Bioconjugate Chemistry

Article

pubs.acs.org/bc

Synthesis and Biological Investigation of PIM Mimics Carrying Biotin or a Fluorescent Label for Cellular Imaging

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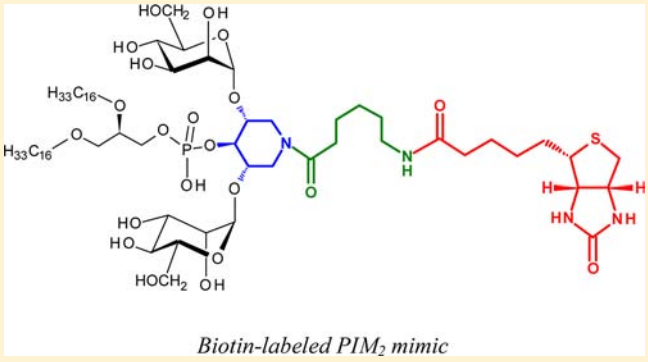
Supporting Information

ABSTRACT: Phosphatidyl inositol mannosides (PIMs) are constituents of the mycobacterial cell wall; these glycolipids are known to exhibit potent inhibitory activity toward the LPS-induced production of cytokines by macrophages, and therefore have potential as anti-inflammatory agents. Recently, heterocyclic analogues of PIMs in which the inositol is replaced by a piperidine (aza-PIM mimics) or a tetrahydropyran moiety (oxa-PIM mimics) have been prepared by short synthetic sequences and shown to retain the biological activity of the parent PIM structures. In this investigation, the aza-PIM analogue was used as a convenient scaffold to link biotin or a fluorescent label (tetramethyl-rhodamine) by way of an aminocaproyl spacer, with the goal of using these conjugates for intracellular localization and for the study of the mechanism of their antiinflammatory action. The synthesis of these compounds is reported, as well as the evaluation of their activities as inhibitors of LPS-induced cytokine production by macrophages (TNFα, IL12p40); preliminary investigations by FACS and confocal microscopy indicated that PIM−biotin conjugate binds to macrophage membranes with rapid kinetics.



Biotin-labeled PIM_2 mimic

INTRODUCTION

Mycobacteria express a number of molecules modulating the host immune system. Among them, mannose-capped lipoarabinomannan (ManLAM), lipomannans (LM), and PIM are recognized by various pattern recognition receptors present on macrophages and dendritic cells such as Toll-like receptors (TLRs)[1−5] or C-type lectins like mannose receptor (CD206) and DC-SIGN (CD209).[6−9] Several molecules of mycobacterial origin down-modulate the immune system, including the ESAT-6 and ManLAM, but also diacylated forms of LM and their glycosyl-phosphatidylinositol (GPI) anchor structure phosphatidyl-*myo*-inositol mannosides (PIM).[10−12] Dimannoside and hexamannoside PIM (PIM_2 and PIM_6), the two most abundant classes of PIM found in *M. tuberculosis* H37Rv and *M. bovis* BCG, inhibit endotoxin-induced proinflammatory responses, and we could reproduce this activity with synthetic PIM_1 and a mimetic of PIM_2.[12] We showed that the inhibition of TNFα and IL-12p40 expression in response to TLR2 or TLR4 agonists by synthetic PIM_1 and PIM_2 mimetic could be partially explained by an inhibition of LPS binding to CD14 in murine macrophages.[13] However, some inhibitory activities of PIM_1 and PIM_2 mimetic were independent of CD14, as seen for the inhibition of IL-12p40 release in response to rough-LPS stimulation of TLR4 pathway independent of CD14.[13] The mechanisms involved in PIM biological actions are thus likely to be complex, not clearly understood, and may involve both receptor recognition and direct interaction with the cell membranes. There is a clear need to perform experiments with labeled derivatives, for in-cell imaging and localization studies.

The synthesis of PIM_1 or PIM_2 derivatives carrying appropriate substituents for labeling would require the differentiation of the OH groups of the inositol moiety. Although efficient methodologies are available for selective protection of the OH groups in *myo*-inositol,[14−16] the synthesis of such compounds would require long sequences of reactions. This difficulty could be avoided by taking advantage of our PIM mimics based on an aza-cyclitol core. We have recently shown[17] that the inositol moiety in PIM_2 could be advantageously replaced by a saturated heterocyclic unit, such as a piperidine (as in **IA**, Figure 1) or a tetrahydropyran (as in **IB**): these compounds retain the biological activity of the parent PIM structures, provided the nitrogen group in **IA** is acylated. The aza-cyclitol moiety present in **IA** and related compounds provides thus a point of attachment readily available for tethering groups which can be used to conjugate PIM mimics

Received: September 4, 2012
Revised: November 6, 2012
Published: November 29, 2012

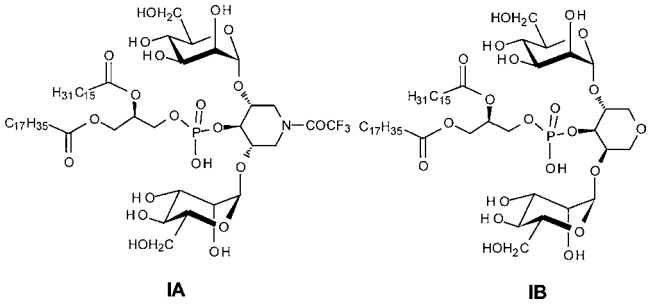


Figure 1. PIM_2 mimics built on an heterocyclic core.[13]

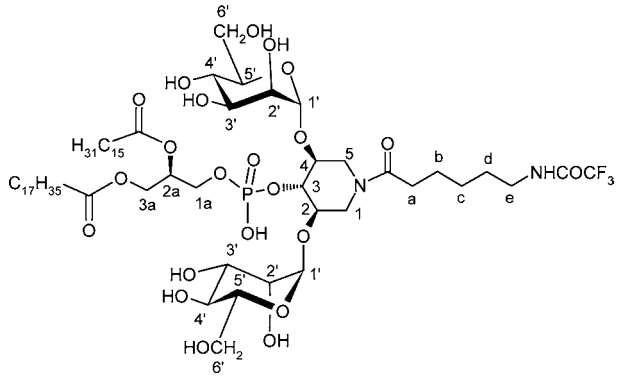
with various markers or carriers. We report in this article the synthesis of aza-PIM mimics carrying biotin or a fluorescent label, as well as initial biological investigations of the immunomodulating activity of the new compounds and of their utility as imaging probes.

EXPERIMENTAL PROCEDURES

Synthesis—General Procedures. Unless otherwise stated, all reactions requiring anhydrous conditions were carried out under dry Ar. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride (CaH_2). Diethyl ether (Et_2O) was distilled from CaH_2 and stored at 4 °C, over 4 Å molecular sieves, under Ar. All reagent-grade chemicals were obtained from commercial suppliers (Sigma-Aldrich, Acros) and were used as received. ^1H, ^{13}C, ^{19}F, and ^{31}P NMR spectra were recorded on a Bruker DPX250 or a Bruker Avance 400 spectrometer at 25 °C. Proton chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si (δ 0.000 ppm) as internal standard. Carbon chemical shifts are referenced to the central line of the $CDCl_3$ triplet (δ 77.16 ppm), unless otherwise stated. ^{31}P and ^{19}F chemical shifts are reported in ppm downfield from the external standard, phosphoric acid and boron trifluoride diethyl etherate, respectively. Coupling constants (J) are reported in hertz (Hz). Spectral splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br, broad. Carbon multiplicities were assigned by DEPT experiments. ^1H and ^{13}C NMR signals were assigned with the help of H−H and H−C correlations (COSY and HSQC spectra). Flash chromatography was performed according to the established protocol on silica gel 60 (40−63 μm). The purification of final compounds was performed on Sephadex LH-20 from Sigma. Reactions were monitored by thin layer chromatography (TLC) (silica gel $60F_{254}$ precoated aluminum plates) and the compounds were detected under UV light and by staining with phosphomolybdic acid solution. Electron spray-ionization mass spectrometry (ESI-MS) was performed with a Perkin-Elmer SCIEX API 300 spectrometer. High-resolution mass spectra were recorded on a Bruker maXis UHR-Q-TOF spectrometer in Orléans.

***N*-Benzyl-3-*O*-benzyl-1,5-dideoxy-1,5-iminoxylitol (5).** This was obtained from diacetone glucose as described recently;[18] purification of **5** by silica gel column chromatography using petroleum ether/ethyl acetate 2/1 as the eluent was required for efficient selective *N*-debenzylation in the next step. (Yield ∼31% over 4 steps, from 3-*O*-benzyl-1,2;5,6-diisopropylidene-α-D-glucofuranose).

3-*O*-Benzyl-1,5-dideoxy-1,5-iminoxylitol (6). The procedure was improved from the one previously published.[17] Compound **5** previously purified (582 mg, 1.85 mmol) was dissolved in EtOH (15 mL). Palladium hydroxide ($Pd(OH)_2$)



(80 mg) was added and the reaction mixture was stirred at rt under H_2 atmosphere for 18 h. The catalyst was removed by filtration through a Millipore membrane and washed with EtOH (2 × 10 mL). The organic phases were combined and the solvent was evaporated under reduced pressure to give essentially pure compound **6** (413 mg, 100%) as a white solid. ^1H NMR (400 MHz, CD_3OD): δ 2.90 (dd, 2H, H1A/5A, J = 12.8, 6.9 Hz), 3.24 (dd, 2H, H5B/1B, J = 12.8, 3.5 Hz), 3.47 (t, 1H, H3, $J_{3,4} = J_{3,2}$ = 6.2 Hz), 3.87 (ddd, 2H, H2/4), 4.78 (s, 2H, CH_2Ph), 7.22−7.46 (m, 5H, Ph). ^{13}C NMR (101 MHz, CD_3OD): δ 48.68 (C1/5), 68.38 (C2/4), 74.65 (CH_2Ph), 81.04 (C3), 128.70, 128.98, 129.30 (CHAr), 139.70 (CqAr). MS: calcd for $C_{12}H_{16}NO_3$: 223.12; (+)IS-MS: m/z 224.5 $[M+H]^+$, 246.5 $[M+Na]^+$.

