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FULL PAPER

Synthesis of mannosylglycerate derivatives as immunostimulating agents

Nadège Hamon,*[a] Caroline C. Mouline,[a] and Marion Travert[a]

Abstract: Mannosylglycerate (MG) is a compatible solute extracted from some red algae and bacteria. Thanks to its ability to protect proteins and stabilize enzymes, MG has been widely studied for its uses against neurodegenerative diseases and in biotechnologies. More recently, its immunostimulating properties against Chronic Lymphocytic Leukaemia have been investigated. Herein we report the synthesis of MG derivatives and a preliminary biological study on their capacity to behave as immunostimulating agents. We investigated their toxicity on Peripheral Blood Mononuclear Cells from healthy donors and their ability to increase the phagocytosis of opsonised bacteria by polynuclear neutrophils, resulting in the discovery of two new molecules as potential immunostimulating agents.

Introduction

Cancer immunotherapy is described by the National Cancer Institute (NCI) as any "biological therapy that uses substances to stimulate or suppress the immune system to help the body fight cancer". This domain has been the subject of intense scientific interest over the past three decades. In fact, numbers of immunotherapies used today were reported to fight cancer such as monoclonal antibody, bi-specific T cell engager, immune checkpoint inhibitor, adoptive cellular immunotherapy and immunomodulatory agent.[1] Among immunomodulatory agents, several classes of compounds are found such as proteins or peptides, glycoproteins, lipids derivatives and polysaccharides.[2] Polysaccharides have a large spectrum of therapeutic properties,[3] in particular the ability to modulate macrophage function, by enhancing either phagocytic or secretory activity. These activities are triggered by inducing the production of ROS (reactive oxygen species), NO (nitric oxide) and cytokines (TNFα, IL-1 and IL-6).[3] The best known immunostimulating polysaccharides are β-glucans.[2,4] β-glucans are glucose polymers extracted from fungi, yeast, bacteria or cereals and promote macrophages proliferation and various activities such as phagocytic activity, NO and cytokines production.[2,4,5] Polysaccharides can also be extracted from other sources such as algae,[2,4,5] which represent an important source of polysaccharides and low molecular weight carbohydrates, potentially exhibiting immunomodulation capacity. This is the case of 2-*O*-α-D-galactopyranosylglycerol, also called floridoside (molecule **1**, Figure 1), which is extracted from *Rhodymenia palmate* or *Mastocarpus stellatus*.[6] Courtois et al. have demonstrated that floridoside is a mediator in the classical complement pathway and causes recruitment and activation of natural immunoglobulin M (IgM).[6] The complement system is part of the innate immunity and is activated to defend the organism against pathogens. Floridoside promotes the recognition of cancer cells by the immune system, and their elimination by macrophages.

Among all the molecules produced by algae, we were interested in mannosylglycerate **2** (MG, Figure 1). MG, also named digeneaside, is a compatible solute extracted for the first time in 1939 from the red algae *Polysiphonia fastigiata.*[7] Since then the presence of MG has been demonstrated in several members of (hyper)thermophilic archaea, thermophilic bacteria and also in red algae.[8] In these organisms, the concentration of MG increases most often in response to osmotic or heat stress.[9-15] In 1955 Bouveng et al. elucidated its structure using methylation or hydrolysis reactions[16] and MG was fully characterized by NMR[17] and X-Ray diffraction[18] in 2006 and 2009 respectively. The synthesis of MG derivatives was reported by Santos in 2008,[19] while the first chemical synthesis of MG itself was only described in 2016.[20] Its originality relies on the use of protected mannitol as a precursor of the glycerate moiety. The condensation of two mannosyl units with the protected mannitol, followed by an oxidative cleavage of the mannitol yields to two equivalents of MG Because of the well documented ability of MG to protect proteins and stabilize enzymes,[19,21-27] this molecule has been considered as a potential drug candidate against neurodegenerative diseases.[28] In particular MG was shown to reduce the Alzeihmer's β-amyloid aggregation and neurotoxicity in human neuroblastomas cells.[29] In Parkinson's disease models, MG inhibited α-synuclein fibril formation by 50% at 100 mM[28] and by 30% at 30 mM, leading to the reduction of α-synuclein toxicity.[30] In addition, MG improves the quality of DNA microarrays. [31]

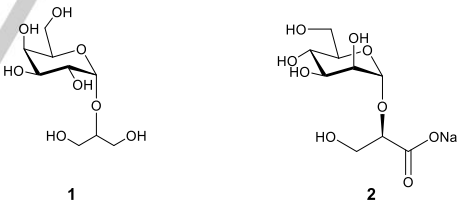

Figure 1 : Structure of floridoside **1** and mannosylglycerate **2**.

More recently, MG was shown to stimulate the human immune system in *in vitro* tests,[32] by activating the anti-tumoral activity of monocytes and polynuclear neutrophils and raising the activity of macrophages, leading to enhanced bacteria's phagocytose. Moreover in a mice model, MG used both alone and in combination with the immunotherapeutic antibody Rituximab, was able to increase mice survival from 25% (for 2mg of MG alone) to 75% (for 2mg or 4mg of MG in combination with 12mg/kg of Rituximab).

To the best of our knowledge no structure-activity relationship study has been reported so far in order to understand the key structural features of MG linked to its immunostimulating activity. Herein we describe the synthesis of MG derivatives and their preliminary biological evaluation as immunostimulating agents. In particular three parts of the molecule were modified. We first wanted to study the influence of the sugar moiety, hence we synthesised glucosylglycerate and galatosylglycerate in which the mannosyl moiety was replaced by glucosyl and galactosyl sugars, respectively. 2α-mannobiosylglycerate was also synthesized to

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test the influence of the number of mannosyl units on the activity. Finally our attention was focused on the carboxylate group: Ryu et al. previously reported that the carboxylate group of MG could be responsible of the inhibition of β-amyloid peptide aggregation since analogues lacking this group were devoid of activity.[29] We decided therefore to synthesize MG derivatives lacking of the carboxylate group (mannosyl ethylene glycol), or where the same group was replaced by bioisosteres such as phosphonate or hydroxamic acid groups.[33]

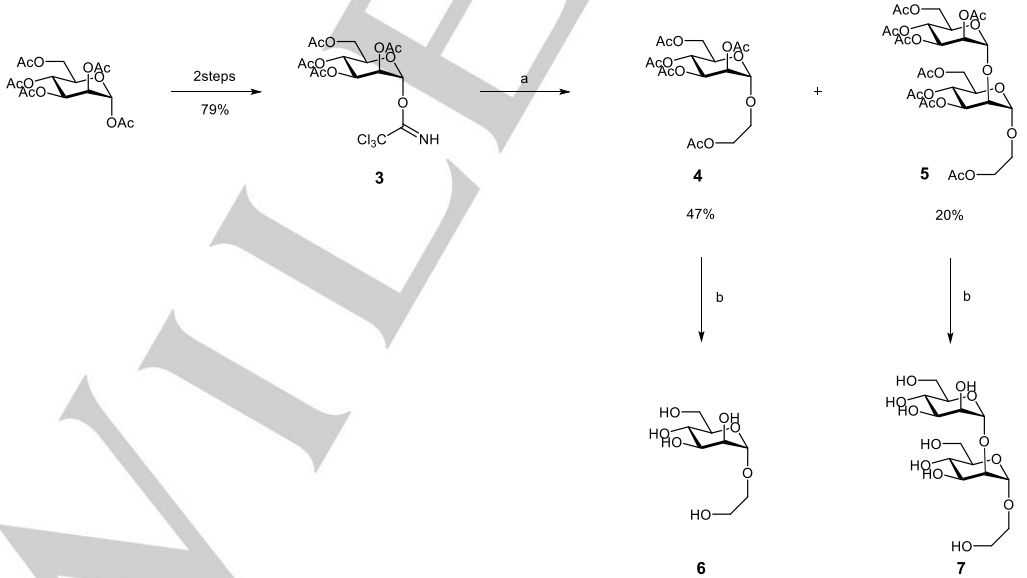
Results and Discussion

Synthesis of MG derivatives

Mannosylglycerate **2** has been synthesized according to the procedure described by Morere et al. [20] 1-(α-D-mannopyranosyl) ethylene glycol **6** (Scheme 1), 1'-hydroxy-2'-(α-D-mannopyranosyl)ethyl phosphonate **13** (Scheme 2) and *N*-hydroxy-3'-hydroxy-2'-(α-D-mannopyranosyl) propanamide **16** (Scheme 3) are new analogues of MG lacking of the carboxylate group (compound **6**) or where the same group has been replaced by bioisosteres (compounds **13** and **16**). For the synthesis of mannosyl ethylene glycol **6**, the α-mannose trichloroacetimidate derivative **3** was synthesised from peracetylated mannose in two steps according to the literature[34-35] and was then used in a glycosylation reaction with 1-*O*-acetyl-ethylene glycol and TMSOTf as a catalyst.[36] As expected we obtained the desired α-anomer of compound **4** in moderate yield (47%) but we also observed the formation of 20% of the 2α-mannobioside **5**. These compounds were easily separated by chromatography on silica gel. Deprotection of compound **4** was performed by an aminolysis reaction in order to obtain mannosyl ethylene glycol **6** in almost quantitative yield. The anomeric configuration of compound **6** was confirmed by NMR analysis, with a coupling constant $J_{C1,\ H1}$ = 170.9 Hz typical from the α-anomer.[37] The same conditions were applied to the bimannoside **5** to yield the mannobiosyl ethylene glycol **7**. The (α-mannopyranosyl)-(1->2)-(α-D-mannopyranosyl) skeleton was confirmed by 2D NMR experiments (see spectra in the supporting information).

In order to synthesize 1'-hydroxy-2'-(α-D-mannopyranosyl)ethyl phosphonate **13**, diethyl vinylphosphonate was submitted to a dihydroxylation reaction according to a procedure described by Sharpless[38] (Scheme 2). Selective protection of the primary alcohol as a silyl ether yielded to compound **9**. The glycosylation reaction of the latter with the glycosyl donor **3** gave the desired α-anomer of compound **10** in 69% yield but we also observed the formation of the bi-mannoside **11**. We postulated that partial deprotection of the hydroxyl methyl group happened during the glycolysation reaction, and that the resulted free alcohol participated to a second non-desired glycosylation reaction. Compounds **10** and **11** were easily isolated by chromatography on silica gel. Removal of the acetyl groups of compound **10** was performed using concentrated ammonia solution in methanol. Final deprotection of the phosphonate group in the presence of TMSBr[39] was accompanied by the removal of the silyl ether group, leading to the desired compound **13** in 52% yield. Once again, the coupling constant $J_{C1,\ H1}$ = 170.8 Hz was typical from the α-anomer.[37]

The synthesis of hydroxy-3'-hydroxy-2'-(α-D-mannopyranosyl) propanamide **16** is depicted in scheme 3. Compound **14** was first synthesized in five steps from peracetylated mannose according to the procedure described by Morere et al.[20] Esterification of the carbocylic acid[40] led to the desired methyl ester. Treatment of compound **15** with hydroxylamine[41,42] yielded the hydroxamic acid derivative **16** and allowed the deprotection of the mannose at the same time.



Scheme 1 : Synthesis of mannosyl ethylene glycol **6** and 2α-mannobiosyl ethylene glycol **7**. Reagents and conditions (a) 1-*O*-acetyl-ethylene glycol, TMSOTf, molecular sieves, CH_2Cl_2, -20 °C, 1 h, r.t., 6h. (b) NH_4OH, MeOH, r.t., 30 h, 99% for **6**, r.t., 6 h, 77% for **7**.

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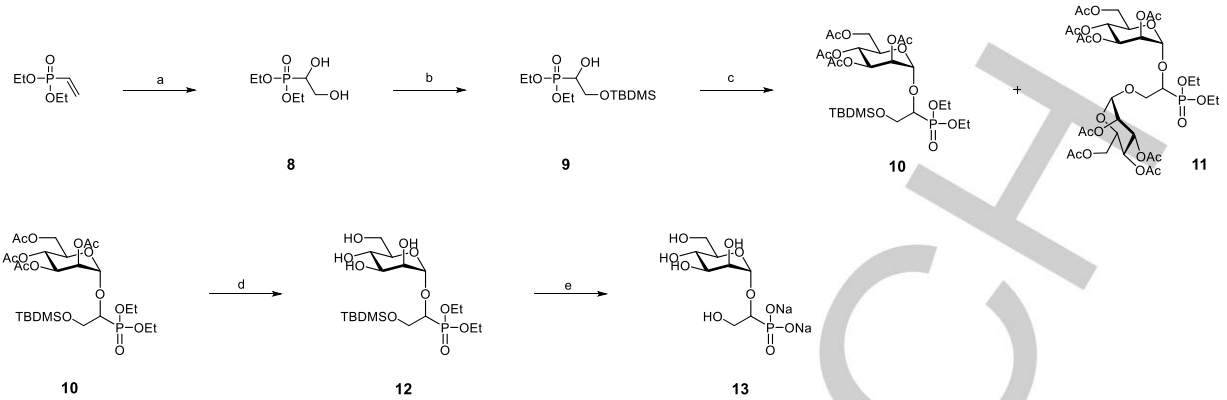
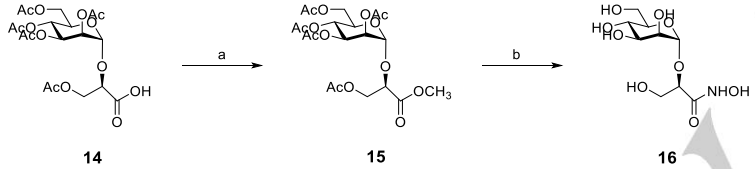


Scheme 2 : Synthesis of 1'-hydroxy-2'-(α-D-mannopyranosyl)ethyl phosphonate **13** as its sodium salt. Reagents and conditions (a) OsO_4, TMO, citric, acid, *t*-BuOH/H_2O, r.t., 29 h, 96%. (b) TBDMSCl, imidazole, DMF, r.t., 5.5 h, 80%. (c) **3**, TMSOTf, CH_2Cl_2, -20 °C, 1h, r.t., 1.5 h. (d) NH_4OH, MeOH, r.t., 64 h, 51%. (e) i. TMSBr, DMF, r.t., 26 h, ii. Dowex Na^+, 52%.



Scheme 3 : Synthesis of hydroxy-3'-hydroxy-2'-(α-D-mannopyranosyl) propanamide **16**. Reagents and conditions (a) K_2CO_3, MeI, DMF, r.t., 2.5 h, 72%. (b) NH_2OH, KCN, MeOH, r.t., 3.5 h, 44%.

