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ARTICLE TYPE

## Synthesis and evaluation of 1,2-*trans* alkyl galactofuranosides mimetics as mycobacteriostatic agents.

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The simple octyl β-D-galactofuranoside was previously described as good bacteriostatic agent against *Mycobacterium smegmatis*, a non-pathogenic model of *M. tuberculosis*. In order to decipher its mechanism of action STD NMR on whole *M. smegmatis* cells was implemented. It outlined the crucial role of the alkyl chain and the possibility of modulation on the furanoside. From there, 16 new alkyl furanosides were then synthesized in order to optimize the mycobacteriostatic activity. They all present the pending alkyl chain in a 1,2-*trans* configuration relative to the sugar ring. Three families were built, that differ by the substituent on the primary position of the galactofuranose ring, the series or the alkyl chain pending. Four showed growth inhibition inferior to the parent octyl β-D-galactofuranoside. Double alkyl chains at C-1 and polar substituent on the primary position of the furanose seemed to favor the activity. Finally, a mixed biantennary alkyl/aryl β-D-galactofuranoside exhibited the best growth inhibition concentration at 90 μM.

### Introduction

Tuberculosis is a medical, social, and economic disaster of immense magnitude that occurs over the world, reaching a dramatic level of 9 million people infected and 1.5 million deaths each year.<sup>1</sup> This deadly infection is difficult to cure and doing so successfully requires long treatment periods with multiple antibiotics.<sup>2</sup> Recent first-line drugs against tuberculosis include ethambutol and isoniazid in association with rifampicine and pyrazinamide. Such Directly Observed Therapy, Short course treatments (DOTS) present multiple shortcomings, like resistance and side-effects.<sup>3</sup> Therefore novel therapeutic approaches, particularly those aimed at exploring new horizons for the development of efficient anti-tuberculosis molecules are required. The use of poly- and monosaccharides with bactericidal or bacteriostatic activities is nowadays gaining momentum regarding to their natural distribution in particular in crucial cellular architecture of pathogenic microorganisms.<sup>4</sup> For example, the cell wall of *Mycobacterium tuberculosis*, the bacteria responsible for tuberculosis, contains a complex mixture of polysaccharides named arabinogalactanes.<sup>5,6</sup>

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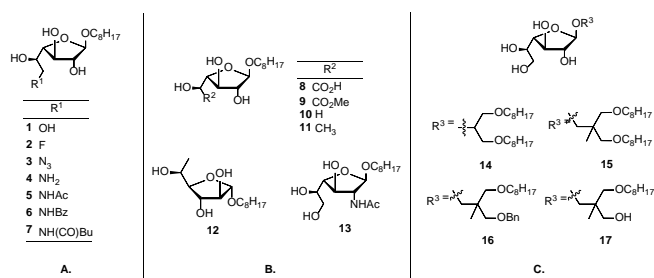
This structure incorporates in particular D-galactofuranose (D-Galf), thermodynamically less stable than its pyranose counterparts and exogenous in mammals. In the light of the crucial role played by such galactofuranosyl containing conjugates, molecules that are able to act on their biosynthesis, catabolism or degradation will certainly interfere with the proliferation, virulence and even survival of the corresponding cell. Such molecule will thus have all the characteristic of a therapeutic agent directed towards Galf presenting cells with less toxicity toward other organisms.<sup>7</sup> performed in the understanding of RNA–ligand recognition principles in recent years, the design of RNA-specific ligands remains a difficult task. RNA is a dynamic target and mutual interactions can greatly modify their conformations.<sup>8</sup> The most significant contributions related to association phenomena result from electrostatic interactions between the protonated amino groups and the negatively charged phosphates of the oligoribonucleotide backbone.<sup>9</sup> Keeping this in mind, we decided to maintain these key functional groups and have developed a synthetic strategy that allows the easy introduction of polar substituents on the 5- or 6-*O*-position of the neamine core. Two compounds **1** and **2** possessing respectively a cyclic carbonate or a sulfate moiety at the 5,6-positions were selected as key building blocks. (Scheme 1).<sup>10</sup> These five-membered rings are likely to undergo a variety of ring-opening reactions in the presence of various nucleophiles.<sup>11</sup>

