Immunomodulatory effects of *Pseudomonas aeruginosa* quorum sensing small molecule probes on mammalian macrophages†

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Pseudomonas aeruginosa produces the quorum sensing signalling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). This natural product not only coordinates production of virulence factors by the bacterium, but also has immunomodulatory effects on the host organism. Immunomodulatory small molecules are valuable for immunology research and are potential therapeutics for autoimmune diseases such as rheumatoid arthritis, and immunosuppressive drugs following organ transplants. We describe the total synthesis of OdDHL using solid-supported reagents and scavengers, which has the potential to be used for automated analogue synthesis. OdDHL and four analogues were tested for their ability to activate or inhibit release of the pro-inflammatory mediators tumour necrosis factor alpha (TNFα) and nitric oxide (NO) from equine or murine macrophages (immune cells). Two of the analogues showed substantial immunomodulatory activity with these macrophages. One analogue showed differing species selectivity, being a potent antagonist in mouse cells, but a partial agonist in horse-derived macrophages. These compounds have the therapeutic potential to be used for protecting animals from bacterial septic shock.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is pathogenic (disease-causing) to animals including humans. In fact, *P. aeruginosa* is a major cause of nosocomial (hospital-acquired) infections and the main contributor to progressive lung degeneration in cystic fibrosis patients.[1] Like many Gram-negative bacteria, *P. aeruginosa* uses *N*-acyl homoserine lactones as its intercellular signal in order to coordinate behaviour in a cell density dependant manner.[2] This phenomenon is known as quorum sensing and enables the organism to express specific genes in a coordinated fashion leading to a rapid and full blown virulence cascade. *P. aeruginosa* has two quorum sensing systems, known as Las and Rhl. The Las system synthesizes and senses *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL, Fig. 1) while the Rhl system synthesizes and senses *N*-butyryl-L-homoserine lactone (BHL).[3] Both systems are involved in the coordinated production of virulence factors and biofilms by the bacterium, allowing it to cause disease in the host organism. However, another role in infection has been implicated for the small molecule OdDHL. Recently it has been shown that OdDHL also affects the immune response of the host organism, and therefore may have additional roles in the pathogenesis of *P. aeruginosa*.[4] It is currently unclear whether OdDHL is an activator or suppresser of the immune response. For example, OdDHL induces production of the cytokines interleukin-1α (IL-1α), IL-6 and gamma interferon (IFNγ) in mice;[4e] whereas, OdDHL suppresses the production of the pro-inflammatory mediator tumour necrosis factor alpha (TNFα)[4b] by mouse cells that have been treated with lipopolysaccharide (LPS). Immunomodulatory small molecules are extremely useful for immunology research and, crucially, are potential therapeutics for autoimmune diseases such as rheumatoid arthritis. Immunosuppressive natural products such as cyclosporin A, FK506 and rapamycin have been used as drugs and are largely responsible for the massive increase in successful organ transplant operations.

Although *N*-acyl L-homoserine lactone (AHL) analogue synthesis has been an area of intense research for quorum

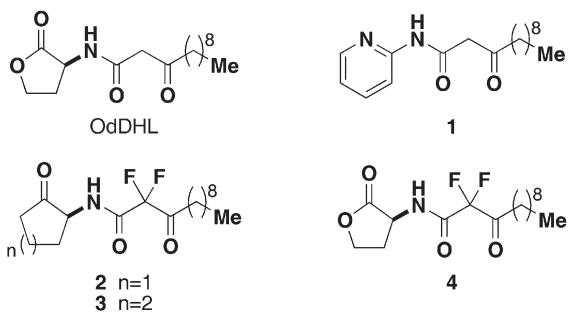

Fig. 1 The natural product OdDHL and four analogues used in this study.

