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Synthesis of the Tetrasaccharide Cap Domain of the Antigenic Lipophosphoglycan of *Leishmania donovani* Parasite

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Abstract—In this paper we report a new synthesis of the immunologically important tetrasaccharide terminal cap domain [Gal*p*(1-4)-β-[Man*p*-(1-2)-α-Man*p*-(1-2)-α]-Man*p*] of lipophosphoglycan (LPG); the major cell surface GPI molecule and key virulence factor of the protozoan parasite *Leishmania donovani*. The synthetic approach provided a short convergent route for the LPG cap motif from lactose and mannose starting materials. The synthesis was then applied for the preparation of a radiolabelled cap epitope for macrophage receptor binding and immunological studies.

Introduction

The protozoan parasite *Leishmania donovani* causing fatal visceral leishmaniasis (kala azar) has a remarkable ability to survive and proliferate in extreme environments during its digenetic life cycle in sandfly vector and human host. Upon deposition of the promastigote form of the parasite into human tissue after a sandfly bite, rapid attachment and entry into the macrophage and transformation to the amastigote form are critical for the parasite. The major parasite cell surface molecule lipophosphoglycan (LPG) expressed[1] abundantly on the promastigote has been implicated in the binding of the parasite in epithelial cells of sandfly midgut and receptor mediated phagocytosis by macrophage via direct interaction with carbohydrate binding sites. There is substantial evidence[1] that LPG is an antigenic and multifunctional virulence factor essential for infectivity and survival of the parasite by inhibition[2] of protein kinase C mediated signal transduction and related gene transcription[3] of the host. LPG deficient mutants of *Leishmania* cannot survive in the sandfly vector or infect mammalian macrophages but both the functions can be restored on insertion of exogenous LPG into the plasma membrane of deficient strains. The intriguing structure[4,5] of LPG (Fig. 1) consists of four distinct domains: (i) a neutral oligosaccharide cap at the terminal reducing end; (ii) variable [6Gal*p*-β1,4-Man*p*-α1-phosphate]$_n$ phosphoglycan repeat units; (iii) a conserved phosphosaccharide core with an internal galactofuranose residue and (iv) a 1-*O*-alkyl$_{(24/26:0)}$-2-*lyso*glycosylphosphatidylinositol (GPI) anchor domain. The unique features of the structure include phosphoglycan repeats and a cryptic tetrasaccharide cap with 1,4-β linkage between galactose and mannose residues, unique among eukaryotic carbohydrates. Structure–activity relationships have been proposed[1,3] for each of the domains; the neutral oligosaccharide cap contains a signal for termination of phosphoglycan assembly[1b] and is thought to attach the parasite to the digestive tract of the sandfly and human macrophage and may also contain an epitope for recognition by macrophage receptors,[6] the phosphoglycan repeats form a helical structure[5a] providing resistance[1d] to hydrolases and antibodies, and the GPI core functions as the anchor[7] to the plasma membrane.

The structure of LPG and its role in host–parasite interaction has led to significant interest and its biosynthetic pathway[8] is viewed as a new target for chemotherapeutic and vaccine design. However, the synthetic efforts towards LPG and its structural domains have been minimal so far and reported work includes synthesis[9] of phosphoglycan fragments and the first synthesis of the tetrasaccharide cap reported by Fraser–Reid[10] from monosaccharide building blocks using the *n*-pentenyl glycosidation strategy. In continuation to our work[11–14] on the chemistry and biosynthesis of cell surface GPI molecules of *L. donovani* parasite, we recently reported[14] a synthesis of the phosphodisaccharide repeat of LPG. Keeping in view the immunological importance of the tetrasaccharide cap of the LPG as a potential epitope for designing synthetic antigens and to study receptor binding on the macrophage, the access to sufficient quantity of structurally defined cap was required. Herein we report a new and efficient synthesis of this tetrasaccharide cap of LPG using lactose and mannose as starting materials. The synthesis has been designed to enable preparation of a radiolabelled cap. The important features of this approach include gluco→manno transformation via glycal

Keywords: carbohydrates; glycolipids; lipophosphoglycan; *Leishmania donovani*.