***N*-(6-Trifluoroacetamidohexanoyl)-3-*O*-benzyl-1,5-dideoxy-1,5-iminoxylitol (7).** To a solution of compound **6** (413 mg, 1.85 mmol) in dry CH_2Cl_2 (10 mL), 6-trifluoroacetamidohexanoic acid (462 mg, 2.03 mmol, 1.1 equiv) and 1-hydroxybenzotriazole (HOBt) (300 mg, 2.22 mmol, 1.2 equiv) were added. The mixture was cooled down to 0 °C and dicyclohexylcarbodiimide (DCC) (458 mg, 2.22 mmol, 1.2 equiv) was added. After having been stirred at rt for 18 h, the reaction mixture was diluted with CH_2Cl_2 (50 mL). The organic phase was washed with 1 N aqueous HCl (20 mL), then with brine (20 mL), dried over $MgSO_4$, and evaporated under vacuum. The residue was purified by silica gel column chromatography (CH_2Cl_2/acetone 3/1, R_f = 0.65) to give compound **7** (480 mg, 60%). ^1H NMR (400 MHz, $CDCl_3$; slow amide bond rotation promotes differentiation of 1/5 and 2/4 positions in the iminoxylitol ring): δ 1.25−1.39 (m, 2H, 2H*c*), 1.50−1.68 (m, 4H, 2H*b*, 2H*d*), 2.30−2.48 (2 td, J_{AB} = 15 Hz, 2H, 2H*a*), 3.31 (app q, 2H, 2H*e*, J = 6.6 Hz), 3.36 (dd, 1H, H1A, $J_{1A,2}$ = 1.5, $J_{1A,1B}$ = 13.6 Hz), 3.51−3.67 (m, 3H, H5A, H5B, H3), 3.78 (br, 1H, H4), 3.89 (br, 1H, H2), 4.15 (dd, 1H, H1B, $J_{1B,2}$ = 4 Hz), 3.34 (br d, 1H, 4-OH, J = 7.2 Hz), 4.58 (br s, 1H, 2-OH), 4.66 (AB, 2H, CH_2Ph, J = 11.6 Hz), 7.26−7.37 (m, 5H, CHar), 7.53 (t, 1H, N*H*, J = 4.8 Hz). ^{13}C NMR (101 MHz, $CDCl_3$): δ 24.32 (C*b*), 26.15 (C*c*), 28.32 (C*d*), 32.69 (C*a*), 39.56 (C*e*), 44.25 (C1), 48.47 (C5), 68.24 (C2, C4), 72.78 (CH_2Ph), 76.98 (C3), 116.04 (q, CF_3, J = 289 Hz), 127.66, 128.01, 128.57 (CHAr), 137.96 (CqAr), 157.51 (q, $COCF_3$, J = 37 Hz), 173.90 (CO). ^{19}F NMR (376 MHz, $CDCl_3$): δ - 75.74. HRMS (ESI): $[M+H]^+$ calcd for $C_{20}H_{28}F_3N_2O_5$: m/z = 433.19448; found m/z = 433.19491. $[M+Na]^+$ calcd for $C_{20}H_{27}F_3N_2NaO_5$: m/z = 455.17643; found m/z = 455.17693.

***N*-(6-Trifluoroacetamidohexanoyl)-3-*O*-benzyl-2,4-bis-*O*-(2,3,4,6-tetra-*O*-methoxyacetyl-α-D-manno-pyranosyl)-1,5-dideoxy-1,5-iminoxylitol (9).** Compound **7** (481

dx.doi.org/10.1021/bc3004974 | *Bioconjugate Chem.* 2013, 24, 72−84

mg, 1.11 mmol) was previously dried overnight under high vacuum and then dissolved under Ar in CH_2Cl_2 (10 mL). 4 Å Molecular sieves were added and the mixture was stirred for 30 min; a solution of 2,3,4,6-tetra-*O*-methoxyacetyl-α-D-manno-pyranosyl trichloroacetimidate (**8**)[17] (2.081 g, 3.38 mmol, 3 equiv) in CH_2Cl_2 (5 mL) was added under argon flux. After having stirred the mixture for 15 min, trimethylsilyl triflate (TMSOTf) (266 μL, 1.35 mmol, 40% mol equiv vs the donor) was added and the mixture was stirred for 1 h at rt under Ar. The reaction was stopped by the addition of triethylamine (376 μL, 2 equiv vs TMSOTf), and the mixture was filtered through a sintered glass filter containing Celite. The solvent was removed under vacuum and the residue was purified by silica gel column chromatography (toluene/acetone 2.5/1 to 1/1, difficult separation) to give a pure fraction of compound **9** (553 mg, 37%) as a colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$; presence of amide rotamers and of nonequivalent mannosyl residues): δ 1.32−1.45 (m, 2H, 2H*c*), 1.52−1.75 (m, 4H, 2H*d*, 2H*b*), 2.25−2.45 (m, 2H, 2H*a*), 2.98 (dd, 0.5 H, *J* = 9.4, 13.0 Hz), 3.07 (dd, 0.5 H, *J* = 8.8, 12.8 Hz), 3.13 (dd, 0.5 H, *J* = 9.2, 13.2 Hz), 3.27 (dd, 0.5H, *J* = 9.0, 13.8 Hz) (4 dd for H1A and H5A), 3.31−3.53 (m, 26H, 8 × CH_3, 2H*e*), 3.55−3.76 (m, 3H, H2, H3, H4), 3.77−4.21 (21H, 8 × CH_2, 2H5′, 2H6′, 2 × 0.5 H1B/5B), 4.21−4.29 (m, 1H, H6′), 4.30−4.35 (m, 0.5H, 0.5 H1B/5B), 4.36−4.46 (m, 1H, H6′), 4.47−4.54 (m, 0.5H, 0.5 H1B/5B), 4.73−4.88 (m, 2H, CH_2Ph), 5.01 (s, 0.5H, H1′), 5.06 (s, 0.5H, H1′), 5.11 (s, 0.5H, H1′), 5.15 (s, 0.5H, H1′), 5.21−5.52 (m, 6H, 2H2′, 2H3′, 2H4′), 7.11−7.21 (m, 0.7H, NH), 7.23−7.44 (m, 5H, 5CHar). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 23.91 and 23.98 (C*b*), 26.03 and 26.16 (C*c*), 28.33 (C*d*), 32.39 and 32.49 (C*a*), 39.44 (C*e*), 41.23, 44.19, 45.63, 47.97 (C1 and C5, 4 signals), 59.34 (CH_3 MAc), 59.42 (CH_3 MAc), 59.46 (CH_3 MAc), 61.78 and 61.88 (C6′), 62.38 and 62.55 (C6′), 65.50, 65.69, 66.05, 66.71, 68.35, 68.60, 68.74, 68.92, 69.07 (2C2′, 2C3′, 2C4′), 69.18−69.80 (6 signals for MeOCH_2), 71.38 and 72.96 (C2 or 4), 75.08 and 75.21 (CH_2Ph), 76.57 (C3), 80.35 and 81.91 (C2 or 4), 94.38 and 94.82 (C1′), 98.18 and 98.66 (C1′), 116.05 (q, CF_3, *J* = 289 Hz), 127.7, 128.04, and 128.62 (CHAr), 137.31 (CqAr), 157.27 (q, $COCF_3$, *J* = 36 Hz), 169.20, 169.34, 169.45, 169.78, 169.90, 170.01, 170.04, 170.19, 171.59, and 171.84 (CO). ^{19}F NMR (376 MHz, $CDCl_3$): δ −75.81. HRMS (ESI): $[M+H]^+$ calcd for $C_{56}H_{80}F_3N_2O_{31}$: *m*/*z* = 1333.46916; found *m*/*z* = 1333.46832.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(2,3,4,6-tetra-*O*-methoxyacetyl-α-D-mannopyranosyl)-1,5-dideoxy-1,5-iminoxylitol (10).** Compound **9** (542 mg, 0.407 mmol) was dissolved in a mixture of CH_2Cl_2 (15 mL) and EtOH (20 mL). 10% Palladium on carbon (100 mg) was added and the reaction mixture was vigorously stirred at rt under an atmosphere of H_2 for 18 h. The reaction mixture was then filtered through a Millipore membrane to remove the catalyst; the black solid was washed with a 1:1 mixture of CH_2Cl_2 and EtOH (2 × 20 mL). The solvents were then evaporated to give compound **10** (496 mg, 98%). R_f = 0.59 (CH_2Cl_2/acetone 2:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.30−1.48 (m, 2H, 2H*c*), 1.53−1.76 (m, 4H, 2H*b*, 2H*d*), 2.29−2.48 (m, 2H, 2H*a*), 2.49−2.71 (m, 1H, 0.5 H1A, 0.5 H5A), 2.90−3.21 (m, 1H, 0.5 H1A, 0.5 H5A), 3.30−3.60 (m, 27H, 8 × CH_3 MAc + 2H*e* + 1H), 3.60−3.75 (m, 1H), 3.81−4.40 (m, 22H, 8 × CH_2 MAc, 4H6′, H5′, H1B/H5B), 4.40−4.53 (m, 1H, H5′), 4.60−4.78 (m, 1H, H1B/H5B), 5.02 (s, 0.5H, H1′), 5.03 (s, 0.5H, H1′), 5.20 (s, 0.5H, H1′), 5.23 (s, 0.5H, H1′), 5.25−5.52 (m, 6H, 2H2′, 2H3′, 2H4′), 7.12−7.22 (m, 0.5H, 0.5 NH), 7.23−7.34 (m, 0.5H, 0.5NH). ^{1}H NMR (400 MHz, DMSO-d_6, 90 °C): δ1.30 (quint, 2H, H*c*), 1.54 (2 overlapping quint, 4H, 2H*b*, 2H*d*), 2.36 (t, 2H, 2H*a*), 2.6−3 (very broad, 1 × H1, 1 × H5), 3.18 (q, 1H, $J_{He,Hd}$ ∼6.7 Hz, $J_{He,NH}$ ∼6.7 Hz, 2H*e*), 3.30, 3.31, 3.32, 3.325, 3.35, 3.36, 3.40, 3.41 (8s, 8 × 3H, 8 × OMe), 3.30−3.50, (br signals, 1 × H1, 1 × H5, H2, H4), 3.58 (dt, 1H, $J_{3,OH}$ = 5.2 Hz, $J_{2,3}$ = $J_{3,4}$ = 8.8 Hz, H3), 3.86−4.13 (several s and AB systems, 16H, 8 CH_2OMe), 4.17 (m, 1H, H5′), 4.2−4.26 (m, 3H, 2H6′A, H6′B), 4.29 (dd, 1H, $J_{5',6'A}$ = 5.2 Hz, $J_{6'A,6'B}$ = 12 Hz, H6′B), 4.53 (dt, 1H, $J_{4'5'}$ =10 Hz, $J_{5',6'A}$= $J_{5',6'B}$ = 3.6 Hz, H5′), 5.15 (br s, 1H, H1′), 5.18−5.27 (overlapping signals, 4H: 2H4′, H2′, H1′), 5.45 (dd, 1H, $J_{2',3'}$ = 3.2 Hz, $J_{3',4'}$ = 10 Hz, H3′), 5.41 (dd, 1H, $J_{2',3'}$ = 3.2 Hz, $J_{3',4'}$ = 10 Hz, H3′), 5.43 (1 narrow dd, H2′), 5.95 (d, 1H, $J_{3,OH}$ = 5.2 Hz, HO-3), 9.02 (br signal, 1H, NH). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 24.05 (C*b*), 26.04 and 26.12 (C*c*), 28.38 (C*d*), 32.50 and 32.57 (C*a*), 39.42 and 39.48 (C*e*), 42.66, 44.08, 46.38, 47.91 (C1, C5, 4 signals), 59.39, 59.45 (CH_3 MAc), 62.30, 62.38, 62.60 (2H6′, 3 signals), 66.00, 66.10, 66.18, 66.60, 68.53, 68.70, 68.38, 69.00, 69.13, 69.61, 69.63, 69.92 (2C2′, 2C3′, 2C4′, 2C5′), 69.27, 69.31, 69.41, 69.52 (8 CH_2 MAc), 74.99, 75.72, 76.03, 76.07, 67.98, 78.38 (C2, C3, and C4, 6 signals), 95.93, 95.95, 98.80, 98.98 (2C1′, 4 signals), 118.50 (q, CF_3, *J* = 288 Hz), 157.29 (q, $COCF_3$, *J* = 37 Hz), 169.25, 169.26, 169.27, 169.30, 169.38, 169.41, 169.43, 169.50, 169.53, 169.59, 169.77, 170.08, 170.19, 170.22 (8 COOR), 171.50, 171.70 (CONH). ^{19}F NMR (376 MHz, $CDCl_3$): δ −75.82, −75.81. HRMS (ESI): $[M+H]^+$ calcd for $C_{49}H_{74}F_3N_2O_{31}$: *m*/*z* = 1243.42221; found *m*/*z* = 1243.42145.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(2,3,4,6-tetra-*O*-methoxyacetyl-α-D-mannopyranosyl)-3-*O*-[(2*R*)(2-hexadecanoyloxy-3-octadecanoyloxypropyl)-(benzyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (12).** Phosphoramidite **11**[17] (197 mg, 0.236 mmol, 1.4 equiv) was added to a solution of compound **10** (210 mg, 0.169 mmol) in dry CH_2Cl_2 (11 mL). The mixture was cooled down to 0 °C and a commercial solution of 1*H*-tetrazole in acetonitrile (∼0.40 M), previously dried over 3 Å molecular sieves, was added (1.27 mL, 0.507 mmol, 3 equiv). The reaction mixture was stirred for 2 h at rt and then was cooled down to −17 °C. A solution of *m*CPBA (purity ∼50%) (175 mg, 0.507 mmol, 3 equiv) in CH_2Cl_2 (4 mL) dried over some $MgSO_4$ was then added dropwise. After keeping the mixture for 10 min at −10 to −15 °C and 1 h at rt, the reaction was quenched by the addition of saturated aqueous $Na_2S_2O_3$ (20 mL) and the entire mixture was extracted with Et_2O (2 × 80 mL). The organic phases were combined and washed successively with saturated aqueous $Na_2S_2O_3$ (3 × 15 mL), aqueous $NaHCO_3$ (15 mL), and brine (15 mL), and then dried over $MgSO_4$. The solvent was removed in vacuum and the residue was purified by silica gel chromatography (toluene/acetone 3:1) to give compound **12** (160 mg, 47%). $R_{f\ Phosphite}$ = 0.41 and $R_{f\ Phosphate}$ = 0.31 (toluene/acetone 2:1). Note: *the NMR data of **12** are complex because of amide rotamers, P*-stereoisomers (≠1:1), and the nonequivalence of the mannosyl residues.* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 6H, 2CH_3, *J* = 6.6 Hz), 1.03−1.50 (m, 54H, 26 CH_2, 2H*c*), 1.51−1.78 (m, 8H, 2H*d*, 2H*b*, 2CH_2 acyl chain), 2.14−2.51 (m, 6H, 2H*b*, 2CH_2 acyl chain), 3.19−3.55 (m, 27H including 8 OCH_3), 3.55−4.49 (32H, including 8 CH_2OMe), 4.95−5.59 (m, 11H), 7.27−7.54 (m, 6H, 5CHar, NH). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.12 (CH_3), 22.69 (CH_2), 23.77 and 23.87, 25.96 and 26.13, and 28.23 (C*b*, C*c*, C*d*), 24.85

