JOC Article

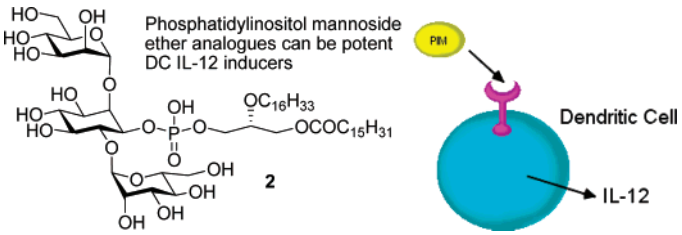
Phosphatidylinositol Mannoside Ether Analogues: Syntheses and Interleukin-12-Inducing Properties

Gary D. Ainge,[†,§] Natalie A. Parlane,[‡] Michel Denis,[‡] Blake S. Dyer,[§] Andrea Härer,[†] Colin M. Hayman,[†] David S. Larsen,[§] and Gavin F. Painter*[,†]

Carbohydrate Chemistry Team, Industrial Research Limited, P.O. Box 31-310, Lower Hutt, New Zealand, AgResearch, Hopkirk Research Institute, Private Bag 11 222, Palmerston North, New Zealand, and University of Otago, P.O. Box 56, Dunedin, New Zealand

g.painter@irl.cri.nz

Received April 5, 2007



Phosphatidylinositol mannosides (PIMs) isolated from mycobacteria have been identified as an important class of glycolipids with significant immune modulating properties. We present here the syntheses of phosphatidylinositol dimannoside ether analogues **2** and **3** and evaluate their interleukin-12 (IL-12)-inducing properties along with dipalmitoyl PIM2 (**1**) in an *in vitro* bovine dendritic cell assay. Both synthetic PIM analogues and synthetic dipalmitoyl PIM2 (**1**) were effective at enhancing IL-12 production by immature bovine dendritic cells. Unexpectedly, ether analogue **2** was significantly more active than dipalmitoyl PIM2 (**1**) which indicates that modified PIM compounds can be strongly immunoactive and may have significant adjuvant activities.

Introduction

Mycobacteria and their products have strong inherent immunomodulatory properties.[1] The bulk of the stimulatory activity resides in the cell walls of mycobacteria, with various products such as lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs), and a plethora of other mycobacterial compounds being able to modulate the immune response.[2] Consequently, mycobacterial products have been exploited to produce formulations to modulate the immune response.[3]

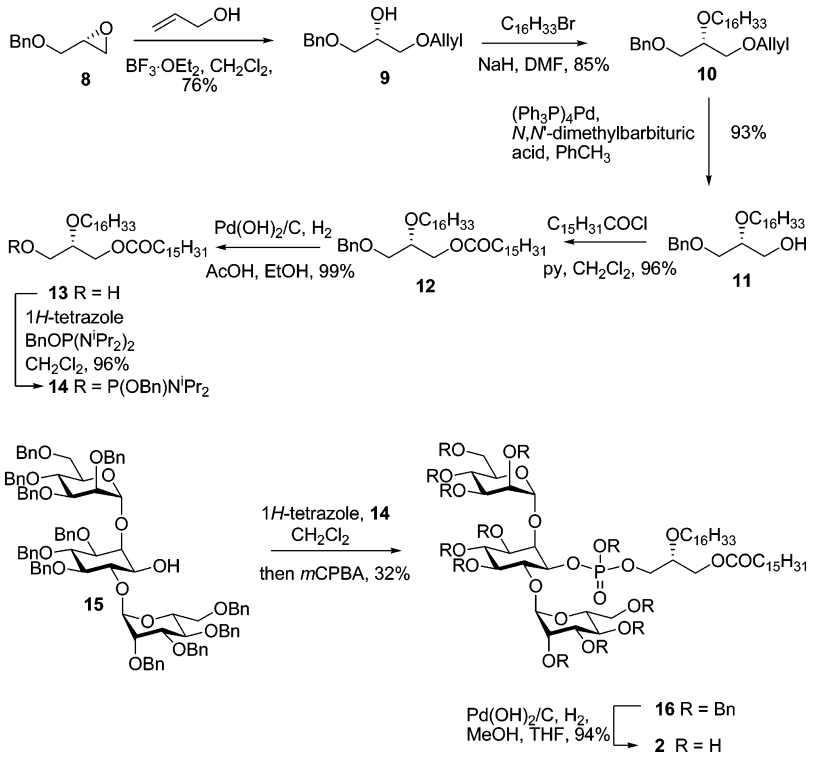
Adjuvants stimulate the immune system to respond to an antigen or a set of antigens and are used extensively to enhance the efficacy of vaccines. Numerous natural and synthetic products act as adjuvants; their structures, the cell types on which they act, and their modes of action cover wide spectra.[4] Adjuvants are particularly important in the case of vaccines that consist of chemically defined components, such as subunit vaccines, which have significant advantages over live attenuated vaccines in terms of their reproducibility, safety, and concomitant ease of registration.[5] However, discrete antigens often have poor immunogenicity compared to live attenuated vaccines, and this is a major drawback to their use. In order to elicit a strong and robust cell-mediated immune response, the coadministration of an adjuvant is required,[6] and there is at present a scarcity of effective adjuvants which are safe and reliable. A canonical adjuvant based on mycobacterial products is Complete Freund's Adjuvant (CFA), which is a formulation of heat-killed mycobacteria in an oil emulsion[7] that has been used extensively for

* Author to whom correspondence should be addressed. Phone: 64 4 931 3103. Fax: 64 4 931 3055.
† Industrial Research Limited.
‡ AgResearch.
§ University of Otago.