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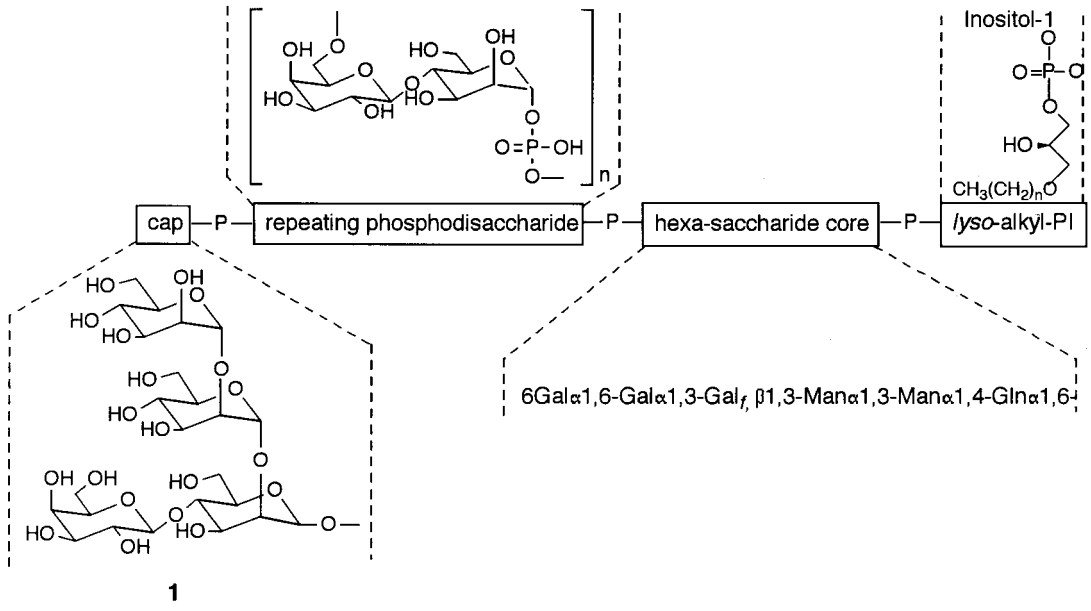
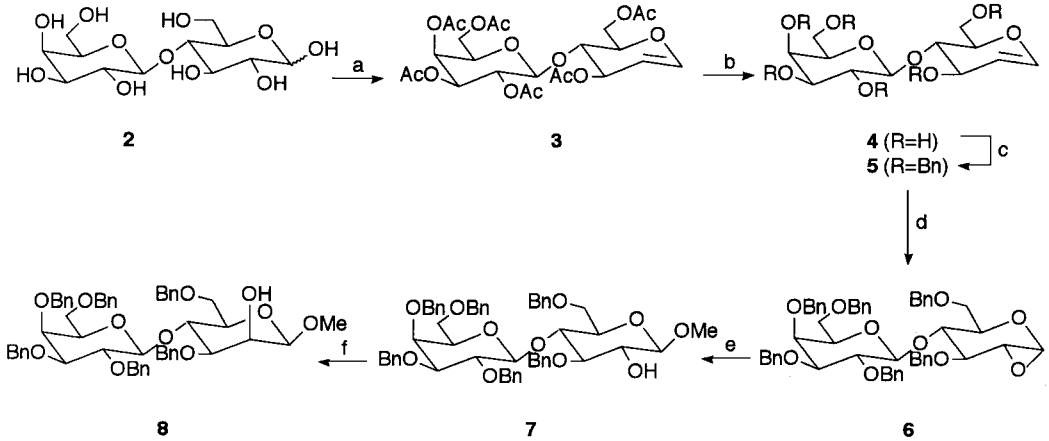


Figure 1. Structural domains of lipophosphoglycan (LPG) of *Leishmania donovani* (n=16–32).

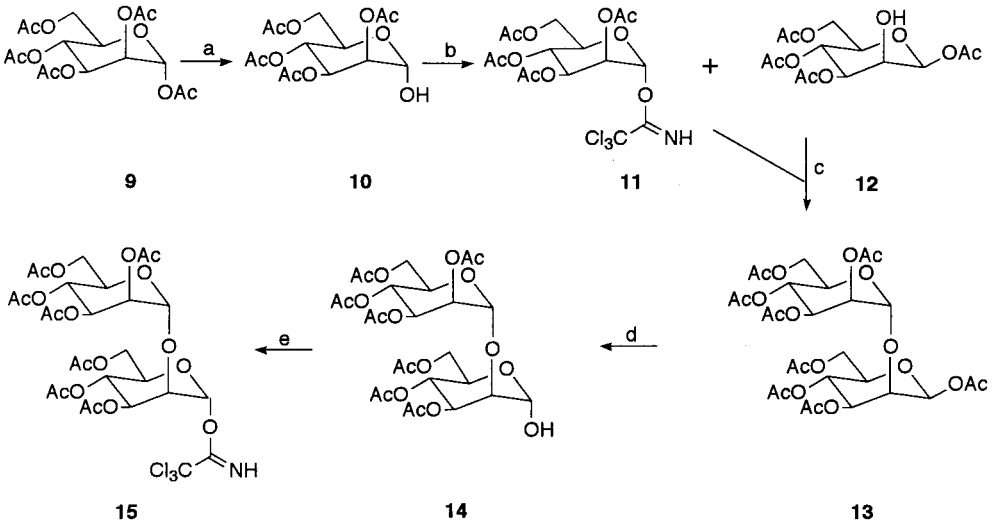
and dimethyldioxirane chemistry to convert protected lactose (Gal-β1→4-Glu) into a suitably protected Gal-β1→4-Man intermediate with a free C_2–OH group available for stereoselective coupling with a mannobiose donor. This avoided several protection/deprotection/glycosidation steps required in the synthesis from monosaccharide building blocks, and provided an opportunity to radiolabel the cap.

Result and Discussion

The key intermediate hexa-*O*-acetyl-lactal (**3**)[15] was prepared from lactose (**2**) in three steps involving acetylation, anomeric bromination and reductive elimination mediated by zinc and Vitamin-B_{12} (Scheme 1). It is worth mentioning here that high yield (more than 90%) could be obtained in the last step of this sequence by the application of a recent method[16] of Forbes and Franck who demonstrated the unusual catalytic efficiency of the Vitamin B_{12} radical in zinc mediated reductive elimination reactions. This provided an easy and multigram access to the required hexa-*O*-acetyl-lactal (**3**) with straightforward work up without any chromatographic purification. The deacetylation of **3** to lactal (**4**) followed by benzylation led to hexa-*O*-benzyl-lactal (**5**) in high yield; this replacement of participatory protecting groups (i.e. acetyl) by the non-participatory protecting benzyl groups was necessitated[17] for the control of the stereochemistry during epoxidation in the forthcoming step. This stereoselective α-epoxidation of hexa-*O*-benzyl-lactal (**5**) was carried out by freshly prepared 2,2-dimethyldioxirane[18] soltution in acetone at 0°C leading exclusively to the 1α,2α-oxirane (**6**). The methanolysis of **6** with excess of anhydrous MeOH at room temperature led to epoxide ring opening to provide the corresponding β-glucoside (**7**) in quantitative yield. Since this selective epoxide ring opening generated a unique free C-2 hydroxyl group in the β-glucoside (**7**), we exploited



Scheme 1. Reagents and conditions: (a) (i) Ac_2O, NaOAc, 100°C; (ii) HBr–AcOH, 4°C, 16 h; (iii) Zinc, Vitamin B_{12}, NH_4Cl, MeOH, 10 min 87% for three steps; (b) Na_2CO_3, MeOH, rt, 2 h, 97%; (c) NaH, BnBr, TBAI, DMF, rt, 3 h 57%; (d) dimethyl dioxirane, CH_2Cl_2, 0°C, 2 h, 90%; (e) MeOH, rt, 4 h, 96%; (f) (i) $(COCl)_2$, DMSO, CH_2Cl_2, −78°C; (ii) $NaBH_4$, rt, 4 h, 64% for two steps.