dx.doi.org/10.1021/bc3004974 | *Bioconjugate Chem.* 2013, 24, 72−84

(CH_2), 29.12, 29.17, 29.33, 29.36, 29.55, and 29.71 (CH_2), 31.92 (CH_2), 32.26 (C*a*), 33.97 and 34.12 (CH_2), 39.39 (C*e*), 39.87, 43.09, 44.45, and 47.14 (C1 and C5), 59.12, 59.17, 59.23, 59.31, 59.33, 59.37, 59.42, 59.47 (8 signals OCH_3), 61.71 (CH_2), 61.89 (CH_2), 62.16−62.58 (broad signal, CH_2), 65.35 (CH), 65.97 (broad, CH_2), 66.88 (CH), 68.45 (CH), 68.79 (CH), 68.92 (CH), 69.15 (CH), 69.25 (CH_2), 69.33 (CH_2), 69.43 (CH_2), 69.67 (CH), 69.70 (CH), 70.02 (CH), 70.26−70.59 (broad signal, CH_2), 71.41 (broad, CH), 73.69−73.85 (broad, CH), 74.07−74.29 (broad, CH), 74.29−74.58 (broad, CH), 75.25−75.50 (broad, CH), 75.63−75.96 (broad, CH), 76.72 (CH), 76.94 (CH), 94.38 and 94.40, 94.99 and 95.03, 97.32 and 97.44, 98.34 and 98.40 (C1′, 4 × 2 signals), 116.05 (q, CF_3, J = 288 Hz), 128.30, 128.35, 128.44, 128.77, 128.81, 128.87, 128.88, 128.97 (CHAr), 135.49, 135.57 (CqAr), 157.21 (q, $COCF_3$, J = 36 Hz), 169.15, 169.23, 169.31, 169.37, 169.43, 169.52, 169.78, 169.82, 169.95, 170.04, 170.13 (CO), 171.86 and 171.89, 172.08 and 172.14, 172.86 and 172.89, 173.24 and 173.26 (CONH, COOR). ^{31}P NMR (162 MHz, $CDCl_3$): δ −1.30, −1.08, −1.03, −1.01. ^{19}F NMR (376 MHz, $CDCl_3$): δ −75.83, −75.80. HRMS (ESI): $[M+H]^+$ calcd for $C_{93}H_{151}F_3N_2O_{38}P$: m/z = 1991.96291; found m/z = 1991.96052.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(2,3,4,6-tetra-*O*-methoxyacetyl-α-D-mannopyranosyl)-3-*O*-[(2*R*)(2-hexadecanoyloxy-3-octadecanoyloxypropyl)-phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (13).** Compound **12** (158 mg, 0.079 mmol) was dissolved in a mixture of CH_2Cl_2 (6 mL) and EtOH (8 mL). 10% Palladium on charcoal (50 mg) was added and the reaction mixture was stirred at rt under H_2 atmosphere for 4 h. The catalyst was removed by filtration through a Millipore membrane. The solid was washed with a 1:1 mixture of CH_2Cl_2 and EtOH (2 × 10 mL). The organic phases were combined and the solvents were evaporated to give compound **13** as a white solid (142 mg, 95%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (t, 6H, CH_3, J = 6.8 Hz), 1.17−1.36 (m, 52H, 26CH_2), 1.36−1.52 (m, 2H, 2H*c*), 1.52−1.78 (m, 8H, 2H*d*, 2H*b*, 2CH_2 acyl chain), 2.23−2.54 (m, 6H, 2H*a*, 2CH_2 acyl chain), 3.21−3.51 (m, 26H, 8xOCH_3, 2H*e*), 3.51−4.48 (m, 33H), 5.04 (s, 0.5H), 5.10 (s, 0.5H), 5.11 (s, 0.5H), 5.17 (s, 0.5H) (2 × H1′), 5.21−5.54 (m, 7H, 2H2′, 2H3′, 2H4′, H2a), 7.34 (br, 0.5 NH), 7.49 (br, 0.5 NH). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.15 (CH_3), 23.83 and 23.95, 26.03 and 26.20, 28.23 (br) and 32.27 (br) (4CH_2, C*a*-C*d*), 22.72, 24.91, 24.98, 29.2−29.8, 31.96, 32.27, 34.06, and 34.20 (CH_2 acyl chains), 39.43 and 39.45 (C*e*), 39.70−39.89, 42.97−43.14, 44.28−44.67, 47.86−47.31 (4 broad signals for C1,C5), 59.11, 59.15, 59.27, 59.30, 59.39, 59.44 (6 signals for OCH_3), 62.10−62.56, 64.98−65.34, 65.74−66.05, 66.74, 68.42−70.42 (several signals), 71.55−71.74, 73.71−73.98, 74.60−74.97, 94.36 and 95.06 (C1′), 97.06 and 98.31 (C1′), 116.10 (q, CF_3, J_{C-F} = 288 Hz), 157.25 (q, $COCF_3$, J_{C-F} = 37 Hz), 169.35−169.78, 170.18, 170.25 (CO), 172.12, 172.42, 173.13, 173.43 (CONH, COOR). ^{31}P NMR ({^{1}H}, 162 MHz, $CDCl_3$): δ −1.57 −1.44. ^{19}F NMR (376 MHz, $CDCl_3$): δ −75.78, −75.75. HRMS (ESI): $[M+H]^+$ calcd for $C_{86}H_{145}F_3N_2O_{38}P$: m/z = 1901.91596; found m/z = 1901.91178.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)(2-hexadecanoyloxy-3-octadecanoyloxypropyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (1).** Compound **13** (135 mg, 0.071 mmol) was dissolved in a 1:4 mixture of $CHCl_3$ and MeOH (2 mL). The reaction mixture was cooled down to 0 °C and *t*-butylamine (164 μL) was added. The mixture was stirred at 0 °C for 10 min then warmed up to rt and stirred for an additional 2 h; solvents were evaporated without heating and the residue was purified by flash chromatography ($CHCl_3$/MeOH/H_2O 70/40/1) to give compound **1** (73 mg, 78%) as an amorphous solid. *Note*: Under these conditions, the $COCF_3$ group is not cleaved. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL; lock on CD_3OD; presence of amide rotamers in a ratio of about 2:1): δ 0.86 (t, 6H, CH_3, J = 6.8 Hz), 1.17−1.35 (m, 52H, 26 CH_2), 1.35−1.43 (m, 2H, 2H*c*), 1.52−1.69 (m, 8H, 2H*d*, 2H*b*, 2CH_2 acyl chain), 2.31 (2 t, 4H, 2CH_2 acyl chain, J = 7.6 Hz), 2.44 (t, 2H, 2H*a*, J = 7.2 Hz), 3.28 (app t, 2H, H*e*, J = 7.1 Hz), 3.39−3.95 (m, 16H), 3.95−4.03 (m, 2H, 2H1a), 4.08−4.28 (m, 2H incl. H3aA), 4.28−4.41 (m, 2H), 4.44 (dd, 1H, H3aB, J = 2.6, 12.1 Hz), 4.95 (s, 0.3H, H1′), 4.98 (s, 0.7H, H1′), 5.02 (s, 0.3H, H1′), 5.09 (s, 0.7H, H1′), 5.18−5.29 (m, 1H, H2a). ^{13}C NMR (62.9 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL; lock on CD_3OD): δ 14.39 (CH_3), 25.45 and 25.50, 27.09, 29.13, 33.16, and 33.30 (C*a*-C*d*), 23.33, 25.59, 25.64, 29.8−30.4, 32.63, 34.73, 34.88 (acyl CH_2), 40.27 and 40.32 (C*e*), ∼48−49 (broad, C1/C5), 61.89 (broad, C6′), 62.27 (broad, C6′), 63.44 (C3a), 64.36 (broad, C1a), 67.61, 68.26, 71.05, 71.19, 71.30, 71.69, 71.92,72.27, 74.06, 74.29, 74.73 (all CH), 97.57 (C1′), 99.25 (C1′), 99.81 (C1′), 101.39 (C1′), 116.94 (q, CF_3, J_{C-F} = 287 Hz), 158.61 (s, $COCF_3$, J_{C-F} = 37 Hz), 174.12, 174.41, 174.60, 174.76 (CO′s). ^{31}P NMR (162 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL): δ −0.49. ^{19}F NMR (376 MHz, $CDCl_3$): δ −76.66, −76.78. HRMS (ESI): $[M+H]^+$ calcd for $C_{62}H_{113}F_3N_2O_{22}P$: m/z = 1325.74692; found m/z = 1325.74709.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(2,3,4,6-tetra-*O*-methoxyacetyl-α-D-mannopyranosyl)-3-*O*-[(2*R*)(2,3-bis(hexadecyloxy)propyl)(benzyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (15).** Compound **14**[19] (318 mg, 0.409 mmol, 1.4 equiv) and compound **10** (358 mg, 0.288 mmol) were separately dried overnight under vacuum over P_2O_5. CH_2Cl_2 (6 mL) was added to compound **10** and CH_2Cl_2 (3 mL) was added to compound **14** under Ar. The solution of compound **14** was added to the solution of compound **10** and the flask rinsed with 1 mL of CH_2Cl_2. Molecular sieves (3 Å) were added, and after 15 min, the solution was cooled down to 0 °C. A commercial solution of 1*H*-tetrazole in acetonitrile (∼0.45 M) (1.92 mL, 0.864 mmol, 3 equiv), previously dried over 3 Å molecular sieves, was then added. The reaction mixture was stirred for 2 h at rt and was then cooled to −17 °C. A solution of *m*CPBA (purity ∼50%) (298 mg, 0.864 mmol, 3 equiv) in CH_2Cl_2 (4 mL) dried over some $MgSO_4$ was then added dropwise. After having been stirred for 10 min at −15 to −10 °C and 1 h at rt, the reaction was quenched by the addition of saturated aqueous $Na_2S_2O_3$ (20 mL). The whole mixture was extracted with Et_2O (2 × 90 mL). The organic phases were combined and washed successively with saturated aqueous $Na_2S_2O_3$ (3 × 20 mL), aqueous $NaHCO_3$ (20 mL), and brine (20 mL), and then dried ($MgSO_4$). The solvent was removed in vacuum and the residue was purified by silica gel column chromatography (CH_2Cl_2/acetone 3/1) to give compound **15** (238 mg, 43%) as a syrup. $R_{f\ phosphite}$ = 0.42 and $R_{f\ phosphate}$ = 0.27 (CH_2Cl_2/acetone 4/1). *NMR data are complex because of amide rotamers, P*-isomers, and non-equivalence of the mannosyl residues.* ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.12 (CH_3), 23.72 and 23.82, 25.94 and 26.1, 28.12 and 28.25, and 32.16 (C*a*-C*d*), 22.68, 26.05, 26.09, 29.4−30.0, 31.92 (CH_2 acyl chains), 39.38 (C*e*), 38.92−39.20 (broad),

