A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201806871
Angew. Chem. 10.1002/ange.201806871

Link to VoR: http://dx.doi.org/10.1002/anie.201806871
http://dx.doi.org/10.1002/ange.201806871

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10.1002/anie.201806871

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Tailor-Made Polysaccharides with Defined Branching Patterns by Enzymatic Polymerization of Arabinoxylan Oligosaccharides

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Abstract: Polysaccharides from plant biomass are explored extensively as renewable resources for the production of materials and fuels. However, the heterogeneous nature of non-cellulosic polysaccharides such as arabinoxylan makes it difficult to correlate molecular structure with macroscopic properties. To study the impact of specific structural features of the polysaccharides on e.g. crystallinity or affinity to other cell wall components, collections of polysaccharides with defined repeating units are required. Herein, a chemo-enzymatic approach towards artificial arabinoxylan polysaccharides with systematically altered branching patterns is described. The polysaccharides were obtained by glycosynthase-catalyzed polymerization of glycosyl fluorides derived from arabinoxylan oligosaccharides that were procured either chemically, chemo-enzymatically, or from a commercial source. X-ray diffraction and adsorption experiments on cellulosic surfaces revealed that the physico-chemical properties of the synthetic polysaccharides strongly depend on the specific nature of their substitution patterns. The presented strategy of combining sophisticated carbohydrate synthesis with glycosynthase technology offers access to artificial polysaccharides for structure-property relationship studies that are not accessible by other means.

Cellulose and xylan are the major polysaccharides in lignocellulosic biomass and as such are promising renewable resources for the production of materials and fuels.[1] While cellulose is regularly utilized for these purposes, the exploration of xylan lags behind due to its degree of structural complexity.[2] In all higher plants xylans possess a backbone consisting of β-1,4-linked D-xylopyranoses that are decorated with arabinose, glucuronic acid, and acetyl groups (Fig. 1). The degree and type of substitution differs substantially among plant species. In grasses and cereals for instance, L-arabinofuranose residues are the major substituents, while in soft- and hardwoods D-glucuronic acids or its 4-*O*-methyl derivatives are mostly found.[3] Xylans are used in the food industry as stabilizers, additives, and as ingredients in functional foods.[4] Non-food applications range from their use as oxygen barrier films for packaging[5] to strengtheners in paper production.[6]

The molecular composition of xylans has a strong impact on their macroscopic properties. A decrease in arabinose-substitution is accompanied by a decrease in solubility[7] and crystallinity as the substituents disrupt the interactions between individual xylan chains.[8] Adsorption of xylan to cellulose also typically increases with lower degrees of substitution.[7, 9] Interestingly, the substitution pattern in glucuronoxylans is not random and recently it was shown by solid-state NMR spectroscopy of intact plant cell walls[10] and molecular dynamics simulations[11] that only xylan domains with evenly spaced substituents interact with the hydrophilic surface of cellulose microfibrils. This implies that the discrete substitution pattern in xylan strongly affects the ability of xylan to bind to cellulose, which determines the strength of the xylan-cellulose network in the cell wall and thus of the respective plant material.

Detailed investigations into structure-property relationships of complex polysaccharides are hampered by their inhomogeneity as they are obtained by extraction from natural sources. Polysaccharides with defined branching patterns can only be obtained synthetically using a bottom-up approach. Enzymatic polymerizations using glycosynthases[12] offer a fast and convenient synthetic route towards artificial polysaccharides. Glycosynthases are mutated glycosyl hydrolases in which the catalytic nucleophile in the active site is exchanged for a non-nucleophilic residue, thus inactivating the hydrolytic reaction. Instead, the enzyme is able to irreversibly catalyze the ligation of activated donors, such as α-glycosyl fluorides, to suitable acceptor substrates, forming new glycosidic bonds. The yields provided by glycosynthases are generally higher than with the respective wild-type glycoside hydrolases.[7, 9a, 13]