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sensing modulatory effects,[5] the immunomodulatory effects of these compounds have been frequently overlooked. The OdDHL acyl chain has been established as optimal for immune suppressive activity.[4f] Instead of varying the acyl chain we chose to vary the homoserine lactone head group to the 2-pyridyl analogue **1** (a LasR antagonist)[5b] and to the non-hydrolysable cyclic ketones **2** and **3** (Fig. 1).[5c] The fluorinated acyl chain in **2** and **3** was required for compound stability in aqueous buffer,[5c] therefore fluorinated OdDHL analogue **4** was synthesised and used as a control.[6] Although the traditional solution-phase synthesis of OdDHL and analogues has been reported,[3a,4f–g] we wanted to develop a synthesis using solid-supported reagents and scavengers.[7] This strategy has become increasingly popular in organic chemistry as it bypasses the purification difficulties associated with traditional solution-phase reactions whilst retaining the beneficial aspects, such as ease of reaction monitoring (TLC, HPLC, LCMS, NMR). Simple filtration, washing and solvent removal is all that is required to isolate the desired material, which allows the possibility for automated high throughput analogue synthesis.

Cross-linked polystyrene resins are ideal as polymer-supports for reagents and scavengers for organic synthesis due to their compatibility with and insolubility in a wide range of organic solvents. High quality, functionalised polystyrene resins are readily synthesised by metallation and electrophile interception, as we have reported previously.[8] The polymer-bound boronic acid and triphenylphosphine used in this study were prepared by this method.

Results and discussion

In this account we describe the synthesis of the quorum sensing natural product OdDHL utilizing solid-supported reagents and scavengers. The synthesised OdDHL and four analogues were screened (i) for their ability to activate pigment production in a *P. aeruginosa* mutant, and (ii) for their ability to activate or inhibit release of the pro-inflammatory mediators TNFα and nitric oxide (NO) from equine and murine macrophages (immune cells).

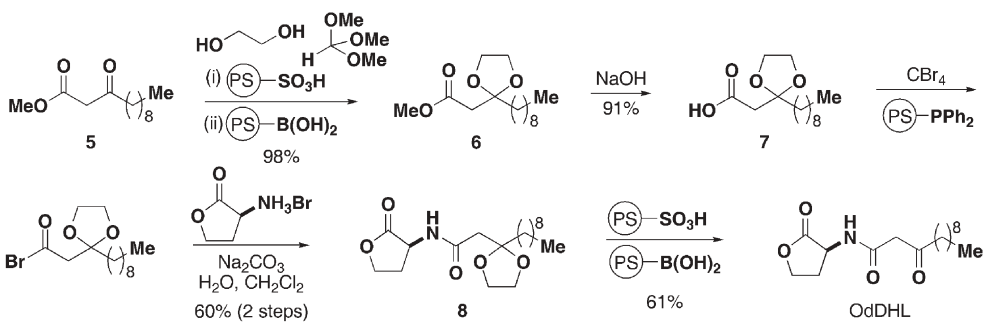
OdDHL synthesis

The synthesis of OdDHL is shown in Scheme 1. β-Keto ester **5** was protected as a cyclic ketal (**6**) using ethylene glycol. After an aqueous work-up and concentration of the dried organic solvent, the crude reaction mixture was only contaminated with excess ethylene glycol. This could be scavenged away from the desired product by adding boronic acid polystyrene resin to the reaction mixture before work-up. The protected product was obtained in excellent yield without need for chromatographic purification (>95% pure by HPLC and ^{1}H NMR). Saponification of the methyl ester gave **7**, which was purified by washing the aqueous reaction mixture with Et_2O, acidification and extraction with Et_2O.

