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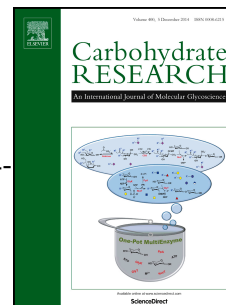
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Characterization of the LM5 pectic galactan epitope with synthetic analogues of β -1,4-D-galactotetraose

Mathias C.F. Andersen, Irene Boos, Susan E. Marcus, Stjepan K. Kračun, Maja Gro Rydahl, William G.T. Willats, J. Paul Knox, Mads H. Clausen



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Graphical Abstract

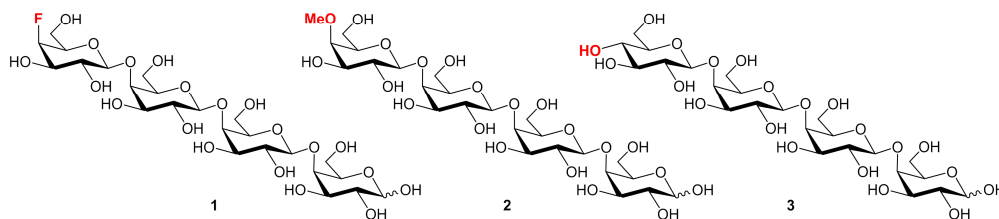
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Characterization of the LM5 pectic galactan epitope with synthetic analogues of β -1,4-D-galactotetraose

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ABSTRACT

Plant cell wall glycans are important polymers that are crucial to plant development and serve as an important source of sustainable biomass. The study of polysaccharides in the plant cell wall relies heavily on monoclonal antibodies for localization and visualization of glycans, using e.g. Immunofluorescent microscopy. Here, we describe the detailed epitope mapping of the mab LM5 that is shown to bind to a minimum of three sugar residues at the non-reducing end of linear β -1,4-linked galactan. The study uses de novo synthetic analogs of galactans combined with carbohydrate microarray and competitive inhibition ELISA for analysis of antibody-carbohydrate interactions.

ARTICLE INFO

1. Introduction

Plant carbohydrates are crucial biomass polymers that we either use directly or process into other products. They are an important source of food and feed ingredients, and constitute a feedstock for bio-based materials and fuels.^[1-3] Plant glycans show an extraordinary diversity and complexity leading to a variety of biological functions as well as physical properties.^[4,5] A major class of highly complex polysaccharides in plant cell walls are pectins, which can be classified into three types: homogalacturonan (HG) and rhamnogalacturonan (RG) I and II. RG-I is a heteropolymer with a backbone of alternating α -linked L-rhamnose and D-galacturonic acid residues with extensive branching of galactans, arabinogalactans and arabinans.^[4,5] Biological studies of individual polysaccharides in plant organs and plant-derived materials are immensely challenging due to their heterogeneity and diversity. Structurally defined oligosaccharides are useful tools as models for the more complex glycan polymers and can be used in investigations of a range of processes, such as cell wall biosynthesis and degradation as well as protein-carbohydrate interactions in general.

Monoclonal antibodies with epitopes found in cell wall polysaccharides are important tools for studying plant glycans *in situ* with immunofluorescence microscopy. For example, this technique has been applied to determine changes in cell wall polysaccharide distributions during plant development.^[6,7] The frequently used rat monoclonal antibody LM5 is known to bind β -1,4-linked galactans.^[8] LM5 has been applied in immunohistochemical analysis, immunofluorescence microscopy, live cell labeling and for glycan microarray screening.^[8-11] The antibody was generated by immunization of rats with a β -1,4-Gal₄-BSA neoglycoprotein. It has been reported that for LM5-binding at least four consecutive β (1 \rightarrow 4) galactosyl units are required as previous studies indicated no interaction with β -1,4-galactobiose and -triose.^[8] Furthermore, we have previously shown that LM5 does not tolerate β -1,6 branching in order to bind to defined galactans.^[12] We were interested in more detailed insight into the binding mode of LM5. From previous work, it

