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Modular Synthesis and Immunological Evaluation of Suspected Allergenic Galactooligosaccharides

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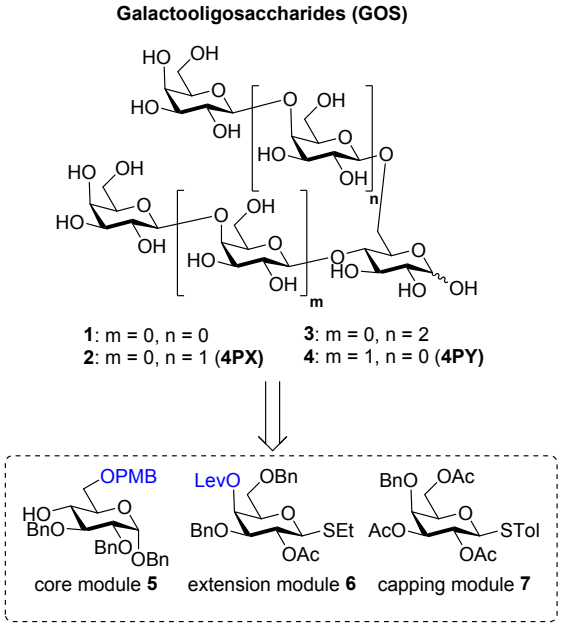
Galactooligosaccharides (GOS) are widely used in the food industry as prebiotics and in very rare cases, can lead to an allergic reaction. Due to the microheterogeneity of GOS it is very difficult to extract pure and well defined oligosaccharides to establish which component is responsible for the observed allergenicity. Herein, we report the chemical synthesis of a suspected allergen 4PX and three closely related oligosaccharides based on a modular approach. The fact that synthesized 4PX and a regioisomer did not cause basophil activation in subjects with confirmed GOS-allergy excludes both tetrasaccharides as key-epitopes in GOS-allergenicity in Singapore.

Introduction

Galactooligosaccharides (GOS) are used in the food industry as prebiotics since the 1990's. GOS are an important component of infant formula to boost the low oligosaccharide content of bovine milk (~0.05g/L) compared to human milk (~20g/L)[1]. Additionally, GOS have been shown to be beneficial to infant health by promoting the growth of benign micro-organisms such as *Bifidobacteria*[2, 3], improving stool consistency and frequency[4, 5], enhancing natural defenses[6] and improving the uptake of minerals[7, 8]. GOS are prepared enzymatically using a β-galactosidase, an enzyme that hydrolyzes lactose into galactose and glucose[9]. However, at high lactose concentration, transgalactosylation becomes the dominant reaction pathway. Under these conditions, the galactose residue is cleaved and transferred to another lactose molecule at the galactose and/or glucose residue, giving rise to a complex mixture consisting of linear- and branched GOS that also differ in chain length. GOS have a safe history of use and are Generally Recognized As Safe (GRAS) by the US FDA.[10,11,12]. In very rare cases reported from the Southeast Asian region, consumption of GOS is reported to lead to an allergic reaction in subjects with pre-existing allergies[13-16]. However, due to the microheterogeneity of GOS, it is very difficult to extract pure and well defined oligosaccharides to identify the allergen. A recent study attributes GOS-related allergenicity in Japanese oyster workers to the branched tetrasaccharide Galβ(1-4)Galβ(1-6)-[Galβ(1-4)]-Glc referred to as 4PX (**2**, Scheme 1)[17].



Scheme 1: Structures of the oligosaccharides prepared in this study from three monosaccharide modules.

4PX was extracted as a 2-aminopyridine(PA) conjugate after extensive purification by size exclusion of GOS-mixtures, chemical conversion to PA-conjugates by chemical modification and again HPLC purification, emphasizing the effort needed to obtain pure GOS components. 4PX was recently synthesized by Saito and coworkers using a chemo-

