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Tetrahedron Letters
Tetrahedron Letters 48 (2007) 2915–2918

Synthesis of β-1,4-di-D-mannuronic acid glycosides as potential ligands for toll-like receptors

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Received 1 September 2006; revised 10 February 2007; accepted 16 February 2007
Available online 22 February 2007

Abstract—Toll-like receptors (TLRs) play important roles in host immune defense, and synthetic TLR ligands are useful therapeutic agents for a variety of diseases including infection, inflammation, and cancers. Alginates are strong immune stimulants mediated by TLR2/4. Reported here are the design and chemical synthesis of two glycosides (**1** and **2**) containing β-1,4-di-D-mannuronic acid moiety derived from alginate. The synthesis features the preparation of β-1,4-D-mannobiose derivatives through diastereoselective β-glycosylation of 4,6-di-*O*-benzylidene protected thiomannoside donor **7**, followed by an oxidation step using TEMPO/BAIB to provide the β-1,4-di-D-mannuronic acid moiety.

The recently discovered toll-like receptors (TLRs) are innate pattern recognition receptors, which play important roles in host immune defense.[1,2] Activation of TLRs and downstream signaling pathways lead to the expression of pro- and anti-inflammatory mediators which have a great impact on human physiology. Synthetic ligands capable of regulating TLR signaling pathways are useful therapeutic agents for treating various diseases including infection, chronic inflammation, and cancers.[3–7] Our interest in synthetic vaccine development[8] has led us to the design and synthesis of a few TLR4 ligands which are potent immune stimulatory molecules potentially useful as vaccine adjuvants.[9,10]

Alginates are un-branched linear polysaccharides consisting of 1,4-linked β-D-mannuronic acid (M) and its C5-epimer α-L-guluronic acid (G) residues (Fig. 1) produced by marine macroalgae and some bacteria belonging to the genera *Azotobacter* and *Pseudomonas*.[11] Their compositions are highly variable, including homo-polymeric (poly-M or poly-G) or hetero-polymeric (poly-GM) structures, depending on the sources from which they are isolated. Alginates from bacteria also display certain degree of acetylation at *O*-2- and *O*-3-position of D-mannuronic acid residues.[12]

Alginates are potent immune stimulating agents. In terms of cytokine production by monocytes, the potency increases with the content of M residues.[13] Poly-G blocks do not induce cytokine production from monocytes, and they also reduce the cytokine production level induced by poly-M blocks, indicating that poly-G blocks may act as antagonists of poly-M blocks. In contrast, other reports show that enzymatically depolymerized alginate oligosaccharides, both oligo-M and oligo-G with a double bond (Δ^4) in the nonreducing end, cause cytotoxic cytokine production in human mononuclear cells[14] and tumor necrosis factor-alpha (TNF-α) in RAW264.7 cells.[15,16] TLR2 and TLR4 have been shown to mediate the cytokine production induced by alginate polymers as well as alginate oligosaccharides,[15,17] indicating that short oligosaccharides of alginate may well serve as agonists or antagonists for these receptors. Therefore, we have designed glycosides **1** and **2** containing β-1,4-di-D-mannuronic acid moiety as potential ligands for TLR2/4 (Fig. 2).

Despite the various industrial applications[18] and medical significance[19] of alginates, the chemical synthesis of alginate oligosaccharides has not been reported, possibly due to the notorious challenge of accessing β-glycosidic linkage of D-mannuronic acid and L-guluronic acid building blocks. In the present Letter, we report the syntheses of compounds **1** and **2**.

