Galactose-derived phosphonate analogues as potential inhibitors of phosphatidylinositol biosynthesis in mycobacteria†

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Galactose-based phosphonate analogues of *myo*-inositol-1-phosphate and phosphatidylinositol have been synthesized from methyl β-D-galactopyranoside. Michaelis–Arbuzov reaction of isopropyl diphenyl phosphite or triisopropyl phosphite with a 6-iodo-3,4-isopropylidene galactoside afforded the corresponding phosphonates. Deprotection of the diphenyl phosphonate afforded methyl β-D-galactoside 6-phosphonate, an analogue of *myo*-inositol-1-phosphate. The diisopropyl esters of the diisopropyl phosphonate were selectively deprotected and the corresponding anion was coupled with 1,2-dipalmitoyl-*sn*-glycerol using dicyclohexylcarbodiimide. Deprotection afforded a methyl β-D-galactoside-derived analogue of phosphatidylinositol. The galactose-derived analogues of phosphatidylinositol and *myo*-inositol-1-phosphate were not substrates for mycobacterial mannosyltransferases (at concentrations up to 1 mM) involved in phosphatidylinositol mannoside biosynthesis in a cell-free extract of *Mycobacterium smegmatis*. The galactose-derived phosphonate analogue of phosphatidylinositol was shown to be an inhibitor at 0.01 mM of PimA mannosyltransferase involved in the synthesis of phosphatidylinositol mannoside from phosphatidylinositol, and a weaker inhibitor of the next mannosyltransferase(s), which catalyzes the mannosylation of phosphatidylinositol mannoside.

Introduction

myo-Inositol-1-phosphate (IP) and phosphatidylinositol (PI) are important metabolites in their own right and are elaborated to a variety of products that play crucial signaling and structural roles in biology.[1,2] In mycobacteria, a group of pathogenic bacteria that includes *Mycobacterium tuberculosis*, *myo*-inositol is a precursor for PI, which leads to the structurally complex phosphatidylinositol mannosides (PIMs) and lipoarabinomannan (LAM), and other related molecules, all of which share a common IP core (Fig. 1).[3–5] The diacylglycerol lipid anchor of the PIMs and LAM allows these conjugates to anchor into membranes and their biosynthesis is compartmentalized within the plasma membrane.[6] Both PIMs and LAM are potent immunogens and modulators of the interactions of the tubercle bacillus and the host immune system.[7] The early steps in the biosynthesis of PIMs and LAM have now been described in considerable detail.[7] In particular, one of the first steps in PIM and LAM biosynthesis, the transfer of a mannosyl group from GDP-mannose to the 2-position of PI to give PIM1 catalyzed by the mannosyltransferase PimA, has been shown to be essential for mycobacterial survival (Fig. 1).[8] Inhibition of this enzyme therefore could provide new anti-tuberculosis drugs.

The successful exploitation of PimA as a drug target will require the development of alternative substrates with which simpler assays for PimA can be established, and the identification of inhibitors as lead compounds for further development. We therefore became interested in the synthesis of a phosphonate analogue of PI. Phosphonates are well-established as surrogates for phosphate groups, yet are hydrolytically stable.[9,10] To this end we noted the surprising stereochemical congruence of D-galactose and *myo*-inositol (Fig. 1).‡ The 6-phosphonate derivative **1** of methyl β-D-galactoside is an analogue of IP, with the β-configuration of the glycoside ensuring that the stereochemistry of the anomeric centre matches that of the inositol. Elaboration of **1** to the corresponding diacylglycerol derivative **2** would give an analogue of PI. Both **1** and **2** could be evaluated as potential substrates and inhibitors of PIM biosynthesis.

Results and discussion

Our synthetic route to the galactose phosphonate **1** envisioned the introduction of a phosphonate moiety using either Michaelis–Arbuzov or Michaelis–Becker reactions of a protected galactoside equipped with a leaving group at C6. Substitution at C6 of galactose derivatives is notoriously difficult owing to steric obstruction of nucleophiles by the axial C4 substituent.[11] The poor reactivity of galactos-6-yl electrophiles can allow a variety of side reactions to intervene including: intramolecular cyclizations to 3,6-anhydro sugars, elimination reactions and, in the case

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† Electronic supplementary information (ESI) available: NMR spectra of **1–3**, **7**, **8**, **11–13**; HRMS of **2**. See DOI: 10.1039/b616450a

‡ Vasella and co-workers have applied the stereochemical similitude of D-galactose and *myo*-inositol to the synthesis of a phosphonate analogue of *myo*-inositol 1,4,5-triphosphate.[38]

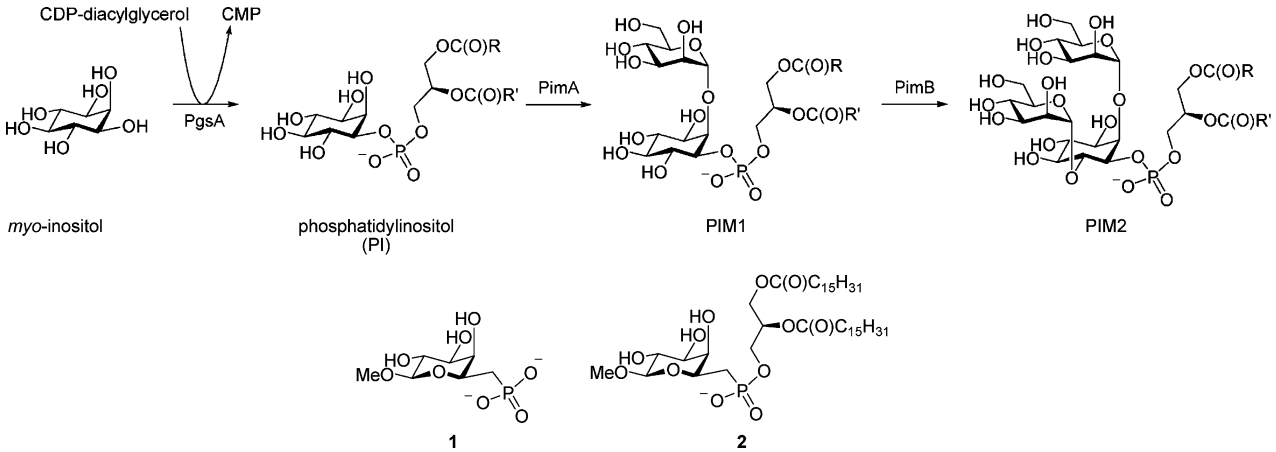
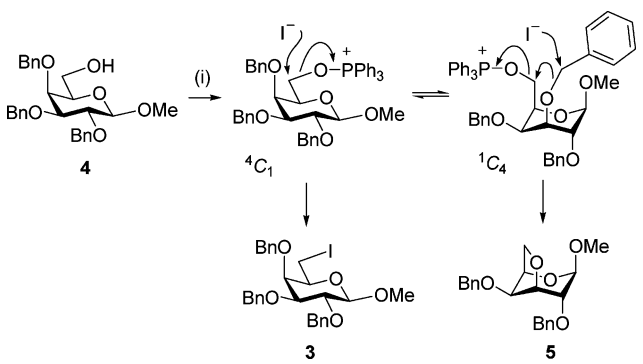


