H⁺) *m/z* 201.1127, found *m/z* 201.1123.

Benzyl O⁻⁻dodecanoylglycolate

Following general procedure 1, the crude reaction mixture was filtered and evaporated under reduced pressure to afford the title benzyl ester as a colourless oil (96%). ¹H NMR (300 MHz, CDCl₃): δ 7.37 (m, 5H), 5.19 (s, 2H), 4.65 (s, 2H), 2.41 (t, *J* 7.5, 2H), 1.65 (quintet, *J* 7.5 Hz, 2H), 1.26 (m, 16H), 0.88 (t, *J* 6.8, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.1, 167.8, 135.0, 128.6, 128.5, 128.3, 67.0, 60.5, 33.7, 31.9, 29.6, 29.4, 29.3, 29.2, 29.0, 24.8, 22.6, 14.1; MS (EI, +ve) *m/z* 348 (M⁺, 1%), 183 (77), 148 (71), 107 (52), 91 (100); HRMS (EI, +ve) calcd for C₂₁H₃₂O₄ (M⁺) *m/z* 348.2301, found 348.2297.

O[•]-Dodecanoylglycolic acid (5c)

Following general procedure 2, the suspension of the crude product was filtered through celite, and the filtrate was concentrated under reduced pressure, to give a colourless solid, which was subjected to silica gel flash column chromatography (ethyl acetate/hexanes/acetic acid) to give the title compound **5c** as colourless crystals (64%). **mp.** 72.5-73.1 °C; ¹H **NMR** (300 MHz, CDCl₃): δ 4.66 (s, 2H), 2.42 (t, *J* 7.5 Hz, 2H), 1.66 (quintet, *J* 7.5 Hz, 2H), 1.30 (m, 16H), 0.88 (t, *J* 6.8 Hz, 3H); ¹³C **NMR** (75 MHz, CDCl₃): δ 173.7, 173.2, 60.0, 33.7, 31.9, 29.6, 29.4, 29.3, 29.2, 29.0, 24.7, 22.7, 14.1; **Elemental Analysis:** C, 64.93; H, 9.90 (C₁₄H₂₆O₄ requires C, 65.09; H, 10.14%); **MS** (ESI, -ve) *m/z* 257.1 ([M-H⁺], 20%), 199 (50), 75 (100); **HRMS** (ESI, -ve) calcd. for C₁₄H₂₅O₄ (M-H⁺) *m/z* 257.1753, found *m/z* 257.1743.

Benzyl *O*^a-tetradecanoylglycolate

Following general procedure 1, the crude reaction mixture was filtered and evaporated under reduced pressure to afford the title benzyl ester as colourless, low melting point crystals (93%). ¹H NMR (300 MHz, CDCl₃): δ 7.36 (m, 5H), 5.19 (s, 2H), 4.65, (s, 2H), 2.41 (t, *J* 7.5 Hz, 2H), 1.64 (quintet, *J* 7.5 Hz, 2H), 1.25 (m, 20H), 0.88 (t, *J* 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.2, 167.8, 135.0, 128.6, 128.5, 128.4, 67.0, 60.5, 33.8, 31.9, 29.6, 29.4, 29.3, 29.2, 29.0, 24.8, 22.7, 14.1; MS

(EI, +ve) *m/z* 376 (M⁺, 1%), 211 (80), 148 (80), 120 (41), 107 (44), 91 (100); **HRMS** (EI, +ve) calcd. for C₂₃H₃₆O₄ *m/z* 376.2614, found *m/z* 376.2627.

O[«]-Tetradecanoylglycolic acid (5d)

Following general procedure 2, the crude reaction mixture was filtered through celite and evaporated under reduced pressure to give a colourless solid. This was subjected to silica gel flash column chromatography (ethyl acetate/hexanes/acetic acid) to afford the glycolate **5d** as colourless crystals (38%). **mp.** 77.6-79.0 °C; ¹**H NMR** (300 MHz, CDCl₃): δ 4.67 (s, 2H), 2.42 (t, *J* 7.5 Hz, 2H), 1.66 (quintet, *J* 7.5 Hz, 2H), 1.29 (m, 20H), 0.88 (t, *J* 6.8 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 173.3, 173.1, 59.9, 33.7, 31.9, 29.7, 29.6, 29.4, 29.3, 29.2, 29.1, 29.0, 24.7, 22.7, 14.1; **Elemental Analysis:** C, 67.21; H, 10.33 (C₁₆H₃₀O₄ requires C, 67.10; H, 10.56%); **MS** (ESI, -ve) *m/z* 285.1 ([M-H⁺], 25%), 227 (20), 75 (100); **HRMS** (ESI, -ve) calcd. for C₁₆H₃₀O₄ *m/z* 285.2066, found *m/z* 285.2055.