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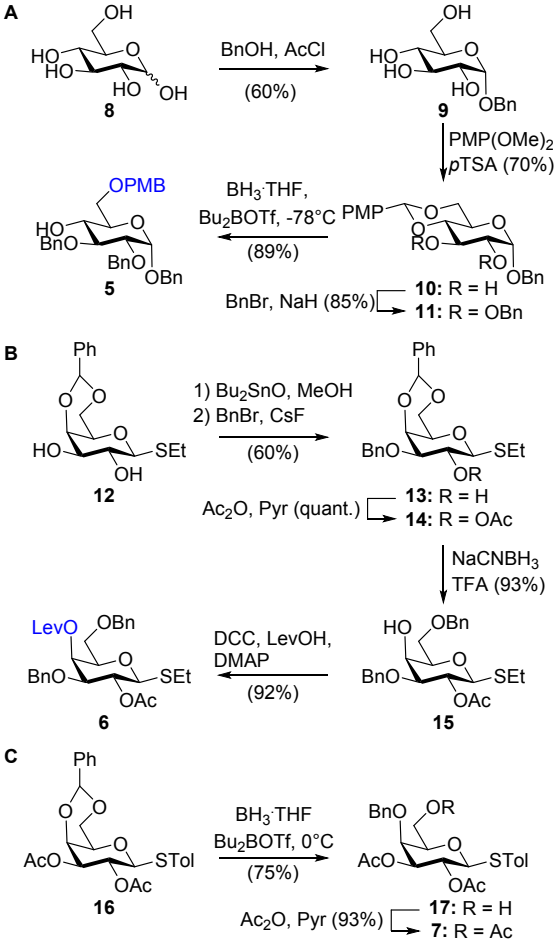
enzymatic approach[18]. However, this approach does not allow for the synthesis of a broad collection of GOS derivatives.

Herein we report the modular chemical synthesis of suspected allergenic GOS component 4PX and three closely related oligosaccharides. The use of orthogonally protected modules in our approach is potentially applicable to the synthesis of a much larger collection of GOS. To test their allergenicity, two of the synthesized GOS structures were tested as pure tetrasaccharides in subjects with confirmed GOS-related allergy in Singapore.

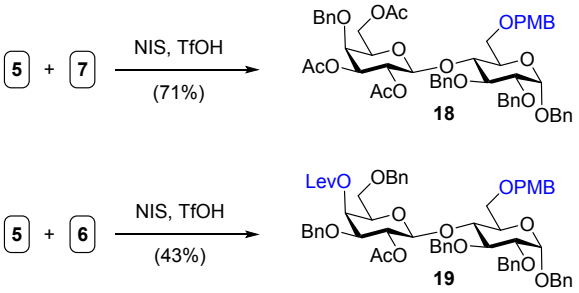
Results and Discussion

The galactooligosaccharides shown in Scheme 1 are made-up of a lactose core carrying β-1,4-galactose extensions at C-4 of the galactose and/or C-6 of the glucose residue. We therefore designed three monosaccharide modules to enable the synthesis of such GOS components (**1**-**4**) in a modular fashion. The synthesis of the glucose core module **5** was achieved in four steps starting from glucose (Scheme 2). Fischer glycosylation using BnOH/AcCl proceeded uneventfully to afford α-benzylglucoside **9**. Installation of a 4,6-*p*-methoxybenzylidene was carried out next, followed by benzylation of the 2,3-diol to provide **11**.



Scheme 2: Synthesis of the monosaccharide modules.

Regioselective opening of the *p*-methoxybenzylidene to the C-4 hydroxyl using Bu_2BOTf/BH_3·THF under previously reported conditions at -78°C afforded **5** in excellent yield (89%)[19]. Extension module **6** was designed to enable the synthesis of the β-1,4-galactose repeats. C-2 *O*-acetyl participation will ensure β-selective glycosylation while the C-4 Lev ester allows for orthogonal deprotection and subsequent extension. C-3 and C-6 benzyl ethers were used to exclude the possibility of protecting group migration towards the C-4 position during glycosylation. Starting from known compound **12**[20], selective benzylation provided compound **13** in moderate yield (60%). Acetylation of the C-2 alcohol followed by regioselective opening of the 4,6-benzylidene using $NaCNBH_3$/TFA afforded alcohol **15**. Esterification with levulinic acid afforded module **6** in excellent yield (92%). Finally, galactose capping module **7** was prepared from known galactoside **16**[21] by regioselective opening of the 4,6-benzylidene followed by acetylation of the primary alcohol (Scheme 2).



Scheme 3: Synthesis of lactoside precursors **18** and **19**.

With the building blocks in hand, the modular assembly of **1**-**4** was explored next. Two lactose derivatives **18** and **19** were prepared to enable the synthesis of **1**-**3** and **4**, respectively. Glycosylation of **5** with **7** was achieved using the NIS/TfOH promoter system affording **18** in good yield. Under the same conditions, glycosylation of **5** with **6** afforded lactoside **19** in a fair yield.

