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Ilona Chlubnová, Blanka Králová, Hana Dvořáková, Vojtěch Spiwok, Dominik Filipp, et al.. Graphical Abstract Biocatalyzed synthesis of difuranosides and their ability to trigger production of TNF-D D Biocatalyzed synthesis of difuranosides and their ability to trigger production of TNF-D D. Bioorganic and Medicinal Chemistry Letters, 2016, 26 (6), pp.1550-1553. 10.1016/j.bmcl.2016.02.018 . hal-01272616

HAL Id: hal-01272616

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Submitted on 11 Feb 2016

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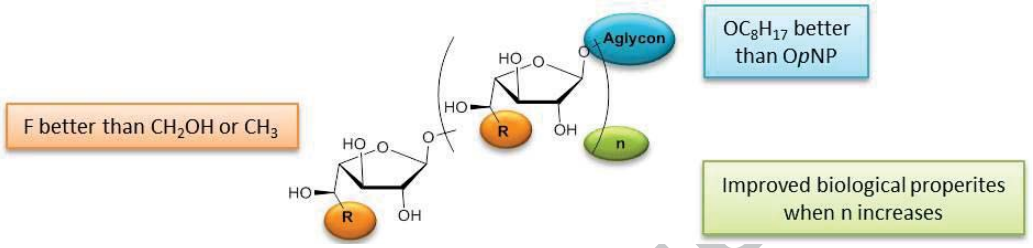
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Biocatalyzed synthesis of difuranosides and their ability to trigger production of TNF-α

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ABSTRACT

Transglycosylation reactions biocatalyzed by the native arabinofuranosidase Ara*f*51 and using D-galactosyl, D-fucosyl and 6-deoxy-6-fluoro-D-galactosyl derivatives as donors and acceptors provided di-to pentahexofuranosides. The immunostimulatory potency of these compounds, and more especially their ability to induce production of TNF-α, was evaluated on the murine macrophage cell line, Raw 264.7. The results obtained showed concentration-dependent and most importantly, structure-dependent responses. Interestingly, oligoarabinofuranosides belonging to the oligopentafuranoside family displayed concentration-, chain length and aglycon-dependent bioactivities irrespective of their fine chemical variations. Thus, neo-oligofuranosides in D-Gal*f* series, as well as their D-Fuc*f* and 6-fluorinated counterparts are indeed potential sources of immunostimulating agents.

Keywords: Carbohydrates Furanosides Immunostimulation

1. Introduction

Rare hexofuranosides occur in many microorganisms but are absent from the mammalian kingdom and invertebrates. That is why they constitute nowadays a source of inspiration to develop new treatments to fight pathogenic microorganisms for which the biosynthesis of these carbohydrates is vital.[1-4] Indeed, immunological, antiviral and antiparasitic properties of furanosyl-containing compounds are increasingly evidenced.[5-13] In this context, many efforts have been investigated to prepare and to mimic glycofuranoconjugates by both conventional chemical approaches and original enzymatic tools. Considering the latter, two classes of biocatalysts can be exploited. The usefulness of furanosyl transferases is dampened by its limited access to both these enzymes as well as the required UDP- or polyprenyl-furanosyl donors. On another hand, glycosyl hydrolases are more easily available and robust, therefore are more interesting as alternative synthetic tools, provided they are used under kinetic conditions, i.e. as a synthetic catalyst and not as a hydrolytic one. Surprisingly, reported results in this domain focused on only two arabinofuranosidases: Ab*f*D3 produced by ~~*Thermo*~~*cellum xylanilyticus*,[14-17] and an enzyme exploited by our group Ara*f*51 from *Clostridium thermocellum*.[18-21] Ab*f*D3 led to disaccharides and allowed Ara*f* transfer to xylobiose and xylotriose. As for Ara*f*51, we have demonstrated that it is involved in autocondensation reactions yielding di- to pentasaccharides. Both enzymes catalyzed the synthesis of furanosyl-containing glycosides starting from *p*-nitrophenyl L-arabinofuranosides (*p*NP Ara*f*) but also from structurally related D-galacto- (D-Gal*f*) and D-fucofuranosyl (D-Fuc*f*) series: Ab*f*D3 led to disaccharides and allowed Ara*f* transfer to xylobiose and xylotriose, while Ara*f*51 was involved in the transfer of di- to pentasaccharides in autocondensation reactions.