Benzyl *O*^{*}-phenylacetylglycolic acid

To a solution of 2-phenylacetic acid (0.50 g, 3.67 mmol) in acetone (50 mL) was added potassium carbonate (1.02 g, 7.34 mmol) and benzyl bromoacetate (0.58 mL, 3.67 mmol) and the mixture was heated at reflux overnight under $N_{2(g)}$, then evaporated under reduced pressure. The residue was filtered and evaporated under reduced pressure. The crude oil was partitioned between *n*-hexane (100 mL) and water (100 mL). The organic fraction was dried over magnesium sulfate, filtered and evaporated under reduced pressure to give the title compound (1.03 g, 99%) as a yellow oil. ¹H NMR (300 MHz, CDCl₃): δ 7.30 (m, 10H), 5.13 (s, 2H), 4.61 (s, 2H), 3.69 (s, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 170.7, 169.2, 134.8, 133.1, 129.4, 128.7, 128.6, 128.5, 128.4, 127.3, 66.8, 60.8, 40.4; MS (ESI, +ve) *m/z* 307.3 ([M+Na⁺], 100%); HRMS (ESI, +ve) calcd. for C₁₇H₁₆O₄Na (M+Na⁺) *m/z* 307.0946, found *m/z* 307.0947.

O[«]-Phenylacetylglycolic acid (2a)

To a solution of benzyl *O*-phenylacetylglycolic acid (0.16 g, 0.563 mmol) in dry methanol (10 mL) was added Pd/C (16 mg, 10% wt), and the mixture was stirred under an atmosphere of hydrogen (balloon) at room temperature for 24 h. The reaction mixture was filtered through celite. The residue was evaporated under reduced pressure and the resulting solid was subjected to Sephadex LH-20 flash column chromatography (CHCl₃/methanol) to give the title compound **2a** (107 mg, 98%) as a colourless solid. **mp.** 71.2-71.7 °C; ¹**H NMR** (300 MHz, CDCl₃): δ 10.90 (bs, 1H), 7.25-7.38 (m, 5H), 4.68 (s, 2H), 3.76 (s, 2H); ¹³C **NMR** (75 MHz, CDCl₃): δ 173.6, 171.1, 133.2, 129.4, 128.7, 127.4, 60.5, 40.6; **Elemental Analysis:** C, 61.69; H, 5.41 (C₁₀H₁₀O₄ requires C, 61.85; H, 5.19%); **MS** (ESI, -ve) *m/z* 193.0 ([M-H⁺], 50%), 167.0 (100); **HRMS** (ESI, -ve) calcd. for C₁₀H₉O₄ *m/z* 193.0501, found *m/z* 193.0498.

tert-Butyl *O*^{*a*}-((*S*)-*N*-acetylphenylalanyl)glycolate

To a solution of *(S)-N*-acetylphenylalanine (0.46 g, 2.25 mmol) in acetone (50 mL) was added potassium carbonate (0.62 g, 4.49 mmol) and *tert*-butyl bromoacetate (0.33 mL, 2.25 mmol). The mixture was heated at reflux overnight under N_{2(g)}, and evaporated under reduced pressure. The crude oil was partitioned between *n*-hexane (100 mL) and water (100 mL). The organic fraction was dried over MgSO₄, filtered and evaporated under reduced pressure to give the title compound (0.602 g, 75%) as a yellow oil. ¹H NMR (300 MHz, CDCl₃): δ 7.26 (m, 5H), 5.95 (bs, 1H), 4.95 (m, 1H), 4.55 (*J*_{AB} 15.9 Hz, 2H), 3.26 (dd, *J* 14.1 and 5.4 Hz, 1H), 3.10 (dd, *J* 14.1 and 6.3 Hz, 1H), 1.94 (s, 3H), 1.38 (m, 9H); ¹³C NMR (75 MHz, CDCl₃): δ 171.2, 169.7, 166.3, 135.8, 129.4, 128.6, 127.1, 82.8, 61.7, 52.9, 37.5, 28.0, 23.0; MS (ESI, +ve) *m/z* 344.3 ([M+Na⁺], 100%); HRMS (ESI, +ve) calcd. for C₁₇H₂₃NO₅Na *m/z* 344.1474, found *m/z* 344.1474.