Amide formation was a key step to optimise as it has the potential to be used for OdDHL analogue synthesis in an automated fashion. Attempts to transform **7** to an acid chloride[9] with polymer-supported triphenylphosphine[8] and CCl_4 were less successful than synthesis of the acid bromide with CBr_4,[10] which proved to be robust. A slight excess of CBr_4 was stirred in CH_2Cl_2 with acid **7** and an excess (2 equiv.) of polymer-supported triphenylphosphine, which acts as a reagent and scavenger. After 3 hours at room temperature the reaction mixture was filtered and concentrated to yield acid bromide, which was pure by ^{1}H NMR. The acid bromide was used immediately in a two phase reaction with homoserine lactone. The separation of the aqueous soluble base from the product and acid bromide in the organic layer proved crucial to avoid lactone hydrolysis and decomposition. The organic layer contained the amide product, but was contaminated with excess amine. In order to characterise amide **8** the reaction mixture was cleaned up by filtration through a pad of silica. However, this crude solution could be used in the following step also, since the acidic amberlyst resin, used in excess, not only deprotected the ketal but also scavenged the amine. The synthesis proved operationally simple, reproducible and the OdDHL produced was pure (>95% by HPLC, ^{1}H NMR).

Effects on *P. aeruginosa* pigment production

The synthesis of coloured virulence factors such as pyoverdin and pyocyanin is at least partly controlled by quorum sensing, so the pigments are not produced in *lasI* or *rhlI* mutants. Restoration of pigment production in a *P. aeruginosa lasI rhlI* double mutant (PAO-JP2) was a simple first screen of the bioactivity of the analogues. Bacterial cells were grown for 3 days in alanine–glucose–salts medium in the presence of 10 μM BHL, and OdDHL or an analogue (Fig. 2). In the absence of BHL, no pigment production was seen (data not shown). OdDHL, the natural LasR ligand, gave the most intense response. The analogues activity followed the order **2**, **4** > **3** > **1**. Interestingly, the six membered ring ketone **3** was less active



Scheme 1 Synthesis of the natural product OdDHL using polymer-supported (PS = polystyrene) reagents and scavengers.

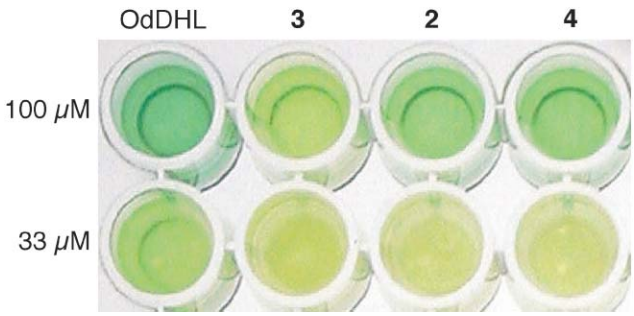


Fig. 2 *P. aeruginosa* pigment production assay. The *lasI rhlI* mutant was supplemented with 10 μM of BHL and the indicated concentration of additives shown above the microtitre wells. Compounds **2** and **4** strongly activated pigment production in *P. aeruginosa*.

than the five membered ring analogue **2**, which is in contrast to BHL analogue structure activity relationships.[5a–c,e]

Effects on the inflammatory response of mammalian macrophages.

The effects of the compounds **1–4** on the inflammatory response of macrophages was determined by stimulating either murine (RAW264.7) macrophage-like cells or equine (eCAS) macrophage-like cells with the OdDHL analogues in either the presence or absence of LPS (from *Escherichia coli* 0157). None of the OdDHL analogues, with the exception of **3** on eCAS cells, stimulated the release of inflammatory meditors from macrophage-like cells when added to the cells for 24 h. OdDHL, **1** and **4** had an insignificant effect on LPS-induced NO or TNFα release with RAW264.7 or eCAS cell lines. In contrast, **2** and **3** essentially abolished NO production in murine cells and reduced it in equine cells (Fig. 3). Moreover, **2** and **3** reduced TNFα production from RAW264.7 (Fig. 4) and eCAS cells when administered 1 h prior to LPS stimulation. Interestingly, in eCAS cells, **3** appeared to have partial agonist activity because it stimulated a low level of production of NO as well as inhibiting LPS-induced NO production (Fig. 3, lower panel). This effect was not seen with TNFα production.[11]