The higher synthetic potential of native Ara*f*51 was demonstrated for autocondensation reactions[20] and in transglycosylation with pyranosidic acceptors.[21] Herein, this potency was applied in transglycosylation involving two furanosidic substrates, D-galactofuranosyl donor and various furanosidic acceptors [D-Gal*f*, D-Fuc*f*, 6-deoxy-6-fluoro-D-galactofuranoside (6-F-D-Gal*f*), Figure 1]. The choice of D-Gal*f* as the donor was based on its occurrence in glycoconjugates of numerous pathogenic species.[22, 23] As for the furanosidic acceptors, we examined the ability of the enzyme to accept

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structural analogues of β-D-Gal*f* in the +I-subsite as well as the possible effect on the enzyme regioselectivity in the absence of a hydroxyl function on C-6 of the acceptor. Regarding the enzyme preferences toward formation of the (1→6) linkage in β-D-Gal*f* series, we were interested in the effect of restriction of C-6 participation in the bond formation. To this end, β-D-Gal*f* moiety was modified at C-6 by the replacement of the hydroxyl group with either hydrogen (β-D-Fuc*f*), or fluorine (6-F-β-D-Gal*f*). Moreover, the set of furanosidic acceptors were also represented by an octyl group at the anomeric position. First, we anticipated that the alkoxyl chain is a more inferior leaving group in comparison to the nitrophenolate, hence octyl furanosides would mainly play the role of acceptors. Second, numerous studies have highlighted the importance of alkyl chains in various biological activities.[7, 24-26] Using alkyl furanosides as acceptors will therefore allow the direct synthesis of glycolipidomimetics. Finally, immunological properties of the neo-oligofuranosides will be assessed, with a particular focus on the expression of tumor necrosis factor-α (TNF-α).

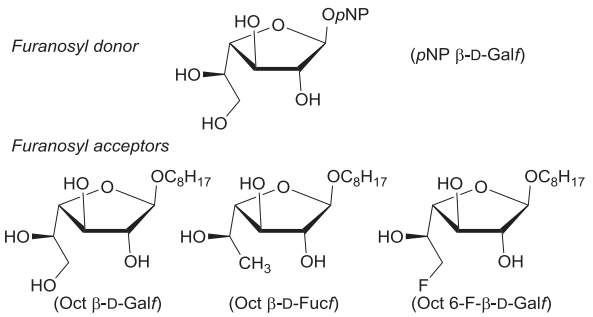


Figure 1. Structures of furanosyl donor and acceptors for biocatalyzed transglycosylation reactions.

Transglycosylation was first performed with Oct β-D-Gal*f* as the acceptor (Table 1, family **A**). Using size exclusion chromatographic purification, fractions of disaccharides (entries 1 and 2, 45%), trisaccharides (entries 3 and 4, 18%), tetrasaccharides (entry 5, 2%), and pentasaccharides (entry 6, 1%) were obtained. As expected, autocondensation of the donor *p*NP β-D-Gal*f* proceeded simultaneously resulting in a 34% yield of *p*NP glycosides. After 90 minutes, the conversion of *p*NP β-D-Gal*f* was nearly complete with only 4% of the donor remaining.

While the donor molecule was nearly entirely consumed, only 55% of acceptor Oct β-D-Gal*f* was transformed. The detailed molar balance of reaction products clearly indicates that the acceptor Oct β-D-Gal*f* simultaneously acted as a donor. Overall, an octyl aglycon initially intended as a protecting group turned out to be a good leaving group in the Ara*f*51-mediated transglycosylation. The nature of the superior nucleofugality of the *p*-nitrophenate group versus the octanolate anion has shown not to be determinant enough. Besides tryptophan residue in the +I-subsite of Ara*f*51 (Trp^{178}) forming a hydrophobic stacking with an acceptor, the funnel of the +I-subsite is furnished with a number of other aromatic/hydrophobic residues[27] which could provide a complementary environment, facilitating the leaving of the alkoxyl chain.