was not clear whether the non-reducing end of linear β -1,4-galactans is part of the LM5 epitope. In order to answer this question, we designed three β -1,4-tetragalactoside analogues **1-3** (Figure 1). In the three synthetic oligosaccharides, the axial C4-OH in the non-reducing end residue was replaced with C4-F, C4-OMe and the C4-epimer, respectively. We envisioned that by either replacing the C4 hydroxy group with the bioisosteric fluorine, blocking it by methylation or changing the orientation by making the *gluco*-analogue, we would be able to learn more about the LM5 epitope. The chemical synthesis of β -1,4-D-galactans can be problematic due to low reactivity of the axially disposed C4-OH of the corresponding galactosyl acceptors. Thus, few approaches have been reported so far.^[13-15] Our group recently published a convergent strategy for the synthesis of linear and branched β (1 \rightarrow 4)-D-galactans.^[12] This approach inspired our pursuit of the three different analogues required for studying the LM5 epitope.

2. Results and Discussion

It was intended to synthesize the analogues with the building blocks **7-11** shown in Figure 1. Thereby, *N*-phenyltrifluoroacetimidate donors could be reacted with pentenyl glycosides as acceptors. Pentenyl glycosides were chosen as intermediates, due to their stability under methylation and fluorination conditions.

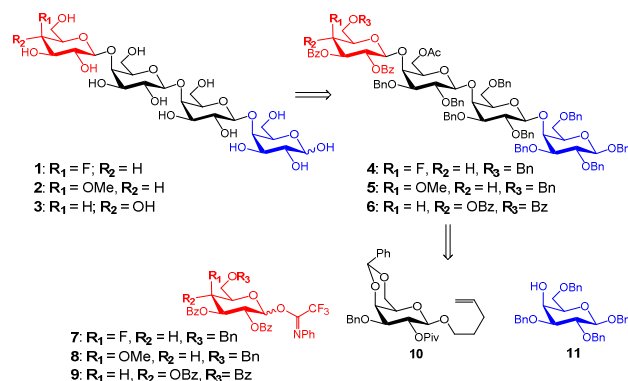
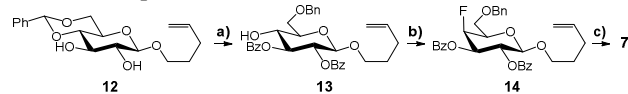


Figure 1: Retrosynthetic analysis of the desired tetragalactan analogues **1–3**.

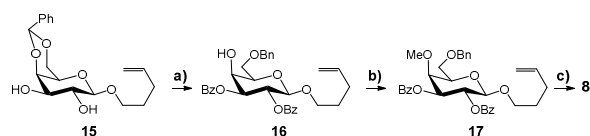
Fluorinated galactoside **14** was prepared in four steps from the known pentenyl glucoside **12** (Scheme 1).^[16,17] The common way of synthesizing fluorinated sugars is the use of diethylaminosulfur trifluoride (DAST).^[18] Unfortunately, this method was not applicable for C4-*F* galactose analogues. Instead, the C4-*OH* of **13** was converted to the corresponding triflate and fluorine was introduced by an S_N2 substitution with tetrabutylammonium fluoride (TBAF) to give **14** in 59% (over two steps).



Scheme 1: a) i. BzCl, Et₃N, DMAP, CH₂Cl₂, 89%, ii. Et₃SiH/TFA, CH₂Cl₂, 74%; b) i. Tf₂O, pyridine, CH₂Cl₂, ii. TBAF, CH₂Cl₂, 85% over two steps; c) i. NBS, MeCN/H₂O (20:1), ii. 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride, Cs₂CO₃, CH₂Cl₂, 5 °C, 59% over two steps.