LPS is a key constituent of the outer membrane of Gram-negative bacteria such as *Salmonella typhimurium* and *E. coli*. The recognition of LPS, as well as other bacterial products, by macrophages is at least partially mediated through the Toll-like receptors (TLRs) which comprise a family of key regulatory proteins in mammals. Currently at least ten different TLRs have been recognised and cloned. TLR4 responds principally to LPS and Gram-negative bacteria, TLR9 to bacterial DNA, TLR5 to bacterial flagellin and TLR2 to a wide range of ligands including bacterial lipoproteins, mycoplasmal proteins, fungal products and Gram-positive bacteria.[12] LPS stimulation of TLR4 plays a critical role in the immune response of animals to infection with Gram-negative bacteria. Animals with either defective or no TLR4 are resistant to LPS-induced endotoxaemia, but show increased susceptibility to infection with *S. typhimurium*.[13] Inappropriate over-stimulation of TLR4 will lead to lethality whilst a lower-level stimulation initiates early responses that control the infection. The degree of

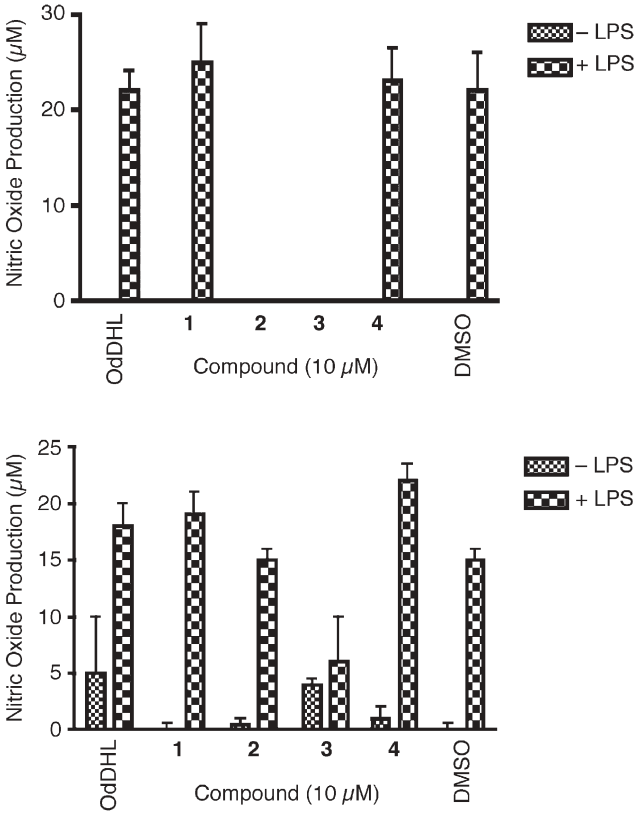


Fig. 3 The effects of OdDHL analogues on lipopolysaccharide-induced nitric oxide production from macrophage-like cells. *Top Panel*: RAW264.7 murine macrophage-like cells were incubated with OdDHL analogues (10 μM in DMSO) were incubated for 1 h prior to stimulation with LPS (100 ng ml^{-1}) or sterile water. Medium was collected after 24 h and nitric oxide production measured using the Griess reaction. These data represent the mean ± standard deviation of at least 3 separate experiments. The results of NO production in the absence of LPS are not seen on the vertical scale displayed. *Lower Panel*: eCAS equine macrophage-like cells were incubated with OdDHL analogues (10 μM in DMSO) for 1 h prior to stimulation with LPS (100 ng ml^{-1}) or sterile water. Medium was collected after 24 h and nitric oxide production measured using the Griess reaction. These data represent the mean ± standard deviation of at least 3 separate experiments.

inflammatory response to bacterial ligands is therefore critical in determining whether the host develops a protective immune response or overwhelming disease. Development of compounds that modulate TLR4 function may therefore produce drugs with a broad therapeutic potential from protecting animals from the effects of endotoxaemia to stimulation of immunity as an adjuvant in vaccinology.