Fig. 1 Early steps in the biosynthesis of PIMs in mycobacteria. *myo*-Inositol is converted to phosphatidylinositol (PI), and then to phosphatidylinositol mannoside (PIM1) and PIM2 by the GDP-mannose dependent mannosyltransferases PimA and an as-yet undefined mannosyltransferase(s), respectively. R,R′ = various acyl groups derived from palmitic, stearic, tuberculostearic and 12-methoxypropanoyloxystearic acids. **1** and **2** are D-galactose-derived phosphonate analogues of *myo*-inositol-1-phosphate and PI, respectively.

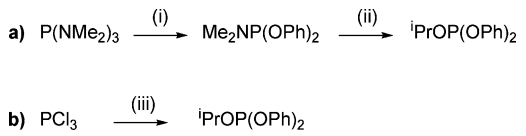
of the Michaelis–Arbuzov reaction, rearrangement of trialkyl phosphites to dialkyl alkylphosphonates. Indeed, preliminary studies aimed at performing a Michaelis–Becker reaction of a benzylated galactoside bearing an iodide or mesylate at C6, with the anion of dibenzyl phosphonate, were not encouraging. Palcic and co-workers have reported difficulties in the preparation of iodide **3** from **4**,[12] observing only cyclization to the 3,6-anhydro sugar **5**.[13] In our hands we found that treatment of the alcohol **4** with triphenylphosphine, iodine and imidazole in toluene at 90 °C afforded the iodide **3** in moderate yield (35%) (Scheme 1). Also isolated was the same 3,6-anhydro sugar **5**, arising from intramolecular attack by the benzyl ether at O3, by way of a ring inversion to the $^1C_4$ conformation encouraged by the establishment of a favourable anomeric effect in that conformer. Such an occurrence forecast difficulties in the Michaelis–Becker reaction. In the event, treatment of either the iodide **3** or methyl 2,3,4-tri-*O*-benzyl-6-methylsulfonyl-β-D-galactoside[13] with the sodium salt of dibenzyl phosphorous acid in THF at reflux or with dibenzyl phosphorous acid/caesium carbonate/Bu_4NI at 120 °C in DMF resulted in no reaction. Higher temperatures lead only to the 3,6-anhydro sugar **5**. With these dissatisfying results an alternative approach was planned that required careful choice of protecting groups to ensure sufficient reactivity of the sugar electrophile



Scheme 1 *Reagents and conditions*: (i) I_2, imidazole, triphenylphosphine, toluene, 90 °C, 35% of **3**, 57% of **5**.

in a phosphonate substitution reaction, whilst at the same time mitigating side reactions.

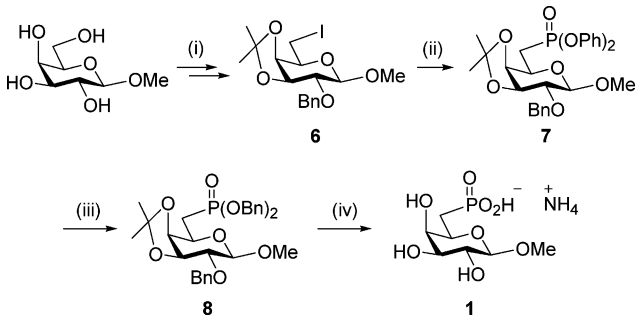
A 3,4-*O*-isopropylidene protecting group has been widely used in substitution reactions at the 6-position of galactose.[11,14] The cyclic acetal restricts ring inversion, preventing intramolecular cyclization, and distorts the conformation of the carbohydrate ring altering the position of the C4-axial substituent and thereby opens up C6 to nucleophilic attack. However, even with an 3,4-*O*-isopropylidene group in place, Golding and co-workers have reported that the Michaelis–Arbuzov reaction of 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene-α-D-galactose with trimethyl phosphite occurs in poor yield, owing to the undesirable rearrangement of this reagent to dimethyl methylphosphonate.[14] These authors have pioneered the use the modified Arbuzov reagents, methyl or isopropyl diphenyl phosphite, to afford improved yields of the diphenyl phosphonates. When methyl diphenyl phosphite was used, the reaction proceeded best under vacuum to remove the methyl iodide by-product, which is a reactive electrophile that can consume the phosphite reagent.[14] In seminal studies, Nesterov and Arbuzov have reported that isopropyl diphenyl phosphite is unreactive towards isopropyl iodide at 200 °C,[15] and Golding and co-workers have shown this phosphite is a superior reagent for the Michaelis–Arbuzov reaction with poorly electrophilic 6-iodo galactose derivatives.[14] While Golding and co-workers have reported the synthesis of isopropyl diphenyl phosphite from hexaethylphosphorous triamide,[14,16] we chose to use hexamethylphosphorous triamide (HMPT) as a more economical starting material. Thus, phenol and HMPT in dimethoxyethane at reflux afforded diphenyl *N*,*N*-dimethylphosphoramidite, which was treated with isopropanol and 1*H*-tetrazole in dimethoxyethane to afford isopropyl diphenyl phosphite (Scheme 2a). While this method was effective for the synthesis of moderate amounts of isopropyl diphenyl phosphite, for larger scale preparations a simpler route was desirable that avoided the need for large amounts of HMPT and 1*H*-tetrazole. A direct one-pot procedure was developed by treating phosphorus trichloride with triethylamine and isopropanol, and then phenol, to afford isopropyl diphenyl phosphite, isolated by distillation in



Scheme 2 *Reagents and conditions*: (i) phenol, 1,2-dimethoxyethane, reflux, 95%; (ii) isopropanol, 1*H*-tetrazole, 1,2-dimethoxyethane, 75%; (iii) PCl_3, isopropanol, Et_3N, Et_2O, then phenol, 30%.

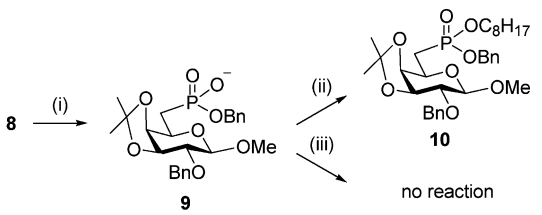
sufficient purity (Scheme 2b). The yield of our one-step process (30%) is lower than the two-step approach but, owing to its simplicity and the inexpensive nature of the reagents, is superior and can be easily scaled to 10–20 g quantities.