Different families of carbohydrate derivatives have been already screened against strains of mycobacteria. For example, uridine diphosphate galactofuranoses modified at the primary position (C-6) of Galf were able to inhibit *M. tuberculosis* growth.<sup>8</sup> However their low availability and stability

limited their therapeutic potential. Another family was built around alkyl D-arabinofuranoside. Such alkyl furanosides are able to inhibit the growth of different strains of *Mycobacterium* (*smegmatis*, *bovis*, *tuberculosis*) and even to destroy it at concentrations around micromolar.<sup>12,9-17</sup> A model proposed by Sucheck *et al.* hypothesized a specific recognition of the alkyl arabinofuranoside motif by mycolyl transferases.<sup>13,13</sup> It is worth noting that such transferase inhibition is the key mechanism linked with the action of ethambutol. Finally, a last class of molecules was built on the alkyl galactofuranoside model. The best results described so far concerned a biantennary thiogalactofuranose developed by von Iztein *et al.*<sup>18</sup> In our laboratory, we conceived a simpler version, the octyl  $\beta$ -D-galactofuranoside **1** (Figure 1) that is able to inhibit the proliferation of *M. smegmatis*, the non pathogenic analogs of *M. tuberculosis* at a concentration of 0.86 mM. Furthermore when it was embedded into a polymeric matrix PBAT/sodium caseinate, a bactericidal effect was observed.<sup>19,20</sup>

The mechanism of action associated with such galactofuranolipids remains unclear and it is of importance to decipher structure-activities relationships. To do so, we first compared the affinity of both octyl  $\beta$ -D-galactofuranoside **1** and its octyl pyranoside counterpart with the microorganism's surface using Saturated Transfer Difference (STD) NMR technique on whole cell. This preliminary experiment confirmed the importance of the nature and length of the lipid chain but also pointed out the weak interaction of the sugar with the cell membrane and so the possibility for modulation.

In this context, a wider range of glycofuranosides bearing an alkyl chain at the anomeric position in a 1,2-*trans* configuration were synthesized. To tune the lipophilic nature of the alkyl furanoside scaffold, three modulations were introduced: i) substitution of the hydroxyl group at primary position (A-family); ii) modification of the osidic series (B-family) and iii) doubling of the octyl chain at the anomeric position (C-family) (Figure 1). The resulting compounds were eventually evaluated against their inhibition of *M. smegmatis* growth.



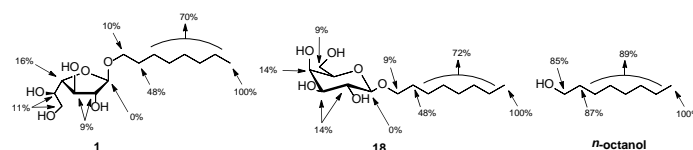
**Figure 1** Targeted alkyl furanosides **2-17**.

## 2. Results and discussion

### 2.1. STD-NMR experiment

We first investigated the action mechanism of octyl  $\beta$ -D-galactofuranoside **1** as mycobacteriostatic. **1** was described as a mycobacteriostatic at a concentration of 0.8 mM. This value is

well below its Critical Micellar Concentration (CMC) that reaches 6.1 mM.<sup>21</sup> Consequently, **1** is unable to form some micelles in the considered inhibitory concentration and we can rule out a possible surfactant effect as action mechanism. It is possible nowadays to map the part of the drug specifically involved in the interaction with the targeted receptor thanks to Saturated Transfer Difference (STD) NMR technique.<sup>22,23</sup> However the exact biological target of our galactofuranolipid is yet unknown. Consequently the STD NMR was here performed on the whole cell of *M. smegmatis*. Using this technique, we compared the interaction profile of **1**, its octyl galactopyranoside counterparts **18** and *n*-octanol (Figure 2 and supplementary information). All compounds were used at a concentration of 5 mM in D<sub>2</sub>O. This concentration was higher than **18**'s growth inhibition value but lower than the CMC of each molecules (6.1 mM for **1** and 16 mM for **18**) in order to avoid again any surfactant effect. When incubated with *M. smegmatis* cell, a strong STD effect was observed at the extremity of the octyl chain of **1**. The percentage of STD effect decreased when moving toward the furanose. There was also some detectable effect on the furanoside ring. The same profile was found for the octyl pyranoside **18** and the octanol. For the latter one, the STD effect was identical along the alkyl chain.