In order to study the influence of the mannosyl moiety on the activity of MG, we decided to replace this sugar by glucosyl or galactosyl units. The main challenge in the synthesis of glucosylglycerate and galactosylglycerate consists in the selective formation of the α-glycosidic bond. A new approach had to be used since the formation of beta-anomers is usually favoured with carbohydrates bearing participating groups such as acetate at the 2-position. Synthesis of (2'R)-2'-(α-D-glucopyranosyl)-D-glycerate has been described by Santos[19] and Ventura.[43] These syntheses relied on a glycosylation step between a thioglucoside donor and a protected glycerate moiety, leading to the desired protected α-glucosylglycerate. However several deprotection steps were then necessary to obtain the final product, leading to an overall twelve steps required for the synthesis of glucosylglycerate **25**. In this work we decided to adapt the methodology used by Morere to synthesize mannosylglycerate[20] and to transpose it to the synthesis of glucosylglycerate. This new strategy relies on the condensation of two glycosyl moieties on a protected mannitol. The key step of the strategy is an oxidative cleavage of the mannitol chain, yielding to the formation of two equivalents of the glycosylglycerate. In this goal thioglucoside **17** was synthesized in four steps from D-glucose in 62% yield (Scheme 4).[44,45] The glycosylation reaction between two equivalents of compound **17** and protected mannitol **18**[32] led to the desired dimer **19**, but also to the monomer **21** and 2,3,4,6-tetrabenzylglucopyranose **20**. These compounds were isolated by chromatography on silica gel and compound **21** could be recycled in a second glycosylation reaction to afford more dimer **19**. Isopropylidene group removal of compound **19** was carried out using a TFA/H_2O mixture, leading to the diol **22**. Oxidative cleavage was performed in the presence of TEMPO and (diacetoxyiodo)benzene in CH_2Cl_2/H_2O and led to two equivalents of the glucosylglyceric acid **23** in 89% yield. Finally deprotection of the glycerate chain under basic conditions followed by removal of the benzyl ethers protecting groups gave the glucosylglycerate **25.**

Synthesis of (2R)-2-(1-*O*-α-D-galactopyranosyl) glycerate **33** has already been described by Maycock et al:[46] a glycosylation reaction between a thiogalactoside and protected glycerate followed by several deprotection steps led to the galactosylglycerate. However once again we decided to apply our strategy based on an oxidative cleavage of mannitol to obtain galactosylglycerate. With this aim thiogalactoside **26** was synthesized in three steps according to the method described by Magnusson[45] (Scheme 5). The glycosylation reaction of compound **26** with protected mannitol **18** led to the desired dimer **27**, in which both the galactosyl moieties have the α-configuration at the anomeric position. However we also observed the formation of its stereoisomer **28**, in which one galactosyl unit has the α-configuration and the second one is linked to the mannitol *via* a β bond. Despite our efforts these compounds were not separable by chromatography on silica gel, therefore the removal of the isopropylidene protecting group was performed on the mixture of compounds **27** and **28**, leading to the diols **29** and **30** which were however easily isolated by chromatography on silica gel. The α,α-dimer **29** was then submitted to an oxidative cleavage, leading to galactosyl glyceric acid **31** in 86% yield. Removal of the acetyl groups followed by a hydrogenation of the benzyl ethers gave the α-galactosyl glycerate **33** as its sodium salt. The anomeric configuration was confirmed by ^1H NMR analysis thanks to the coupling constant $J_{1\text{-}2}$ = 3.8 Hz, typical for the α-anomer. [47] This three-steps sequence was then applied to the α,β-dimer **30** and we were able to isolate galactosyl glycerate **34**. The coupling constant $J_{1\text{-}2}$ = 6.5 Hz confirmed that compound **34** was the β-anomer.

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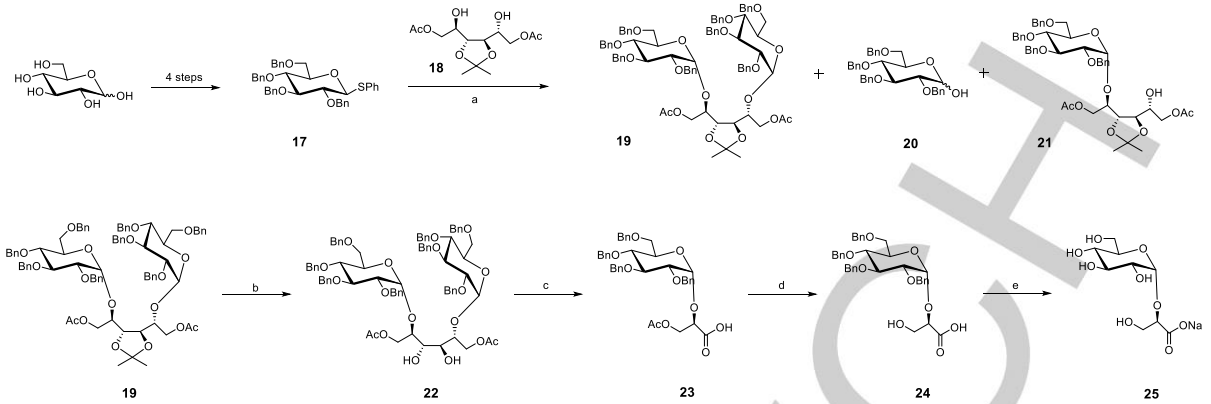
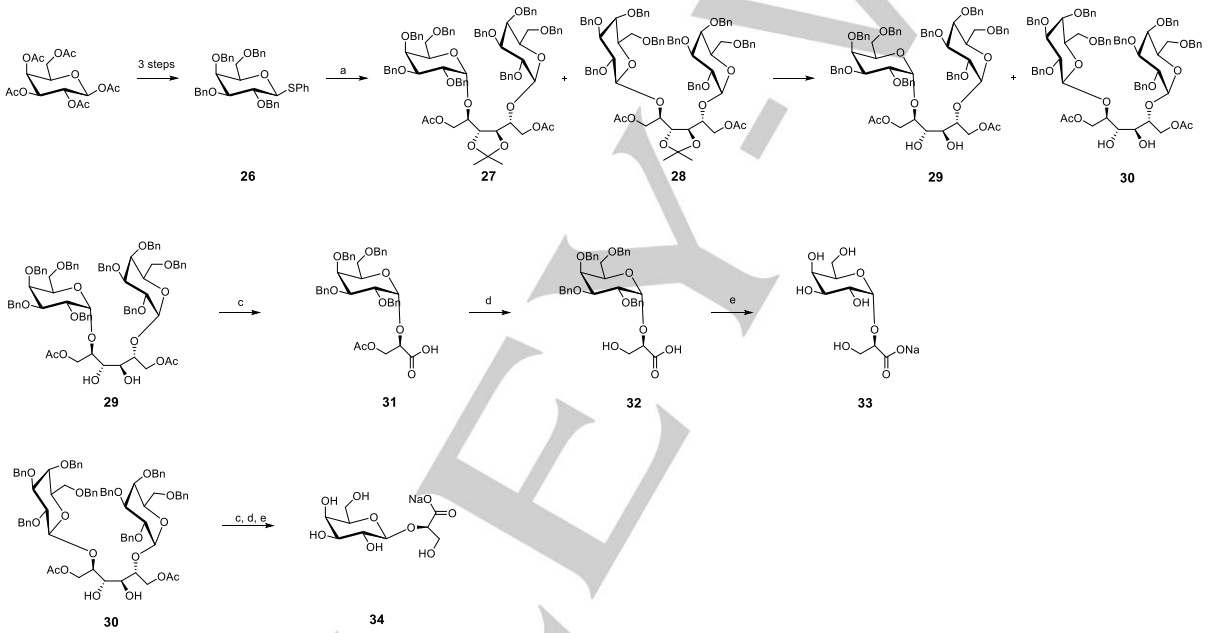
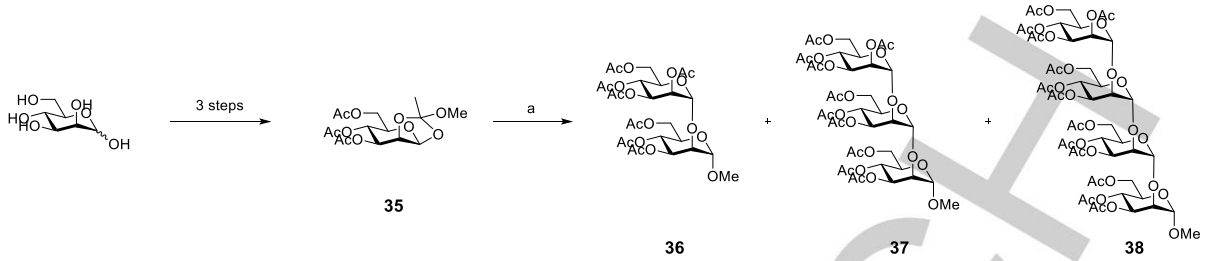


Scheme 4 : Synthesis of (2'R)-2'-(α-D-glucopyranosyl)-D-glycerate **25**. Reagents and conditions: (a) NBS, TMSOTf, DCM/Et_2O, -55 °C, 6 h (b) TFA/H_2O, r.t., 30 min, 15% over two steps (c) TEMPO, BAIB, r.t., 5.5 h, 89% (d) NH_4OH, MeOH, r.t., 18.5 h, 89% (e) i. Pd/C, AcOEt/MeOH/AcOH 5/5/1, 20 bars H_2, r.t., 1 week, ii. Dowex Na^+, 88%.



Scheme 5 : Synthesis of (2R)-2-(1-*O*-α-D-galactopyranosyl)glycerate **33** and (2R)-2-(1-*O*-β-D-galactopyranosyl)glycerate **34**. Reagents and conditions: (a) **18**, NBS, TMSOTf, CH_2Cl_2/Et_2O, -55 °C, 2 h (b) TFA/H_2O, r.t., 30 min, **29**: 29% over two steps, **30**: 16% over two steps (c) TEMPO, BAIB, CH_2Cl_2/H_2O 1/1, r.t., 5.5 h, 86% for **31** (d) NH_4OH, MeOH, r.t., 18.5 h, 32% for **32** (e) i. Pd/C, AcOEt/MeOH/AcOH 5/5/1, 20 bars H_2, r.t., 1 week, ii. Dowex Na^+, 84% for **33**, 27% over three steps for **34**.

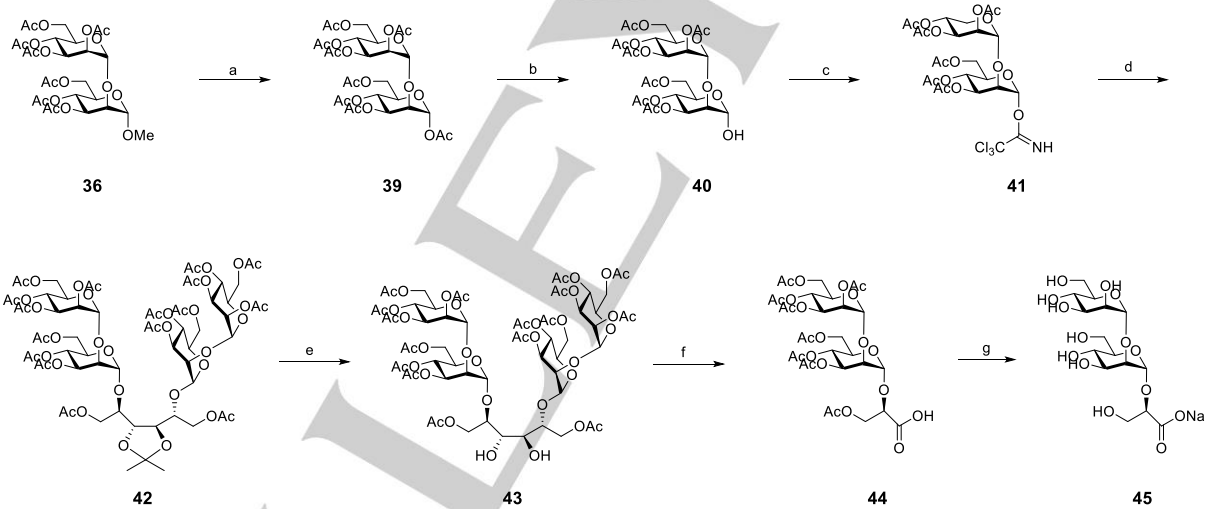
The influence of the number of mannosyl units on the activity of the molecule has also been investigated by synthesising (2'R)-2'-[(α-D-mannopyranosyl)-(1→2)-(α-D-mannopyranosyl)] glycerate **45** (Scheme 7). We also noticed that mannobiosyl ethylene glycol **7** showed promising biological activity whereas mannosyl ethylene glycol **6** was not active (see the biological evaluation below, table 3), hence we were interested in studying the addition of one more mannosyl unit to this structure, generating trimannosyl ethylene glycol **48** (Scheme 8). So we aimed at establishing a chemical route which could allow rapid access to methyl dimannoside **36** and trimannoside **37**. We were in particular impressed by the efficient synthetic approach leading simultaneously to methyl di- and trimannosides **36** and **37** from methyl 1,2-ortho ester **35** described by Tan[48] and Fraser-Reid[49] (Scheme 6). To our delight, treatment of compound **36** with TMSOTf in dichloromethane afforded the desired di- and tri-saccharides **36** and **37** with 42% and 29% yields respectively, along with the formation of 8% of the methyl tetramannoside **38**. These compounds were separated by chromatography on silica gel using a slow gradient of petroleum ether in ethyl acetate (6/4 to 2/8). Even if Fraser-Reid tried to explain the formation of these saccharides,[49] the mechanism of generation of these compounds still remains unclear. However we were able to confirm that this approach led simultaneously and in only one step to the desired di- and trimannosides **36** and **37** and also allowed us to isolate tetramannoside **38**.



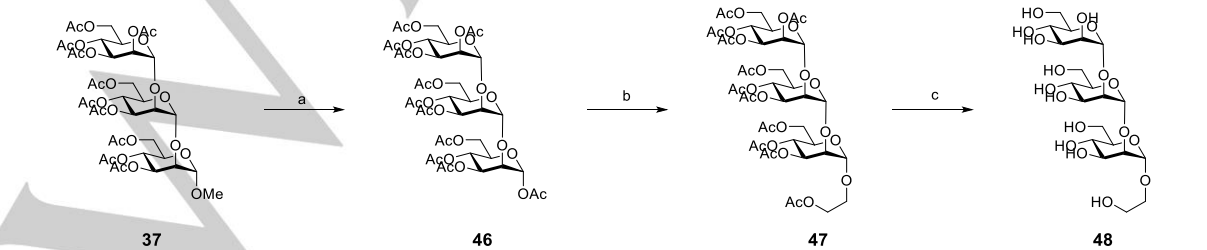
Scheme 6 : synthesis of methyl di- tri- and tetramannosides **36**, **37** and **38**. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, 5 min, -30 °C, **36**: 42%, **37**, 29%, **38**: 8%.

Disaccharide **36** was then used in the synthesis of mannobiosyl glycerate **45** (scheme 7). Conversion of the disaccharide **36** into its 1-*O*-acetylated derivative **39** was performed using a catalytic amount of sulfuric acid in acetic anhydride.[48] The anomeric position of peracetate **39** was deacylated using ammonium acetate[50] and then converted under standard conditions to the activated trichloroacetimidate **41**. Glycosylation reaction of this latter with protected mannitol **18** led to the desired compound **42** in 78% yield. Removal of the isopropylidene group in the presence of TFA/H_2O afforded the diol **43** which was then submitted to an oxidative cleavage, giving the disaccharide **44** in 74% yield. Final deprotection in the presence of NH_4OH (30-33% solution) in methanol gave the desired mannobiosyl glycerate **45**. The (α-mannopyranosyl)-(1->2)-(α-D-mannopyranosyl) skeleton was confirmed by 2D NMR experiments and we also observed the presence of 8% of (2'R)-2'-[(α-D-mannopyranosyl)-(1→2)-(β-D-mannopyranosyl)] glycerate (see spectra in the supporting information).

Methyl trimannoside **37** was used in the synthesis of mannotriosyl ethylene glycol **48** (Scheme 8). Acetylation of compound **37** followed by a glycosylation reaction of the resulting peracetylated trisaccharide **46** with 1-*O*-acetyl-ethylene glycol gave the mannotrioside **47** in 50% yield. Final removal of the acetyl groups under basic conditions afforded the desired mannotriosyl ethylene glycol **48**. The α-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl skeleton was fully confirmed by 2D NMR experiments (see supporting information).