Conclusions

The total synthesis of OdDHL was achieved using polymer-supported reagents and scavengers and avoiding the need for chromatographic purification. This route has the potential to be used for automated analogue synthesis. OdDHL and four analogues were tested for their ability to activate or inhibit release of the pro-inflammatory mediators TNFα and NO from equine and murine macrophages. OdDHL analogues **2**

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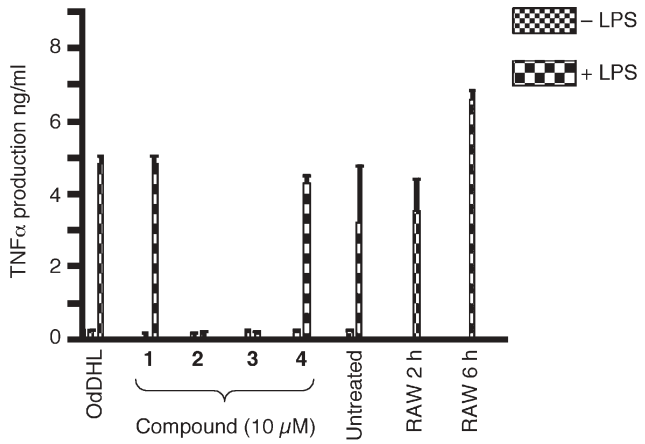

Fig. 4 The TNFα production of LPS stimulated RAW cells was prevented in the presence of compounds **2** and **3**. These data represent the mean ± standard deviation of at least 3 separate experiments and was verified by ELISA.

and **3** showed substantial immunomodulatory activity with different mammalian species. Indeed **3** also showed differing species selectivity, being a potent antagonist in mouse cells, but a partial agonist in horse-derived macrophages. These compounds have the therapeutic potential to be used for protecting animals from bacterial septic shock and are being investigated further.

Experimental

Experimental techniques and apparatus for synthesis

Experimental techniques and apparatus are standard except as otherwise indicated, reactions were carried out under nitrogen with dry, freshly distilled solvents. All reagents were purified in accordance with the instructions in *Purification of Laboratory Chemicals*[14] or used as obtained from commercial sources. Yields refer to spectroscopically pure compounds. Compounds used in biological assays were >95% pure by HPLC. Melting points were obtained using a Reichert hot plate microscope with a digital thermometer attachment and are uncorrected. Infrared spectra were recorded neat on a Perkin–Elmer Spectrum One spectrometer with internal referencing. Absorption maxima (υ_{max}) are reported in wavenumbers (cm^{-1}) and the following abbreviations are used: w, weak; m, medium; s, strong; br, broad. Proton magnetic resonance spectra were recorded on Bruker Ultrashield 500. Chemical shifts (δ_H) are quoted in ppm and are referenced to the residual non–deuterated solvent peak. Coupling constants (J) are reported in Hertz to the nearest 0.5 Hz. Data are reported as follows: chemical shift, integration, multiplicity [br, broad; s, singlet; d, doublet; t, triplet; q, quartet; qui, quintet; sept, septet; m, multiplet; or as a combination of these (*e.g.* dd, dt, *etc.*)], coupling constant(s) and assignment. Diastereotopic protons are assigned as X and X′, where the ′ indicates the higher field proton. Carbon magnetic resonance spectra were recorded on a Bruker Ultrashield 500 spectrometer. Chemical shifts (δ_C) are quoted in ppm to the nearest 0.01 ppm, and are referenced to the deuterated solvent. LCMS spectra were recorded on an HP/Agilent MSD LCMS APCI. High resolution mass measurements were made by the EPSRC mass spectrometry service (Swansea) and reported mass values are within the error limits of ±5 ppm mass units.

