Enzymatic synthesis of oligo-D-galactofuranosides and L-arabinofuranosides: from molecular dynamics to immunological assays†‡

Ilona Chlubnová,[a,b,c] Dominik Filipp,[d] Vojtech Spiwok,[c] Hana Dvořáková,[e] Richard Daniellou,*[a,b] Caroline Nugier-Chauvin,*[a,b] Blanka Králová[c] and Vincent Ferrières[a,b]

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D-Galactofuranosyl-containing conjugates are ubiquitous in many pathogenic microorganisms, but completely absent from mammals. As they may constitute interesting pharmacophores, recent works have been dedicated to their preparation. Besides well-reported chemical procedures, enzymatic approaches are still limited, mainly due to the lack of the corresponding biocatalysts. Based on the similarity between chemical structures, the arabinofuranosyl hydrolase Ara*f*51 from *Clostridium thermocellum* was expected to recognize both the L-Ara*f* motif and its D-Gal*f* analogue. Molecular dynamics and STD-NMR were firstly used to confirm this hypothesis and increase our knowledge of the active site. Interestingly, this arabinofuranosidase was not only able to hydrolyze galactosyl derivatives, but was also really efficient in catalyzing oligomerisations using *p*-nitrophenyl furanosides as donors. The structures of the products obtained were determined using mass spectrometry and NMR. Amongst them, all the possible regioisomers of di-arabino and -galactofuranosides were synthesized, and the ratio of each regioisomer was easily tuned with respect to the reaction time. Especially, the galactofuranobioside displaying the biologically relevant sequence β-D-Gal*f*-(1,6)-β-D-Gal*f* was enzymatically prepared for the first time. All fractions going from di- to penta-arabino- and galactofuranosides were tested for their ability in eliciting the production of TNF-α. Interesting immunological properties were observed with arabinofuranosides as short as three sugar residues.

Introduction

D-Galactose is by far the most widespread hexose found in the furanose form in naturally occurring glycoconjugates from many bacteria, protozoa, fungi, plants and archaebacteria.[1] One impressive example is that of *Mycobacterium tuberculosis*. Despite several decades of successful chemotherapeutic treatment, this microorganism has re-emerged through multidrug resistance to become a major cause of mortality, with an annual rate of approximately three million deaths over the world. The cell wall complex in this species is largely composed of two polysaccharides, a lipoarabinomannan (LAM) and an arabinogalactan (AG), in which all of the D-galactose and the D-arabinose residues are present as five-membered rings.[2] The AG portion of mycobacteria comprises a linear chain of approximately 30 alternating β-(1,5)- and β-(1,6)-linked D-Gal*f* residues. Moreover, the latter has been described to be essential for the growth and survival of mycobacteria.[3] Both AG and LAM have critical roles in the pathogenicity of mycobacterial diseases, including tuberculosis.[2,4] More precisely, inhibition of enzymes that assemble these polysaccharides prevents proliferation of mycobacteria.[5] Noteworthily, these hexofuranosides are not present in mammals. Therefore, structurally well-defined synthetic analogues are of great interest for the development of new pharmacophores and new therapies.[6–9] Some of them have been proven to present antigenic properties. For instance, β-D-Gal*f* *O*-linked oligosaccharides, found in *Penicillium* and *Aspergillus* species, appeared to be immunodominant.[10,11] Many efforts were then dedicated to the chemical synthesis of fragments of natural galactofuranose-containing glycosides.[1,12] Disaccharidic sequences such as β-D-Gal*f*-(1,5)-β-D-Gal*f* and β-D-Gal*f*-(1,6)-β-D-Gal*f*,[13–18] as well as trisaccharidic units of the galactan produced by *M. tuberculosis*[19] have been synthesized. However, the number of methods involving biocatalysts is still quite limited, probably due to the low availability of the required specific enzymes. Lowary and colleagues have recently studied a galactofuranosyl transferase and performed a small elongation of a galactofuranosyl chain.[20] Nevertheless, and to the best of our knowledge, no galactofuranosyl hydrolase has been involved in chemo-enzymatic approaches. In this context, and owing to the structural similarity between residues of D-Gal*f* and L-Ara*f* (Fig. 1), biocatalyzed furanosylation has been recently developed. The wild type arabinofuranosyl hydrolase Ab*f*D3 (E.C.3.2.1.55) from *Thermobacillus xylanolyticus* was obviously efficient enough to recognize and transfer 4-nitrophenyl

[a]*Ecole Nationale Supérieure de Chimie de Rennes, CNRS, UMR 6226, Avenue du Général Leclerc, CS 50837, 35708 Rennes Cedex 7, France. E-mail: richard.daniellou@ensc-rennes.fr, caroline.nugier@ensc-rennes.fr; Fax: (+) 33-2-23-23-80-46; Tel: (+) 33-2-23-23-80-96*
[b]*Université européenne de Bretagne, France*
[c]*Department of Biochemistry and Microbiology, Institute of Chemical Technology of Prague, Techniká 3, 166 28 Prague 6, Czech Republic*
[d]*Laboratory of Immunobiology, Institute of Molecular Genetics AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic*
[e]*Laboratory of NMR spectroscopy, Institute of Chemical Technology of Prague, Techniká 5, 166 28 Prague 6, Czech Republic*

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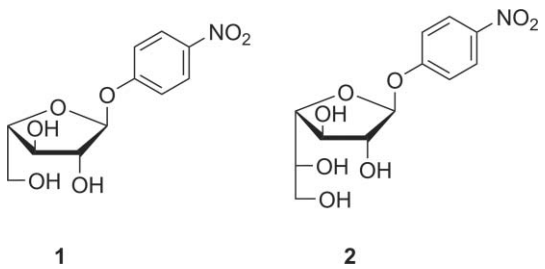


Fig. 1 Structural similarities between *p*NP-L-Ara*f* **1** and *p*NP-D-Gal*f* **2**.

L-arabinofuranoside **1** (*p*NP-Ara*f*), but also the corresponding D-galactofuranoside **2** (*p*NP-Gal*f*) to either pyranosyl acceptors in transglycosylation reactions or to *p*NP-Gal*f* itself in auto-condensation dimerizations.[21,22] The versatility of this enzyme was further extended to the preparation of non-natural disaccharides obtained from 5-deoxy-L-arabinosyl and D-fucofuranosyl, as well as 6-fluoro-D-galactofuranosyl donors.[23,24] Despite these interesting results towards the synthesis of (1,2)- and (1,3)-difuranosides, further biocatalysts with quite different reactivity (regioselectivity, capability of oligomerization and/or branching) deserve to be studied and developed.

In this paper, we present our recent exploration of another α-L-arabinofuranosyl hydrolase, Ara*f*51, from *Clostridium thermocellum*.[25] This particular enzyme, belonging to the GH51 family, is known to remove α-(1,2)-, α-(1,3)- and α-(1,5)-linked L-arabinofuranosyl moieties from arabinans and xylans. We were thus really inclined to evaluate the potential synthetic ability of this enzyme for the preparation of both L-arabinofuranosyl- and D-galactofuranosyl-containing oligosaccharides. Molecular modelling was first performed to anticipate how the target pentose and hexose moieties fit within the active site of the enzyme. Subsequently, the scope of interactions evaluated *in silico* was completed in aqueous solution by a Saturation Transfer Difference NMR (STD-NMR) study. The Ara*f*51-catalyzed oligomerizations using either **1** or **2** as favoured substrates were finally performed and the resulting oligofuranosides were examined as potent immunostimulating agents.

Results and discussion

Unlike Ab*f*D3, the arabinofuranosidase Ara*f*51 has never been used for synthetic purposes up to now, and even *p*NP-Gal*f* **2** has never been reported to act as a substrate for this enzyme. However, since the amino acid sequences of these two biocatalysts share 26% of their identity, and since a similar active site has been revealed through X-ray determination of both crystal structures,[25,26] we were likely to envisage Ara*f*51 as a strong candidate for the enzymatic synthesis of oligogalactofuranosides.

Molecular dynamics and STD-NMR

Firstly, we compared the *in silico* behaviour of the natural ligand α-L-arabinofuranosyl-(1,4)-α-D-xylopyranoside (Ara*f*-Xyl*p*) to that of its Gal*f* analogue (Gal*f*-Xyl*p*). Therefore, one subunit of the homohexameric enzyme containing the co-crystallized substrate was extracted from the 2C8N pdb file. The 6-C side chain of one of the furanosyl parts was modified manually to obtain the analogue. Both complexes of enzyme and ligand were surrounded with explicit water in a periodic box, several molecules of water being changed for Na^+ in order to keep the box neutral. Finally, after optimization of the geometry, the simulations of molecular dynamics (MD) were first performed with fixed chosen atoms (restrained) for 0.2 + 0.2 ns, in order to equilibrate each system, and then followed by a 5 ns unrestrained MD. Final pictures are depicted in Fig. 2. In both cases, the xylopyranoside derivative was rather flexible in the +1 subsite. Its major interaction with the protein seemed to occur through stacking with a tryptophan residue, thus confirming results previously obtained during the structural determination and explaining the substrate versatility naturally observed for such enzymes. Consequently, we inferred that the +1 subsite was likely to interact with a panel of aglycons, such as the furanosyl entity or aromatic rings. On the contrary, the Ara*f* part of the natural ligand was quite stable during the simulation. This behaviour was mainly ascribed to a strong anchorage of the arabinosyl unit in the −1 subsite, due to the participation of multiple hydrogen bonds with at least six different amino acid residues. Interestingly, the Gal*f* residue also stabilized in a similar position, thanks to an equivalent network of direct polar interactions, despite increased steric hindrance. Still, it is noteworthy that the hydroxymethyl group finally settled in a non-polar cavity. Moreover, there was no indication that water binds to this cavity when an arabinoside substrate is in the active site of Ara*f*51. However, such computer modelling clearly demonstrated that the enzyme could be able to recognize the Gal*f* motif, potentially even better than the Ara*f* disaccharide. Therefore, this hypothesis encouraged us to pursue our study.