O^{*}-((S)-N-Acetylphenylalanyl)glycolic acid (3a)

A solution of *tert*-butyl *O*-(*(S)*-*N*-acetylphenylalanyl)glycolate (0.40 g, 1.24 mmol) in neat trifluoroacetic acid (2 mL) was stirred at room temperature under $N_{2(g)}$ for 30 min, before evaporation of solvent under reduced pressure. Diethyl ether was added and the mixture was evaporated to dryness under reduced pressure to give a colourless solid which was subjected to Sephadex LH-20 flash column chromatography (CHCl₃/methanol) to give the title compound (280 mg, 85%) as a clear oil, spectroscopically the same as that reported in the literature.⁴ ¹**H NMR** (300 MHz, CDCl₃): δ 7.20 (m, 5H), 6.00 (d, *J* 7.5 Hz, 1H), 4.88 (m, 1H), 4.64 (*J*_{AB} 16.5 Hz, 2H), 3.20 (dd, *J* 14.1, 6.0 Hz, 1H), 3.06 (dd, *J* 14.1, 6.6 Hz, 1H), 1.92 (s, 3H); ¹³**C NMR** (75 MHz, CDCl₃): δ 171.0, 170.9, 170.3, 135.4, 129.3, 128.7, 127.3, 61.0, 53.2, 37.3, 23.0; **Elemental Analysis:** C, 58.48; H, 5.93; N, 5.22 (C₁₃H₁₅NO₅ requires C, 58.86; H, 5.70; N, 5.28%); **MS** (ESI, -ve) *m/z* 264.1 ([M-H⁺], 100%); **HRMS** (ESI, -ve) calcd. for C₁₃H₁₅NO₅ *m/z* 264.0872, found *m/z* 264.0870.

Synthesis and Analytical Data for S-Acylthioglycolic Acid Inhibitors

General Procedure 3

To a solution of the acylating acid (2.41 mmol) in DCM (8 mL) was added BOP (2.41 mmol) and diisopropylethylamine (9.66 mmol). The mixture was allowed to stir at 25 °C under $N_{2(g)}$ for 15 min before the addition of thioglycolic acid (0.167 mL, 1.85 mmol). Stirring of the mixture was allowed to continue at 25 °C under $N_{2(g)}$ for 18 h before it was partitioned between EtOAc (100 mL) and brine (100 mL). The brine solution was acidified with 0.3 M citric acid solution and extracted with EtOAc (3 x 30 mL), and the combined organic extracts were washed with 0.3 M citric acid (1 x 30 mL) and brine (2 x 30 mL), dried (MgSO₄) and concentrated *in vacuo*. The crude residue was absorbed onto silica gel and subjected to flash silica gel column chromatography (ethyl acetate/hexanes/acetic acid).

General Procedure 4

To a solution of thioglycolic acid (14.4 mmol) in a mixture of toluene (10 mL) and water (10 mL) was added sodium hydroxide (33 mmol). The mixture was cooled to 0 °C and the acylating acid chloride (14.4 mmol) diluted in water (10 mL) was slowly added, and stirring was continued for 30 min at 0 °C and for an additional 30 min at 25 °C. The organic layer was separated and washed with water (2 x 30 mL), and the combined aqueous phases were acidified to pH 3 through the addition of conc. HCl. The resultant precipitate was filtered and dried under vacuum before recrystallisation from ethyl acetate.

