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Synthesis of new sulfated disaccharides for the modulation of TLR4-dependent inflammation

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Natural sulfated glycans are key players in inflammation through TLR4 activation, and therefore synthetic exogenous sulfated saccharides can be used to downregulate inflammation processes. We had designed and synthesized new sulfated compounds based on small and biocompatible carbohydrates able to cross the BBB. Suitable protected donor and acceptor, obtained from a unique precursor, have been stereoselectively glycosylated to give an orthogonally protected cellobiose disaccharide. Selective deprotection and sulfation allowed to synthesize four differentially sulfated disaccharides, which have been characterized by NMR, HRMS and MS/MS. Together with their partially protected precursors, the new compounds were tested on HEF-TLR4 cells. Our results show the potential of small oligosaccharides to modulate TLR4 activity, confirming the need of sulfation and the key role of the 6-sulfate groups to trigger TLR4 signalization.

Introduction

Toll-like receptors (TLRs) are a type of pattern recognition receptors (PRR) that identify molecules shared by pathogens but distinguishable from host molecules collectively referred to as pathogen-associated molecular patterns (PAMPs). TLRs play a critical role by triggering the molecular activation cascade that regulates the innate immune response/inflammatory process.[1,2,3] The upregulation of TLR with the development of TLR agonists could be useful for various tumor treatment,[4] allergies,[5] infectious disease (HBV, malaria).[6] The downregulation with antagonists is implicated in pathologies such as sepsis,[7] type 1&2 diabetes,[8,9] or neuroinflammation.[10]

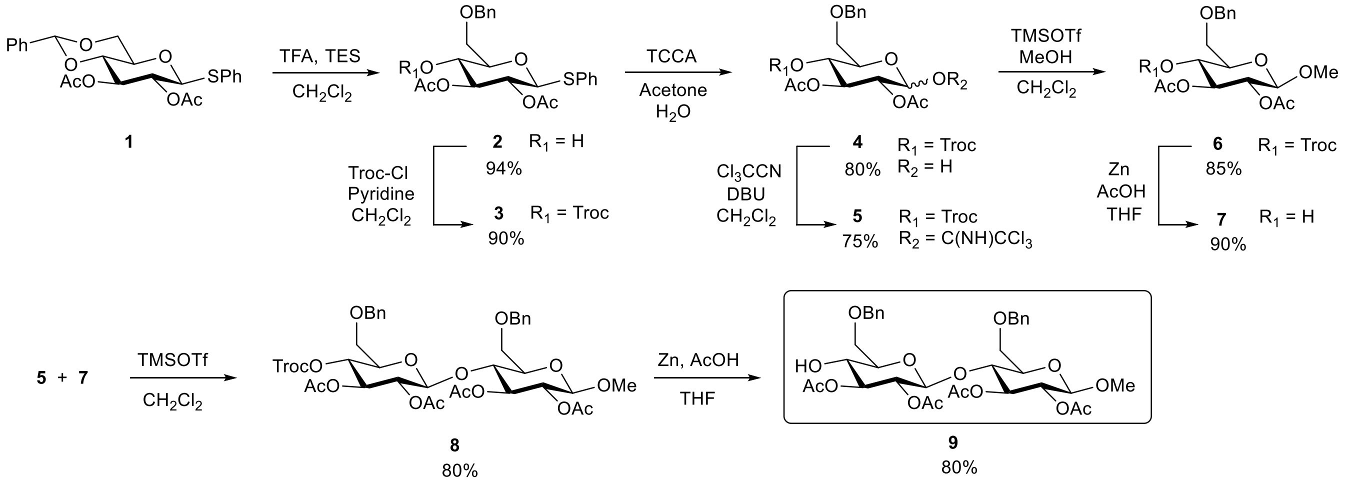
Among TLRs, TLR4 is a key cell surface receptor involved in innate and adaptive immune response. This receptor is activated through the exposure of the lipopolysaccharide (LPS), lipid A or lipooligosaccharides (LOS) and initiate the production of a number of inflammatory mediators, including IL-1β, TNF-α, and macrophage inflammatory protein 1 alpha (MIP1α) *via* TLR4-dependent activation through the MyD88 adaptor and the NF-kB pathway.[11] It can be found on the surface of microglial cells in the central nervous system (CNS), brain or spinal cord. Interestingly, neuroinflammation is known to play a decisive role in neurological diseases such as neurodegeneration.[10] Thus, targeting brain inflammation represents a potential clinical intervention strategy for such pathologies but the blood brain barrier (BBB) is one of the main hurdles to access the CNS. Some molecules able to modulate the TLR4 response, have been tested previously and are often too big and/or not specific enough. Nevertheless, some small molecule modulators, able to cross the BBB, have been reported to have antagonist activities. [12,13,14,15,16]