With ample supplies of isopropyl diphenyl phosphite in hand, we next turned to the Michaelis–Arbuzov reaction, with the immediate aim of preparing the IP analogue **1** (Scheme 3). The iodide **6** was synthesized from methyl β-D-galactopyranoside according to the literature.[17–19] Treatment of **6** with 5 equivalents of isopropyl diphenyl phosphite at 180 °C afforded the diphenyl phosphonate **7** in excellent yield (81%). Successful formation of the phosphonate was revealed by ^{31}P NMR, with a characteristic signal at δ_P 20.63 ppm. Deprotection of **7** to afford the analogue **1** was achieved in three steps. Base-catalyzed transesterification of the phenyl ester of **7** with benzyl alcohol, using the method of Billington and co-workers,[20] afforded the dibenzyl phosphonate **8**. Hydrolysis of the isopropylidene acetal of **8** with TFA–water, and then debenzylation by hydrogenolysis (Pd/C), afforded the galactose phosphonate IP analogue **1**, isolated as the ammonium salt after ion exchange chromatography (51%).



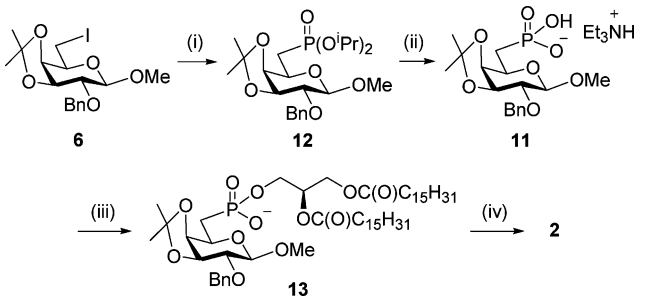
Scheme 3 *Reagents and conditions*: (i) see ref. 17–19; (ii) $^{i}PrOP(OPh)_2$, 180 °C, 81%; (iii) BnOH, NaH, THF, 74%; (iv) a) TFA–H_2O (9 : 1), b) Pd/C, THF, H_2O, 51%.

We next targeted the PI analogue **2**. Our initial synthetic plan considered a dehydrative coupling of a monobenzyl phosphonate such as **9** with 1,2-dipalmitoyl-*sn*-glycerol (Scheme 4). While dipalmitoyl PI has not been isolated from mycobacteria, authentic mycobacterial PIs and their derivatives are modified with long chain acyl groups including palmityl, stearyl, tuberculostearyl and 12-methoxypropanoyloxystearyl groups, suggesting that the PI-modifying enzyme PimA can tolerate unnatural combinations of long chain acyl groups. In addition, PimA can act on PI from alternative sources such as soy-bean.[21] Thus, the dibenzyl phosphonate **8** was monodebenzylated to afford **9** by prolonged treatment with 2-mercaptobenzothiazole[22] or, preferably, by treatment with DABCO at reflux in toluene.[23] While **9** could be condensed with simple alcohols such as 1-octanol under the agency of BOP or DCC to give **10**, even after extensive investigation we were unable



Scheme 4 *Reagents and conditions*: (i) 2-mercaptobenzothiazole, $Et^{i}Pr_2N$, DMF, 94%; (ii) octanol, DCC, pyridine; (iii) 1,2-dipalmitoyl-*sn*-glycerol, DCC, pyridine.

to effect efficient condensation with 1,2-dipalmitoyl-*sn*-glycerol using any of a range of coupling reagents (BOP,[24] PyBOP,[25] HATU, DCC, EDC, triisopropylbenzenesulfonyl chloride[26] or trichloroacetonitrile[27]). This troublesome road block therefore demanded an alternative approach. It was forecast that the phosphonate **11** would act as a better electrophile than **9** in condensations with the poorly nucleophilic 1,2-dipalmitoyl-*sn*-glycerol. However, the selective debenzylation of the phosphonate of **8** or, alternatively, the selective dephenylation of the diphenyl phosphonates of **8**, in the presence of a 2-*O*-benzyl ether, presented a particular challenge.[28] Therefore, we revisited our approach to **11** by way of the diisopropyl phosphonate **12**, anticipating the chemoselective cleavage of the isopropyl phosphonate esters using TMSBr (Scheme 5).[29–31] Treatment of the iodide **6** with triisopropyl phosphite at 180 °C cleanly afforded the diisopropyl phosphonate **12** in excellent yield (87%). Cleavage of the isopropyl groups of **12** in the presence of the isopropylidene moiety proceeded without incident by treatment of **12** with TMSBr and Et_3N, affording the anion **11**. Confirming our predictions, **11** proved a much better electrophile than the monoester **9**: treatment of **11** with 1,2-dipalmitoyl-*sn*-glycerol and DCC in pyridine gave the protected galactose phosphonate **13**. Finally, removal of the isopropylidene group (TFA–H_2O) and hydrogenolysis using $Pd(OH)_2$ proceeded without incident to afford the phosphonate **2** in 75% yield.



Scheme 5 *Reagents and conditions*: (i) triisopropyl phosphite, 180 °C, 87%; (ii) TMSBr, Et_3N, CH_2Cl_2, 92%; (iii) 1,2-dipalmitoyl-*sn*-glycerol, DCC, pyridine, 59%; (iv) a) TFA–H_2O (9 : 1), b) $Pd(OH)_2$, MeOH, EtOAc, 75%.

IP analogue **1** and PI analogue **2** were assessed for their ability to act as substrates and/or inhibitors in a *Mycobacterium smegmatis* cell-free system obtained by nitrogen cavitation of log phase cells. This cell-free system is capable of the synthesis of PIMs, which can be assessed using GDP-[^{3}H]mannose as a mannosyl donor, partitioning of lipophilic products into 1-butanol, and separation of radiolabelled products by HPTLC (Fig. 2).[6] The products seen are radiolabelled by [^{3}H]mannosyl-transfer in the cell-free system,

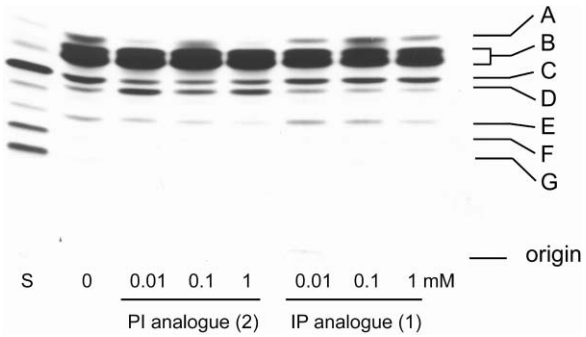


Fig. 2 HPTLC analysis of IP analogue **1** and PI analogue **2** as substrates/inhibitors of *M. smegmatis* mannosyltransferases. *M. smegmatis* cell lysates were incubated with GDP-[^{3}H]mannose in the absence of any analogue (lane 0) or the presence of different concentrations of either PI analogue **2** or IP analogue **1**. S = authentic PIM standards derived from *in vivo* [^{3}H]mannose labelling; A = Ac_2PIM1; B = PPM, Ac_2PIM2, AcPIM1; C = AcPIM2; D = PIM1; E = PIM2; F = AcPIM5, AcPIM5′; G = AcPIM6.[39] The abbreviations used are: Ac_xPIM_y, PIM species with x (1 or 2) fatty acyl chains, linked to either the core α-1,2-linked Man or the *myo*-inositol head group, and y Man residues; PPM, polyprenol (C_{35}/C_{50}) phosphomannose.