S-Decanoylthioglycolic acid (1b)

Using general procedure 3, the acylthioglycolic acid **1b** was isolated following column chromatography (ethyl acetate/hexanes/acetic acid) as a colourless solid (77%). **mp.** 59.4-60.0 °C; ¹H NMR (300 MHz, CDCl₃): δ 11.19 (bs, 1H), 3.72 (s, 2H), 2.61 (t, *J* 7.2 Hz, 2H), 1.67 (quintet, *J* 7.2 Hz, 2H), 1.33-1.25 (m, 12H), 0.87 (t, *J* 7.2 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 197.6, 175.1, 43.6, 31.8, 30.9, 29.3, 29.19, 29.16, 28.8, 25.4, 22.6, 14.1; **Elemental Analysis:** C, 58.48; H, 9.08; S, 13.09 (C₁₂H₂₂O₃S requires C, 58.50; H, 9.00; S, 13.01%); **MS** (ESI, +ve) *m/z* 269.1 ([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd. for C₁₂H₂₂NaO₃S (M+Na⁺) *m/z* 269.1187, found *m/z* 269.1185.

S-(Phenylacetyl)thioglycolic acid (2b)

Following general procedure 3, the acylthioglycolic acid **2b** was isolated through flash silica gel column chromatography (ethyl acetate/hexanes/acetic acid) as a colourless crystalline solid (67%). **mp.** 54.3-56.6 °C; ¹H NMR (300 MHz, CDCl₃): δ 10.65 (bs, 1H), 7.36-7.29 (m, 5H), 3.89 (s, 2H), 3.70 (s, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 195.8, 174.7, 132.6, 129.7, 128.7, 127.7, 49.8, 31.3; **Elemental Analysis:** C, 57.34; H, 4.67 (C₁₀H₁₀O₃S requires C, 57.13; H, 4.79%); **MS** (ESI, +ve) m/z 233.0 ([M+Na⁺], 30%), 102 (50), 91 (100); **HRMS** (ESI, +ve) calcd. for C₁₀H₁₀NaO₃S m/z 233.0248, found m/z 233.0252.

S-((*S*)-*N*-Acetylphenylalanyl)thioglycolic acid (3b)

Following general procedure 3, the crude product was subjected to flash silica gel column chromatography (ethyl acetate/hexanes/acetic acid) providing the title acylthioglycolic acid **3b** as a viscous oil which formed a colourless solid upon addition of chloroform (73%). A portion of the solid was recrystallised from chloroform/methanol to give a colourless crystalline solid. **mp.** 122.2-123.3 °C; ¹H NMR (400 MHz, CD₃OD): δ 7.28-7.20 (m, 5H), 4.79 (dd, *J* 10.4 and 4.8 Hz, 1H), 3.70 (*J*_{AB} 16.0 Hz, 2H), 3.26 (dd, *J* 14.0 and 4.8 Hz, 1H), 2.86 (dd, *J* 14.0 and 10.4 Hz, 1H), 1.91 (s, 3H); ¹³C NMR (75 MHz, CD₃OD): δ 200.6, 173.6, 172.0, 138.1, 130.1, 129.5, 127.9, 61.9, 38.3, 32.0, 22.3; **Elemental Analysis:** C, 55.25; H, 5.67; N, 5.23; S, 11.03 (C₁₃H₁₅NO₄S requires C, 55.50; H, 5.37; N, 4.98; S, 11.40%); **MS** (ESI, +ve): *m/z* 304.1 ([M+Na⁺], 100%). **HRMS** (ESI, +ve) calcd. for C₁₃H₁₅NNaO₄S (M+Na⁺) *m/z* 304.0620, found *m/z* 304.0621.

S-Benzoylthioglycolic acid (4b)

Using general procedure 4, recrystallisation of the crude product from ethyl acetate provided the acylthioglycolic acid **4b** as a colourless crystalline solid (49%), spectroscopically identical to that reported previously.⁵ **mp.** 104-105 °C (lit.⁵ 102-103 °C); ¹**H NMR** (300 MHz, CDCl₃): δ 7.99 (d, *J* 7.2 Hz, 2H), 7.61 (t, *J* 7.2 Hz, 1H), 7.48 (t, *J* 7.2 Hz, 2H), 3.92 (s, 2H); ¹³C **NMR** (75 MHz, CDCl₃): δ 190.1, 174.2, 135.9, 134.0, 128.8, 127.5, 31.1; **Elemental analysis:** C, 55.30; H, 4.04 (C₉H₈O₃S requires C, 55.09; H, 4.11%); **MS** (EI) *m/z* 196.0 (M⁺, 15%) 105 (100), 77(95) 51 (80); **HRMS** (EI) calcd. for C₉H₈O₃S (M⁺) *m/z* 196.0194, found *m/z* 196.0192.