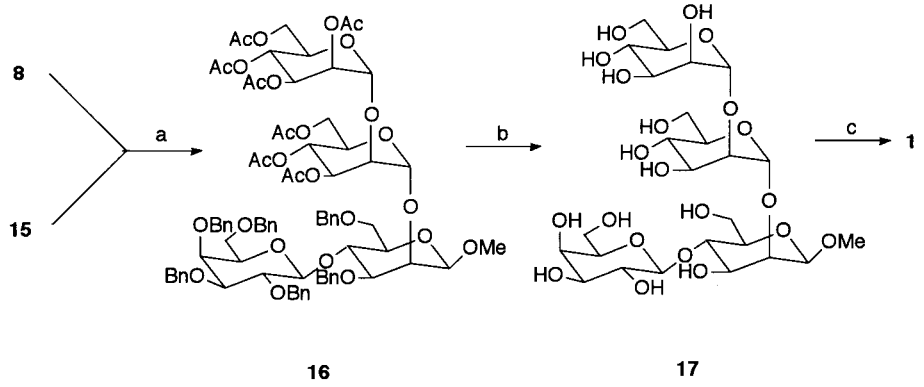


Scheme 2. Reagents and conditions: (a) Me_2NH, CH_3CN, −20°C, 1 h, quant.; (b) CCl_3CN, DBU, CH_2Cl_2, 0°C, 70%; (c) TMSOTf, CH_2Cl_2, 4A MS, 45 min, −30°C, 60%; (d) Me_2NH, CH_3CN, −20°C, 5.5 h, 91%; (e) CCl_3CN, DBU, CH_2Cl_2, 60%.

Danishefsky's approach[19] to convert β-glucoside to β-mannoside. For this the compound **7** was first oxidised by Swern oxidation to give a 2-ulose intermediate which was directly reduced with $NaBH_4$ in CH_2Cl_2–MeOH to provide key lower half intermediate methyl *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1-4)-3,6-di-*O*-benzyl-β-D-mannopyranoside (**8**) in 65% yield with free C-2 hydroxyl group in the mannosyl moiety ready for coupling with a suitable mannobiosyl donor. To confirm complete gluco→manno transformation during the oxidation–reduction sequence, a small part of compound **8** was converted to its C-2 acetate, the ^1H NMR spectrum of which showed C2 α-proton as doublet (3 Hz) at 5.5 ppm.

The upper half mannobiose donor **15** was prepared from mannose (Scheme 2) by the following steps. For this the known mannose trichloroacetimidate (**11**)[20] was prepared from mannose pentaacetate (**9**) by selective anomeric deacetylation with dimethylamine at −20°C followed by reaction with trichloroacetonitrile/DBU using Schmidt method[21] to obtain corresponding trichloroacetimidate (**11**) in 70% yield; the α-mannosyl stereochemistry was established by NMR analysis. The trimethylsilyltriflate (TMSOTf) mediated coupling of this donor with freshly prepared 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose (**12**) at −30°C led to the fully protected mannobiose octaacetate (**13**); the coupling conditions led to formation of the major 1→2α isomer which could be isolated by silica column in 60% yield. Selective anomeric deacetylation of **13** with dimethylamine at −20°C led to the corresponding mannobiose heptaacetate (**14**) with free C-1α hydroxyl group; the α-stereochemistry confirmed by NMR showing a doublet at δ 5.36 (J=1.5 Hz). The required dimannan α-trichloroacetimidate donor **15** was prepared from **14** by the Schmidt method (CCl_3CN, DBU, CH_2Cl_2, −30°C, 75 min) in 60% yield after column purification.

Having secured access to both the lower half Gal-Man acceptor (**8**) and mannobiose trichloroacetimidate donor (**15**) in high yielding steps, final glycoside coupling of the two intermediates was carried out using TMSOTf at −30°C which led to the fully protected tetrasaccharide cap (methyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-manno pyranosyl)-(1-2)-*O*-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1-2)-*O*-[(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1-4)]-3,6-di-*O*-benzyl-α-D-mannopyranoside, **16**) in 63 % yield after repeated silica column chromatography. This was followed by global deprotection in two steps of debenzylation (palladium



Scheme 3. Reagents and conditions: (a) TMSOTf, DCM, 45 min, −30°C, 67%; (b) (i) $Pd(OH)_2$, H_2, 4 h, (ii) NaOMe, MeOH, quant; (c) (i) Ac_2O/AcOH/H_2SO_4, rt, (ii) Na_2CO_3, MeOH, rt, 41% for two steps.

hydroxide, H_2) and deacetylation (NaOMe, MeOH) to provide the methyl glycoside of the tetrasaccharide cap (**17**) in quantitative yield (Scheme 3). Finally the acetolysis ($Ac_2O/AcOH/H_2SO_4$) and decetylation (NaOMe, MeOH) led to the fully deprotected tetrasaccharide Cap domain (41% yield) of the LPG of *L. donovani* parasite.

For the radiochemical synthesis of the Cap, the protected disaccharide **7** was oxidised by Swern oxidation to obtain the corresponding 2-ulose intermediate which was then reduced with radiolabelled NaB^3H_4 (specific activity 600 μCi/mmol) in diglyme solution to give labelled [2-^{3}H]-methyl-*O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1-4)-3,6-di-*O*-benzyl-β-D-mannopyranoside (**8**). This labelled reduced material was coupled with protected mannobiose trichloroacetimidate (**15**) to provide lebelled protected tetrasaccharide **16** in 67% yield. The rest of the deprotection steps were carried out essentially as described above for unlabelled synthesis. The labelled materials were visualised on TLC plates by autoradiography and analysed by scintillation counting. This provided labelled LPG cap of high specific activity (600 μCi/mmol) which is now being used by us for macrophage receptor binding and epitope recognition experiments.