largely from an existing pool of the direct intermediates, rather than by *de novo* synthesis from PI. In the absence of exogenously added **1** or **2** (lane 0), a family of products are seen, which were assigned as various PIMs and PPM based on previous studies (see Fig. 2 legend).[6] At concentrations up to 1 mM neither **1** nor **2** acted as a substrate, with no additional products observed even at the highest concentration tested (1 mM).

The same analyses allowed assessment of the ability of **1** and **2** to inhibit the formation of each of the individual PIMs, as each labelled compound is formed largely from their direct precursors. Careful inspection of the resulting chromatogram reveals a clear reduction in the labelling of PIM1 (band D) and PIM2 (band E) in samples treated with IP analogue **1**. That this result does not arise from an indirect effect of the detergent-like nature of **2** was shown in control experiments where the detergent octyl α-D-mannoside was included in the cell free assay and resulted in no observable change in the appearance of the spectrum of products formed even at 1 mM (see ESI†). The IP analogue **1** thus appears to be an inhibitor of PimA, the first mannosyltransferase in the pathway of PIM biosynthesis (Fig. 1). The decrease in labelling of PIM2 could reflect reduced levels of *de novo*-synthesized PIM1, or inhibition of the second mannosyltransferase in the PIM biosynthesis pathway.[32,33] This transfer, which may be catalyzed by more than one mannosyltransferase, adds a mannosyl group to the 6-position of *myo*-inositol in PIM1 and must therefore recognize structural features of the IP core of PIM1, and the IP analogue **1**. In contrast, PI analogue **2** had less effect on PIM1 biosynthesis, while partially inhibiting PIM2 biosynthesis at concentrations up to 1 mM. Therefore it appears that PimA distinguishes between endogenous PI and the PI analogue **2**, whereas the second mannosyltransferase does not.

Conclusions

In summary, we describe the synthesis of galactose-derived phosphonate analogues of *myo*-inositol-1-phosphate and phosphatidylinositol. Notable steps include the introduction of the phosphonate moiety using modified Michaelis–Arbuzov reagents and a 6-iodo-3,4-isopropylidene-protected galactoside; the selective deprotection of a diisopropyl phosphonate in the presence of a 3,4-*O*-isopropylidene group; and the DCC-mediated condensation of the phosphonate **11** with 1,2-dipalmitoyl-*sn*-glycerol. The IP analogue **1** and the PI analogue **2** were investigated as substrates and inhibitors of PIM mannosyltransferases in a mycobacterial cell-free system. Neither compound acted as a substrate, demonstrating that these analogues are not effective bioisosteres for native PI, and that these structural modifications are too dramatic for recognition by PimA. The IP analogue **1** was shown to be an effective inhibitor of PimA, the mannosyltransferase that catalyzes the synthesis of PIM1 from PI, and a partial inhibitor of the mannosyltransferase that converts PIM1 to PIM2. The PI analogue **2** was shown to be a weak inhibitor of this second mannosyltransferase. Further analysis of the inhibition of both mannosyltransferases by **1** and **2** will require the development of improved assays for these two enzymes.

Experimental

Pyridine was distilled before use. Diethyl ether, 1,2-dimethoxyethane and THF were dried over sodium before use. Reactions were monitored using thin layer chromatography (TLC), performed with Merck Silica Gel 60 F_{254}. Detection was effected by charring in a mixture of 5% sulfuric acid in methanol, vanillin stain (6% vanillin, 1% H_2SO_4 in EtOH), 10% phosphomolybdic acid in EtOH, and/or visualising with UV light. Flash chromatography was performed according to the method of Still *et al.* using Merck Silica Gel 60.[34] Melting points were obtained using a Reichert–Jung hotstage microscope (corrected). Optical rotations were obtained using a JASCO DIP-1000 polarimeter (Melbourne, Australia). $[\alpha]_D$ values are given in 10^{-1} cm^2 g^{-1}. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded using Unity Plus 400 or Inova 400 or 500 instruments (Melbourne, Australia). For ^{31}P NMR spectra, 85% orthophosphoric acid (^{31}P NMR: δ 0.00 ppm) was used as a reference. Elemental analyses were conducted by C.M.A.S. (Belmont, Victoria). Refractive indices were obtained on a Bellingham & Stanley refractometer (Melbourne, Australia). Low resolution mass spectrometry was performed by Sioe See Volaric (Melbourne University, Australia). High resolution mass spectrometry was performed by Mr Hadi Loie on a Finnigan hybrid LTQ-FT mass spectrometer (Thermo Electron Corp.).

Methyl 2,3,4-tri-*O*-benzyl-6-deoxy-6-iodo-β-D-galactoside 3 and methyl 3,6-anhydro-2,4-di-*O*-benzyl-β-D-galactoside 5

A suspension of the alcohol **4**[12] (233 mg, 501 μmol), imidazole (93.8 mg, 1.38 mmol) and triphenylphosphine (214 mg, 817 μmol) in toluene (5 mL) was azeotropically dried (Dean–Stark). The reaction mixture was cooled to 90 °C, iodine (210 mg, 829 μmol) was added and the mixture was stirred under N_2. After 40 min, TLC indicated the formation of two compounds of lower polarity than the starting material. The reaction was quenched with MeOH (5 mL) and then diluted to 100 mL with toluene, washed with water (8 × 25 mL), $Na_2S_2O_3$ (0.25 M, 2 × 10 mL), dried ($MgSO_4$) and evaporated to yield a dark orange oil. The crude oil was purified by flash chromatography (10–20% EtOAc/pet. spirits) to yield first

the iodide **3** as an orange syrup (100 mg, 35%). Next to elute was the 3,6-anhydro derivative **5** as a yellow syrup (101 mg, 57%).

