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Synthesis and biological evaluation of novel lipid A antagonists

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Abstract—A mimetic of Lipid A with a β-*N*(*O*Me) glycosidic linkage, four linear C-14 hydrophobic chains and without phosphate groups has been prepared together with its β-O-linked analogue. Both these molecules were active in inhibiting the inflammatory action of *Escherichia coli* lipid A on MT2 macrophages in a dose-dependent manner, while they were completely devoid of inflammatory activity.

1. Introduction

Septic shock is a serious syndrome associated with high mortality rates and is caused by a systemic inflammatory and immune response due to the presence of lipopolysaccharides (LPS) in the blood of affected patients.[1–5] LPS, a component of Gram-negative bacteria cell wall, consist of a hydrophilic heteropolysaccharide and a covalently bound disaccharide phospholipid component, termed Lipid A.[6,7] Lipid A (Fig. 1A), the biologically active part of the entire LPS, initiates the production of multiple endogenous mediators of the inflammatory response, including cytokines (e.g., tumour necrosis factor TNFα), arachidonic acid metabolites and tissue factors. The molecular pathway associated to LPS action is initiated by the extra-cellular association of LPS with membrane-bound CD14[8] followed by formation of the LPS receptor complex by recruitment of MD-2[9,10] and TLR4 receptors, the latter being responsible for the intracellular transmission of the signal leading to the induction of cytokine gene expression.[11,12] Lipid A has received worldwide scientific attention being one of the most potent pro-inflammatory substances known and several synthetic lipid A mimetics have been developed with both agonistic and antagonistic properties. While synthetic agonists have been used as important tools for characterizing LPS–receptor interactions and for elucidating the entire LPS signal transduction system, synthetic inhibitors of LPS are interesting lead compounds for the development of anti-sepsis drugs.

Depending on the different bacterial origin, Lipid A exists in a variety of natural forms that differ by slight chemical modifications mainly involving the lipophilic chains. Some structural features have been recognized to be essential for the endotoxic activity of different Lipid A species, including the presence of a β(1-6)-linked glucosamine backbone, diphosphorylation at the anomeric C-1 and C-4′ positions and a suitable number and location of appropriately long 3-acyloxyacyl groups per disaccharide. These general rules have allowed the design of synthetic analogues that have shown potent LPS agonist activities. Compound ER-112022 (Fig. 1B) is an interesting but unique exception, being a phospholipid dimer connected via an acyclic spacer. Although lacking the disaccharide scaffold, it presents a potent pro-inflammatory activity.[13] Recently, the bioactivity of Lipid A has been related to its three-dimensional conformation, that is, mainly influenced by the length, number and symmetry of acyl chains, as well as the number and distribution of negative charges.[14] Lipid A species having an asymmetric (4+2) distribution of lipophilic chains adopt a conical shape and are highly active, whereas lipid A species with a symmetric (2+2) distribution have a cylindrical conformation and have antagonistic properties. Conical-shaped lipid A species are believed to trigger the conformational change that

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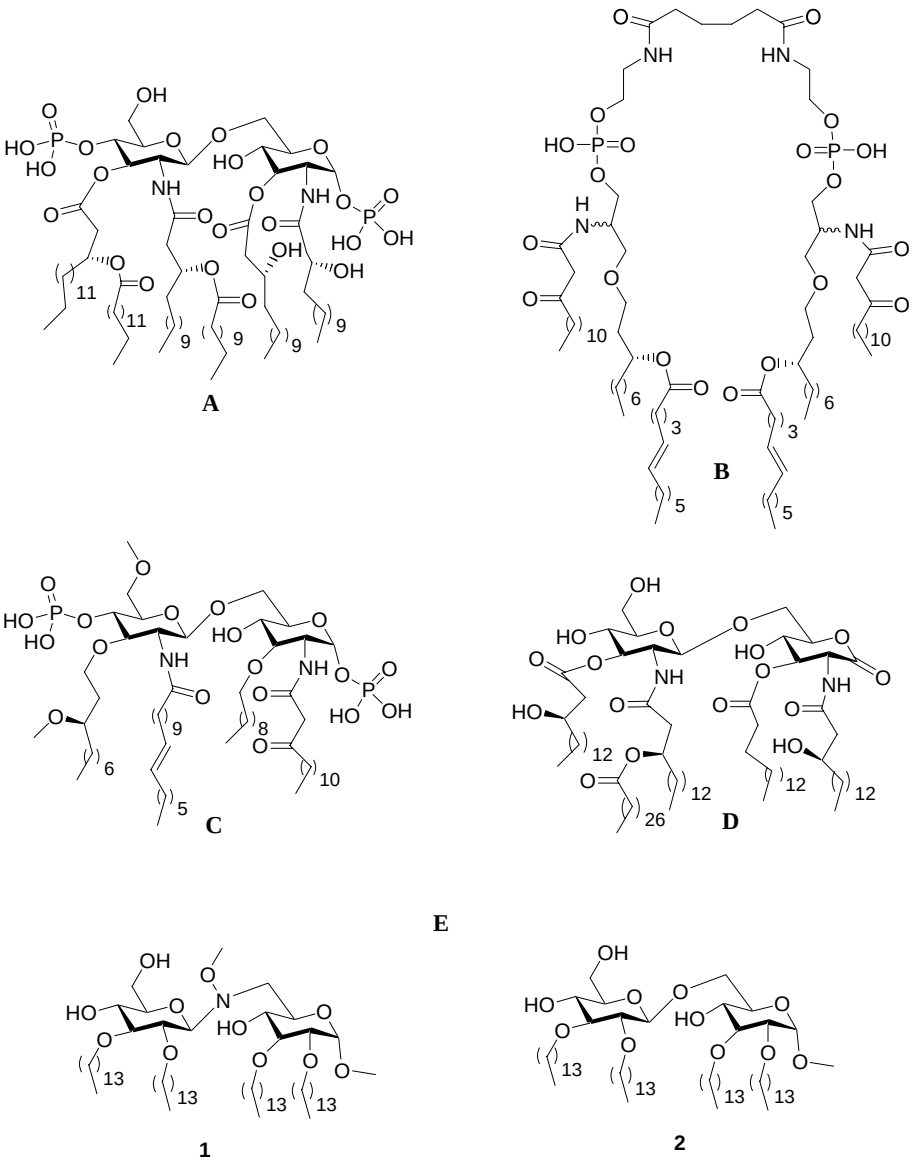


Figure 1. (A): *Escherichia coli* lipid A; (B): ER-112011 (synthetic agonist); (C): E5564 (synthetic antagonist); (D): synthetic antagonist from *R. sin*-1 lipid A; (E): molecules **1** and **2**.

allows the intracellular signal transmission, and thus interact with the TLR4 binding site in an optimal way. Cylindrical Lipid As bind to the same receptor without causing downstream target activation and cytokine production. Finally, lipid As adopting an intermediate slight conical shape, deriving from a (3+2) chain distribution such as in *Porphyromonas gingivalis*, have weak pro-inflammatory activity deriving from TLR2 activation, and in some cases, for example, in *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, lipid As have antagonistic properties. The two latter species of lipid A, the so-called non-toxic lipid A, lack a pro-inflammatory activity but are able to inhibit the action of *Escherichia coli* lipid A in vitro and in vivo.[15,16] The chemical structures of both these compounds inspired the design of lipid A antagonists with a symmetrical (2+2) chain arrangement, among which included compound E5564 (Fig. 1C) that showed a potent LPS antagonist activity and is now under development as a therapeutic compound.[17] Boons and co-workers have recently shown that a synthetic analogue of the lipid A from the nitrogen-fixing bacterium *R. sin-1* (Fig. 1D) is able to antagonize the effect of *E. coli* lipid A in in vitro experiments on human monocytic cell lines.[18] Noteworthy, the disaccharide has unique and interesting features, being devoid of phosphate groups and containing a 2-aminogluconolactone monosaccharide unit.