S-(*(S)*-*N*-Acetylleucyl)thioglycolic acid (6a)

Following general procedure 3, the crude product was subjected to flash silica gel column chromatography (ethyl acetate/hexanes) to give a colourless solid, which recrystallised from ethyl acetate/hexanes to give the title compound **6a** as shiny colourless crystals (74%). **mp.** 118.1-119.2 $^{\circ}$ C; ¹H NMR (300 MHz, CD₃OD): δ 4.58-4.52 (m, 1H), 3.66 (J_{AB} 16.2 Hz, 2H), 2.02 (s, 3H), 1.74-1.55 (m, 3H), 0.95 (d, *J* 6.0 Hz, 3H), 0.90 (d, *J* 6.0 Hz, 3H); ¹³C NMR (75 MHz, CD₃OD): δ 201.9, 173.8, 172.1, 59.1, 41.5, 31.8, 25.8, 23.4, 22.4, 21.5; **Elemental Analysis:** C, 48.36; H, 6.80; N, 5.62; S, 12.84 (C₁₀H₁₇NO₄S requires C, 48.57; H, 6.93; N, 5.66; S, 12.96%); **MS** (ESI, +ve) *m/z* 270.1 ([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd for C₁₀H₁₇NNaO₄S (M+Na⁺) *m/z* 270.0776, found *m/z* 270.0775.

S-(*(S)*-*N*-Acetylmethionyl)thioglycolic acid (6b)

Following general procedure 3, the crude product was subjected to flash silica gel column chromatography (ethyl acetate/hexanes/acetic acid) to give the title compound **6b** as a colourless crystalline solid (58%). **mp.** 124.0-125.2 °C; ¹**H NMR** (300 MHz, CD₃OD): δ 4.69 (dd, *J* 9.6 and 4.8 Hz, 1H), 3.68 (*J*_{AB} 16.2 Hz, 2H), 2.65-2.42 (m, 2H), 2.20-2.00 (m, 1H), 1.98-1.80 (m, 1H), 2.07 (s, 3H), 2.03 (s, 3H); ¹³C NMR (75 MHz, CD₃OD): δ 201.2, 173.8, 172.0, 59.5, 32.1, 31.9, 30.9, 22.4, 15.1; **MS** (ESI, +ve) *m/z* 288.0 ([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd. for C₉H₁₅NNaO₄S₂ (M+Na⁺) *m/z* 288.0340, found *m/z* 288.0343.

Synthesis and Analytical Data for N-Acylated Glycine Substrates

N-Decanoylglycine methyl ester

To a solution of decanoic acid (0.518 g, 3.01 mmol) in DCM (10 mL) was added BOP (1.33 g, 3.01 mmol) and diisopropylethylamine (2.10 mL, 12.0 mmol). The mixture was allowed to stir at 25 °C under $N_{2(g)}$ for 30 min before the addition of glycine methyl ester hydrochloride (0.415 g, 3.31

mmol). Stirring of the reaction mixture was allowed to continue at 25 °C under $N_{2(g)}$ for 16 h before it was partitioned with hexanes (50 mL) and 1 M HCl (50 mL). The acidic layer was extracted with hexanes (3 x 30 mL) and ethyl acetate (1 x 30 mL). The combined organic extracts were then washed with 0.3 M citric acid (2 x 50 mL), saturated NaHCO₃ (3 x 30 mL) and brine (1 x 30 mL), and dried (MgSO₄) and concentrated *in vacuo* to give the title compound as a colourless solid (0.67 g, 92%). ¹H NMR (300 MHz, CDCl₃): δ 6.01 (bs, 1H), 4.04 (d, *J* 5.1 Hz, 2H), 3.75 (s, 3H), 2.23 (t, *J* 7.5 Hz, 2H), 1.63 (quintet, *J* 7.5 Hz, 2H), 1.35-1.20 (m, 12H), 0.86 (t, *J* 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃): δ 173.3, 170.6, 52.3, 41.1, 36.4, 31.8, 29.4, 29.3, 29.23, 29.20, 25.5, 22.6, 14.1; MS (ESI, +ve) *m/z* 243 ([M+H⁺], 25%), 55.0 (100).