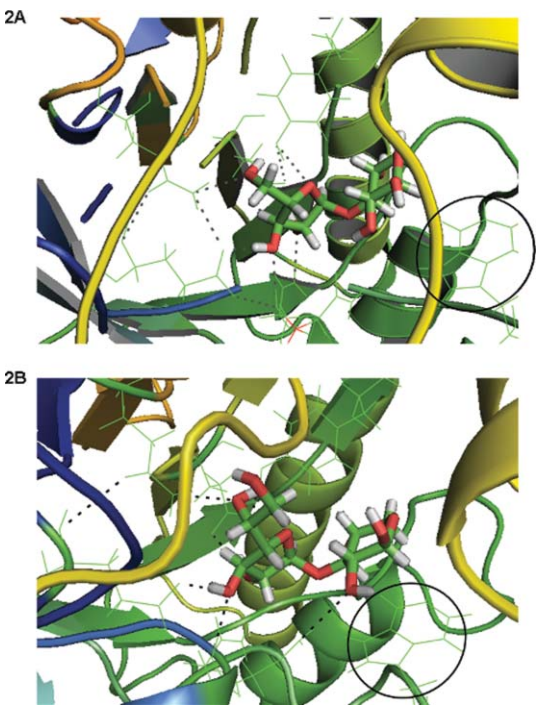


Fig. 2 Molecular modelling of arabinofuranoside (2A) and galactofuranoside (2B) in the active site or Ara*f*51 (the circle highlights the interesting tryptophan residue).

In order to complement our *in silico* findings, we also considered a physical method. Saturation Transfer Difference NMR (STD-NMR) nowadays constitutes a reliable and cheap tool for

observing the interactions between ligand and protein.[27] Particularly, this method allows the quantification of the relative importance of the interaction between each proton of a substrate and the active site. Because the previously used disaccharides were not commercially available, experiments were performed using both furanosides **1** and **2**. To ensure observation of the STD effect, the E173A mutant[25] was preferred to the wild type Ara*f*51, in order to prevent hydrolysis of the substrates and, therefore, for simplifying NMR analysis. The resultant effects were expressed as percentages, giving 100% to be the highest signal, *i.e.* the proton in the *meta* position of the aromatic aglycon (Fig. 3). The *p*NP rings in both **1** and **2** showed the strongest signals and, therefore, were in close contact with the active site of Ara*f*51, surely through a strong π–stacking interaction involving the tryptophan residue previously shown as responsible for the recognition of the xylopyranoside ring. Probably despite this particular effect caused by the artificial aglycon, the sugar ring displayed moderate signals. Nevertheless, all protons of the furanosyl moiety show STD effects. Particularly, 1-H with its 12 or 11% effect for **1** and **2**, respectively, appeared to have good interaction with residue(s) in the active site. Even more interestingly, the hindered 6-C side chain of **2** exhibited the same value as the hydroxymethyl group of **1** and did not disturb at all the way the sugar fit into the protein. Consequently, STD data complemented well *in silico* modelling and strengthened the thought that both molecules **1** and **2** exhibited a similar pattern of interactions with the arabinofuranosidase Ara*f*51, and could be engaged in biocatalytic reactions. However, these two studies needed to be complemented by the measurement of the specificity of the enzymes towards both substrates.

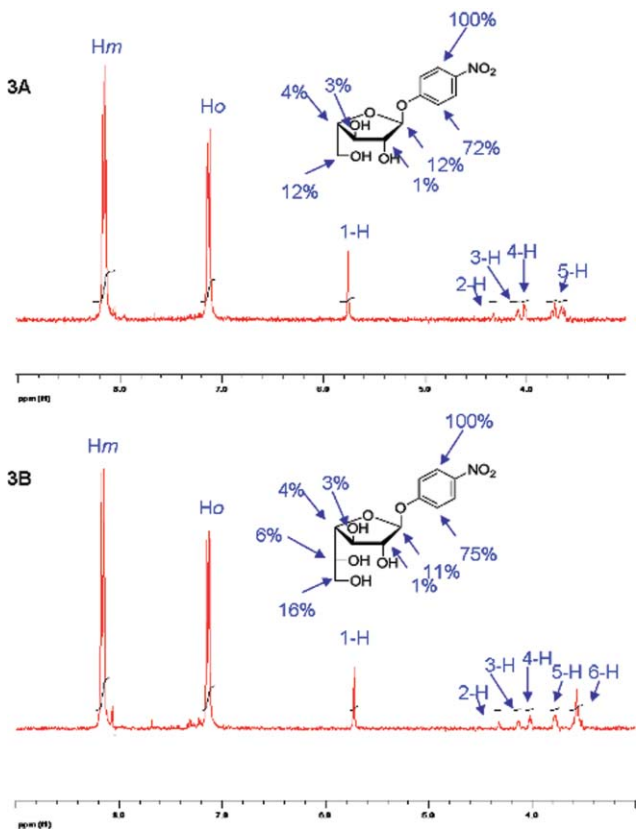


Fig. 3 STD-NMR spectra of **1** (3A) and **2** (3B).

Kinetic parameters of Ara*f*51

The kinetic parameters of Ara*f*51 were evaluated by looking at the hydrolytic activity of the enzyme towards the Gal*f* derivative **2**. Whereas Ara*f*51 is a thermophilic enzyme with $T_{opt} \sim 82$ °C, using *p*NP-Ara*f* **1** as a substrate,[25] we determined its activity at 25 °C, for practical reasons. The value obtained for K_m for the hydrolysis of **1** under standard conditions (100 mM PBS pH 7, 1 mg mL^{-1} BSA) was 0.15 mM (0.25 mM at 37 °C). Optimum pH was determined for this reaction using both 0.65 and 1 mM as the substrate concentrations, and thus, appeared to be in the range of 7 to 8. Evaluation of K_m for the hydrolysis of **2** under the same conditions showed a significantly greater value of 53 mM, similar to the one previously observed during Ab*f*D3-catalyzed hydrolysis.[23] Moreover, Ara*f*51-mediated hydrolysis of **2** was characterized by a significantly lower activity ($V_m = 0.32$ μmol min^{-1}) compared with that of **1** ($V_m = 2.7$ μmol min^{-1}). Despite a 6000-fold lower specificity (V_m/K_m), the D-galactofuranoside **2** is still recognized as a substrate by the enzyme, as previously shown through the molecular modelling and the STD-NMR study. Nevertheless, measurement of the kinetic parameters clearly showed that Gal*f* derivative **2** can be hydrolyzed, demonstrating that it is in a favourable position in the active site of Ara*f*51, and might act as an efficient donor in auto-condensation reactions.

Analytical study of the auto-condensation reactions

Furanosides **1** and **2** (5 mM) were incubated with Ara*f*51 (2376 UI) at 60 °C and pH 7.4. Reactions were quenched by enzyme denaturation at 100 °C for three minutes before HPLC analysis. All products were separated thanks to size exclusion chromatography, and also detected and quantified by UV absorbance at 280 nm. Newly observed peaks at shorter retention times than those corresponding to the substrates **1** (77.7 min) and **2** (78.7 min) were isolated. Mass spectrometric analyses allowed simple identification of four fractions, respectively containing penta- (10 min), tetra- (13–15 min), tri- (18–20 min) and difuranosides (30–55 min). In a second experiment, aliquots were withdrawn at increasing reaction times (2, 5, 10, 15 and 20 min) in order to evaluate the impact of time on the ratio of all synthesized oligofuranosides. Starting from arabinoside **1**, the HPLC profile revealed that the substrate was completely consumed after less than 10 min (Fig. 4). Moreover, the overall conversion yield

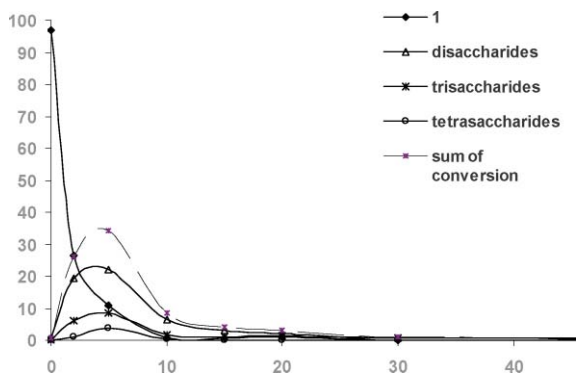


Fig. 4 Kinetics of the *p*NP-Ara*f* **1** auto-condensation products catalyzed by Ara*f*51. The conversions were calculated as a percentage of the initial quantity of **1** from 0 to 45 min.

observed for the desired auto-condensation reaction reached its maximum at 35% after approximately 5 min. Interestingly, the di-, tri- and tetrasaccharides were produced right from the beginning of the Ara*f*51-catalyzed incubation with maximum conversions of 22.1, 8.4 and 3.6%, respectively, within less than 5 min of reaction. Thereafter, decrease of the auto-condensation products were observed, suggesting the straightforward partial hydrolysis of these oligofuranosides.