dx.doi.org/10.1021/bc3004974 | *Bioconjugate Chem.* 2013, 24, 72−84

42.39−42.91 (broad), 43.83−44.21 (broad), 46.61−47.07 (broad) (C1 and C5), 59.00−59.45 (6 signals for OCH_3), 61.56−61.97 (broad, CH_2, C6′), 62.30 and 62.53 (CH_2, C6′), 65.29 (CH), 65.89 (CH), 67.04 (CH), 67.73−68.07 (broad, CH_2), 68.16−68.36 (broad, CH), 68.85 (CH), 69.08 (CH), 69.21 (CH_2), 69.32 (CH_2), 69.42 (CH_2), 69.90 (CH), 70.06 (CH_2), 70.55 (CH_2), 70.63 (CH_2), 71.19 (CH), 71.82 (CH_2O alkyl chain), 72.0−75.0 (several broad signals, CH), 76.99 (CH), 77.36 (CH), 94.36 (C1′), 95.14 (C1′), 96.28 and 96.47 (C1′), 97.80 and 98.01 (C1′), 116.08 (q, CF_3, J = 288 Hz), 128.1−128.8 (CHAr), 135.56 and 135.60 (CqAr), 135.65 and 135.68 (CqAr), 157.19 (q, $COCF_3$, J = 36 Hz), 157.22 (q, $COCF_3$, J = 36 Hz), 169.07−170.08 (12 lines for MAc-CO), 172.19 and 172 25 (CONH), 172.40 and 172.49 (CONH). ^{31}P NMR (162 MHz, $CDCl_3$): δ −1.04. ^{19}F NMR (376 MHz, $CDCl_3$): δ −75.79, −75.75, and −75.74. HRMS (ESI): $[M+Na]^+$ calcd for $C_{91}H_{150}F_3N_2NaO_{36}P$: m/z = 1957.95502; found m/z = 1957.95012.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)(2,3-bis(hexadecyloxy)-propyl)(benzyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (16).** Compound **15** (56 mg, 0.029 mmol) was dissolved in a 1:4 mixture of $CHCl_3$ and MeOH (1 mL). The reaction mixture was cooled down to 0 °C and *t*-butylamine (164 μL) was added. After having been stirred at 0 °C for 45 min, the mixture was evaporated without heating and the residue was purified by flash chromatography ($CHCl_3$/MeOH 8/1 to 4/1) to give compound **16** (32 mg, 82%) as an amorphous solid. R_f (**15**) = 0.81 and R_f (**16**)= 0.38 (CH_2Cl_2/MeOH 6:1). *NMR data are complex because of amide rotamers, P*-isomers, and nonequivalence of the mannosyl residues.* ^1H NMR (400 MHz, $CDCl_3/CD_3OD$ 0.35/0.2 mL, lock on CD_3OD, calibration TMS ; amide rotamers: ∼2:1 ratio): δ 0.89 (t, 6H, $2CH_3$, J = 6.6 Hz), 1.18−1.48 (m, 58H), 1.48−1.70 (m, 8H), 2.34−2.48 (m, 2H, 2H*a*), 3.30 (t, 2H, 2He, J = 6.8 Hz), 3.38−3.96 (m, 25H) 3.97−4.09 (m, 1.7H), 4.09−4.18 (m, 1H), 4.23 (m, 0.36H), 4.37−4.48 (m, 1H), 4.85−5.01 (6 lines, 2H, 2H1′), 5.11−5.20 (m, 2H, CH_2Ph), 7.33−7.48 (m, 5H, 5CHAr). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ 0.35/0.2 mL; sm = small signals, probably due to minor rotamer form): δ 14.29 (CH_3), 25.01 and 25.07, 26.70, 28.79 and 28.84, 32.87, and 33.00 (C*a*-C*d*), 23.09, 26.45, 26.48, 26.50, 29.79, 30.00, 30.13, 30.36, 32.37 (CH_2 acyl chain), 39.96 and 40.02 (C*f*), 40.76, 43.8 (broad), 45.66, 47.85 (4 signals, C1/5), 62.05, 62.12, 62.35, and 62.05 (4 signals, 2 × C6′), 67.46 (sm, CH), 67.56 (CH), 67.67 (CH), 68.07 (CH_2), 68.14 (CH_2), 68.20 (CH_2), 68.74 (CH_2), 69.90 (CH_2), 69.93 (CH_2), 70.12 (sm, CH), 70.17 (sm, CH), 70.22 (sm, CH), 70.27 (sm, CH), 70.52 (CH), 70.69−70.75 (broad, CH_2Ph), 70.93 (CH), 71.19 (CH_2O alkyl chain), 71.28 (sm, CH), 71.39 (CH), 71.60 (CH), 72.04 (sm, CH), 72.09 (sm, CH), 72.14 (sm, CH), 72.34 (CH_2O alkyl chain), 73.36 (broad, CH), 73.97 (CH), 74.05 (CH), 74.15 (CH), 74.26 (sm, CH), 74.62 (CH), 74.67 (CH), 74.75 (sm, CH), 74.81 (sm, CH), 74.85 (sm, CH), 76.42 (sm, CH), 76.48 (sm, CH), 76.58 (sm, CH), 76.66 (sm, CH), 77.26 (sm, CH), 77.50 (CH), 77.57 (CH), 97.42 and 97.47, 99.17, 100.70 and 100.72, 102.27 and 102.33 (7 signals, C1′), 116.62 (q, CF_3, J = 288 Hz), 128.73, 128.78, 128.27, 129.19, 129.25, 129.38, 129.42 (CHAr) 135.88−135.96 (CqAr), 158.40 (q, $COCF_3$, J = 37 Hz), 173.57 and 174.03 (CONH). ^{31}P NMR (162 MHz, $CDCl_3$): δ −2.17 ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ −76.43 and −76.44 (∼2:1). HRMS (ESI): $[M+H]^+$ calcd for $C_{67}H_{119}F_3N_2O_{20}P$: m/z = 1359.80404; found m/z = 1359.80231.

***N*-(6-Trifluoroacetamidohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)-(2,3-bis(hexadecyloxy)-propyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (2).** Compound **16** (31 mg, 0.023 mmol) was dissolved in a 2:1 mixture of CH_2Cl_2/EtOH (6 mL). 10% Palladium on charcoal (50 mg) was added and the reaction was stirred at rt under H_2 atmosphere for 3.5 h. The solid catalyst was removed by filtration through a Millipore membrane. The catalyst was washed with a 1:1 mixture of CH_2Cl_2 and EtOH (2 × 20 mL). The solvents were evaporated to give compound **2** (26 mg, 90%) as a solid. R_f (**16**) = 0.65, R_f (**2**) = 0.20 (CH_2Cl_2/MeOH 4:1). *The NMR data are complex because of amide rotamers (∼70/30) and nonequivalence of the mannosyl residues.* ^1H NMR (400 MHz, $CDCl_3/CD_3OD$ 0.35/0.2 mL + 1 drop D_2O − lock on CD_3OD): δ 0.85 (t, 6H, $2CH_3$, J = 6.8 Hz), 1.14−1.41 (m, 69H), 1.48−1.67 (m, 9H), 2.33−2.44 (m, 2H, 2H*a*), 3.26 (app t, 2H, 2He, J = 6.6 Hz), 3.39−3.93 (m, 28H), 3.93−4.08 (m, 2H), 4.31−4.46 (m, 1H), 4.98 (several br s, 2H, 2H1′). ^{13}C NMR (101 MHz, *J*-Mod, $CDCl_3/CD_3OD$ 0.35/0.2 mL + 1 drop D_2O − lock on CD_3OD): δ 14.33 (CH_3), 25.12 and 25.17, 26.79, 28.84 and 28.84, 32.93, and 33.06 (C*a*-C*d*), 23.14, 26.52, 26.57, 29.84, 30.02, 30.14, 30.41, 30.89, 32.41 (CH_2 acyl, alkyl chains), 40.04 and 40.10 (C*e*), 40.59, 43.85, 45.69, and 47.87 (4 signals, C1 and C5), 61.94 and 62.14 (2C6′), 66.87 (CH_2), 67.48, 67.56 (CH), 67.84 (small broad CH), 70.50 (CH), 70.55 (CH_2), 70.64 (CH), 70.98 and 71.02 (CH), 71.32 (OCH_2), 71.40 (CH), 71.65 (CH), 72.38 (OCH_2), 72.90 (CH), 73.91 (CH), 74.14 (CH), 74.57 (CH), 78.11 (C3), 97.42, 99.14, 100.06, and 101.77 (4 signals, C1′), 116.67 (q, CF_3, J = 288 Hz), 158.46 (q, $COCF_3$, J = 37 Hz), 173.79 and 174.28 (CONH). ^{31}P NMR (162 MHz, $CDCl_3$): δ −1.45 ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ −76.45 (small pic) and −76.44. HRMS (ESI): $[M-H]^-$ calcd for $C_{60}H_{111}F_3N_2O_{20}P$: m/z = 1267.74254; found m/z = 1267.74310.