**Figure 2.** Quantification of molecular interactions of **1**, **18** and octanol with *M. smegmatis* by STD NMR. The largest STD effect in each spectrum was set to 100%.

From this result, it appeared first that the alkyl chain on the sugar played a crucial role on the activity thus explaining previous results on such galactofuranolipids. Mycobacteria present a unique cell surface made of mycolic acids that are grafted on arabinogalactan. This very hydrophobic cell wall allows the bacteria to reduce its sensitivity against antibiotics and the recognition by the immune cells.<sup>24</sup> It can nevertheless interact with other lipid through weak van der Waals interactions. We can subsequently hypothesize that the alkyl chain strongly contributes to the interaction of the glycolipid with the cell membrane. On the other hand, such experiment did not allow concluding on the importance of the furanoside ring as no difference occurred between pyranoside and furanoside. It might mean that the furanose recognition was either too low to be measured or that it took place into the cell membrane after penetration and was thus impossible to detect by such NMR technique. If the nature of the sugar moiety did not reduce the measured affinity, we can also hypothesize that modification on the carbohydrate scaffold should not deter the interaction between the cell membrane and the glycolipids. Interestingly, previous screening on eukaryote parasites of the *leishmania* genus concluded on the importance of the substituent on the galactofuranoside scaffold to measure some growth inhibition.<sup>25</sup> In this context and in an attempt to improve the biological activity of the parent furanolipid, we decided to

modulate the lipophilicity of the alkyl chain and the polarity of the sugar head.

## 2.2. Synthesis

5 5-Membered ring hexofuranoses generally tend to isomerise into 6-membered one, the most thermodynamically stable form. It is thus critical to first fix the ring size and then modulate the functional groups on the scaffold. To access to the A-family, we started from the known octyl  $\beta$ -D-galactofuranoside  
10 **1**<sup>26,27</sup> whose C-6 position could be tuned and whose anomeric configuration was already installed, thus avoiding complex purification process.

Four functional groups were envisioned at the primary position of the galactofuranose, the 6-fluoro, 6-azido, 6-amino  
15 and three 6-amido. They can be easily obtained thanks to selective nucleophilic attacks of the corresponding nucleophiles on position 6 of the cyclic sulfate **19** (Scheme 1). **19** was synthesized from octyl  $\beta$ -D-galactofuranoside according to literature procedure.<sup>28</sup> Ring-opening by tetrabutylammonium  
20 fluoride (TBAF), followed by the hydrolysis of the resulting sulfate led to the intermediate **20**. In a similar way, **21** was prepared from **19** using sodium azide in dimethylformamide (DMF). Its subsequent palladium-catalyzed hydrogenolysis gave the amine **22** in a moderate yield. It can be purified by column  
25 chromatography on silica gel or directly converted to *N*, *O*-diacyl derivatives **23-25** by action of acetyl chloride, benzoyl chloride or valeroyl chloride in presence of DMAP. Finally, all ester groups on compounds **20-25** were cleaved under basic conditions to give final products **2-7**. All resulting compounds were obtained as  
30 pure 1,2-*trans* anomer as confirmed by the value of the NMR coupling constant between *H*-1 and *H*-2 ( $J_{1,2} < 2$  Hz).

In order to investigate the importance of the D-galactofuranose scaffold, other series presenting common  
35 skeleton with Galf were also sought. Octyl  $\beta$ -D-galactofuranoside uronic acid (Octyl D-GalfA) **8** could be obtained according to reported strategy of furanosylation of octanol in presence of calcium chloride.<sup>22</sup> The same procedure was implemented to access the octyl (methyl  $\beta$ -D-galactofuranosid)uronate **9** using  
40 (methyl  $\beta$ -D-galactopyranosid)uronate **26**<sup>29</sup> (Scheme 2). The resulting uronate **9** was obtained as a mixture of anomers with a slight excess of the  $\beta$ -one. The targeted anomer could be separated from the  $\alpha$ -isomer by purification on C18-grafted silica column. As for the L-arabinose (L-Ara) and L- and D-fucose (L- and D-Fuc) series, a two steps strategy starting from the  
45 corresponding per-*O*-acetyl furanose **27-29** was used. Such activated furanoses were easily obtained from the parent sugars as described previously.<sup>29</sup> Glycosylation of octanol by **27**, **28** or **29**, in presence of trifluoroboron etherate complex, generated  
50 again the 1,2-*trans* anomers as the major isomers. They were purified first to remove the 1,2-*cis* anomer before subsequent acetate cleavage to give **10**, **11** or **12**, respectively. The overall yields were generally low due to the relative low reactivity of the anomeric acetate.