Conclusion

The biologically important terminating cap domain of LPG has been synthesised from lactose and mannose in a high yielding convergent approach. This synthesis was designed to enable radio isotope labelling of the cap structure for its application in macrophage receptor binding and immunological studies. The current approach provided an opportunity for tritium labelling at non-exchangeable C-2 position of the lower mannose residue by using labelled NaB^3H_4 in the reduction of **7** to **8** step to obtain a labelled acceptor. Since the radiolabel come at a fairly late stage of the synthesis, the access to deprotected cap required only three more steps. Considering the obvious difficulties encountered in handling labelled intermediates in a multistep synthesis, it was desirable to incorporate radiolabel at the final steps of synthesis. Therefore, the present approach is practically suitable for preparation of the labelled cap of LPG. The work on the macrophage receptor binding using radiolabelled cap will be published in due course.

Experimental

General methods

The reagents used for synthesis were from Aldrich and Fluka, and organic solvents were of analytical purity. The radiolabelled NaB^3H_4 (5–10 Ci/mmol) was obtained from Amersham and was diluted to the specific activity of 0.6 mCi/mmol for synthetic experiments. Thin layer chromatography was performed on Merck Kieselgel 60 F_{254} plates, compounds visualised by ammonium molybdate–ceric sulfate developing reagent. The spots of radioactive compounds were observed by exposure of TLC plates to X-ray films and fluorography. Flash chromatography was carried out with silica gel 60 (200–400 mesh). Liquid reagents and anhydrous solvents were introduced by syringes through septa-isolated flasks under nitrogen atmosphere. The NMR spectra (^{1}H, ^{13}C, two-dimensional ^{1}H–^{1}H COSY, DQFCOSY, TOCSY, gradient-HMQC) were obtained on a Bruker 300 MHz spectrometer (Avance DRX-300); the spectra recorded in $CDCl_3$ unless stated otherwise. Chemical shifts are expressed in δ (ppm) relative to residual solvent resonance, and coupling constants J expressed in Hz. Electrospray ionisation mass spectra were obtained on a quadrupole mass spectrometer (VG Platform-II) using acetonitrile–water (1:1) mobile phase. High-resolution FAB mass spectra were performed at the Indian Institute of Chemical Technology, Hyderabad. Optical rotations (Na D line) were measured on a Perkin–Elmer 241 polarimeter.

Hexa-*O*-acetyl lactal (3). A solution of Vitamin B_{12} (310 mg, 0.228 mmol) in anhyd MeOH (80 mL) was thoroughly purged with nitrogen for 30 min and zinc powder (17.5 g, 267.6 mmol) and ammonium chloride (14.2 g, 266.25 mmol) were added to the solution. The reaction mixture was stirred for another 45 min and heptaacetyl lactosyl bromide (9.4 g, 13.5 mmol), freshly prepared from lactose by a known method,[15] dissolved in MeOH (30 mL) was added. Immediately after addition of the bromide, the dark red solution changed to reddish yellow and then back to dark red in 5 min. The solution was filtered through celite to remove zinc, the celite pad washed with MeOH and the filtrate was concentrated to give a white and red solid. This was dissolved in water (100 mL) and extracted with CH_2Cl_2 (3×60 mL). Organic extracts were combined, dried over Na_2SO_4, and concentrated to provide hexa-*O*-acetyl lactal (**3**, 7.2 g, 87%) as amorphous solid, mp 113° (lit.[15] mp 114°); $[\alpha]_D=-18°$ (*c* 1.0, $CHCl_3$) (lit.[15] −18°, *c* 1.0, $CHCl_3$).

Hexa-*O*-benzyl lactal (5). A solution of hexa-*O*-acetyl lactal (**3**, 7.26 g, 13.0 mmol) and freshly dried sodium carbonate (9.0 g, 85 mmol) in anhyd MeOH (150 mL) was stirred at room temperature for 90 min. The suspension was filtered to remove excess Na_2CO_3 and the filtrate was concentrated under reduced pressure to give deacetylated lactal **4** as an amorphous solid (3.87 g, 98%); mp 191–193°; R_f=0.2 in 7:3 $CHCl_3$–MeOH; $[\alpha]_D=+27°$ (*c* 1.6, H_2O) (lit.[15] +27°, *c* 1.6, H_2O). The compound **4** (500 mg, 1.62 mmol) dissolved in anhyd DMF (5 mL) was added dropwise at 0°C to a suspension of NaH (1.3 g, 60% dispersion in paraffin) in DMF (5 mL), followed by addition of benzyl bromide (2 mL, 16.8 mmol) and few crystals of tetrabutyl ammonium iodide. The reaction mixture was brought to room temperature and stirred for 3 h. After completion of the reaction, the mixture was cooled to 0°C and quenched with MeOH (5 mL), diluted with cold water (50 mL) and extracted with diethyl ether (3×30 mL). The ethereal layer was dried over Na_2SO_4 and concentrated to give a crude product which was flash chromatographed using 5% ethyl acetate in hexane to provide compound **5** as colourless oil (792 mg, 58%); R_f=0.6 in 50% ethyl acetate–hexane; $[\alpha]_D=-2.1$ (*c* 0.73, $CHCl_3$); IR ν_{max} (film) 2910, 2850, 1450, 1375, 1250, 1210, 1150, 1100 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39–7.27 (m, 30H, Ph), 6.43 (dd, J=6.2, 1.1 Hz, 1H, H-1), 4.92 (d, J=10.8 Hz, 1H), 4.86 (dd, J=6.3, 3.6 Hz, 1H, H-2), 4.82 (d, J=11.7 Hz, 1H), 4.71