***N*-(6-Aminohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)(2,3-bis(hexadecyloxy)propyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (17).** Compound **2** (23 mg, 0.018 mmol) was dissolved under Ar in MeOH (2 mL). A solution of MeONa was prepared (∼8 mg Na in MeOH (2 mL)) under Ar. This solution was transferred to the solution of compound **2**. The reaction mixture was then stirred for 40 h at 25 °C under vacuum (∼0.4 bar) to trap methyl trifluoroacetate (bp = 43−43.5 °C) until complete deprotection. The base was neutralized with AcOH (2 drops) and the solvent was evapored to give compound **17** containing sodium acetate. R_f (**2**) = 0.78, R_f (**17**) ∼0.1 (CH_2Cl_2/MeOH/D_2O 70/40/6) (pink in ninhydrin spot test). ^1H NMR (400 MHz, $CDCl_3/CD_3OD$ 0.4/0.3 mL − lock on CD_3OD): δ 0.85 (CH_3), 1.15−1.34 (m, 52H), 1.34−1.47 (m, 2H, 2H*c*), 1.47−1.59 (m, 4H, $2CH_2$ alkyl chain), 1.59−1.74 (m, 4H, 2H*b*, 2H*d*), 2.25−2.39 (m, 1H, H*a*A), 2.42−2.63 (m, 1H, H*a*B), 2.80−3.00 (m, 2H, 2He), 3.01−3.16 (m, 1H), 3.39−3.50 (m, 4H, OCH_2 alkyl chain +2H), 3.50−3.99 (several m, 21H), 4.26−4.63 (m, 2H), 4.93−5.03 (2 br s, 1.4H, H1′), 5.08−5.16 (m, 0.6H, H1′). ^{31}P NMR (162 MHz, $CDCl_3$): δ 0.197 ppm. ^{19}F NMR (376 MHz, $CDCl_3/CD_3OD$ 0.4/0.3): δ −76.39 (trace of fluorinated impurity, probably CF_3COONa). HRMS (ESI): $[M+H]^+$ calcd for $C_{58}H_{114}N_2O_{19}P$: m/z = 1173.77479; found m/z = 1173.77413.

***N*-(6-Biotinylamidohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)(2,3-bis(hexadecyloxypropyl)-**

dx.doi.org/10.1021/bc3004974 | *Bioconjugate Chem.* 2013, 24, 72−84

phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (3). Compound **17** (20 mg, 0.0167 mmol) was dissolved in dry DMF (0.5 mL) under Ar; 0.5 mL of CH_2Cl_2 was then added to increase solubility. Et_3N (116 μL, 0.084 mmol, 5 equiv) was added, then (+)-biotin *N*-hydroxysuccinimide ester (6.9 mg, 0.02 mmol, 1.2 equiv). The mixture was stirred for 5 h under Ar. The solvents were evaporated under vacuum. The residue was purified by gel permeation chromatography on Sephadex LH-20 (diam = 25 mm, H = 400) with MeOH/CH_2Cl_2 2/1 as the eluent (V_0 = 15 mL, fractions = 5 mL, collecting compound in tubes 14 to 19) to give the expected biotinylated derivative **3** (17 mg, 74%) as a white solid. R_f (**3**) = 0.35 (CH_2Cl_2/MeOH/D_2O 70/40/6). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL + 1 drop D_2O − lock on CD_3OD; amide rotamers in ratio ∼2:1): δ 0.85 (CH_3), 1.15−1.45 (m, 58H), 1.45−1.75 (m, 10H), 2.19 (t, 2H, $2H_I$), 2.26−2.49 (m, 2H, 2H*b*), 2.71 (d, 1H, H_DA, J = 12.4 Hz), 2.9 (dd, 1H, H_DB, J = 4.8, 12.4 Hz), 3.06−3.22 (m, 4H, 2H*f*, $2H_E$), 3.39−4.0 (several m, 28H), 4.26−4.34 (m, 1H, H_B), ∼4.5 (hidden by HOD, H_C), 4.92 (s, 0.31H, H1′), 4.95 (s, 0.69H, H1′), 5.01 (s, 0.3 H, H1′), 5.09 (s, 0.7H, H1′). ^{13}C NMR (62.9 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL + 1 drop of D_2O − lock on CD_3OD): δ 14.37 (CH_3), 23.14, 25.23, 26.53, 26.53, 26.60, 27.06, 28.60, 28.86, 29.35, 29.84, 30.06, 30.20, 30.88, 32.41, 32.92, 36.15, 39.74 and 39.81, 40.72 (all CH_2), 56.24 (CH), 60.81 (CH), 61.87 (broad, CH_2), 62.51, 65.74 (broad), 67.35 (CH), 68.11 (broad, CH), 68.83 (CH_2), 70.17−70.45 (broad, CH_2), 70.85 (broad, CH_2), 71.13 (CH), 71.22 (OCH_2 alkyl), 71.38 (CH), 71.53 (CH), 71.71 (CH), 72.32 (OCH_2 alkyl), 74.00 (CH), 74.48 (CH), 78.16 (CH), 97.36 (C1′), 98.28 (C1′), 100.57 (C1′), 103.50 (C1′), 165.07 and 165.10 (CO biotin), 174.61 (CONH), 175.34 (CONH). ^{31}P NMR (162 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL + 1 drop of D_2O): δ − 1.856 ppm. ^{19}F NMR (376 MHz): no signal. HRMS (ESI): $[M+H]^+$ calcd for $C_{68}H_{128}N_4O_{21}PS$: m/z = 1399.85239; found m/z = 1399. 85097.

Tetramethylrhodamine-Labeled *N*-(6-aminohexanoyl)-2,4-bis-*O*-(α-D-mannopyranosyl)-3-*O*-[(2*R*)(2,3-bis(hexadecyloxy)propyl)phosphoryl]-1,5-dideoxy-1,5-iminoxylitol (4). Compound **17** (21 mg, 0.0176 mmol) was dissolved in dry DMF (1 mL) under Ar. Et_3N (100 μL, 0.724 mmol, 41 equiv) was added followed by tetramethylrhodamine isothiocyanate (TRITC, mixture of 5- and 6-isomers; 10 mg, 0.02 mmol, 1.1 equiv). The mixture was stirred for 15 h under Ar. The solvent was then removed under vacuum. The residue was first submitted to gel permeation chromatography on Sephadex LH-20 (diam = 25 mm, H = 400 mm) with MeOH/CH_2Cl_2 2/1 as the eluent. A second purification by flash chromatography (CH_3Cl_3/MeOH/D_2O 70/40/5) was necessary to give pure compound **4** (15 mg, 52%) as an amorphous magenta solid. R_f ∼0.63 (CH_3Cl_3/MeOH/D_2O 70/40/6). ^{1}H NMR (250 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL; lock $CDCl_3$; calibration TMS): δ 0.88 (t, 6H, $2CH_3$, J = 6.5 Hz), 1.11−1.46 (m, 54H), 1.47−1.74 (m, 8H), 2.27−2.55 (m, 2H, 2H*a*), 3.00−3.31 (m, 12H, $4CH_3$−Rh), 3.39−4.00 (30H), 4.96 (br s, 0.3H, H1′), 4.99 (br s, 0.7H, H1′), 5.08 (br s, 0.3H, H1′), 5.18 (br s, 0.7H, H1′), 6.67 (s, 2H, 2CH-Rh), 6.80−6.87 (m, 2H, 2CH-Rh), 7.20−7.45 (m, 3H, 3CH-Rh), 7.87−8.18 (2H, 2CH-Rh). ^{13}C NMR (126 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL− lock $CDCl_3$ − Calibration $CDCl_3$): δ 13.58 (CH_3), 22.34, 24.59, 24.78, 25.78, 25.82, 26.43, 28.12, 29.0−29.7, 31.62, 32.14 (C*b*, major rotamer), 32.39 (C*a*, minor rotamer), 38.93, 40.09 (CH_3−Rh), 43.84, 46.70, 60.23, 61.11, 61.33, 64.98, 66.22, 66.41, 67.29, 68.05−68.47 (broad), 69.74, 69.91, 70.14, 70.33, 70.38 (OCH_2), 70.50, 70.69, 70.91, 71.47 (OCH_2), 72.93, 73.13, 73.55, 77.35, 77.65, 77.71, 96.23 (CHAr-Rh), 96.33 (broad, C1′), 97.54 (broad, C1′), 112.72 (CHAr-Rh), 121.79 (broad, CAr-Rh), 122.58 (broad, CAr-Rh), 129.77 (CHAr-Rh), 131.34 (CHAr-Rh), 141.88 (broad, CAr-Rh), 156.05 (minor rotamer), 156.14 (major), 156.61 (minor), 156.69 (major) (CAr-Rh), 171.09 (COOH-Rh), 173.11 (CONH minor rotamer), 173.57 (CONH major), 180.24 (CS). ^{31}P NMR (162 MHz, $CDCl_3$/CD_3OD 0.35/0.2 mL − lock $CDCl_3$): δ 4.05 (minor rotamer), 4.26 (major rotamer). ^{19}F NMR (376 MHz): no signal. HRMS (ESI): $[M+H]^+$ calcd for $C_{83}H_{135}N_5O_{22}PS$: m/z = 1616.905155; found m/z = 1616.903886.

BIOLOGICAL PROCEDURES

Bioassays. Murine bone marrow cells harvested from femurs were cultivated (10^6/mL) for 7 days in Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, 20% horse serum, and 30% L929 cell-conditioned medium as a source of M-CSF. After three further days in fresh medium, the bone marrow-derived macrophages (BMDM; 10^5 cells/well) in DMEM supplemented with 2 mM L-glutamine, 2 × 10^{-5} M β-mercapto-ethanol, and 0.1% fetal calf serum (FCS) were stimulated with 100 ng/mL of LPS (*Escherichia coli*, serotype O111:B4, Sigma, St. Louis, MO) in the presence of IFN-γ (500 U/mL). Lyophilized PIM preparations were solubilized in DMSO and used at a noncytotoxic, maximum 1% DMSO final concentration. PIM or DMSO vehicle controls were added 30 min prior to LPS stimulation (100 ng/mL). Absence of cytotoxicity was controlled using MTT incorporation. Cell culture supernatants were harvested after 24 h at 37 °C and TNFα and IL-12p40 cytokine content were measured using commercially available ELISA Kits (Duoset R&D Systems, Abingdon, U.K.). Nitrite was determined using the Griess reaction (3% phosphoric acid, 1% *p*-aminobenzenesulfonamide, 1% *N*-(1-naphthyl)ethylenediamine). Results are expressed as percent of inhibition as compared with full release in the presence of LPS plus DMSO vehicle.

Immunoassay. Biotinylated PIM_2 derivative **3** at the indicated concentrations in PBS (100 μL/well) was coated on a microtiter plate and incubated overnight at room temperature. After saturation with 1% BSA in PBS (200 μL/well) for 1 h at room temperature and three washings with 0.05% Tween 20-PBS, the bound PIM−biotin was revealed by incubation with horseradish peroxidase−streptavidin D conjugate (1/2000, Vector Laboratories) diluted in 1% BSA in PBS, for 20 min at room temperature. After addition of the substrate (ABTS, 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt, at 0.3 g/L in 0.1 M anhydrous citric acid containing 0.3% H_2O_2) for 20 min at room temperature, absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instrument, Inc.).

Flow Cytometry. Macrophages (1 × 10^6 cells in 50 μL DMEM containing 0.2% FCS) were incubated with biotinylated PIM_2 derivative **3** at the indicated concentrations in PBS (100 μL/well) for 1−30 min at 37 °C under gentle agitation. Cells were washed with ice-cold PBS and stained with streptavidin−Alexa A532 conjugate (diluted 100× in cold PBS) for 15 min. After further washings and fixation with 3% paraformaldehyde (PFA), fluorescent cells were analyzed on a FACS Canto II equipment (BD Bioscience, San Jose, Ca, USA) using a Tree Star software entitled *FlowJo* (Trustees of Leland

dx.doi.org/10.1021/bc3004974 | *Bioconjugate Chem.* 2013, 24, 72−84

Stanford Jr. University, Stanford, CA, USA). Cytotoxicity was controlled by 7-amino-actinomycin (7-AAD) staining.