(1) Goren, M. B. *Am. Rev. Respir. Dis.* **1982**, *125*, 50−69.
(2) Briken, V.; Porcelli, S. A.; Besra, G. S.; Kremer, L. *Mol. Microbiol.* **2004**, *53*, 391−403.
(3) Ivanyi, J.; Sharp, K.; Jackett, P.; Bothamley, G. *Springer Semin. Immunopathol.* **1988**, *10*, 279−300.
(4) Petrovsky, N.; Aguilar, J. C. *Immunol. Cell Biol.* **2004**, *82*, 488−96.
(5) Francis, J. N.; Larche, M. *Curr. Opin. Allergy Clin. Immunol.* **2005**, *5*, 537−43.
(6) Clark, T. G.; Cassidy-Hanley, D. *Dev. Biol. (Basel)* **2005**, *121*, 153−63.
(7) Claassen, E.; de Leeuw, W.; de Greeve, P.; Hendriksen, C.; Boersma, W. *Res. Immunol.* **1992**, *143*, 478−83.

10.1021/jo070639m CCC: $37.00
Published on Web 06/09/2007

SCHEME 1. Synthesis of Monoether 2

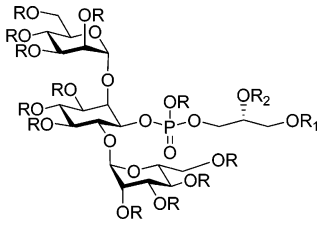


experimental purposes for many decades. CFA, however, has toxic side effects which preclude its use in humans, and there is an urgent need for the development of novel immune modulators that are safe, potent, and based on chemically defined entities.

A major challenge in this field is therefore to isolate or synthesize compounds that have adjuvant activities (i.e., stimulate a pro-inflammatory response), with limited side effects,[4] and are chemically well defined. Phosphatidylinositol mannosides (PIMs) are glycolipids, embedded in the cell walls of mycobacteria, that anchor an array of more complex glycolipids to the cell membrane.[8] Native PIMs generally occur with two (PIM2) or six mannose (PIM6) residues attached, singly or as oligosaccharides, to their inositol component. We and others have demonstrated that the smaller natural PIM compounds, by themselves, retain immunomodulatory activities[9−12] and that synthetic PIM samples also retain these activities.[13,14] In particular, they stimulate cytokine production via key cells of the immune system and generate an inflammatory response.[10]

The precise acylation state of the PIM molecule appears crucial in the type and magnitude of the observed bioactivity,[10] and we have already demonstrated[14] that synthetic PIM2 (**1**) is sensitive to degradation by deacylation. In particular, hydrogenolytic debenzylation of the perbenzylated compound **6** using solvent mixtures that contained some water led to a small amount of the monodeacylated product **7**. The chemical instability of the *sn*-2 acyl linkage of phospholipids is known; indeed, specific loss of this group is used as a means of structural characterization by mass spectral techniques.[15] Inasmuch as acyl ester groups are also subject to enzymatic removal, we postulated that PIM analogues containing more stable linkages may be better immune modulators than natural PIMs.

We present here the synthesis of the PIM ether analogues **2** and **3** and assess their ability to induce bovine dendritic cells (DC) to secrete interleukin-12 (IL-12).[16]



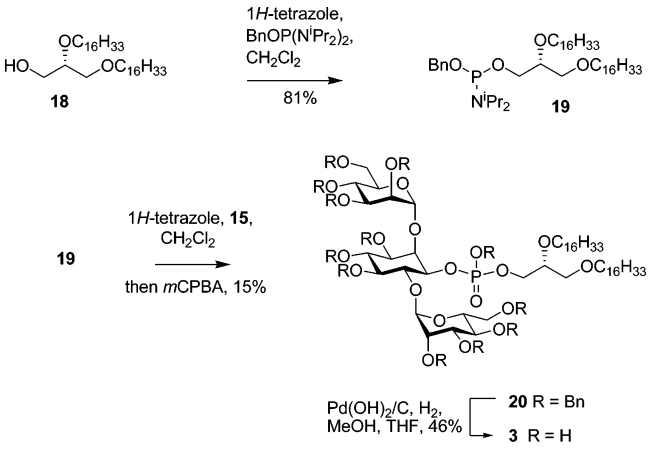
1 R = H, $R_1 = R_2 = COC_{15}H_{31}$
2 R = H, $R_2 = C_{16}H_{33}$, $R_1 = COC_{15}H_{31}$
3 R = H, $R_1 = R_2 = C_{16}H_{33}$
6 R = Bn, $R_1 = R_2 = COC_{15}H_{31}$
7 R = R_2 = H, $R_1 = CO_{15}H_{31}$