N-Decanoylglycine (1c)

N-Decanoylglycine methyl ester (0.199 g, 0.818 mmol) was dissolved in 10 mL of dry THF. To this was added methanol (2 mL) and LiOH.H₂O (0.5 M, 6.5 mL), and the mixture was stirred at room temperature (25 °C) for 16 h. The mixture was then acidified to *ca*. pH 1 with 1 M HCl, and the aqueous layer was extracted with ethyl acetate (3 x 20 mL). The combined organic fractions were washed with brine (1 x 20 mL) and dried (MgSO₄). Concentration *in vacuo* yielded a colourless solid which was subjected to flash silica gel column chromatography (acetone/hexanes/acetic acid) to give the title *N*-acylated glycine **1c** as a colourless solid (0.179 g, 96%). **mp.** 114.2-114.8 °C; ¹H **NMR** (400 MHz, CD₃OD): δ 3.89 (s, 2H), 2.25 (t, *J* 7.6 Hz, 2H), 1.62 (quintet, *J* 7.6 Hz), 1.35-1.25 (m, 12H), 0.90 (t, *J* 6.8 Hz, 3H); ¹³C **NMR** (100 MHz, CD₃OD): δ 176.7, 173.1, 41.8, 36.8, 33.0, 30.6, 30.5, 30.4, 30.2, 26.8, 23.7, 14.4; **Elemental Analysis:** C, 62.77; H, 10.27; N, 6.16 (C₁₂H₂₃NO₃ requires C, 62.85; H, 10.11; N, 6.11%); **MS** (ESI, +ve) *m/z* 252.1 ([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd for C₁₂H₂₃N Na O₃ *m/z* 252.1576, found *m/z* 252.1573.

N-(Phenylacetyl)glycine methyl ester

To a solution of phenylacetic acid (0.298 g, 2.19 mmol) in DCM (10 mL) was added BOP (0.969 g, 2.19 mmol) and diisopropylethylamine (1.53 mL, 8.76 mmol). The mixture was allowed to stir at 25 °C under N_{2(g)} for 30 min before the addition of glycine methyl ester hydrochloride (0.302 g, 2.40 mmol). Stirring of the reaction mixture was allowed to continue at 25 °C under N_{2(g)} for 16 h before it was diluted with ethyl acetate (50 mL). The organic layer was washed with 1 M HCl (3 x 40 mL), saturated NaHCO₃ (2 x 40 mL), H₂O (1 x 40 mL) and brine (1 x 40 mL), and dried (MgSO₄) and concentrated *in vacuo* to give the title compound as a colourless solid (0.31 g, 68%). ¹H NMR (400 MHz, CDCl₃): δ 7.31-7.20 (m, 5H), 5.96 (bs, 1H), 3.92 (d, *J* 5.2 Hz, 2H), 3.64 (s, 3H), 3.55 (s, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 171.1, 170.2, 134.4, 129.4, 129.0, 127.4, 52.2, 43.4, 41.2; MS (ESI, +ve) *m/z* 230.3 ([M+Na⁺], 100%); HRMS (ESI, +ve) calcd. for C₁₁H₁₃NNaO₃ (M+Na⁺) *m/z* 230.0793, found *m/z* 230.0793.

N-(Phenylacetyl)glycine (2c)

N-(Phenylacetyl)glycine methyl ester (0.273 g, 1.32 mmol) was dissolved in 11 mL of dry THF. To this was added LiOH.H₂O (0.5 M, 11.0 mL), and the mixture was stirred at room temperature (25 °C) for 16 h. The mixture was then acidified to *ca*. pH 1 with 1 M HCl, and the aqueous layer was extracted with ethyl acetate (3 x 20 mL). The combined organic fractions were washed with brine (1 x 20 mL) and dried (MgSO₄). Concentration *in vacuo* yielded the title compound **2c** as a colourless solid (0.173 g, 68%). **mp.** 142-146 °C; ¹H NMR (400 MHz, Acetone-*d*₆): δ 7.40 (bs, 1H), 7.35-7.20 (m, 5H), 3.93 (d, *J* 6.0 Hz, 2H), 3.57 (s, 2H); ¹³C NMR (100 MHz, Acetone-*d*₆): δ 170.7, 170.5, 136.0, 129.2, 128.2, 126.5, 42.4, 40.7; **Elemental Analysis:** C, 62.10; H, 6.02; N, 7.17 (C₁₀H₁₁NO₃ requires C, 62.17; H, 5.74; N, 7.25); **MS** (ESI, +ve) *m/z* 216.3 ([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd for C₁₀H₁₂NO₃ (M⁺) *m/z* 194.0817, found *m/z* 194.0818.