Confocal Microscopy. Macrophages adsorbed on glass microslides (1×10^5 cells in 200 μL DMEM containing 5% FCS) overnight at 37 °C and rinsed once with PBS were incubated with biotinylated-PIM_2 conjugate **3** (5 μg/mL in DMEM containing 1% FCS for 10 min at 37 °C). After rapid fixation (1 min at RT in 4% PFA) and rinsing in PBS, cells were incubated with Streptavidin−Alexa 532 fluorescent conjugate (10 μg/mL for 15 min at RT) and fixed (10 min at RT in 4% PFA). The microslides were then washed twice with PBS, mounted in Vectashield medium (Vector Laboratories, Inc., Burlingame, CA, USA), and analyzed by confocal microscopy.

RESULTS AND DISCUSSION

Synthesis of a *N*-ω-Aminocaproyl Derivative of aza-PIM_2 in Ester Series (1). The first objective of the project was

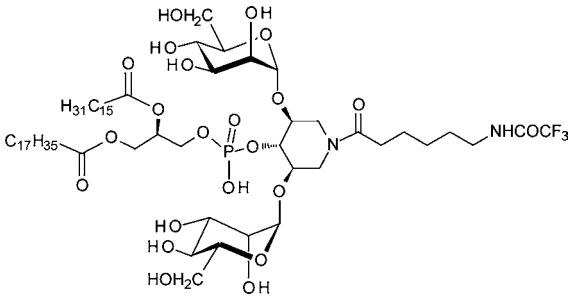
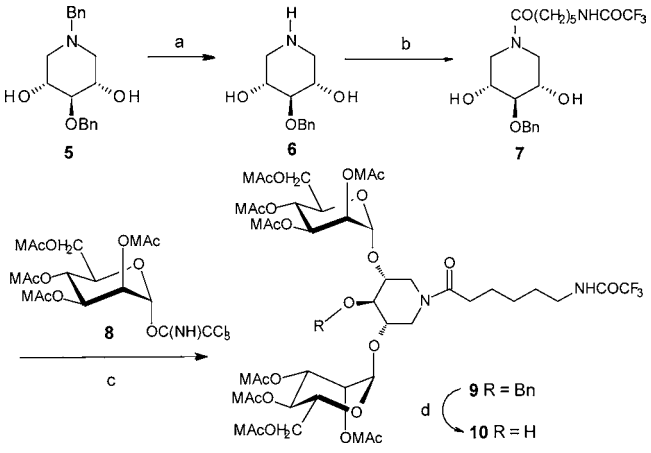


Figure 2. Ester series: N-TFAC (*N*-ω-TriFluoroAcetamidoCaproyl) precursor **1**.

Scheme 1. Synthesis of Precursor 10 (Diester Series)

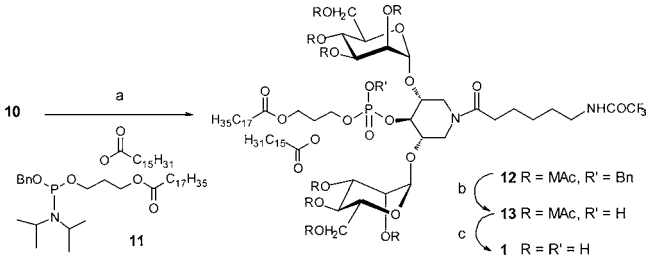


Conditions: (a) EtOH, H_2, 20% $Pd(OH)_2/C$, quant.; (b) $CF_3CONH(CH_2)_5COOH$, CH_2Cl_2, HOBT, DCC, 60%; (c) **8** (2 equiv), TMSOTf, CH_2Cl_2, 37%; (d) CH_2Cl_2/EtOH, H_2, 10% Pd/C, 98%.

the synthesis of an aza-PIM_2 mimic carrying an *N*-aminocaproyl residue protected with a $COCF_3$ group (N-TFAC precursor **1**, Figure 2) which could be used to tether various reporter groups after deprotection. This compound was prepared from iminoxylitol **6** by the sequence of reactions described in Schemes 1 and 2.

The synthesis of the previously described iminoxylitol derivative **6**[17,18] was significantly improved: selective cleavage of the *N*-benzyl group[20] could be achieved quantitatively by catalytic hydrogenolysis of purified **5** using Pearlman's catalyst.

Scheme 2. Synthesis of Diester Precursor 1



Conditions: (a) **11**, CH_2Cl_2, 1*H*-tetrazole, then *m*CPBA, 47%; (b) H_2, 10% Pd/C, CH_2Cl_2/EtOH, 95%; (c) MeOH/CH_2Cl_2, *t*BuNH$_2$, 45 min, −17 °C, 78%.

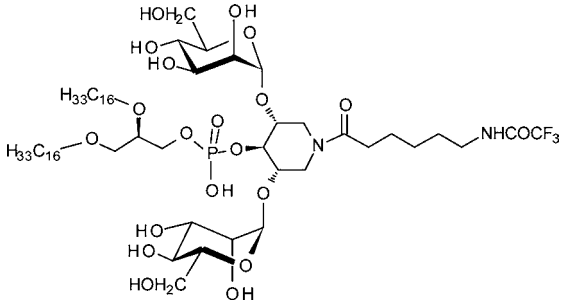
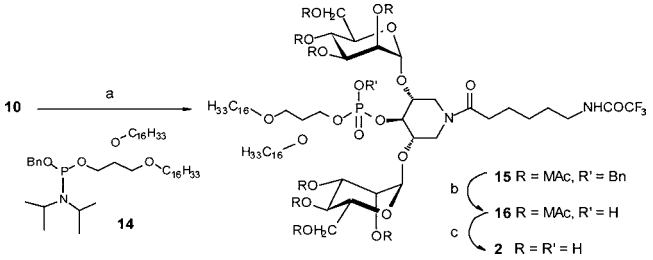


Figure 3. Ether series: N-TFAC precursor **2**.

Scheme 3. Synthesis of Diether Precursor 2



Conditions: (a) **14**, CH_2Cl_2, 1*H*-tetrazole, then *m*CPBA, 43%; (b) MeOH/$CHCl_3$, *t*BuNH$_2$, −17 °C, 82%; (c) H_2, CH_2Cl_2/EtOH, 10% Pd/C, 90%.

N-Acylation followed by double glycosylation using the trichloroacetimidate of tetra-*O*-methoxyacetyl-α-D-mannopyranose (**8**) and *O*-debenzylation afforded the protected precursor **10** in good yield (Scheme 1).

Compound **10** was then phosphorylated by reaction with phosphoramidite **11**[17] and oxidation of the resulting phosphite to give phosphotriester **12** in 47% yield[a] (Scheme 2). The benzyl phosphate group was then cleaved by hydrogenolysis and the mannosyl residues deprotected from methoxyacetate groups by reaction with *t*-butylamine in methanol, to provide the diester precursor **1** in 78% yield.

We showed earlier that a mimetic of PIM_2 in which the cyclitol is replaced by a 3-C glycerol scaffold could recapitulate the inhibitory activity of natural PIM_2 purified from *M. bovis* BCG on LPS-induced macrophage release of IL-12p40 and IL-10.[12] PIM_2 mimetic also reproduced the inhibitory activity of natural PIM_2 on TNF and NO release, while being non-cytotoxic (data not shown). Aza-PIM_2-NTfa (**IA**) and Oxa-PIM_2 (**IB**) retained the activity of PIM_2 mimetic and were at least as active as PIM_2 mimetic for inhibition of TNF, IL-12 p40, and NO release by LPS-induced macrophages, with **IB**

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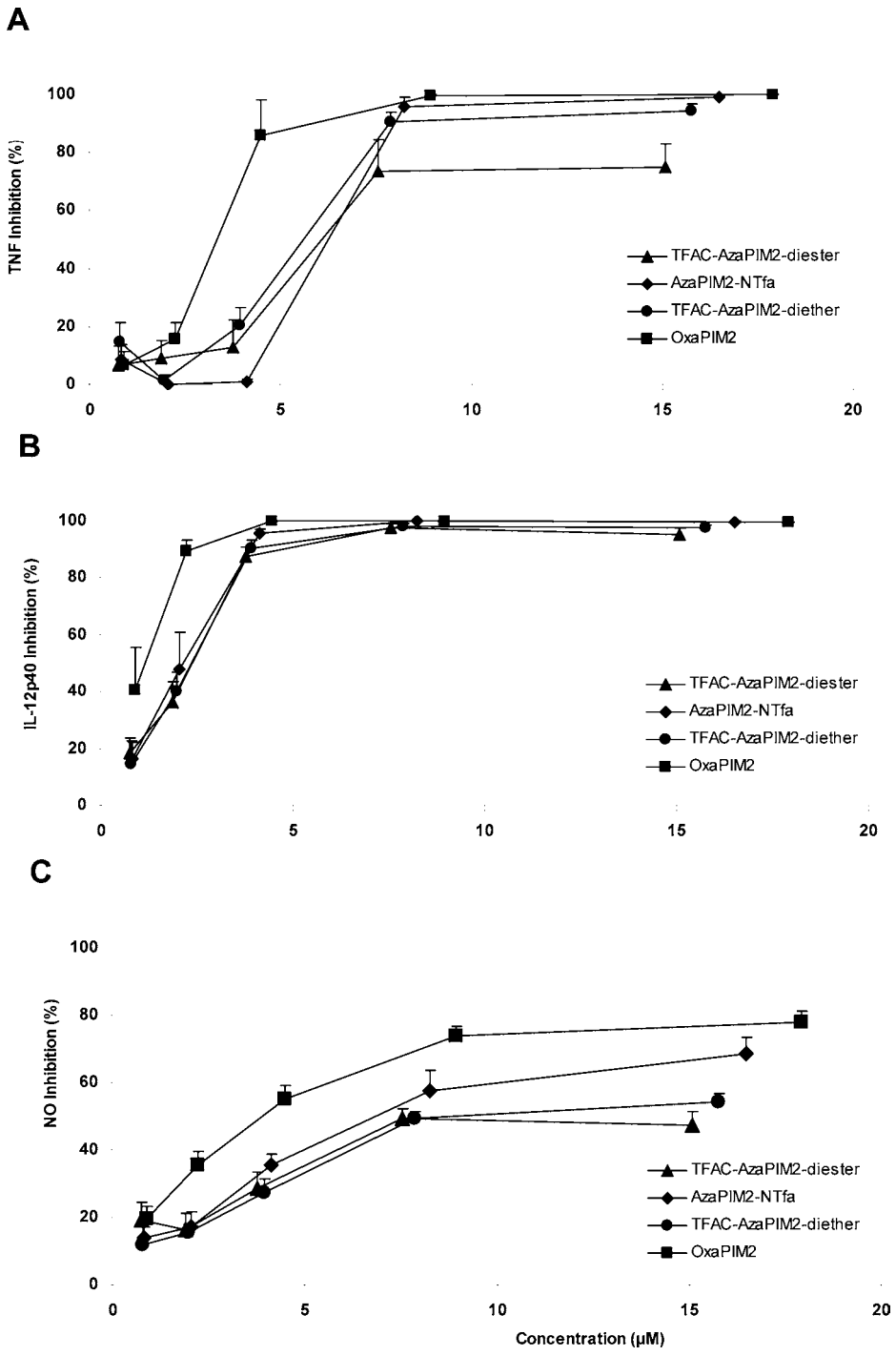

Figure 4. Both ester and ether forms of PIM_2 heterocyclic analogues inhibit TNFα, IL-12p40, and NO release by LPS-stimulated macrophages. Bone marrow-derived macrophages were incubated for 24 h with LPS in the presence of increasing concentrations of oxa-PIM_2 analogue (**IB**), aza-PIM_2 analogue (**IA**), compound **1** (TFAC-AzaPIM_2-diester), and compound **2** (TFAC-AzaPIM_2-diether). TNFα (A), IL-12p40 (B), and NO (C) concentrations were measured in the supernatants. Results are expressed as percent of inhibition of the control stimulation performed with LPS plus vehicle and represent the mean ± SEM of $n = 4$ values from two independent experiments.