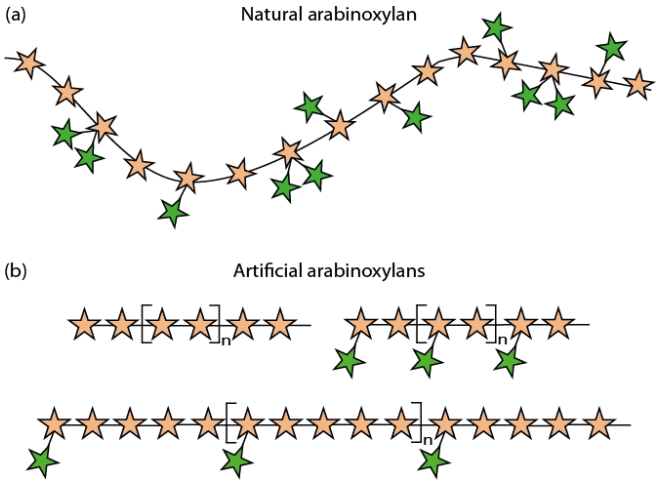

Figure 1. Schematic representation of (a) a natural xylan with random branching pattern and (b) artificial arabinoxylans with defined branching patterns.

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10.1002/anie.201806871

When glycosynthases based on *endo*-glycosylases are used, the activated glycosyl donor acts as both the donor and acceptor, resulting in a polymerization reaction. In this way polysaccharides of different classes,[13a, 14] with molar masses up to 60 kDa, have been produced.[14a, 14b, 15] Xylanase-derived glycosynthases have also been reported and used to synthesize homopolymers of xylan.[14d-f] Substituted xylan polysaccharides have not been synthetically prepared. Herein, we present the synthesis of artificial arabinoxylan polysaccharides with defined branching patterns by glycosynthase-catalyzed polymerization of a number of different oligosaccharide fluorides.

To catalyze the polymerization of the arabinoxylan oligosaccharide fluorides, we chose the glycosynthase XynAE265G that is able to produce xylan homopolymers containing more than 100 monosaccharide units.[14d] This glycosynthase is derived from a xylanase belonging to the glycoside hydrolase (GH) family 10 (Carbohydrate Active Enzyme database).[16] GH10 xylanases typically release small oligosaccharides that carry arabinose substituents in the 2- and/or 3-position of the non-reducing xylose residue as hydrolysis products from natural arabinoxylan polysaccharides.[17] Based on this specificity, we designed seven arabinoxylan oligosaccharides (Fig. 2) to serve as monomers for the polymerization reactions.

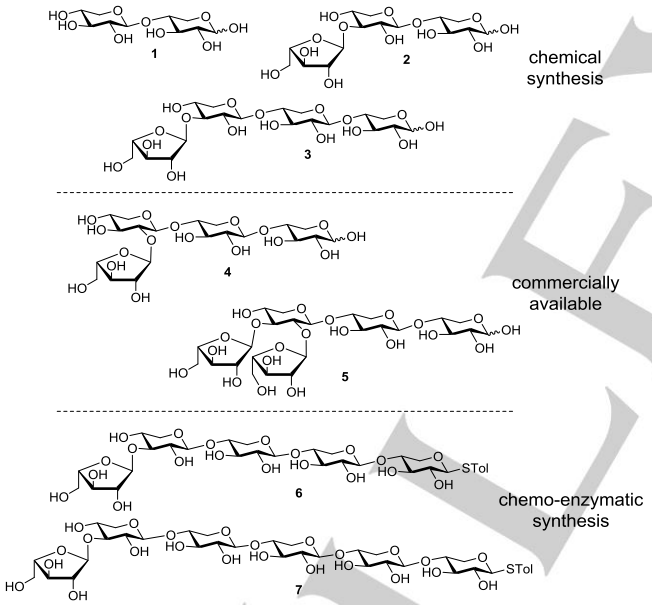
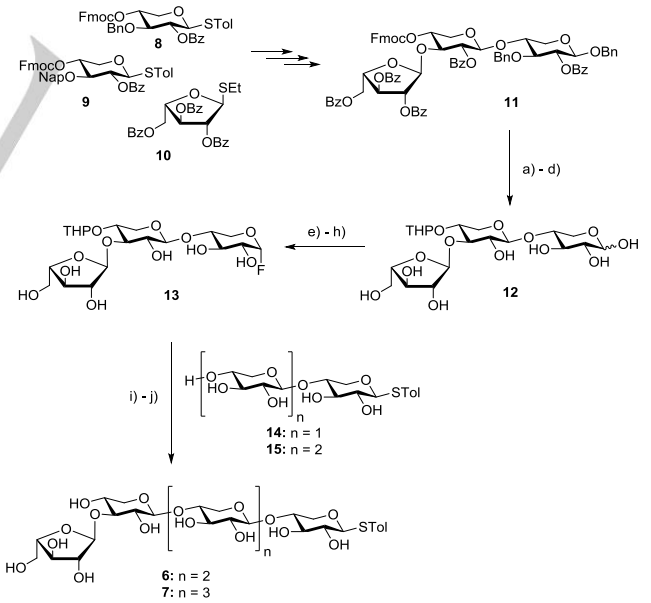