Data for **3**: mp 60.5–62.0 °C (colourless needles from petroleum spirit); $[\alpha]_D^{23}$ +3.46 (*c* 0.305, $CHCl_3$) (Found: C, 58.58; H, 5.38. $C_{28}H_{31}O_5I$ requires C, 58.55; H, 5.44); ^{1}H NMR (400 MHz; $CDCl_3$) δ 3.17 (1H, dd, $J_{5,6}$ 7.2, $J_{6,6}$ 9.8 Hz, H6), 3.29 (1H, dd, $J_{5,6}$ 6.8, $J_{6,6}$ 9.8 Hz, H6), 3.51 (1H, ddd, $J_{4,5}$ 0.8, $J_{5,6}$ 6.8, 7.2 Hz, H5), 3.52 (1H, dd, $J_{2,3}$ 9.6, $J_{3,4}$ 2.9 Hz, H3), 3.57 (3H, s, OCH_3), 3.80 (1H, dd, $J_{1,2}$ 7.7 Hz, H2), 4.01 (1H, dd, $J_{3,4}$ 2.9, $J_{4,5}$ 0.8 Hz, H4), 4.27 (1H, d, $J_{1,2}$ 7.7 Hz, H1), 4.75, 4.82, 4.91 (4H, 3 d, *J* 11.0, 11.8 Hz, 2 × PhCH_2), 4.67, 5.04 (2H, 2 d, *J* 11.3 Hz, PhCH_2), 7.23–7.42 (15H, m, Ph); ^{13}C NMR (100 MHz; $CDCl_3$) δ 3.08 (C6), 57.11 (OCH_3), 73.34, 73.86, 74.75, 75.02, 75.06, 79.04, 82.08 (C2,3,4,5, 3 × Ph*C*H_2), 104.67 (C1), 127.51–128.41, 138.15, 138.25, 138.61 (Ph).

Data obtained for **5** were in accordance with the literature.[35]

N,*N*′-Dimethyl diphenylphosphoramidite

Hexamethylphosphorous triamide (10.0 mL, 55.0 mmol) was added to a solution of phenol (10.4 g, 110 mmol) in dry 1,2-dimethoxyethane (20 mL). The mixture was refluxed under a N_2 atmosphere for 20 h, after which time TLC (1% $KMnO_4$ in H_2O) indicated conversion of the starting materials to a compound of lower polarity. The solvent was removed by rotary evaporation, and the residue was purified by distillation to afford *N*,*N*′-dimethyl diphenylphosphoramidite as a colourless liquid (13.8 g, 96%); n_D^{20} 1.5698 (lit.[36] n_D^{20} 1.5660); ^{1}H NMR (400 MHz; $CDCl_3$) δ 2.79 (6H, d, $J_{H,P}$ 9.6 Hz, 2 × CH_3), 7.05–7.35 (10H, m, Ph); ^{13}C NMR (100 MHz; $CDCl_3$) δ 34.83 (d, J_{CNP} 82 Hz, 2 × CH_3), 120.10 (d, $J_{C,P}$ 34 Hz, Ph), 120.74 (d, $J_{C,P}$ 27 Hz, Ph), 123.02, 124.25 (2s, Ph), 129.54, 129.70 (2s, Ph), 151.61 (d, $J_{C,P}$ 15 Hz, Ph), 153.69 (d, $J_{C,P}$ 27 Hz, Ph); ^{31}P (161.8 MHz; $CDCl_3$) δ 139.02 (lit.[37] 139.09 in C_6H_6).

Isopropyl diphenyl phosphite

Method A. 2-Propanol (1.88 mL, 24.6 mmol) was added dropwise to a solution of *N*,*N*′-dimethyl diphenylphosphoramidite (6.43 g, 24.6 mmol) and 1*H*-tetrazole (3.35 g, 47.9 mmol) in 1,2-dimethoxyethane (25 mL) at 0 °C. The mixture was stirred and allowed to warm to r.t. After 7 h TLC (vanillin stain) indicated conversion of the phosphoramidite to a material of lower polarity. The solvent was evaporated and the solid residue was suspended in a small amount of pet. spirits. The suspension was filtered through a short plug of silica, and was washed with pet. spirits (4 × 100 mL). Concentration of the eluant *in vacuo* afforded a pale yellow liquid. The residue was distilled to afford isopropyl diphenyl phosphite as a colourless liquid (5.13 g, 75%); n_D^{20} 1.5369 (lit.[15] n_D^{21} 1.5394); ^{1}H NMR (500 MHz; $CDCl_3$) δ 1.34 (6H, d, *J* 6.0 Hz, 2 × CH_3), 4.93 (1H, m, C*H*(CH_3)$_2$), 7.07–7.33 (10H, m, Ph); ^{13}C NMR (125 MHz; $CDCl_3$) δ 24.61 (d, $J_{C,P}$ 2.7 Hz, 2 × CH_3), 68.28 (d, $J_{C,P}$ 3.6 Hz, *C*H(CH_3)$_2$), 120.59, 120.67, 121.05, 121.12, 123.99, 124.64, 130.02, 130.09 (Ph), 152.79 (d, $J_{C,P}$ 7.4 Hz, Ph); ^{31}P (161.8 MHz; $CDCl_3$) δ 129.82.

Method B. Triethylamine (48.0 mL, 346 mmol) followed by isopropanol (4.50 mL, 58.8 mmol) in anhydrous ether (25 mL) was added dropwise to a chilled solution of phosphorus trichloride (5.00 mL, 57.3 mmol) in anhydrous ether (500 mL). The reaction mixture was stirred at room temperature for 1 h, then a solution of recrystallised phenol (10.8 g, 115 mmol) in anhydrous ether (50.0 mL) was added dropwise to the reaction mixture. The resultant mixture was stirred at room temperature overnight, then filtered (Celite) and concentrated to produce a yellow oil. The crude material was filtered through a plug of silica and eluted with petroleum spirits (5 × 100 mL). The eluant was concentrated and purified by vacuum distillation (120–126 °C at 5 Torr) to yield the phosphite as a colourless liquid (4.74 g, 30%).

Methyl 2-*O*-benzyl-6-deoxy-6-diphenoxyphosphinyl-3,4-*O*-isopropylidene-β-D-galactoside 7