Next, transglycosylation was performed in the presence of Oct β-D-Fuc*f*. After purification, mainly di- and trisaccharides were isolated at 20% (Table 1, entries 7-9, family **B**) and 11% yields (entries 10-12), respectively. It is noteworthy that (i) the conversion of *p*NP β-D-Gal*f* in the presence of Oct β-D-Fuc*f* proceeded much slower than in the case of Oct β-D-Gal*f* (48% vs. 96 % after 90 min), (ii) the conversion of Oct β-D-Fuc*f* (42%) was comparable to that of the donor *p*NP β-D-Gal*f*, in contrast to the transglycosylation reaction with Oct Gal*f* as acceptor, (iii) the degree of hydrolysis was very low, with a yield of less than 3% of the products. Therefore, these observations indicate that the presence of Oct β-D-Fuc*f* reduces *p*NP β-D-Gal*f* autocondensation. Because of the low solubility of Oct β-D-Fuc*f* in water, the corresponding kinetic parameters could not be determined. Nevertheless, we hypothesized that the acceptor Oct β-D-Fuc*f* is significantly retained within the donor –I-subsite, thus limiting the access of the donor *p*NP β-D-Gal*f*. To corroborate this hypothesis, analysis of isolated products revealed the presence of a fucosyl residue at the non-reducing end of a number of resulting saccharides. This clearly demonstrated that Ara*f*51 is able to use Oct β-D-Fuc*f* simultaneously and efficiently as a donor and an acceptor.

Finally, transglycosylation with Oct 6-F-β-D-Gal*f* (entries 15-18, family **C**) as the acceptor was characterized by the slow rate of the reaction: (i) conversion of *p*NP β-D-Gal*f* reached only 43% after 90 minutes and (ii) Oct 6-F-β-D-Gal*f* was barely converted to the reaction products; its consumption did not exceed 20%. Nevertheless, there was a very low degree of hydrolysis (2%), and similarly to the reaction with Oct β-D-Fuc*f*, *p*NP β-D-Gal*f* acted as a better acceptor than Oct 6-F-β-D-Gal*f*. It is interesting to note that *p*NP β-D-Gal*f* residue was more frequently found in the reducing position of products. Taking together the very low degree of hydrolysis and *p*NP β-D-Gal*f* autocondensation as well as the unwillingness of Oct 6-F-β-D-Gal*f* to participate in the formation of transglycosylation products, this implies that the presence of fluorine has an inhibitory effect on enzyme activity. It seems that 6-F-β-D-Gal*f* residue is strongly retained in the donor subsite resulting in a more rapid process of the transfer of the β-D-Gal*f* residue to an acceptor.

Table 1. Oligofuranosides produced with Ara*f*51.

Entry	R	Oligosaccharides (Yield)	
1	OH	**Adi1**	Gal*f*-Gal*f*-O*p*NP (26%)
2	(Oct β-D-Gal*f*)	**Adi2**	Gal*f*-Gal*f*-OC_8H_{17} (19%)
3		**Atri1**	Gal*f*-Gal*f*- Gal*f*-O*p*NP (8%)
4		**Atri2**	Gal*f*-Gal*f*- Gal*f*-OC_8H_{17} (9%)
5		**Atetra**	Gal*f*-Gal*f*- Gal*f*- Gal*f*-OC_8H_{17} (2%)
6		**Apenta**	Gal*f*-Gal*f*- Gal*f*- Gal*f*-Gal*f*-OC_8H_{17} (1%)
7	H	**Bdi1**	Fuc*f*-Gal*f*-O*p*NP (12%)
8	(Oct β-D-Fuc*f*)	**Bdi2**	Fuc*f*-Fuc*f*-OC_8H_{17} (4%)
9		**Bdi3**	Gal*f*-Fuc*f*- OC_8H_{17} (4%)
10		**Btri1**	Fuc*f*-Fuc*f*-Gal*f*-O*p*NP (5%)
11		**Btri2**	Fuc*f*-Gal*f*-Gal*f*-O*p*NP or Gal*f*-Fuc*f*-Gal*f*-O*p*NP (5%)
12		**Btri3**	Gal*f*- Gal*f*- Fuc*f*-OC_8H_{17} (1%)
13	F	**Cdi1**	Gal*f*- Gal*f*-O*p*NP (13%)
14	(Oct	**Cdi2**	6-F-Gal*f*- Gal*f*-O*p*NP (3%)
15	6-F-β-D-Gal*f*)	**Cdi3**	Gal*f*-6-F-Gal*f*-OC_8H_{17} (6%)
16		**Cdi4**	6-F-Gal*f*-6-F-Gal*f*-OC_8H_{17} (3%)
17		**Ctri1**	6-F-Gal*f*-Gal*f*-Gal*f*-O*p*NP or Gal*f*-6-F-Gal*f*- Gal*f*-O*p*NP (1%)
18		**Ctri2**	Gal*f*-Gal*f*-6-F-Gal*f*-OC_8H_{17} (1%)

