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1-Oxabicyclic β-lactams as new inhibitors of elongating MPT–a key enzyme responsible for assembly of cell-surface phosphoglycans of *Leishmania* parasite†

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New iminosugars (1-oxabicyclic β-lactam disaccharides) have been synthesized as inhibitors of elongating α-D-mannosyl phosphate transferase (eMPT), a key enzyme involved in the iterative biosynthesis of cell-surface phosphoglycans of the *Leishmania* parasite. The design is based on a transition-state model for this remarkable enzyme that transfers intact α-D-mannosyl-phosphate from GDP-Man. Since these phosphoglycans are unique to *Leishmania* and are essential for its infectivity and survival, their biosynthetic pathway has emerged as a novel target for anti-leishmanial drug and vaccine design.

Introduction

The protozoan parasite *Leishmania*, responsible for multiple human diseases, survives in extremely hostile milieu throughout its life cycle in both the sand-fly vector and the human host. To accomplish this, all *Leishmania* species synthesize[1] a unique class of molecules termed phosphoglycans (PGs), including the membrane-bound lipophosphoglycan (LPG) and secreted proteophosphoglycan (PPG). Substantial evidence has been accumulated showing that the PGs are essential virulence factors, being responsible for (a) infectivity and survival of the parasite in the human host; (b) attachment and maturation in the sand-fly mid-gut; (c) recognition of the specific carbohydrate binding sites on macrophages; (d) inhibition of normal signalling of the immune system and (e) pathological lesions caused by subsequent disease. The role(s) of PGs in parasitic virulence has been a topic of intense debate[2] in recent years, but it has now been established[3] that the principal determinant of virulence consists of the PG domain and is independent of the molecular platform. The intriguing structure (Fig. 1) of LPG of *L. donovani*, the species causing fatal visceral leishmaniasis, consists of four distinct domains: an alkyl-*lyso*-glycosylphosphatidylinositol (GPI) anchor, a conserved glycan core with an internal Gal_f residue, variable PG repeats and a neutral oligosaccharide cap.

† Electronic supplementary information (ESI) available: Copies of ^{1}H and ^{13}C NMR spectra for new compounds (**6**, **1**, **7**, **2** and **9–13**). See http://www.rsc.org/suppdata/ob/b4/b418247b/

The most distinct feature of the LPG/PPG structure is the variable PG domain composed of [6Gal*p*-β1,4-Man*p*-α1-PO_4]$_n$ repeats linked to each other *via* phosphodiester bonds between the anomeric-OH of the mannose of one repeat and 6-OH of the galactose of the adjoining repeat, a structure unique to *Leishmania* among all the carbohydrates known in nature. The PG repeats form a spring-like helical supramolecular assembly around the parasite that provides resistance to host enzymes and antibodies, and constitutes the epitopes for recognition of macrophage receptors. Although the biosynthetic pathway of LPG has not been elucidated in detail, a cell-free system using *Leishmania* microsomal membranes has been reported[4] and parasite mutants have been isolated[3] that are deficient in specific genes and produce truncated LPGs. The current data[4,5] suggest that the PG domain is assembled by the sequential action of the α-D-mannosyl phosphate transferase (MPT) and 1,4-β-galactosyltransferase (GalT) enzymes (Fig. 2). At least two types of MPTs are present, one with specificity for the GPI-anchor and the second with specificity for the [6Gal*p*-β1,4-Man*p*-α1-PO_4] repeat. These enzymes, described as the initiating-MPT and elongating-MPT respectively, have not been purified or cloned.

The MPTs are unique enzymes that can transfer an intact α-D-mannose-phosphate moiety from the nucleotide sugar donor GDP-Man to the glycan substrate. It should be mentioned here that in normal biology a mannose residue is transferred from GDP-Man with the release of a GDP unit, whereas in *Leishmania* intact α-D-mannose-phosphate is transferred with the release of GMP (Fig. 2). Since there are no such MPT

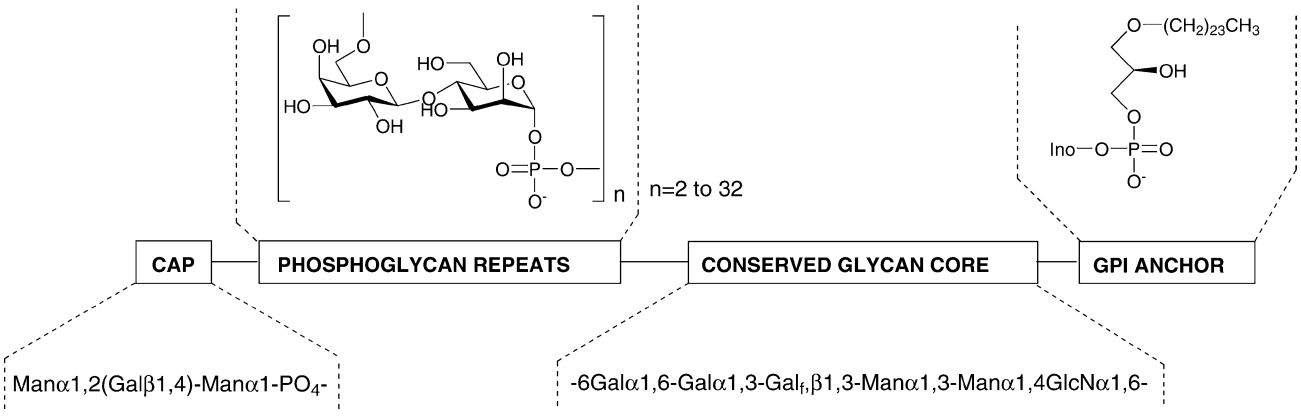

Fig. 1 Structure of LPG of *Leishmania donovani*.