(d, J=9.6 Hz, 1H), 4.70 (brs, 2H), 4.55 (m, 7H), 4.35 (dd, J=4.2, 1.0 Hz, 2H), 4.29 (m, 1H), 4.14 (m, 2H), 3.86 (brs, 1H), 3.83–3.74 (m, 2H), 3.65 (m, 1H), 3.54–3.41 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.76–137.85 (6 *ipso* C), 128.3–127.4 (30 ArC's), 102.79, 99.75, 82.26, 79.4, 75.12, 75.85, 75.54, 73.47, 73.43, 73.25, 73.12, 72.92, 72.31, 70.30, 68.57, 67.97; HRMS(FAB): calcd for $(M+Na)^+$ $C_{54}H_{56}O_9Na$ 871.382204, found 871.386586.

Hexa-*O*-benzyl-lactal-1,2α-epoxide (6). A solution of 2,2-dimethyl dioxirane in acetone was freshly prepared by the reported method[18] from potassium monoperoxy sulphate (Oxone, DuPont), and 15 mL of this was added dropwise to a solution of compound **5** (250 mg, 0.3 mmol) in dry CH_2Cl_2 (15 mL) at 0°C. After 2 h the reaction mixture was concentrated with a stream of nitrogen gas, and further dried under vacuum to provide a semisolid hexa-*O*-benzyl lactal 1,2α-epoxide **6** (232 mg, 90%); R_f=0.16 in 50% ethyl acetate–hexane; $[\alpha]_D$=+21.8 (*c* 0.24, $CHCl_3$); IR ν_{max} (film) 3022, 2900, 1500, 1450, 1375, 1210, 1100, 1070 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz, COSY) δ 7.36–7.23 (m, 30H, Ph), 5.03 (d, J=11.8 Hz, 1H), 4.99 (d, J=1.96 Hz, 1H, H-1), 4.90 (t, J=12 Hz, 1H), 4.76 (m, 5H), 4.70–4.52 (m, 3H), 4.45–4.32 (m, 4H), 3.90 (m, 3H), 3.89–3.76 (m, 3H), 3.65 (m, 2H), 3.51–3.37 (m, 3H), 3.11 (d, J=1.97 Hz, 1H, H-2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.8–138.9 (6 *ipso* C), 128.3–127.4 (30 ArC's), 102.51, 82.4, 79.7, 76.6, 75.2, 74.6, 73.5, 73.47, 73.41, 73.0, 72.69, 72.61, 69.17, 68.36; HRMS(ESMS): calcd for $(M+Na)^+$ $C_{54}H_{56}O_{10}Na$ 887.3771, found 887.3761.

Methyl *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-benzyl-β-D-glucopyranoside (7). The α-epoxide (**6**, 232 mg, 0.268 mmol) was dissolved in dry MeOH (150 mL) and allowed to stir at room temperature for 4 h. The solvent was evaporated and the residue dried under vacuum to yield β-methyl lactoside **7** (231 mg, 96%) as semisolid material; R_f=0.37 in 50% ethyl acetate in hexane; $[\alpha]_D$=+12 (*c* 0.20, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3500–3300 (br), 2900, 2850, 1490, 1450, 1370, 1210, 1100, 1075 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz, COSY) δ 7.32–7.20 (m, 30H, Ph), 5.07 (d, J=11.1 Hz, 1H), 4.90 (d, J=11.4 Hz, 1H), 4.78 (dd, J=11.1, 4.5 Hz, 2H), 4.70 (dd, J=11.7, 1.8 Hz, 4H), 4.54 (d, J=10.5 Hz, 2H), 4.44–4.25 (m, 4H), 4.20 (d, J=9 Hz, 1H), 3.95 (m, 1H), 3.90 (d, J=2.7 Hz, 1H), 3.83–3.70 (m, 3H), 3.54 (s, 3H, OMe), 3.48–3.36 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.92, 138.82, 138.68, 138.39, 138.22, 137.92 (6 *ipso* C), 128.29–127.35 (30 ArC's), 103.40, 102.69, 82.65, 82.38, 79.85, 76.14, 75.40, 74.62, 74.54, 73.36, 73.32, 73.06, 72.94, 72.52, 68.09, 56.87; ESMS *m/z* 919.5 $(M+Na)^+$; HRMS(FAB): calcd for $(M+Na)^+$ $C_{55}H_{60}O_{11}Na$ 919.4033, found 919.4023.