Sulfated glycans such as glycosaminoglycans on proteoglycans are key players in both molecular and cellular events of inflammation through TLR4 activation. For example, it has been shown that heparan sulfate is degraded in inflammation to become a potent TLR4 ligand, and that TLR4 can be activated by small soluble fragments of heparan sulfate. Therefore, exogenous sulfated glycans of various structures and origins can be used to interventionally downregulate inflammation processes.[17,18,19] As example, disaccharides like Lipid A and analogs have proven to establish interaction with TLR4 and could be potent interesting modulators.[20, 21]

Herein, we propose to modulate TLR4 activity to reduce neurodegeneration by the modulation of the neuroinflammation. We have designed and synthesized new sulfated saccharides acting as inflammation regulator in order to restrain the neuroinflammation. Those modulators are based on small and biocompatible carbohydrates and then, would be able to pass the BBB.

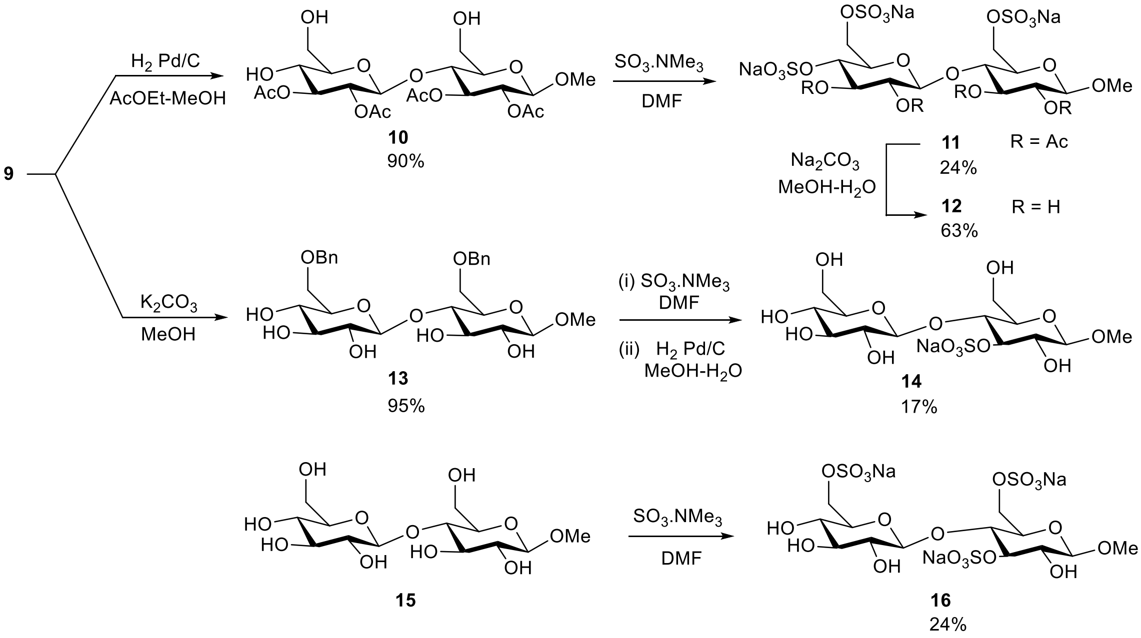
Results and discussion

The synthetic route is depicted in Scheme 1. Regioselective reductive opening of the benzylidene acetal of known phenyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (**1**)[22] was performed using trifluoroacetic acid (TFA) and triethylsilane (TES) to give the 6-*O*-benzyl derivative **2** in 94 % yield. The 4-hydroxyl was temporarily protected as a 2,2,2-trichloroethoxycarbonyl (Troc), followed by the selective removal of the thiophenyl group of compound **3** in the presence of trichloroisocyanuric acid (TCCA)[23] affording **4** in 80 % yield. This method is cleaner than the traditional use of NBS to cleave the C-S linkage. Adding dichloromethane at the end of the reaction produces the precipitation of the isocyanuronic acid and makes the workup very simple.



Scheme 1. Synthesis of orthogonally protected disaccharide **9**.