Methyl-3-oxododecanoate (5)[15]

To a solution of Meldrum's acid (2.0 g, 13.9 mmol) in CH_2Cl_2 (20 ml) at 0 °C under nitrogen was added pyridine (2.24 ml, 27.8 mmol) dropwise over 20 min, followed by decanoyl chloride (3.16 ml, 15.3 mmol) and the reaction stirred for 1 h at 0 °C and then allowed to warm to room temperature for 1 h. The orange reaction was diluted with CH_2Cl_2 (20 ml) and poured into ice and HCl (2 M, 20 ml). The organic layer was separated, washed with HCl and brine, then dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was refluxed in methanol (55 ml) under nitrogen for 4 h and concentrated *in vacuo*. The yellow oil was purified by vacuum distillation (125 °C, 2 mbar) and column chromatography to yield the title compound as a colourless liquid (2.6 g, 82%); R_f 0.31 (SiO_2; 9 : 1 hexane : ethyl acetate); ν_{max} (neat)/cm^{-1} 2924s, 2854s, 1748s (ester), 1717s (ketone); δ_H (400 MHz; $CDCl_3$) 3.68 (3H, s, OCH_3), 3.39 (2H, s, C(2)H_2), 2.48 (2H, t, *J*7.5, C(4)H_2), 1.54–1.52 (2H, m, C(5)H_2), 1.21 (12H, br m, alkyl CH_2), 0.83 (3H, t, *J*7.5, CH_2CH_3); δ_C (125 MHz, $CDCl_3$) 202.69 and 167.61 (C=O), 52.16 (CH_3), 48.91, 42.97, 31.78, 29.32, 29.28, 29.17, 28.93, 24.40 and 23.58 (CH_2), 13.99 (CH_3); *m*/*z* (APCI) 229 (MH^+).

Methyl-(2-nonyl-[1,3]-dioxolan-2-yl)-acetate (6)

To methyl-3-oxododecanoate (**5**, 30 mg, 0.13 mmol) and Amberlyst™ 15 resin (300–550 μm, 4.70 mequiv./g, 5 mg) was added trimethyl orthoformate (50 μl, 0.46 mmol) and ethylene glycol (0.06 ml, 1.0 mmol) under nitrogen. The reaction was stirred 4 h and then polymer-bound boronic acid (150–300 μm, 1.95 mequiv./g, 1.0 g) was added. The suspension was stirred overnight and quenched by addition of 5% w/v NaH_2PO_4 solution (0.5 ml) and stirred for 15 min. Ether (5 ml) was added and the beads removed by filtration. The organic layer was washed with water and brine, then dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield the title compound as a colourless oil (35 mg, 98%); R_f 0.34 (SiO_2, 10 : 1 hexane : ethyl acetate); ν_{max} (neat)/cm^{-1} 2924s, 1744s (ester), 1234s (ether); δ_H (400 MHz, $CDCl_3$) 3.98–3.90 (4H, m, OCH_2CH_2O), 3.66 (3H, s, OCH_3), 2.63 (2H, s, C(2)H_2), 1.77–1.73 (2H, m, C(4)H_2), 1.37–1.34 (2H, m, alkyl CH_2), 1.23 (12H, br m, alkyl CH_2), 0.83 (3H, t, *J*7.5, CH_2CH_3); δ_C (125 MHz, $CDCl_3$) 169.96 (C=O), 109.38 (C), 65.05 (CH_2), 51.64 (CH_3), 42.37, 37.73, 31.84, 29.65, 29.51, 29.47, 29.25, 23.47 and 22.62 (CH_2), 14.04 (CH_3); *m*/*z* (APCI) 273 (MH^+); HRMS (ES) found 295.1900 $C_{15}H_{28}O_4Na$ (MNa^+) required 295.1885.

(2-Nonyl-[1,3]dioxolan-2-yl)-acetic acid (7)