The described data prompted towards the design and synthesis of two new lipid A mimetics, a β(1-6) disaccharide with a polar methoxyamino group between two glucose units (**1**) and its O-linked analogue (**2**) with potential LPS antagonistic properties (Fig. 1). In physiological condition, at neutral pH values, compound **1** would be protonated to the intra-glycosidic nitrogen thus presenting a group with a positive charge between the sugar units.[19] As addressed above, the presence of negatively charged phosphate groups is a common fea-

ture in the majority of mimetics synthesized so far. On the other hand, the influence of positively charged groups in lipid A mimetics is to our knowledge a new interesting feature still to be explored and investigated.

2. Results and discussion

2.1. Synthesis

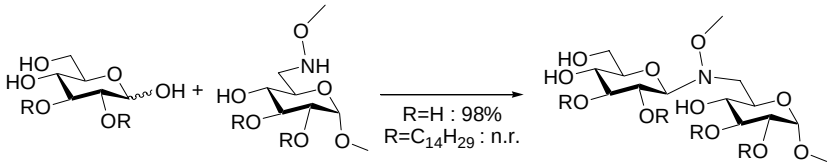
Disaccharide **1** possesses an oxymethyl interglycosidic bridge that can be introduced in a chemoselective fashion by reacting two unprotected monosaccharide units bearing, respectively, an aldehyde and a hydroxylamine group in the proper position. The methodology, recently described as a very efficient and convergent route for the synthesis of β(1–6) disaccharide analogues,[20,21] leads to the formation of *N*(*O*Me) disaccharides with good glycosylation yields and total stereoselectivity in favour of the β-anomeric configuration. In order to exploit the straightforward synthetic approach for the preparation of lipid A mimetics, monosaccharides **9** and **11** derived from D-glucose were prepared, each bearing two hydrophobic chains on C-2 and C-3. Monosaccharide **11** has a free anomeric carbon and **9** has a methoxyamino group on C-6 (Scheme 1). Unfortunately, and in contrast with previous observation on monosaccharides with free C-2 and C-3 hydroxyls, the condensation between the two monosaccharides with lipidic chains did not take place in a reasonable time (up to 24 h) in a series of experimental conditions of solvents and temperature. Unlike analogue compounds, saccharides **9** and **11** showed to be mutually unreactive probably because of the presence of the long lipophilic chains on C-2 and C-3.[22] Therefore, the desired N-linked disaccharide product **1**, as well as its O-linked analogue **2**, was synthesized according to more traditional condensation strategies. Both synthetic pathways share the common key intermediate **4** that bears a *p*-methoxybenzylidene on C-4,6 and two lipophilic chains on C-2 and C-3 (Scheme 2) that can easily give access to the required monomers through a series of functional group interconversions and selective deprotection reactions. In the case of target molecule **1**, the key glycosylation step was successfully achieved with a Koenigs–Knorr condensation of bromide **12** with the hydroxylamino glycoside **8**, with a 100% conversion yield (Scheme 3).[23] Noteworthy, the stereochemical outcome of the reaction gave interesting mechanistic information. The disaccharide was formed as a mixture of α and β anomers, in a 1:2 ratio in favour of the β, as determined by NMR characterization of the two products, isolated by chromatography. While the major (β) anomer showed good stability,[†] its α counterpart spontaneously interconverted into the β when dissolved in solvents in neutral environment.[‡] The interconversion of α into β-*N*(*O*Me) disaccharide was observed here for the first time and probably takes place through the intermediate formation of the positively charged oxiiminium ion.[21] The β isomer being the thermodynamic favoured product gets accumulated in time course. In the case of the O-linked disaccharide **2**, intermediate **13** was activated as anomeric trichloroacetimidate **16** and used for the glycosylation reaction with glycosyl acceptor **5** in the presence of trimethylsilyltriflate as catalyst. The reaction was found to be regioselective in favour of the thermodynamic more stable β isomer (ratio α:β = 1:2). Final deprotection of the hydroxyl group on C-4 and de-acetylation afforded the target disaccharide in good overall yields.

2.2. Disaccharide antagonistic activities

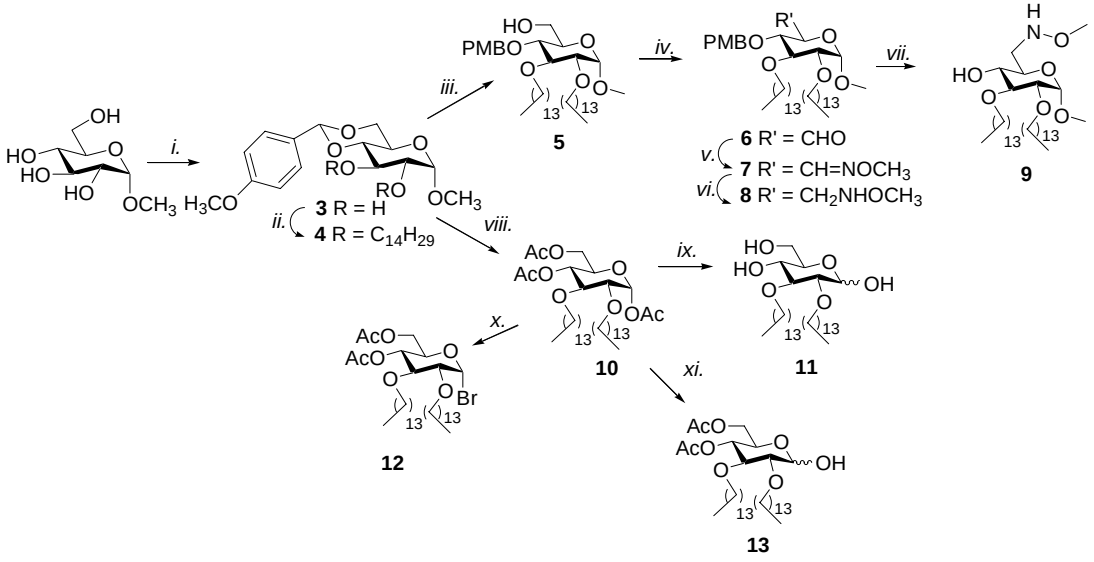
To test the antagonistic activity of the disaccharides **1** and **2**, we took advantage of the well-characterized macrophage cell line MT2 from mice.[24,25] MT2 cells express TLR4 and, upon stimulation with LPS, produce TNFα, one of the principal mediators of the septic shock, and the anti-bacterial product nitrogen oxide (NO). We first investigated whether the lipid A itself could be sufficient to activate MT2 cells. As shown in Figure 2, lipid A used at a concentration ranging from 2 to 6 ng/mL was able to stimulate TNFα and NO production by MT2 cells, while it was toxic at higher concentrations. Since the lipid A showed its maximal activity at the concentration of 2 nM, we used this concentration in the subsequent experiments. Before testing if the two disaccharides could interfere with the lipid A activity, we excluded the fact that they could show any possible inflammatory function. For this reason we directly incubated them with MT2 cells and evaluated their capacity to elicit TNFα and NO. As expected no activation of macrophages could be observed in these conditions (Fig. 3a). We then analyzed the antagonistic function of the two disaccharides. MT2 cells were, thus, stimulated with the lipid A after a pre-exposure to the compounds **1** and **2**, and the amounts of NO and TNFα produced in these conditions were measured. As shown in Figure 3 both the disaccharides interfered with the lipid A function in a dose-dependent manner. The antagonistic activity shown by the two disaccharides specifically involved the TLR4 since no effect on TNFα and NO production was observed when the MT2 cells were activated in the presence of an oligo-DNA containing the unmethylated CpG motif of bacterial DNA that is recognized by TLR9[26] and the synthetic double-stranded RNA poly I:C that exerts its function through the TLR3[27](Fig. 4). Both disaccharides **1** and **2** antagonized the inflammatory effect of *E. coli* Lipid A on MT2 macrophages, while they were devoid of pro-inflammatory effects on the same cell lines In the same experiments, monosaccharides **9** and **11** were tested on murine MT2 cells (data not shown) and, as expected, were inactive both as agonist and antagonist. Taken together, these results suggest that the disaccharide core is essential to the antagonistic activity. Anyway, the chemical structures of disaccharides **1** and **2** are quite different from those of natural Lipid As and other known synthetic antagonists, so the finding that both these compounds lacking phosphate groups inhibited cytokine production initiat-

