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Chemo-enzymatic synthesis of clickable xylooligosaccharide monomers from hardwood 4-*O*methyl-glucuronoxylan

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Abstract

A chemo-enzymatic pathway was developed to transform 4-*O*-methylglucuronic acid (MeGlc*p*A) containing xylo-oligosaccharides from beechwood into clickable monomers capable of polymerizing at room temperature and in aqueous conditions to form unique polytriazoles. While the gluco-oligosaccharide oxidase (GOOX) from *Sarocladium strictum* was used to oxidize C6-propargylated oligosaccharides, the acid-amine coupling reagents EDAC and DMT-MM were employed and compared for their ability to append click functionalities to carboxylic acid groups of enzyme-treated oligosaccharides. While DMT-MM was a superior coupling reagent for this application, a triazine side product was observed during C-1 amidation. Resulting bifunctional xylo-oligosaccharide monomers were polymerized using a Cu(I) catalyst, forming a soft gel which was characterized by ¹H NMR, confirming the triazole product.

Introduction

Hemicelluloses comprises 20-30% of plant biomass, and while particular composition and structure varies depending on the botanical source, hemicelluloses are thought to function as bridging macromolecules between cellulose fibrils imparting flexibility to plant fibre.¹ This property of hemicelluloses has been harnessed to control the barrier characteristics of cellulosic materials; hemicelluloses have also been used as hydrogels, food additives, and packaging films.^{2–}

The pretreatment of lignocellulosic biomass with pressurized hot water (autohydrolysis) can generate liquid fractions enriched in mono- and oligosaccharides of hemicellulose.⁷ For example, when processing deciduous hardwood, both neutral and acidic xylo-oligosaccharide fragments of 4-*O*-methylglucuronoxylan (GX)⁸ are released. At present, corresponding fractions are typically fermented to commodity chemicals or fuels,⁹ often sacrificing the structure and properties of the native compounds. Alternatively, oxidation of specific hydroxyl groups within terminal sugars of oligosaccharide substrates would generate hemicellulose fragments that are primed for reassembly into novel biopolymers.

While carbohydrates can be oxidized or otherwise modified using chemical techniques, certain features such as low regioselectivity, requirement for protection/deprotection steps, and the use of rare metal catalysts can pose significant challenges.^{10–12} On the other hand, enzymatic oxidation presents an attractive option for regio-selective modification of carbohydrates, especially for more complex structures found in oligosaccharides.

Carbohydrate oxidoreductases are presently classified into various auxiliary activity (AA) families within the Carbohydrate-Active enZYmes database (www.cazy.org).¹³ One such enzyme, gluco-oligosaccharide oxidase (GOOX, SstAA7, EC 1.1.3.-) from *Sarocladium strictum*, catalyzes

the reducing end C-1 oxidation of a wide range of mono- and oligo-pyranose sugars to the corresponding lactone with the concomitant reduction of molecular oxygen to H_2O_2 (Scheme 1).¹⁴ GOOX is a flavoenzyme containing a double covalent linkage to its FAD cofactor. ¹⁵ A variety of GOOX mutants have been produced, in some cases leading to new recombinant enzymes with enhanced substrate specificity, higher catalytic efficiency, and reduced substrate inhibition. ^{16,17}



The pathway presented here employs both existing and enzymatically introduced carboxylic acid groups as platforms for the site-specific introduction of new functionalities via amide linkages.



Scheme 2. Proposed chemo-enzymatic pathway to clickable hemicellulose monomers.