Scheme 7 : Synthesis of (2'R)-2'-[(α-D-mannopyranosyl)-(1→2)-(α-D-mannopyranosyl)] glycerate **45**. Reagents and conditions: (a) H_2SO_4, Ac_2O, 0 °C, 3.5 h, 95%. (b) ammonium acetate, DMF, r.t., 3.5 days, 90%. (c) CCl_3CN, DBU, CH_2Cl_2, r.t., 23 h, 70%. (d) **18**, TMSOTf, CH_2Cl_2, -30 °C, 4.5 h, 78%. (e) TFA/H_2O 16/1, r.t., 30 min, 45%, 57% based on recovered starting material. (f) TEMPO, BAIB, CH_2Cl_2/H_2O 1/1, r.t., 20 h, 74%. (g) NH_4OH, MeOH, r.t., 2.5 days, 88%.



Scheme 8 : Synthesis of α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl ethylene glycol **48**. Reagents and conditions: (a) H_2SO_4, Ac_2O, 0 °C, 3 h, 83%. (b) 1-*O*-acetyl-ethylene glycol, $BF_3.Et_2O$, CH_3CN, r.t., 16.5 h, 50%. (c) NH_4OH, MeOH, r.t., 40 h, 80%.

Toxicity of mannosylgycerate

In a previous study Berthou et al. demonstrated that the natural mannosylglycerate isolated from red algae, also called digeneaside, was not toxic at concentrations up to 10mM on neither tumor cell lines Daudi nor on primary leukaemia cells isolated from patients with Chronic Lymphocytic Leukaemia (CLL).[32] Two sources of MG were used in the study: the first form of MG, isolated from the red algae *soliera chordalis* and *Ceramium botryocarpum*, was obtained as its sodium salt and is called digeneaside; the second source of MG, isolated from the extremophilic bacteria *Rhodothermus marinus* was obtained as its potassium salt and is called firoin.

Table 1 : Cytotoxicity of digeneaside, firoin and synthetic mannosylglycerate at 10 mM on primary leukaemia cells and PMBC isolated from healthy donors.

	Viability of cells (%)	
	B LLC	PMBC from healthy donors
Control (untreated cells)	70.2[a]	75.4[e]
Digeneaside 24h	69.2[b]	-
Digeneaside 48h	41.1[b]	-
Firoin 24h	82.5[c]	69.6[d]
Firoin 48h	82.7[c]	69.3[d]
Synthetic mannosylglycerate sodium salt, 24h	78.1[d]	76.1[d]
Synthetic mannosylglycerate sodium salt, 48h	64.5[d]	74.8[d]

a: study performed on cells from 5 different donors.
b: study performed on cells from 1 donor.
c: study performed on cells from 3 different donors.
d: study performed on cells from 2 different donors.
e: study performed on cells from 4 different donors.

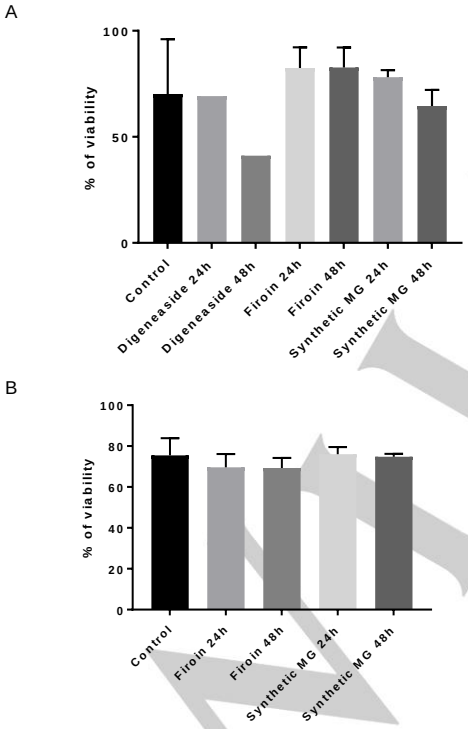


Figure 2: Toxicity of mannosylgycerate. A: B cells isolated from patients with CLL were isolated and treated with 10 mM of digeneaside, firoin or synthetic MG for 48h and viability was evaluated by annexin V/propidium iodide by flow cytometry. B: Peripheral Mononuclear Blood Cells (PBMC) isolated from healthy donors were incubated with 10 mM of firoin or synthetic MG for 48h and viability was evaluated by annexin V/propidium iodide by flow cytometry.

Our first goal was to confirm the bioequivalence of our synthetic MG relative to digeneaside and firoin. In this aim B cells isolated from patients with CLL were treated with 10 mM of digeneaside, firoin or synthetic MG for 48h. As shown on figure 2 and table 1, our synthetic MG was devoid of toxicity, leading to only 5% of the cells death after 48h. In order to confirm that MG was not toxic on healthy cells, peripheral Mononuclear Blood Cells (PBMC) isolated from healthy donors were incubated with 10 mM of synthetic MG for 48h. As shown in table 1, the sodium salt of synthetic MG was not toxic at 10 mM on PMBC. In fact, no increase of the cells death was observed compared to untreated cells. These results suggested the bioequivalence of the synthetic MG with the natural digeneaside and firoin.

Evaluation of the immunostimulating power of synthetic mannosylgycerate

The ability of synthetic MG to stimulate the immune system, and in particular innate immune cells like neutrophils, monocytes and NK cells, was also investigated by exploring different activities of these cells such as phagocytosis and ADCC (antibody dependent cell cytotoxicity) after treatment with synthetic MG. Phagocytosis is a process in which polymorphonuclear neutrophils cells and macrophages capture bacteria in order to eliminate the pathogen. In the test, bacteria were labelled with a fluorochrome. Non-phagocytosed bacteria were eliminated and the fluorescence of phagocytosed bacteria in the polymorphonuclear neutrophils cells was measured by flow cytometry. Berthou *et al.* showed that treatment of human whole blood with 10mM of natural MG for 24 hours enhanced the phagocytosis of polymorphonuclear neutrophils cells from 42.5% in the control study to 77.4% after digeneaside treatment.[32] In order to demonstrate the bioequivalence of our synthetic MG, human whole blood was treated with 10mM of synthetic MG or firoin (as a positive control) for 24 hours and the phagocytosis was investigated by phagotest kit and flow cytometry. As shown in figure 3A, firoin induced 12% more phagocytosis compared to control. However synthetic MG did not induce phagocytosis in comparison with untreated whole blood. These results suggest that synthetic MG lacks of the ability to enhance the phagocytosis of polymorphonuclear neutrophils cells, in contrast with natural MG.
In the previous study firoin was able to increase the number of monocytes from 13.9% compared to untreated PBMC to 21.9% in 5 days.[32] So PBMC from healthy donors were treated with 10mM of synthetic MG for 9 days and the absolute number of monocytes CD14+ and dendritic cells CD11b+ were evaluated by flow cytometry. No increase in the number of monocytes or dendritic cells modification was observed after 5 days of treatment with 10mM of synthetic MG. However, as shown in figure 3B, the number of monocytes CD14+ and dendritic cells CD11b+ raised after 9 days in the presence of synthetic MG, along with an increase of monocytes CD14+ after 7 days (data not shown). Thereby the immunostimulating power of synthetic MG was demonstrated, although with a delay of 2-4 days when compared to firoin.

ADCC is a process in which an effector cell (monocytes, NK cells, polymorphonuclear neutrophils cells) actively destroys a targeted cell presenting a specific antibody. In a previous study the potential of natural MG to augment ADCC in the presence of Rituximab was demonstrated.[32] So PBMC from healthy donors were treated with 10 mM of synthetic MG for 5 days and specific lysis was determined by flow cytometry both in the presence or absence of 400 ng/ml of rituximab. As illustrated in figure 3C, treatment of PBMC with 10mM of synthetic MG enhanced the

ADCC from 30% for untreated cells to 40%. Furthermore, in the absence of Rituximab, a natural cell cytotoxicity of PBMC after treatment with 10 mM of synthetic MG was observed. In order to understand what kind of cells was in charge of cell cytotoxicity, magnetic cell sorting was performed: NK cells and monocytes were magnetic cell sorted from PBMC from healthy donors and treated with 10 mM of synthetic MG for 5 days and specific lysis was determined by flow cytometry in the presence or absence of 400 ng/ml of rituximab. Treatment of NK cells with synthetic MG for 5 days did not increase ADCC (Figure S1 in the supplementary data). However, as illustrated in figure 3D, treatment of monocytes with 10 mM of synthetic MG and 400 ng/ml of rituximab enhanced ADCC specific cell cytotoxicity from 25% for untreated cells to 35% for treated monocytes. Furthermore, in the absence of Rituximab, we observed a natural cell cytotoxicity of monocytes after treatment.

To summarize, it appeared that synthetic MG was unable to enhance the phagocytosis of polymorphonuclear neutrophils cells in comparison with natural MG. However synthetic MG showed the same immunostimulating properties than natural MG with an increase in both the number of monocytes and the ADCC of PBMC and monocytes.

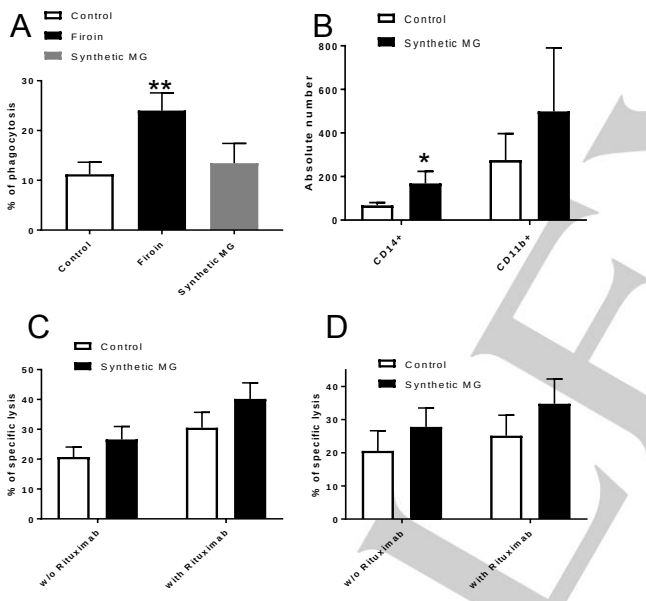


Figure 3: Evaluation of the immunostimulating power of synthetic mannosylgycerate. A: Human whole blood was treated with 10mM of synthetic MG or firoin (positive control) for 24 hours and the phagocytosis was investigated by phagotest kit and flow cytometry (study performed on cells from 10 different donors.). B: PBMC from healthy donors were treated with 10mM of synthetic MG for 9 days and the absolute number of monocytes CD14+ and dendritic cells CD11b+ were evaluated by flow count by flow cytometry (study performed on cells from 11 different donors.). C: PBMC from healthy donors were treated with 10 mM of synthetic MG for 5 days and specific lysis of Daudi cells was determined by flow cytometry after addition or not of 400 ng/ml of rituximab for 4 hours (study performed on cells from 8 different donors.). D: Monocytes were magnetic cell sorted from PBMC from healthy donors and treated with 10 mM of synthetic MG for 5 days and specific lysis of Daudi cells was determined by flow cytometry after addition or not of 400 ng/ml of rituximab for 4 hours (study performed on cells from 5 different donors.). Statistical analyses were performed with the Mann-Whitney test using GraphPad Prism software. The significant is shown as follows: *, $p \leq 0.05$; **, $p \leq 0.01$. w/o = without.

Evaluation of the immunostimulating power and toxicity of mannosylglycerate derivatives

The toxicity of mannosylglycerate analogues has been evaluated at 10 mM on PMBC isolated from healthy donors. In detail, Peripheral Mononuclear Blood Cells (PBMC) isolated from healthy donors were incubated with 10 mM of synthetic MG or its derivatives and the results are given in table 2. To evaluate the capacity of MG derivatives to behave as immunostimulating agents, we measured their capacity to increase the phagocytic activity of polymorphonuclear neutrophils from healthy donors (Table 3). As shown in table 2, replacement of the mannosyl moiety of MG by a galactosyl or a glucosyl did not have an influence on the toxicity of the molecule since all these compounds were not toxic on PBMC cells from healthy donors. Glucosylglycerate **25** was less active than MG since the phagocytosis induced by compound **25** was 2.6% weaker than the phagocytosis induced by MG (table 3). When the mannosyl moiety was replaced by a galactosyl, we noticed a slight increase in the activity of the α-galactosylglycerate **33** compared to MG, whereas the β-galactosylglycerate **34** induced 4.6% more phagocytosis than MG. Therefore the anomeric configuration of the sugar seems to be important for the activity of the molecule. Removal of the carboxylate group of the glycerate chain (compound **6**) or its replacement by bioisosteres such as either the phosphonate group (compound **13**) or the hydroxamic group (compound **16**) led to an acute toxicity on PMBC from healthy donors compared to MG. These results suggest that the carboxylate group seems to be crucial for the activity of MG. To our delight compound **7**, obtained from a side reaction in one of our syntheses, induced more phagocytosis than MG, although with similar toxicity (4.5% more compared to MG). Encouraged by these results, we synthesized compound **45** which combines the 2α-mannobiosyl skeleton with the glycerate chain. As shown in tables 2 and 3, mannobiosylglycerate **45** did not show any toxicity, however it did not seem to increase phagocytosis compare to untreated cells. Finally, the activity of trimannosyl ethylene glycol **48** was compared to the activity of mannobiosyl ethylene glycol **7**: once again the introduction of one more mannosyl unit on the molecule did not increase the toxicity on PBMC cells, and trimannosyl ethylene glycol **48** induced 3% more phagocytosis than bimannosyl ethylene glycol **7** and 10% more phagocytosis than untreated cells, making it a promising immunostimulating agent.

Table 2 : Cytotoxicity of mannosylglycerate and mannosylglycerate derivatives. Viability of PMBC cells from healthy donors treated with 10 mM of MG or derivatives.

	Viability of cells (%)				
	Donor 1	Donor 2	Donor 3	Donor 4	Average %
Control (untreated cells)	79.7	71.0	68.5	69.2	72.1
2	73.2	61.4	69.2	-	67.9
6	17.8	28.7	-	-	23.3
7	65.7	59.1	65.5	-	63.4
13	22.7	25.7	-	-	24.9
16	36.9	19.2	-	-	28.1
25	-	-	69.3	-	69.3
33	-	-	74.3	-	74.3
34	-	-	64.3	-	64.3
45	-	-	-	69.6	69.6
48	-	-	-	69.5	69.5

Table 3: Percentage of phagocytosis observed on opsonised bacteria by PNN after 48h in the presence of 10 mM of MG or its derivatives.

	% of phagocytosis					
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Average %
Control (untreated cells)	29.0	9.3	9.3	17.3	6.6	14.3
2	31.9	-	3.2	10.8	-	15.3
6	25.7	-	-	-	-	25.7
7	40.1	19.5	16.5	17.3	13.5	21.4
13	48.0	21.4	-	-	-	34.7
16	21.0	-	-	-	-	21.0
25	-	-	-	8.2	-	8.2
33	-	-	4.4	-	-	4.4
34	-	-	7.8	-	-	7.8
45	-	-	-	-	7	7
48	-	-	-	-	16.5	16.5

Conclusion

In summary, we have synthesized and fully characterized nine derivatives of MG. Thanks to the new method described by Morere et al., based on a glycosylation reaction between an activated sugar and protected mannitol followed by an oxidative cleavage of the mannitol chain,[20] we bypassed some deprotection steps in the synthesis of glucosylglycerate **25** and galactosylglycerate **33** and we successfully synthesized 2α-mannobiosyl glycerate **45**. We then demonstrated that synthetic MG was not toxic on LLC cells and on PMBC cells from healthy donors, as was the natural digeneaside. Moreover synthetic MG was able to stimulate the immune system since it increased the number of monocytes and raised the ADCC of PBMC and monocytes. A preliminary biological study was performed on MG derivatives and we observed that variation of the sugar moiety or the number of mannosyl units does not seem to improve the activity of the molecule. Replacement of the carboxylate group of the glycerate chain led to a strong toxicity on PBMC from healthy donors. However preliminary biological results showed that two new molecules, mannobiosyl ethylene glycol **7** and mannotriosyl ethylene glycol **48**, could behave as promising immunostimulating agents. It is worth noting that compound **7** results from a side–reaction in one of our syntheses and that we obtained compound **48** thanks to a strategy leading simultaneously to di-and trisaccharides. Now that we have highlighted the immunostimulating properties of these two compounds, new optimized synthetic routes are under consideration. Moreover, further biological experiments, such as the increase of monocytes and dendritic cells, need to be performed to confirm these first results and investigate on the mechanism behind the biological activity of these molecules.