[†] No degradation product was detected in a sample solution in organic solvents after one week at room temperature and light exposure.

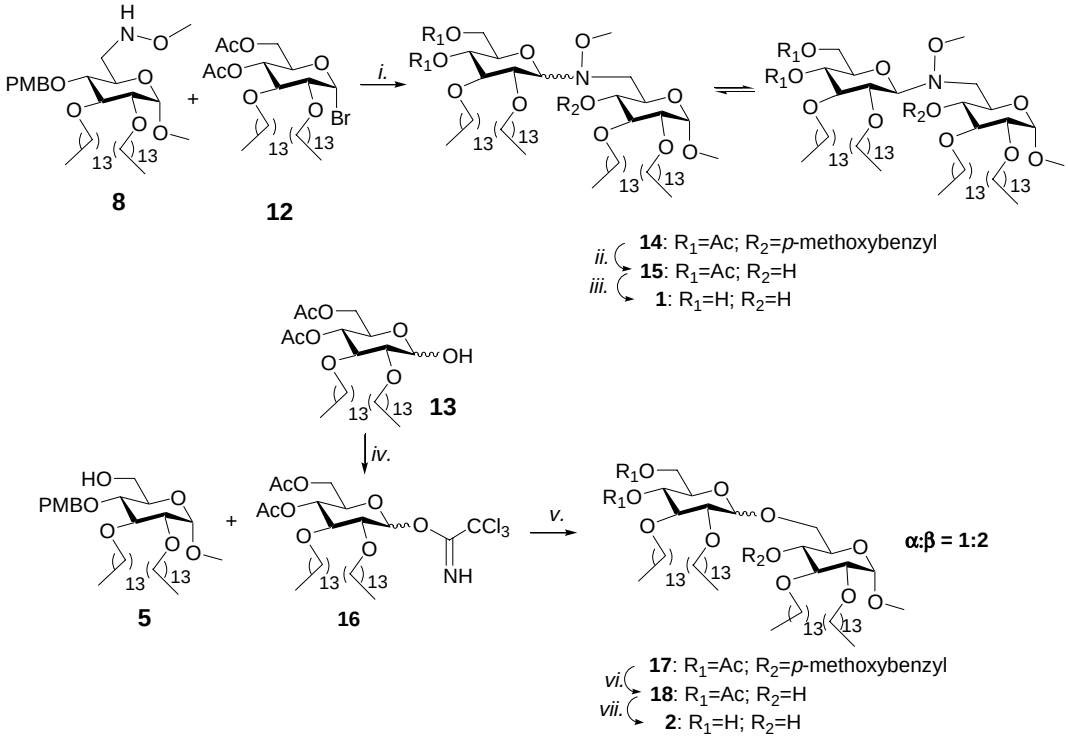
[‡] As a consequence, no ^{13}C NMR of the pure α-*N*(*O*Me) disaccharide could be recorded, the interconversion rate being higher than the time required for spectra acquisition.



Scheme 1. Chemoselective aminooxy-aldehyde condensation for *N*(OMe) disaccharide preparation.



Scheme 2. Reagents: (i) anisaldehyde dimethylacetal, CSA, DMF, 94%; (ii) $C_{14}H_{29}Br$, NaH, DMF, 74%; (iii) $LiAlH_4$, $AlCl_3$, CH_2Cl_2, Et_2O, 86%; (iv) Dess Martin periodinane, CH_2Cl_2; (v) H_2NOCH_3, pyridine, 65% (over two steps); (vi) $NaBH_3CN$, AcOH, 92%; (vii) TFA, CH_2Cl_2, 90%; (viii) Ac_2O, TFA, 83%; (ix) $NaOCH_3$, CH_3OH, 87%; (x) HBr, AcOH, CH_2Cl_2, quant.; (xi) $H_2NNH_3^+$ AcO^-, DMF, quant.



Scheme 3. Reagents and conditions: (i) $AgCO_3$, AgOTf, CH_2Cl_2 (quant.); (ii) DDQ, H_2O, CH_2Cl_2 (90%); (iii) $NaOCH_3$, MeOH (quant.); (iv) CCl_3CN, $CsCO_3$, CH_2Cl_2, then (v) TMSOTf, CH_2Cl_2, −30 °C (41%); (vi) DDQ, H_2O, CH_2Cl_2 (83%); (vii) $NaOCH_3$, MeOH (quant.).

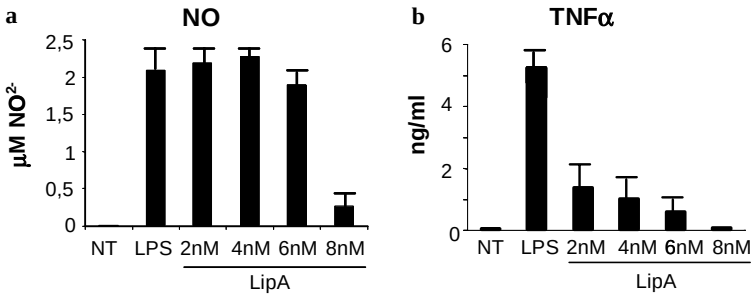


Figure 2. Lipid A inflammatory activity. MT2 cells (2.5×10^5) were incubated with LPS (10 ng/mL) or in presence of the indicated concentration of lipid A. After 24 h NO_2^- accumulation (a) or TNFα (b) production were tested in the supernatants. NO_2^- accumulation was measured as NO production by Greiss assays. NT: untreated cells; Lip A: cells treated with lipid A; LPS: cells treated with LPS.