Discussion

The phosphoramidite **14** was prepared from (*R*)-benzyl glycidol (**8**) which was obtained by hydrokinetic resolution of the racemate using the methodology of Jacobsen et al. (Scheme 1).[17] The ee of **8** was estimated as greater than 95% from the

(8) Stadelmaier, A.; Schmidt, R. R. *Carbohydr. Res.* **2003**, *338*, 2557−69.
(9) Sprott, G. D.; Dicaire, C. J.; Gurnani, K.; Sad, S.; Krishnan, L. *Infect. Immun.* **2004**, *72*, 5235−46.
(10) Gilleron, M.; Quesniaux, V. F.; Puzo, G. *J. Biol. Chem.* **2003**, *278*, 29880−9.
(11) Torrelles, J. B.; Azad, A. K.; Schlesinger, L. S. *J. Immunol.* **2006**, *177*, 1805−16.
(12) Sayers, I.; Severn, W.; Scanga, C. B.; Hudson, J.; le Gros, G.; Harper, J. L. *J. Allergy Clin. Immunol.* **2004**, *114*, 302−309.
(13) Ainge, G. D.; Hudson, J.; Larsen, D. S.; Painter, G. F.; Gill, G. S.; Harper, J. L. *Bioorg. Med. Chem.* **2006**, *14*, 5632−42.
(14) Ainge, G. D.; Parlane, N. A.; Denis, M.; Hayman, C. M.; Larsen, D. S.; Painter, G. F. *Bioorg. Med. Chem.* **2006**, *14*, 7615−7624.
(15) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 986−99.
(16) Boyaka, P. N.; Lillard, J. W., Jr.; McGhee, J. *Immunol. Res.* **1999**, *20*, 207−17.

SCHEME 2. Synthesis of Diether 3



^{1}H and ^{19}F NMR spectra of a corresponding Mosher ester derivative.[18] Here, Lewis acid-promoted opening of epoxide **8** with allyl alcohol provided allyl ether **9** which had an optical rotation consistent with that reported by Mikkilineni et al.[19] The *sn*-2 hydroxyl group in compound **9** was alkylated with 1-bromohexadecane without incident, affording ether **10** in good yield. Removal of the allyl group was effected by treatment with a catalytic amount of tetrakis(triphenylphosphine)palladium-(0) and *N*,*N*′-dimethylbarbituric acid[20] to give alcohol **11** in excellent yield. The specific optical rotation was equal, but opposite in sign, to that reported for the enantiomer by Duclos.[21] Esterification of compound **11** with palmitoyl chloride afforded monoester **12** in good yield. The benzyl ether group was removed with $Pd(OH)_2$/C in the presence of acid to give alcohol **13,** and the phosphoramidite **14** was prepared from it by use of benzyloxy-bis(diisopropylamino)phosphine activated by 1*H*-tetrazole. Coupling of compound **14** with the pseudo-trisaccharide **15**[14,22] gave the fully substituted **16**, and deprotection afforded the monoether **2** in good yield. The ^{1}H NMR spectrum showed all the expected signals in the appropriate ratios. In particular, the two anomeric protons were evident as broad singlets at δ 5.14 and 5.11, and the *sn*-2 proton now resonated at higher field as expected (for PIM2 **1**, the *sn*-2 proton appears at δ 5.3). The ^{31}P NMR displayed a single peak at δ −3.5.

The phosphoramidite **19** was prepared from alcohol **18**[23,24] (Scheme 2) by phosphitylation with benzyloxy-bis(diisopropylamino)phosphine. Coupling with the pseudo-trisaccharide **15** afforded the benzyl-protected compound **20** from which the required diether **3** was obtained after deprotection. The ^{1}H NMR spectrum showed all the expected signals in the appropriate ratios. In particular, the two anomeric protons were evident at δ 5.16 and 5.11, and as expected the *sn*-2 proton was absent from this region.

The Th1-inducing activities of PIM compounds **1**, **2**, and **3** were tested in an *in vitro* bovine dendritic cell (DC) assay, and the results were compared with responses induced by lipopolysaccharide (LPS), a known potent IL-12 inducer (Figure 1). Products which direct the immune responses toward a Th1 profile are IL-12 inducers, and specialized antigen presenting cells such as DCs are excellent bioprobes for the detection of factors likely to direct an immune response toward a Th1 profile, via their high potential for IL-12 release.

Interestingly, the three synthetic compounds **1**, **2**, and **3** all induced significant levels of IL-12 from bovine DCs, suggesting that these compounds are good adjuvant candidates and, in particular, have potential for stimulating a Th1 cell-mediated immune response, which is important for protection against intracellular pathogens. The diether **3** and dipalmitoyl PIM2 (**1**) induced IL-12 production to a similar level, indicating that the acyl linkages in PIM2 are not required for the observed activity. On the other hand, the mono-ether **2** was significantly more active than either the diacyl **1** or diether **3** compounds. This would indicate that the acyl linkage attached at the *sn*-1 carbon center together with a stable linkage at the *sn*-2 carbon center are both important for inducing higher levels of IL-12.