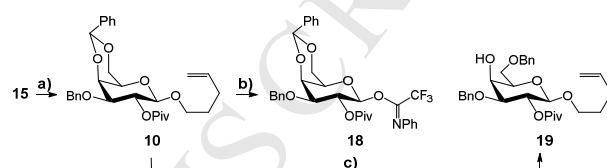
The *O*-4 methylated pentenyl galactoside donor **17** was obtained in three steps from the known diol **15** (Scheme 2).^[19] Alkylation of the C4-*OH* of **16** turned out to be challenging due to the low reactivity and the tendency of the neighboring benzoyl group to either migrate or undergo hydrolysis. A combination of Meerwein's salt and Proton-sponge® gave smooth conversion to **17** in 84% yield without side product formation.^[20] Unfortunately, neither pentenyl donor **14** nor **17** could be used as donors, as they proved to have low reactivity and afford unsatisfactory coupling yields in glycosylation reactions. Instead, the pentenyl glycosides were converted to the more reactive imidate donors **7** and **8** in two steps. The perbenzoylated glucosyl imidate **9** was synthesized following a literature procedure.^[16]

Next step was the synthesis of the trisaccharide acceptor **22**. Starting from pentenyl glycoside **15**, regioselective benzylation of C3-*OH* was performed via the stannylene acetal in a yield of 79% (Scheme 3). Piv-protection of C2-*OH* afforded donor **10**, which was transformed to galactosyl imidate **18** in two steps.



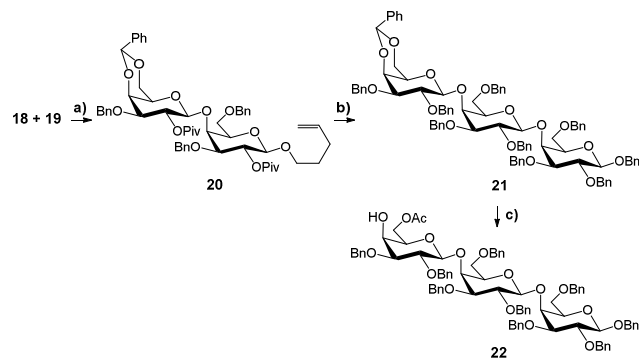
Scheme 2: a) i. BzCl, Et₃N, DMAP, CH₂Cl₂, 89 %, ii. Et₃SiH/TFA, CH₂Cl₂, 0 °C, 72%; b) Me₃OBf₄, Proton sponge, CH₂Cl₂, 84%; c) i. NBS, MeCN/H₂O (20:1), ii. 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride, Cs₂CO₃, CH₂Cl₂, 0 °C, 74% over two steps.

Acceptor **19** was obtained from **10** by regioselective opening of the benzylidene acetal with triethylsilane/trifluoroacetic acid (Et₃SiH/TFA) in 80%.^[21] The regioselectivity was validated by acetylating the liberated C4-*OH*, which resulted in a characteristic downfield shift from 3.98 ppm to 5.08 ppm of the H-4 proton in the NMR spectrum, whereas the H-6 protons resonated at the same frequencies as for the starting material.^[22,23]



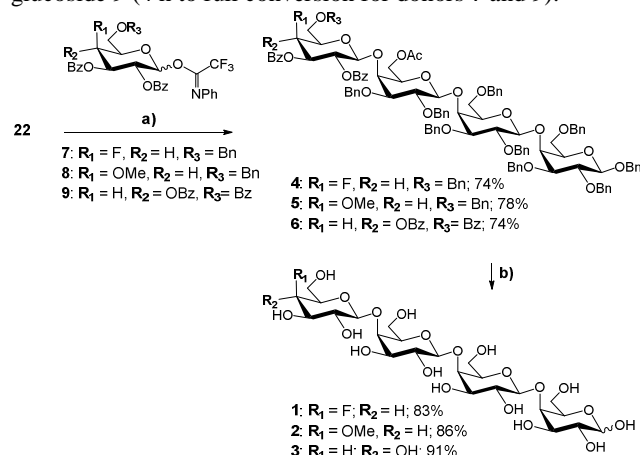
Scheme 3: a) i. Bu₃SnO, then BnBr, CsF, toluene, 79%, ii. PivCl, Et₃N, DMAP, CH₂Cl₂, 92 %; b) i. NBS, acetone/H₂O (9:1), 2,6-lutidine, 50 °C, ii. 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride, Cs₂CO₃, CH₂Cl₂, 75% over two steps; c) Et₃SiH/TFA, CH₂Cl₂, 80%.