Further chromatographic separation led to disaccharides **1-11** (Figure 2) which were identified by mass spectrometry and their structures elucidated by 1D and 2D NMR. The β-configuration of the newly formed furanosidic linkages were based on a small H1-

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H2 coupling constant less than 1.0 Hz. The five-membered ring size of the transferred glycosyl entity was deduced from the latter value, which is specific to furanosides,[28] and from ^{13}C NMR data, i.e. 107.4 ppm for C1, a value that also corroborates the β-configuration, and a chemical shift upper to 80 ppm for C-4.[29] Finally, the regioselectivity of the glycosidic coupling was established by comparing the chemical shifts of the carbon atoms of the reducing entity with those of the starting acceptor since *O*-glycosylation results in downfield chemical shifts.[29] For instance, δ_{C2a} increased from 81.6 ppm to 87.5 ppm in compound **1** while δ_{C6a} rose 6.2 ppm (from 62.9 ppm to 69.1 ppm) in disaccharide **2**. For disaccharides **4** and **10**, downfield chemical shifts from 76.6 ppm to 82.3 ppm, and from 62.8 ppm to 69.1 ppm, corroborated (1→3) and (1→6) new glycosidic linkages, respectively.

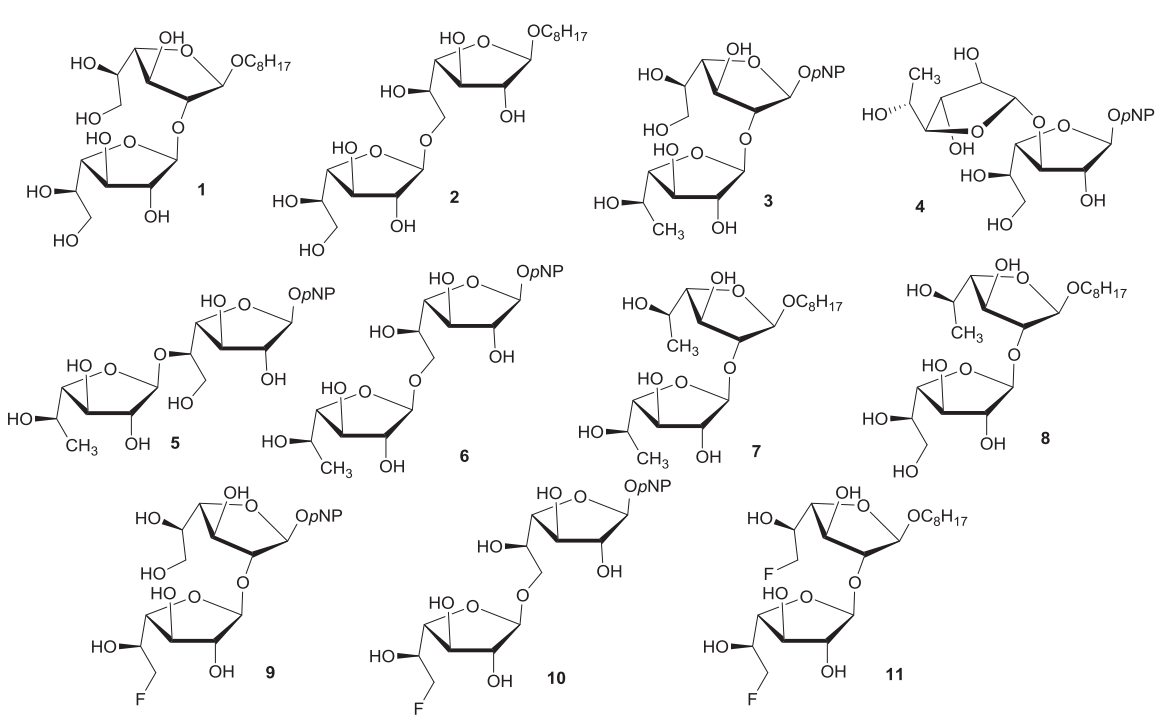


Figure 2. Elucidated structures of isolated difuranosides.

To evaluate the immunomodulatory potency of enzymatically synthesized neofuranosides, these molecules were incubated with the murine macrophage cell line, Raw 264.7. All glycoconjugates were tested at concentrations of 1, 10, 100 and 1000 ng/mL. *E. coli* lipopolysaccharide (LPS) was used as a positive control. After 18 hours of incubation, the production of tumor necrosis factor-α (TNF-α) in the culture medium was assessed using TNF-α-specific ELISA. While in the unstimulated control sample, Raw 264.7 secreted only background levels of TNF-α (4.4 ± 3.4 pg/mL, not shown), these levels in positive control samples stimulated with 100 ng/mL of LPS reached 880 ± 53 pg/mL. Importantly, significant TNF-α responses (up to 800 pg/mL) were elicited upon