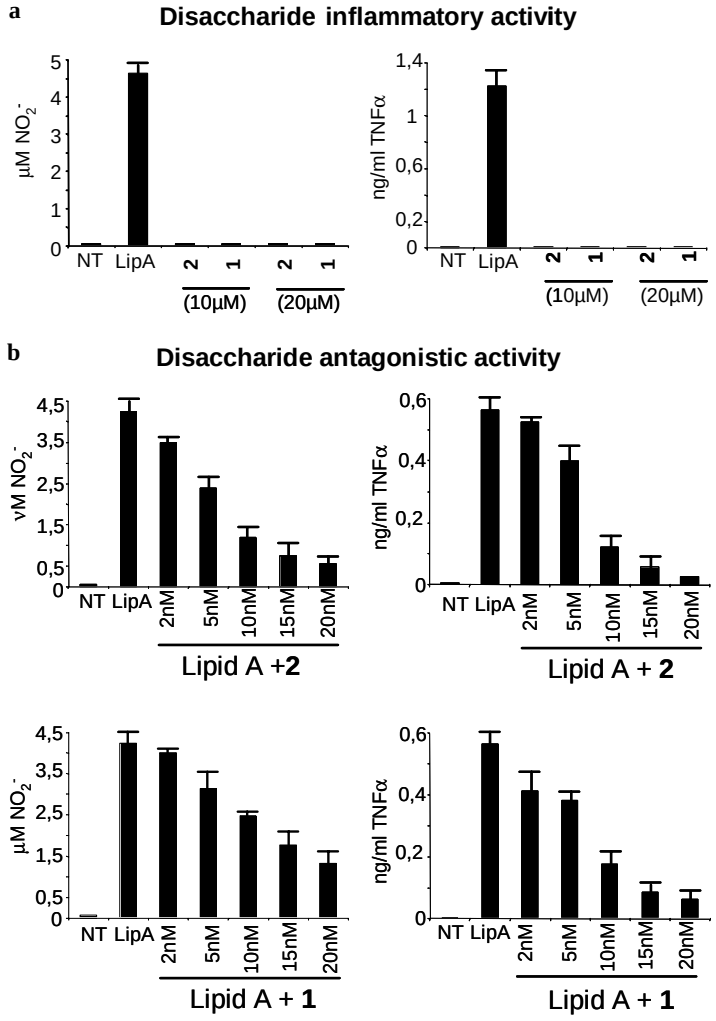


Figure 3. Disaccharide antagonistic activities. (a) The inflammatory activity of the two disaccharides was tested by incubating MT2 cells (2.5×10^5) in the presence of the indicated concentration of disaccharides **1** and **2** or lipid A as control. After 24 h, NO_2^- accumulation or TNFα production was measured in the supernatants. (b) Inhibition of the lipid A function induced by the disaccharides **1** and **2**. MT2 cells were treated with lipid A alone or lipid A in the presence of increasing concentrations of the two disaccharides. NO_2^- accumulation or TNFα production was measured in the supernatants 24 h later. NT: untreated cells; Lipid A: cells treated with lipid A alone; Lipid A + **2**: cells treated with lipid A together with increasing concentrations of disaccharide **2**; Lipid A + **1**: cells treated with lipid A together with increasing concentrations of disaccharide **1**.

ed by *E. coli* Lipid A is significant. The fact that N- and O-linked disaccharides have very similar activities indicates that the chemical nature of the interglycosidic bridge does not influence in a relevant way their antagonistic activities. The N-linked disaccharide was much more soluble in water and in aqueous buffers that its O-linked counterpart, this property due to the presence of a positive charge in the protonated methoxyamino

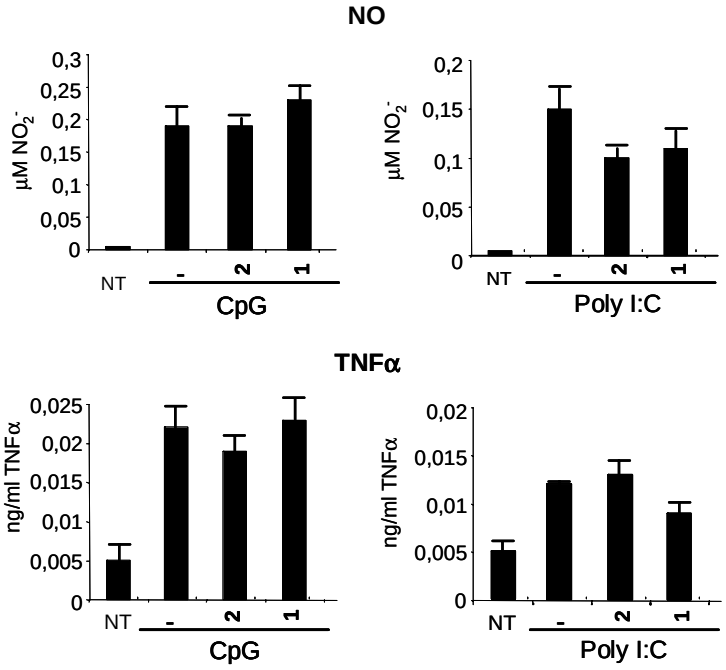


Figure 4. Specificity of disaccharide **1** and **2** antagonistic activities. MT2 cells were incubated for 24 h with CpG (1μM) or Poly I:C (20 ng/mL) in the presence or absence of disaccharide **1** or **2** (20 nM each). The amounts of NO_2^- or TNFα were then measured in the supernatants. NT: untreated cells; -: cells treated with CpG or poly I:C alone; **2**: cells treated with CpG or poly I:C in the presence of the disaccharide **2**; **1**: cells treated with CpG or poly I:C in the presence of the disaccharide **1**.

group at neutral pH, facilitated solubilization of compound **1** and its use in biological tests. We are currently investigating the activity of both disaccharides on dendritic cells (DCs), a special type of leukocyte able to alert the immune system for the presence of infections and responsible for the activation of both innate and adaptive immune responses. Given the central role of DCs in priming immunity, blocking their activation could result in a profound downregulation of initial inflammatory responses.

3. Experimental

3.1. General

All solvents were dried over molecular sieves (Fluka), for at least 24 h prior to use. When dry conditions were required, the reactions were performed under Ar atmosphere. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Merck) with detection with UV light when possible, or charring with a solution containing concd H_2SO_4/EtOH/H_2O in a ratio of 5/45/45 followed by heating at 180 °C. Column flash chromatography was performed on silica gel 230–400 mesh (Merck). The boiling range of petroleum ether used as eluent in column chromatography is 40–60 °C. ^1H and ^{13}C NMR spectra were recorded at 400 MHz on a Varian MERCURY instrument at 300 K. Chemical shifts are reported in parts per million downfield from TMS as an internal standard. In the proton assignment, H-α and H-β refer to the positions of C-14 chains. Mass spectra were recorded on a MALDI2-TOF Kompakt Kratos instrument, using gentisic acid (DHB) as matrix. Optical rotations were measured at ambient temperature on a Perkin-Elmer 241 polarimeter.

3.1.1. Methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside (3).[28,29] To a solution of methyl α-D-glucopyranoside (10 g, 51.5 mmol) in DMF (50 mL), camphorsulfonic acid (cat.) and anisaldehyde dimethylacetal (10 mL, 51.5 mmol) were added and the resulting mixture was stirred for 40 min, concentrated and quenched by addition of $NaHCO_3$ (satd aq solution, 100 mL) and stirring for an additional hour. The precipitate was filtered and washed with cold $NaHCO_3$ (satd aq, 100 mL). Trituration with hexane afforded **3** (15 g, 94%). Compound **3**: $R_f = 0.31$ (EtOAc/MeOH/H_2O 7:2:1).