Experimental Section

Chemistry

General Methods. Reagents used for the synthesis were purchased from Acros Organics, and Sigma-Aldrich and used without further purification. Solvents and water were distilled before use. The following instruments were used for the characterization. For NMR: ^{1}H and ^{13}C NMR spectra were recorded with Bruker Avance 500 (500 MHz for ^{1}H), Bruker Avance 400 (400 MHz for ^{1}H), or Bruker DRX 300 (300 MHz for ^{1}H, 75 MHz for ^{13}C) spectrometers and data were listed in parts per million (ppm). High-resolution mass spectrometry was realized on an HRMS Q-Tof MaXis, sources ESI, APCI, APPI, and nano-ESI (at the Institute of Organic and Analytic Chemistry −ICOA in Orleans). Compounds were purified by chromatography on silica gel (VWR, ref 1.09385.5000). The resin dowex was purchased from Aldrich (ref 44462, Dowex 50WX2, Na^+ form) All the final compounds were desalted on Sephadex G10 resin (Aldrich, ref GE17-0010-01) before biological evaluation.

1-O-Acetyl-ethylene glycol. To a solution of ethylene glycol (1 mL, 17.9 mmol) in CH_2Cl_2 (179 mL) was added trimethyl orthoacetate (3.42 mL, 26.9 mmol, 1.5 eq) and *p*-toluenesulfonic acid (68 mg, 0.3 mmol, 0.02 eq). The reaction mixture was stirred at room temperature for 14.5 h before addition of 1.5 eq of H_2O. The reaction mixture was stirred at room temperature for 30 min and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: CH_2Cl_2/MeOH 100/1) gave 1-*O*-Acetyl-ethylene glycol (1.63 g, 15.6 mmol, y = *87*%) as a colourless oil. Rf (CH_2Cl_2/MeOH 100/5) = 0.48. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.20 (dd, J = 5.5, 3.7 Hz, 2H), 3.83 (dd, J = 5.5, 3.8 Hz, 2H), 2.10 (s, 3H), 1.97 (s, 1H). ESI-HR-MS (positive): *m/z* calculated for $C_4H_9O_3$: 105.0546 found: 105.0548, calculated for $C_4H_8NaO_3$: 127.0366, found: 127.0370.

2-O-Acetyl-ethyl 2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranoside (**4**) *and 2-O-Acetyl-ethyl ((2',3',4',6'-tetra-O-acetyl-α-D-mannopyranosyl)-(1->2)-(2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranoside))* (**5**). To a solution of compound **3** (500 mg, 1.02 mmol, 1.21 eq) and molecular sieves 4Å (707 mg) in CH_2Cl_2 (8.83 mL) was added 1-*O*-acetyl-ethylene glycol (88 mg, 0.84 mmol). The reaction mixture was stirred at room temperature for 45 min and then cooled down to -20 °C. A solution of TMSOTf (38 µL, 0.21 mmol, 0.25 eq) in CH_2Cl_2 (1.75 mL) was added dropwise and the reaction mixture was then stirred at -20 °C for 1h and at room temperature for 6h before addition of a saturated solution of $NaHCO_3$ (1.5 mL). The mixture was then washed with H_2O (3 * 10 mL) and the organic phase was dried over $MgSO_4$ before evaporation of solvents to dryness. Purification of the residue by chromatography on silica gel (eluent: hexane/AcOEt 6/4 to 4/6) gave **4** (174 mg, 0.4 mmol, y = 47%) as a colourless oil and **5** (128 mg, 0.18 mmol, y = 21%) as a colourless oil. Data for **4**: Rf (Hexane/AcOEt 1/1) = 0.48. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.35 (dd, J = 10.1, 3.4 Hz, 1H, H3'), 5.27 (t, J = 10.0 Hz, 1H, H4'), 5.26 (dd, J = 3.5, 1.8 Hz, 1H, H2'), 4.85 (d, J = 1.4 Hz, 1H, H1'), 4.31 – 4.21 (m, 3H, H6'a, CH_2OAc), 4.11 (dd, J = 12.2, 2.4 Hz, 1H, H6'b), 4.01 (ddd, J = 9.9, 5.4, 2.3 Hz, 1H, H5'), 3.86 (ddd, J = 11.4, 5.4, 3.9 Hz, 1H, CH_2CH_2OAc), 3.72 (ddd, J = 11.4, 5.7, 4.1 Hz, 1H, CH_2CH_2OAc), 2.15 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 170.7, 170.0, 169.9, 169.7 (C=O), 97.5 (C1'), 69.4 (C3'), 68.9 (C2'), 68.6 (C5'), 66.1 (C4'), 66.0 (CH_2OAc), 62.9 (CH_2CH_2OAc), 62.5 (C6'), 20.09, 20.07 (CH3). ESI-HR-MS (positive): *m/z* calculated for $C_{18}H_{26}NaO_{12}$: 457.1316, found: 457.1316. Data for **5:** Rf (Hexane/AcOEt 1/1) = 0.21. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (dd, J = 10.0, 3.3 Hz, 1H), 5.36 – 5.22 (m, 5H), 4.98 (d, J = 1.6 Hz, 1H), 4.93 (d, J = 1.5 Hz, 1H), 4.3-4.10 (m, 8H), 4.09 – 4.00 (m, 1H), 3.94 (m, 1H), 3.86 (m, *1*H), 3.75 – 3.64 (m, 1H), 2.15 (s, 3H), 2.13 (s, 3H), 2.08 (s, 6H), 2.04 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 170.4, 169.8, 169.4, 99.1, 98.1, 70.1, 69.7, 69.1, 68.7 (s), 68.4, 66.4, 66.2, 65.8, 62.7, 62.5, 62.1, 20.8, 20.6. ESI-HR-MS (positive): *m/z* calculated for $C_{30}H_{42}NaO_{20}$: 745.2162, found: 745.2165.

1-O-(α-D-Mannopyranosyl) Ethylene Glycol (**6**). [51] To a solution of compound **4** (120 mg, 0.28 mmol) in MeOH (3.95 mL) was added NH_4OH (30-33% solution, 1.32 mL). The reaction mixture was stirred at room temperature for 30 h before evaporation of solvents to dryness. The residue was purified on silica gel

chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and desalted on sephadex® G10 to give **6** (61 mg, 0.27 mmol, y = 98%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.2. ^{1}H NMR (500 MHz, D_2O) *δ* 4.90 (d, *J* = 1.5 Hz, 1H, H1'), 3.99 (dd, *J* = 3.4, 1.7 Hz, 1H, H2'), 3.90 (dd, *J* = 12.1, 1.5 Hz, 1H, CH_2*CH_2*OH), 3.87 – 3.80 (m, 2H, H3', *CH_2*CH_2OH), 3.80 – 3.73 (m, 3H, H6', CH_2*CH_2*OH), 3.68 (m, 2H, H5', H4'), 3.62 (ddd, *J* = 10.2, 5.7, 3.1 Hz, 1H, *CH_2*CH_2OH). ^{13}C NMR (126 MHz, D_2O) *δ* 102.6 (C1'), 75.4 (C5'), 73.2 (C3'), 72.7 (C2'), 71.2 (*CH_2*CH_2OH), 69.5 (C4'), 63.7 (CH_2*CH_2*OH), 63.1 (C6'). ESI-HR-MS (positive): *m/z* calculated for $C_8H_{17}O_7$: 225.0969, found: 225.0969, calculated for $C_8H_{16}NaO_7$: 247.0788, found: 247.0788.

(α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl) Ethylene Glycol (**7**). To a solution of compound **5** (100 mg, 0.14 mmol) in MeOH (2 mL) was added NH_4OH (30-33% solution, 660 µL). The reaction mixture was stirred at room temperature for 6 h before evaporation of solvents to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and desalted on sephadex® G10 to give **7** (41 mg, 0.11 mmol, y = 77%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.38. ^{1}H NMR (500 MHz, D_2O) *δ* 5.14 (d, *J* = 1.3 Hz, 1H, H1'a), 5.05 (d, *J* = 1.4 Hz, 1H, H1'b), 4.09 (dd, *J* = 3.2, 1.8 Hz, 1H, H2'b), 4.02 (dd, *J* = 3.3, 1.7 Hz, 1H, H2'a), 3.97 (dd, *J* = 8.9, 3.3 Hz, 1H, H3'a), 3.92 (s, 1H, H6'a ou H6'b), 3.89 (s, 1H, H6'a ou H6'b), 3.86 (dd, *J* = 9.7, 3.3 Hz, 1H, H3'b), 3.84 – 3.66 (m, 8H, *CH_2*CH_2OH, CH_2*CH_2*OH, H5'a, H5'b, H4'a ou b, H6'a, H6'b), 3.66 – 3.58 (m, 2H, *CH_2*CH_2OH, H4'a ou b). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 105.2 (C1'b), 101.2 (C1'a), 81.5 (C2'a), 76.1 (C5'a ou b), 75.6 (C5'a ou b), 73.2 (C3'b), 73.0 (C3'a), 72.8 (C2'b), 71.5 (*CH_2*CH_2OH), 69.8 (C4'a ou b), 69.7 (C4'a ou b), 63.9, 63.8, 63.3 (C6'a, C6'b, CH_2*CH_2*OH) . ESI-HR-MS (positive): *m/z* calculated for $C_{14}H_{27}O_{12}$: 387.1497, found: 387.1497, calculated for $C_{14}H_{26}NaO_{12}$: 409.1316, found: 409.1312.

Diethyl (1,2-dihydroxethyl)phosphonate (**8**).To a solution of diethyl vinylphosphonate (1 mL, 6.51 mmol) in *tert*-butanol/H_2O (1/1 v/v, 11 mL) was added trimethylamine N-oxide (723 mg, 6.51 mmol, 1eq), citric acid (625 mg, 3.25 mmol, 0.5 eq) and osmium tetroxide (6.6 mg, 26 µmol, 0.004 eq). The reaction mixture was stirred at room temperature for 29 h before addition of amberlite (OH form, 13g) and the reaction mixture was stirred for one more hour before filtration of amberlite. The reaction mixture was extracted with CH_2Cl_2 (5*50 mL) and the combined organic phases were dried over $MgSO_4$ before evaporation of solvents to dryness. Purification of the residue by chromatography on silica gel (eluent: AcOEt/MeOH 1/0 à 9/1) gave compound **8** (1.202 g, 6.1 mmol, y = 93%, 96% BORSM) as a colourless oil. Rf (AcOEt/MeOH 9/1) = 0.37. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 4.20 (dd, *J* = 9.3, 4.5 Hz, 4H, CH_2CH_3), 4.03 – 3.95 (m, 1H), 3.95 – 3.87 (m, 2H), 3.38 (s, 2H, OH), 1.34 (t, *J* = 7.0 Hz, 6H, CH_2*CH_3*). ^{31}P NMR (162 MHz, $CDCl_3$) δ 21.14. ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 69.0 (d, *J* = 158.7 Hz, C1), 63.1 (d, *J* = 7.0 Hz, *CH_2*CH_3), 62.9 (d, *J* = 7.0 Hz, *CH_2*CH_3), 62.7 (d, *J* = 2.8Hz, C2), 16.4 (d, *J* = 5.5 Hz, CH_2*CH_3*). ESI-HR-MS (positive): *m/z* calculated for $C_6H_{16}O_5P$: 199.0730, found: 199.0731, calculated for $C_6H_{15}NaO_5P$: 221.0549, found: 221.0550.

*Diethyl (2-((tert-butyldimethylsilyl)oxy)-1-hydroxyethyl)phosphonate (***9**). To a solution of compound **8** (1.18 g, 5.95 mmol) in DMF (22 mL) was added imidazole (1.256 g, 18.4 mmol, 3.1 eq) at 0°C was added *tert*-butyldimethylchlorosilane (1.121 g, 7.43 mmol, 1.25 eq) portionwise. The reaction mixture was stirred at room temperature for 5.5 h and was then diluted with AcOEt (20 mL) and H_2O (20 mL). Phases were separated and the organic phase was washed with H_2O (20 mL), 0.1M HCl (20 mL), saturated NaCl (2 * 20 mL) and dried over $MgSO_4$. Solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (hexane/AcOEt 6/4 to 4/6) gave **9** (1.494 g, 4.8 mmol, y = 80%) as a white solid. Rf (Hexane/AcOEt 6/4) = 0.16. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 4.18 (pd, *J* = 7.2, 1.7 Hz, 4H, *CH_2*CH_3), 4.02 – 3.83 (m, 3H, H1, H2), 1.34 (td, *J* = 7.0, 2.5 Hz, 6H, CH_2*CH_3*), 0.90 (s, 3H, *t*Bu), 0.09 (s, 6H, Si*$(CH_3)_2$*). ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 22.45. ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 68.9 (d, *J* = 161.5 Hz, C1), 62.9 (d, *J* = 5.1 Hz, C2), 62.6 (t, *J* = 7.3 Hz, *CH_2*CH_3), 25.8 (C*$(CH_3)_3$*), 16.5 (d, *J* = 5.3 Hz, CH_2*CH_3*), -5.3, -5.9 (Si*$(CH_3)_2$*). ESI-HR-MS (positive): *m/z* calculated for $C_{12}H_{30}O_5PSi$: 313.1595, found: 313.1595, calculated for $C_{12}H_{29}NaO_5PSi$: 335.1414, found: 335.1412.