stimulation with the indicated oligofuranosides when used at concentrations of 100 and 1000 ng/mL (Figure 3). It is of note that only trace and non-stimulatory levels of LPS were detected in the samples used, as admixing LPS inhibitor polymyxin B to cell cultures resulted in the production of comparable levels of TNF-α (data not shown).

TNF-α protein levels produced by regioisomers of galactofuranosides (**A**) and their structural analogues fucofuranosides (**B**) or 6-deoxy-6-fluorogalactofuranosides (**C**) clearly demonstrated that certain structural features play an important role in their bioactivity. First, as depicted in figure 3, oligofuranosides bearing a *p*NP aglycon exhibited a weaker effect on TNF-α production than that induced by octyl oligosaccharides, provided that the number of glycosyl units was equivalent. Nevertheless, oligofuranosides from families **A** (Gal*f*) and **C** (Gal*f*, 6-F-Gal*f*) are better inducers of the production of target cytokine than those of family **B** (Gal*f*, Fuc*f*). Second, although more noticeable with the octyl series, the length of the carbohydrate chain was of great importance since biological activity significantly increased with the number of furanosyl rings, irrespectively to the nature of the glycosyl units (Gal*f*, Fuc*f*, or 6-F-Gal*f*). This chain length-dependent activity has been previously described with synthetic oligoarabinofurnaosides.[20] Third, fluorine-containing di- or trisaccharides **C** seem to be much more efficient than their hydroxylated or deoxy counterparts. Indeed, the disaccharide **11** displayed a similar biological effect than the tested pentasaccharide (TNF-α>200 pg/mL), which represents approximately 2.5-times greater immunostimulatory potential. While it is difficult to predict the impact of the number of fluorine atoms as well as the position of fluorinated units along the octyl oligofuranoside on the biological activity, adding a furanosidic unit can significantly increase the production of TNF-α which reached 600 pg/mL for the fraction 6-F-Gal*f*-6-F-Gal*f*-OC_8H_{17}.