Figure 2. Oligosaccharide monomers for the synthesis of arabinoxylan polysaccharides. **1**-**3** and **6** and **7** were produced by chemical and chemo-enzymatic synthesis, respectively. **4** and **5** were commercially available.

Xylobiose **1** and α-1,3-arabinofuranosyl substituted xylan tri- and tetrasaccharides **2** and **3** were chemically synthesized following the same protecting group strategy as we have used previously for the automated glycan assembly of arabinoxylan oligosaccharides[17d, 18] (see Supporting Information). α-1,2-Arabinofuranosyl- and α-1,2-α-1,3-arabinofuranosyl-disubstituted oligosaccharides **4** and **5** were procured in limited amounts commercially. Larger α-1,3-arabinofuranosyl-substituted xylan oligosaccharides **6** and **7** were obtained by a chemo-enzymatic approach (Scheme 1), in which arabinoxylan trisaccharide donor **13** was ligated to di- and trixyloside acceptors **14** and **15** using the glycosynthase XynAE265G.[14d] To prevent enzyme-catalyzed self-condensation, **13** was equipped with a tetrahydropyranyl (THP) ether in the C4-position of the non-reducing xylose residue. The THP group had successfully been used in the step-wise enzymatic synthesis of cellodextrins[19] and xyloglucan oligosaccharides previously.[20]

Glycosyl donor **13** was prepared from the monosaccharide building blocks **8**-**10** that already served as starting materials for the synthesis of oligosaccharides **1**-**3**. After assembly of trisaccharide **11**, the Fmoc-protecting group was exchanged by a THP-ether, and subsequent methanolysis of the benzoyl esters and hydrogenolysis of the benzyl ethers provided 4'-*O*-THP-protected trisaccharide **12**. Peracetylation of **12** was then followed by fluorination with HF/pyridine at low temperature to provide the corresponding glycosyl fluoride.[21] Reaction temperature and time were carefully adjusted to ensure formation of the thermodynamically favored α-fluoride without cleavage of the rather acid-labile arabinofuranose substituent. The THP-group did not stay intact under these reaction conditions and was subsequently reintroduced at the C4'-position using 3,4-dihydro-2*H*-pyran (DHP) and catalytic amounts of *p*TsOH.[22] Removal of the acetate groups by treatment with 1 equiv sodium methoxide at 0 °C finally afforded THP-protected glycosyl fluoride **13**. Overnight-incubation of glycosyl fluoride **13** with either acceptor **14** or **15** and the



Scheme 1. Chemo-enzymatic synthesis of penta- and hexaarabinoxylosides **6** and **7**. Reagents and conditions: a) NEt_3, DCM, 2 h, rt, 90%; b) DHP, *p*-TsOH, DCM, 4 h, rt; c) NaOMe, MeOH/DCM, 16 h, rt, 66% over 2 steps; d) H_2, Pd/C, MeOH/EtOAc/$Na_xH_xPO_4$ buffer, rt, 24 h; e) Ac_2O, py, 4 h, rt; f) HF/py, DCM, -30 °C → -20 °C, 2 h, 60% over 3 steps; g) DHP, *p*-TsOH, DCM, 4 h, rt, 91%; h) NaOMe, MeOH, 0 °C, 96%; i) **14** or **15**, XynAE265G, rt, $Na_xH_xPO_4$ buffer, 16 h; j) 1 M HCl, rt, 30 min, **6**: 78%, **7**: 76% over 2 steps. For the synthesis of **14** and **15**, see Supporting Information.