3.1.2. Methyl 4,6-*O*-(4-methoxybenzylidene)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (4).[30] To a solution of **3** (8 g, 25.6 mmol) in DMF (80 mL), NaH (60% suspension in mineral oil, 6.4 g, 160 mmol) was carefully added in small portions. Tetradecylbromide (38 mL, 128 mmol) was added dropwise and the resulting mixture was stirred at 60 °C overnight. After cooling to room temperature, methanol (20 mL) was carefully added and the solution was stirred for 20 min to hydrolyze excess of sodium hydride. Solvents were then evaporated and the residue diluted with AcOEt (500 mL). Citric acid (satd aq soln, 400 mL) was added, the layers were separated, the organic layer was washed with water (3×400 mL), dried (Na_2SO_4) and evaporated. Flash column chromatography on silica gel of the residue (petroleum ether/AcOEt 85:15) afforded **4** (13.3 g, 74%). Compound **4**: $R_f = 0.32$ (petroleum ether/AcOEt

9:1); $[\alpha]_D$ = +23 (*c* 1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.40–6.85 (A_2X_2q, 4H, aromatics), 5.48 (s, 1H, OCH_3), 4.77 (d, *J* = 3.7 Hz, H-1), 4.24 (dd, *J* = 9.7, 4.4 Hz, 1H, H-6a), 3.80 (s, 3H, OCH_3), 3.77–3.60 (m, 8H), 3.47 (t, *J* = 9.3 Hz, 1H), 3.42 (s, 3H, OCH_3), 3.34 (dd, *J* = 9.3, 3.7 Hz, 1H, H-2), 1.5–1.6 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, *J* = 5.8 Hz, 6H, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 161.2 (C_{ar}), 127.4 (C_{ar}), 128.4 (C_{ar}), 114.0(C_{ar}), 105.1, 98.4 (C-1), 83.4, 80.8, 74.5 (OCH_2), 73.5 (OCH_2), 71.6 (OCH_2), 71.2, 70.2 (C-6), 69.6, 57.7 (OCH_3), 55.6 (OCH_3), 32.4, 30.8, 30.5, 30.1, 30.1, 30.0, 29.9, 29.8, 26.4, 23.1, 14.6. MS (MALDI-TOF): *m*/*z*: 705.6 $[M+H]^+$, 727.5 $[M+Na]^+$.

3.1.3. Methyl 4-*O*-(4-methoxybenzyl)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (5). Compound **4** (1.0 g, 1.41 mmol) was dissolved in a mixture of diethylether/CH_2Cl_2 2:1 (70 mL) under argon atmosphere. $LiAlH_4$ (1 M in THF, 7.2 mL) and $AlCl_3$ (1.16 g, 8.72 mmol) in diethylether (25 mL) were added dropwise and the resulting mixture was refluxed for 4 h. After cooling to room temperature, AcOEt (300 mL) and water (300 mL) were added and the layers separated. The organic layer was washed with brine (3 × 200 mL), dried over sodium sulfate and evaporated. Flash column chromatography of the residue (petroleum ether/AcOEt 7:3) afforded **5** (860 mg, 86%). Compound **4**: R_f = 0.25 (petroleum ether/AcOEt 8.5:1.5). $[\alpha]_D$ = +46 (*c* 1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.36 (m, 2H, aromatic), 6.92 (m, 2H, aromatic), 4.86–4.57 (ABq, 2H, *J* = 10.6 Hz, C*H*$_2$–(4-*O*MePh)), 4.75 (d,1H, *J* = 3.5 Hz, H-1), 3.90–3.54 (m, 11H, H-3, H-5, H-6a, H-6b, H-α), 3.41 (dd, 1H, *J* = 9.8, 9.0 Hz, H-4), 3.37 (s, 3H, OCH_3), 3.27 (dd, 1H, *J* = 3.6, 9.7 Hz, H-2), 1.5–1.6 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, 6H, *J* = 5.8 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 159.2 (C_{ar}), 130.2 (C_{ar}), 129.47 (C_{ar}), 113.9(C_{ar}), 98.3 (C-1), 81.3, 80.7, 73.9 (OCH_2), 73.5 (OCH_2), 71.6 (OCH_2), 71.1, 70.2 (C-6), 69.6, 55.6 (OCH_3), 55.6 (OCH_3), 32.4, 30.8, 30.5, 30.1, 30.1, 30.1, 30.0, 29.9, 29.8, 26.4, 23.2, 14.6 (CH_2). MS (MALDI-TOF): *m*/*z*: 707.4 $[M+H]^+$, 729.4 $[M+Na]^+$, 745.3 $[M+K]^+$.

3.1.4. Methyl 4-*O*-(4-methoxybenzyl)-2,3-di-*O*-tetradecyl-α-D-gluco-hexodialdo-1,5-pyranoside (6). To a solution of compound **5** (800 mg, 1.13 mmol) in dry dichloromethane (35 mL), Dess Martin periodinane (720 mg, 1.70 mmol) was added under argon atmosphere. After 1 h, crude was diluted with dichloromethane (200 mL) and saturated solutions of $NaHCO_3$ and $Na_2S_2O_3$ (1/1, 200 mL) were added. The layers were separated, the organic layer was washed with water (200 mL), dried on sodium sulfate and evaporated. The residue was used without further purification for the next reaction. Compound **6**: R_f = 0.30 (petroleum ether/AcOEt 7.5:2.5).

3.1.5. Methyl 4-*O*-(4-methoxybenzyl)-2,3-di-*O*-tetradecyl-α-D-gluco-hexodialdo-1,5-pyranose-6-*O*-methyloxime (7). Crude aldehyde **6** (800 mg, 1.13 mmol) was dissolved in pyridine (12 mL) and *O*-methyl hydroxylamine hydrochloride (142 mg, 1.7 mmol) was added. After 1.5 h, the solvent was evaporated. Flash column chromatography of the residue (petroleum ether/AcOEt 9:1) afforded **7** (540 mg, 65%). Compound **7**: R_f = 0.70 (petroleum ether/AcOEt 7.5:2.5); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.17 (d, 1H, *J* = 7.6 Hz, H-6), 7.13–6.77 (A_2X_2q, 4H, *J* = 8.6 Hz, aromatic), 4.71 (d, 1H, *J* = 3.4 Hz, H-1), 4.65, 4.44 (ABq, 2H, *J* = 10.6 Hz, C*H*$_2$–(4-*O*MePh)), 4.10 (dd, 1H, *J* = 9.9, 6.3 Hz, H-3), 3.82 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.33 (s, 3H, OCH_3), 3.45–3.78 (m, 5H), 3.20–3.28 (m, 2H), 1.50–1.60 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, 6H, *J* = 5.8 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 159.4 (C_{ar}), 147.3 (C_{ar}), 130.3 (C_{ar}), 129.9 (C_{ar}), 113.9 (C6), 98.4 (C1), 81.4, 80.4, 79.7, 76.0, 76.5, 72.1, 68.5, 62.1 ($NOCH_3$), 55.9 (OCH_3), 55.9 (OCH_3), 32.3, 31.0, 30.4, 30.1, 30.1, 30.1, 30.0, 30.0, 29.9, 29.8, 26.7, 26.4, 23.1, 14.6 ($O(CH_2)_{13}CH_3$). MALDI-TOF (DHB): *m*/*z*: 757.1 $[M+Na]^+$, 773.3 $[M+K]^+$.