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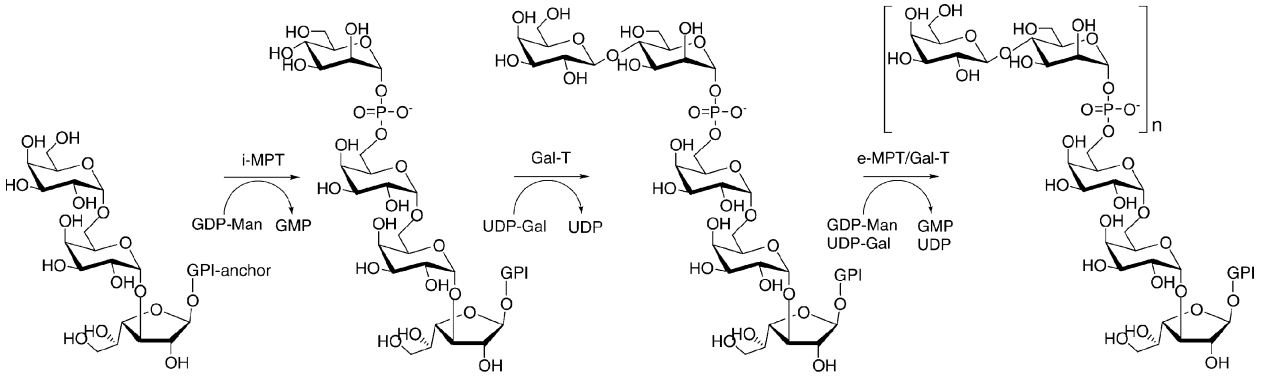


Fig. 2 Proposed biosynthetic pathway for the assembly of phosphoglycan.

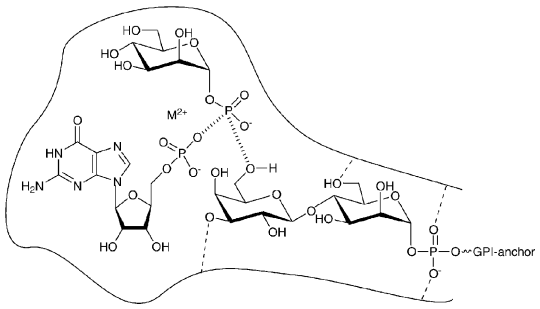


Fig. 3 Transition state model for the reaction of eMPT.

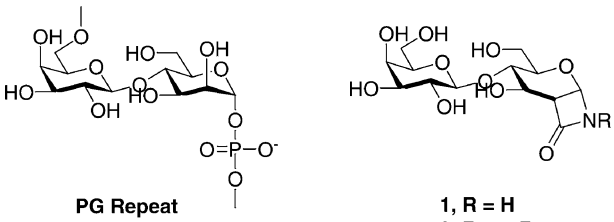
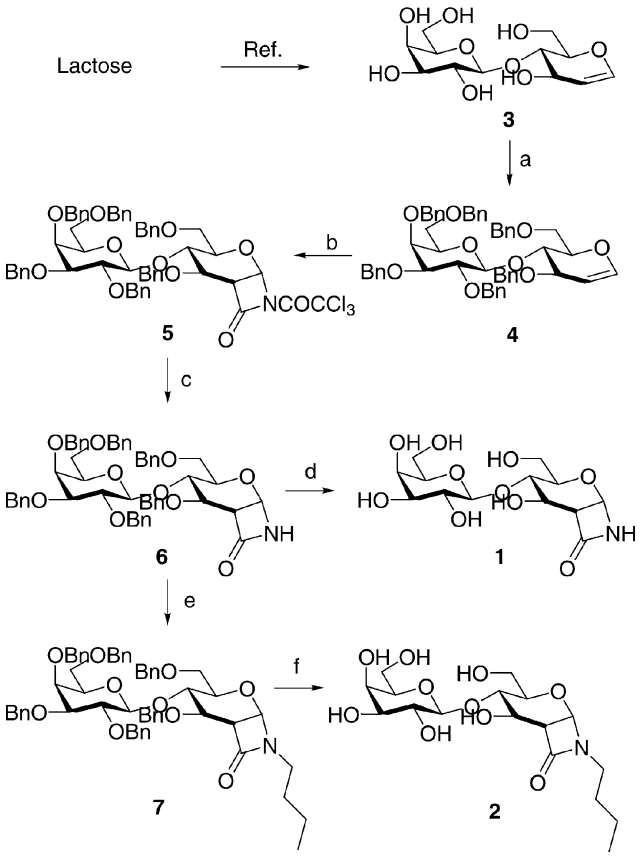


Fig. 4 A comparison of the structure of a PG repeat and the designed inhibitors.