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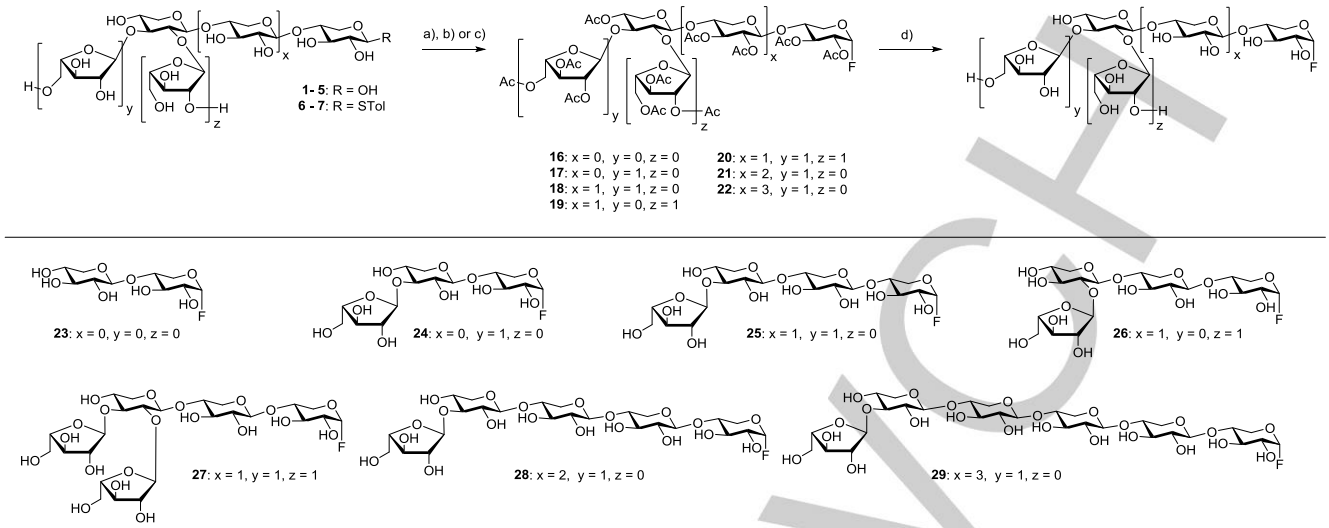
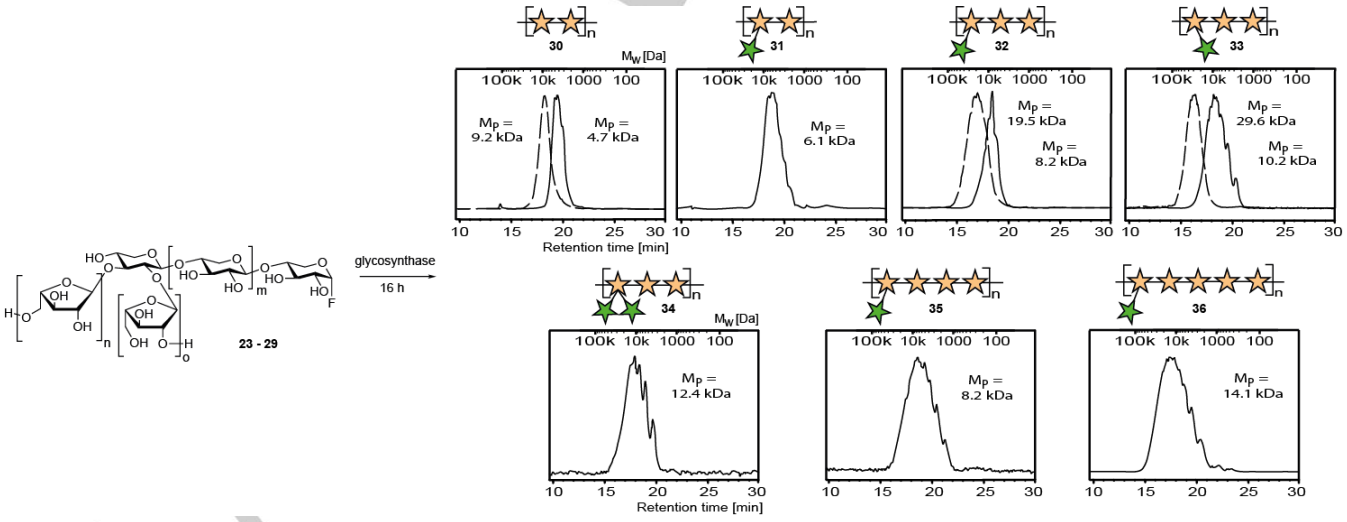