3.1.6. Methyl 6-methoxyamino-4-*O*-(4-methoxybenzyl)-2,3-di-*O*-tetradecyl-6-deoxy-α-D-glucopyranoside (8). To a solution of *O*-methyloxime **7** (500 mg, 0.68 mmol) in glacial acetic acid (35 mL), sodium cyanoborohydride (214 mg, 3.4 mmol) was added and the resulting mixture was stirred for 4 h. The solvent was evaporated and the residue was purified by flash column chromatography on silica gel (petroleum ether/AcOEt 8:2) to yield **8** (460 mg, 92%). Compound **8**: R_f = 0.42 (petroleum ether/AcOEt 7.5:2.5); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.25 (m, 2H, aromatic), 6.83 (m, 2H, aromatic), 5.60 (br s, 1H, NH), 4.82, 4.55 (ABq, 2H, *J* = 10.6 Hz, C*H*$_2$–(4-*O*MePh)), 4.72 (d, 1H, *J* = 3.5 Hz, H-1), 3.90–3.55 (m, 5H, 4H-α, H-3), 3.80 (s, 3H, OCH_3), 3.49 (s, 3H, OCH_3), 3.38 (s, 3H, OCH_3), 3.30–3.20 (m, 4H, H-2, H-4, H-5, H-6a), 2.84 (dd, 1H, *J* = 13.2, 7.7 Hz, H-6b). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 159.4, 130.7, 129.8, 114.5, 98.0, 97.9, 82.0, 81.3, 79.9, 74.9, 74.1, 72.1, 67.1, 61.6, 61.6, 55.6, 55.6, 55.4, 55.4, 52.7, 32.3, 31.0, 30.5, 30.1, 30.1, 29.9, 29.7, 26.46, 23.15, 14.6. MALDI-TOF: *m*/*z*: 758.1$[M+Na]^+$, 774.1 $[M+K]^+$.

3.1.7. Methyl 6-methoxyamino-2,3-di-*O*-tetradecyl-6-deoxy-α-D-glucopyranoside (9). Monosaccharide **8** (200 mg, 0.27 mmol) was dissolved in TFA and CH_2Cl_2 (1/1, 20 mL) at 0 °C and the solution was stirred for 1 h at this temperature. The solvent was evaporated, and the residue was purified by flash column chromatography on silica gel (35% ethyl acetate in petroleum ether) (150 mg, 90%). Compound **9**: R_f = 0.42 (petroleum ether/AcOEt 7.5:2.5); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.76 (d, 1H, *J* = 3.5 Hz, H-1), 3.89 (m, 1H, H-α), 3.82 (m, 1H, H-5), 3.63 (m, 1H, H-α), 3.60–3.50 (m, 2, H-3, H-4), 3.54 (s, 1H, OCH_3), 3.32 (dd, 1H, *J* = 3.1, 13.5 Hz, H-6a), 3.27 (dd, 1H, *J* = 3.5, 9.5 Hz, H-2), 3.00 (dd, 1H, *J* = 7.2, 13.5 Hz, H-6b), 1.5–1.6 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, 6H, *J* = 5.8 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 98.2 (C-1), 81.2, 80.9, 74.9, 73.4, 72.1, 67.3, 61.8, 55.5, 53.7, 32.3, 31.0, 30.5, 30.1, 30.1, 29.9, 29.7, 26.46, 23.15, 14.6. MALDI-TOF (DHB): *m*/*z*: 639.4 $[M+Na]^+$, 655.1 $[M+K]^+$.

3.1.8. 1,4,6-Tri-*O*-acetyl-2,3-di-*O*-tetradecyl glucopyranoside (10). The 4-methoxybenzylidene derivative **4**

(200 mg, 0.28 mmol) was dissolved in acetic anhydride (12 mL) in the presence of TFA (3 mL). After 4 h, the reaction was quenched by adding water and ice (150 mL). Chloroform (200 mL) was added and the layers were separated. The organic layer was washed with saturated aqueous $NaHCO_3$ (200 mL), water (2×200 mL), then dried over sodium sulfate and evaporated. Flash column chromatography of the residue on silica gel (petroleum ether/AcOEt 8.5:1.5) afforded **10** (162 mg, 83%). Compound **10**: $R_f = 0.35$ (petroleum ether/AcOEt 9:1), mixture of α and β anomers (1:2 ratio in favour of the α-anomer): $[\alpha]_D = +32$ (*c* 0.50 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 6.30 (d, 1H, $J = 3.5$ Hz, H-1α anomer), 5.51 (d, 1H, $J = 8.0$ Hz, H-1β anomer), 5.00 (t, 1H, $J = 9.5$ Hz, H-4), 4.23 (dd, 1H, $J = 4.4$, 12.4 Hz, H-6a), 4.00 (dd, 1H, $J = 2$, 12.3 Hz, H-6b), 3.94 (m, 1H, H-5), 3.80–3.70 (m, 2H, H-2, H-3), 3.60–3.30 (m, 4H, H-α), 2.05 (3s, 9H, CH_3CO), 1.5–1.6 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, 6H, $J = 5.8$ Hz, CH_3). ^{13}C NMR (400 MHz, MeOH-*d*): δ (ppm) = 94.9 (C-1), 88.6, 87.3, 85.2, 84.8, 79.9, MALDI-TOF (DHB): *m*/*z*: 721.8 $[M+Na]^+$, 737.6 $[M+K]^+$.

3.1.9. 2,3-Di-*O*-tetradecyl glucopyranoside (11). The triacetyl derivative **10** (150 mg, 0.21 mmol) was dissolved in methanol (15 mL). Sodium (catalytic) was added and the mixture was stirred for 20 min. At the end of the reaction, the pH was adjusted to 7 by addition of IR 120 H^+. After filtration, the solvent was evaporated and the product was isolated by flash column chromatography on silica gel (petroleum ether/AcOEt 1:1) (104 mg, 87%). Compound **11**: $R_f = 0.20$ (petroleum ether/AcOEt 1:1). Compound **11** was almost exclusively in the α-anomeric configuration. ^1H NMR (400 MHz, MeOH-*d*): δ (ppm) = 4.80 (d, 1H, $J = 3.5$ Hz, H-1), 4.00–3.40 (m 9H), 3.52 (t, 1H, $J = 9.3$ Hz, H-3), 3.28 (dd, 1H, $J = 3.5$, 9.3 Hz, H-2), 1.5–1.6 (m, 4H, H-β), 1.22 (br s, 44H, CH_2), 0.85 (t, 6H, $J = 5.8$ Hz, CH_3). MALDI-TOF (DHB): *m*/*z*: 573.5 $[M+H]^+$, 596.3 $[M+Na]^+$, 612.2 $[M+K]^+$.

3.1.10. 4,6-Di-*O*-acetyl-2,3-di-*O*-tetradecyl glucopyranosyl bromide (12). The triacetyl derivative **10** (150 mg, 0.21 mmol) was dissolved in dichloromethane (5 mL). At 0 °C, hydrobromic acid (33% in acetic acid, 220 μL, 1.26 mmol) was added dropwise and the resulting mixture was stirred for 1.5 h. Dichloromethane (100 mL) was added and the reaction was quenched by careful addition of ice and water (50 mL). The layers were separated, the organic layer was washed with $NaHCO_3$ (satd aq soln, 100 mL), brine (100 mL), dried over sodium sulfate and evaporated. The crude product was pure according to TLC analysis and was used without further purification for the next reaction. Compound **12**: $R_f = 0.50$ (petroleum ether/AcOEt 8:2).

3.1.11. 4,6-Di-*O*-acetyl-2,3-di-*O*-tetradecyl glucopyranose (13). To a solution of triacetyl derivative **10** (150 mg, 0.21 mmol) in DMF (8 mL), hydrazynium acetate (40 mg, 0.42 mmol) was added and the resulting mixture was stirred for 40 min. AcOEt (150 mL) and a saturated aqueous solution of $NaHCO_3$ (150 mL) were added. The layers were separated, the organic phase was washed with 0.1 M hydrochloric acid (150 mL), water (3×150 mL), dried over sodium sulfate and evaporated. Flash column chromatography on silica gel of the residue (petroleum ether/AcOEt 7.5:2.5) afforded **13** (139 mg, quant. yield). Compound **13**: $R_f = 0.30$ (petroleum ether/AcOEt 8:2). MALDI-TOF (DHB): *m*/*z*: 657.8 $[M+H]^+$, 680.2 $[M+Na]^+$.