Compound **1** is designed as a glycolipid for the reason that naked small carbohydrates are rapidly cleared away

Keywords: Alginate; D-Mannuronic acid; β-1,4-Di-D-mannuronic acid; Oligosaccharide; Glycolipid; TLR ligand.
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doi:10.1016/j.tetlet.2007.02.086

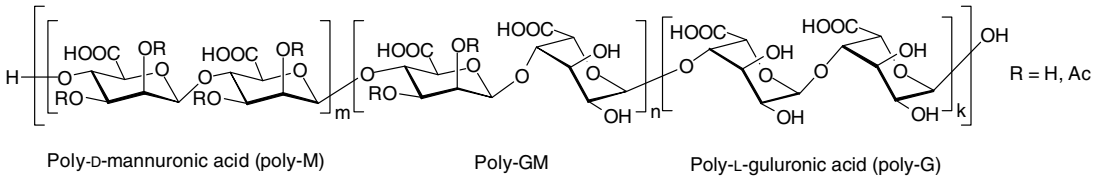


Figure 1. Structures of alginates.

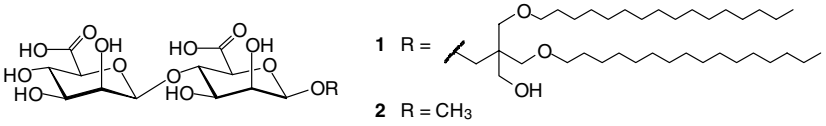
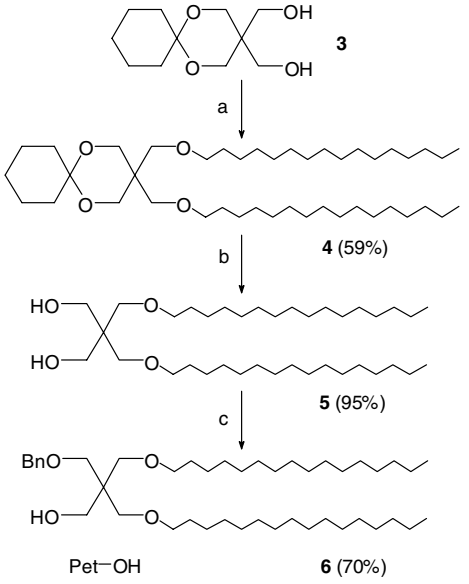


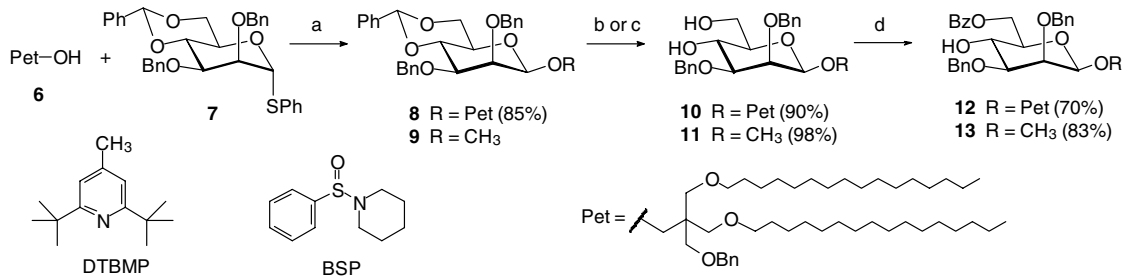
Figure 2. β-1,4-Di-D-mannuronic acid glycosides **1** and **2** designed as potential ligands for TLR2/4.

from the biological system,[20] but the conjugation with lipids may enhance their stability, rendering them better drug candidates than naked carbohydrates. Glycolipids are also suitable for liposome formulation, a drug delivery system proven to be efficient in reducing toxicity and side effects.[21] Thus, lipidated pentaerythritol derivative **6** (Pet–OH) is prepared as the lipid anchor (Scheme 1). Two C_{16}–lipid chains are introduced to the readily available pentaerythritol derivative **3**[22] by treating with 1-bromohexadecane and sodium hydride in DMF to provide **4** in 59% yield. Trans-ketal reaction of **4** with neopentyl glycol in the presence of camphorsulfonic acid (CSA) liberates diol **5** in 95% yield. Monobenzylation of **5** through treatment with dibutyltin oxide (Bu_2SnO) followed by benzyl bromide and tetrabutylammonium bromide gives compound **6** in 70% yield.