To prepare **1**-**3**, lactoside **18** was extended and the C-6 position of the glucose residue. To this end, DDQ oxidation was used to deprotect the PMB ether and reveal the C-6 alcohol. Disaccharide **20** was obtained in a moderate yield and a more polar byproduct was also recovered. NMR analysis showed that the C-4 benzyl on the galactose residue had also been removed which is a known side reaction in literature[22]. By limiting the amount of DDQ an improved yield of 61% was obtained. Subsequent glycosylation with **6** proceeded smoothly to afford trisaccharide **21** which after removal of the Lev ester using hydrazine acetate afforded **22** in good yield. Further deprotection by deacetylation and hydrogenation afforded trisaccharide **1**. An additional cycle of glycosylation with **6** and Lev deprotection afforded tetrasaccharide **23**. Lev deprotection using the aforementioned conditions gave rise to acceptor **24** in good yield. Global deprotection of **24** afforded 4PX tetrasaccharide **2** which was in perfect agreement with the synthesized structure by Saito and coworkers[18]. Although the deacetylation as final deprotection step in Saito's study

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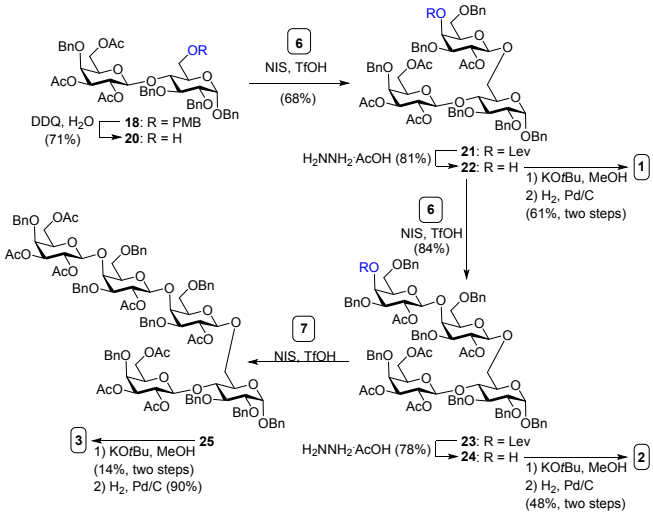
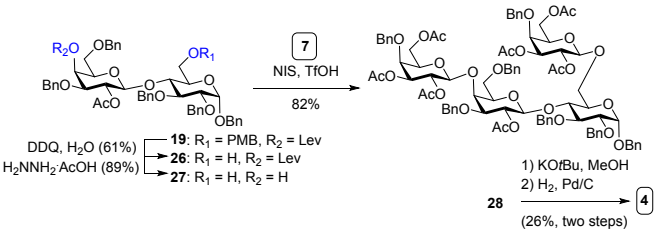
gave a higher yield, our strategy allows for access of various other GOS-epitopes.



Scheme 4: Synthesis of GOS components **1**-**3** using a modular approach.

Alternatively, glycosylation of **24** with **7** using NIS/TfOH afforded pentasaccharide **25**. Though the glycosylation went smoothly, inseparable trisaccharide and trehalose derived byproducts complicated purification. The impurities were removed upon deprotection of **25** using KOtBu/MeOH and subsequent column chromatography. Final deprotection by hydrogenation using Pd/C, H_2 afforded the pure pentasaccharide **3**.

A study by Van Leeuwen *et al.* aimed to identify the oligosaccharides present in a mixture of commercial Vivinal® GOS up to the pentasaccharide[23]. Through extensive chromatographical separation and NMR analysis, over 40 structures were identified, including 4PX (referred to as DP4f1a). The tetrasaccharide in van Leeuwen's study was isolated as a mixture with one other saccharide present, 4PY (**4**). However, initial comparison of our 4PX (**2**) NMR data showed a mismatch with the proposed characterization of 4PX by van Leeuwen *et al*. Extensive 2D-NMR studies of our synthesized 4PX (**2**) confirmed the regiochemistry and a correct structural assignment (see supplementary information), suggesting the isolated compound by Van Leeuwen *et al.* was erroneously assigned as 4PX. The misassignment of isolated compounds from crude Vivinal® GOS underlines the difficulty of characterizing complex mixtures of closely related oligosaccharides and emphasizes the need for the chemical preparation of analytical standards.



Scheme 5: Synthesis of 4PX regioisomer 4PY (**4**) using a double coupling approach.

Next, we set out to prepare regioisomer 4PY (**4**, Scheme 5). Because GOS exists as a microheterogeneous mixture, it is very difficult to reliably establish which component is responsible for biological activity. This is especially true for isobaric components that are regioisomeric. Due to our modular approach, tetrasaccharide **4** was accessible by simply switching the order of assembly without the need to prepare additional monosaccharide building blocks. Furthermore, to shorten the synthesis procedure we opted to install the β-1,4-galactose extensions on the lactose scaffold in a single step. To this end we first prepared the appropriate acceptor diol **27** by a stepwise deprotection of precursor **19** (Scheme 5). Subsequent glycosylation with **7** and NIS/TfOH afforded the desired