As far as the *p*NP-Gal*f* **2** auto-condensation reaction was concerned, consumption of the substrate was nearly complete after 10 min (Fig. 5). Maximum conversion was indeed observed from 5 min and, thanks to mass spectrometric analysis, three fractions corresponding to di-, tri- and tetrasaccharides were identified, representing 34.6, 8.6 and 1.1% yield, respectively. Thereafter, the amount of disaccharide significantly decreased, while those of the upper tri- and tetrasaccharides still increased to reach a plateau at nearly 10 and 4%, respectively. These analytical results clearly showed different behaviour between the arabinose and the galactose series towards Ara*f*51. They could be ascribed (i) to a very interesting ability of the Ara*f*51 to recognize the galactofuranoside **2** and to use it as a donor in the glycosylation reaction, and (ii) to a lower capability of the biocatalyst for hydrolyzing the synthesized galactofuranosides, thus resulting in higher conversion yields starting from **2**.

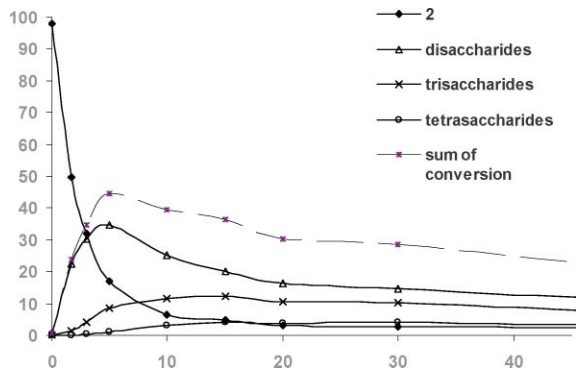


Fig. 5 Kinetics of the *p*NP-Gal*f* **2** auto-condensation products catalyzed by Ara*f*51. The conversion ratios were calculated as a percentage of the initial quantity of **2** from 0 to 45 min.

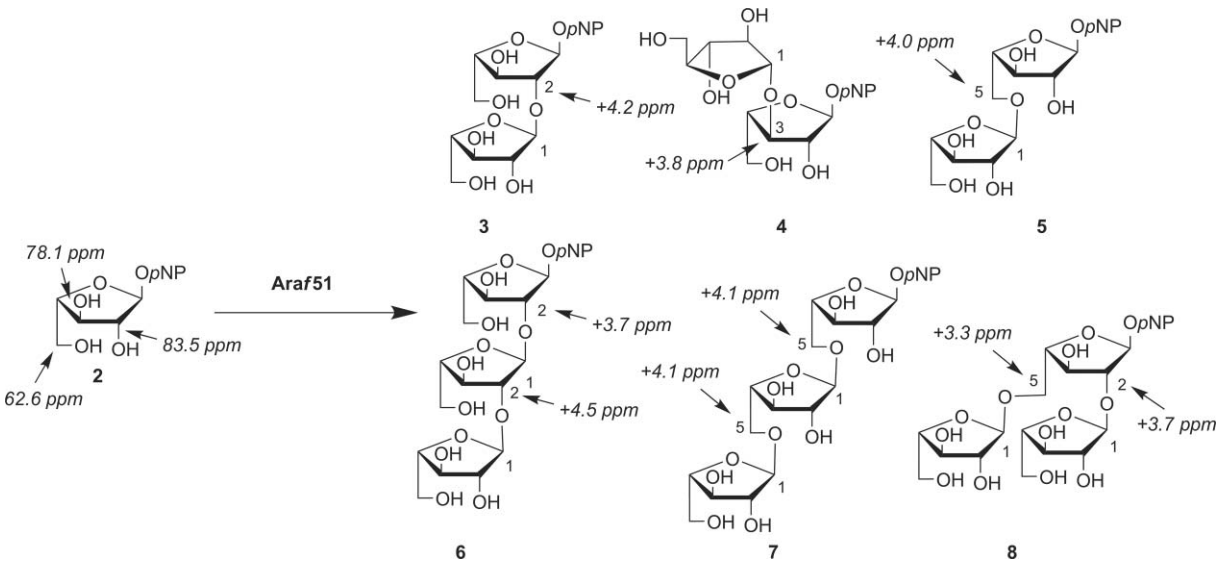
Preparative scale of enzymatic reaction

Analytical results further needed to be sharpened in order to precisely elucidate the number of regioisomers formed, as well as the nature of the glycosidic linkages. When auto-condensation reactions were performed on larger scale (100 mM of substrate, *i.e.* 30 mg of **1** or 100 mg of **2**) but with lower quantity of the biocatalyst, times had to be adjusted to 25 and 90 min, respectively, in order to reach the maximum conversion. After denaturation of the enzyme at 100 °C for 10 min, products were analyzed by usual TLC methods (see the ESI‡) and carefully separated thanks to the use of P2 biogel. Such size-exclusion chromatography allowed us to rapidly quantify the amounts of the different oligosaccharides, once again going from di- to pentafuranosides. Sugars with similar retention properties were then pooled together and finally lyophilized. Purified compounds were then identified using mass spectrometry and their structures were finally assessed through 1D and 2D NMR analysis.

Scale-up was first performed from the arabinoside **1**. In accordance with the analytical studies, arabinofuranobiosides constituted the major fraction (22.6%), followed by the trisaccharides (7.9%) and the tetrasaccharides (3.1%) (Table 1, entries 1–3). Moreover, even in low yield, we were also able to isolate pentasaccharides (entry 4), and prove their presence thanks to mass spectrometry. A more detailed analysis of the NMR data of the fraction containing the disaccharides permitted us to characterize the three different and possible regioisomers **3**, **4** and **5**. Because the spectra were similar to those previously published,[21] disaccharide **3**, which was obtained with the best isolated yield of 12.6%, was unambiguously identified as the α-(1,2)-linked furanobioside. This result already constitutes a significant improvement on the enzymatic synthesis of this compound, as it was only isolated in 5.8% when using Ab*f*D3. Subsequently, the small coupling constants observed between 1b-H and 2b-H ($J_{1b,2b}$ = 1.8 and 1.2 Hz for **4** and **5**, respectively) clearly indicated that the newly formed glycosidic bonds exhibited α-L-stereochemistry. As **4** is concerned, its ^{13}C NMR spectra showed a downfield shift of the 3a-C ($\Delta\delta$ = +3.8 ppm, compared to 3a-C of **1**) of the reducing arabinofuranosyl moiety, as well as intense three-bond coupling in the ^{1}H–^{13}C (HMBC) spectra between 3a-C (δ = 81.9 ppm) and 1b-H (δ = 5.06 ppm). This indicated that the linkage is (1,3). For disaccharide **5**, similar results were obtained, but involving 5a-C ($\Delta\delta$ = +4.0 ppm compared to **1**, δ = 66.6 ppm) and 1b-H (δ = 4.92 ppm). These data demonstrated the presence of an α-L-(1,5) bond between both furanosyl residues. Consequently, the three possible isomers could be synthesized thanks to the use of the Ara*f*51 biocatalyst. With structural data in hand, we also established the evolution profile according to reaction time (Fig. 6). Thus, between 2 and 10 min, the ratio of **3**, **4** and **5** was significantly modified. Indeed, the (1,2)-linked disaccharide **3** was kinetically produced after 2 min, but disappeared after 10 min. On the other side, its (1,5)-linked counterpart was the major one after this time. As a consequence, and with regard to the observed regioselectivity of the reaction, as well as the amount obtained for each disaccharide, we can easily deduce that the α-L-(1,2) linkage is kinetically formed (but rapidly hydrolyzed thereafter), and that the α-L-(1,5), and the α-L-(1,3) but to a lesser extent, are thermodynamically favored. Such kinetic observations are in complete accordance with the hydrolytic preferences of Ara*f*51 towards arabinoxylane in nature, and emphasize the fact that frequently, information on the hydrolytic substrate specificity of glycosidases can be very valuable in order to predict the regioselectivity of transglycosylation reactions.[28]

Having elucidated the structures of disaccharides and their behaviour under the described conditions, we expected Ara*f*51 to be able to produce all of the trisaccharidic regioisomers, but in various amounts. In this context, three different trifuranosides **6–8** were chromatographically separated and isolated in 2.7, 0.6 and 2.2% yield, respectively. On the assumption of small coupling constants between 1b-H and 2b-H ($J_{1b,2b}$ = 1.2–1.8 Hz), and also between 1c-H and 2c-H ($J_{1c,2c}$ = 1.2 Hz), 1,2-*trans* configurations between arabinosyl entities were easily established. The nature of the linkages was subsequently deduced from ^{13}C and ^{1}H–^{13}C NMR spectra. The arabinotrioside **6** was demonstrated to have two (1,2) linkages, since the two carbon atoms exhibited signals