The synthetic route employed to produce bifunctional monomers from acidic xylooligosaccharide fragments capable of click polymerization is shown in Scheme 2. Briefly, the presence of pendant 4-*O*-methylglucuronic acid (MeGlc*p*A) residues in acidic GX fragments (i.e, $U^{4m^2}XX$), together with GOOX-mediated C-1 oxidation, allows for the generation of bifunctional oligosaccharides bearing functionalities for polymerization. Depending on the desired method of

polymerization, potential chemical groups appended to carboxylic acid positions in the sugar structure could include amines or alcohols (polycondensation), vinyl groups (radical polymerization), or even primers for reverse glycoside hydrolase activity. ^{18–20}

In this work, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction was chosen as a mode of polymerization for its high rate of reaction under mild aqueous conditions, and minimal cross reactivity with other functionalities.²¹ In addition to desirable reaction characteristics, the resulting triazole moiety can serve as a hydrogen bond acceptor, facilitate π - π interactions with aromatic rings,²² and strongly coordinate transition metals.²³ These features, together with the attractive properties of native oligosaccharides, could lead to carbohydrate-based polytriazoles with high biocompatibility, solubility, and binding capabilities to cellulosic ²⁴ or metallic ²⁵ surfaces.

Accordingly, primary amines bearing terminal azide or alkyne groups were added to U^{4m2}XX through dehydrocondensation with carboxylic acids at the reducing and non-reducing ends of the oligosaccharide. Whereas acid-amine condensation can be straightforward using high temperature and pressure to drive the removal of water, such processes are not amenable to sugar chemistry due to their relatively low decomposition temperatures (220-315 °C for hemicellulose).²⁶ In addition, other common techniques for amide synthesis employing organic solvents and anhydrous conditions (eg. acyl chlorides, anhydrides) are not compatible with sugars due to their limited solubility in most organic solvents.²⁷ Therefore, amide linkages were synthesized in this pathway using the water-soluble coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). Although EDAC is the most widely applied reagent for aqueous acid-amine coupling,²⁸ reactions are often hindered by poor yields, side product formation²⁹ and the requirement for tight pH

control.³⁰ Comparatively, DMT-MM has garnered little attention despite several publications claiming superior performance to EDAC under certain conditions.^{31–33}

In this study, U^{4m2}XX (see Scheme 2) was used as a model substrate for acidic GX fragments, and was purified from commercial beechwood xylan (BWX) according to previously published protocols.³⁴ Subsequent modifications were performed under mild aqueous conditions without the need for protecting groups on the oligosaccharide structure. The resultant bifunctional monomers were then investigated for their ability to polymerize in the presence of a Cu(I) catalyst, and were found to generate a soft gel material at relatively low monomer concentrations. This pathway illustrates a potential route to the valorization of underutilized hemicellulosic side streams produced during the processing of hardwood biomass, and presents both chemical and enzymatic modification methods which are generally applicable to carbohydrate and polysaccharide chemistry.

Experimental section

Reagents

Sodium acetate (98%), formic acid (88%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 99%), propargylamine (98%), N-hydroxysulfosuccinimide (98%), beechwood xylan (BWX), 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 97%), N-methylmorpholine (NMM, 98%), tetrahydrofuran (99%), *tert*-butanol (99.5%, anhydrous), aniline (99.5%), diphenylamine (99%), acetone (99%), phenol, 4-aminoantipyrine (4-AA), and horseradish peroxidase (HRP, 1840 U/mg) were purchased from Sigma (USA) and used as received. 2-(N-morpholino)ethanesulfonic acid (MES, 99.5%), methanol (99%), and CuSO₄ (98.5%) were purchased from Bioshop Inc. (CA) and used as received. Acetic acid (99.7%, Caledon Labs, CA) ethyl acetate (HPLC grade, Caledon

Labs, CA), isopropanol (99.5%, Caledon Labs, CA), ammonium hydroxide (EMD Millipore), azidopropylamine (Click Chemistry Tools, USA), β-xylanase GH10 from *Cellvibrio japonicas* (EC 3.2.1.8, Megazyme, IRL), sodium ascorbate (98%, Bio-Basic Inc., CA) and xylotriose (95%, Megazyme, IRL) were also used as received.

Gluco-oligosaccharide oxidase (GOOX) was obtained from a previously published study. ¹⁷ The recombinant His₆-tagged protein was expressed in *Pichia pastoris* and purified using a GE HisTrapTM column (GE Healthcare, UK). Protein purity was evaluated by SDS-PAGE and protein concentration determined using gel densitometry.