N-((*S*)-*N*-Acetylphenylalanyl)glycine methyl ester

To a solution of *(S)-N*-acetylphenylalanine (0.35 g, 1.69 mmol) in DCM (20 mL) was added BOP (0.747 g, 1.69 mmol) and diisopropylethylamine (1.20 mL, 6.76 mmol). The mixture was allowed to stir at 25 °C under N_{2(g)} for 30 min before the addition of glycine methyl ester hydrochloride (0.234 g, 1.86 mmol). Stirring of the reaction mixture was allowed to continue at 25 °C under N_{2(g)} for 16 h before it was diluted with ethyl acetate (50 mL). The organic layer was washed with 1 M HCl (3 x 30 mL), saturated NaHCO₃ (2 x 30 mL) and brine (1 x 40 mL), and dried (MgSO₄) and concentrated *in vacuo* to give the title compound as a colourless solid (0.120 g, 27%). ¹H NMR (400 MHz, CDCl₃): δ 7.22-7.12 (m, 5H), 6.92 (bs, 1H), 6.61 (bd, *J* 7.2 Hz, 1H), 4.73 (dd, *J* 10.8 and 6.8 Hz, 1H), 3.94-3.81 (m, 2H), 3.63 (s, 3H), 3.06-2.92 (m, 2H), 1.87 (s, 3H); ¹³C NMR (100 MHz, CD₃OD): δ 171.6, 170.4, 169.8, 136.5, 129.2, 128.5, 126.8, 54.2, 52.2, 41.1, 38.1, 22.9; MS (ESI, +ve) *m/z* 301.4 ([M+Na⁺], 100%); HRMS (ESI, +ve) calcd. for C₁₄H₁₉N₂O₄ (M⁺) *m/z* 279.1345, found *m/z* 279.1345.

N-((*S*)-*N*-Acetylphenylalanyl)glycine (3c)

To a solution of *N*-(*(S)*-*N*-acetylphenylalanyl)glycine methyl ester (99 mg, 0.372 mmol) in THF (5 mL) and H₂O (5 mL) was added LiOH.H₂O (62 mg, 1.49 mmol), and the mixture was stirred at room temperature (25 °C) for 48 h. The mixture was acidified to *ca*. pH 1 with 0.5 M HCl, and the aqueous layer was extracted with ethyl acetate (4 x 30 mL). The combined organic fractions were washed with brine (1 x 50 mL), dried (MgSO₄), and concentrated *in vacuo* giving a colourless solid. The solid was triturated with ethyl acetate and ether to give the title compound as a colourless solid (0.08 g, 85%), spectroscopically the same as that previously reported.⁴ ¹H NMR (400 MHz, CD₃OD): δ 7.29-7.17 (m, 5H), 4.67 (dd, *J* 9.6 and 5.2 Hz, 1H), 3.90 (s, 2H), 3.20 (dd, *J* 14.0 and 5.2 Hz, 1H), 2.87 (dd, *J* 14.0 and 9.6 Hz), 1.89 (s, 3H); ¹³C NMR (100 MHz, CD₃OD): δ 174.1, 173.2, 172.8, 138.6, 130.2, 129.4, 127.7, 55.9, 41.9, 38.8, 22.4; **Elemental Analysis:** C, 58.75; H, 6.27; N, 10.27 (C₁₃H₁₆N₂O₄ requires C, 59.08; H, 6.10; N, 10.60); **MS** (ESI, +ve) *m/z* 287.4

([M+Na⁺], 100%); **HRMS** (ESI, +ve) calcd. for $C_{13}H_{17}N_2O_4$ (M⁺) m/z 265.1188, found m/z 265.1188.

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