3.1.12. Methyl 6-methoxyamino-4-*O*-(4-methoxybenzyl)-2,3-di-*O*-tetradecyl-6-deoxy-6-*N*-(4,6-di-*O*-acetyl-2,3-di-*O*-tetradecyl-β-D-glucopyranosyl)-α-D-glucopyranoside (14). Silver carbonate (55 mg, 0.2 mmol) and silver triflate (8 mg, 0.03 mmol) were dissolved in dry CH_2Cl_2 (5 mL) in the presence of 4 Å molecular sieves under argon atmosphere and the resulting mixture was stirred for 40 min in the absence of light. A solution of bromide **12** (90 mg, 0.14 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise, followed by a solution of the methoxyamino monosaccharide **8** (55 mg, 0.075 mmol) in dichloromethane (5 mL) and the resulting mixture was stirred overnight. CH_2Cl_2 (50 mL) was added and the organic layer was washed with brine (2×100 mL), dried over sodium sulfate and evaporated. Flash column chromatography of the residue (petroleum ether/AcOEt 9:1) afforded **14** (66 mg of α-anomer, 36 mg of β-anomer, quant.). The α-anomer was converted into β by dissolving it in $CHCl_3$ and stirring the solution for 24 h. Evaporation of the solvent and filtration on silica gel afforded **14**, $R_f = 0.42$ (petroleum ether/AcOEt 8:2). Compound **14**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.20$,6.80 (A_2X_2q, 4H, H_{arom}), 4.83 (t, 1H, $J = 9.5$ Hz, H-4′), 4.78, 4.44 (ABq, 2H, CH_2–PMB), 4.66 (d, 1H, $J = 3.4$ Hz, H-1), 4.16 (dd, 1H, $J = 12.0$, 5.0 Hz, H-6′a), 4.08 (d, 1H, $J = 8.2$ Hz, H-1′), 4.02 (dd, 1H, $J = 12.0$, 2.5 Hz, H-6′b), 3.80 (m, 1H, H-α), 3.71 (s, 1H, OCH_3–PMB), 3.65 (m, 2H, H-5 and H-α), 3.60 (t, 1H, $J = 9.4$ Hz, H-3), 3.60–3.50 (m, 2H, H-6), 3.51 (s, 3H, $NOCH_3$), 3.40 (m, 1H, H-5′), 3.35 (m, 1H, H-3′), 3.33 (s, 3H, OCH_3), 3.28 (m, 1H, H-2′), 3.20 (dd, 1H, $J = 9.7$, 3.4 Hz, H-2), 3.09 (t, 1H, $J = 8.9$ Hz, H-4), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, $J = 8.4$ Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 98.6 (C-1), 95.1 (C-1′), 84.4, 82.4, 81.5, 80.5, 79.2, 75.6, 74.0, 74.1, 73.9, 73.6, 72.4, 71.7, 70.8, 63.5 (C-6), 61.0, 56.1, 56.0. MALDI-TOF (DHB): *m*/*z*: 1376.4 $[M+H]^+$, 1398.5 $[M+Na]^+$.

3.1.13. Methyl 6-methoxyamino-2,3-di-*O*-tetradecyl-6-deoxy-6-*N*-(4,6-di-*O*-acetyl-2,3-di-*O*-tetradecyl-β-D-glucopyranosyl)-α-D-glucopyranoside (15). To a solution of disaccharide **14** (pure β-anomer) (70 mg, 0.051 mmol) in CH_2Cl_2 (10 mL), DDQ (10 mg, 0.044 mmol) and water (0.2 mL) were sequentially added and the resulting mixture was stirred overnight. Dichloromethane (20 mL) and $NaHCO_3$ (satd aq soln, 30 mL) were added and the layers were separated. The organic layer was washed with brine (2×50 mL), dried over sodium sulfate and evaporated. Flash column chromatography of the residue on silica gel (petroleum ether/AcOEt 9:1) afforded **15** (58 mg, 90%). Compound **15**: $R_f = 0.25$ (petroleum ether/AcOEt 8:2). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.84$ (t, 1H, $J = 9.5$ Hz, H-4′), 4.66 (d, 1H,

J = 3.5 Hz, H-1), 4.16 (dd, 1H, J = 11.6, 4.6 Hz, H-6′a), 4.10 (d, 1H, J = 8.7 Hz, H-1′), 3.99 (dd, 1H, J = 11.6, 2.2 Hz, H-6′b), 3.78 (m, 1H, H-α), 3.65 (m, 2H, H-6), 3.70 (m, 1H, H-5), 3.51 (s, 3H, $NOCH_3$), 3.50 (m, 1H, H-5′), 3.40 (m, 2H, H-3′, H-2′), 3.33 (s, 3H, OCH_3), 3.24 (m, 1H, H-2), 3.20 (dd, 1H, J = 9.7, 3.5 Hz, H-2), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, J = 8.4 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 105.4 (C-1), 100.4 (C-1′), 91.2, 87.9, 87.4, 85.4, 81.5, 80.9, 80.8, 80.7, 80.2, 78.8, 77.2, 76.2, 70.9, 69.8, 67.6, 62.7. MALDI-TOF (DHB): *m/z*: 1256.2 $[M+H]^+$, 1277.5 $[M+Na]^+$.

3.1.14. Methyl 2,3-di-*O*-tetradecyl-6-deoxy-6-methoxyamino-6-*N*-(2,3-di-*O*-tetradecyl-β-D-glucopyranosyl)-α-D-glucopyranoside (1). Disaccharide **15** (20 mg, 0.016 mmol) was dissolved in methanol (3 mL). A freshly prepared solution of sodium methanoate (1 M, 20 μL, 0.02 mmol) was added dropwise. After 20 min the reaction was quenched by adding IRA 120 H^+ to a pH value of 7. The resin was filtered and the solvent was evaporated. Flash column chromatography of the residue (petroleum ether/AcOEt 6:4) afforded **1** (18 mg, quant.). 1: R_f = 0.45 (petroleum ether/AcOEt 1:1). $[\alpha]_D$ = −58.26 (*c* 0.30 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.64 (d, 1H, J = 2.6 Hz, H-1), 4.14 (d, 1H, J = 7.4 Hz, H-1′), 4.0–3.3 (m, 17H), 3.44 (s, 3H, $NOCH_3$), 3.36 (s, 3H, OCH_3), 3.20 (m, 1H, H-2′), 3.10 (dd, 1H, J = 7.9, 2.7 Hz, H-2), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, J = 8.4 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 98.0 (C-1), 93.8 (C-1′), 86.0, 81.8, 80.9, 79.7, 76.8, 73.2, 70.1, 69.7, 62.7, 56.0 (OCH_3). MS(ESI): *m/z*: 1193.02 $[M+Na]^+$.