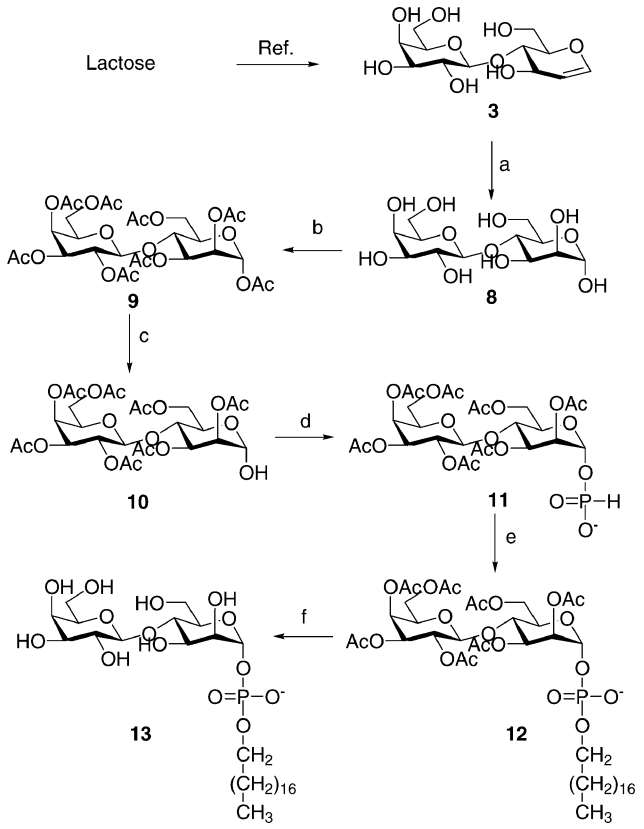
enzymes in human biology, the *Leishmania* MPTs present novel opportunities[6] for drug design. A transition state model has been proposed[4,5] for the eMPT reaction (Fig. 3). According to this model, a negatively charged anomeric phosphate of the first PG repeat is the key recognition element for eMPT. Other essential features include (a) 6-OH and 3-OH on the Man residue, (b) the configuration of the Gal residue and (c) divalent cations (Mg^{2+} and Mn^{2+}). The model suggests that there should be at least three binding domains in eMPT involved in a ternary complex, the first to recognize the mannosyl-1-α-phosphate, the second to correctly position GDP-Man with two of its phosphodiesters complexed with divalent metal ion and the third to hold the acceptor galactose. Since the iminosugars, the analogues of the monosaccharides where either the ring oxygen or the exocyclic oxygen of the anomeric carbon is replaced by nitrogen, are known to be excellent inhibitors of carbohydrate enzymes (glycosyltransferases and glycosidases), we decided to examine this approach to target the eMPT and PG biosynthesis of the *Leishmania* parasite. For this, we decided to mimic the α-D-mannose-phosphate motif, leaving the acceptor part untouched, by placing a nitrogen at the anomeric position linked directly to a C=O, isosteric to the P=O of the phosphate. Since the orientation of the 2-OH of the Man residue is not critical[5] for recognition, a constraint was designed between positions 1 and 2. This analysis suggested that the construction of a 1-oxabicylic β-lactam ring between positions 1 and 2 of a PG substrate would satisfy the above design requirements. Although, in a strict sense, the designed β-lactam moiety did not fully mimic the anomeric phosphate, the presence of an anomeric NCO linkage (isosteric to OPO) was considered to be interesting as a probe of MPT inhibition. As a continuation of our investigations[7] into the chemistry and biology of *Leishmania* phosphoglycans and related GPI molecules, we now report the synthesis and evaluation of compound **1** and its *N*-alkyl analogue **2** (Fig. 4) as new eMPT inhibitors.

Results and discussion

From a synthetic point of view, a [2 + 2] heteroatom cycloaddition on a suitable disaccharide–glycal scaffold bearing the PG-substrate features appeared to be a good option to access these compounds. Such cycloadditions on monosaccharide glycals have been reported,[8] where a glycal with non-polar protecting groups undergoes reversible cycloaddition with electron-deficient isocyanates to give a mixture of [2 + 2] and [4 + 2] adducts. However, many such cycloadditions are reversible, require high pressure conditions and give very low yields *e.g.* cycloaddition of tri-*O*-benzyl-D-glucal with trichloroacetyl-isocyanate is reported to give 24% yield. However, despite the low yields, this chemistry holds great potential for the synthesis of aza-sugars. Despite the known concerns, we attempted this chemistry with an appropriate disaccharide-glycal, hexa-*O*-benzyl-D-lactal (**4**, Scheme 1). To our surprise, the cycloaddition of compound **4** with trichloroacetyl-isocyanate progressed extremely well at room temperature and pressure, providing 87% yield of exclusively the [2 + 2] product **6**, after *in situ* removal of the *N*-trichloroacetyl group from adduct **5**. This is a novel observation, where a sugar substituent (galactose) at the 4-position of a glycal moiety plays a remarkable role in stabilizing [2 + 2] cycloaddition products to a great extent (from 24 to 87% yield). The same reaction when repeated with the monosaccharide glycal, tri-*O*-benzyl-D-glucal, under identical conditions gave only 24% yield, as reported previously.[8] This efficient cycloaddition provided high yielding access to the new 1-oxabicyclic β-lactams, designed as eMPT inhibitors. The synthesis of **1** and **2** started from known hexa-*O*-benzyl lactal (**4**). The construction of the β-lactam ring involved [2 + 2] cycloaddition of trichloroacetyl isocyanate to **4** at room temperature for 18 h, progress of the reaction monitored by following a parallel reaction in a NMR tube and observing the spectra at different time points. The *N*-chloroacetyl group was removed *in situ* using benzylamine at −20 °C, providing 2-carboxy-2-deoxy-3,6-di-*O*-benzyl-4-(2,3,4,6-tetra-*O*-benzyl-β-galactopyranosyl)-α-D-glucopyranosyl-β-aminolactam **6** in 87% yield. The reaction proceeded regio- and stereospecifically, with formation of β-lactam *anti* to the substituent at C-3. The gluco configuration of the β-lactam ring was confirmed by ^{1}H NMR analysis (doublet at 5.4, J = 4.5 Hz for H-1), and also by NOE experiments (H-1/H-2 and H-4 across the β-face of the pyranose ring). The removal of benzyl protecting groups from compound **6** by transfer hydrogenation provided