The lipid antigen-presenting molecule CD1d has been implicated in the observed activity of PIM compounds *in vivo*,[25] and the crystal structure of a PIM2−CD1d complex has been resolved.[26] However, it has recently been discovered[27] that the bovine CD1 family contains group 1 CD1 proteins but no functional CD1d, suggesting that cattle have no functional NKT cells. In addition, synthetic dipalmitoyl PIM2 (**1**) does not stimulate mouse NKT cells in the same way, if at all, as do lipid products such as α-galactosylceramide.[28] Therefore, receptors of the innate immune system, such as Toll receptors[10] or perhaps the group 1 CD1 proteins (CD1a, CD1b, and CD1c), may be more relevant here, when purified DCs are used as a readout system. It is emphasized that PIMs have been reported to have multiple effects on a variety of cell types, including binding to integrins present on CD 4 + T cells, which is likely to activate these cells.[29] It therefore is unclear which mechanism-(s) of action is responsible for the complete immunomodulatory actions of PIMs *in vivo*, and the possibility that multiple mechanisms are implicated (binding to integrins on T cells, engagement to TLR receptors on antigen-presenting cells, activation of NKT cells) remains a possibility.

We have prepared synthetic samples of PIM2 ether analogues **2** and **3** and together with dipalmitoyl PIM2 (**1**) evaluated their relative abilities to induce IL-12 in bovine DCs as a model for Th1-inducing activity. The three synthetic compounds are good IL-12 inducers with mono-ether **2** being significantly more potent than either the diacylated (**1**) or diether (**3**) counterparts. Hence, non-natural PIMs can be at least as active as natural PIMs at inducing IL-12 from DCs *in vitro*, and may identify a

(17) Schaus, S. E.; Brandes, B. D.; Larrow, J. F.; Tokunaga, M.; Hansen, K. B.; Gould, A. E.; Furrow, M. E.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2002**, *124*, 1307−1315.
(18) Dyer, B. S.; Jones, J. D.; Ainge, G. D.; Denis, M.; Larsen, D. S.; Painter, G. F. *J. Org. Chem.* **2007**, *72*, 3282−3288.
(19) Mikkilineni, A. B.; Kumar, P.; Abushanab, E. *J. Org. Chem.* **1988**, *53*, 6005−6009.
(20) Tsukamoto, H.; Kondo, Y. *Synlett* **2003**, *7*, 1061−1063.
(21) Duclos, R., Jr. I. *Chem. Phys. Lipids* **1993**, *66*, 161−170.
(22) Elie, C. J. J.; Verduyn, R.; Dreef, C. E.; Van der Marel, G. A.; Van Boom, J. H. *J. Carbohydr. Chem.* **1992**, *11*, 715−39.
(23) Bhattacharya, S.; De, S. *Chem. Eur. J.* **1999**, *5*, 2335−2347.
(24) Browne, J. E.; Freeman, R. T.; Russel, J. C.; Sammes, P. G. *J. Chem. Soc., Perkin Trans. 1* **2000**, 645−652.
(25) Fischer, K.; Scotet, E.; Niemeyer, M.; Koebernick, H.; Zerrahn, J.; Maillet, S.; Hurwitz, R.; Kursar, M.; Bonneville, M.; Kaufmann, S. H.; Schaible, U. E. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10685−10690.
(26) Zajonc, D. M.; Ainge, G. D.; Painter, G. F.; Severn, W. B.; Wilson, I. A. *J. Immunol.* **2006**, *177*, 4577−4583.
(27) Rhijn, I. V.; Koets, A. P.; Im, J. S.; Piebes, D.; Reddington, F.; Besra, G. S.; Porcelli, S. A.; van Eden, W.; Rutten, V. P. M. G. *J. Immunol.* **2006**, *176*, 48884893.
(28) Kinjo, Y.; Tupin, E.; Wu, D.; Fujio, M.; Garcia-Navarro, R.; Zajonc, D. M.; Ben-Menache, G.; Ainge, G. D.; Painter, G. F.; Khurana, A.; Hoebe, K.; Behar, S. M.; Beutler, B.; Wilson, I. A.; Tsuji, M.; Sellati, T. J.; Wong, C. H.; Kronenberg, M. *Nat. Immunol.* **2006**, *7*, 978−986.
(29) Rojas, R. E.; Thomas, J. J.; Gehring, A. J.; Hill, P. J.; Belisle, J. T.; Harding, C. V.; Boom, W. H. *J. Immunol.* **2006**, *177*, 2959−68.

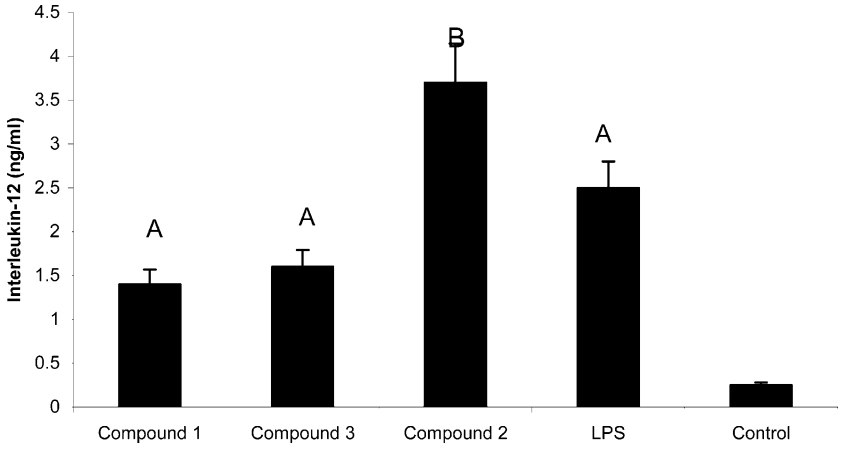


FIGURE 1. Induction of IL-12 by synthetic PIM compounds, **1**, **2**, and **3** on bovine dendritic cells (50 μg of each compound) compared with saline control (Control). Supernatants were collected 48 h after stimulation and IL-12 levels then measured. Columns with different letters are significantly different from each other ($P < 0.05$).

novel avenue for creating more stable and potent adjuvants. We are currently pursuing this possibility.