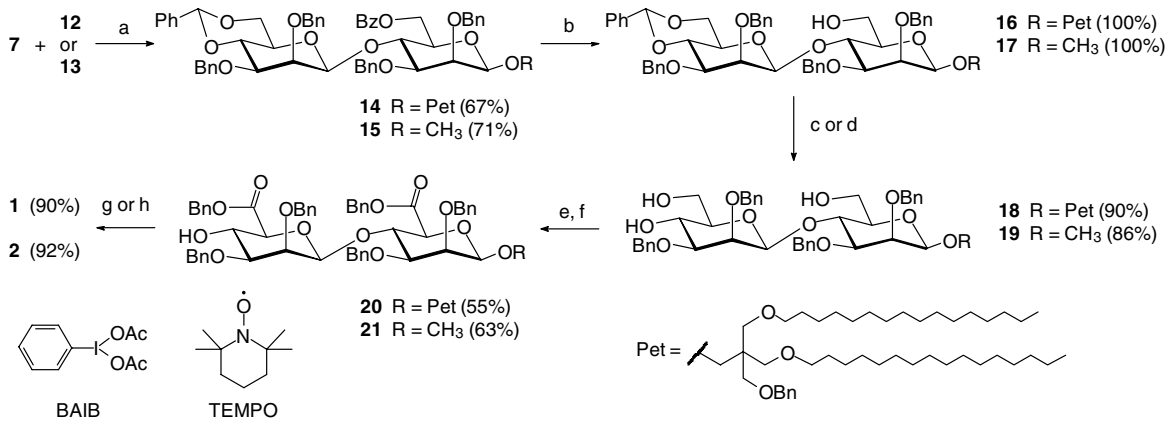


Scheme 1. Reagents and conditions: (a) *n*-$C_{16}H_{33}Br$, DMF, NaH, 50 °C; (b) neopentyl glycol, CSA, CH_2Cl_2, 35 °C; (c) (i) Bu_2SnO, benzene, 90 °C; (ii) BnBr, Bu_4NBr, 90 °C.

The strategy we employ to make the β-1,4-di-D-mannuronic acid moiety is to first synthesize the β-1,4-D-mannobiose unit, followed by an oxidation step to convert both mannose residues to the mannuronic acid residues. Diastereoselective synthesis of β-D-mannopyranoside has been a long standing challenge in carbohydrate chemistry.[23] The 4,6-di-*O*-benzylidene protected methodology recently developed by Crich and co-workers[24,25] offers an efficient method for the direct synthesis of β-D-mannosides, as demonstrated by the synthesis of β-1,2-D- and β-1,4-D-mannan.[26,27] Here we use a similar strategy to construct the β-1,4-D-mannobiose moiety (Schemes 2 and 3). Thus, activation of the readily accessible thiomannoside **7**[27] by 1-benzenesulfinyl piperidine and trifluoromethanesulfonic anhydride (BSP/Tf_2O)[25] in the presence of the hindered base 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) in dichloromethane at −60 °C followed by the addition of acceptor **6** at −78 °C provides the desired β-mannoside **8** in 85% yield. The α-isomer is also formed in about 8% yield, but an



Scheme 2. Reagents and conditions: (a) BSP, DTBMP, Tf_2O, CH_2Cl_2, −78 °C to 0 °C; (b) neopentyl glycol, CSA, CH_2Cl_2, 35 °C, for **10**; (c) HOAc–H_2O (4:1), 75 °C, for **11**; (d) BzCl, pyridine, DMAP, CH_2Cl_2, −25 °C to 0 °C.



Scheme 3. Reagents and conditions: (a) BSP, DTBMP, Tf_2O, CH_2Cl_2, −78 °C to 0 °C; (b) NaOMe, MeOH–CH_2Cl_2 (1:1), rt; (c) neopentyl glycol, CSA, CH_2Cl_2, 35 °C, for **18**; (d) HOAc–H_2O (4:1), 75 °C, for **19**; (e) BAIB, TEMPO, CH_2Cl_2–H_2O (2:1), rt; (f) BnBr, KF, DMF, rt; (g) H_2, Pd/C, THF–H_2O (4:1), rt, for **1**; (h) H_2, Pd/C, MeOH–H_2O (3:1), rt, for **2**.