Scheme 1 *Reagents and conditions*: (a) BnBr, TBAI, NaH, DMF, rt, 4 h, 85%; (b) CCl_3CONCO, $CHCl_3$, rt, 18 h; (c) benzylamine, −20 °C, 87%; (d) Pd/C, HCOOH, MeOH, 50 °C, 12 h, 95%; (e) butyl bromide, NaH, 0 °C, 3 h, 80%; (f) Pd/C, HCOOH, MeOH, 50 °C, 12 h, 95%.



Scheme 2 *Reagents and conditions*: (a) mCPBA, H_2O–ether, 0 °C, 4 h; (b) Ac_2O, py, rt, 16 h, 90%; (c) Me_2NH, CH_3CN, −20 °C, 3 h, 92%; (d) PCl_3, imidazole, CH_3CN, 0 °C, 2 h; TEAB workup, 86%; (e) stearyl alcohol, adamantane carbonyl chloride, 1 h; I_2 oxidation, 30 min; TEAB workup, 85%; (f) Na_2CO_3, MeOH, 2 h, 93%.

2-carboxy-2-deoxy-4-(β-galactopyranosyl)-α-D-glucopyranosyl β aminolactam (**1**), ^{1}H NMR showing doublets at 5.48 (J = 4.2 Hz, H-1) and 4.37 (J = 7.8 Hz, H-1′) for the two anomeric protons.

Since the eMPT enzyme in *Leishmania* is known to be associated with the Golgi membrane, success of a potential inhibitor critically depends on its accessibility to the membrane environment. We reasoned that placement of a small lipid tail in the β-lactam **1** would enhance its intercalation with the Golgi vesicles and, hence, the inhibition of eMPT. To test this proposition an *N*-alkylated analogue of **1** was synthesized by the coupling of the compound **6** with *n*-butyl bromide to afford intermediate **7**, which on deprotection gave water soluble **2** showing doublets at 5.38 (J = 4.2 Hz, H-1) and 4.31 (J = 7.8 Hz, H-1′) by ^{1}H NMR.

For biological evaluation of the above designed inhibitors **1** and **2**, an efficient eMPT assay with the microsomal membranes of *Leishmania* was required. For this we synthesized a new lipid-linked phosphoglycan **13** (Scheme 2) as an exogenous substrate for membrane-bound eMPT. The rationale behind attachment of a lipid anchor was that it should help intercalation of the substrate to *Leishmania* membranes during the assay and facilitate isolation of biosynthetic products by solid-phase extraction on reversed-phase Sep-Pak cartridges, thus enabling a clean and high throughput eMPT assay. The synthesis of the substrate mimic **13** started from a known[7a] disaccharide Gal1,4βMan (**8**), which, on acetylation to the octa-acetate **9** followed by selective deprotection of the anomeric acetyl group (Me_2NH, −20 °C), provided the hepta-*O*-acetyl compound **10**. This, on reaction with anhyd PCl_3 and imidazole, led to the *H*-phosphonate **11** (characteristic doublet at 6.95 for J_{HP} = 637 Hz). Compound **11** was coupled with stearyl alcohol by adamantane carbonyl chloride, followed by *in situ* oxidation to give 1-*O*-stearyl-2,3, 6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranosyl phosphate (**12**). Final deprotection of the above provided **13**, which proved to be a good exogenous substrate of the eMPT enzyme in the bioassay.

The microsomal membranes equipped with the machinery for LPG biosynthesis (MPT, Gal-T and GDP-Man transporter) were prepared from *Leishmania donovani* promastigotes using a reported method.[4] For the control eMPT assay, the cell lysate was suspended in buffer (50 mM HEPES–NaOH pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM TLCK, 1 μg mL^{-1} leupeptin, 1 mM ATP and 0.5 mM DTT). Each control assay contained 23 μM GDP-Man with 1 μCi of GDP-[^{3}H]Man and 25 nmol of synthetic substrate **13** and was incubated at 28 °C for 20 min. The products were purified using Sep-Pak (C-18) cartridges by sequential elution with 100 mM NH_4OAc (to remove unused GDP-Man), 5, 20, 40 and 60% *n*-PrOH in 100 mM NH_4OAc, respectively. The required [^{3}H]-labelled product was eluted in 60% *n*-PrOH and analyzed by scintillation counting as well as by TLC (visualized by phosphorimaging). The biochemical control assay, in triplicate, showed 1.4% incorporation from labelled GDP-Man. Having a control eMPT assay in hand, inhibition experiments with synthetic β-lactams **1** and **2** were carried out at a concentration range from 0.05 mM to 1 mM (Fig. 5). Compound **1** showed inhibition of eMPT with an IC_{50} of 0.6 mM whereas the *N*-alkylated compound **2** showed better inhibition with an IC_{50} of 0.20 mM. The maximum inhibition of 70% was reached at 0.80 mM, beyond which saturation was observed.