Experimental Section

1-*O*-Allyl-3-*O*-benzyl-*sn*-glycerol (9). $BF_3{\cdot}OEt_2$ (50 μL, 0.4 mmol) was added to a stirred solution of (*R*)-benzyl glycidol (**8**) (260.8 mg, 1.6 mmol) and allyl alcohol (1.1 mL, 16 mmol) in dry CH_2Cl_2 (10 mL). After 1h, the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **9** (269 mg, 1.20 mmol, 76%) as a clear oil. $[\alpha]_D^{28} = +0.76$ (*c* 1.00, EtOH). {lit.[19] $[\alpha]_D = +0.74$ (*c* 3.53, EtOH)}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40−7.26 (m, 5H), 5.97 (ddt, J = 17.3, 10.4, 5.7 Hz, 1H), 5.27 (dq, J = 17.2, 1.6 Hz, 1H), 5.19 (dq, J = 10.4, 1.4, 1H), 4.57 (s, 2H), 4.39−4.23 (m, 3H), 3.60−3.45 (m, 4H), 2.52 (d, J = 4.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.0, 134.5, 128.5, 127.8, 127.8, 117.3, 73.5, 72.4, 71.4, 71.3, 69.6. HRMS-ESI $[M + Na]^+$ calcd for $C_{13}H_{18}O_3Na$: 245.1154. Found 245.1163.

1-*O*-Allyl-3-*O*-benzyl-2-*O*-hexadecyl-*sn*-glycerol (10). 1-Bromohexadecane (685 μL, 2.2 mmol) was added to a stirred suspension of **9** (248 mg, 1.1 mmol) and sodium hydride (60% dispersion in mineral oil, 150 mg, 3.8 mmol) in dry DMF (10 mL) under nitrogen. After being stirred for 16 h, the reaction was quenched by addition of 1 M HCl (100 mL). The mixture was extracted with CH_2Cl_2 (2 × 100 mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with Et_2O/light petroleum (0:1 to 1:19) afforded the title compound **10** (426 mg, 0.95 mmol, 85%) as an oil.$[\alpha]_D^{28} = +0.60$ (*c* 1.00, EtOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36−7.24 (m, 5H), 5.89 (ddt, J = 17.3, 10.2, 5.6 Hz, 1H), 5.26 (dq, J = 17.2, 1.6 Hz, 1H), 5.17 (dq, J = 10.4, 1.5 Hz, 1H), 4.56 (s, 2H), 4.00 (dt, J = 5.6, 1.5 Hz, 2H), 3.67−3.48 (m, 7H), 1.63−1.52 (m, 2H), 1.39−1.20 (m, 26H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.5, 134.9, 128.4, 127.64, 127.57, 116.9, 78.0, 73.4, 72.4, 70.7, 70.3, 70.2, 32.0, 30.2, 29.8, 29.73, 29.70, 29.6, 29.4, 26.2, 22.8, 14.2. HRMS-ESI $[M + Na]^+$ calcd for $C_{29}H_{50}O_3Na$: 469.3658. Found 469.3651.

3-*O*-Benzyl-2-*O*-hexadecyl-*sn*-glycerol (11). A mixture of the ether **10** (403 mg, 0.90 mmol), *N*,*N*′-dimethylbarbituric acid (373 mg, 2.4 mmol), and tertakis(triphenylphosphine)palladium (60.8 mg, 0.05 mmol)[20] in dry THF (4 mL) was heated at 90 °C under nitrogen in a sealed tube. After being stirred at the same temperature for 40 h, the reaction mixture was cooled to rt and poured into a saturated $NaHCO_3$ solution and extracted with CH_2Cl_2 (2 × 100 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **11** (342 mg, 0.84 mmol, 93%) as an oil. $[\alpha]_D^{28} = +10.8$ (*c* 1.23, CH_2Cl_2). {lit.[21] for *ent*-**11**, 1-*O*-benzyl-2-*O*-hexadecyl-*sn*-glycerol $[\alpha]_D^{28} = -10.2$ (*c* 5.00, CH_2Cl_2)}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38−7.22 (m, 5H), 4.54 (m, 2H), 3.77−3.42 (m, 7H), 2.13 (t, J = 5.7 Hz, 1H), 1.61−1.52 (m, 2H), 1.39−1.19 (m, 26H), 0.92−0.82 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.1, 128.5, 127.8, 127.7, 78.6, 73.6, 70.5, 70.1, 63.0, 32.0, 30.2, 29.8, 29.7, 29.5, 29.4, 26.2, 22.8, 14.2. HRMS-ESI $[M + Na]^+$ calcd for $C_{26}H_{46}O_3Na$: 429.3345. Found 429.3351.