Reaction with trichloroacetonitrile in the presence of DBU led to the trichloroacetimidate donor **5** (75 % yield). The ^{1}H NMR spectrum revealed its α configuration (δ 6.50, d, *J* = 3.6 Hz, 1H, H-1). The glycosyl acceptor was prepared from compound **5** in two steps. First, glycosylation with methanol was achieved using TMSOTf as promotor to give the methyl glucopyranoside **6** with total β stereoselectivity shown by the H-1 doublet at δ 4.39 (*J* = 7.9 Hz) in the ^{1}H NMR spectrum, as expected in the presence of an acetate participating group at C-2. Second, the Troc protecting group was selectively removed by activated Zn/AcOH in THF. The acceptor **7** was obtained in 90 % yield.[24]



Scheme 2. Synthesis of diversely sulfated products.

Glycosylation between the donor **5** and acceptor **7** promoted by TMSOTf proceeded smoothly at room temperature affording the disaccharide **8** in 80 % yield. In the ^{1}H NMR spectrum, the anomeric protons appeared at 4.42 ppm (d, *J* = 8.0 Hz, H-1) and 4.33 ppm (d, *J* = 7.9 Hz, 1H, H-1), whereas the ^{13}C NMR spectrum showed the signals of C-1′ and C-1 at δ 101.0 and 100.0, respectively. Treatment of compound **8** in the same conditions used above to remove the Troc group gave the key disaccharide **9** in 80 % yield.

The disaccharide **9** can be orthogonally deprotected in order to obtain regioselective sulfated products (Scheme 2). Catalytic hydrogenation over 10 % Pd/C allowed to cleavage the benzyl groups, leading to the triol **10**. Sulfation using SO_3-trimethylamine complex (5 equiv./OH) afforded **11**, which by deacetylation gave the compound **12**. On the other hand, deacetylation of **9** afforded compound **13** which has five free hydroxyls. Sulfation of **13** as above followed by hydrogenolysis led to compound **14**. For comparison, known methyl cellobioside **15** was also sulfated to give compound **16**.

All the sulfation reactions proceeded well, but the extensive purifications had to be performed to completely eliminate the excess of sulfating agent on Sephadex LH-20, and the isolated yields were poor (24 %). Nevertheless, the sequence allowed us to obtain a family of differently sulfated disaccharides useful to analyze the structure-activity relationships.

We expected some regioselectivity of the sulfation reaction. However, the analysis of sulfated disaccharides was not straightforward, and a combination of NMR and MS analysis were necessary to determine the structure of compounds **11**, **12**, **14** and **16**.