Methyl-(2-nonyl-[1,3]-dioxolan-2-yl)-acetate (**6**, 1.00 g, 3.68 mmol) was stirred in 2 M sodium hydroxide solution (1.84 ml, 3.68 mmol) for 3 h. The aqueous layer was diluted with water (5 ml) and washed with ether (×3). The aqueous layer was acidified and extracted using ether (×3). The organic

layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield the title compound as a white solid (864 mg, 91%); mp 38–42 °C; ν_{max} (neat)/cm^{-1} 2913s, 1705s (acid), 1056s (ether); δ_H (500 MHz, $CDCl_3$) 4.08–4.01 (4H, m, OCH_2CH_2O), 2.74 (2H, s C(2)H_2), 1.82–1.78 (2H, m, C(4)H_2), 1.42–1.37 (2H, m, alkyl CH_2), 1.31–1.23 (12H, br m, alkyl CH_2), 0.91 (3H, t, *J*7.0, CH_2CH_3); δ_C (125 MHz, $CDCl_3$) 174.53 (C=O), 109.32 (C), 65.13, 42.34, 37.60, 31.89, 29.63, 29.53, 29.50, 29.20, 23.53 and 22.69 (CH_2), 14.13 (CH_3).

(*S*)-2-(2-Nonyl-[1,3]dioxolan-2-yl)-*N*-(2-oxo-tetrahydrofuran-3-yl)-acetamide (8)

A mixture of **7** (50 mg, 0.19 mmol), polymer-bound triphenylphosphine (150–300 μm, 1.36 mequiv./g, 0.42 mequiv.) and carbon tetrabromide (70.6 mg, 0.21 mmol) in dry CH_2Cl_2 was stirred under nitrogen for 3 h. The beads were removed by filtration and the solvent removed *in vacuo* to yield the acid bromide. To a solution of (*S*)-(−)-α-amino-γ-butyrolactone hydrobromide (39 mg, 0.29 mmol) and Na_2CO_3 (3 equiv.) in water (3 ml) was added the bromide in CH_2Cl_2 (3 ml) and stirred overnight at room temperature. The organic layer was separated and washed with 1 M Na_2CO_3 solution (×2), brine (×2), then dried ($MgSO_4$), filtered through a pad of silica and concentrated *in vacuo* to yield the title compound as a beige solid (38.9 mg, 60%); $[\alpha]_D^{25}$ +26.0 (*c* 0.1 in $CHCl_3$); mp 69–72 °C; R_f 0.28 (SiO_2, ethyl acetate); ν_{max} (neat)/cm^{-1} 1767s (lactone), 1654s (amide), 1184s (ether); δ_H (500 MHz, $CDCl_3$) 7.01 (1H, d, *J*6.0, NH), 4.59–4.57 (1H, m, NHC*H*), 4.45 (1H, td, *J*9.0, 1.0, CH*H*′O) 4.28–4.25 (1H, m, C*H*H′O), 4.09–3.98 (4H, m, OCH_2CH_2O), 2.80–2.78 (1H, m, NHCHCH*H*′), 2.64 (2H, s, C(2)H_2), 2.14 (1H, dq, *J*11.5, 9.0, NHCHC*H*H′), 1.69–1.66 (2H, m, C(4)H_2), 1.38–1.31 (2H, m, CH_2 alkyl), 1.25 (12H, br m, CH_2 alkyl), 0.87 (3H, t, *J*7.0, CH_3); δ_C (125 MHz, $CDCl_3$) 175.19 and 169.77 (C=O), 109.61 (C), 65.88 and 65.08 (CH_2), 48.93 (CH), 44.17, 37.51, 31.85, 30.31, 29.65, 29.47, 29.27, 23.67 and 22.64 (CH_2), 14.08 (CH_3); *m*/*z* (APCI) 342 (MH^+); HRMS (ES) found 364.2114 $C_{18}H_{31}O_5NNa$ (MNa^+) required 364.2100.