Trimethylsilyl trifluoromethanesulfonate (TMSOTf) catalyzed coupling of donor **18** and acceptor **19** afforded disaccharide **20** in an excellent yield of 81% (Scheme 4). Disaccharide **20** was used as donor in the following glycosylation of monosaccharide acceptor **11**, synthesized according to Andersen *et al.*^[12] After the glycosylation reaction, the pivaloyl groups were exchanged by benzyl ethers in a two-step procedure in 82% to give trisaccharide **21**. This maneuver was elected, because we were concerned that the relatively harsh basic conditions needed for deesterification could lead to elimination of hydrogen fluoride for the fluorinated analogue. Regioselective opening of the benzylidene acetal of **21** proved challenging. No conversion was observed upon treatment with NaCNBH₃/HCl^[24] or NaCNBH₃/I₂^[25], whereas Et₃SiH/TFA^[21] and Et₃SiH/Cu(OTf)₂^[26] only resulted in slow hydrolysis. Instead, the acetal was hydrolyzed with ethanethiol and *p*-toluenesulfonic acid (*p*-TSA) followed by a selective acetylation of C6-*OH* to yield **22** (Scheme 4).



Scheme 4: a) TMSOTf, CH₂Cl₂, -40 °C, 81%; b) i. **11**, NIS/TESOTf, CH₂Cl₂/MeCN (1:1), -40 °C, 86%, ii. KOH, DMSO, iii. BnBr, NaH, TBAI, DMF, 82% over two steps; c) i. ethanethiol, *p*-TSA, CH₂Cl₂, 94%, ii. Ac₂O, Et₃N, CH₂Cl₂, 88%.

TMSOTf-catalyzed glycosylation of acceptor **22** with the three donors **7-9** afforded the corresponding tetrasaccharides **4**, **5** and **6** in good yields of 74-78% (Scheme 5). The experiments were performed under identical reactions conditions in dichloromethane with 5% TMSOTf as promoter. The three reactions were allowed to reach 0 °C, since no conversion was observed at lower temperatures. The methylated donor **8** reacted faster (2.5 h to full conversion) than the electron-poor, fluorinated galactoside **7** and the glucoside **9** (4 h to full conversion for donors **7** and **9**).



Scheme 5: a) TMSOTf, CH₂Cl₂, -20 °C → 0 °C; b) i. NaOMe, MeOH, ii. H₂, Pd(OH)₂, MeOH, THF.

Transesterification with sodium methoxide in methanol followed by hydrogenolysis with Pearlman's catalyst gave the unprotected target molecules **1**, **2** and **3** (Scheme 5). In order to remove traces of Pd(OH)₂/C, purification by C18 reverse-phase column chromatography on C18-modified silica was necessary.

The compounds **1-3** were printed and analyzed using microarrays as described previously.^[12] The result of the microarray analysis is shown in Table 1. The galactans β-1,4-D-Gal₃ and β-1,4-D-Gal₄, previously synthesized in our lab, were used as controls.^[27] β-1,4-D-Gal₄ was used to normalize the signals (highest fluorescence intensity set at 100). The controls showed interaction with LM5, but not with LM6. LM6 is an antibody with high affinity against α-1,5-L-Ara and was used as control. We have previously shown that LM5 does not tolerate branching when binding to galactans and that introduction of arabinose as the terminal, non-reducing end residue precludes binding.^[12] According to the original report, LM5 requires at least four consecutive galactose units to bind.^[8] Surprisingly, we could see binding with the trisaccharide β-1,4-D-Gal₃. This result shows that three galactose units are enough for antibody recognition. Binding studies with the three synthesized analogues **1-3** showed noteworthy differences. Moderate intensity was detected with C4-*F* galactan **1**. As an isostere of the β-1,4-D-Gal₄ similar binding behavior was expected and could be seen even though lesser in intensity than β-1,4-D-Gal₄. Similar results were seen with the methylated galactan **2**. It was proposed that methylation will block the C4 position and thus hinder the antibody binding. That could not be confirmed. Interestingly, no binding was detected with the epimeric analogue **3**. This result suggest that the non-reducing end residue of the galactans is a pivotal part of the binding epitope. The configuration at the terminal C4 appears to be important for

the LM5-binding, which explains the similar behavior of the fluorinated and methylated analogues **1** and **2** compared to epimeric analogue **3**.