Scheme 2. Synthesis glycosyl fluoride donors **23**–**29**. Reagents and conditions: a) Ac_2O, py, rt,; b) only for **18** and **20**: i) N_2H_4·AcOH, DMF, 0 °C, 5 h, ii) DAST, DCM, 0 °C, 1 h, c) NIS, cat. HF/py, -50 °C (for **21** and **22** only), HF/py, DCM, -30 °C → -10 °C, 2-5 h, **16**: 79%, **17**: 89%; **18**: 77% over 3 steps, **19**: 43%, **20**: 44% over 3 steps, **21**: 53% over 2 steps, **22**: 52% over 2 steps; h) NaOMe, MeOH, 0 °C, **23**: quant., **24**: quant.; **25**: quant., **26**: quant., **27**: 96%, **28**: 99%, **29**: quant.

glycosynthase XynAE265G, followed by removal of the THP-protecting group, gave the desired penta-and hexasaccharides **6** and **7** in good yields. The thioether moiety in **6** and **7** provided a good hydrophobic handle for purification and permitted their later conversion into the corresponding glycosyl fluorides.

The conversion of arabinoxylan oligosaccharides **1**-**7** into glycosyl fluorides **23**-**29** was performed by a three step sequence consisting of peracetylation, fluorination, and deacetylation (Scheme 2).[21] In the case of **16**, **17** and **19**, fluorination was performed using HF/pyridine at low temperature. Activation of the peracetylated oligosaccharides derived from thioglycosides **6** and **7** required the admixture of NIS to activate the thioether leaving group.[23] A milder procedure was applied to oligosaccharides **3** and **5**, as the usage of a larger excess of HF/pyridine provoked cleavage of the arabinofuranose. The hydroxyl group at the anomeric center was selectively deprotected using hydrazine acetate first, and the resulting hydroxyl group converted with *N*,*N*'-diethylaminosulfur trifluoride (DAST) into the corresponding β-fluoride. The β-fluorides were finally equilibrated into the desired α-fluorides **25** and **27** using only small amounts of HF/pyridine.[24]



Scheme 3. Synthesis of artificial arabinoxylan polysaccharides by enzymatic polymerization of arabinoxylan oligosaccharide fluorides. The molecular mass distribution of the resulting polysaccharides was analyzed by HPSEC chromatography using a TSK gel 3000 column and an ELSD detector. Chromatograms with two ELSD traces were obtained when a water-insoluble polysaccharide fraction (dashed line) and a water-soluble fraction (solid line) was obtained. These fractions were analyzed separately.