3-*O*-Benzyl-1-*O*-hexadecanoyl-2-*O*-hexadecyl-*sn*-glycerol (12). Palmitoyl chloride (1.10 mL, 3.64 mmol) was added dropwise to a stirred solution of alcohol **11** (1.34 g, 3.31 mmol) and pyridine (1.34 mL, 16.6 mmol) in CH_2Cl_2 (20 mL) cooled to 0 °C. After being stirred for 12 h at rt, the reaction mixture was quenched with H_2O (100 mL). The mixture was extracted with Et_2O (2 × 150 mL) and the ethereal extract washed with a 0.5M HCl solution (100 mL) and saturated $NaHCO_3$ solution (100 mL) and dried ($MgSO_4$). After filtration, the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (0:1 to 1:9) afforded the title compound **12** (2.04 g, 3.18 mmol, 96%) as an oil. $[\alpha]_D^{20} = +5.3$ (*c* 0.92, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37−7.22 (m, 5H), 4.54 (m, 2H), 4.27−4.10 (m, 2H), 3.66 (quintet, J = 5.2 Hz, 1H), 3.57−3.52 (m, 4H), 2.29 (t, J = 7.4 Hz, 2H), 1.67−1.50 (m, 4H), 1.38−1.17 (m, 50H), 0.92−0.85 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 138.2, 128.4, 127.7, 76.7, 73.5, 70.7, 69.7, 63.7, 34.3, 32.0, 30.1, 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 26.1, 25.0, 22.8, 14.2. HRMS-ESI $[M + Na]^+$ calcd for $C_{42}H_{76}O_4Na$: 667.5641. Found 667.5632.

1-*O*-Hexadecanoyl-2-*O*-hexadecyl-*sn*-glycerol (13). A mixture of the benzyl ether **12** (1.00 g, 1.55 mmol) and $Pd(OH)_2$/C (20%, 300 mg) in EtOH (100 mL)/HOAc (10 mL) was stirred under hydrogen for 14 h. The hydrogen was removed and the mixture filtered through Celite. The filtrate was concentrated *in vacuo* and the residue purified by column chromatography on silica gel. Elution with EtOAc/CH_2Cl_2 (1:24) afforded the title compound **13** (850 mg, 1.54 mmol, 99%) as an oil. $[\alpha]_D^{20} = -4.8$ (*c* 1.20, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.18−4.15(m, 2H), 3.65−3.43 (m, 5H), 2.32 (d, J = 7.4 Hz, 2H), 2.09−2.02 (m, 1H), 1.65−1.52 (m, 4H), 1.36−1.21 (m, 50H), 0.92−0.85 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 77.8, 70.6, 62.8, 62.8, 34.3, 32.0, 30.1, 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 26.1, 25.0, 22.8, 14.2. HRMS-ESI $[M + Na]^+$ calcd for $C_{35}H_{70}O_4Na$: 577.5172. Found 577.5172.

Benzyl (1-*O*-Hexadecanoyl-2-*O*-hexadecyl-*sn*-glycero)-diisopropylphosphoramidite (14). 1*H*-Tetrazole (55 mg, 0.79 mmol) was added to a stirred solution of alcohol **13** (395 mg, 0.714 mmol)

and benzyloxy-bis(diisopropylamino)phosphine (482 mg, 1.43 mmol) in dry CH_2Cl_2 (10 mL). After 1 h at rt, the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with Et_3N/EtOAc/light petroleum (1:3:16) afforded the title compound **14** (544 mg, 0.689 mmol, 96%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37−7.23 (m, 5H), 4.79−4.62 (m, 2H), 4.30−4.20 (m, 1H), 4.15−4.05 (m, 1H), 3.70−3.48 (m, 7H), 2.29 (t, J = 7.3 Hz, 2H), 1.65−1.50 (m, 4H), 1.30−1.16 (m, 62H), 0.90−0.83 (m, 6H). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 149.4, 149.2. HRMS-ESI, $[M + Na]^+$ calcd for $C_{48}H_{90}NO_5NaP$: 814.6454. Found 814.6469.