Table 1 Auto-condensation of **1**



Entry	Products	Yield (%)	Elucidated structures (yield, %)		
1	Disaccharides	22.6	**3** (12.6)	**4** (2.2)	**5** (5.1)
2	Trisaccharides	7.96	**6** (2.7)	**7** (0.6)	**8** (2.2)
3	Tetrasaccharides	3.1			
4	Pentasaccharides	0.1			

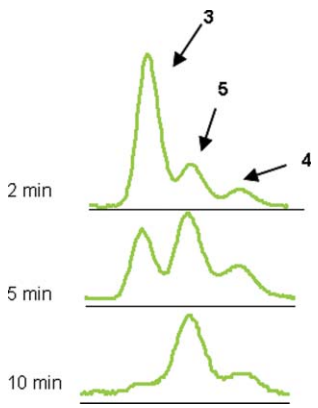
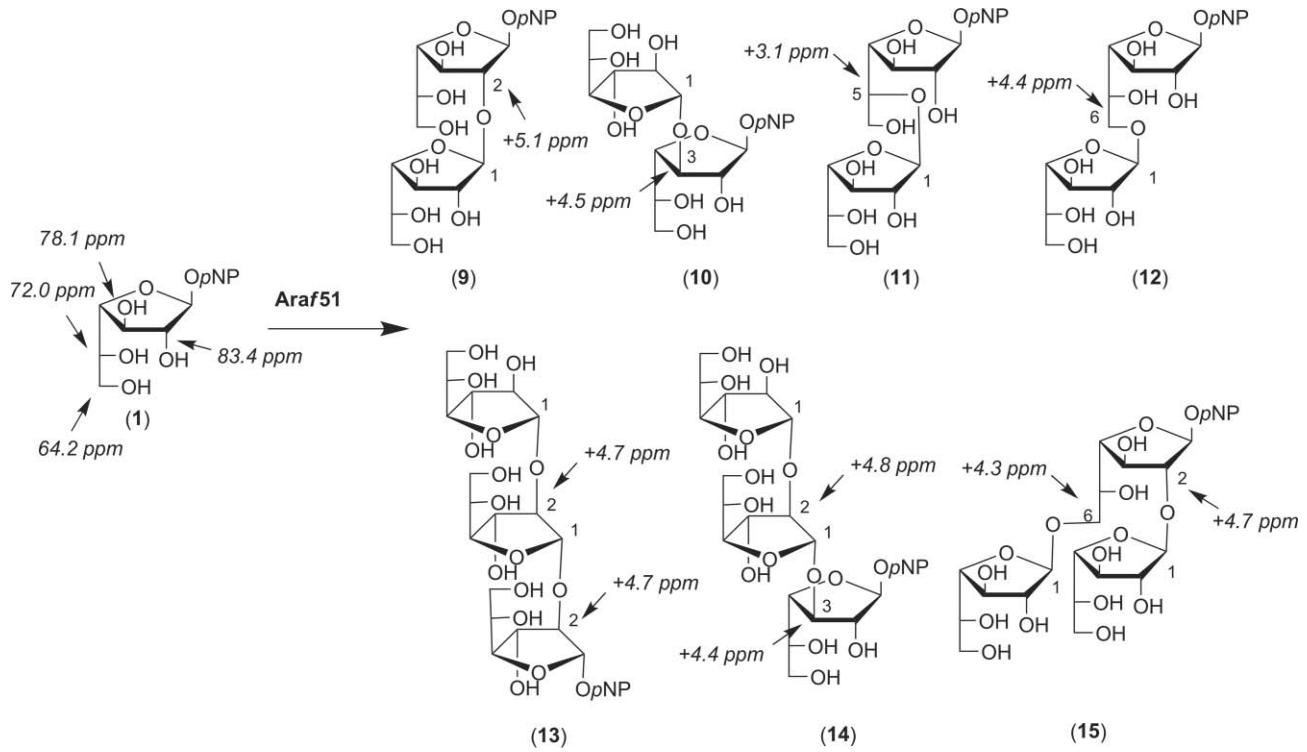


Fig. 6 HPLC profiles showing the modulation of the ratio of disaccharides **3**, **4** and **5** in the presence of 2376 units of Araf51.

with a downfield shift compared to **1** (for 2a-C, $\Delta\delta = +3.7$ ppm, and for 2b-C, $\Delta\delta = +4.5$ ppm, Table 1). They also showed an intense correlation in the HMBC experiment with 1b-H ($\delta = 5.19$ ppm) and 1c-H ($\delta = 5.02$ ppm), respectively. The structure of **7** was identified as the (1,5) linear analogue as its 5a-C and 5b-C showed upshifted signals ($\Delta\delta = +4.1$ ppm), and correlations with 1b-H ($\delta = 4.93$ ppm) and 1c-H ($\delta = 4.92$ ppm). Finally, **8** was deduced to be the branched (1,2)- and (1,5)-triarabinofuranoside using a similar reasoning, and the signals corresponding to 2a-C ($\Delta\delta = +3.7$ ppm) and 5a-C ($\Delta\delta = +3.3$ ppm). Therefore, Araf51 showed very interesting versatility, even for the production of both linear and branched oligoarabinofuranosides.

Looking at the selectivity of Araf51 during reactions involving *p*NP-Araf **1**, we were really confident in isolating a great variety of oligogalactofuranosides starting from donor **2** (Table 2). As expected, disaccharides represented the major part of the isolated compounds, with a yield of 34.5%, even higher than the one obtained in the arabinose series (entry 1). Tri- and tetrasaccharides were also obtained but in somewhat lower yields (entries 2 and 3). Only small amounts of derivatives with five sugar units were detected by mass spectrometry (entry 4). Unsurprisingly with regards to the structural similarity between L-Araf and D-Galf, ^{1}H NMR spectra of all of the compounds showed signals for 1-H with small $J_{1,2}$ coupling constants close to 1.2 Hz, representative of a 1,2-*trans* β-D-glycodic linkages. Moreover, all of the possible regioisomers, *i.e.* (1,2), (1,3), (1,5) and (1,6), could be observed in the galactofuranobiosides fraction (entry 1). Notably, **9**, which was previously enzymatically synthesized in an isolated yield of 6.0% using Abf D3, was obtained, herein, in 23.9% yield.[22] All of the other structures were determined through careful analysis of the ^{13}C and the HMBC NMR spectra. Although the majority of these compounds were isolated in moderate quantities, the Araf51-biocatalyzed reaction gave **12** in 7.8% yield. Moreover, it is important to note the influence of reaction time on the ratios of **9–12** (Fig. 7). Indeed, the (1,2)-linked digalactoside **9** was rapidly formed but also quickly disappeared from the reaction mixture. On the contrary, (1,3), (1,5) and (1,6) disaccharides were concomitantly obtained and their amounts remained constant under a large range of time. It is noteworthy that **12**, the major thermodynamic disaccharide, displays the important sequence β-D-Galf-(1,6)-β-D-Galf. This is found in many pathogenic microorganisms, and was here enzymatically prepared for the first time. Finally, amongst the variety of possible oligogalactofuranosides, three trisaccharides were clearly characterized: (i) **13** presented a linear chain built up with (1,2) glycosidic bonds, (ii) **14** exhibited

Table 2 Auto-condensation of **2**



Entry	Products	Yield (%)	Elucidated structures (yield, %)			
1	Disaccharides	34.5	**9** (23.9)	**10** (0.6)	**11** (1.5)	**12** (7.8)
2	Trisaccharides	4.6	**13** (1.2)	**14** (0.8)	**15** (0.3)	
3	Tetrasaccharides	2.5				
4	Pentasaccharides	0.1				

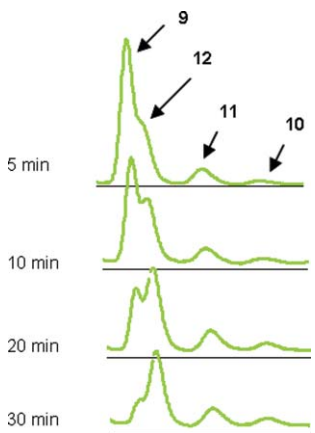


Fig. 7 HPLC profiles showing the modulation of ratio of disaccharides **9–12** in the presence of 2376 units of Ara*f*51.

(1,2) and (1,3) glycosidic linkages, and (iii) **15**, a branched (1,2) and (1,6) galactotrioside, resulted from the two favoured regiochemical preferences of Ara*f*51.

Biological evaluation

The presence of galactofuranosyl residues in *M. tuberculosis* constitutes an interesting and original target in the fight against mycobacteria species.[12] Therefore, the synthesis of small and well-defined oligofuranosides with immuno-modulatory properties represents a promising challenge.[29,30] To assess the biological properties of the enzymatically synthesized neofuranosides described in this study, we incubated a murine macrophage cell line Raw 264 with either oligo-arabino or galactofuranosides. Their stimulatory effects measured by the TNF-α production were compared to those of a lipopolysaccharide (LPS) used as a positive control (Fig. 8). It is of note that trace levels of contaminating LPS in the samples used, measured by limulus assay, had no significant effect on the stimulatory activity of neofuranosides, as the addition of LPS inhibitor polymyxin B to cell cultures resulted in production of comparable levels of TNF-α (data not shown).