The iodide **6**[17–19] (1.61 g, 3.71 mmol) and isopropyl diphenyl phosphite (5.13 g, 18.6 mmol) was heated at 180 °C for 4 days. Unreacted phosphite was recovered by diluting the crude reaction mixture with acetonitrile (50 mL) and washing with petroleum spirits (12 × 50 mL). The petroleum phase was back-extracted with MeCN (2 × 50 mL), and the combined petroleum extracts were concentrated *in vacuo*. The crude phosphite (3.69 g) was distilled for reuse. The MeCN extracts were evaporated to dryness to afford a colourless solid. The solid was purified by flash chromatography (20–60% EtOAc–pet. spirits) to afford the diphenyl phosphonate **7** as a colourless solid (1.62 g, 81%), mp 94–96 °C (EtOAc–pet. spirits); $[\alpha]_D^{25}$ 22.7 (*c* 0.950, $CHCl_3$) (Found: C, 64.37; H, 6.09. $C_{29}H_{33}O_8P$ requires C, 64.44; H, 6.15%); ^{1}H NMR (400 MHz; $CDCl_3$) δ 1.30, 1.38 (6H, 2 s, 2 × CH_3), 2.56 (1H, ddd, $J_{5,6}$ 5.6, $J_{6,6}$ 16.0, $J_{H,P}$ 19.0 Hz, H6), 2.70 (1H, ddd, $J_{5,6}$ 8.0, $J_{H,P}$ 18.0 Hz, H6), 3.37–3.45 (1H, m, H5), 3.47 (3H, s, OCH_3), 4.19–4.32 (3H, m,), 4.27 (1H, d, $J_{1,2}$ 8.0 Hz, H1), 4.79 (2H, ABq, CH_2Ph), 7.15–7.39 (15H, m, Ph); ^{13}C NMR (100 MHz; $CDCl_3$) δ 26.34, 27.72 (2 × C(*C*H_3)$_2$), 28.44 (d, $J_{C6,P}$ 144.2 Hz, C6), 56.88 (OCH_3), 68.04, 73.48, 75.28 (d, $J_{C,P}$ 10.6 Hz), 78.99, 79.28 (C2,3,4,5,*C*H_2Ph), 103.99 (C1), 109.96 (*C*(CH_3)$_2$$OCH_3$), 120.52–120.72 (2d, Ph), 125.26, 128.12, 128.24, 129.77, 129.84, 138.29, 150.25, 150.30, 150.33, 150.39 (Ph); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 20.63 (1P).

Methyl 2-*O*-benzyl-6-dibenzyloxyphosphinyl-6-deoxy-3,4-*O*-isopropylidene-β-D-galactoside 8

Benzyl alcohol (distilled and stored over 3 Å molecular sieves, 62.0 μL, 600 μmol) and sodium hydride (60% in oil, 25.2 mg, 630 μmol) were added to THF (2 mL) in a two-necked flask under N_2. This mixture was stirred until effervescence ceased, and then the diphenyl phosphonate **7** (108 mg, 199 μmol) was added *via* the second neck. After 5 min the reaction was quenched with sat. aq. NH_4Cl (2 mL) and the mixture was partitioned between water and dichloromethane. The aqueous phase was separated and re-extracted with dichloromethane (4 × 10 mL). The combined organic extracts were then washed with H_2O (2 × 25 mL) and sat. aq. NaCl (25 mL), dried ($MgSO_4$) and evaporated to afford a crude brown syrup. The crude material was purified by flash chromatography (40–80% EtOAc–pet. spirits) to yield the dibenzyl phosphonate **8** as yellow crystals (83.3 mg, 74%). Recrystallisation from EtOAc–pet. spirits afforded colourless needles, mp 73–74 °C; $[\alpha]_D^{25}$ 23.5 (*c* 0.495, $CHCl_3$) (Found: C, 65.51; H, 6.63. $C_{31}H_{37}O_8P$ requires C, 65.48; H, 6.56%); ^{1}H NMR (500 MHz; $CDCl_3$) δ 1.21, 1.32 (6H, 2 s, 2 × CH_3), 2.29–2.41 (2H, m, H6,6), 3.32 (1H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 7.0 Hz, H2), 3.44 (3H, s, OCH_3), 4.01–4.10 (3H, m,

H3,4,5), 4.14 (1H, d, $J_{1,2}$ 8.0 Hz, H1), 4.76 (2H, dd, CH_2Ph), 4.94–5.08 (4H, m, 2 × POCH_2Ph), 7.30–7.37 (10H, m, Ph); ^{13}C NMR (125 MHz; $CDCl_3$) δ 26.20, 27.72 (2 × C(CH_3)$_2$), 27.65 (C6), 56.83 (OCH_3), 67.20–67.50 (m, 2 × POCH_2Ph), 67.96 (C3), 73.45 (CH_2Ph), 75.14 (d, C5), 78.84, 79.19 (C2,4), 103.80 (C1), 109.73 (C(CH_3)$_2$), 127.54–128.61 (Ph), 136.08–136.22 (m, Ph), 138.23 (Ph); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 28.25.

Methyl 6-deoxy-6-dihydroxyphosphinyl-β-D-galactopyranoside, ammonium salt 1

A solution of dibenzyl phosphonate **8** (193 mg, 0.339 μmol) in trifluoroacetic acid–water (9 : 1, 1.0 mL) was stirred for 40 min. The solvent was evaporated by co-evaporation with toluene (4 × 2 mL) to afford a crude brown syrup. A suspension of the crude syrup, Pd/C (10%, 40 mg) in THF–water (2 : 1, 30 mL) was treated with H_2 for 18 h. The mixture was filtered (Celite), pyridine (87 μL, 1.06 mmol) was added to the filtrate, and the solution was evaporated to dryness to afford the pyridinium salt as pale yellow syrup. Cyclohexylamine (0.39 mL, 3.39 mmol) in toluene (5 mL) were added to the crude pyridinium salt and the mixture was evaporated to dryness. Excess cyclohexylamine was removed by co-evaporation with toluene (4 × 4 mL), yielding a colourless solid. The crude material was purified by flash chromatography (7 : 2 : 1 then 2 : 2 : 1 EtOAc–MeOH–H_2O), followed by size-exclusion chromatography (Bio-Gel P2, 250 mM NH_4HCO_3 buffer). The buffer was removed by lyophilisation to afford a colourless fluffy solid. This material was passed through an ion exchange column (Dowex 50WX8-400, NH_4^+ form) and freeze-dried to afford the ammonium salt of **1** as a colourless, hygroscopic powder (54.8 mg, 51%); $[\alpha]_D^{25}$ −11.4 (*c* 0.71, H_2O) (Found: C, 27.98; H, 6.72; N, 4.85. $C_7H_{18}NO_8P{\cdot}1.5H_2O$ requires C, 27.82; H, 7.00; N, 4.63%); ^{1}H NMR (400 MHz; D_2O) δ 1.89–2.04 (2H, m, H6,6), 3.44 (1H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 10 Hz, H2), 3.53 (3H, s, OCH_3), 3.65 (1H, dd, $J_{2,3}$ 10, $J_{3,4}$ 3.2 Hz, H3), 3.85 (1H, m, H5), 3.92 (1H, d, $J_{3,4}$ 3.2 Hz. H4), 4.29 (1H, d, $J_{1,2}$ 8.0 Hz, H1); ^{13}C NMR (100 MHz; D_2O) δ 23.91, 24.41, 29.13, 30.45, 48.97, 50.47, 57.18, 70.64, 71.54, 73.03, 103.67 (C1); ^{31}P NMR (161.8 MHz; D_2O) δ 22.03.