In conclusion, novel substrate mimics of eMPT enzymes of the *Leishmania* parasite have been designed and synthesized using high yielding [2 + 2] cycloaddition, which showed good (μM range) inhibition. These are the first functional inhibitors of this remarkable enzyme and provide lead structures for further optimization of activity for use in drug development.

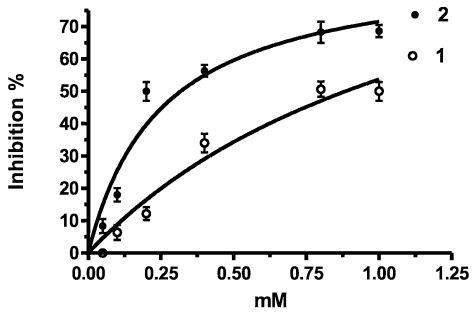


Fig. 5 Inhibition of *Leishmania* eMPT activity by **1** and **2**.

Experimental

Solvents were purified according to standard procedures and all reagents used were of highest purity available. The NMR spectra (^{1}H, ^{13}C, ^{31}P, 2D ^{1}H–^{1}H COSY and ^{1}H–^{13}C HETCOR, HMQC and HMBC) were recorded on a 300 MHz spectrometer (Bruker, Avance series) fitted with a pulse-field gradient probe. Trimethylsilane (TMS) or residual resonance of deuterated solvent were used as internal reference. For ^{31}P NMR spectra, phosphoric acid was used as external reference. ^{13}C NMR spectra were broadband ^{1}H decoupled or inverse HMQC experiments. Chemical shifts are expressed in ppm and coupling constants (J) in Hz. Where appropriate, signal assignments were made by DEPT, ^{1}H–^{1}H COSY and ^{1}H–^{13}C HETCOR experiments. Low and high resolution mass spectra were recorded on Platform-II or LCT spectrometer (Micromass-Waters) respectively using an acetonitrile–water (1 : 1) mobile phase. Optical rotations were measured at ambient temperature with a digital Perkin-Elmer 141 polarimeter. Thin layer chromatography was performed on Merck Kieselgel 60 F_{254} plates, compounds visualized either by viewing with a UV lamp (254 nm) or by dipping into ammonium-molybdate–cericsulfate developing reagent, followed by heating. Silica column chromatography was carried out with silica gel 60 (60–120 mesh).

2-Carboxy-2-deoxy-3,6-di-*O*-benzyl-4-(2,3,4,6-tetra-*O*-benzyl-β-galactopyranosyl)-α-D-glucopyranosyl β-aminolactam 6

To a solution of hexa-*O*-benzyl lactal[7b] (**4**, 300 mg, 0.36 mmol) in $CHCl_3$ (0.36 cm^3) was added trichloroacetyl isocyanate (90 mm^3, 0.74 mmol). The mixture was stirred at rt for 18 h to afford **5** which was characterized by ^{1}H NMR: δ 6.04 (1H, d, J = 5.4 Hz, H-1, gluco-isomer, [2 + 2] adduct). The reaction was cooled to −20 °C, treated with benzylamine (0.13 cm^3, 1.17 mmol) and the flask was warmed to rt. The organic phase was washed with water, dried (Na_2SO_4), concentrated and the residue purified by silica column chromatography (30% EtOAc in hexane) to give the product **6** (275 mg, 87%); R_f = 0.33 in 50% EtOAc in hexane; δ_H (300 MHz, $CDCl_3$) 3.37–3.46 (5 H, m, H-2, H-6, H-6′), 3.58–3.70 (3 H, m, H-3, 4, 5), 3.77–3.89 (3 H. m, H-2′, 3′, 5′), 4.34 (1 H, m, H-4′), 4.47 (1 H, d, J 7, H-1′), 4.58–4.97 (12 H, m, 6 × OCH_2Ph), 5.40 (1 H, d, J 4.5, H-1), 6.24 (1 H, s, NH), 7.22–7.36 (30 H, m, 6 × Ph); δ_C (75 MHz, $CDCl_3$) 54.27, 68.43, 69.39, 71.48, 72.65, 73.06, 73.12, 73.37, 74.56, 75.05, 75.08, 75.35, 75.95, 76.58, 79.47, 82.31, 102.98, 127.42–128.33 (multiple peaks), 138.07–138.83 (multiple peaks), 166.90; m/z (ES) 914.5 [M + Na]$^+$; (HRMS, found: [M + Na]$^+$ 914.3921. $C_{55}H_{57}O_{10}NNa$ requires m/z 914.3880).

2-Carboxy-2-deoxy-4-(β-galactopyranosyl)-α-D-glucopyranosyl β-aminolactam 1

To a solution of **6** (240 mg, 0.28 mmol) in CH_3OH (24 cm^3) was added Pd–C (10%, 800 mg) and formic acid (2.4 cm^3). The mixture was stirred at 50 °C overnight, catalyst filtered and solvent evaporated to afford compound **1** (90 mg, 95%); R_f = 0.27 in 40% CH_3OH in CH_2Cl_2; δ_H (300 MHz, CD_3OD) 3.39–3.69 (7 H, m), 3.78–3.84 (6 H, m), 4.17 (1 H, m, H-4′), 4.37 (1 H, d, J 7.8, H-1′), 5.48 (1 H, d, J 4.2, H-1), 8.02 (1 H, br s, NH); δ_C (75 MHz, CD_3OD) 55.36, 61.0, 68.47, 68.87, 69.47, 71.15, 73.36, 75.52, 75.95, 76.50, 79.91, 104.03, 169.37; m/z (ES) 374.33 [M + Na]$^+$; (HRMS found: [M + Na]$^+$ 374.1095. $C_{13}H_{21}O_{10}NNa$ requires m/z 374.1063).