As depicted in Fig. 8 and in sharp contrast to LPS, none of the galactofuranosides were able to produce any significant response, even when used at concentrations up to 1 μg mL^{-1}. This observation may be assigned to both the short length of the oligosaccharide chains and to their high structural heterogeneity in the mixtures used. Importantly, significant TNF-α responses were elicited upon stimulation with oligoarabinofuranosides when used at concentrations of 100 ng mL^{-1} and higher. Cytokine responses were concentration- and length of the oligosaccharide-dependent (see columns Tri-, Tetra- and Penta-Ara). Stimulatory effects comparable to the Tri-Ara mixture were observed using isolated **6**, **7** and **8**. These data strongly suggest that the nature

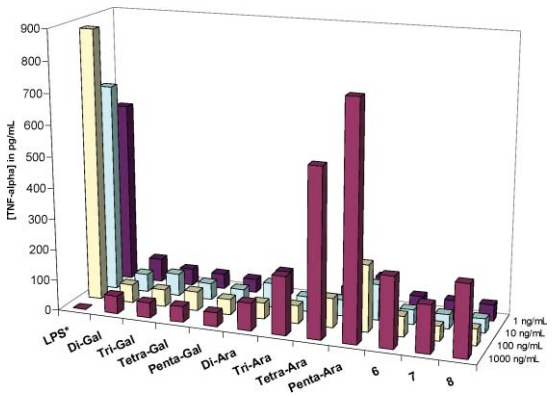


Fig. 8 Production of TNF-α cytokine upon macrophage stimulation with enzymatically synthesized oligoarabinofuranosides (Ara) and oligogalactofuranosides (Gal). (* Effect of LPS at 1000 ng mL^{-1} was not assessed.)

of the glycosidic linkages between furanosyl units, *i.e.* (1,2), (1,3) and/or (1,5), had no significant impact on the production of TNF-α. Thus, even if immune activities of polysaccharides containing furanosyl entities were already reported,[31,32] this is the first time to the best of our knowledge that such short oligofuranosides, mimics of natural oligosaccharides derived from pathogens, are able to induce a cytokine response in immunocompetent cells.

Conclusions

Besides the (1,2) and (1,3) linkages already yielded by the arabinofuranosyl hydrolase Ab*f*D3, the linkages (1,5) and (1,6), reported to be present in some clinically important pathogens, were obtained thanks to the assistance of Ara*f*51. These results also confirmed preferences of this easily available enzyme to transfer the furanosyl residue kinetically onto the 2-OH and thermodynamically to the primary hydroxyl function of the furanosyl acceptor, whatever the Ara*f* or the Gal*f* series. Moreover, the regioselectivity of the reaction was modulated with respect to reaction time. It also has to be pointed out that the isolated yields were significantly improved in all cases and thus, this enzymatic procedure is now at least as competitive as the corresponding multistep chemical reactions. Interestingly, Ara*f*51 appeared to be much more efficient as a synthetic tool than its counterpart Ab*f*D3: (i) the ratio of hydrolysis over auto-condensation of substrates as well as reaction times being reduced, (ii) auto-condensation reactions being not limited to the preparation of disaccharides. These results encourage us to pursue the study of the synthetic potency of Ara*f*51 and will lead us to the evaluation of its versatility towards original furanosyl-based substrates. Finally, immunostimulating responses obtained with short oligoarabinofuranosides are very promising, and this opens very interesting opportunities in this field,[33] especially for further development on poly-L-arabinan directly extracted from biomass.

Experimental

General procedures

^{1}H, ^{13}C, HSQC, HMBC and COSY NMR spectra were recorded on a Bruker 600 spectrometer equipped with a cryoprobe at 600 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are given in δ-units (ppm) measured downfield from Me_4Si. Coupling constants J are given in Hz. Electrospray-ionisation mass spectra (ESI-MS) were recorded on samples dissolved in MeOH injected in a volume of 2–5 μL into a flow (100 μL min^{-1}) of MeOH using a Q-TOF Micro (Waters, USA). Sample cone voltage was 42 V and the source temperature was 150 °C. Measurements were performed in positive ($[M + Na]^+$ ion detection) mode in the range of 100–1000 Da.

Molecular dynamics

All simulations were performed in GROMACS 3.3.2 package.[34] A single subunit of arabinofuranosidase complexed with β-L-Ara*f*-(1,3)-β-D-Xyl*p* (PDB ID: 2C8N) was taken as the starting structure.[25] The protein was modelled using the AMBER99SB force field.[35] Protonation states of dissociable residues were assigned using the output from the H++ tool.[36] Force field parameters of β-L-Ara*f*-(1,3)-β-D-Xyl*p* were generated using Glycam server.[37] Force field parameters of β-D-Gal*f*-(1,3)-β-D-Xyl*p* were generated from those of β-L-Ara*f*-(1,3)-β-D-Xyl*p* by prolonging the hydroxymethyl moiety (missing parameters were taken from *N*-acetylneuraminic acid). Partial charges were taken from β-D-Gal*f*-OMe calculated using the RESP[38] method at the HF/6-31G* level of theory using Gaussian03 package.[39] Glycam topologies were then converted to GROMACS format using a modified *amb2gmx.pl* script (it was necessary to modify the script because in the original it ignores periodic functions in dihedral terms if they are multiplied by negative coefficients).[40] The starting structure of the complex with β-L-Ara*f*-(1,3)-β-D-Xyl*p* was taken from the crystal structure. An additional moiety of the complex with β-D-Gal*f*-(1,3)-β-D-Xyl*p* was fitted manually. Structures were solvated by 13069 TIP3P[41] water molecules and seven sodium ions to neutralize its net charge. The system was energy minimized and then equilibrated by 400 ps molecular dynamics simulation with harmonic restraints applied to non-hydrogen atoms of the protein (200 ps strong and 200 ps weak restraints). Finally, a 5 ns unrestrained molecular dynamics simulation was performed.

STD-NMR experiments

Samples were prepared in 0.5 mL of D_2O and contained ~30 μmol of *p*NP-sugars. STD-NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Processing of all data was performed on a PC with Bruker Topspin v2.0 software. After the determination of the optimal conditions, *i.e.* temperature, delay between pulse (d_{20}) and molecular ratio (protein : ligand), STD-NMR experiments were performed at 283 K as followed. The protein (1 : 100 ratio) was saturated on resonance at 0.7 ppm and off resonance at 40 ppm with a cascade of 40 selective gaussian-shaped pulses of 50 ms duration with a 100 μs delay between each pulse. The total duration of the saturation time was set to 2 s. A total of 256 scans/STD-NMR experiment was acquired. A WATERGATE sequence was used to suppress residual HOD signal. A spin lock filter with strength of 5 kHz and duration of 10 ms was also applied to suppress the protein background. A similar experiment with no enzyme was used as a reference in order to verify the absence of STD effects in these experimental conditions. Intensities of all STD effects were calculated though

integrals over the respective signals in ^{1}H NMR reference spectra. The largest STD effect in each spectrum was set to 100% and relative intensities were determined, as common for non-refined STD effects. Hence, sufficient comparisons of relative STD effects between sugars were possible, but absolute binding intensities could not be determined.

Production of the enzyme and determination of its kinetic parameters

Recombinant arabinofuranosidase Ara*f*51 expressed from the plasmid-borne Ara*f*51 gene was produced and purified from *Escherichia coli* cells as described previously.[25] The hydrolytic activity of Ara*f*51 was quantified by incubation of the enzyme with **1** or **2** (5 mM) in 100 mM potassium phosphate buffer pH 7.4 at 60 °C. Continuous release of *p*-nitrophenol was measured at 405 nm (Microplate Spectrophotometer PowerWave XS/XS2, BioTek) and data evaluated with Gen5 Data Analysis Software (BioTek). One unit of activity corresponds to the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. Initial rate conditions and suitable substrate concentrations (0.03–3 mM and 0.625–40 mM for **1** and **2**, respectively) were used in order to determine the kinetic parameters K_m and k_{cat}.

Analytical scale of auto-condensation reactions

Reactions were performed in buffered conditions (100 mM potassium phosphate buffer, pH 7.4) at 60 °C with shaking (INC Orbital Mixing Chilling/Heating Plate, Torrey Pines Scientific) in the presence of *p*NP-glycosides (50 mM) and 2376 U of enzyme. The total reaction volume was 360 μL. Aliquots (20 μL) of the reaction mixture were withdrawn at different times and the reaction was quenched by enzyme denaturation at 100 °C for 3 min. Samples were mixed with 50 μL of deionized water, passed through an ultrafiltration membrane (VectaSpin Microtubes, MWCO 12 kDa, Whatman) at 20000 × g for 30 min (Mikro 22R, Hettich) to remove protein, and applied in a volume of 25 μL to a calcium carbohydrate HPLC column. The HPLC system was consisting of a solvent delivery system 600 LCD HPLC Pump (Waters), an UV/VIS 486 Tunable Absorbance Detector (Waters) and Differential Refractometer RIDK 101 (Laboratorní Přístroje Praha), equipped with a column (Supelcogel Ca, 30 cm × 7.8 mm, Supelco) and an appropriate guard column (Supelcogel Ca and C611, 5 cm × 4.6 mm, Supelco) maintained at 80 °C during the analysis. Deionized and filtrated (0.22 μm PVDF membrane, Millipore) water was used as a mobile phase in isocratic mode with a constant flow rate of 0.5 mL min^{-1}. The substrate, products of auto-condensation and *p*-nitrophenol were detected and quantified by UV absorbance at 280 nm.