Methyl 2-*O*-benzyl-6-benzyloxyphosphinyl-6-deoxy-3,4-*O*-isopropylidene-β-D-galactoside, pyridinium salt 9

A solution of dibenzyl phosphonate **8** (267 mg, 470 μmol, 94 mM), 2-mercaptobenzothiazole (1.26 g, 7.36 mmol, final concentration 1.5 M) and ethyldiisopropylamine (1.3 mL, 7.6 mmol, 1.5 M) in DMF (5 mL) was stirred at 50 °C under N_2 for 19 h. The solvent was evaporated under reduced pressure, and the crude orange solid was purified by flash chromatography (100% EtOAc then 7 : 2 : 1 EtOAc–MeOH–H_2O) to afford a clear colourless glass (248 mg). Pyridine (2 mL) was added to the glass, and the excess solvent removed by rotary evaporation. The residue was dissolved in MeOH and passed through an ion exchange column (Dowex 50W X8-400, pyridinium form) to afford the pyridinium salt **9** as a clear yellow syrup (247 mg, 94%); ^{1}H NMR (500 MHz; $CDCl_3$) δ 1.23, 1.32 (2 × 3H, 2 × s, C(CH_3)), 2.27–2.42 (2H, m, H6,6), 3.37 (1H, dd, $J_{1,2}$ 7.0, $J_{2,3}$ 7.0 Hz, H2), 3.45 (3H, s, OCH_3), 4.08–4.16 (3H, m, H3,4,5), 4.19 (1H, m, $J_{1,2}$ 7.0 Hz, H1), 4.74–4.80 (2H, ABq, CH_2Ph), 7.22–7.40 (10H, m, Ph), 7.60–7.70, 8.08–8.18, 8.65–8.80 (3 × m, pyr); ^{13}C NMR (125 MHz; $CDCl_3$) δ 25.92, 27.49 (C(CH_3)$_2$), 28.57 (d, $J_{6,P}$ 138.1 Hz, C6), 56.32 (OCH_3), 66.02 (d, $J_{C,P}$ 2.0 Hz, POCH_2Ph), 68.33, 72.88, 78.54, 79.06 (C2,3,4,CH_2Ph), 75.02 (d, $J_{5,P}$ 6.8 Hz, C5), 103.32 (C1), 109.10 (C(CH_3)$_2$), 125.88, 127.11, 127.15, 127.51, 127.72, 127.81, 128.02, 137.10, 137.16, 137.99, 142.52, 143.19 (Ar); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 30.64.

Methyl 2-*O*-benzyl-6-deoxy-6-diisopropoxyphosphinyl-3,4-*O*-isopropylidene-β-D-galactoside 12

A mixture of the iodide **6** (603 mg, 1.39 mmol) and triisopropylphosphite (1.6 mL, 7.0 mmol) was stirred at 180 °C for 20 h, whereupon TLC indicated complete conversion of the iodide to a compound of higher polarity. The reaction mixture was cooled, and the crude material purified by flash chromatography (80–100% EtOAc–pet. spirits) to afford the phosphonate **12** as a yellow syrup (569 mg, 87%), $[\alpha]_D^{26}$ 25.5 (*c* 1.225, $CHCl_3$); ^{1}H NMR (400 MHz; $CDCl_3$) δ 1.29–1.33 (18H, m, 6 × CH_3), 2.16–2.34 (2H, m, H6,6), 3.34 (1H, dd, *J* 6.0, 8.0 Hz, H3), 3.53 (3H, s, OCH_3), 4.04–4.15 (3H, m, H2,3,5), 4.18 (1H, d, $J_{1,2}$ 8.0 Hz, H1), 4.66–4.74 (2H, m, 2 × CH(CH_3)$_2$), 4.77 (2H, ABq, CH_2Ph), 7.20–7.37 (5H, m, Ph); ^{13}C NMR (100 MHz; $CDCl_3$) δ 24.23, 24.26, 24.31 (CH(CH_3)$_2$), 26.60, 27.98 (CH_3), 29.65 (d, $J_{C6,P}$ 143.5 Hz, C6), 57.04 (OCH_3), 70.46, 70.66 (2d, $J_{C,P}$ 6.9 Hz), 68.53, 73.64, 75.41 (d, $J_{C,P}$ 6.6 Hz), 79.14, 79.53 (C2,3,4,5, 2 × CH(CH_3)$_2$,CH_2Ph), 104.07 (C1), 109.88 (CMe$_2$), 127.71, 128.31, 128.40, 138.51 (Ph); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 25.01; HRMS (ESI$^+$, *m/z*) calc. for $C_{23}H_{37}NaO_8P$ $[M + Na]^+$ 495.2118, found 495.2114.

Methyl 2-*O*-benzyl-6-deoxy-6-dihydroxyphosphinyl-3,4-*O*-isopropylidene-β-D-galactoside, triethylammonium salt 11

TMSBr (280 μL, 2.16 mmol) was added to a solution of the diisopropyl phosphonate **12** (254 mg, 0.538 mmol) and triethylamine (1.00 mL, 7.17 mmol) in dry dichloromethane (9 mL) at 0 °C and the resultant mixture was stirred for 21 h. Additional TMSBr (280 μL, 2.16 mmol) was added and stirring was continued for a further 6 h. The reaction mixture was then concentrated *in vacuo.* The residue was purified by flash chromatography (7 : 2 : 1 then 5 : 2 : 1 EtOAc–MeOH–H_2O) to give the salt **11** as a yellow oil (263 mg, 92%), $[\alpha]_D^{23}$ +22.6 (*c* 1.20, $CHCl_3$); ^{1}H NMR (400 MHz; D_2O) δ 1.07 (9H, t, *J* 7.2 Hz, CH_3CH$_2$N), 1.15, 1.17 (2 × 3H, 2s, C(CH_3)$_2$), 1.84 (1H, dd, $J_{5,6}$ 5.6, $J_{6,P}$ 5.6 Hz, H6), 1.89 (1H, dd $J_{5,6}$ 5.6, $J_{6,P}$ 5.6 Hz, H6), 2.98 (6H, q, *J* 7.2 Hz, CH_3CH_2N), 3.17 (1H, dd, $J_{1,2}$ 8.2, $J_{2,3}$ 8.2 Hz, H2), 3.37 (3H, s, OCH_3), 3.95–4.01 (1H, m, H5), 4.07 (1H, dd, $J_{2,3}$ 6.0, $J_{3,4}$ 6.0 Hz, H3), 4.17 (1H, d, $J_{3,4}$ 5.2 Hz, H4), 4.22 (1H, d, $J_{1,2}$ 8.4 Hz, H1), 4.59 (2H, s, CH_2Ph), 7.18–7.26 (5H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.91 (CH$_3$CH$_2$N), 25.94, 27.58 (2C, C(CH_3)$_2$), 30.65 (d, $J_{C6,P}$ 134 Hz, C6), 47.30 (CH_3CH$_2$N)), 57.54 (OMe), 69.74, 73.91, 78.91, 79.62 (C2,3,4, CH$_2$Ph), 76.62 (d, $J_{C5,P}$ 7.3 Hz, C5), 103.39 (C1), 111.11 (C(CH_3)$_2$), 129.07, 129.33, 129.60, 137.60 (4C, Ph); ^{31}P NMR (161.8 MHz, $CDCl_3$) δ 25.46 (1P); HRMS (ESI$^-$, *m/z*) calcd for $C_{17}H_{25}O_8P$ $[M - H]^-$ 387.1203, found 387.1201.