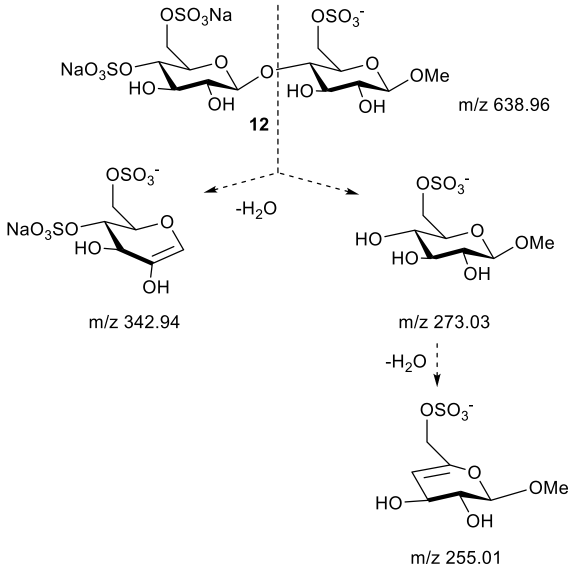
The simplest case was the sequence **10** → **11** → **12**. HRMS (negative mode) of compound **11** showed unambiguously the presence of three sulfates, m/z 807.0028 $[M - 3H + 2Na]^-$. Thus, in our conditions, the sulfation of the primary positions was accompanied by the sulfation of the 4-position. In the ^{13}C NMR of compound **11**, the resonances of the sulfated primary positions C-6 and C-6’ appeared at δ 65.5 and 66.5, a deshielding of about 7 ppm when compared to those of compound **10**. The corresponding signal of C-4’ shifted from δ 69.2 to 73.9 upon sulfation.

The deacetylation step led to compound **12**, and the presence of the three sulfate groups was confirmed on positions 6-, 4’- and 6’- by NMR. The HRMS at m/z 638.9576 fitted well with the trisulfated disaccharide structure. MS/MS of this ion showed main fragments at m/z 519.00 (corresponding to the loss of $NaSO_3$ and water) and the peaks arising from cleavages at the interglycosidic linkage (Scheme 3). The peak at m/z 342.94 (C cleavage, loss of water) is consistent with a monosaccharide fragment carrying two sulfate groups, whereas the other sulfate is found in the reducing end fragment (Z cleavage, m/z 273.03).

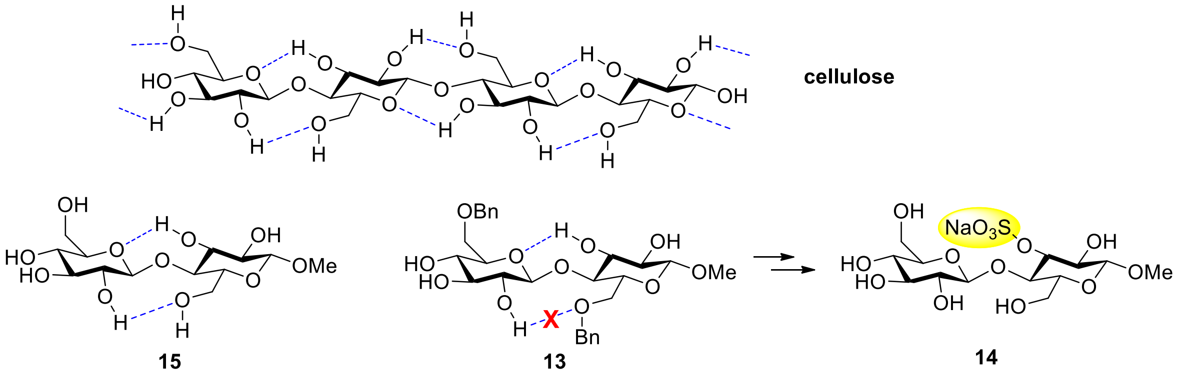
Sulfation of compound **13** was performed in the same conditions used above. Unfortunately, different purifications by flash chromatography (reverse phase) of the sulfated product did not allow to obtain this intermediate in pure form, hydrogenolysis of the benzyl groups were accomplished and the deprotected sulfated molecule **14** was finally purified by Sephadex LH20. HRMS of compound **14** showed the presence of one sulfate group, as the molecular ion $[M - H]^-$ appeared at m/z 435.0860. As the secondary hydroxyls are less reactive than the primary positions (blocked as benzyl ethers in compound **13**), the sulfation did not lead to an oversulfated product. From the five available positions, the question is to determine the exact location of the sulfate group in compound **14**.

MS/MS of the molecular ion gave limited information. The main fragments observed were at m/z 273.00 (Y interglycosidic cleavage), m/z 254.99 and m/z 240.98 (loss of water and methanol, respectively, from the precedent ion), consistent with the location of the sulfate group at the reducing end.