With the desired arabinoxylan glycosyl fluorides in hand, enzymatic polymerizations were performed by overnight incubation of the respective glycosyl fluoride with XynAE265G, and arabinoxylan polysaccharides in amounts up to 55 mg (**31**) in one batch were obtained (Scheme 3). In some cases, precipitates of water-insoluble polysaccharides (**30a**, **32a**, and **33a**) were formed during the reactions while in the other cases the reaction products remained fully soluble. When precipitated polysaccharides were formed, they were collected by centrifugation and the remaining solution was passed through a short C18 cartridge to isolate the soluble polysaccharide fraction (**30b**, **32b**, and **33b**). Water-soluble polysaccharide products were directly subjected to C18 reversed-phase chromatography. MALDI mass spectrometry was used to confirm the formation of oligomers of the starting oligosaccharides (see Supporting Information). Further conformation was obtained by digestion of the synthetic polysaccharides with xylanases,[25] which resulted, unlike in the case of natural rye arabinoxylan, in the formation of very simple and defined hydrolysis products, a fact that may be exploited for determining the substrate specificity of newly discovered xylan-degrading enzymes (see Supporting Information). The molecular mass distribution of the products was derived from high-performance size-exclusion chromatography (HPSEC) calibrated with pullulan standards (Fig. 2) since it cannot be deduced from mass spectrometry experiments due to the size dependent molecule desorption from the MALDI matrix.[26] We found that the molecular weight at the peak maximum (M_P) of the different polysaccharide products ranged from 4.7 kDa for the water-soluble fraction of linear xylan **30** to 29.6 kDa for the water-insoluble fraction of arabinoxylan **33**. These values correspond to 36 (n = 18) and 224 (n = 56) monosaccharides, respectively. The observed differences in the degree of polymerization (DP) of the artificial polysaccharides are most likely a result of the substrate specificity of the enzyme that transfers some of the arabinoxylan oligosaccharides more efficiently than others.

There is an inherent limitation for the maximum achievable lengths of the synthetic xylan polysaccharides that is determined by their solubility. Thus, the M_p of the linear xylan polysaccharide **30** for example did not exceed 9.2 kDa. Besides for the linear xylan, water-insoluble polysaccharide fractions were obtained for arabinoxylans **32** and **33** which carry the arabinofuranosyl substituents at the 2- and 3-position of every third xylose residue, respectively. In line with this, for arabinoxylans **32** and **33** significant crystallinity was observed whereas **31**, **35**, **36**, as well as natural rye arabinoxylan having differently spaced substituents were completely amorphous, as qualitatively determined by X-ray diffraction (Fig. 3a). The specific substitution pattern of arabinoxylans **32** and **33** might favor a stable threefold helical screw conformation, with the substituents all pointing in the same direction.[27] Such a conformation would enable interactions between the xylan backbones of individual molecules, explaining the low solubility and the crystallinity of **32** and **33.** This is contradicting the commonly accepted notion that solubility and crystallinity of xylans directly correspond with the degree of substitution.[7, 9a]

Recent studies show that in intact plant cell walls xylan partially flattens from the typical threefold helical screw into a twofold helical screw ribbon to enable intimate binding to cellulose microfibrils.[10a, 11, 28] An even pattern of xylan substitution with acetyl and/or glucuronic acid residues appears to be essential for this interaction to occur and consequently for the development of normal plant secondary cell walls.[29] In order to study the ability of synthetic xylan polysaccharides **30**-**32** to adsorb and bind to cellulose, we performed QCM-D experiments on spin-coated cellulose surfaces (Fig. 3b). As expected, unsubstituted xylan **30** showed the highest adsorption of the synthetic polysaccharides to the cellulose surface. More interestingly, we observed that arabinoxylan **31** adsorbed irreversibly, i.e. only a minor desorption upon rinsing, to the cellulose surface while the water-soluble fraction of arabinoxylan **32**, despite the fact that it is less substituted, only adsorbed weakly with a large desorption upon rinsing (both polysaccharides having similar molecular weights). Since both these polymers should have similar driving forces for their adsorption, this result indicates that, once adsorbed, **31** has experienced a conformational change from a threefold to a twofold helical screw, permitting strong interactions between **31** and the cellulose surface. Unlike **31**, **32** cannot adopt a twofold helical screw conformation with the substituents all pointing in the same direction. Natural rye arabinoxylan, which was enzymatically hydrolyzed to a similar size as the synthetic xylan polysaccharides, showed stronger adsorption properties than **32** (more comparable to synthetic xylan **30**), indicating that it does not contain large amounts of unevenly substituted xylan domains. Our study provides direct support by physical *in vitro* experiments outside the cell wall for the observation that specific substitution patterns are required for strong xylan-cellulose interactions and the first hint that, besides acetylation and