analytically pure material has not been obtained. The removal of 4,6-di-*O*-benzylidene group in **8** is effected by treating with neopentyl glycol in the presence of CSA to give compound **10** in 90% yield. Compound **10** is converted to 6-*O*-benzoate **12** through regioselective benzoylation with benzoyl chloride and pyridine in the presence of 4-*N*,*N*-dimethylpyridine (DMAP) in 70% yield. The β-glycosidic linkage, originally formed in compound **8**, has been confirmed by the value of $^1J_{C,H}$ coupling constant of the anomeric carbon in **12** ($^1J_{C1,H1} = 154.6$ Hz).[28] Similarly, the previously reported methyl β-D-mannoside **9**[27] is treated with acetic acid–water (4:1) at 75 °C to give diol **11**,[27] which is then converted to its 6-*O*-benzoate **13** in 83%.

By using the same glycosylation protocol, both **12** and **13** are glycosylated with **7** to provide disaccharides **14** and **15** in 67% and 71% yield, respectively (Scheme 3). Again the α-isomers for both reactions are formed in negligible amount and therefore are not characterized. The newly formed β-linkage in **14** and **15** is confirmed[29] by the characteristic H-5 signal[24,27] of the sugar residue with the 4,6-di-*O*-benzylidene group and the $^1J_{C,H}$ coupling constant of the anomeric carbon. Compounds **14** and **15** are converted to triols **18** and **19**, respectively, in high yield through deacylation with sodium methoxide in dichloromethane–methanol (→**16**, **17**) and removal of the 4,6-di-*O*-benzylidene group. Regioselective oxidation of the primary hydroxyl function in **18** and **19** to carboxylic acid is achieved by the combination of 2,2,6,6-tetramethylpiperidinyloxy free radical (TEMPO) and [bis(acetoxyiodo]benzene (BAIB)[30,31] (Scheme 3). Thus, triols **18** and **19** are converted into their corresponding dicarboxylic acids, which are subsequently treated with benzyl bromide in the presence of potassium fluoride in DMF to give benzyl esters **20**[32] and **21** in 55% and 63% yield, respectively. The TEMPO/BAIB oxidation system seems to be effective in simultaneous oxidation of multiple primary hydroxyl groups to carboxylic acid functions in the presence of secondary hydroxyl groups. Hydrogenolytic debenzylation of **20** and **21** in the presence of palladium on charcoal under hydrogen atmosphere produces the final products **1** and **2** in good yield. The structures of **1** and **2** have been confirmed by spectroscopy data.[33,34]

The initial biological evaluation of compounds **1** and **2** with respect to the immune stimulating and modulating properties is under way. The result will be reported in due course.

In brief summary, we have described an efficient route for the synthesis of β-1,4-di-D-mannuronic acid glycosides **1** and **2** designed as potential ligands for TLR2/4. The method shall be applicable for synthesizing glycoconjugates containing larger mannuronic acid oligosaccharides. Further research is being devoted to the synthesis and biological investigation of neo-glycoconjugates containing oligo-D-mannuronic acid as well as oligo-L-guluronic acid.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada, NSERC (312630), and Lakehead University.