*Diethyl (2-((tert-butyldimethylsilyl)oxy)-1-(tetra-O-acetyl-α-D-mannopyranosyl)ethyl)phosphonate (***10***) and Diethyl (2-(2',3',4',6'-tetra-O-acetyl-α-D-mannopyranosyl)-1-(2',3',4',6'-tetra-O-acetyl-α-D-mannopyranosyl)ethyl)phosphonate (***11***).* To a solution of compound **3** (1.908 g, 3.87 mmol, 1.21 eq) and molecular sieves 4Å (2.723 g) in CH_2Cl_2 (34 mL) was added compound **9** (1 g, 3.20 mmol). The reaction mixture was stirred at room temperature for 30 min and then cooled down to -15 °C. A solution of TMSOTf (145 µL, 0.8 mmol, 0.25 eq) in CH_2Cl_2 (6.7 mL) was added dropwise and the reaction mixture was then stirred at -15 °C for 1h30 and at room temperature for 1 h before addition of Et_3N (3 mL).). After filtration of the molecular sieves, solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: hexane/AcOEt 6/4 to 1/9) gave **10** (1.433 g, 2.2 mmol, y = 69%, mixture of diastereoisomers *ratio* 1/1.1) as a pale yellow oil and **11** (523 mg, 0.61 mmol, y = 19%, mixture of diastereoisomers, *ratio* 1/0.8) as a yellow foam. Data for **10**: Rf (Hexane/AcOEt 2/8) = 0.60. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.39-5.28 (m, 4H), 4.51 (m, 0.6H), 4.44 (m, *J* = 9.1 Hz, 0.5H), 4.35 (dd, *J* = 12.5, 4.2 Hz, 0.6 H), 4.27 (dd, *J* = 12.5, 4.2 Hz, 0.4H), 4.24 – 4.09 (m, 5.4H), 4.05 (dd, *J* = 12.4, 2.1 Hz, 0.6H), 3.96 (m, 1H), 3.93 – 3.81 (m, 1H), 2.15, 2.13, 2.11, 2.10, 2.03, 2.01, 1.99, 1.98 (s, OAc), 1.35 (m, CH_2*CH_3*), 0.92 (s, 4H), 0.85 (s, 5H), 0.10 (s, 47H), 0.08 – 0.03 (m, 99H). ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 19.84, 19.73. ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 170.6, 170.5, 169.8, 169.7, 169.7, 169.6, 169.5, 169.4 (C=O), 98.9 (d, *J* = 8.9 Hz, C1' of one diastereoisomer), 96.2 (C1' of one diastereoisomer), 73.3 (d, *J* = 164.2 Hz, C1 of one diastereoisomer), 72.7 (d, *J* = 156.0 Hz, C1 of one diastereoisomer), 69.0, 68.9, 68.1 (C5', C3', C2'), 65.7 (C4' of one diastereoisomer), 65.5 (C4' of one diastereoisomer), 63.6 (d, *J* = 11.2 Hz, C2), 62.7 – 62.4 (m, *CH_2*CH_3), 62.1 (C6'), 25.9 (*$C(CH_3)_3$* of one diastereoisomer), 25.6 (C*$(CH_3)_3$* of one diastereoisomer), 20.7 (OAc of one diastereoisomer), 20.6 (OAc of one diastereoisomer), 18.3 (*$C(CH_3)_3$* of one diastereoisomer), 17.9 (*$C(CH_3)_3$* of one diastereoisomer), 16.4 (CH_2*CH_3* of one diastereoisomer), 16.3 (CH_2*CH_3* of one diastereoisomer), -5.54 (d *J* = 9.1 Hz, Si*$(CH_3)_2$* of one diastereoisomer), -5.75 (Si*$(CH_3)_2$* of one diastereoisomer). ESI-HR-MS (positive): *m/z* calculated for $C_{26}H_{48}O_{14}PSi$: 643.2545, found: 643.2543, calculated for $C_{26}H_{47}NaO_{14}PSi$: 665.2365, found: 665.2364. Data for **11**: Rf (Hexane/AcOEt 2/8) = 0.24. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.40 – 5.16 (m, 8H), 5.12 (s, 0.8H), 4.87 (s, 1H), 4.53 – 3.83 (m, 19H), 3.75 (ddd, *J* = 14.5, 11.8, 6.9 Hz, 1H), 2.15, 2.14, 2.12, 2.10, 2.09, 2.05, 2.04, 2.03, 2.01, 1.99, 1.98, 1.97, 1.96), 1.84 (s, 3H, OAc), 1.41 – 1.31 (m, 10H, CH_2*CH_3*). ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 18.59, 18.52. ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 170.6, 170.5, 169.9, 169.9, 169.8, 169.8, 169.7, 169.7, 169.5, 169.5, 169.4, 169.4 (C=O), 98.5-96.8 (m, C1', C1''), 71.1 (d, *J* = 168 Hz, C2 of one diastereoisomer), 70.6 (d, *J* = 168 Hz, C2 of one diastereoisomer),

69.6 – 68.6 (m, C2', C2'', C3', C3'', C5', C5''), 67.8 (d, *J* = 9.5 Hz, C1 of one diastereoisomer), 66.7 (d, *J* = 8.6 Hz, C1 of one diastereoisomer), 65.9 (C4' of one diastereoisomer), 65.6 (C4' of one diastereoisomer, C4''), 63.58 – 62.57 (CH_2CH_3, C6', C6''), 62.4-62.0 (m, CH_2CH_3, C6', C6''), 20.6 (OAc), 16.4, 16.3 (CH_2CH_3). ESI-HR-MS (positive): *m/z* calculated for $C_{34}H_{51}NaO_{23}P$: 881.2451, found: 881.2451.

*Diethyl (2-((tert-butyldimethylsilyl)oxy)-1-(α-D-mannopyranosyl)ethyl)phosphonate (***12***)* . To a solution of compound **10** (410 mg, 0.64 mmol) in MeOH (9.1 mL) was added NH_4OH (30-33% solution, 3 mL). The reaction mixture was stirred at room temperature for 64 h before evaporation of solvents to dryness. The residue was partitioned between H_2O (10 mL) and AcOEt (10 mL). The aqueous phase was extracted with AcOEt (3*10 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporate to dryness. Purification of the residue on silica gel chromatography (Eluent: CH_2Cl_2/MeOH 1/0 to 9/1) gave **12** (157 mg, 0.33 mmol, y = 51%, mixture of diastereoisomer, *ratio* 1/1.2) as a yellow oil. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.66. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.30 (s, 1H, C1' of one diastereoisomer), 5.20 (s, 1.2 H, C1' of one diastereoisomer), 4.25 – 4.08 (m, 9H, CH_2CH_3), 4.08 – 3.67 (m, 13H, C2', C3', C4', C5', C6', H1, H2), 2.90 (br. s, OH), 1.46 – 1.22 (m, 13H, CH_2CH_3), 0.90 (t, *J* = 6.6 Hz, 19H, $C(CH_3)_3$), 0.08 (s, 5H, $CH_3)_2$ of one diastereoisomer), 0.07 (d, *J* = 2.0 Hz, 4H, $CH_3)_2$ of one diastereoisomer). ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 21.01, 20.76. ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 100.9 (C1' of one diastereoisomer), 100.1 (C1' of one diastereoisomer), 74.7, 73.9, 72.8, 73.2, 72.8, 72.6, 71.7, 71.4, 70.7, 70.5 (C2', C3', C5', C2), 66.6 (C4' of one diastereoisomer), 66.8(C4' of one diastereoisomer), 63.2 , 62.6, 61.1, 60.8 (C2, C6'), 25.9 ($C(CH_3)_3$ of one diastereoisomer), 25.8 ($C(CH_3)_3$ of one diastereoisomer), 18.4 ($C(CH_3)_3$ of one diastereoisomer), 18.2 ($C(CH_3)_3$ of one diastereoisomer), 16.44 (CH_2CH_3), -5.43 ($Si(CH_3)_2$ of one diastereoisomer), -5.52 ($Si(CH_3)_2$ of one diastereoisomer). ESI-HR-MS (positive): *m/z* calculated for $C_{18}H_{40}O_{10}PSi$: 475.2123, found: 475.2122, calculated for $C_{18}H_{39}NaO_{10}PSi$: 497.1942, found: 497.1941.

2-Hydroxy-1-(α-D-mannopyranosyl)ethyl phosphonate, sodium salt (**13**). To a solution of compound **12** (106 mg, 0.22 mmol) in DMF (3.2 mL) at 0 °C was added TMSBr (295 µL, 2.2 mmol, 10 eq). The reaction mixture was stirred at room temperature for 26h and the reaction mixture was quenched with NH_4OH. Solvents were evaporated to dryness and the residue was diluted in H_2O (10 mL) and extracted with CH_2Cl_2 (3*10 mL). The combined organic phase were dried over $MgSO_4$ and solvents were evaporated to dryness. The residue was purified by flash chromatography (C18 column, 100% H_20) to give **13** as a white solid (52%, mixture of diastereoisomers *ratio* 1/1.4). ^{1}H NMR (400 MHz, D_2O) *δ* 5.20 (s, 1H), 5.15 (s, 1.4H), 4.07 – 4.02 (m, 2.4H), 4.02 – 3.70 (m, 17H), 3.68 (d, *J* = 9.5 Hz, 1.4H, H5' first diastereoisomer), 3.63 (d, *J* = 9.8 Hz, 1.4H). ^{31}P NMR (162 MHz, D_2O) δ 14.89. ^{13}C NMR (126 MHz, D_2O) *δ* 102.6 (C1', first diastereoisomer), 102.3 (d, *J* = 7.4 Hz, C1', second diastereoisomer), 79.3 (d, *J* = 147.7 Hz, C1 first diastereoisomer), 77.5 (d, *J* = 152.6 Hz, C1, second diastereoisomer), 75.4 (C5' first diastereoisomer), 75.3 (C5' second diastereoisomer), 72.9 (C2', C3'), 69.8 (C4' first diastereoisomer), 69.5 (C4' second diastereoisomer), 65.0 (d, *J* = 7.5 Hz, C2 first diastereoisomer), 64.7 (d, *J* = 7.5 Hz, C2 second diastereoisomer), 63.7 (C6' first diastereoisomer), 63.66 (C6' second diastereoisomer). ESI-HR-MS (negative): *m/z* calculated for $C_8H_{16}O_{10}P$: 303.0487, found: 303.0488, calculated for $C_8H_{15}NaO_{10}P$: 325.0306, found: 325.0304.

Methyl 3-O-acetyl-2-O-(2',3',4',6'-tetra-O-acetyl-α-D-mannopyranosyl)-D-glycerate (**15**). To a solution of compound **14** (280 mg, 0.58 mmol) in DMF (5.8 mL) at 0 °C was added K_2CO_3 (161 mg, 1.17 mmol, 2eq) and MeI (168 µL, 2.7 mmol, 4.6 eq) dropwise. The reaction mixture was stirred at room temperature for 2.5 h and was then diluted in AcOEt (20 mL). The organic phase was washed with H_2O (3*20 mL) and a saturated solution of NaCl (3*20 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the crude by chromatography on silica gel (eluent: hexane/AcOEt 6/4) gave **15** (208 mg, 0.42 mmol, y = 72%) as a yellow oil. Rf (hexane/AcOEt 1/1) = 0.42. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.34 (dd, *J* = 3.3, 1.8 Hz, 1H, H2'), 5.28 (dd, *J* = 10.0, 3.4 Hz, 1H, H3'), 5.20 (t, *J* = 10.0 Hz, 1H, H4'), 4.97 (d, *J* = 1.5 Hz, 1H, H1'), 4.47 (dd, *J* = 11.6, 3.1 Hz, 1H, H2), 4.42 (dd, *J* = 6.6, 3.2 Hz, 1H, H3a), 4.22 (dd, *J* = 12.0, 4.0 Hz, 1H, H6'a), 4.19 (dd, *J* = 12.0, 8.0 Hz, 1H, H3b), 4.08 – 3.98 (m, 2H, H6'b, H5'), 3.72 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.03 (s, 6H, OAc), 1.98 (s, 3H, OAc), 1.92 (s, 3H, OAc). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 170.4, 170.1, 169.6, 169.5, 169.5, 168.5 (C=O), 96.5 (C1'), 72.5 (C2), 69.1 (C3'), 68.9 (C2'), 68.5 (C5'), 65.8 (C4'), 63.5 (C3), 62.2 (C6'), 52.5 (CH_3), 20.6 (OAc), 20.5 (OAc), 20.4 (OAc). ESI-HR-MS (positive): *m/z* calculated for $C_{20}H_{28}NaO_{14}$: 515.1371, found: 515.1370.

N-hydroxy-3-hydroxy-2-(α-D-mannopyranosyl) propanamide (**16**). To a solution of compound **15** (450 mg, 0.91 mmol) in MeOH (9.1 mL) at 0 °C was added dropwise hydroxylamine (840 µL, 13.7 mmol, 15 eq) and KCN (30 mg, 0.46 mmol, 0.5 eq). The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 3.5 h and was then quenched with H_2O (15 mL). The reaction mixture was extracted with AcOEt (2*10 mL) and CH_2Cl_2 (2*10 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and desalted on sephadex® G10 to give **16** (72 mg, 0.26 mmol, y = 44%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.10. ^{1}H NMR (500 MHz, D_2O) *δ* 4.90 (d, *J* = 1.5 Hz, 1H, H1'), 4.35 (t, *J* = 4.3 Hz, 1H, H2), 4.08 (dd, *J* = 3.3, 1.7 Hz, 1H, H2'), 3.90 (2 d, *J* = 9.2, 8.75 Hz, 2H, H6'a, H3'), 3.87 (d, *J* = 4.3 Hz, 2H, H3), 3.74 (m, 2H, H6'b, H5), 3.68 (t, *J* = 9.5 Hz, 1H, H4'). ^{13}C NMR (126 MHz, D_2O) *δ* 171.0 (C=O), 101.98 (C1'), 78.9 (C2), 76.1 (C5'), 72.9 (C3'), 72.5 (C2'), 69.3 (C4'), 64.7 (C3), 63.6 (C6'). ESI-HR-MS (positive): *m/z* calculated for $C_9H_{18}NO_9$: 284.0976, found: 284.0978, calculated for $C_9H_{17}NNaO_9$: 306.0796, found: 306.0795.

1,6-Di-O-acetyl-2,5-Di-O-(2',3',4',6'-tetra-benzyl-α-D-glucopyranosyl)-3,4-O-isopropylidene-D-mannitol (**19**). To a solution of protected mannitol **18** (1 g, 3.26 mmol), compound **17** (4.958 g, 7.84 mmol, 2.4 eq) and N-Bromosuccinimide (1.395 g, 7.84 mmol, 2.4 eq) in CH_2Cl_2 (33 mL) and Et_2O (65 mL) at -55 °C was added dropwise TMSOTf (590 µL, 3.26 mmol, 1 eq). The reaction mixture was stirred at 0 °C for 4 h before addition of Et_3N (5 mL) and the reaction mixture was stirred for one more hour before addition of CH_2Cl_2 (100 mL). The organic phase was then washed with a saturated solution of $NaHCO_3$ (3*100 mL) and H_2O (100 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: Petroleum ether/AcOEt 9/1) gave **19** (1.844 g, 1.36 mmol, contaminated with succinimid derivatives) as a yellow solid, but also compound **20** (1.382 g, y = 32%) and compound **21** (1.305 g, y = 48%). Data for **19**: Rf (Petroleum Ether/AcOEt 7/3) = 0.65. See ^{1}H NMR spectra in experimental part. ESI-HR-MS (positive): *m/z* calculated for $C_{81}H_{91}O_{18}$: 1351.6199, found: 1351.6185, calculated for $C_{81}H_{90}NaO_{18}$: 1373.6019, found:

1373.6012. Data for **21**: Rf (Petroleum Ether/AcOEt 7/3) = 0.29. See ^{1}H NMR spectra in experimental part. ESI-HR-MS (positive): *m/z* calculated for $C_{47}H_{56}NaO_{13}$: 851.3613, found: 851.3609.