2-Carboxy-2-deoxy-3,6-di-*O*-benzyl-4-(2,3,4,6-tetra-*O*-benzyl-β-galactopyranosyl)-α-D-glucopyranosyl *n*-butyl β-aminolactam 7

Compound **6** (200 mg, 0.22 mmol) was dissolved in DMF (4 cm^3) at 0 °C and sodium hydride (60% dispersion, 12 mg, 0.33 mmol) was added followed by *n*-butyl bromide (48 mm^3, 60 mg, 0.44 mmol). The mixture was stirred at 0 °C for 3 h, quenched with CH_3OH (1 cm^3) to destroy excess NaH, diluted with CH_2Cl_2 and washed with water. The organic phase was dried (Na_2SO_4), concentrated and purified by silica column chromatography (15% EtOAc in hexane) to afford compound **7** (172 mg, 80%); R_f = 0.57 in 50% EtOAc in hexane; δ_H (300 MHz, $CDCl_3$) 0.93–1.03 (3 H, t, J 7.2, CH_3), 1.36–1.45 (4 H, m, NCH_2CH_2 CH_2CH_3), 3.25 (2 H, m, NCH_2), 3.36–3.56 (5 H, m, H-2, 6, 6′), 3.60–3.75 (2 H, m, H-3, 5), 3.80 (2 H, m, H-5′, 4′), 3.82 (2 H, m, H-2′, 3′), 4.43 (1 H, m, H-4′), 4.55 (1 H, d, J 7.5, H-1′), 4.68–5.15 (12 H, m), 5.46 (1 H, d, J 4.2), 7.33–7.56 (30 H, m); δ_C (75 MHz, $CDCl_3$) 13.45, 20.22, 29.65, 40.29, 53.82, 68.37, 69.05, 69.40, 71.50, 72.60, 72.99, 73.04, 73.01, 73.35, 73.36, 74.54, 75.07, 75.50, 75.58, 79.44, 75.55, 82.32, 127.38–128.30 (multiple peaks), 138.18–138.77 (multiple peaks), 166.67; m/z (ES) 970.90 [M + Na]$^+$; (HRMS found: [M + Na]$^+$ 970.4520. $C_{59}H_{65}O_{10}NNa$ requires 970.4506).

2-Carboxy-2-deoxy-4-(β-galactopyranosyl)-α-D-glucopyranosyl *n*-butyl β-amino lactam 2

To a solution of compound **7** (48 mg) in CH_3OH (4 cm^3) was added 10% Pd/C (0.14 g) and formic acid (0.4 cm^3) and was stirred at 50 °C overnight, catalyst filtered and solvent evaporated to give compound **2** (19 mg, 95%); δ_H (300 MHz, CD_3OD) 0.72–0.77 (3 H, t, J 7.2, CH_3), 1.35–1.42 (4 H, m, $NCH_2CH_2CH_2CH_3$), 3.05–3.09 (2 H, m, NCH_2), 3.30–3.37 (3 H, m), 3.48–3.76 (11 H, m), 4,16 (1H, m, H-4′), 4.31 (1 H, d, J 7.8, H-1′), 5.38 (1 H, d, J 4.2, H-1); δ_C (75 MHz, CD_3OD) 12.72, 19.69, 28.77, 40.65, 52.84, 60.95, 61.19, 67.48, 68.50, 70.88, 70.92, 72.56, 75.17, 77.66, 79.12, 103.19, 169.83; m/z (ES) 430.37 [M + Na]$^+$; (HRMS found: [M + Na]$^+$ 430.1710. $C_{17}H_{29}O_{10}NNa$ requires 430.1689).

1,2,3,6-Tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-manno pyranose 9

Acetic anhydride (4 cm^3) was added dropwise to a stirring solution of known compound **8**[7a] (700 mg, 2.04 mmol) in anhydrous pyridine (6 cm^3) at 0 °C. The reaction mixture was brought to rt and stirred for 16 h. The mixture was poured over ice and the product crystallized to afford compound **9** (1.25 g, 90%); δ_H (300 MHz, $CDCl_3$) 1.8–2.2 (24 H, m, 8 × $COCH_3$), 3.86–3.99 (2H, m, H-6′), 3.93 (1 H, br dd, H-5′), 4.15 (1 H, m, H-5), 4.05–4.25 (2 H, m, H-6), 4.44 (1 H, dd, $J_{4,5}$ $J_{3,4}$ 9.6, H-4), 4.53 (1 H, d, $J_{1',2'}$ 7.8, H-1), 4.96 (1 H, dd, $J_{3',4'}$ 3.4, H-3′), 5.10 (1 H, m, H-2′), 5.40 (1 H, dd, H-2), 5.42 (1 H, d, $J_{3,2}$ 3, H-3), 5.47 (1 H, br d, H-4′), 6.00 (1 H, d, J 1.9, H-1); δ_C (75 MHz, $CDCl_3$) 20.38–20.77, 60.73, 62.10, 66.50, 68.33, 69.06, 69.08, 70.43, 70.82, 70.89, 73.83, 90.31, 101.17, 169.14–169.98 (multiple peaks); m/z (ES) 701.5 [M + Na$^+$]; (HRMS found: [M + Na]$^+$ 701.1985. $C_{28}H_{38}O_{19}Na$ requires 701.1905).