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29. Compound **14**: ^{1}H NMR (500 Hz, $CDCl_3$): δ 3.05 (ddd, $J = 10.0$, 10.0, 4.5 Hz, 1H, H-5); ^{13}C NMR (125 Hz, $CDCl_3$): δ 102.2 ($^1J_{C,H} = 155.5$ Hz), 101.9 ($^1J_{C,H} = 154.5$ Hz), 101.3 ($^1J_{C,H} = 157.9$ Hz). **15**: ^{1}H NMR ($CDCl_3$): δ 3.06 (ddd, $J = 10.0$, 10.0, 4.5 Hz, 1H, H-5); ^{13}C NMR ($CDCl_3$): δ 102.3 ($^1J_{C,H} = 154.3$ Hz), 101.8 ($^1J_{C,H} = 152.4$ Hz), 101.3 ($^1J_{C,H} = 158.1$ Hz).
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32. Compound **20**: $R_f = 0.69$ (hexane–ethyl acetate–methanol–acetic acid, 2:1:0.05:0.05). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, $J = 7.0$ Hz, 6H, $2CH_3$), 1.25 (br s, 52H, $26CH_2$), 1.48 (m, 4H, $2CH_2$), 2.77 (d, $J = 2.5$ Hz, 1H, OH), 3.10 (dd, $J = 10.0$, 3.0 Hz, 1H, H-3), 2.28–3.49 (m, 13H), 3.64 (d, $J = 3.0$ Hz, 1H, H-2), 3.65 (d, $J = 3.0$ Hz, 1H, H-2), 3.81 (d, $J = 9.5$ Hz, 1H, H-5), 4.00 (d, $J = 9.5$ Hz, 1H, H-5), 4.18 (ddd, $J = 10.0$, 9.5, 2.5 Hz, 1H, H-4), 4.32 (s, 1H, H-1), 4.40 (m, 3H), 4.45 (m, 2H), 4.46 (s, 1H, H-1), 4.49 (d, $J = 12.0$ Hz, 1H), 4.47 (d, $J = 12.0$ Hz, 1H), 4.64 (d, $J = 12.0$ Hz, 1H), 4.68 (d, $J = 12.0$ Hz, 1H), 4.71 (d, $J = 12.0$ Hz, 1H), 4.85 (d, $J = 12.0$ Hz, 1H), 5.00 (d, $J = 12.5$ Hz, 1H), 5.01 (d, $J = 12.0$ Hz, 1H), 5.10 (d, $J = 12.0$ Hz, 1H), 5.20 (d, $J = 12.5$ Hz, 1H), 7.25 (m, 35 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.1, 168.1, 138.9, 138.7, 138.6, 138.0, 135.3, 135.1, 128.6, 128.5, 128.4, 128.3, 128.27, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.4, 127.3, 127.2, 102.9, 102.1, 80.1, 79.4, 75.1, 75.0, 74.8, 74.6, 74.2, 73.7, 73.2, 72.3, 71.6, 70.5, 69.6, 68.0, 67.1, 66.9, 45.2, 31.9, 29.7, 29.6, 29.5, 29.3, 26.2, 22.7, 14.1.
33. Compound **1**: $R_f = 0.46$ ($CHCl_3$–CH_3OH–H_2O–HOAc, 4:2:0.4:0.4). MS (MALDI) calcd for $C_{49}H_{92}O_{16}$ 936.63 $[M]^+$, found 959.63 $[M+Na]^+$, 981.62 $[M-H+2Na]^+$. A well resolved NMR spectrum was not obtained.
34. Compound **2**: $R_f = 0.44$ (isopropanol–H_2O–HOAc, 4:2:1). ESI-MS (negative mode) calcd for $C_{13}H_{20}O_{13}$ 384.08 $[M]^-$, found 383.10 $[M-H]^-$. ^{1}H NMR (500 MHz, D_2O): δ 3.54 (s, 3H, OCH_3), 3.68 (dd, $J = 9.5$, 3.5 Hz, 1H, H-3), 3.79 (dd, $J = 10.0$, 9.5 Hz, 1H, H-4), 3.81 (dd, $J = 8.5$, 3.5 Hz, 1H, H-3), 3.92 (d, $J = 10.0$ Hz, 1H, H-5), 3.95 (d, $J = 9.0$ Hz, 1H, H-5), 3.99 (dd, $J = 9.0$, 8.5 Hz, 1H, H-3), 4.00 (d, $J = 3.0$ Hz, 1H, H-2), 4.05 (d, $J = 3.0$ Hz, 1H, H-2), 4.66 (s, 1H, H-1), 4.74 (s, 1H, H-1). ^{13}C NMR (125 MHz, D_2O): δ 173.2, 173.1, 101,3, 100.4, 78.3, 74.9, 74.3, 72.5, 71.5, 70.3, 69.6, 68.0, 57.3.