1,6-Di-O-acetyl-2,5-Di-O-(2',3',4',6'-tetra-benzyl-α-D-glucopyranosyl)-D-mannitol (**22**). A solution of compound **19** (1.721 g, 1.27 mmol) in TFA/H_2O (16/1 v/v, 3.24 mL) was stirred at room temperature for 30 min before addition of AcOEt (60 mL) and a saturated $NaHCO_3$ solution (60 mL). The organic phase was extracted with H_2O (30 mL) and the aqueous phase was extracted with AcOEt (2*30 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: petroleum ether/AcOEt 9/1 to 6/4) gave **22** (652 mg, 050 mmol, y = 15% over two steps, contaminated with succinimid derivatives) as a colourless oil. Rf (petroleum ether/AcOEt 7/3) = 0.09. ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 7.36 – 7.21 (m, 35H), 7.16 (dd, *J* = 8.0, 5.6 Hz, 5H), 4.90 (m, *J* = 11.4, 12.9 Hz, 6H, 1**CH*$_2$Ph,2*H1'), 4.88 (d, J = 10.9 Hz, 2H, *CH*$_2$Ph), 4.83 (d, *J* = 10.4 Hz, 2H, *CH*$_2$Ph), 4.80 (d, *J* = 11 Hz, 2H, *CH*$_2$Ph), 4.67 (d, *J* = 12.7 Hz, 2H, *CH*$_2$Ph), 4.62 (d, *J* = 12.1 Hz, 2H, *CH*$_2$Ph), 4.51 (d, *J* = 10.8 Hz, 2H, *CH*$_2$Ph),4.46 (d, *J* = 12.1 Hz, 2H, *CH*$_2$Ph), 4.29 – 4.21 (m, 4H), 4.01-3.90 (dd, m, 6H), 3.87 – 3.82 (m, 2H), 3.75 (dd, *J* = 10.6, 2.9 Hz, 2H), 3.70 (t, *J* = 9.5 Hz, 2H), 3.61 – 3.52 (m, 4H), 1.98 (s, 6H, OAc). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 170.7, 138.4, 138.1, 137.8, 137.1, 128.6, 128.4, 128.3, 128.2, 127.9, 127.7, 127.7, 127.6, 99.8, 82.3, 80.0, 79.1, 77.56, 75.6, 74.9, 74.2, 73.5, 71.1, 68.3, 68.0, 64.3, 20.9. ESI-HR-MS (positive): *m/z* calculated for $C_{78}H_{87}O_{18}$: 1311.5887, found: 1311.5888, calculated for $C_{78}H_{86}NaO_{18}$: 1333.5706, found: 1333.5708.

3-O-acetyl-2-O-(2',3',4',6'-tetra-O-benzyl-α-D-glucopyranosyl)-D-glyceric acid (**23**). To a solution of compound **22** (575 mg, 0.44 mmol) in CH_2Cl_2/H_2O (1/1 v/v, 6.3 mL) at 0°C was added TEMPO (7 mg, 0.04 mmol, 0.1 eq) and BAIB (424 mg, 1.31 mmol, 3 eq). The reaction mixture was stirred at room temperature for 5.5 h and was then diluted in CH_2Cl_2 (10 mL). Phases were separated and the organic phase was washed with H_2O (10 mL). The aqueous phases were combined, acidified with HCl 1N (10 mL) and extracted with CH_2Cl_2 (5*10 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue (eluent CH_2Cl_2/MeOH 100/1 to 90/10) gave **23** (526 mg, 1.28 mmol, y = 89%) as a yellow foam. Rf (CH_2Cl_2/MeOH 100/2) = 0.20. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.29 (dd, *J* = 18.7, 10.6 Hz, 17H), 7.14 (s, 3H), 4.98 – 4.76 (m, 6H), 4.59 – 4.30 (m, 7H), 4.06 (s, 1H), 3.89 (d, *J* = 9.2 Hz, 1H), 3.78 – 3.49 (m, 4H), 1.96 (s, 3H, OAc). ESI-HR-MS (positive): *m/z* calculated for $C_{39}H_{42}NaO_{10}$: 693.2670, found: 693.2672.

2-O-(2',3',4',6'-tetra-O-benzyl-α-D-glucopyranosyl)-D-glyceric acid (**24**). To a solution of compound **23** (483 mg, 0.72 mmol) in MeOH (10 mL) was added NH_4OH (30-33% solution, 3.432 mL). The reaction mixture was stirred at room temperature for 18.5 h before evaporation of solvents to dryness. The residue was purified on silica gel chromatography (Eluent: CH_2Cl_2/MeOH 95/5 to 7/3) to give **24** (370 mg, 0.59 mmol, y = 81%) as a white solid. Rf (CH_2Cl_2/MeOH 95/5) = 0.10. ^{1}H NMR (500 MHz, MeOD) *δ* 7.40 (d, *J* = 6.5 Hz, 2H), 7.33 – 7.19 (m, 18H), 7.13 – 7.08 (m, 2H), 5.09 (s, 1H), 4.94 (d, *J* = 11.3 Hz, 2H, *CH*$_2$Ph), 4.80 (d (under H_2O), *J* = 11.8 Hz, 2H, *CH*$_2$Ph), 4.71 (d, *J* = 11.0 Hz, 1H, *CH*$_2$Ph), 4.51 – 4.44 (m, 2H, *CH*$_2$Ph), 4.40 (d, *J* = 12.0 Hz, 1H, *CH*$_2$Ph), 4.26 (s, 1H), 4.08 (t, *J* = 9.4 Hz, 1H), 3.94 (d, *J* = 8.6 Hz, 1H), 3.90 (d, *J* = 9.2 Hz, 1H), 3.81 (d, *J* = 8.3 Hz, 1H), 3.62 (dd, *J* = 10.8, 4.1 Hz, 1H), 3.59 – 3.54 (m, 2H), 3.51 (t, *J* = 9.5 Hz, 1H). ^{13}C NMR (126 MHz, MeOD) *δ* 177.9, 140.1, 139.5, 139.2, 138.7, 129.8, 129.6, 129.4, 129.1, 128.9, 128.8, 128.7, 96.7, 82.9, 79.6, 79.1, 76.3, 75.9, 74.3, 74.1, 72.3, 69.7, 64.2. ESI-HR-MS (positive): *m/z* calculated for $C_{37}H_{40}NaO_9$: 651.2565, found: 651.2561.

(2R)-2-(α-D-glucopyranosyl)-D-glycerate, sodium salt (**25**)**.** [19,43] Compound **24** (290 mg, 0.46 mmol) was dissolved in MeOH/AcOEt/AcOH (5/5/1 v/v/v, 11 mL) before addition of Pd/C (450 mg) and the resulting mixture was stirred at room temperature under H_2 atmosphere (20 bars) for 7.5 days. After filtration of the Pd on celite, solvents were evaporated to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and treated with Dowex® 50WX2 (Na^+) and desalted on sephadex® G10 to give compound **25** (121 mg, 0.42 mmol, y = 90%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.23. ^{1}H NMR (500 MHz, D_2O) *δ* 5.02 (d, *J* = 3.8 Hz, 1H, H1'), 4.21 (dd, *J* = 6.1, 3.1 Hz, 1H, H2), 3.92 – 3.74 (m, 6H, H3', H3, H5', H6'), 3.55 (dd, *J* = 9.8, 3.8 Hz, 1H, H2'), 3.44 (t, *J* = 9.5 Hz, 1H, H4'). ^{13}C NMR (126 MHz, D_2O) *δ* 179.9 (C=O), 100.3 (C1'), 81.9 (C2), 76.1 (C3'), 75.1 (C5'), 74.4 (C2'), 72.3 (C4'), 65.9 (C3), 63.3 (C6'). ESI-HR-MS (positive): *m/z* calculated for $C_9H_{17}O_9$: 269.0867, found: 269.0869, calculated for $C_9H_{16}NaO_9$: 291.0687, found: 291.0686.

1,6-Di-O-acetyl-2,5-Di-O-(2',3',4',6'-tetra-benzyl-α-D-galactopyranosyl)-D-mannitol (**29**) and *1,6-Di-O-acetyl-2-O-(2',3',4',6'-tetra-benzyl-β-D-galactopyranosyl)-5-O-(2',3',4',6'-tetra-benzyl-α-D-galacto-pyranosyl)-D-mannitol* (**30**). To a solution of protected mannitol **18** (907 mg, 2.96 mmol), compound **26** (4.5 g, 7.11 mmol, 2.4 eq) and N-Bromosuccinimide (1.265 g, 7.11 mmol, 2.4 eq) in CH_2Cl_2 (30 mL) and Et_2O (59 mL) at -55 °C was added dropwise TMSOTf (428 µL, 2.36 mmol, 0.8 eq). The reaction mixture was stirred at 0°C for 4 h before addition of Et_3N (7.5 mL) and the reaction mixture was stirred for one more hour before addition of CH_2Cl_2 (100 mL). The organic phase was then washed with a saturated solution of $NaHCO_3$ (3*100 mL) and H_2O (100 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: Petroleum ether/AcOEt 8/2) gave a mixture of the non-separable dimers α/α **27** and α/β **28** (4.365g). Rf (Hexane/AcOEt 7/3) = 0.51 and 0.43. This mixture of inseparable compounds **27** and **28** (4.365 g) was dissolved in TFA/H_2O (8 mL, 16/1 v/v) and the reaction mixture was stirred at room temperature for 30 min before dilution in AcOEt (100 mL). The aqueous phase was extracted with AcOEt (2*50 mL) and the combined organic phases were washed with H_2O (2*50 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent hexane/AcOEt 8/2 to 6/4) gave **29** (dimer α/α: 1.242 g, 0.95 mmol, y = 32% over two steps) and **30** (dimer α/β: 689 mg, 0.53 mmol, y = 18% over two steps) as white foams. Data for **29**: Rf (Hexane/AcOEt 6/4) = 0.46. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.38 – 7.20 (m, 40H), 4.95 (d, *J* = 3.7 Hz, 2H), 4.89 (d, *J* = 11.4 Hz, 2H), 4.82 (d, *J* = 11.6 Hz, 2H), 4.72 (s, 4H), 4.66 (d, *J* = 11.6 Hz, 2H), 4.53 (d, *J* = 11.4 Hz, 2H), 4.44 (d, *J* = 11.7 Hz, 2H), 4.37 (d, *J* = 11.7 Hz, 2H), 4.20 – 4.08 (m, 4H), 4.08 – 3.96 (m, 6H), 3.90 – 3.81 (m, 4H), 3.76 (m, 2H), 3.56 (t, *J* = 8.5 Hz, 2H), 3.42 (dd, *J* = 8.8, 5.2 Hz, 2H), 1.94 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 170.7, 138.5, 138.3, 137.8, 137.4, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7, 127.5, 127.5, 127.2, 100.0, 79.5, 79.4, 75.9, 74.7, 74.4, 74.3, 73.4, 72.4, 69.8, 68.5, 68.3, 64.5, 20.7. ESI-HR-MS (positive): *m/z* calculated for $C_{78}H_{87}O_{18}$: 1311.5887, found: 1311.5881, calculated for $C_{78}H_{86}NaO_{18}$: 1333.5706, found: 1333.5698. Data for **30**: Rf (Hexane/AcOEt 6/4) = 0.43. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.46 – 7.28 (m, 40H), 5.12 (d, *J* = 3.6 Hz, 1H), 4.98 – 4.90 (m, 3H), 4.79 (dtd, *J* = 23.7, 11.7, 6.2 Hz, 8H), 4.55 (ddd, *J* = 18.1, 16.2, 8.2 Hz, 5H), 4.50 – 4.33 (m, 6H), 4.23 – 4.17 (m, 3H), 4.11 – 4.04 (m, 3H), 4.01 –

3.94 (m, 4H), 3.91 – 3.79 (m, 2H), 3.76 (d, *J* = 2.7 Hz, 1H), 3.70 (s, 1H), 3.65 – 3.56 (m, 4H), 3.55 – 3.46 (m, 2H), 3.37 (q, *J* = 8.4 Hz, 1H), 1.96 (d, *J* = 5.2 Hz, 3H), 1.85 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 170.9, 170.7, 138.6, 138.5, 138.3, 138.1, 138.1, 137.8, 137.5, 137.2, 128.2, 128.1, 128.0, 127.9, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.1, 104.2, 99.7, 81.8, 78.9, 78.8, 76.8, 75.9, 74.8, 74.6, 74.4, 74.3, 73.9, 73.7, 73.4, 73.4, 73.2, 73.1, 72.3, 69.6, 69.0, 68.8, 68.3, 66.9, 65.63, 64.4, 20.5, 20.5.

3-O-acetyl-2-O-(2',3',4',6'-tetra-O-benzyl-α-D-galactopyranosyl)-D-glyceric acid (**31**). To a solution of compound **29** (dimer α/α, 1.229 g, 0.94 mmol) in CH_2Cl_2/H_2O (1/1 v/v, 13 mL) at 0 °C was added TEMPO (15 mg, 0.09 mmol, 0.1 eq) and BAIB (905 mg, 2.8 mmol, 3 eq). The reaction mixture was stirred at room temperature for 5 h and was then diluted in AcOEt (80 mL) and washed with a 10% solution of $Na_2S_2O_3$ (10* 25 mL). The combined aqueous phases were evaporated to dryness to give a white solid which was dissolved in H_2O (50 mL) and acidified with AcOEt (50 mL) containing HCl 1M (10 mL). The aqueous layer was extracted with AcOEt (7*50 mL). All the combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue (eluent CH_2Cl_2/MeOH 100/2 to 100/6) gave **31** (1.083 g, 1.61 mmol, y = 86%) as a yellow oil. Rf (CH_2Cl_2/MeOH 95/5) = 0.43. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 11.08 (s, 1H), 7.46 – 7.14 (m, 20H), 4.96 (d, *J* = 11.4 Hz, 1H), 4.91 (d, *J* = 11.3 Hz, 1H), 4.82 – 4.69 (m, 4H), 4.56 (d, *J* = 11.4 Hz, 1H), 4.52 (dd, *J* = 2.3, 11.7 Hz , 1H), 4.47 – 4.37 (m, 2H), 4.35 (dd, *J* = 6.7, 2.3 Hz, 1H), 4.28 (dd, *J* = 11.7, 6.8 Hz, 1H), 4.13 (dd, *J* = 10.0, 3.8 Hz, 1H), 4.06 (m, 2H), 3.96 (dd, *J* = 10.0, 2.4 Hz, 1H), 3.55 (t, *J* = 8.4 Hz, 1H), 3.47 (dd, *J* = 9.0, 5.5 Hz, 1H), 1.97 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 169.9, 138.3, 137.9, 137.6, 136.4, 128.9, 128.7, 128.5, 128.4, 128.3, 128.1, 127.9, 127.9, 127.8, 127.8, 127.4, 100.7, 79.3, 77.6, 75.2, 74.9, 74.7, 73.8, 73.5, 72.4, 70.8, 68.2, 64.1, 20.5. ESI-HR-MS (positive): *m/z* calculated for $C_{39}H_{42}NaO_{10}$: 693.2670, found: 693.2668.

(2R)-2-(α-D-galactopyranosyl)-D-glycerate, sodium salt (**33**). [46] To a solution of compound **31** (1.3 g, 1.94 mmol) in MeOH (27 mL) was added NH_4OH (30-33% solution, 9 mL). The reaction mixture was stirred at room temperature for 70 h before evaporation of solvents to dryness. The residue was dissolved in AcOEt (30 mL) and washed with H_2O (30mL). The organic phase was dried over $MgSO_4$ and solvents were evaporated to dryness. The residue was purified on silica gel chromatography (Eluent: CH_2Cl_2/MeOH 95/5) to give a yellow foam (390 mg) corresponding to the de-acylated product **32**. Rf (CH_2Cl_2/MeOH 95/5) = 0.35. This foam (300 mg) was dissolved in MeOH/AcOEt/AcOH (5/5/1 v/v/v, 11 mL) before addition of Pd/C (450 mg) and the resulting mixture was stirred at room temperature under H_2 atmosphere (20 bars) for 4.5 days. After filtration of the Pd on celite, solvents were evaporated to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and treated with Dowex® 50WX2 (Na^+) and desalted on sephadex® G10 to give **33** (117 mg, 0.40 mmol, y = 21% over 2 steps) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.18. ^{1}H NMR (400 MHz, D_2O) *δ* 5.04 (d, *J* = 3.8 Hz, 1H, H1'), 4.20 (dd, *J* = 6.0, 3.1 Hz, 1H, H2), 4.06 (t, *J* = 6.2 Hz, 1H), 4.01 – 3.92 (m, 2H), 3.88 (dd, *J* = 12.1, 3.0 Hz, 1H), 3.85 – 3.76 (m, 2H), 3.76 – 3.69 (m, 2H). ^{13}C NMR (75 MHz, D_2O) *δ* 180.1, 100.5, 81.8, 74.2, 72.5, 72.2, 71.3, 67.8, 66.0, 64.1. ESI-HR-MS (positive): *m/z* calculated for $C_9H_{17}O_9$: 269.0867, found: 269.0869, calculated for $C_9H_{16}NaO_9$: 291.0687, found: 291.0687.