3,4,5-Tri-*O*-benzyl-2,6-di-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-1-*O*-(1-*O*-Hexadecanoyl-2-*O*-hexadecyl-*sn*-glycero-3-benzylphosphoryl)-D-*myo*-inositol (16). 1*H*-Tetrazole (19 mg, 0.27 mmol) was added to a stirred solution of 3,4,5-tri-*O*-benzyl-2,6-di-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-D-*myo*-inositol (**15**) (81 mg, 0.054 mmol) and phosphoramidite **14** (214 mg, 0.271 mmol) in dry CH_2Cl_2 (8 mL) cooled to 0 °C under argon. After being stirred at rt for 2 h, the reaction mixture was cooled to −40 °C and a solution of *m*-CPBA (50%, 93 mg, 0.27 mmol) in CH_2Cl_2 (10 mL) was transferred by cannula into the reaction mixture. After being stirred at rt over 2 h, the reaction was quenched by addition of a 10% Na_2SO_3 solution (50 mL) and the combined mixture extracted with Et_2O (100 mL). The ethereal extract was washed with a saturated $NaHCO_3$ solution (3 × 50 mL) and dried ($MgSO_4$). After filtration, the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **16** (38 mg, 0.017 mmol, 32%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38−7.01 (m, 60H), 5.53−5.47 (m, 1H), 5.37−5.31 (m, 1H), 5.04 (ap t, J = 7.2 Hz, 2H), 4.92−4.37 (m, 21H), 4.30−3.75 (m, 17H), 3.54−3.20 (m, 9H), 2.21−2.10 (m, 2H), 1.58−1.41 (m, 4H), 1.31−1.15 (m, 50H), 0.89−0.82 (m, 6H). ^{13}C (75 MHz, $CDCl_3$) selected signals δ 173.3, 99.6, 98.6. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 1.17, 1.13. HRMS-ESI $[M + Na]^+$ calcd for $C_{137}H_{173}O_{22}NaP$: 2224.2054. Found 2224.2051.

2,6-(Di-*O*-α-D-mannopyranosyl)-1-*O*-(1-*O*-hexadecanoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (2). Pd(OH)$_2$/C (20%, 25 mg) was added to a stirred solution of the fully substituted **16** (38 mg, 0.017 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under hydrogen for 2.5 h at rt and the hydrogen was replaced with argon. The mixture was filtered through Celite and the filtrate concentrated *in vacuo*. The residue was lyophilized to afford **2** (19 mg, 0.16 mmol, 94%) as a white powder. $[\alpha]_D^{20} = +34$ (*c* 0.20, $CHCl_3/CH_3OH/H_2O$, 70:40:6). ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD/D_2O$, 70:40:6) δ 5.14 (br s, 1H), 5.11 (br s, 1H), 4.50−3.20 (m, 25H), 2.35 (t, J = 7.2 Hz, 2H), 1.65−1.55 (m, 4H), 1.33−1.22 (m, 50H), 0.86 (t, J = 6.9 Hz, 6H). ^{31}P NMR (121.5 MHz, $CDCl_3/CD_3OD/D_2O$, 70:40:6) δ −3.5. HRMS-ESI $[M - H]^-$ calcd for $C_{53}H_{101}O_{22}P$: 1119.6444. Found 1119.6453. Anal. Calcd for $C_{53}H_{101}O_{22}P\cdot 4H_2O$: C, 53.34; H, 9.21. Found: C, 53.30; H, 8.92.

Benzyl (1,2-Di-*O*-hexadecyl-*sn*-glycero)-diisopropylphosphoramidite (19). Benzyloxy-bis(diisopropylamino)phosphine (250 mg, 0.74 mmol) in dry CH_2Cl_2 (10 mL) was transferred by cannula into a stirred mixture of alcohol **18** (200 mg, 0.37 mmol) and 1*H*-tetrazole (26.0 mg, 70.1 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C. The ice-bath was removed after 15 min, and after the solution had been stirred for 2 h at rt under argon the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with Et_3N/EtOAc/light petroleum (0.3:1:9) afforded the title compound **19** (233 mg, 0.30 mmol, 81%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37−7.25 (m, 5H), 4.79−4.63 (m, 2H), 3.68−3.40 (m, 11H), 1.57−1.53 (m, 4H), 1.25−1.17 (m, 64H), 0.88 (t, 6.43, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.7, 128.3, 127.2, 127.0, 78.6, 71.7, 71.0, 70.7, 65.5, 65.2, 63.3, 63.1, 43.2, 43.0, 32.0, 30.2, 29.8, 29.6, 29.4, 26.2, 24.7, 24.7, 22.8, 14.2. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 148.9, 148.8. HRMS-ESI $[M + Na]^+$ calcd for $C_{48}H_{92}O_4NPNa$: 800.6662. Found: 800.6664.

3,4,5-Tri-*O*-benzyl-2,6-di-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-1-*O*-(1,2-di-*O*-hexadecyl-*sn*-glycero-3-benzylphosphoryl)-D-*myo*-inositol (20). 1*H*-Tetrazole (16 mg, 0.23 mmol) was added to a stirred solution of pseudo-trisaccharide **15** (90 mg, 0.06 mmol) and phosphoramidite **19** (181 mg, 0.23 mmol) in CH_2Cl_2 (9 mL) cooled to −10 °C. The ice bath was removed after 15 min and after being stirred at rt for 2 h under argon the reaction mixture was cooled to −40 °C and a solution of predried *m*-CPBA (50%, 55.0 mg, 0.32 mmol) in CH_2Cl_2 (5 mL) was transferred by cannula into the reaction mixture. After being warmed to rt over 3 h, the reaction was quenched by the addition of a 10% Na_2SO_3 solution (50 mL) and the mixture was extracted with Et_2O (2 × 30 mL). The combined organic extracts were washed with a saturated $NaHCO_3$ solution (3 × 50 mL) and brine (50 mL). After drying ($MgSO_4$) and filtration, the solvent was removed *in vacuo* and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (2:8 to 2.5:7.5) followed by two further purifications on fresh silica gel eluted with Et_2O/CH_2Cl_2 (0.25:9.75) afforded the title compound **20** (20 mg, 0.0091 mmol, 15%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30−6.95 (m, 60H), 5.44 (d, J = 7.3 Hz, 1H), 5.28 (d, J = 9.7 Hz, 1H), 4.99 (t, J = 7.9 Hz, 2H), 4.86−3.16 (m, 49H), 1.49−1.31 (m, 4H), 1.30−1.10 (m, 52H), 0.83−0.79 (m, 6H). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 1.29, 1.25. HRMS-ESI $[M + Na]^+$ calcd for $C_{137}H_{175}O_{21}PNa$: 2210.2364. Found: 2210.2314.