55 We previously described the formation of the oxazoline derivative of 2-acetamido-2-deoxy-D-galactofuranose (D-

Gal/NHAc) **30** thanks to the acetylation of per-*O*-silylated Gal/NHAc in presence of acetic anhydride and a large excess of  
60 *p*-toluenesulfonic acid (*p*TSA).<sup>29</sup> Such oxazoline could nevertheless undergo nucleophilic attack by methanol to give selectively the methyl  $\beta$ -D-Gal/NHAc in excellent yield. We thus decided here to use the same strategy. Unfortunately no reaction occurred when using octanol as nucleophile in presence of various Lewis acid like BF<sub>3</sub>.Et<sub>2</sub>O or TMSOTf. Only copper(II)  
65 chloride, a weak Lewis acid, allowed obtaining octyl Gal/NHAc as a mixture of anomers.<sup>30</sup> The 1,2-*trans* anomer was the major product of the reaction. Transesterification of the ester finally gave the targeted octyl  $\beta$ -D-Gal/NHAc **13** with 30% yield over two steps.

70 Four biantennary substituents were envisioned at the C-1 position of galactofuranose. For that, three acceptors **32**, **34** and **38** were synthesized (Scheme 3). Glycerol derivative **32** was first prepared by reaction of epichlorohydrin with sodium octanoate according  
75 to literature procedure.<sup>31</sup> Dialkylation of 2-(hydroxymethyl)-2-methyl-1,3-propanediol **33** with sodium hydride and bromooctane in presence of sodium hydride and tetrabutylammonium iodide afforded the desired compound **34**. On the other hand, the protection of two alcohol functions of the triol **33** was easily  
80 achieved by treatment with acetone in presence of *p*TSA leading to the acetal **35**. After benzylation with benzylbromide and sodium hydride, the ketal moiety was hydrolyzed by treatment with sulfuric acid in methanol. Monoalkylation of diol **37** was then accomplished in good yield using conditions identical to  
85 those employed for the synthesis of **34**.<sup>32</sup>

Next, glycosylation of alcohols **32**, **34** and **38** with 2,3,5,6-tetra-*O*-acyl  $\square$ -thiogalactofuranoside **39**<sup>33</sup> in presence of silver triflate and *N*-iodosuccinimide gave the corresponding  
90 intermediates **40**, **41** and **42**, respectively (Scheme 4).<sup>34</sup> If the biantennary galactofuranosides **41** and **42** were obtained as a mixture of anomers, respectively  $\square/\square\square = 1:4$  and  $1:19$ , **40** was only isolated as a pure  $\beta$ -anomer. Acyl groups were then cleaved under basic conditions to give final products **14-16**. Due to the  
95 presence of an asymmetric carbon on the alkyl chain, **16** was obtained as a complex mixture. Finally, debenylation of **16** by hydrogenolysis gave the last product **17** as a non-separable diastomeric mixture but with 1,2-*trans* configuration at the anomeric carbon.

## 2.3. Biological evaluation

The resulting library of alkyl furanosides was then evaluated for their bacteriostatic properties against a mutated strain of *M. smegmatis* (mc<sup>2</sup> 155), one of the non-pathogenous model of  
105 mycobacteria. The Minimum Inhibition Concentration (MIC) responsible for 99% of bacteria growth inhibition was reported for each compound in table 1. The determination of the MIC values was based on a resazurine modified assay in which the deep blue resazurine was oxidized into the fluorescent pink  
110 resorufine in the presence of living bacteria.<sup>35</sup> The aim of this study was to confirm i) the influence of the alkyl chain at the anomeric position (chain length, linear vs. split); to complete the study, methyl and butyl galactofuranoside **43** and **44**<sup>36</sup> were also

evaluated, ii) the impact of C-6 hydroxyl group on the activity, iii) the impact of the cycle (furanoside vs. pyranoside); octyl galactopyranoside **18** was thus included in the evaluation and iv) the importance of the nature and configuration of the carbohydrate (GalF vs. others, D vs. L). Ethambutol, a known antituberculosis molecule was used as positive control.