(2R)-2-(β-D-galactopyranosyl)-D-glycerate, sodium salt (**34**). To a solution of compound **30** (dimer α/β, 689 mg, 0.53 mmol) in CH_2Cl_2/H_2O (1/1 v/v, 10 mL) at 0°C was added TEMPO (8 mg, 0.05 mmol, 0.1 eq) and BAIB (507 mg, 1.58 mmol, 3 eq). The reaction mixture was stirred at room temperature for 4 h and was then diluted in AcOEt (40 mL) and washed with a 10% solution of $Na_2S_2O_3$ (10* 10 mL). The combined aqueous phases were evaporated to dryness to give a white solid which was dissolved in H_2O (30 mL) and acidified with AcOEt (30 mL) containing HCl 1M (6 mL). The aqueous layer was extracted with AcOEt (7*30 mL). All the combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue (eluent CH_2Cl_2MeOH 100/0 to 95/5) gave **31** (322 mg, 0.48 mmol, y = 46%) as a yellow oil, and 353 mg (0.53mmol, y = 50%) of an inseparable mixture of compounds **31** and **its β anomer.** Rf (CH_2Cl_2/MeOH 95/5) = 0.40. To the mixture of compounds **31** and **its β anomer** (260 mg, 0.39 mmol) in MeOH (5.5 mL) was added NH_4OH (30-33% solution, 1.84 mL). The reaction mixture was stirred at room temperature for 3 days before evaporation of solvents to dryness. The residue was purified on silica gel chromatography (Eluent: CH_2Cl_2/MeOH 95/5) to give a yellow foam (213 mg, y = 87%) corresponding to a mixture of inseparable compounds **32** and **its β anomer.** Rf (CH_2Cl_2/MeOH 95/5) = 0.21. This foam (210 mg) was dissolved in MeOH/AcOEt/AcOH (5/5/1 v/v/v, 11 mL) before addition of Pd/C (315 mg) and the resulting mixture was stirred at room temperature under H_2 atmosphere (20 bars) for 4.5 days. After filtration of the Pd on celite, solvents were evaporated to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and treated with Dowex® 50WX2 (Na^+) and desalted on sephadex® G10 to give **34** (117 mg, 0.40 mmol, y = 22%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.17. ^{1}H NMR (500 MHz, D_2O) *δ* 4.51 (d, *J* = 6.9 Hz, 1H, H1'), 4.20 (dd, *J* = 6.5, 3.1 Hz, 1H, H2), 3.92 (d, *J* = 2.9 Hz, 1H, H4'), 3.88 (dd, *J* = 12.2, 3.1 Hz, 1H, H3a), 3.83 – 3.75 (m, 2H,H3b, H6'a), 3.71 (dd, *J* = 11.8, 4.3 Hz, 1H, H6'b), 3.69 – 3.62 (m, 3H, H3', H5', H2'). ^{13}C NMR (75 MHz, D_2O) *δ* 180.8, 105.5, 84.2, 77.9, 75.5, 73.7, 71.6, 65.5, 63.8. ESI-HR-MS (positive): *m/z* calculated for $C_9H_{17}O_9$: 269.0867, found: 269.0865 calculated for $C_9H_{16}NaO_9$: 291.0687, found: 291.0686.

*Methyl 3,4,6-tri-O-Acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (***36***), Methyl (2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1->2)-O-(3,4,6-tri-O-acetyl-α-D-mannopyranosyl)-(1->2)-3,4,6-tri-O-acetyl-α-D-mannopyranoside (***37***) and Methyl (2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1->2)-O-(3,4,6-tri-O-acetyl-α-D-mannopyranosyl)-(1->2)-3,4,6-tri-O-acetyl-α-D-mannopyranoside (***38***).* To a solution of compound **35** (500 mg, 1.38 mmol) in CH_2Cl_2 (17 mL) at -30 °C was added dropwise TMSOTf (750 µL, 4.14 mmol, 3 eq). The reaction mixture was stirred at -30 °C for 5 min before addition of CH_2Cl_2 (20 mL) and was then quenched with a saturated $NaHCO_3$ solution. The aqueous phases were extracted with CH_2Cl_2 (3*30 mL) and the combined organic phases were washed with brine (3*50 mL) and dried over $MgSO_4$. Solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (hexane/AcOEt 1/ to 3/7) gave **36** (189 mg, 0.29 mmol, y = 42%) as a colourless oil, **37** (125 mg, 0.13 mmol, y =29%) as a colourless oil and **38** (34 mg, 0.03 mmol, y =8%) as a colorless oil. Data for **36**: Rf (hexane/AcOEt 3/7) = 0.69. NMR in agreement with the literature.[48-49] Data for **37**: Rf (hexane/AcOEt 3/7) = 0.48. NMR in agreement with the literature.[48-49] Data for **38**: Rf (hexane/AcOEt 3/7) = 0.4. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.39 (dd, *J* = 10.0, 3.4 Hz, 1H), 5.36 - 5.24 (d, *J* = 9.6 Hz, 8H), 5.13 (d, *J* = 1.7 Hz, 1H), 5.07 (d, *J* = 2.1 Hz, 1H), 4.96 (d, *J* = 1.4 Hz, 1H), 4.86 (d, *J* = 1.7 Hz, 1H), 4.27 – 4.07 (m, 14H), 4.04 – 4.02 (m, 1H),

3.90 (ddd, *J* = 9.4, 4.2, 2.4 Hz, 1H), 3.41 (s, 3H), 2.15 (s, 3H), 2.12 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 2.08 (s, 6H), 2.06 (s, 6H), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 170.5, 170.3, 169.9, 169.6, 169.5, 169.3,169.3, 169.2, 169.2, 99.6, 99.4, 99.1, 99.0, 76.8, 76.2, 70.2, 69.6, 69.5, 69.4, 69.3, 69.0, 68.3, 68.2, 66.3, 66.1, 66.0, 65.9, 62.3, 62.1, 61.9, 55.0, 20.6, 20.4. ESI-HR-MS (positive): *m/z* calculated for $C_{51}H_{71}O_{34}$: 1227.3821, found: 1227.3823, calculated for $C_{51}H_{70}NaO_{34}$: 1249.3641, found: 1249.3637.

*1,3,4,6-tetra-O-Acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranose (***39***)*.[52] To a solution of compound **36** (788 mg, 1.21 mmol) in acetic anhydride (15 mL) at 0 °C was added dropwise concentrated H_2SO_4 (41 µL). The reaction mixture was stirred at 0 °C for 3.5 h and was then diluted in CH_2Cl_2 (15 mL) and quenched with a few drops of a saturated $NaHCO_3$ solution. The reaction mixture was then washed with H_2O (2 * 15 mL) and brine (2*30 mL). The combined aqueous phases were extracted with CH_2Cl_2 (30 mL). The combined organic phases were finally dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: petroleum ether/AcOEt 1/1) gave **39** (784 mg, 1.16 mmol, y = 95%) as a white foam. Rf (petroleum ether/AcOEt 1/1) = 0.17. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.23 (d, *J* = 1.7 Hz, 1H), 5.46 – 5.36 (m, 2H), 5.32 – 5.21 (m, 3H), 4.94 (d, *J* = 1.3 Hz, 1H), 4.27 – 4.15 (m, 4H), 4.11 (dd, *J* = 12.5, 2.0 Hz, 1H), 4.04-3.98 (m, 2H), 2.14 (s, 6H), 2.13 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.03 (s, 6H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 170.7, 170.5, 169.9, 169.7, 169.4, 169.2, 168.2, 99.2, 91.4, 75.8, 70.7, 69.8, 69.7, 69.6, 68.3, 66.2, 65.4, 62.4, 61.7, 20.9, 20.8, 20.6. In agreement with the literature.

*3,4,6-tri-O-Acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranose (***40***).* To a solution of compound **39** (1.33 g, 1.95 mmol) in DMF (13 mL) was added ammonium acetate (453 mg, 5.88 mmol, 3eq). The reaction mixture was stirred at room temperature for 3.5 days and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: hexane/AcOEt 1/1) gave **40** (1.117 g, 1.75 mmol, 90%) as a white foam. Rf (petroleum ether/AcOEt 1/1) = 0.12. NMR in agreement with the literature.[53]

*3,4,6-tri-O-Acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl trichloroacetimidate (***41***).* To a solution of compound **40** (1.59 g, 2.5 mmol) in CH_2Cl_2 (7 mL) at 0°C were slowly added CCl_3CN (977 µL, 9.74 mmol, 3.9 eq) and DBU (122 µL, 0.75 mmol, 0.3 eq). The reaction mixture was stirred at room temperature for 23 h and 4.9 eq of CCl_3CN and 0.3 eq of DBU were added over this period. The reaction mixture was washed with brine (2*20 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent hexane/ACOEt 7/3 + 0.1% Et_3N) gave **41** (1.371 g, 1.76 mmol, y = 70%) as a white foam. Rf (hexane/AcOEt 1/1) = 0.38. NMR in agreement with the literature.[53]

1,6-Di-O-acetyl-2,5-Di-O-(3',4',6'-tri-O-Acetyl-2'-O-(2'',3'',4'',6''-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-3,4-O-isopropylidene-D-mannitol (**42**). A solution of protected mannitol **18** (177 mg, 0.58 mmol), molecular sieves 4Å (441 mg) and compound **41** (1.35 g, 1.73 mmol, 3 eq) in CH_2Cl_2 (8.3 mL) was stirred at room temperature for 30 min and then cooled down to -30 °C. A solution of TMSOTf (63 µL, 0.35 mmol, 0.6 eq) in CH_2Cl_2 (450 µL) was added dropwise and the reaction mixture was then stirred at -30 °C/-15 °C for 4.5 h before addition of a saturated solution of $NaHCO_3$ (2 mL) at 0°C. The mixture was then washed with H_2O (3 * 20 mL) and the organic phase was dried over $MgSO_4$ before evaporation of solvents to dryness. Purification of the residue by chromatography on silica gel (eluent: CH_2Cl_2/MeOH 100/0.2 to 100/2) gave **42** (698 mg, 0.45 mmol, y = 78%) as a white foam. Rf (CH_2Cl_2/MeOH 100/1) = 0.34. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (dd, *J* = 10.0, 3.2 Hz, 2H), 5.37 (d, *J* = 9.8 Hz, 2H), 5.33 (d, *J* = 8.8 Hz, 1H), 5.31 – 5.25 (m, 5H), 5.22 (s, 2H), 4.97 (s, 2H), 4.36 (dd, *J* = 12.1, 2.6 Hz, 2H), 4.27 – 4.11 (m, 14H), 4.11 – 4.04 (m, 4H), 4.02 (s, 2H), 2.14 (s, 6H), 2.14 (s, 6H), 2.09 (s, 6H), 2.07 (s, 12H), 2.03 (s, 12H), 2.00 (s, 6H), 1.36 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 170.6, 170.3, 169.7, 169.4, 169.3, 109.6, 99.5, 99.4, 77.9, 77.2, 69.9, 69.7, 69.0, 68.3, 66.7, 65.9, 63.8, 62.8, 62.0, 27.0, 20.8, 20.6. ESI-HR-MS (positive): *m/z* calculated for $C_{65}H_{91}O_{42}$: 1543.4979, found: 1543.4993, calculated for $C_{65}H_{90}NaO_{42}$: 1565.4799, found: 1565.4784.

1,6-Di-O-acetyl-2,5-Di-O-(3',4',6'-tri-O-acetyl-2'-O-(2'',3'',4'',6''-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-D-mannitol (**43**). A solution of compound **42** (680 mg, 0.44 mmol) in TFA/H_2O (16/1 v/v, 1.1 mL) was stirred at room temperature for 30 min before addition of AcOEt (15 mL) and a saturated $NaHCO_3$ solution (15 mL). The organic phase was extracted with H_2O (2*20 mL) and the aqueous phase was extracted with AcOEt (2*20 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: CH_2Cl_2/MeOH 100/1) gave **43** (298 mg, 0.19 mmol, y = 45%, y_{BORSM} = 57%) as a white solid. Rf (CH_2Cl_2/MeOH 100/2) = 0.24. ^{1}H NMR (400 MHz, D_2O) δ 5.40 – 5.30 (m, 6H), 5.26-5.22 (m, 4H), 5.13 (s, 2H), 4.94 (d, *J* = 1.1 Hz, 2H), 4.45 – 4.35 (m, 4H), 4.21 (m, 6H), 4.15 – 4.05 (m, 6H), 4.03 (s, 2H), 3.90 (d, *J* = 8.7 Hz, 2H), 3.71 (d, m, 2H), 3.30 (d, *J* = 5.9 Hz, 2H), 2.13 (s, 6H), 2.13 (s, 6H), 2.10 (s, 6H), 2.07 (s, 12H), 2.03 (2s, 12H), 2.00 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 171.2, 170.9, 170.5, 169.8, 169.7, 169.5, 169.4, 100.2, 99.5, 78.8, 77.2, 70.2, 69.7, 69.2, 69.0, 68.3, 67.9, 66.6, 65.8, 64.1, 62.4, 62.1, 20.8, 20.7, 20.6, 20.6. ESI-HR-MS (positive): *m/z* calculated for $C_{62}H_{87}O_{42}$: 1503.4666, found: 1503.4658, calculated for $C_{62}H_{86}NaO_{42}$: 1525.4486, found: 1525.4479.

3-O-acetyl-2-O-(3',4',6'-tri-O-acetyl-2'-O-(2'',3'',4'',6''-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-D-glyceric acid (**44**). To a solution of compound **43** (322mg, 0.21 mmol) in CH_2Cl_2/H_2O (1/1 v/v, 3 mL) at 0 °C was added TEMPO (3 mg, 0.02 mmol, 0.1 eq) and BAIB (206 mg, 0.64 mmol, 3 eq). The reaction mixture was stirred at room temperature for 20 h and was then diluted in CH_2Cl_2 (10 mL). Phases were separated and the organic phase was washed with H_2O (10 mL). The aqueous phases were combined, acidified with HCl 1N (10 mL) and extracted with CH_2Cl_2 (4*10 mL). The combined organic phases were dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue (eluent CH_2Cl_2/MeOH 100/2 to 100/5) gave **44** (244 mg, 0.32 mmol, y = 74%) as a white solid. Rf (CH_2Cl_2/MeOH 100/5) = 0.1. ^{1}H NMR (400 MHz, MeOD) δ 5.39 – 5.24 (m, 6H), 5.05 (s, 1H), 4.52 (d, *J* = 10.6 Hz, 1H), 4.44 – 4.29 (m, 3H), 4.27 – 4.06 (m, 6H), 2.15 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 2.06 (s, 6H), 2.04 (s, 6H), 1.99 (s, 3H). ^{13}C NMR (75 MHz, MeOD) δ 172.6, 172.4, 172.3, 171.8, 171.5, 171.4, 171.3, 100.4, 98.1, 78.4, 71.5, 70.9, 70.7, 70.3, 67.5, 67.3, 66.1, 63.5, 63.2, 20.8, 20.7, 20.7, 20.6. ESI-HR-MS (positive): *m/z* calculated for $C_{31}H_{42}NaO_{22}$: 789.2060, found: 789.2068.