(Oxa-PIM_2) being slightly more active than **IA** (AzaPIM_2-NTfa) (Supporting Information Figure 1). The ability of AzaPIM_2-diester derivative **1** to inhibit LPS-induced pro-inflammatory response was tested on murine bone marrow-derived macrophages (see Figure 4A–C). Compound **1** inhibited the release of TNFα, IL-12p40, and NO essentially as strongly as precursor **IA**. The aza-derivatives were slightly less potent than the oxa PIM_2 analogue **IB**, which is shown as a reference. Thus, the addition of the aminocaproyl spacer did not alter the immuno-modulating activity of the parent aza-PIM_2.

Further elaboration of **1** revealed, however, that the chemical synthesis of conjugated derivatives from **1** would not be successful: indeed under the basic conditions required to remove the N-$COCF_3$ group,[21] the cleavage of the fatty acid esters occurred more rapidly. We therefore decided to replace

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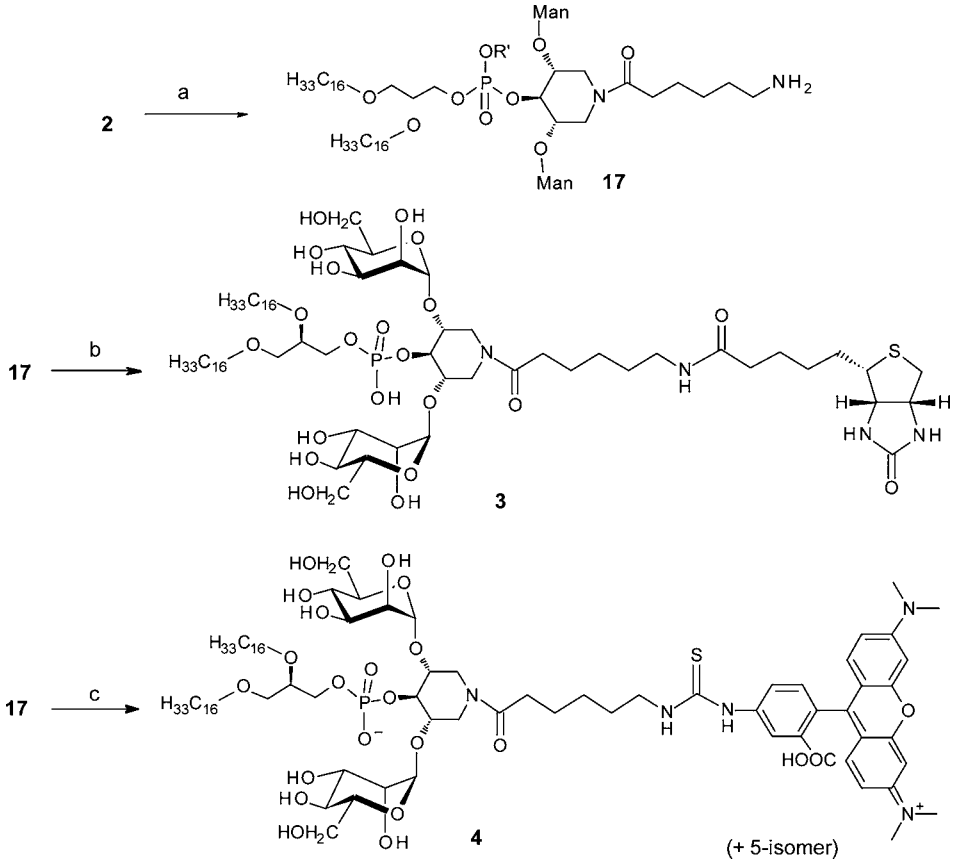
Scheme 4. Synthesis of 3 and 4 from 2



Conditions: (a) MeOH, NaOMe, 40 h, 25 °C, 0.4 bar; (b) DMF, Et_3N, CH_2Cl_2, (+)-biotin *N*-succinimide ester, 5 h, 74% ; (c) DMF, Et_3N, TRITC, 15 h, 52%.

the glycerol esters with ethers and to proceed with PIM mimics carrying a di-*O*-hexadecyl glycerol unit as in compound **2** (Figure 3). Previous work has already shown that esters can be replaced by ether linkages in PIMs without loss of biological activity;[16,19] subtle differences, however, have been recently observed between PIM_2 analogues carrying one alkyl chain at *sn*-2 or at *sn*-1 glycerol position or two alkyl chains in their ability to induce cytokine (IL-6) production by macrophages (agonist effect), but all three types of compounds retained the ability of the diester to inhibit LPS-induced production of this cytokine by the same immune system cells.[16]

Synthesis of a *N*-ω-Aminocaproyl Derivative of aza-PIM_2 in Ether Series (2). The synthesis of the di-*O*-hexadecyl analogue of **1**, compound **2**, was achieved from **10** by coupling with phosphoramidite **14**,[19] oxidation, and deprotection of the resulting benzyl phosphate to afford compound **16** (Scheme 3). The methoxyacetyl groups of **16** could be cleaved selectively without affecting the trifluoroacetamido group in high yield (82%) using *t*-butylamine in MeOH, thus providing diether precursor **2**. The biological activity of AzaPIM_2-diether **2** was tested on LPS-stimulated macrophages, and a clear, dose-dependent inhibition of TNFα, IL-12p40, and NO release documented (Figure 4A−C). Therefore, diether **2** retained the immuno-modulating activity of the corresponding diester **1** and of the parent aza-PIM_2 (**IA**).

Synthesis of 3 and 4 from 16. Cleavage of the trifluoroacetyl group from **2** required the use of sodium methoxide in MeOH. This reaction was performed under reduced pressure to eliminate methyl trifluoroacetate. This provided the key aza-PIM_2 derivative **17** functionalized for conjugation or coupling with various markers (Scheme 4). The fully deprotected PIM_2 mimic **17** could be coupled in one step to (+)-biotin using biotin *N*-succinimidyl ester derivative to give conjugate **3** in 74% yield. Compound **17** was also engaged in a coupling reaction with TRITC (tetramethylrhodamine isothiocyanate) and gave the corresponding labeled derivative **4** (as a mixture of two isomers at the phenyl group). Both compounds were fully characterized before they were engaged in biological studies.

The biological activity of biotinylated-PIM_2 conjugate **3** was first tested in terms of inhibition of pro-inflammatory cytokine release by LPS-stimulated macrophages. The dose-dependent inhibition of TNFα, IL-12p40, and NO release was at least as good as the activity measured with the diether precursor **2** (Figure 5A−C). Dipalmitoyl phosphatidyl inositol PI, used as a non-mannosylated, negative control, exhibited strongly reduced inhibitory activity. None of the compounds induced cytotoxicity at the concentrations investigated, as measured by MTT assay (data not shown). Therefore, biotinylated PIM_2 mimics **3** retain the immuno-modulating activity of the parent PIM_2 diether **2**.

In contrast, the derivative labeled with rhodamine (**4**) appeared to be cytotoxic for macrophages in our culture conditions, as observed by microscopy, while standard MTT or NO release assays were hampered by the strong absorption of the compound. Although IL-12p40 production seemed to be

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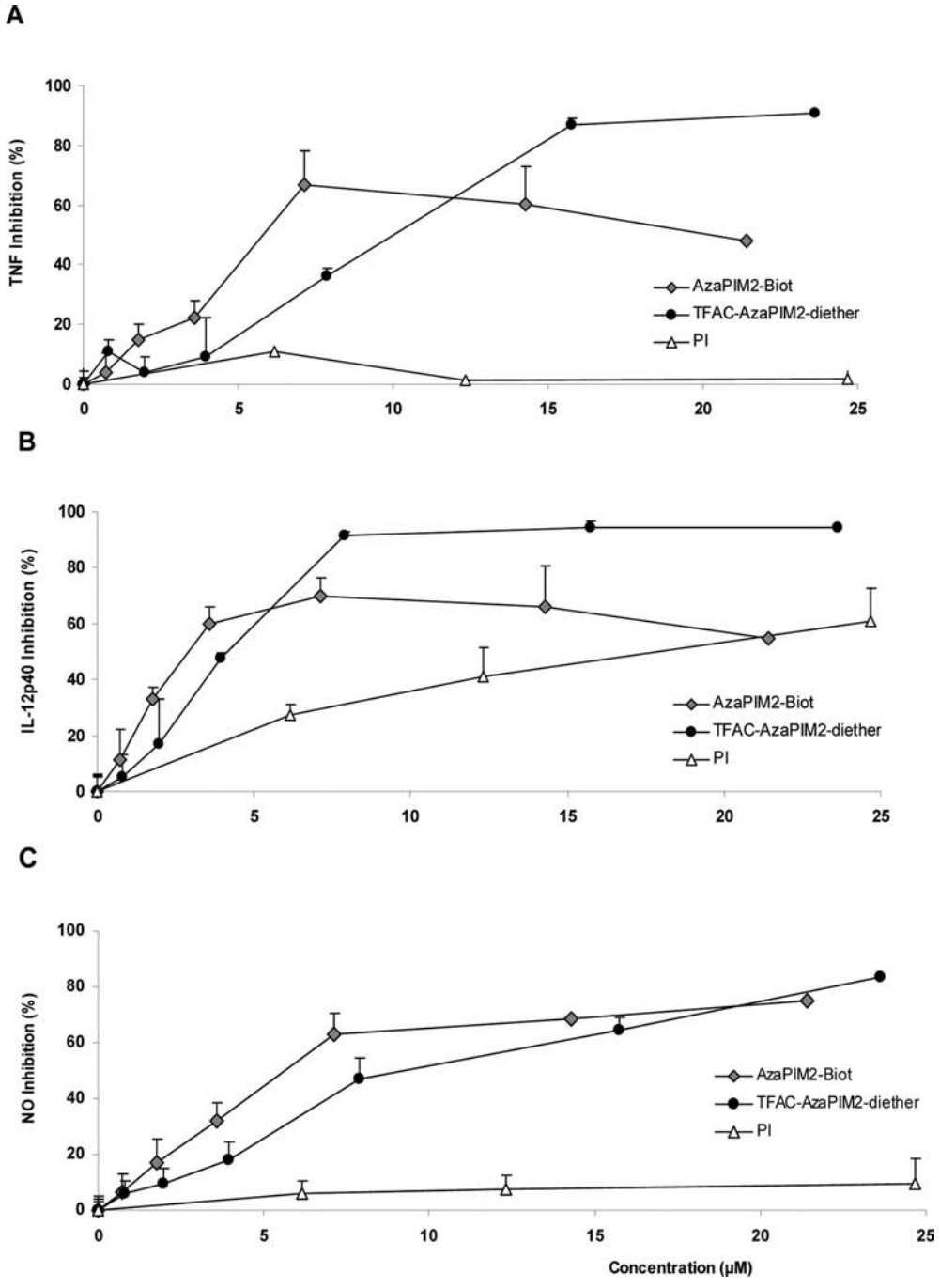

Figure 5. Biotinylated PIM_2 derivative **3** retains inhibitory activity on TNFα, IL-12p40, and NO release by LPS-stimulated macrophages. Bone marrow-derived macrophages were incubated for 24 h with LPS in the presence of increasing concentrations of biotinylated-PIM_2 conjugate **3** (AzaPIM$_2$-Biot), precursor **2** (TFAC-AzaPIM$_2$-diether), and dipalmitoyl phosphatidyl inositol (PI) as a negative control. TNFα (A), IL-12p40 (B), and NO (C) concentrations were measured in the supernatants. Results are expressed as percent of inhibition of the control stimulation performed with LPS plus vehicle and represent the mean ± SEM of $n = 6$ values from 3 independent experiments representative of 4 independent experiments.

slightly decreased in the presence of labeled derivative **4**, an effect which may be linked to the cytoxicity of this compound, it is worth noting that TNFα release was not inhibited at concentrations up to 20 μg/mL (data not shown). Further titration and kinetic studies will be needed to fully assess how this compound might be used. PIM cellular labeling was thus further investigated with biotinylated-PIM_2 conjugate **3**.