Preparative scale reactions

Experiments were performed in 100 mM phosphate buffer, pH 7.4 at 60 °C in the presence of *p*NP-glycosides (100 mM) with 1440 U of Ara*f*51 in a total reaction volume of 1.05 mL for reaction with **1**, and with 4800 U of enzyme in a total reaction volume of 3.5 mL in the case of **2**. Reaction mixtures were monitored by TLC (Kieselgel 60 F_{254} (Merck), ethyl acetate–acetic acid–water, 7 : 2 : 2) and visualized under UV light and by exposure to 2% orcinol in 20% H_2SO_4–ethanol. Auto-condensation reactions were stopped after 25 min or 90 min in the case of **1** and **2**, respectively, by enzyme denaturation at 100 °C for 10 min. The reaction products were separated by gel permeation chromatography on P-2 Bio-Gel (Bio-Rad) using an FPLC system consisting of a solvent delivery system Biologic F40 DuoFlow, Biologic QuadTec UV-Vis Detector and Biologic BioFrac Fraction Collector (all Bio-Rad). Deionized filtrated (0.22 μm PVDF membrane, Millipore) water was used as a mobile phase with a flow rate of 0.15 mL min^{-1}. Separation was monitored by UV absorbance at 280 and 405 nm by operating software Biologic DuoFlow. Collected fractions were lyophilized (FreeZone Freeze Dry System, Labconco) and submitted to structural analyses.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,2)-α-L-arabinofuranoside (3).** ^{1}H NMR (600 MHz, D_2O): δ = 8.11 (2H, d, J = 9.6, H_m C_6H_4), 7.08 (2H, d, J = 9.6, H_o C_6H_4), 5.81 (1H, d, $J_{1a,2a}$ = 1.5, 1a-H), 5.08 (1H, d, $J_{1b,2b}$ = 1.5, 1b-H), 4.30 (1H, dd, $J_{2a,3a}$ = 4.0, 2a-H), 4.09 (1H, dd, $J_{3a,4a}$ = 6.2, 3a-H), 4.02 (1H, ddd, $J_{4a,5a}$ = 3.3, $J_{4a,5'a}$ = 5.1, 4a-H), 4.00 (1H, dd, $J_{2b,3b}$ = 3.3, 2b-H), 3.87 (1H, ddd, $J_{4b,5b}$ = 3.3, $J_{3b,4b}$ = $J_{4b,5'b}$ = 6.2, 4b-H), 3.79 (1H, dd, 3b-H), 3.68 (1H, dd, $J_{5a,5'a}$ = 12.8, 5a-H), 3.59 (1H, dd, 5′a-H), 3.55 (1H, dd, $J_{5b,5'b}$ = 12.8, 5b-H), 3.30 (1H, dd, 5′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): δ = 161.4 (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.7 (C_o C_6H_4), 107.6 (1b-C), 104.5 (1a-C), 87.3 (2a-C), 84.2 (4b-C, 4a-C), 81.3 (2b-C), 76.6 (3b-C), 74.5 (3a-C), 61.0 (5b-C), 60.4 (5a-C) ppm. MS (ESI): m/z calcd for $C_{16}H_{21}NNaO_{11}$ $[M + Na]^+$ 426.1012; found 426.0892.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,3)-α-L-arabinofuranoside (4).** ^{1}H NMR (600 MHz, D_2O): δ = 8.11 (2H, d, J = 9.6, H_m C_6H_4), 7.08 (2H, d, J = 9.6, H_o C_6H_4), 5.73 (1H, d, $J_{1a,2a}$ = 1.2, 1a-H), 5.06 (1H, d, $J_{1b,2b}$ = 1.8, 1b-H), 4.43 (1H, dd, $J_{2a,3a}$ = 2.4, 2a-H), 4.13 (1H, ddd, $J_{4a,5a}$ = 3.0, $J_{4a,5'a}$ = 5.4, 4a-H), 4.03 (1H, dd, $J_{3a,4a}$ = 5.4, 3a-H), 4.00 (1H, dd, $J_{2b,3b}$ = 3.0, 2b-H), 3.88 (1H, ddd, $J_{4b,5b}$ = 3.0, $J_{3b,4b}$ = $J_{4b,5'b}$ = 6.0, 4b-H), 3.80 (1H, dd, 3b-H), 3.71 (1H, dd, $J_{5a,5'a}$ = 12.6, 5a-H), 3.67 (1H, dd, $J_{5b,5'b}$ = 12.0, 5b-H), 3.63 (1H, dd, 5′a-H), 3.54 (1H, dd, 5′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): δ = 161.1 (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.8 (C_o C_6H_4), 107.2 (1b-C), 105.6 (1a-C), 84.4 (4a-C), 84.0 (4b-C), 81.9 (3a-C), 81.2 (2b-C), 79.5 (2a-C), 76.6 (3b-C), 61.1 (5b-C), 60.8 (5a-C) ppm. MS (ESI): m/z calcd for $C_{16}H_{21}NNaO_{11}$ $[M + Na]^+$ 426.1012; found 426.1002.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,5)-α-L-arabinofuranoside (5).** ^{1}H NMR (600 MHz, D_2O): δ = 8.11 (2H, d, J = 9.6, H_m C_6H_4), 7.07 (2H, d, J = 9.6, H_o C_6H_4), 5.71 (1H, d, $J_{1a,2a}$ = 1.8, 1a-H), 4.92 (1H, d, $J_{1b,2b}$ = 1.2, 1b-H), 4.27 (1H, dd, $J_{2a,3a}$ = 3.0, 2a-H), 4.13 (1H, ddd, $J_{4a,5a}$ = 3.0, $J_{4,5'a}$ = 5.4, 4a-H), 4.00 (1H, dd, $J_{3a,4a}$ = 5.4, 3a-H), 3.95 (1H, dd, $J_{2b,3b}$ = 3.0, 2b-H), 3.92 (1H, ddd, $J_{4b,5b}$ = 3.0, $J_{3b,4b}$ = $J_{4b,5'b}$ = 6.0, 4b-H), 3.78 (1H, dd, 3b-H), 3.75 (1H, dd, $J_{5a,5'a}$ = 12.0, 5a-H), 3.65 (2H, dd, $J_{5b,5'b}$ = 12.0, 5b-H, 5′a-H), 3.54 (1H, dd, 5′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): δ = 161.2 (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.7 (C_o C_6H_4), 107.4 (1b-C), 105.4 (1a-C), 84.0 (4b-C), 83.7 (4a-C), 81.0 (2a-C), 80.9 (2b-C), 76.5 (3b-C, 3a-C), 66.6 (5a-C), 61.2 (5b-C) ppm. MS (ESI): m/z calcd for $C_{16}H_{21}NNaO_{11}$ $[M + Na]^+$ 426.1012; found 426.1663.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,2)-α-L-arabinofuranosyl-(1,2)-α-L-arabinofuranoside (6).** ^{1}H NMR (600 MHz, D_2O): δ = 8.11 (2H, d, J = 9.6, H_m C_6H_4), 7.08 (2H, d, J = 9.6, H_o C_6H_4),

5.82 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 5.19 (1H, d, $J_{1b,2b} = 1.2$, 1b-H), 5.02 (1H, d, $J_{1c,2c} = 1.8$, 1c-H), 4.30 (1H, dd, $J_{2a,3a} = 3.6$, 2a-H), 4.12 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 4.03 (1H, ddd, $J_{4a,5a} = 3.0$, $J_{4a,5'a} = 5.4$, 4a-H), 4.00 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.97 (1H, dd, $J_{2c,3c} = 3.0$, 2c-H), 3.92 (2H, m, 3b-H, 4c-H), 3.86 (1H, ddd, $J_{3b,4b} = 3.6$, $J_{4b,5b} = 3.0$, $J_{4b,5'b} = 5.4$, 4b-H), 3.80 (1H, dd, $J_{3c,4c} = 6.0$, 3c-H), 3.68 (2H, m, 5a-H, 5c-H), 3.56 (3H, m, 5′a-H, 5b-H, 5′c-H), 3.47 (1H, dd, $J_{5b,5'b} = 12.0$, 5′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.3$ (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.7 (C_o C_6H_4), 107.7 (1c-C), 106.2 (1b-C), 104.5 (1a-C), 88.0 (2b-C), 87.2 (2a-C), 84.4 (4a-C), 84.2 (3b-C), 82.8 (4b-C), 81.2 (2c-C), 76.5 (3c-C), 75.0 (4c-C), 74.5 (3a-C), 66.8 (5c-C), 60.6 (5a-C, 5b-C) ppm. MS (ESI): m/z calcd for $C_{21}H_{29}NNaO_{15}$ $[M + Na]^+$ 558.1435; found 558.2307.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,5)-α-L-arabinofuranosyl-(1,5)-α-L-arabinofuranoside (7).** ^{1}H NMR (600 MHz, D_2O): $\delta =$ 8.11 (2H, d, $J = 9.6$, H_m C_6H_4), 7.08 (2H, d, $J = 9.6$, H_o C_6H_4), 5.72 (1H, d, $J_{1a,2a} = 1.2$, 1a-H), 4.93 (1H, d, $J_{1b,2b} = 1.2$, 1b-H), 4.92 (1H, d, $J_{1c,2c} = 1.2$, 1c-H), 4.27 (1H, dd, $J_{2a,3a} = 3.0$, 2a-H), 4.13 (1H, ddd, $J_{4a,5a} = 3.0$, $J_{4a,5'a} = 5.4$, 4a-H), 4.04 (1H, ddd, $J_{4b,5b} = 3.0$, $J_{4b,5'b} = 6.0$, 4b-H), 3.99 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 3.96 (2H, m, 2c-H, 2b-H), 3.93 (1H, ddd, $J_{3c,4c} = 3.6$, $J_{4c,5c} = 3.0$, $J_{4c,5'c} = 6.0$, 4c-H), 3.84 (1H, dd, $J_{2b,3b} = 3.0$, $J_{3b,4b} = 6.0$, 3b-H), 3.78 (1H, dd, $J_{2c,3c} = 3.0$, $J_{3c,4c} = 6.0$, 3c-H), 3.76 (1H, dd, $J_{5a,5'a} = 11.8$, 5′a-H), 3.67 (2H, m, 5a-H, 5c-H), 3.64 (2H, m, 5b-H, 5′b-H), 3.54 (1H, dd, $J_{5a,5b} = 12.6$, 5′c-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta =$ 161.3 (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.2 (C_o C_6H_4), 107.6 (1b-C), 107.4 (1c-C), 105.4 (1a-C), 83.9 (4c-C), 83.7 (4a-C), 82.4 (4b-C), 80.8 (2a-C, 2b-C, 2c-C), 76.5 (3a-C, 3b-C, 3c-C), 66.7 (5a-C, 5b-C), 61.1 (5c-C) ppm. MS (ESI): m/z calcd for $C_{21}H_{29}NNaO_{15}[M+Na]^+$ 558.1435; found 558.2434.