2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-manno pyranose 10

Compound **9** (600 mg, 0.89 mmol) was dissolved in anhydrous CH_3CN saturated with dimethylamine (40 cm^3) at −20 °C and stirred for 3 h, after which TLC confirmed the disappearance of the starting material. Excess dimethylamine was removed under

a reduced pressure, below 30 °C, and the reaction mixture was concentrated to provide the desired product **10** (517 mg, 92% yield); δ_H (300 MHz, $CDCl_3$) 1.8–2.2 (21 H, m, 7 × $COCH_3$), 3.80–3.90 (2H, m, H-6′), 3.91 (1 H, br dd, H-5′), 4.15 (1 H, m, H-5), 4.2 – 4.0 (2 H, m, H-6), 4.43 (1 H, dd, $J_{4,5}$ $J_{3,4}$ 9.69, H-4), 4.53 (1 H, d, $J_{1',2'}$ 7.9, H-1), 4.95 (1 H, dd, $J_{3',4'}$ 3.4, H-3′), 5.11 (1 H, m, H-2′), 5.22 (1 H, dd, $J_{1,2}$ 1.5, $J_{1,OH}$ 3, H-1), 5.38 (1 H, m, H-2), 5.42 (1 H, d, $J_{3,2}$ 3, H-3), 5.45 (1 H, br d, H-4′); *m/z* (ESMS) 636 [M$^+$]; (HRMS found: [M]$^+$ 636.1982. $C_{26}H_{36}O_{18}$ requires 636.1902).

2,3,6-Tri-*O*-acetyl-4-*O*-[2,3,4,6-tetra-*O*-acetyl–β-D-galactopyranosyl]-α-D-mannopyranosyl *H*-phosphonate 11

To a stirred solution of imidazole (1 g, 14.68 mmol) in anhydrous CH_3CN (20 cm^3) at 0 °C was added PCl_3 (0.8 cm^3, 9.14 mmol) and triethylamine (2.4 cm^3, 0.86 mmol). The mixture was stirred for 20 min and a solution of compound **10** (500 mg, 0.786 mmol) in anhydrous CH_3CN (20 cm^3) was added. The mixture was stirred at 0 °C for 2 h and quenched with 1 M TEAB (10 cm^3) and further stirred for 15 min, diluted with CH_2Cl_2, organic layer washed with ice cold water (2 × 10 cm^3) and cold 1 M TEAB buffer (2 × 10 cm^3), dried over Na_2SO_4 and concentrated to yield compound **11** (500 mg, 86%) as a triethylammonium salt. R_f = 0.35 in 20% CH_3OH in CH_2Cl_2; δ_H (300 MHz, $CDCl_3$) 1.90–2.08 (21 H, m, 7 × $OCOCH_3$), 3.96 (2 H, m, H-5′, H-5), 3.80–3.95 (2 H, m, H-6′), 4.24–4.13 (2 H, m, H-6), 4.47 (1 H, d, *J* 7.8, H-4), 4.54 (1 H, d, *J* 7.8, H-1′), 4.96 (1 H, dd, *J* 3.3 and 7.8, H-3′), 5.05 (1 H, dd, *J* 2.1 and 7.8, H-2′), 5.20–5.40 (2 H, m, H-4′, 3), 5.28 (1 H, dd, *J* 2.1 and 3.6, H-2), 6.95 (1 H, dd, J_{HP} 637, H-1); δ_P (100 MHz, $CDCl_3$) 0.129; *m/z* (ES) 699.2 [M – Et_3N–H]$^-$; (HRMS found: [M – Et_3N–H]$^-$ 699.1593. $C_{26}H_{36}O_{20}P$ requires 699.1538).

Stearyl-2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranosyl phosphate 12

A mixture of the above disaccharide *H*-phosphonate **11** (25 mg, 0.031 mmol) and stearyl alcohol (11 mg, 0.04 mmol) was dried by evaporation of pyridine (2 × 0.5 cm^3). The residue was dissolved in anhydrous pyridine (1 cm^3), adamantane carbonyl chloride (16 mg, 0.08 mmol) was added, and the mixture was stirred at rt for 1 h after which a freshly prepared solution of iodine (16 mg, 0.063 mmol) in 95% aq. pyridine (3 cm^3) was added. After 30 min CH_2Cl_2 was added, the solution washed with 1M $Na_2S_2O_3$ and 1M TEAB, dried (Na_2SO_4) and concentrated. The residue was purified by silica column chromatography (2.5% CH_3OH in CH_2Cl_2 with 1% Et_3N) to afford the coupled product **12** (29 mg, 85%); R_f = 0.46 in 20% CH_3OH in CH_2Cl_2; δ_H (300 MHz, $CDCl_3$) 0.84 (3 H, t, CH_3), 1.23–1.45 (34 H, m, lipid CH_2), 1.85–2.12 (21 H, m, 7 × $OCOCH_3$), 3.84–4.16 (6 H, m, H-5/5′, H-6/6′), 4.51 (1 H, d, *J* 7.8, H-1′), 4.85–5.01 (2 H, m, H-2′, 3′), 5.25 (3 H, m, H-4, 4′, 3), 5.52 (1 H, dd, *J* 2.1 and 3.6, H-2), 5.69 (1 H, dd, J_{HP} 6.8 and $J_{1,2}$ 1.9, H-1); δ_C (75 MHz, $CDCl_3$) 13.99, 20.48–20.77, 22.56, 27.8–29.59, 31.80, 36.44, 38.78, 52.82, 60.69, 68.99, 69.48, 70.23, 70.91, 76.52, 93.26, 100.93, 168.99–170.42 (multiple peaks); δ_P (100 MHz, $CDCl_3$) −2.90; *m/z* (ES) 967.5 [M – H]$^-$; (HRMS found: [M – H]$^-$ 967.4315. $C_{44}H_{72}O_{21}P$ requires 967.4304).