2,6-Di-*O*-(α-D-mannopyranosyl)-1-*O*-(1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (3). Pd(OH)$_2$/C (20%, 7.0 mg) was added to a stirred solution of the fully substituted **20** (20 mg, 0.0091 mmol) in THF/MeOH (2:3, 2.5 mL), and stirring was continued under hydrogen for 3 h at rt after which Et_3N (1 mL) was added. The mixture was filtered through Celite and the filter cake washed with further THF/MeOH (2:3, 10 mL). The filtrate and washings were concentrated *in vacuo*, and the residue was purified by column chromatography on silica gel. Elution with H_2O/MeOH/$CHCl_3$ (0:2:7 to 0:4:7 to 0.2:4:7 to 0.8:4:7) and evaporation of the solvent afforded the title compound **3** (4.6 mg, 0.0042 mmol, 46%) as a white powder. $[\alpha]_D^{20} = +32$ (*c* 0.23, $D_2O/CD_3OD/CDCl_3$, 0.6:4:7). ^{1}H NMR (300 MHz, $D_2O/CD_3OD/CDCl_3$, 0.5:4:7) δ 5.16 (br s, 1H), 5.11 (br s, 1H), 4.05−3.43 (m, 28H), 3.36−3.19 (m, 12H), 1.58 (br s, 5H), 1.27 (br s, 51H), 0.89 (t, J = 6.5 Hz, 6H). ^{31}P NMR (121.5 MHz, $D_2O/CD_3OD/CDCl_3$, 0.5:4:7) δ 0.74. HRMS-ESI $[M + Na]^+$ calcd for $C_{53}H_{103}O_{21}PNa$: 1129.6627. Found: 1129.6664. Anal. Calcd for $C_{53}H_{103}O_{21}P\cdot 4H_2O$: C, 53.97; H, 9.49. Found: C, 53.71; H, 9.21.

Isolation of Bovine Dendritic Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of cattle by density-gradient centrifugation. Cells were adjusted to 10^7/mL in RPMI-1640 medium containing 10% heat-inactivated FCS, 10 mM HEPES, antibiotics, and 5 × 10^{-5} M 2-mercaptoethanol (all obtained from InVitrogen, Auckland, New Zealand). Cells were seeded at 10^7 cells/well of a 24-well plastic tissue dish. Nonadherent cells were removed after 3 h by washing with warm phosphate-buffered saline (PBS). Adherent cells were then incubated in complete medium with 0.2 U/mL of recombinant bovine GM-CSF and 200 U/mL of recombinant bovine IL-4 (obtained from Serotec, Oxford, UK). After 3−4 days, fresh media and cytokines were added to cells. Cells were harvested after 7−10 days of culture, washed three times, and adjusted to give the desired cell numbers. When harvested, cells were more than 90% DCs based on the following: DCs lacked canonical B and T cells markers and had high expression of MHC class 2 and class 1 (Serotec), and had low expression of CD11a (Serotec) and CD14 (Serotec). DCs after 10 days of culture also expressed CD80 and CD86 (Veterinary Medical Research and Development, Inc. (VMRD, Pullman, WA)). In addition, DCs had the characteristic veiled morphology and functional phenotype of DCs. DCs were used immediately by transferring to a 96-well microtiter plate at 2 × 10^5 cells per 200 uL of complete medium. The cells were cultured in RPMI-1640

with 10% FCS, 10 mM HEPES, and 4 mM L-glutamine, with the indicated concentrations of reagents for 48 h prior to measurements of IL-12.

IL-12 ELISA. Plates (Maxisorp, Nunc, Denmark) were coated with 8 μg/mL anti-IL-12 (Serotec) and incubated overnight at rt. The plates were washed in washing buffer, and blocking buffer was added for 1 h. Following a further washing step, samples were added for 1 h. Dilutions of the samples were added to the plates for 1 h. Following washing, biotin-labeled anti-IL-12 (Serotec: 8 μg/mL in blocking buffer) was added for 1 h, followed by washing and addition of SA-HRP for 45 min. Following the final washing step TMB substrate was added, the reaction was stopped by the addition of H_2SO_4 and the absorbance values read at 450 nm.

Acknowledgment. The authors wish to thank the New Zealand Foundation for Research and Technology (C08 × 0209) and the Marsden Fund (Royal Society of New Zealand, IRL0401) for financial support (to G.P.). We thank Lilian Morrison for statistical analyses and Professor Robin Ferrier for assisting in the preparation of the manuscript.

Supporting Information Available: General experimental remarks. NMR spectra for all compounds reported in the Experimental Section. This material is available free of charge via the Internet http://pubs.acs.org.

JO070639M