3.1.15. Methyl 2,3-di-*O*-tetradecyl-4-*O*-*p*-methoxybenzyl-6-*O*-(2,3-di-*O*-tetradecyl-4,6-di-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside (17). To a solution of monosaccharide **13** (100 mg, 0.15 mmol) in dry CH_2Cl_2 (6 mL), trichloroacetonitrile (60 μL, 0.6 mmol) and caesium carbonate (25 mg, 0.075 mmol) were added and the resulting mixture was stirred for 1.5 h at room temperature. AcOEt (100 mL) was added and the organic layer was washed with $NaHCO_3$ (satd aq soln, 100 mL), brine (3 × 100 mL), dried over sodium sulfate and evaporated. To a solution of the crude trichloroacetimidate **16** (116 mg, 0.14 mmol) and glycosyl donor **5** (98 mg, 0.14 mmol) in dry CH_2Cl_2 (6 mL), trimethylsilyl triflate (10 μL) was added at −30 °C and the resulting mixture was stirred for 2 h. Ethyl acetate was added and the organic layer was washed with $NaHCO_3$ (satd aq soln, 50 mL), brine (3 × 50 mL), then dried over sodium sulfate and evaporated, to yield disaccharide **17** as a mixture of α and β isomers. Flash column chromatography on silica gel (petroleum ether/AcOEt 9.5:0.5) afforded **17** as a mixture of α and β isomers (102 mg, 55%). R_f = 0,45 (petroleum ether/AcOEt 9:1). MALDI-TOF (DHB): *m/z*: 1346.8 $[M+H]^+$, 1370.0 $[M+Na]^+$, $[\alpha]_D$ = +21 (*c* 0.5 in $CHCl_3$); The ^{1}H NMR spectrum is composed by the signals of both α and β anomers in 1:3 ratio, we report here the diagnostic signals for each isomer: **17**-α: δ = 5.10 (d, 0.25H, J = 3.0 Hz, H-1), 4.72 (d, 0.25H, J = 2.8 Hz, H-1′); **17**-β: δ = 4.79 (d, 0.75H, J = 3.5 Hz, H-1), 4.26 (d, 0.75H, J = 7.9 Hz, H-1′), 3.80 (s, 3H, OCH_3–PMB), 3.38 (s, 3H, OCH_3), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, J = 8.4 Hz, CH_3). ^{13}C NMR (only the signals of **17**-β carbons have been reported) (400 MHz, $CDCl_3$): δ (ppm): 104.5 (C-1′), 98.4 (C-1), 82.0 (C-2′), 81.8, 81.7, 80.9, 78.2, 77.0, 68.2, 68.0, 66.4, 66.3, 65.8, 65.3, 65.2, 64.8, 62.2 (C-6′), 52.8 (OCH_3).

3.1.16. Methyl 2,3-di-*O*-tetradecyl-6-*O*-(2,3-di-*O*-tetradecyl-4,6-di-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside (18). To a solution of the disaccharide **17** (mixture of α and β isomer, 50 mg, 0.037 mmol) in CH_2Cl_2 (4 mL), DDQ (20 mg, 0.088 mmol) and water (60 μL) were sequentially added and the resulting mixture was stirred for 3 h. Dichloromethane (40 mL) and $NaHCO_3$ (satd aq soln, 50 mL) were added and the layers were separated. The organic layer was washed with brine (2 × 50 mL), then dried over sodium sulfate and evaporated. Flash column chromatography of the residue (petroleum ether/AcOEt 9:1) afforded **18**α (14 mg) and **18**-β (28 mg) (83%). MALDI-TOF (DHB): *m/z*: 1376.4 $[M+H]^+$, 1398.5 $[M+Na]^+$ **18**-α: R_f = 0.30 (petroleum ether/AcOEt 8:2). Compound **18**-β: R_f = 0.25 (petroleum ether/AcOEt 8:2). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 4.84 (t, 1H, J = 9.9 Hz, H-4′), 4.71 (d, 1H, J = 3.5 Hz, H-1), 4.28 (d, 1H, J = 7.9 Hz, H-1′), 4.12 (dd, 1H, J = 11.6, 4.6 Hz, H-6′a), 4.02 (dd, 1H, J = 11.6, 2.2 Hz, H-6′b), 4.01 (m, 1H, H-6a), 3.80 (m, 2H, H-α), 3.70 (m, 2H, H-6b, H-α), 3.60–3.30 (m, 9H), 3.33 (s, 3H, OCH_3), 3.20 (dd, 1H, J = 9.7, 3.5 Hz, H-2), 3.15 (t, 1H, J = 7.9 Hz, H-2′), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, J = 8.4 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 104.5 (C-1′), 98.4 (C-1), 82.0 (C-2′), 81.1, 80.5 (C-2), 70.0 (C-4′), 68.9 (C-6), 62.2 (C-6′), 55.4.

3.1.17. Methyl 2,3-di-*O*-tetradecyl-6-*O*-(2,3-di-*O*-tetradecyl-β-D-glucopyranosyl)-α-D-glucopyranoside (2). Disaccharide **18** (pure β isomer, 20 mg, 0.014 mmol) was dissolved in dry methanol (3 mL) under argon atmosphere. A freshly prepared solution of sodium methanoate (1 M, 20 μL, 0.02 mmol) was added dropwise and the resulting mixture was stirred for 20 min, after which time IRA 120 H^+ was added until pH = 7. The resin was filtered and the solvent was evaporated. Flash column chromatography (petroleum ether/AcOEt 7:3) afforded **1** (16 mg, quant.). Compound **2**: R_f = 0.30 (petroleum ether/AcOEt 8:2). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 4.80 (d, 1H, J = 3.3 Hz, H-1), 4.40 (d, 1H, J = 7.5 Hz, H-1′), 4.10 (bd, 1H, J = 10.0 Hz, H-6a), 4.0–3.3 (m, 16H, H-3′, H-4′, H-5′, 2H-6′, H-4, H-5, H-6b, 8H-α), 3.43 (s, 3H, OCH_3), 3.29 (dd, 1H, J = 8.4, 3.0 Hz, H-2), 3.20 (t, 1H, J = 9.0 Hz, H-3), 3.15 (t, 1H, J = 7.9 Hz, H-2′), 1.60 (m, 8H, H-β), 1.30 (m, 38H, CH_2), 0.90 (t, 12H, J = 8.4 Hz, CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 104.5 (C-1′), 97.8 (C-1), 85.0 (C-3), 83.2 (C-2′), 82.0, 81.0 (C-2), 76.8, 75.2 (C-α), 75.1 (C-α), 73.8 (C-α), 69.5 (C-6), 62.7 (C-6′), 55.4 (OCH_3). MS(ESI): *m/z*: 1163.99 $[M+Na]^+$.

3.1.18. Chemoselective coupling of 7 and 9. Equimolar (50 μmol) amounts of the two monomers were dissolved in THF (0.25 mL). Acetic acid (0.4 mL) was added and the mixture was stirred for 6 days.

3.2. Biological assays

3.2.1. Culture medium and reagents. MT2 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 100 IU of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine (all from Sigma Chem. St. Louis, MO) and 50 μM β-mercaptoethanol.

LPS (*E. coli* 026:B6, used at 10 μg/mL) and lipid A were obtained from Sigma. Oligo CpG (TCCATGA CGTTCCTGATGCT) was purchased from Primm (Milan, Italy) and used at a concentration of 1 μM. Poly I:C (used at 20 μg/mL) was purchased from Amersham.

3.2.2. Determination of nitrite accumulation. NO^{2-} accumulation was measured as NO production by Greiss assays, with a sodium nitrite standard.

3.2.3. TNFα ELISA. TNFα ELISA was performed using the DuoSet kit (R&D, Minneapolis, MN) and following the manufacturer recommendations.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmc.2005.08.047.

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