Methyl 2-*O*-benzyl-6-deoxy-6-[(2*R*)-2,3-dipalmitoyloxypropyloxy]hydroxyphosphinyl-3,4-*O*-isopropylidene-β-D-galactoside 13

The phosphonate salt **11** (192 mg, 392 μmol) was dried by co-evaporation with pyridine (3 × 5 mL). Dipalmitoyl-*sn*-glycerol

(223 mg, 392 μmol) was then added and the reaction mixture dried again by co-evaporation with pyridine (2 × 5 mL). The mixture was dissolved in pyridine (8 mL), treated with DCC (809 mg, 3.92 mmol), and heated to 40 °C for 24 h. The reaction mixture was then quenched with H_2O (0.5 mL) and extracted with chloroform (5 × 30 mL). The combined organic extract was dried ($MgSO_4$) and concentrated *in vacuo*. The resulting residue was purified by flash chromatography (100% EtOAc then 17 : 2 : 1 EtOAc–MeOH–H_2O) to give the protected PI analogue **13** as a colourless solid (215 mg, 59%), mp 77–78 °C; $[\alpha]_D^{23}$ +22.2 (*c* 0.62, $CHCl_3$) (Found: C, 62.42; H, 9.19. $C_{52}H_{90}KO_{12}P.H_2O$ requires C, 62.75; H, 9.32%); ^{1}H NMR (400 MHz; D_2O) δ 0.87 (6H, m, CH_3 of palmitoyl), 1.18–1.34 (54H, m, palmitoyl sidechain, $C(CH_3)_2$), 1.55 (4H, m, CH_2), 1.98–2.30 (6H, m, $CH_2C{=}O$, H6,6), 3.32 (1H, m, H2), 3.52 (3H, s, OCH_3), 4.00–4.39 (8H, m, H1,3,4,5, 2 × CH_2 of glycerol), 4.75 (2H, s, CH_2Ph), 5.22 (1H, br s, CH of glycerol), 7.20–7.40 (5H, m, Ph); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ 14.1, 22.67, 24.84, 24.91, 26.34, 27.82, 29.18, 29.21, 29.36, 29.41, 29.58, 29.60, 29.67, 29.72, 31.91, 34.16 (d, $J_{C6,P}$ 17.7 Hz, C6), 57.15 (OMe), 62.78, 69.15, 70.60, 73.29 (C2,3,4,*C*H_2Ph), 79.31 (d, $J_{C5,P}$ 71.4 Hz, C5), 103.72 (C1), 109.51 (*C*$(CH_3)_2$), 127.42, 127.92, 128.14, 138.35 (Ph); 173.39, 173.67 (C=O); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 19.38 (P); HRMS (ESI$^-$, *m*/*z*) calcd for $C_{52}H_{91}O_{12}P$ $[M - H]^-$ 937.6164, found 937.6171.

Methyl 6-deoxy-6-[(2*R*)-2,3-dipalmitoyloxypropyloxy]-hydroxyphosphinyl-β-D-galactopyranoside 2

The protected PI analogue **13** (37 mg, 39 μmol) was treated with TFA–H_2O (9 : 1, 1.0 mL) for 30 min then concentrated *in vacuo* with co-evaporation with toluene to remove residual solvent. The residue was dissolved in methanol–ethyl acetate (1 : 1, 8 mL), treated with 10% $Pd(OH)_2$ on carbon (40 mg) and shaken under H_2 at 350 kPa for 24 h. The residue was filtered through Celite, concentrated *in vacuo* and purified by flash chromatography (17 : 2 : 1 EtOAc–MeOH–H_2O) to give the PI analogue **2** as a yellow oil (24 mg, 75%); ^{1}H NMR (400 MHz; D_2O) δ 0.88 (6H, m, CH_3 of palmitoyl), 1.25 (48H, m, palmitoyl sidechain), 1.58 (m, CH_2), 1.95–2.40 (6H, m, $CH_2C{=}O$, H6), 3.50 (3H, s, OCH_3), 3.55–4.42 (9H, m, H1,2,3,4,5, 2 × CH_2 of glycerol), 5.23 (1H, bs, CH of glycerol); ^{31}P NMR (161.8 MHz; $CDCl_3$) δ 21.83 (P); HRMS (ESI$^-$, *m*/*z*) calc. for $C_{42}H_{80}O_{12}P$ $[M–H]^-$ 807.5382, found 807.5374.

Biochemical analysis using *M. smegmatis* cell-free system

M. smegmatis mc^{2}155 was grown to mid-exponential phase at 37 °C in 7H9 broth (Difco, Detroit, MI, USA), supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol and 15 mM NaCl. Bacteria were harvested by centrifugation in mid-exponential growth and washed twice with 50 mM Hepes/NaOH (pH 7.4). Pellets were resuspended at 0.2 g of wet pellet per mL using a lysis buffer containing 25 mM Hepes/NaOH (pH 7.4), 25% (w/v) sucrose, 2 mM EGTA and a cocktail of protease inhibitors (Roche). The cells were subjected to three rounds of nitrogen cavitation (Kontes, Vineland, NJ, USA) at 15 MPa with a 30 min equilibration period before the release of pressure. Unlysed cells were removed by centrifugation (2500 *g*, 10 min) and the supernatant was stored at −80 °C. The lysates were supplemented with 5 mM $MgCl_2$ before incubation at 37 °C and addition of analogue **1** or **2** followed by GDP-[^{3}H]mannose (6.3 Ci mmol^{-1}, final concentration 9 mCi mL^{-1}) to initiate labelling. The labelling reaction was terminated at the indicated time points by adding chloroform–methanol (1 : 1, v/v, 6.7 vol.). After sonication, the insoluble material was removed by centrifugation (15 000 *g*, 2 min) and the supernatant was dried under N_2. Labelled lipids were recovered by biphasic partitioning between water and 1-butanol (1 : 2, v/v) and analysed by HPTLC developed in chloroform–methanol–1 M ammonium acetate–13 M ammonia–water (180 : 140 : 9 : 9 : 23 v/v). Radiolabelled lipids were detected by treating the HPTLC sheets with En^{3}Hance (NEN Life Science Products, Boston, MA, USA) and exposing them to a BioMax MR film (Kodak, Rochester, NY, USA) at −80 °C.

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