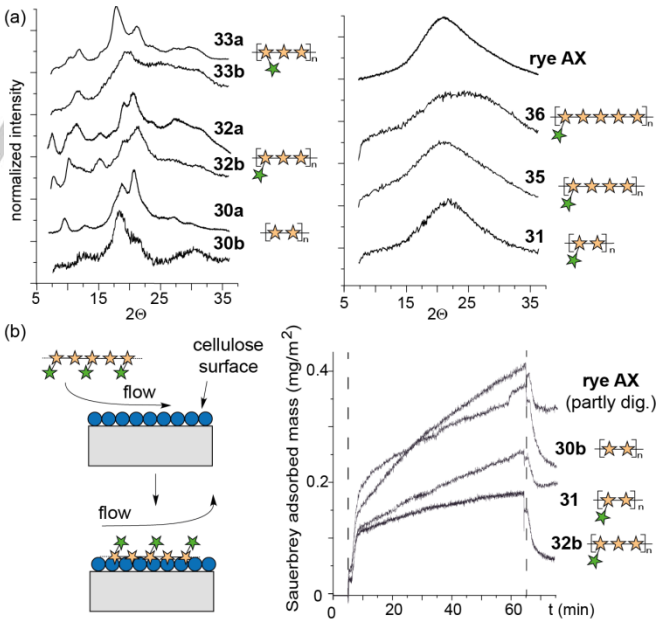


Figure 3. (a) Influence of the arabinose substitution patterns of the synthetic polysaccharides on crystallinity, as determined by x-ray diffraction; **rye AX**: rye arabinoxylan. (b) Adsorbed amounts (g/m^2) (including immobilized water) of **30b**, **31**, **32b**, and partly digested rye arabinoxylan (M_P = 5.5 kDa) on spin-coated model cellulose surfaces, as determined by QCM-D.

substitution by glucuronic acid, also the patterning of arabinose substituents on the xylan backbone may be important. These interactions influence both strength and digestibility of plant cell walls.

In summary, we have explored glycosynthase technology for the polymerization of seven mostly chemically synthesized arabinoxylan oligosaccharides into artificial polysaccharides with well-defined branching patterns. The obtained polysaccharides contained polysaccharide chains with molecular masses up to 80 kDa (606 monosaccharides), which are to the best of our knowledge the largest polysaccharides produced by glycosynthase technology to date. Due to the defined nature of the polysaccharide branching patterns, specific properties were observed for particular members of the prepared arabinoxylan collection rather than the simple linear correlation between e.g. crystallinity and degree of substitution reported previously.[9a] Currently, we evaluate their immunomodulatory properties[30] as well as their potential to serve as substrates in assays that aim at determining the specificities of xylan-degrading enzymes.[25b, 25c]

Acknowledgements

We gratefully acknowledge financial support from the Max Planck Society and the German Research Foundation (DFG, Emmy Noether program PF850/1-1 to FP and SFB 765). The Knut and Alice Wallenberg Foundation is acknowledged for funding the Wallenberg Wood Science Center. We thank Maiko Schulze and Suvrat Chowdhary for experimental help. We thank Ingrid Zenke for recording X-ray diffractograms and Janete Rodriguez and Dr. Luca Bertinetti for helpful discussions. The plasmid for expression of XynAE265G was kindly provided by Prof. Dr. Yuval Shoham (Technion – Israel Institute of Technology).

Keywords: Carbohydrates • Chemo-enzymatic synthesis • Plant cell wall • Xylan • Glycosynthases

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10.1002/anie.201806871

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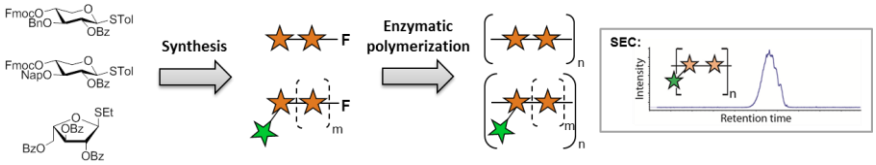

Synthetic designer polysaccharides: Enzymatic polymerization of synthetic arabinoxylan oligosaccharides provided artificial polysaccharides with systematically altered branching patterns. These well-defined arabinoxylans constitute a unique set of tools for structure-property relationship studies.

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