Methyl *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-benzyl-β-D-mannopyranoside (8). A solution of oxalyl chloride (95.8 μL, 0.177 mmol) in anhyd CH_2Cl_2 (7 mL) was cooled to −78°C, and anhyd DMSO (154.3 μL, 0.349 mmol) was added dropwise. The mixture was stirred at −78°C for 10 min and solution of methyl glycoside **7** (231 mg, 0.258 mmol) in CH_2Cl_2 (11.5 mL) was added over 10 min. The cloudy solution was stirred for 40 min followed by addition of triethylamine (5 mL) to give a clear solution. The mixture was brought to room temperature, diluted with cold water (30 mL) and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield the oxidised 2-ulose intermediate (R_f=0.53 in 50% ethyl acetate in hexane). This product was dissolved in 50% CH_2Cl_2 in MeOH (4 mL) and $NaBH_4$ (150 mg, 3.96 mmol) was added at 0°C. The reaction mixture was brought to room temperature and after 4 h it was diluted with CH_2Cl_2 and washed with cold water. The organic layer was collected, dried over Na_2SO_4 and concentrated to give a crude product which was purified by flash chromatography using 20% ethyl acetate in hexane to yield methyl *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-benzyl-β-D-mannopyranoside **8** as colourless gum (149 mg, 64.5%); R_f=0.27 in 50% ethyl acetate–hexane; $[\alpha]_D$=+3.89, (*c* 0.26, $CHCl_3$); IR ν_{max} (film) 3500–3300 (br), 2910, 2850, 1490, 1450, 1370, 1210, 1100, 1075 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz, COSY, TOCSY) δ 7.30–7.21 (m, 30H, Ph), 4.94 (d, J=11.4 Hz, 1H), 4.78 (d, J=11.4 Hz, 1H), 4.74 (d, J=6.6 Hz, 1H), 4.68 (m, 3H), 4.54–4.41 (m, 4H), 4.46 (d, J=5 Hz, 1H, H-1′), 4.35 (d, J=2.1 Hz, 1H, H-1), 4.30 (d, J=11.7 Hz, 1H), 4.08 (t, J=8.7 Hz, 1H), 4.03 (bs, 1H), 3.90 (d, J=2.4 Hz, 1H), 3.84–3.80 (dd, J=2.5, 8.1 Hz, 1H), 3.78–3.73 (dd, J=5.4, 10.4 Hz, 1H), 3.58–3.39 (m, 6H), 3.52 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.85, 138.63, 138.39, 138.33, 137.88, 135.52 (6 *ipso* C), 128.30–127.29 (30 ArC's), 103.07, 100.65, 82.48, 79.75, 79.15, 75.27, 75.06, 74.49, 73.39, 73.07, 72.94, 72.54, 72.33, 68.52, 68.29, 56.87; ESMS *m/z* 919.5 $(M+Na)^+$; HRMS(FAB): calcd for $(M+Na)^+$ $C_{55}H_{60}O_{11}Na$ 919.403333, found 919.400521.

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-trichloroacetimidate (11). The 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**9**, 500 mg, 1.28 mmol) was dissolved in dry CH_3CN saturated with dimethylamine (35 mL) and stirred at −20°C for 1 h, TLC confirmed complete disappearance of the starting material. Extra dimethylamine was removed under reduced pressure at room temperature and the reaction mixture was concentrated. Flash column chromatography (25:75 ethyl acetate–hexane) provided 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose (**10**, 445 mg) in quantitative yield. To a solution of compound **10** (335 mg, 0.962 mmol) in dry CH_2Cl_2 (3 mL) was added trichloroacetonitrile (Cl_3CCN, 1 mL, 10.0 equiv.) and 1,8-diaza bicyclo[5.4.0]undec-7-ene (DBU, 74.7 μL, 0.05 equiv.) at 0°C. After stirring for 75 min, the solvent was evaporated under reduced pressure and the residue purified by flash chromatography with 20% ethyl acetate–hexane to give pure **11** as viscous material (331 mg, 70%); IR ν_{max} (film) 3320 (N–H), 1750, 1680 (C=N), 1430, 1375, 1210, 1080, 1050 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz, COSY) δ 8.77 (s, 1H, NH), 6.26 (d, J=1.8 Hz, 1H, H-1), 5.45 (dd, J=1.8, 3.0 Hz, 1H, H-2), 5.39–5.37 (dd, J=2, 5 Hz, 1H, H-3), 5.42–5.33 (m, 1H, H-4), 4.18 (m, 1H, H-5), 4.29–4.12 (m, 2H, H-6), 2.19 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.0 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.45, 169.68, 169.60, 169.50, 159.62, 94.39, 71.08, 68.67, 68.13, 67.73, 65.26, 61.91, 20.54; HRMS (ESMS): calcd for $(M+H)^+$ $C_{16}H_{21}O_{10}NCl_3$ 491.0153, found 491.0187.

1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-β-D-mannopyranose (13). A solution of mannose trichloroacetimidate donor **11** (985 mg, 2 mmol) and freshly prepared[22] 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose **12** (348 mg, 1 mmol) in anhyd CH_2Cl_2 (20 mL) was stirred with activated molecular sieves (10 g, 4 Å) under nitrogen for 30 min. The reaction mixture was cooled to −30°C and a solution of trimethylsilyltriflate (TMSOTf, 220 μL, 1.2 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise. The temperature was maintained below −30°C for 15 min when TLC indicated completion of the reaction. The mixture was quenched with pyridine (5 mL) and filtered through celite, and the filtrate was co-concentrated with toluene. Flash chromatography of the crude product with 50% ethyl acetate–hexane afforded pure mannobiose octaacetate **13** as amorphous solid (0.406 g, 60%); mp 72°C R_f=0.20 in 50% ethylacetate–hexane; $[\alpha]_D$=+9.0 (*c* 0.25, $CHCl_3$); IR ν_{max} ($CHCl_3$) 1750, 1430, 1380, 1210, 1140, 1090, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, COSY, HMQC) δ 5.78 (d, *J*=0.9 Hz, 1H, H-1), 5.46–5.50 (dd, *J*=3.0, 9.9 Hz, 1H), 5.39–5.32 (t, *J*=9.6 Hz, 2H), 5.31–5.29 (dd, *J*=3.6, 1.95 Hz, 1H), 5.13–5.09 (dd, *J*=9.6, 3.0 Hz, 1H), 5.00 (d, *J*=1.8 Hz, 1H, H-1′), 4.40 (m, 1H), 4.16–4.14 (dd, *J*=3.0, 1.2 Hz, 1H), 4.34–4.01 (m, 4H), 3.80–3.76 (m, 1H), 2.15–2.01 (8×s, 24H, COMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171, 170.7, 170.4, 169.6, 169.5, 169.3, 169.1, 168.2, 98.2, 90.8, 74.5, 73.1, 72.0, 69.8, 68.7, 68.2, 66.0, 65.6, 62.1, 61.6, 20.77–20.30 (8 C); ESMS *m/z* 701.0 (M+Na); HRMS(FAB): Calcd. for $(M+Na)^+$ $C_{28}H_{38}O_{19}Na$ 701.190499, found 701.187839.