Stearyl-4-β-D-galactopyranosyl-α-D-mannopyranosyl-phosphate 13

To a solution of compound **12** (15 mg, 0.014 mmol) in anhydrous CH_3OH (2.5 cm^3) was added anhydrous Na_2CO_3 (16 mg, 0.15 mmol). The mixture was stirred at rt for 2 h, excess Na_2CO_3 removed by filtration and solvent evaporated to yield the target exogenous eMPT substrate **13** (9 mg, 93% yield); R_f = 0.55 in 10 : 10 : 3 CH_3OH–CH_2Cl_2–0.25% KCl; δ_H (300 MHz, D_2O) 0.76 (3H, t, CH_3), 1.16–1.83 (34 H, m, lipid CH_2), 3.22 (2H, m, CH_2), 3.41–3.53 (4 H, m, H-2, 5, 5′, 2′), 3.61–3.87 (12 H, m), 4.34 (1 H, br d, *J* 6.3, H-1′), 5.30 (1 H, dd, *J* 6.8 and 1.9, H-1); δ_H (75 MHz, D_2O) 13.87, 20.03, 29.47–30.06, 31.96, 36.24, 39.50, 60.42, 62.15, 66.28, 68.58, 69.87, 70.17, 70.66, 70.94, 71.28, 72.36, 75.26, 95.75, 104.69; δ_P (100 MHz, D_2O) −1.72; *m/z* (ES) 673.6 [M – H]$^-$; (HRMS found: [M – H]$^-$ 673.3594. $C_{30}H_{58}O_{14}P$ requires 673.3564).

Parasite culture and preparation of microsomal membranes

The promastigotes of *Leishmania donovani* (DD8 strain) were grown at 25 °C in Dulbecco's modified Eagle's medium supplemented with 0.05 mM adenosine, 0.05 mM xanthine, 1 mg L^{-1} biotin, 40 mg L^{-1} Tween-80, 5 mg L^{-1} hemin, 0.5% triethanolamine, 0.3% BSA, 50 mg L^{-1} gentamycin and 10% heat inactivated fetal bovine serum, with the pH maintained at 7.2. The promastigotes were harvested by centrifugation at 3000 g for 10 min at 20 °C, the pellet resuspended in ice cold phosphate buffered saline (20 mM, pH = 7.2) and centrifuged once again. The promastigotes were counted using a Neubauer chamber. For rupturing, the cell pellet (6.5 × 10^9 cells) was suspended in 5 mL of hypotonic buffer (0.1 mM TLCK and 1 μg ml^{-1} leupeptin) and sonicated on ice (6 × 10 s pulses with 3 s intervals), breaking assessed using a light microscope. The above suspension was centrifuged at 3000 g for 5 min to remove debris and the supernatant was centrifuged at 100 000 g for 1 h at 4 °C to obtain a membrane pellet, which was suspended in the buffer (50 mM HEPES–NaOH, pH = 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM TLCK, 1 μg mL^{-1} leupeptin, 1 mM ATP and 0.5 mM DTT). The concentration of the protein present in this solution was 13 mg mL^{-1}.

Elongating-MPT control and inhibition assay

For the control eMPT assay, each tube was prepared by adding 12.5 mm^3 of 1% Chaps, 28 mm^3 of 200 μM GDP-Man, 10 mm^3 of GDP-[^{3}H]Man (1μCi) and 17 μg (25 nmol) of synthetic substrate **13**. The contents were lyophilized and then 250 mm^3 of above membrane suspension (1.4 × 10^8 cell equivalent) was added to each tube. The tubes were incubated at 28 °C for 20 min, cooled to 0 °C and the membranes pelleted at 4 °C for 10 minutes by centrifugation at 3000 g. The supernatants (250 mm^3) were diluted with 500 mm^3 of 100 mM NH_4OAc buffer and loaded onto C18 Sep-Pak cartridges (preactivated by equilibrating and washing with 100 mM NH_4OAc). Each cartridge was washed with 5 cm^3 of 100 mM NH_4OAc followed by 1.5 cm^3 each of 5, 20, 40 and 60% *n*-propanol in 100 mM NH_4OAc. The desired [^{3}H] labelled PG products were eluted in 60% *n*-propanol fraction, the eluates concentrated and redissolved in 100 mm^3 of 60% *n*-propanol and the radioactivity measured by scintillation counting. The control assay was carried out in triplicate and the % incorporation was calculated for eMPT activity. The inhibition with synthetic **1** and **2** was carried using the above assay, in which the inhibitors were added in a concentration range from 0.05 to 1.0 mM, the experiments conducted in triplicate and averaged. The incorporation of [^{3}H]-Man was compared with the cpm present in control assay (incubation of reaction components with the substrate **13** but without **1** and **2**). The IC_{50} values were determined (GraphPad Prism 4.01) from response curves in which concentations ranged from 0.05 to 1 mM.

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