NMR spectra of compound **14** were assigned by comparison with those of methyl cellobioside **15**. The HSQC NMR spectrum of compound **14** showed a downfield shifted triplet at δ 4.35 (that can be assigned to H-3) that correlates with a peak at δ 84.0 (thus assigned to C-3). In addition, the little difference between chemical shifts of C-1 between the non-sulfated and the sulfated product indicated that the sulfate group cannot be at C-2. Therefore, the structure of compound **14** corresponds to a 3-sulfate methyl cellobioside.



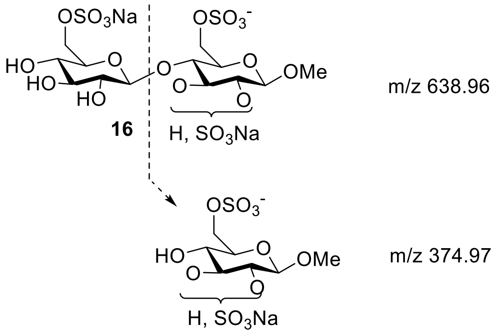
Scheme 3. MS/MS analysis of molecular ion of compound **12**.



Scheme 4. Conformation of cellulose and methyl cellobiosides and regioselectivity of the sulfation of compound **13**.

This regioselectivity for the *O*-3 can be explained assuming a conformation of cellobiose similar to those found in cellulose (Scheme 4). In methyl cellobioside **15**, the reactivity would be enhanced for both *O*-3 (linked through hydrogen bond to the sugar *O*-5) and *O*-2' (linked through hydrogen bond to the *O*-6). However, in compound **13**, the 6-oxygen is blocked with a benzyl group, therefore the *O*-2' is no longer activated for substitution and sulfation takes place on position -3.

Finally, methyl cellobioside **15** was directly sulfated and the major compound **16** was purified and analyzed. The HRMS showed the molecular ion at m/z 638.9604, indicating a trisulfated disaccharide. The preferentially substitution is expected to occur at 6- and 6'-positions. In the ^{13}C NMR spectrum of **16** a shift was observed for the resonances corresponding to C-6 and C-6' when compared to those in the spectrum of **15**, from δ 61.4 and 61.9 to δ 68.9 and 69.3, respectively, confirming the assignment. NMR spectra also showed that this trisulfated disaccharide **16** is different of that compound **12**, which is also sulfated at C-6 and C-6' but possess an additional sulfate at C-4'. MS/MS of the molecular ion showed a diagnostic fragment at m/z 374.97, indicating the presence of the remaining sulfate on the reducing unit (Scheme 5). From the two possible hydroxyls, NMR spectra of compound **16** are consistent with the presence of the additional sulfate group at position C-3.



Scheme 5. MS/MS analysis of molecular ion of compound **16**.

The sulfated molecules synthesized, together with some precursors or partially protected molecules that have been then tested for TLR4 interaction.