The compiled results showed no inhibition for octanol (Entry 21), 6-acetamido-GalF **5** (Entry 5), L-Fucf **12** (Entry 12), Galf/NHAc **13** (Entry 4) and Galf pending with branched alcohol **17** (Entry 17) or with short chains **43** and **44** (Entries 19 and 20) as well as for octyl pyranoside **18** (Entry 18). However, inhibitory values were measured when the microorganism was incubated with the other alkyl glycosides that derived from octyl D-galactofuranose **1** (A-Family, Entries 2-4, 6 and 7), that shared common structural characteristics with **1** (B-Family, Entries 8-11) or Galf with biantennary alkyl chain at the anomeric position (C-Family, Entries 14-16). Their MIC were found to be between 0.1 and 2 mM. Only four neoglycolipids exhibited MIC values below the one found for the parent octyl  $\beta$ -D-GalF **1**. First, octyl 6-amino-GalF **4** and octyl  $\beta$ -D-GalFA **8**, with polar substituent on the pending C-4 arm of the skeleton, exhibited respectively MIC value of 0.3 and 0.16 mM (Entries 4 and 8). Second, biantennary **15** and **16** showed inhibition of *M. smegmatis* growth at 0.5 and 0.09 mM, respectively (Entries 15 and 16).

It therefore appeared that the furanose form was essential for the activity as the pyranoside analog **18** showed no bacteriostatic activity. In addition, no or low activity was measured with most compounds from B-family (**10-13**). There was so a strong impact of the considered series on the activity. The lack of inhibitory activity of methyl and butyl GalF, **43** and **44**, confirmed also that a minimum chain length was compulsory to measure any inhibition. The doubling of the alkyl chain had nevertheless a medium effect. **14**, the *O*-glycoside analogs of von Iztejn's best inhibitor<sup>18</sup> showed poor activity. It might be linked with lower stability of *O*-glycosides when compared with *S*-glycosides. However when the split chain was separated from the sugar by one methyl, the inhibition increased. The best activity was found with an aromatic group on one of the chain (**16**). In addition, structural modulations at C-6 position greatly influenced the biological activity. Indeed compounds **5** (6-NHAc) showed no activity while increasing MIC values were found for furanosides **6** (6-NHBz), **3** (6-N<sub>3</sub>), **9** (methyl D-GalFA), **2** (6-F), **7** (6-NHCOBu), **4** (6-NH<sub>2</sub>) and **8** (D-GalFA). We can so hypothesize that hydrophobic chain on the carbohydrate was detrimental to the activity but polar substituent on GalF like hydroxyl, acid or amine increased the potency of the molecule. The same trend was observed when A-family analogs were incubated in presence of *Leishmania donovani* promastigote.<sup>25</sup>

There is consequently a strong impact of both the nature of the lipid chain and the substituent on the furanose on the development of the mycobacteria. The biantennary molecule **16** was found to be the best growth inhibitor found so far in this family of *O*-alkyl galactofuranosides. Previously, we showed that such family of galactofuranolipids was completely innocuous towards both soil microorganism and human macrophages.<sup>19,25</sup> It confirmed their potential as alternative of DOTS.

### 3. Conclusion

Two strategies were implemented in order to obtain a library of 17 furanolipids bearing one or two alkyl chains, in a 1,2-*trans* configuration of the carbohydrate moiety. After incubation with *M. smegmatis*, five of them revealed good inhibitory values against the microorganism growth. The best molecules are all derived from galactofuranose and bear either a polar group on the galactofuranose moiety or a biantennary alkyl chain at the anomeric position. The biantennary non symmetrical compound **16** with octyl and benzyl group on the bis(hydroxymethyl) linker was found to inhibit the growth of *M. smegmatis* at 90  $\mu$ M. Even if such values were at least eight times higher than the one found with ethambutol, such family of molecules was described as innocuous towards other microorganisms or even macrophages, thus increasing their associated therapeutic index.<sup>25</sup> First attempt to better understand their mechanism of action was achieved by STD NMR experiments that showed a strong affinity between the alkyl chain and the cell wall of mycobacteria. There should be first an anchorage of the alkyl chain to the cell wall followed by the recognition of the furanose by specific receptors. Studies to confirm this second step are under investigation.

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