3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranose (14). The mannobiose octaacetate **13** (298 mg, 0.44 mmol) was dissolved in anhyd acetonitrile saturated with dimethylamine (39 mL) at −20°C and stirred for 5 h after which TLC confirmed disappearance of the starting material. Excess of dimethylamine was removed under reduced pressure at 30°C and the reaction mixture was concentrated. Flash column chromatography of the crude product with 40% ethyl acetate–hexane resulted in pure heptaacetate **14** as viscous solid (254 mg, 91%); R_f=0.09 in 50% ethylacetate–hexane; $[\alpha]_D$=+19.54 (*c* 0.22, $CHCl_3$); IR ν_{max} (thin film) 3500–3300 (br), 2950, 2900, 1750, 1430, 1375, 1220, 1100, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, COSY) δ 5.42–5.37 (dd, *J*=3.3, 9.9 Hz, 1H), 5.36 (d, *J*=1.5 Hz, 1H, H-1), 5.36–5.32 (m, 2H), 5.30–5.23 (m, 2H), 4.92 (d, *J*=1.8 Hz, 1H, H-1′), 4.23–4.19 (m, 2H), 4.17–4.14 (dd, *J*=3.7, 5.1 Hz, 1H), 4.13–4.11 (m, 2H), 4.08–4.05 (ddd, *J*=2.4, 2.7, 2.4 Hz, 1H), 3.65–3.59 (m, 1H), 2.13–1.99 (7×s, 21H, COMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.0, 170.6, 170.3, 169.7, 169.6, 169.4, 169.37, 169.3, 98.6, 92.5, 77.2, 70.4, 69.97, 69.6, 69.5, 68.97, 68.3, 66.2, 66.0, 62.27, 62.1, 20.77-20.54 (7 C); ESMS *m/z* 659.2 $(M+Na)^+$; HRMS(FAB): Calcd for $(M+H)^+$ $C_{26}H_{37}O_{18}$ 637.197990, found 637.200305.

3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-(α-D-mannopyranosyl)-α-D-mannopyranosyl trichloroacetimidate (15). To a solution of heptaacetate **14** (254 mg, 0.4 mmol) in dry CH_2Cl_2 (3 mL) at 0°C was added successively, trichloroacetonitrile (10.0 equiv., 400 μL) and DBU (0.0325 equiv., 20 μL). After stirring for 1 h at 0°C TLC showed completion of the reaction. Solvent was evaporated under reduced pressure and the residue was flash chromatographed (30:70, ethyl acetate–hexane) to give the disaccharide donor **15** as viscous material (185 mg, 60%); $[\alpha]_D$=+31.2, (*c* 1.40, $CHCl_3$); IR ν_{max} (thin film) 3320, 2910, 2850, 1750, 1680 (C=N), 1380, 1210, 1150 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, COSY, HMQC) δ 8.71(s, 1H, NH), 6.41 (d, *J*=1.86 Hz, 1H, H-1), 5.49–5.46 (brd, *J*=9.9 Hz, 1H, H-4), 5.43–5.38 (dd, *J*=3.45, 10.2 Hz, 1H, H-3′), 5.35–5.31 (dd, *J*=3.15, 10.2 Hz, 1H, H-3), 5.28–5.23 (m, 1H, H-4′), 5.28–5.26 (dd, *J*=1.8, 3.3 Hz, 1H, H-2′), 4.98 (d, *J*=1.5 Hz, 1H, H-1′), 4.29–4.27 (dd, *J*=2.55, 5.1, 1H, H-2), 4.24–4.14 (m, 5H), 4.12–4.10 (ddd, *J*=3, 3.6, 3.6 Hz, 1H), 2.14–2.00 (7×s, 21H, COMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.6, 170.5, 170.2, 169.78, 169.59, 169.37, 169.10, 99.1, 95.4, 76.4, 75.4, 74.8, 71.1, 69.7, 69.5, 69.4, 68.2, 66.0, 65.2, 62.1, 61.5, 20.74–20.53; HRMS (ESMS): calcd for $(M+Na)^+$ $C_{28}H_{36}O_{18}NCl_3Na$ 802.0896, found 802.0801.

Methyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→2)-*O*-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→2)-*O*-[(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→4)]-3,6-di-*O*-benzyl-α-D-mannopyranoside (16). A solution of the protected Gal-Man acceptor 8 (18 mg, 0.02 mmol) and mannobiose trichloroacetamidate donor 15 (31 mg, 0.04 mmol) in dry diethyl ether (5 mL) was stirred with freshly activated molecular sieves (4 Å, 150 mg) at room temperature under nitrogen atmosphere for 15 min, and trimethylsilyl triflate (TMSOTf, 3.66 μL) was added. The reaction mixture was stirred for 2 h at room temperature when TLC showed complete disappearance of the starting materials. The mixture was quenched with $NaHCO_3$, filtered through a celite pad and concentrated under reduced pressure. The residue was purified by silica column using ethyl acetate–hexane (38:62) to provide the fully protected tetrasaccharide cap domain (16, 20 mg, 67%); R_f=0.35 in 50% ethyl acetate–hexane; $[\alpha]_D$=+12.1 (*c* 0.06, $CHCl_3$); IR ν_{max} (film) 2910, 2850, 1750, 1460, 1370, 1210, 1110, 1100, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, COSY, HMQC) δ 7.29–7.14 (m, 30H, ArH), 5.33 (d, *J*=1.8 Hz, 1H, H-1), 5.38–5.32 (m, 3H), 5.25 (d, *J*=9.9 Hz, 2H), 5.18 (dd, *J*=1.8, 1.5 Hz, 1H), 4.91 (d, *J*=11 Hz, 2H), 4.75 (d, *J*=2.1 Hz, 1H), 4.66–4.60 (m, 2H), 4.62 (d, *J*=1.64 Hz, 1H), 4.51 (d, *J*=12 Hz, 2H), 4.43 (d, *J*=6.9 Hz, 1H, H-1′), 4.39 (d, *J*=6.1 Hz, 1H), 4.33 (d, *J*=9.9 Hz, 2H), 4.25 (m, 1H), 4.29–4.17 (m, 4H), 4.14–4.07 (m, 4H), 4.04 (m, 2H), 4.00 (m, 2H), 3.89 (d, *J*=2.7 Hz, 1H), 3.83–3.80 (m, 1H), 3.74–3.68 (m, 1H), 3.57–3.52 (m, 1H), 3.46 (s, 3H, OMe), 3.44–3.32 (m, 4H), 2.09, 2.03, 2.01, 2.00, 1.99, 1.97, 1.96 (7×s, 21H, COMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.32, 169.93, 169.67, 169.55, 169.43, 169.34, 139.16, 138.93, 138.67, 138.50, 138.40, 138.09, 128.24–127.32 (30 ArC's), 103.00, 100.61, 99.41, 98.55, 82.46, 79.85, 79.71, 77.57, 77.10, 76.79, 75.82, 75.10, 74.73, 74.53, 73.43, 73.21, 72.95, 72.65, 72.55, 70.33, 69.70, 69.07, 68.71, 68.19, 66.12, 65.90, 62.07, 61.99, 56.81, 30.05–28.85.; ESMS *m/z* 1537.1 $(M+Na)^+$; HRMS (FAB): Calcd. for $(M+Na)^+$ $C_{81}H_{94}O_{28}Na$ 1537.58290, found 1537.58270.