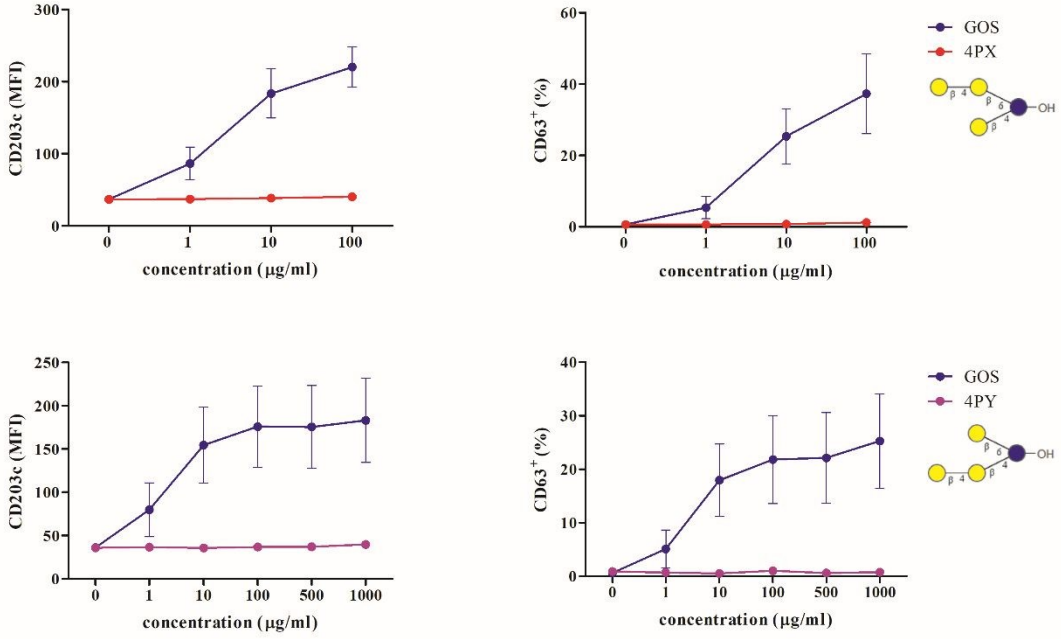


Figure 1: 4PX and 4PY do not induce basophil activation. Expression of CD203c and CD63 on IgE-high cells after stimulating with GOS, 4PX or 4PY were analyzed by using flow cytometry. Results were expressed as mean±SEM. (n=5 in case of 4PX, n=3 in case of 4PY)

tetrasaccharide **28**. Final deprotection as described earlier afforded tertrasaccharide **4** with small aromatic impurities present related to the hydrogenation. The pure compound **4** was obtained after C18-chromatography.

Allergenicity Data

With the synthesized suspected allergenic 4PX (**2**) and regioisomer 4PY (**4**) in hand we set out to study their allergenicity on blood samples from adult subjects with previously confirmed GOS-related allergy in Singapore. The effect of 4PX and 4PY on basophil activation was determined by measuring the expression of the activation markers CD203c and CD63 for each subject. Figure 1 shows that the positive control Vivinal GOS (blue line) induces dose-dependent basophil activation, whereas 4PX (red line) has no effect on basophil activation (top graphs). Interestingly, regioisomer 4PY (bottom graphs) was also found to have no effect on basophil activation. These results indicate that 4PX and 4PY are not the GOS structures causing allergenicity in GOS-allergic subjects in Singapore.

Previous studies in Japanese GOS-allergic subjects revealed that certain tetrasaccharides in GOS induced an allergic response with 4PX identified as the major allergenic component in GOS[17]. Allergenicity of 4PX was not confirmed in our study in Singapore GOS-allergic subjects, suggesting that GOS-related allergy in different regions involves different allergenic epitopes. This may be related to differences in the primary sensitizer, responsible for eliciting the cross-reactivity of GOS; the primary sensitizer is most likely dependent on the local environment. Our research rules out 4PX and 4PY of being the causal allergenic structures in GOS in Singapore, and warrants further research into the causal allergenic epitopes in GOS responsible for GOS-related allergy in Singapore. Finding the causal allergenic epitopes in GOS will facilitate the development of new GOS compositions lacking allergenic structures, to make sure GOS-related allergy will no longer occur in the future.

Conclusions

A modular approach was developed for the efficient synthesis of GOS fragments using three standard building blocks. The method was applied to the successful synthesis of four oligosaccharides and holds the potential to be extended to the synthesis of other GOS fragments consisting of β-1,4-linked galactosides. Extensive NMR analysis of the synthetic oligosaccharides revealed that earlier annotation of extracted GOS components was incorrect, highlighting the necessity of pure analytical standards. More importantly, immunological evaluation of the synthesized tetrassaccharides 4PX and 4PY ruled out both as allergens in GOS-related allergenicity in Singapore, suggesting regional differences in allergenic epitopes.

Conflicts of interest

There are no conflicts to declare.

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