Table 1: Binding of LM5 monoclonal antibody to galactan analogues **1-3** and linear galactans (β-1,4-D-Gal_{3/4}) on microarray; LM6 antibody (affinity to α-1,5-L-Ara) was used as a control.

Galactan	Antibody	
	LM5	LM6
β-1,4-D-Gal ₃	55	0
β-1,4-D-Gal ₄	100	0
1 - C4- <i>F</i>	34	0
2 - C4- <i>OMe</i>	31	0
3 - C4- <i>epimer</i>	0	0
α-1,5-L-Ara ₄	0	36

In order to confirm that the non-reducing end residue of the galactans is recognized by LM5, we designed a competitive inhibition enzyme-linked immunosorbent assay (ciELISA) experiment to demonstrate that the presence of a higher concentration of smaller oligosaccharides more effectively inhibited the binding of LM5 to an immobilized antigen. Firstly, lupin galactan was titrated in the soluble phase to determine its capacity to inhibit binding of LM5 to immobilized antigen (lupin galactan) and 20 μg/ml was chosen as the amount to act as soluble galactan inhibitor. In order to increase the ratio of non-reducing ends in the solution, in a set of separate tubes the soluble galactan was hydrolyzed with a range of concentrations of endo-β-1,4-galactanase for 15 min at 37 °C. Reactions were stopped by heating at 95 °C for 5 min and samples were then decanted to act as the soluble phase in LM5 ciELISAs. Figure 2 shows that at low enzyme concentrations enzyme-treated galactan was a more effective inhibitor than the untreated sample, demonstrating a higher capacity for binding to LM5. Increasing the enzyme concentration leads to less inhibition, this is due to degradation to β-1,4-D-Gal₂, which is not recognized by LM5. These data confirm the microarray results by illustrating a higher inhibitory effect from the presence of a higher concentration of smaller galactans.

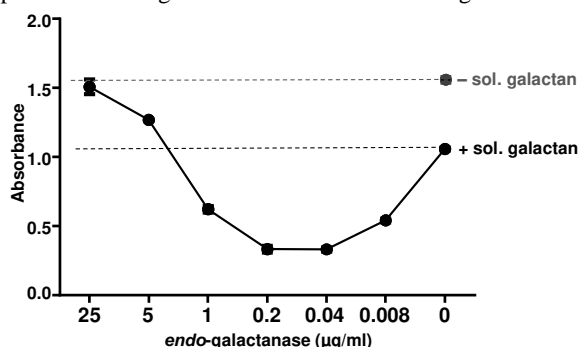


Figure 2: LM5 ELISA showing capacity of the pre-treatment of 20 μg/ml lupin galactan (soluble galactan) with a range of endo-β-1,4-galactanase concentrations to modulate LM5 binding to immobilized antigen. At low concentrations of enzyme the inhibitory capacity of soluble galactan increases. Means and SD of three independent experiments shown.

In conclusion, the synthetic oligosaccharide microarrays and enzyme-ciELISAs indicate that LM5 recognition of pectic 1,4-galactans by LM5 requires a non-reducing end residue and that LM5 does not bind to internal regions of linear galactans. This extended understanding of the LM5 epitope has implications for the interpretation of LM5 binding in *in situ* analyses and other immunoassays.

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Acknowledgments

Characterization of the LM5 pectic galactan epitope with synthetic analogues of β -1,4-D-galactotetraose

Highlights:

- The synthesis of three analogs of β -1,4-D-galactotetraose
- Efficient, mild methylation of Gal 4-OH with Meerwein's salt and Proton-sponge
- Immobilization of oligosaccharides onto microarrays
- Detailed epitope mapping of mAb LM5 using carbohydrate microarrays and ciELISA