Methyl *O*-(α-D-mannopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-[β-D-galactopyranosyl-(1→4)]-α-D-mannopyranoside (17). Solution of the fully protected

tetrasaccharide cap **16** (15 mg, 0.0105 mmol) in absolute EtOH (22 mL) and palladium hydroxide (40 mg, 20 wt%) was stirred under slight pressure of hydrogen for 4 h. The reaction mixture was filtered through celite and the filtrate concentrated under reduced pressure to obtain debenzylated material. This product was dissolved in dry MeOH (12 mL) and treated with anhyd sodium methoxide (6 mg) and the solution was stirred for 2 h at room temperature. The reaction mixture was quenched with few drops of 0.5% HCl solution and excess of MeOH was removed under reduced pressure, and the residue was lyophilised three times with the addition of water (1 mL) to remove traces of HCl. This provided pure methyl glycoside of the tetrasaccharide cap **17** as white powder in quantitative yield; R_f=0.53 in *n*-propanol–acetone–water (9:6:5); IR ν_{max} (KBr) 3600–3200 (br), 2910, 1400, 1250, 1140, 1050 cm^{-1}; ^1H NMR (D_2O, 300 MHz) δ 5.23 (d, J=1.46 Hz, 1H, H-1‴), 5.20 (d, J=1.22 Hz, 1 H, H-1″), 4.86 (bs, 1H, H-1), 4.26 (d, J=8.51 Hz, 1H, H-1′), 3.95 (d, J=1.83 Hz, 1H), 3.93 (m, 1H), 3.9 (d, J=2.53 Hz, 1H), 3.76 (bs, 1H), 3.75 (bs, 1H), 3.54 (d, J=1.8 Hz, 3H), 3.40 (d, J=7.91 Hz, 1H), 3.37 (t, J=2 Hz, 1H), 3.34 (s, 3H, OMe); ^{13}C NMR (D_2O, 75 MHz) δ 103.01, 102.17, 100.70, 99.71, 78.64, 77.46, 77.21, 77.05, 75.50, 75.31, 75.17, 73.79, 72.92, 72.60, 72.48, 72.24, 70.82, 70.23, 69.94, 68.40, 68.08, 66.62, 60.99, 60.79, 60.46, 56.83; ESMS *m/z* 703.0 $(M+Na)^+$; HRMS(FAB): Calcd. for $(M+Na)^+$ $C_{25}H_{44}O_{21}Na$ 703.227279, found 703.226277.

***O*-(α-D-mannopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-[β-D-galactopyranosyl-(1→4)]-β-D-mannopyranoside (1).** A solution of the methyl glycoside of the cap (**17**, 2 mg. 0.0028 mmol) in acetic anhydride (200 μL) and acetic acid–sulfuric acid (20 μL of 41:1 v/v solution) was stirred at room temperature for 3 h and then neutralised with sodium bicarbonate and methanol. The mixture was stirred for 30 min, diluted with water, and extracted with dichloromethane. The organic layer was dried (Na_2SO_4) and purified to get fully acetylated cap (1.5 mg, 41%). This was deacetylated in dry MeOH (1 mL) with Na_2CO_3 (1 mg) by stirring for 2 h at room temperature. The reaction mixture was filtered through celite pad and concentrated by freeze-drying to provide free (0.80 mg) tetrasaccharide cap [Gal*p*(1-4)-β-[Man*p*-(1-2)-α-Man*p*-(1-2)-α]-Man*p*] of LPG, as lyophilised powder; IR ν_{max} (KBr) 3500–3200 (br), 2900, 1500, 1450, 1300, 1100, 1000 cm^{-1}; ^1H NMR (D_2O, 300 MHz) δ 5.23 (d, J=1.46 Hz, 1H, H-1‴), 5.20 (d, J=1.22 Hz, 1 H, H-1″), 4.80 (bs, 1H, H-1), 4.26 (d, J=8.0 Hz, 1H, H-1′), 3.95 (d, J=1.3 Hz 1H), 3.93 (m, 1H), 3.9 (m, 1H), 3.76 (bs, 1H), 3.75 (bs, 1H), 3.54 (d, J=1.8 Hz, 3H), 3.40 (d, J=7.91 Hz, 1H), 3.37 (t, J=2 Hz, 1H); HRMS (ESMS): calcd for $(M+Na)^+$ $C_{24}H_{42}O_{21}Na$ 689.2116, found 689.2125.

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