(2'R)-2'-((α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl))-D-glycerate, sodium salt (**45**). To a solution of compound **44** (130 mg, 0.17 mmol) in MeOH (2.42 mL) was added NH_4OH (30-33%

solution, 810 µL). The reaction mixture was stirred at room temperature for 2.5 days before evaporation of solvents to dryness. The residue was purified on silica gel chromatography (Eluent: *i*PrOH/NH_4OH/H_2O 7/2/1). After evaporation, the residue was diluted in water and treated with Dowex® 50WX2 (Na^+) and desalted on sephadex® G10 to give **45** (67 mg, 0.15 mmol, y = 88%, *ratio* α/β anomers = 100/8) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.16. ^{1}H NMR (500 MHz, D_2O) δ 5.19 (s, 0.08H, β anomer), 5.11 (d, *J* = 1.4 Hz, 1H, α anomer), 5.06 (d, *J* = 1.5 Hz, 1H, α anomer), 4.39 – 4.34 (m, 0.08H, β anomer), 4.24 (d, *J* = 2.8 Hz, 0.08H, β anomer), 4.19 (dd, *J* = 7.0, 2.9 Hz, 1H, α anomer), 4.14 – 4.10 (m, 1.16H, α anomer, β anomer), 4.07 (dd, *J* = 3.2, 1.8 Hz, 1H, α anomer), 4.05 (dd, *J* = 9.1, 3.2 Hz, 1H, α anomer), 4.01 (dd, *J* = 9.7, 3.5 Hz, 1H, β anomer), 3.92 (dd, *J* = 12.3, 2.2 Hz, 0.16H, β anomer), 3.90 – 3.80 (m, 4.16 H), 3.79 – 3.65 (m, 7H), 3.61 (dd, *J* = 20.2, 10.0 Hz, 0.24H, β anomer), 3.39 – 3.34 (m, 1H, β anomer). ^{13}C NMR (126 MHz, D_2O) δ 179.9 (β anomer), 179.4 (α anomer), 105.1 (α anomer), 103.9 (β anomer), 102.7 (β anomer), 99.9 (α anomer), 85.0 (β anomer), 81.4 (α anomer), 80.9 (α anomer), 79.6 (β anomer), 78.5 (β anomer), 76.5 (β anomer), 75.8 (α anomer), 75.2 (α anomer), 73.2 (α anomer), 73.1 (β anomer), 72.9 (β anomer), 72.9 (α anomer), 72.9 (α anomer), 69.9 (β anomer), 69.8 (α anomer), 69.6 (β anomer), 69.5 (α anomer), 65.8 (α anomer), 65.2 (β anomer), 64.1 (β anomer), 63.8 (α anomer), 63.6 (α anomer). ESI-HR-MS (positive): *m/z* calculated for $C_{15}H_{26}NaO_{14}$: 453.1215, found: 453.1215.

(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-(1,3,4,6-tetra-O-acetyl-α-D-mannopyranoside) (**46**). [54] To a solution of compound **37** (500 mg, 0.53 mmol) in acetic anhydride (6.6 mL) at 0 °C was added dropwise concentrated H_2SO_4 (18 µL). The reaction mixture was stirred at 0 °C for 3 h and was then diluted in CH_2Cl_2 (10 mL) and quenched with a few drops of a saturated $NaHCO_3$ solution. The reaction mixture was then washed with H_2O (2 * 10 mL) and brine (2*20 mL). The combined aqueous phases were extracted with CH_2Cl_2 (2*20 mL). The combined organic phases were finally dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: petroleum ether/AcOEt 1/1 to 45/55) gave **46** (431 mg, 0.45 mmol, y = 83%) as a white foam. Rf (petroleum ether/AcOEt 4/6) = 0.22. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.24 (d, *J* = 2.1 Hz, 1H), 5.43 – 5.25 (m, 8H), 5.12 (d, *J* = 2.0 Hz, 1H), 4.94 (d, *J* = 1.7 Hz, 1H), 4.27 – 4.16 (m, 5H), 4.16 – 4.05 (m, 7H), 4.05 – 3.98 (m, 1H), 2.15 (s, 6H), 2.13 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.04 (s, 3H), 2.04 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 170.4, 170.1, 169.9, 169.7, 169.4, 169.2, 169.1, 168.2, 99.8, 99.3, 91.5, 77.2, 75.3, 70.7, 69.9, 69.6, 69.4, 68.3, 66.2, 65.9, 65.4, 62.4, 62.1, 61.6, 20.8, 20.7, 20.5. ESI-HR-MS (positive): *m/z* calculated for $C_{40}H_{54}NaO_{27}$: 989.2745, found: 989.5739.

2-O-Acetyl-ethyl ((2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1->2)-O-(3,4,6-tri-O-acetyl-α-D-mannopyranosyl)-(1->2)-(3,4,6-tri-O-acetyl-α-D-mannopyranoside)) (**47**). A solution of compound **46** (620 mg, 0.64 mmol) and 1-O-Acetyl-ethylene glycol (80 mg, 0.77 mmol, 1.2 eq) in acetonitrile (13 mL) was stirred at room temperature for 10 min and was then cooled down to 0 °C before addition of $BF_3.Et_2O$ (244 µL, 1.92 mmol, 3 eq). The reaction mixture was stirred at room temperature for 16.5 h and solvents were evaporated to dryness. The residue was diluted in AcOEt (50 mL), washed with H_2O (3 * 50 mL), brine (50 mL), dried over $MgSO_4$ and solvents were evaporated to dryness. Purification of the residue by chromatography on silica gel (eluent: Petroleum ether/ethyl acetate 1/1 to 4/6) gave **47** (325 mg, 0.32 mmol, y = 50%) as a white foam. Rf (petroleum ether/AcOEt 4/6) = 0.14. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39 (dd, *J* = 10.0, 3.4 Hz, 1H), 5.35 – 5.24 (m, 6H), 5.11 (d, *J* = 1.8 Hz, 1H), 5.00 (d, *J* = 1.9 Hz, 1H), 4.95 (d, *J* = 1.5 Hz, 1H), 4.31 (ddd, *J* = 12.1, 6.5, 3.2 Hz, 1H), 4.27 – 4.07 (m, 10H), 4.04 (t, *J* = 2.4 Hz, 1H), 3.96 (dd, *J* = 6.5, 2.4 Hz, 1H), 3.91 – 3.84 (m, 1H), 3.72 (ddd, *J* = 11.4, 6.1, 3.2 Hz, 1H), 2.15 (s, 3H), 2.13 (s, 3H), 2.13 (s, 3H), 2.09 (s, 6H), 2.07 (s, 3H), 2.06 (s, 3H), 2.03 (s, 9H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.75, 170.69 170.66, 170.4, 169.99, 169.97, 169.95, 169.7, 169.6, 169.4, 169.37, 169.3, 99.6, 99.3, 98.0, 77.2, 76.3, 70.2, 69.6, 69.5, 69.4, 69.2, 68.7, 68.3, 66.2, 66.1, 65.8, 62.6, 62.4, 62.2, 62.0, 20.8, 20.7, 20.6, 20.57, 20.55. ESI-HR-MS (positive): *m/z* calculated for $C_{42}H_{59}O_{28}$: 1011.3187, found: 1011.3193, calculated for $C_{42}H_{58}NaO_{28}$: 1033.3007, found: 1033.3009.

(α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl) Ethylene Glycol (**48**). To a solution of compound **47** (126 mg, 0.13 mmol) in MeOH (1.78 mL) was added NH_4OH (30-33% solution, 593 µL). The reaction mixture was stirred at room temperature for 40 h before evaporation of solvents to dryness. The residue was dissolved in ultrapure H_2O (1 mL) and subject to dialyse (Membrane Float-A-lyser G2, spectrum lab, cut-off 01-0.5 KDa) in ultrapure H_2O for 28h (ultrapure H_2O was renewed at 4h, 9h and 24h). The 1 mL solution was then removed from the membrane and lyophilised to give **48** (55 mg, 0.10 mmol, y = 80%) as a white solid. Rf (*i*PrOH/NH_4OH/H_2O 7/2/1) = 0.06. ^{1}H NMR (500 MHz, D_2O) δ 5.31 (d, *J* = 1.5 Hz, 1H, $H1_B$), 5.12 (d, *J* = 1.3 Hz, 1H, $H1_A$), 5.05 (d, *J* = 1.5 Hz, 1H, $H1_C$), 4.12 (dd, *J* = 3.1, 1.8 Hz, 1H), 4.08 (dd, *J* = 3.3, 1.8 Hz, 1H), 3.99 (dd, *J* = 3.2, 1.7 Hz, 1H), 3.96 (dt, *J* = 8.8, 3.6 Hz, 2H), 3.93 – 3.87 (m, 3H), 3.85 (dd, *J* = 9.7, 3.4 Hz, 1H), 3.83 – 3.71 (m, 8H), 3.71 – 3.65 (m, 3H), 3.63 (d, *J* = 9.9 Hz, 1H), 3.62 – 3.58 (m, 1H). ^{13}C NMR (126 MHz, D_2O) δ 105.1 ($C1_C$), 103.5 ($C1_B$), 101.1 ($C1_A$), 81.7 ($C2_A$), 81.4 (C2B), 76.1, 75.4, 73.2, 72.9, 72.8, 71.5, 69.9, 69.8, 69.7, 64.0, 63.9, 63.8, 63.3. ESI-HR-MS (positive): *m/z* calculated for $C_{20}H_{37}O_{17}$: 549.2025, found: 549.2028, calculated for $C_{20}H_{36}NaO_{17}$: 571.1844, found: 571.1849

Biological activity analysis

Cells

Daudi (Human Burkitt's lymphoma) cells were obtained from DSMZ and grown in the RPMI medium (Eurobio) supplemented with 1% of L-Glutamine (Eurobio) and 10% of heat inactivated FBS (Eurobio).Venous blood was obtained from EFS in accordance with declaration of Helsinki. PBMC were collected from hemochromatosis donors, who had given their informed consent. For PBMC isolation, the cells were purified by sedimentation on Lymphocyte-Separation Medium (Eurobio), a separation solution made with Ficoll TM 400. Then, after dilution with equal parts of culture medium, heparinized whole blood was carefully poured over the lymphocyte separation solution. Centrifugation of the mixture at 400×g for 20 min led to the concentration of the lymphocytes (70–100% enrichment) in the interphase (white layer) between the plasma and the separation solution. These lymphocytes were subsequently extracted with a sterile pipette and washed twice with the culture medium. Assessment of the cell viability was done with the use of 0.05% Trypan blue, which stains only the non-viable cells. The cell viability percentages were always above 95%. Then, after counting of the cell suspension, PBMC concentration was adjusted to the expected final one in AIMV (ThermoFisher). Then, the PBMCs were used and incubated in 5% CO2 at 37°C to the bioactivity assays. Primary leukemia cells from patients with CLL were obtained through the Hospital CHRU Morvan, Brest, France.

Cytotoxicytic assay
Peripheral Mononuclear Blood Cells (PBMC) isolated from hemochromatosis donors were incubated with 10 mM of Firoin (Bitop AG, Germany), MG or its derivatives for 48h. PBMC were plated at 300 000 cells per well in AIMV medium in 24 well plate and incubated in 5% CO2 at 37°C. After 48h, the number of apoptotic cells was determined after annexin V and IP staining (Clinisciences) using a FC500 flow cytometer (Beckman Coulter, Villepinte, France). Apoptotic cells were evaluated as Annexin V + IP + (propidium iodure).

Phagocytosis assay
After dilution of human whole blood, aliquots were preincubated for 24 h with 10mM of Firoin, MG or analogue of MG to investigate phagocytosis with an assay adapted from the commercially available Phagotest kit (BD Biosciences). This test relies on the measurement by flow cytometry (FC500, Beckman coulter) of the uptake of fluorescence-labeled E. coli by the cells of interest. Briefly, heparinized whole blood is incubated for 10 more minutes with fluorescein-labeled E. coli bacteria at 37 ◦C while a negative control sample is kept on ice. The phagocytosis is stopped by placing the samples on ice. To exclude artifacts of bacteria, or cells, aggregation, a DNA staining solution is added just before the measurement, by flow cytometry, of the number of ingested bacteria.

Determination of monocytes and dentritic cells number
Peripheral Mononuclear Blood Cells (PBMC) isolated from hemochromatosis donors were incubated with or without 10 mM of synthetic MG. PBMC were plated at 2 400 000 cells per well in AIMV medium in 6 well plate and incubated in 5% CO2 at 37°C. After 1, 3, 5, 7 and 9 days after stimulation, all cells were harvested with a cell scraper and the absolute number of $CD14^{pos}$ or $CD11^{pos}$ cells was evaluated using fluorescein (FITC)-conjugated anti-CD14 mAb (Beckman Coulter) or PE-conjugated anti-CD11b mAb (BD Biosciences), and FlowCount beads (Beckman Coulter).

Magnetic cell sorting
10^9 Peripheral Mononuclear Blood Cells (PBMC) isolated from hemochromatosis donors were used to magnetic cell sorting. NK cells were negatively sorted using NK cell isolation kit (Miltenyi Biotech, Germany). Purity of NK cell population was controlled by flow cytometry analysis CD56 expression (90-95% CD56+ cells). Purified NK cells were used to ADCC assays. Monocytes were positively sorted using anti-CD14 labeled magnetic beads (Miltenyi Biotech). Purity of cell population was controlled by flow cytometry analysis of CD14 expression (95-97% CD14+ cells). Purified monocytes were used to ADCC assays.

ADCC
As reported in the literature, [55] this flow cytometric assay is based on two fluorescent dyes and provides a measurement of NK cytotoxicity. PBMC were plated at $3x10^5$ in 200µl of AIMV in 96 wells plate (15/1 ratio), purified monocytes were plated at $2x10^5$ in 200µl of AIMV in 96 wells plate (10/1 ratio) and purified NK cells were plated at $1x10^5$, $2x10^5$ or 2.5x106 in 200µl of AIMV in 96 wells plate (respectively 5/1, 10/1 or 25/1 ratio). These cells were stimulated or not with synthetic MG for 5 days. Target Daudi cells were stained with 300nM calcein-AM, a non-fluorescent substance which is converted by esterase to the green fluorescent calcein in viable cells. The stained Daudi cells (at $2x10^4$ in 50µl of AIMV), the effector one (PBMC, purified monocytes or purified NK cells) and an anti-CD20 antibody (rituximab [400ng/ml]) were mixed in sterile Falcon polystyrene tubes and incubated at 37 ◦C in a humidified 5% CO2 incubator for 4 h. Then, ethidium homodiner-1, a red DNA stain non-permeable to viable cells, was added at the concentration of 10mM, and the reaction was let to develop for 15 min at room temperature prior to the acquisition of the Flow cytometric data. The dead target cells exhibit a green-red staining. Data analysis is performed by gating the regions of living and dead target and living effector cells from appropriate controls. Non-specific events are subtracted from the dead target region, and the ratio of specific dead target events to the total target events gives the percentage of cytotoxicity

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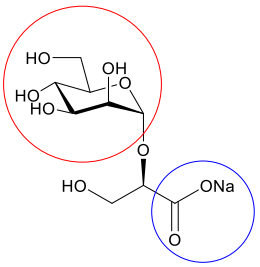
- nature of the sugar (mannosyl, glucosyl, galactosyl)
- number of mannosyl units



Influence of the carboxylate group

Analogs of mannosylglycerate have been synthesized and evaluated as immunostimulating agents

Key Topic: Mannosylgycerate, immunostimulating agent

Nadege Hamon, Caroline Mouline, Marion Travert*

Page No. – Page No.

Synthesis of mannosylglycerate derivatives as immunostimulating agents