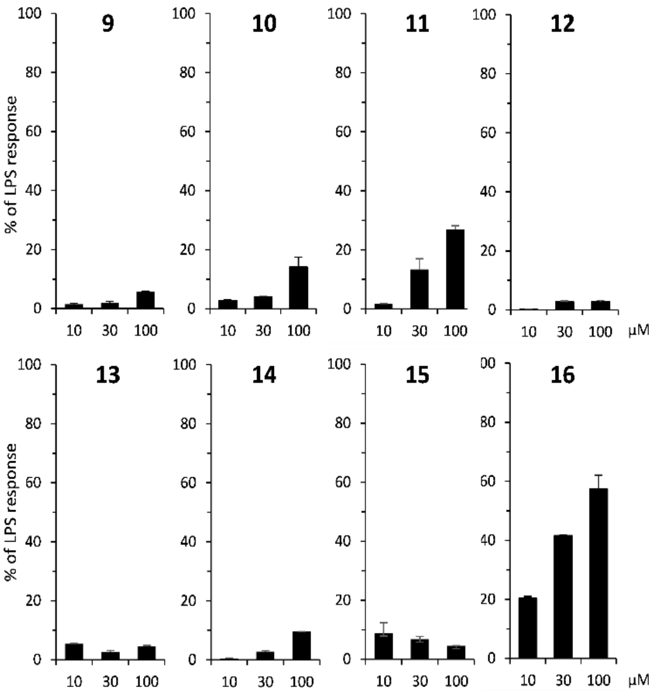


Figure 1. Stimulation of TLR4-expressing HEK cells by compounds ***9*** *to* ***16****. HEK-TLR4 cells were stimulated by the addition of compounds* ***9*** *to* ***16,*** *each at the finale concentrations of 10, 30 and 100 μM. After 16 h of incubation, the production of SEAP related to the reporter gene activation was quantified by measuring the phosphatase activity released in cell-free supernatants with a chromogenic substrate (620 nm). Data of SEAP activity are means values ± SEM from three separate*

To test the capacity of compounds **9** to **16** to trigger signalization through TLR4, we decided to use a TLR4 transfected-cell line. A number of enzymatic bio-assays have been indeed developed, based on the use of stably TLR-transfected cell lines and designed to provide a sensitive method for the detection of TLR agonists.[xxv] Among them, HEK-Blue™/hTLR4 (HEK-TLR4) is a commercially available cell line, which is stably co-transfected to express the human TLR4 gene and a TLR-inducible reporter gene encoding a secreted embryonic alkaline phosphatase (SEAP). Parental HEK-293 cells do not express TLRs on their plasma membrane. Accordingly, TLR4 stimulation can be conveniently monitored through the release of SEAP from HEK-TLR4 cells by using a phosphatase detection assay. In addition, we used the HEK-Blue™/Null1 (HEK-Null) cell line as a negative control. Indeed, this cell line is only transfected with the reporter gene encoding SEAP but is devoid of TLR4. Thus, the way by which compounds **9** to **16** induced the production of SEAP in HEK-TLR4 cells, but not in HEK-Null cells, is informative on their capacity to trigger signalization through TLR4.[xxvi]

First, we checked that compounds **9** to **16** did not induce any activation of HEK cells independently of TLR4 expression. To this end, HEK-Null cells were exposed to compounds **9** to **16** (each at 10, 30 and 100 μM) for 16 h, after which time the activity of SEAP was measured. As expected, we found that these concentrations did not interfere with the production of SEAP from HEK cells that are devoid of TLR4.

Then, we tested the ability of HEK-TLR4 cells to detect the compounds **9** to **16** at the same finale concentrations (Figure 1). In our hands, non-sulfated molecules **9**, **13** and **15** did not show any interaction with TLR4. Only the partially acetylated **10** showed a slight response at high concentration

(100 μM). Compound **11**, bearing both acetate and sulfate esters induced a TLR4 response at 30 μM. Unexpectedly, the trisulfated (and non-acetylated) disaccharide **12** (obtained from **11**) seemed inactive. Monosulfated Compound **14** showed a very low response, no significant difference was observed when compared with the non-substituted analog **15**. The highest response was shown by compound **16**, the 3,6,6'-trisulfated disaccharide.

Clearly, these findings indicate that sulfate groups on the primary positions favored the interaction with the TLR4, thus triggering an inflammatory signalization. It is difficult however to explain the absence of activity of the trisulfated molecule **12**, because the 6,6'-disulfation pattern of **16** is also present. Further investigations and other synthetic derivatives could allow to explain this result and the role of the specific position of sulfate groups for TLR4 binding. Anyway, our results on TLR4 binding make compound **16** a promising starting point for the regulation of the inflammatory response.

Conclusions

Altogether, we showed in this current work the potential of small oligosaccharides to modulate TLR4 activity, and we confirmed the need of sulfation and the key role of the 6-sulfate groups to trigger TLR4 signalization.

Author Contributions

S. T. and J. K. designed and supervised the synthetic work. R. N. performed the synthesis and the characterization of the compounds. F. A. designed the biological test performed by A. D. S. T., J. K. and F. A. wrote the paper and together with J. A. revised its final version.

Conflicts of interest

The authors declare no conflicts of interest.

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