Functional Characterization of Biotinylated PIM Derivative. For functional characterization, aza-PIM-biotin conjugate **3** was first titrated in a solid phase assay (Figure 6A). Biotinylated PIM was adsorbed onto a solid phase in a microtiter plate overnight at room temperature and revealed by incubation with a streptavidin–peroxidase conjugate, followed by substrate addition and spectrophotometric detection of the colored product. Dose-dependent coating of the biotinylated PIM was detected at concentrations from 0.05 to 1 μg/mL. There was no further increase in PIM-biotin adsorption at concentrations of 1–20 μg/mL (not shown). Thus, the biotin moiety grafted on the PIM molecule was stable in our

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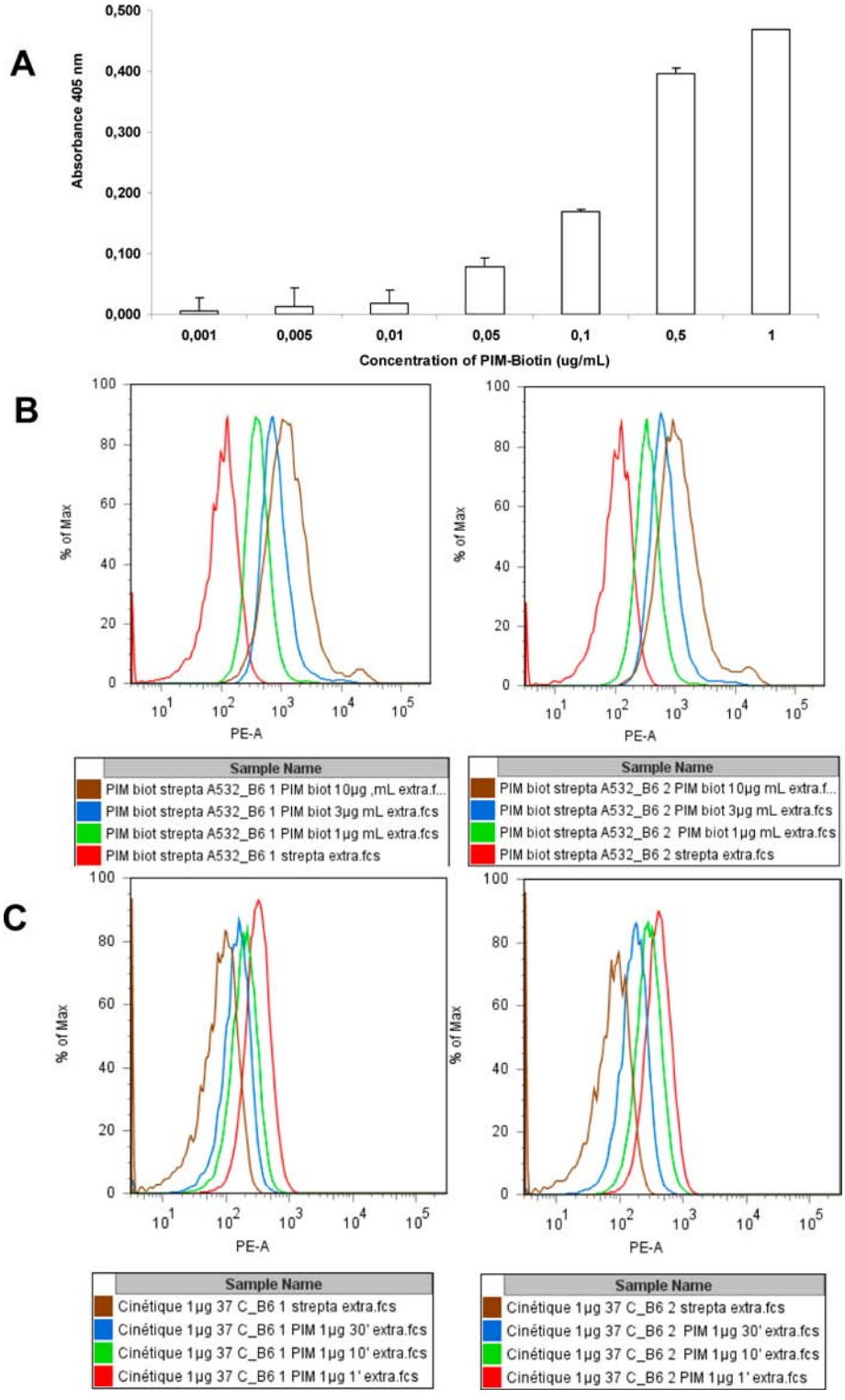

Figure 6. Biotinylated-PIM$_2$ derivative **3** is detected by fluorescent streptavidin conjugate and labels macrophages: (A) Aza-PIM$_2$-biotin conjugate **3** at increasing concentrations (0.001−1 μg/mL) was adsorbed onto a microtiter plate overnight at room temperature. After saturation of the free binding sites and washings, bound PIM-biotin was revealed with a streptavidin−peroxidase conjugate, followed by substrate addition and spectrophotometric detection of the colored product at 406 nm. (B) Macrophages were incubated with biotinylated-PIM$_2$ conjugate **3** (1, 3, and 10 μg/mL) for 30 min at 37 °C and cell-bound biotin detected by flow cytometry using a streptavidin−Alexa 532 fluorescent conjugate (10 μg/mL). (C) Kinetic study of biotinylated-PIM$_2$ binding to macrophages revealed a strong labeling already after 1 min incubation with conjugate **3** (1 μg/mL), which was less pronounced after 10 and 30 min. Results from 2 independent macrophage populations from two different mice are shown in B and C.

experimental conditions and could be detected by binding of a labeled streptavidin conjugate.

To further evaluate whether biotinylated PIM functionally bind immune cells, bone marrow-derived macrophages were

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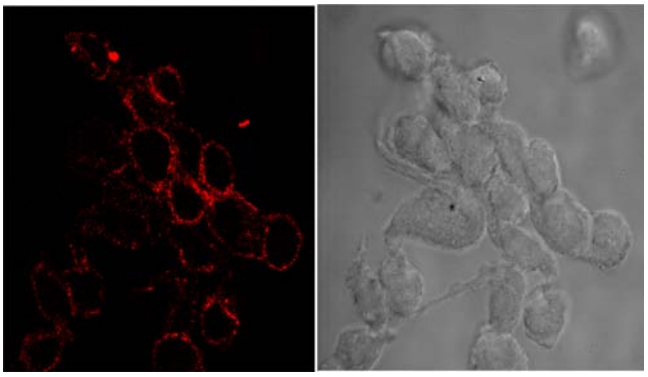

Figure 7. Subcellular localization of biotinylated PIM conjugate **3** on macrophages. Macrophages adsorbed on glass microslides overnight at 37 °C were incubated with biotinylated-PIM_2 conjugate **3** (5 μg/mL for 10 min at 37 °C). Cell-bound biotin was detected using a streptavidin–Alexa 532 fluorescent conjugate (10 μg/mL). Red labeling indicative of a membrane localization of the PIM–biotin conjugate (left) and the corresponding cell morphology (right) are shown. Negative controls with streptavidin conjugate alone showed no background fluorescence (not shown).

incubated in the presence of increasing concentrations of PIM-biotin and cell-associated biotin detected by flow cytometry using a streptavidin–Alexa 488 fluorescent conjugate (Figure 5B). A dose-dependent labeling of macrophages was evident at PIM–biotin conjugate concentrations of 1 to 10 μg/mL after 30 min incubation at 37 °C. No cytotoxicity was associated with incubation at these concentrations of biotinylated PIM derivative **3**, although higher concentration (20 μg/mL) yielded some cell mortality, as detected by 7-AAD labeling (not shown). The kinetics of the association of biotinylated PIM to macrophages (Figure 5C) revealed a strong binding already at 1 min of incubation. PIM-biotin labeling was less pronounced at 10 and 30 min, which might be due to desorption or internalization. Thus, the PIM–biotin conjugate binds macrophages with a rapid kinetics.

Confocal microscopy was then used to localize the biotinylated PIM derivative at the subcellular level. Macrophages incubated with PIM–biotin conjugate **3** were revealed with a streptavidin–Alexa 532 fluorescent conjugate (Figure 7). The clear membrane labeling indicated that under these conditions PIM conjugates associated preferentially with the membrane on macrophages.

In conclusion, we demonstrated here the following:

(1) A biotin-aza-PIM_2 conjugate (compound **3**) and a fluorescent tetramethylrhodamine-aza-PIM_2 conjugate (compound **4**) could be efficiently constructed by chemical synthesis by taking advantage of the reactive function present in the piperidine ring of the aza-PIM_2 mimic (as in **IA**), thus overcoming the difficult problem of labeling PIMs based on an intact, natural inositol scaffold.

(2) The introduction of a functionalized spacer of the acylaminocaproyl (AC) type onto the piperidine ring of the PIM_2 mimic does not alter the immuno-modulating activity of the parent compound.

(3) The conjugation of biotin to the aza-PIM_2 analogue by way of the AC spacer does not hamper its biological activity: this conjugate does not exhibit cytotoxicity at the concentrations used for the assays and it retains the ability to inhibit LPS-induced production of pro-inflammatory cytokines such as TNFα and IL12p40.

(4) In contrast with **3**, the aza-PIM_2 mimic carrying a fluorescent label, compound **4**, exhibits significant cytotoxicity which will limit its use in further biological studies.

(5) In addition, our first experiments with biotin-aza-PIM_2 conjugate **3** clearly showed that PIMs rapidly associate with macrophage cell membrane. This compound will be an invaluable tool to further analyze the mode of action of the PIMs. Among the many questions that are amenable to study with this conjugate or related molecules are the colocalization with cell-surface receptors, co-receptors, co-stimulatory molecules, or with molecules associated with their downstream pathways, the kinetics of internalization, and cellular compartmentalization.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by CNRS, University of Orléans, and European Union FEDER no. 1649-32264.

■ ADDITIONAL NOTE

[a]Because of the presence of slow-exchanging amide rotamers, of *P**-diastereoisomers, and of the nonequivalence of the sugar moieties, the NMR spectrum of **12** is highly complex, exhibiting in particular eight anomeric signals of nearly equal intensities. The ^{1}H NMR spectrum of precursor **10** in DMSO was recorded at 90 °C; at this temperature, the spectrum showed clearly a single species present with signals of two non-equivalent mannosyl units.

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