To conclude, a set of original oligofuranosides which are structurally-related to D-galactofuranose and possess chemical modulations on the primary position was obtained using native type Ara*f*51. This biocatalyst allowed the synthesis of D-Gal*f* derivatives but also oligomers derived from D-fucofuranose and 6-deoxy-6-fluoro-D-galactofuranose. In each case, the transglycosylation process constituted a major reaction with a very low degree of hydrolysis. Importantly, the octyl furanosides first designed as acceptors also played the role of donors, probably thanks to a good recognition within the –I sub-site. All of the compounds were tested on Raw264.7 cells in order to evaluate their immunostimulatory activity. They significantly upregulate TNF-α secretion in a concentration-, chain length-

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and aglycon-dependent fashion. Importantly, the presence of an alkyl chain and/or of fluorine atoms significantly increased biological activity. Consequently, both arabinofuranosyl and galactofuranosyl derivatives are compounds of interest for their immunostimulatory properties and further studies are currently underway to evaluate their potential as adjuvants in vaccine development.

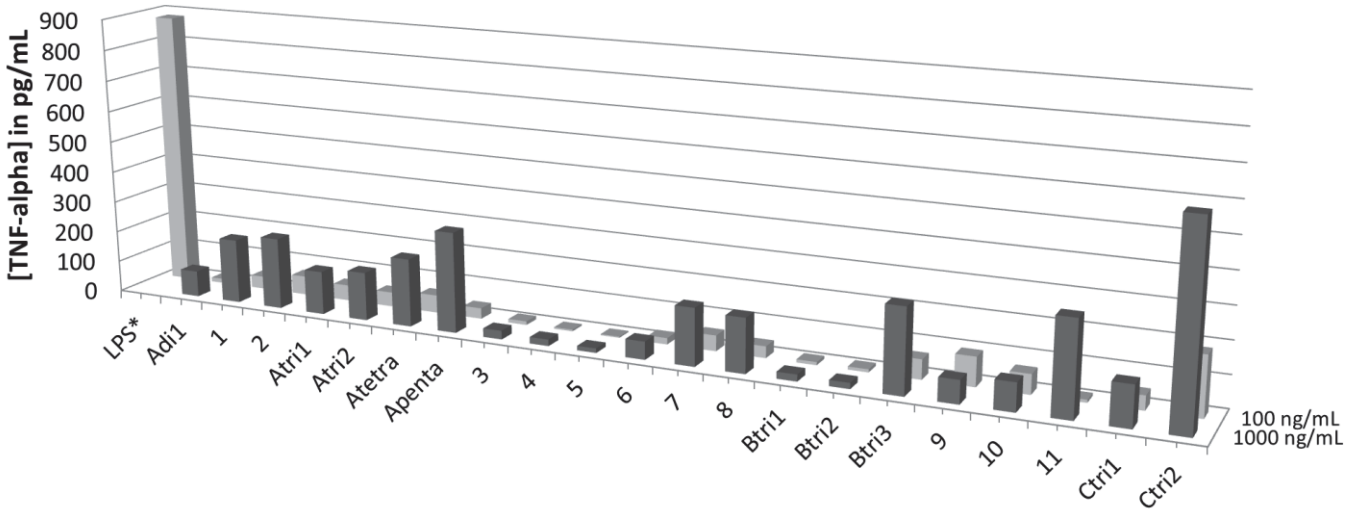


Figure 3. Production of TNF-α cytokine upon macrophage cell line Raw264.7 stimulation with enzymatically synthesized oligohexofuranosides. The bar graph data are presented as mean of duplicate samples for each neo-oligofuranoside tested. One representative result of two independent experiments is shown. Negative (unstimulated) and positive (LPS-stimulated) controls, tested in triplicates, are not shown (see text for details).

Acknowledgments

I.C. thanks the Czech Ministery of Education and the Ministère Français des Affaires Etrangères for a grant (MSM:6046137305) and the Grant 14-17194P from the Grant Agency of Czech Republic (GA ČR). Financial supports by the Programme Hubert Curien (Barrande, Ministère Français des Affaires Etrangères), the Agence Nationale de la Recherche (JCJC06_140075), and the GDR-RDR2 "Aller vers une chimie éco-compatible" are gratefully acknowledged, as well as Prof G. Davies for supplying the plasmid of Ara*f*51. The authors are grateful to the GlycoOuest network, supported by the Région Bretagne and the Région Pays de la Loire.

Supplementary Material

Experimental details, NMR data and description of biological tests are available in Supplementary material.

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