***p*-Nitrophenyl α-L-arabinofuranosyl-(1,5)-[α-L-arabinofuranosyl-(1,2)]-α-L-arabinofuranoside (8).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.11$ (2H, d, $J = 9.6$, H_m C_6H_4), 7.08 (2H, d, $J = 9.6$, H_o C_6H_4), 5.82 (1H, d, $J_{1a,2a} = 1.2$, 1a-H), 5.08 (1H, d, $J_{1c,2c} = 1.2$, 1c-H), 4.91 (1H, d, $J_{1b,2b} = 1.2$, 1b-H), 4.29 (1H, dd, $J_{2a,3a} = 3.0$, 2a-H), 4.15 (2H, m, 3a-H, 4a-H), 4.00 (1H, dd, $J_{2c,3c} = 3.0$, 2c-H), 3.95 (1H, dd, $J_{2b,3b} = 3.0$, 2b-H), 3.90 (1H, ddd, $J_{3c,4c} = 3.6$, $J_{4c,5c} = 3.0$, $J_{4c,5'c} = 6.0$, 4c-H), 3.88 (1H, ddd, $J_{4b,5b} = 3.0$, $J_{4b,5'b} = 6.0$, 4b-H), 3.78 (2H, m, 3b-H, 3c-H), 3.75 (1H, dd, $J_{4a,5a} = 4.8$, $J_{5a,5'a} = 12.0$, 5a-H), 3.66 (2H, m, 5c-H, 5′a-H), 3.54 (2H, m, 5b-H, 5′c-H), 3.46 (1H, dd, $J_{4b,5'b} = 12.0$, $J_{5b,5'b} = 12.0$, 5′b-H). ^{13}C NMR (125 MHz, D_2O): $\delta = 161.3$ (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.7 (C_o C_6H_4), 107.5 (1c-C), 107.4 (1b-C), 104.4 (1a-C), 87.2 (2a-C), 84.1 (4b-C), 83.9 (4c-C), 82.5 (4a-C), 81.4 (2c-C), 81.0 (2b-C), 76.6 (3b-C), 76.5 (3c-C), 74.7 (3a-C), 66.0 (5a-C), 61.1 (5c-C), 61.0 (5b-C) ppm. MS (ESI): m/z calcd for $C_{21}H_{29}NNaO_{15}$ $[M + Na]^+$ 558.1435; found 558.1612.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,2)-β-D-galactofuranoside (9).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.10$ (2H, d, $J = 9.0$, H_m C_6H_4), 7.08 (2H, d, $J = 9.0$, H_o C_6H_4), 5.73 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 5.06 (1H, d, $J_{1b,2b} = 1.2$, 1b-H), 4.27 (1H, dd, $J_{2a,3a} = 4.8$, 2a-H), 4.20 (1H, dd, $J_{3a,4a} = 7.2$, 3a-H), 3.99 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.96 (1H, dd, $J_{4a,5a} = 3.6$, 4a-H), 3.93 (1H, dd, $J_{3b,4b} = 6.2$, 3b-H), 3.79 (1H, dd, $J_{4b,5b} = 3.4$, 4b-H), 3.72 (1H, ddd, $J_{5a,6a} = 7.4$, $J_{5a,6'a} = 5.0$, 5a-H), 3.60 (1H, ddd, $J_{5b,6'b} = 7.8$, $J_{5b,6b} = 4.2$, 5b-H), 3.50 (2H, m, 6a-H, 6′a-H), 3.24 (1H, dd, $J_{6b,6'b} = 12.0$, 6b-H), 3.17 (1H, dd, 6′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.6$ (C_{ipso} C_6H_4), 142.6 (C_p C_6H_4), 126.7 (C_m C_6H_4), 117.3 (C_o C_6H_4), 108.4 (1b-C), 105.1 (1a-C), 88.5 (2a-C), 83.5 (4b-C), 83.2 (4a-C), 82.0 (2b-C), 77.2 (3b-C), 75.4 (3a-C), 70.9 (5b-C), 70.5 (5a-C), 63.3 (6b-C), 63.2 (6a-C) ppm. MS (ESI): m/z calcd for $C_{18}H_{25}NNaO_{13}$ $[M + Na]^+$ 486.1224; found 486.2127.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,3)-β-D-galactofuranoside (10).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.10$ (2H, d, $J = 9.6$, H_m C_6H_4), 7.08 (2H, d, $J = 9.6$, H_o C_6H_4), 5.69 (1H, d, $J_{1a,2a} = 1.2$, 1a-H), 5.05 (1H, d, $J_{1b,2b} = 1.8$, 1b-H), 4.41 (1H, dd, $J_{2a,3a} = 3.0$, 2a-H), 4.14 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 4.06 (1H, dd, $J_{4a,5a} = 3.6$, 4a-H), 3.99 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.93 (1H, dd, $J_{3b,4b} = 6.2$, 3b-H), 3.80 (1H, dd, $J_{4b,5b} = 4.2$, 4b-H), 3.79 (1H, ddd, 5a-H), 3.68 (1H, ddd, $J_{5b,6'b} = 7.8$, $J_{5b,6'b} = 4.8$, 5b-H), 3.50 (4H, m, 6a-H, 6′a-H, 6b-H, 6′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.6$ (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.0 (C_m C_6H_4), 116.8 (C_o C_6H_4), 107.2 (1b-C), 105.6 (1a-C), 83.3 (4a-C), 82.9 (4b-C), 82.6 (3a-C), 81.3 (2b-C), 79.5 (2a-C), 76.6 (3b-C), 70.5 (5b-C), 70.4 (5a-C), 62.7 (6b-C, 6a-C) ppm. MS (ESI): m/z calcd for $C_{18}H_{25}NNaO_{13}$ $[M +$ $Na]^+$ 486.1224; found 486.2245.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,5)-β-D-galactofuranoside (11).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.10$ (2H, d, $J = 9.0$, H_m C_6H_4), 7.08 (2H, d, $J = 9.0$, H_o C_6H_4), 5.65 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 5.06 (1H, d, $J_{1b,2b} = 1.2$, 1b-H), 4.25 (1H, dd, $J_{2a,3a} = 3.6$, 2a-H), 4.09 (1H, dd, $J_{3a,4a} = 6.6$, 3a-H), 4.03 (1H, dd, $J_{4a,5a} = 3.6$, 4a-H), 3.98 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.92 (2H, m, 3b-H, 4b-H), 3.82 (1H, ddd, $J_{5a,6a} = 7.4$, $J_{5a,6'a} = 5.4$, 5a-H), 3.68 (1H, ddd, $J_{5b,6'b} = 7.8$, $J_{5b,6b} = 4.2$, 5b-H), 3.60 (2H, m, 6a-H, 6′a-H), 3.56 (1H, dd, $J_{6b,6'b} = 12.0$, 6b-H), 3.50 (1H, dd, 6′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.6$ (C_{ipso} C_6H_4), 142.3 (C_p C_6H_4), 126.0 (C_m C_6H_4), 116.8 (C_o C_6H_4), 107.0 (1b-C), 105.2 (1a-C), 83.2 (4a-C), 82.7 (4b-C or 3b-C), 81.2 (2a-C, 2b-C), 76.5 (3a-C), 75.9 (3b-C or 4b-C), 75.1 (5a-C), 70.5 (5b-C), 62.7 (6b-C), 60.8 (6a-C) ppm. MS (ESI): m/z calcd for $C_{18}H_{25}NNaO_{13}$ $[M + Na]^+$ 486.1224; found 486.3852.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,6)-β-D-galactofuranoside (12).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.09$ (2H, d, $J = 9.0$, H_m C_6H_4), 7.07 (2H, d, $J = 9.0$, H_o C_6H_4), 5.66 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 4.82 (1H, d, $J_{1b,2b} = 1.8$, 1b-H), 4.25 (1H, dd, $J_{2a,3a} = 3.6$, 2a-H), 4.06 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 3.97 (1H, dd, $J_{4a,5a} = 3.6$, 4a-H), 3.90 (1H, dd, $J_{2b,3b} = 3.0$, 2b-H), 3.85 (2H, m, 3b-H, 5a-H), 3.71 (1H, dd, $J_{4b,5b} = 4.8$, 4b-H), 3.66 (1H, dd, $J_{6a,6'a} = 10.8$, $J_{5a,6'a} = 4.2$, 6a′-H), 3.59 (1H, ddd, $J_{5b,6'b} = 7.2$, $J_{5b,6b} = 4.2$, 5b-H), 3.42 (3H, m, 6a-H, 6b-H, 6′b-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.4$ (C_{ipso} C_6H_4), 142.2 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.7 (C_o C_6H_4), 107.7 (1b-C), 105.3 (1a-C), 84.0 (4a-C), 83.0 (4b-C), 81.2 (2a-C), 81.0 (2b-C), 76.7 (3b-C), 76.5 (3a-C), 70.7 (5b-C), 68.9 (5a-C), 68.6 (6a-C), 62.7 (6b-C) ppm. MS (ESI): m/z calcd for $C_{18}H_{25}NNaO_{13}$ $[M + Na]^+$ 486.1224; found 486.1533.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,2)-β-D-galactofuranosyl-(1,2)-β-D-galactofuranoside (13).** ^{1}H NMR (600 MHz, D_2O): $\delta =$ 8.12 (2H, d, $J = 9.0$, H_m C_6H_4), 7.10 (2H, d, $J = 9.0$, H_o C_6H_4), 5.74 (1H, d, $J_{1a,2a} = 1.2$, 1a-H), 5.16 (1H, d, $J_{1b,2b} = 1.8$, 1b-H), 5.02 (1H, d, $J_{1c,2c} = 1.8$, 1c-H), 4.27 (1H, dd, $J_{2a,3a} = 2.4$, 2a-H), 4.20 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 4.07 (1H, dd, $J_{3b,4b} = 6.0$, 3b-H), 4.02 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.97 (1H, dd, $J_{2c,3c} = 3.6$, 2c-H), 3.95 (2H, m, 3c-H, 4a-H), 3.86 (1H, dd, $J_{4c,5c} = 4.8$, 4c-H), 3.80 (1H,