3-Oxododecanyl-L-homoserine lactone (OdDHL)[3a,4f]

To a solution of the protected ketone **8** (14 mg, 44 μmol) in CH_2Cl_2 was added Amberlyst™ 15 resin (300–550 μm, 4.70 mequiv./g, 9 mg) and water (25 μl). The reaction was stirred for 5 h and then polymer-bound boronic acid (150–300 μm, 1.95 mequiv./g, 45 mg) was added. The resins were removed by filtration and the organic layer dried ($MgSO_4$), concentrated *in vacuo* to yield the title compound as a white solid (8.0 mg, 61%); $[\alpha]_D^{25}$ +32.0 (*c* 0.1 in $CHCl_3$); mp 80–81 °C (lit.[4f] 84–85 °C); R_f 0.30 (SiO_2, 3:1 CH_2Cl_2: ethyl acetate); ν_{max} (neat)/cm^{-1} 3294m (NH), 2921m, 1776s (lactone), 1716s (ketone), 1654s (amide), 1176 (ether); δ_H (500 MHz, $CDCl_3$) 7.72 (1H, d, *J*6.0, NH), 4.64–4.58 (1H, m, NHC*H*), 4.49 (1H, t, *J*9.0, CH*H*′O), 4.32–4.26 (1H, m, C*H*H′O), 3.84 (2H, s, C(2)H_2), 2.78–2.75 (1H, m, NHCHCH*H*′), 2.53 (2H, t, *J*7.5, C(4)H_2), 2.28–2.23 (1H, m, NHCHC*H*H′), 1.61–1.58 (2H, m, C(5)H_2), 1.32–1.23 (12H, br m, CH_2 alkyl), 0.99 (3H, t, *J*7.0, CH_3); δ_C (125 MHz, $CDCl_3$) 206.66, 174.82 and 166.37 (C=O), 65.89 (CH_2), 49.04 (CH), 48.04, 43.95, 31.85, 29.83, 29.38, 29.34, 29.24, 28.99, 23.53 and 22.67 (CH_2), 14.12 (CH_3); *m*/*z* (APCI) 298 (MH^+); HRMS (CI) found 298.2016 $C_{16}H_{28}O_4N$ (MH^+) required 298.2013.

Cell culture

RAW 264.7 murine macrophage-like cells were obtained from the European Cell culture collection. RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum supplemented with 2 μM glutamine, 200 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Equine eCAS cells[16] were cultured in RPMI (Gibco, UK) containing 20% horse serum, 1% penicillin (100 units ml^{-1})/streptomycin (100 μg ml^{-1}), 1% L-glutamine (2 μM), 1% non-essential amino acids (Gibco, UK), 1% sodium pyruvate (100 μM) and 0.05% amphoteracin B (5 μg ml^{-1}) at 37 °C and at 5% CO_2.

Measurement of nitric oxide

NO was only detected from RAW and eCAS cells. To determine nitric oxide synthase (NOS) activity the supernatant of the cultured equine bone marrow cells or RAW 264.7 cells was removed 24 h after addition of LPS and assayed for nitrite accumulation by the Griess reaction as an indication of iNOS activity.[17] Briefly, an equal volume of Griess reagent (4% sulfanilamide and 0.2% naphtylethylenediamine dihydrochloride in 10% phosphoric acid) was added to an equal volume of sample and the colorimetric difference in optical density at 540 nm and 620 nm read immediately. The values obtained were compared to standards of sodium nitrite dissolved in DMEM and the concentration of nitrite released calculated and expressed as concentration (μM).

TNFα cytotoxicity assay

L929 (murine fibroblast) cells were maintained in RPMI 1640 medium supplemented with 10% feta calf serum (FCS), 2 μM glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were collected by trypsin treatment, resuspended in medium and plated at a density of 7×10^4 per 100 ml per well in 96 well plates. Cycloheximide (Sigma, 100 μg ml^{-1} solution in DMSO) was diluted to a concentration of 0.3 μg ml^{-1} in culture medium and 50 μl added per well. After a four to six hour incubation period to allow the cells to adhere to the plate, TNFα was added to wells in triplicate, at a range of concentrations, and the plates incubated overnight. The medium was aspirated and replaced with 100 μl per well of fresh, phenol red-free culture medium. 20 μl of XTT dissolved in phenol red-free RPMI 1640 was added to each well and plates incubated at 37 °C and 5% CO_2 for 2 h. Plates were shaken and absorbance was read at 450 nm with reference at 620 nm. The results were verified by TNF ELISA.

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