dd, $J_{4b,5b} = 4.8$, 4b-H), 3.71 (1H, ddd, 5a-H), 3.55 (6H, m, 5b-H, 5c-H, 6a-H, 6′a-H, 6b-H, 6′b-H) 3.22 (1H, dd, $J_{6c,6'c} = 12.0$, 6c-H), 3.18 (1H, dd, 6′c-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.4$ (C_{ipso} C_6H_4), 142.2 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.6 (C_o C_6H_4), 107.5 (1c-C), 106.7 (1b-C), 104.5 (1a-C), 88.1 (2a-C, 2b-C), 83.2 (4c-C), 82.5 (4a-C), 81.8 (4b-C), 81.3 (2c-C), 76.7 (3c-C), 75.2 (3b-C), 74.7 (3a-C), 70.7 (5a-C), 70.0 (5b-C), 69.9 (5c-C), 62.7 (6a-C, 6b-C, 6c-C) ppm. MS (ESI): *m/z* calcd for $C_{24}H_{35}NNaO_{18}$ $[M + Na]^+$ 648.1752; found 648.1912.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,2)-β-D-galactofuranosyl-(1,3)-β-D-galactofuranoside (14).** ^{1}H NMR (600 MHz, D_2O): $\delta =$ 8.12 (2H, d, $J = 9.6$, H_m C_6H_4), 7.10 (2H, d, $J = 9.6$, H_o C_6H_4), 5.72 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 5.17 (1H, d, $J_{1b,2b} = 1.8$, 1b-H), 5.01 (1H, d, $J_{1c,2c} = 1.8$, 1c-H), 4.44 (1H, dd, $J_{2a,3a} = 2.4$, 2a-H), 4.15 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 4.08 (3H, m, 3b-H, 4a-H, 4b-H), 4.01 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.97 (1H, dd, $J_{2c,3c} = 3.6$, 2c-H), 3.93 (1H, dd, $J_{3c,4c} = 6.0$, 3c-H), 3.86 (1H, dd, $J_{4c,5c} = 4.8$, 4c-H), 3.80 (2H, m, 5a-H, 5b-H), 3.71 (2H, m, 5c-H, 6a-H), 3.61 (5H, m, 6′a-H, 6b-H, 6′b-H, 6c-H, 6′c-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.4$ (C_{ipso} C_6H_4), 142.2 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.8 (C_o C_6H_4), 107.7 (1c-C), 105.9 (1a-C), 105.7 (1b-C), 88.2 (2b-C), 83.6 (4a-C, 4b-C), 83.3 (4c-C), 82.5 (3a-C), 81.2 (2c-C), 79.4 (2a-C), 76.6 (3c-C), 75.4 (3b-C), 70.7 (5b-C), 70.6 (5c-C), 70.2 (5a-C), 62.8 (6a-C, 6b-C, 6c-C) ppm. MS (ESI): *m/z* calcd for $C_{24}H_{35}NNaO_{18}$ $[M + Na]^+$ 648.1752; found 648.2551.

***p*-Nitrophenyl β-D-galactofuranosyl-(1,6)-[β-D-galactofuranosyl-(1→2)]-β-D-galactofuranoside (15).** ^{1}H NMR (600 MHz, D_2O): $\delta = 8.12$ (2H, d, $J = 9.0$, H_m C_6H_4), 7.10 (2H, d, $J = 9.0$, H_o C_6H_4), 5.74 (1H, d, $J_{1a,2a} = 1.8$, 1a-H), 5.05 (1H, d, $J_{1c,2c} = 1.8$, 1c-H), 4.82 (1H, d, $J_{1b,2b} = 1.8$, 1b-H), 4.26 (1H, dd, $J_{2a,3a} = 2.4$, 2a-H), 4.20 (1H, dd, $J_{3a,4a} = 6.0$, 3a-H), 3.98 (1H, dd, $J_{2c,3c} = 3.6$, 2c-H), 3.97 (1H, dd, $J_{4a,5a} = 3.6$, 4a-H), 3.91 (1H, dd, $J_{3c,4c} = 6.0$, 3c-H), 3.89 (1H, dd, $J_{2b,3b} = 3.6$, 2b-H), 3.85 (2H, m, 3b-H, 5a-H), 3.78 (1H, dd, $J_{4c,5c} = 4.8$, 4c-H), 3.71 (1H, dd, $J_{4b,5b} = 4.8$, 4b-H), 3.65 (1H, dd, $J_{6a,6'a} = 10.2$, $J_{5a,6a} = 4.8$, 6a-H), 3.60 (2H, m, 5b-H, 5c-H), 3.49 (1H, m, 6b-H), 3.45 (1H, m, 6′a-H), 3.39 (1H, m, 6′b-H), 3.19 (1H, dd, $J_{6c,6'c} = 12.0$, $J_{5c,6c} = 4.2$, 6c-H), 3.19 (1H, dd, $J_{5c,6'c} = 7.8$, 6′c-H) ppm. ^{13}C NMR (125 MHz, D_2O): $\delta = 161.4$ (C_{ipso} C_6H_4), 142.2 (C_p C_6H_4), 126.1 (C_m C_6H_4), 116.6 (C_o C_6H_4), 107.9 (1c-C), 107.6 (1b-C), 104.4 (1a-C), 88.1 (2a-C), 83.0 (4b-C), 82.9 (4c-C), 82.3 (4a-C), 81.4 (2c-C), 80.9 (2b-C), 76.7 (3b-C), 76.5 (3c-C), 74.7 (3a-C), 70.7 (5c-C), 70.2 (5b-C), 68.5 (6a-C), 68.4 (5a-C), 62.7 (6b-C, 6c-C) ppm. MS (ESI): *m/z* calcd for $C_{24}H_{35}NNaO_{18}$ $[M + Na]^+$ 648.1752; found 648.1688.

Immunostimulation assay

Stock solutions of oligosaccharides were prepared in Iscove's Modified Dulbecco's medium (IMDM) (Gibco). LPS (*E. coli* 0127:B8, Sigma) was used as a positive control, negative controls had no oligosaccharide added. The murine macrophage cell line RAW264.7 (obtained from ATCC, USA) was maintained in IMDM supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37 °C and 5% CO_2. Cells were harvested by centrifugation and resuspended in the same media to a final concentration of 5×10^5 cells mL^{-1}. Thereafter, 5×10^4 RAW264.7 cells in 100 μL of IMDM with 10% FBS per well were incubated with the indicated concentration of oligosaccharides at 37 °C and 5% CO_2. After 18 h, supernatants were collected and cytokine production was assessed by TNF-α-specific ELISA (Mouse TNF-α ELISA Ready-SET-Go kit, eBioscience).

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