DMT-MM was prepared according to previously published protocols. ³⁵ Briefly, 2.12 g of Nmethylmorpholine and 3.86 g of CDMT were dissolved in 60 mL THF and stirred at room temperature for 30 min before collecting the precipitated DMT-MM by suction filtration.

Chromatographic separation and detection of xylo-oligosaccharides

TLC analyses were performed using silica plates on polyester backing (20 cm x 20 cm; Sigma Aldrich, USA) as the stationary phase for mono- and oligosaccharide analysis, with a mobile phase consisting of ethyl acetate/acetic acid/isopropanol/formic acid/water (25:10:5:1:15), or butanol/pyridine/boric acid/acetic acid (6:4:3:1). Carbohydrates were visualized using a diphenylamine-aniline stain,³⁶ made by dissolving 2 g of diphenylamine and 2 mL aniline in 100 mL acetone followed by the addition of 10 mL concentrated phosphoric acid. After staining, plates were developed in an oven at 85 °C for up to 1 h.

Preparative anion exchange chromatography (AEC) was performed using Dowex 1X8 anion exchange resin (50-100 mesh) in a glass column (2.6 cm ID x 30 cm) using gravity flow, or connected to a BioLogic DuoFlow FPLC unit with a Quadtec UV detector (Bio-Rad, USA) with

flow rates ranging from 0.5 - 2.0 mL/min. MilliQ water was used as the primary eluent, and acidic compounds were eluted using a 0 - 0.4 M ammonium acetate (pH 6.5) gradient. Fractions containing eluted products were desalted by multiple rounds of lyophilization or further purified by size exclusion chromatography (SEC).

Samples were concentrated to $\leq 2 \text{ mL}$ by rotary evaporation prior to SEC using Bio-Gel P2 Extra Fine polyacrylamide gel (Bio-Rad, USA) in a glass column (1.5 cm ID x 100 cm) connected to a BioLogic DuoFlow FPLC unit with a Quadtec UV detector (Bio-Rad, USA) using water as the primary eluent at flow rates between 0.1 - 0.3 mL/min.

Preparation of U^{4m2}XX

For production of (U^{4m2}XX, see **Scheme 2**), 10 g of BWX was added to 1 L of 50 mM sodium acetate buffer (pH 5.0) and boiled for 5 min. After cooling, 1 mL of β -xylanase GH10 from *C*. *japonicas* (500 U, 38 U/mg at 40 C) was added and the solution was incubated with mixing at 40 °C for 72 h. After incubation, the solution was centrifuged (5000 g, 2 x 10 min) and the supernatant was frozen at -80 °C before concentrating by lyophilization to approximately 500 mL. The acidic U^{4m2}XX was then purified from solution by AEC and lyophilized, followed by subsequent purification using SEC.

Enzyme activity assay

GOOX activity was assessed by measuring H_2O_2 production using a chromogenic detection method.¹⁴ Assays were performed in a 96-well microplate at a total volume of 250 µL consisting of 50 mM Tris-HCl (pH 8.0), 0.1 mM 4-aminoantipyrine, 1 mM phenol, 0.5 U horseradish

peroxidase, 16 nM GOOX, and 1 mM substrate. Reactions were incubated at 37 °C, and absorbance was measured at 500 nm using an Infinite M200 spectrophotometer (Tecan, CHE).

Synthesis and assembly of bifunctional xylo-oligosaccharides

Oxidation of propargyl- $U^{4m^2}XX(2)$

GOOX-mediated oxidation was performed in 50 mM Tris-HCl (pH 8.0) buffer at a substrate concentration of 2 mM and enzyme concentration of 160 nM. Reactions were incubated at 37 °C with mixing for up to 4 days.

Chemical amidation of activated acids

EDAC mediated amidation reactions were carried out in milliQ water at an EDAC : carboxylic acid ratio of 1.2 - 2. Acids were first activated by incubation with EDAC for 30 min in 50 mM MES buffer (pH 4.5) with or without the addition of sulfo-NHS, prior to addition of amine at a amine : acid ratio of 1.5 - 2. Reactions were incubated at room temperature with mixing for up to 48 h.

DMT-MM mediated amidation reactions at the C-6 position were carried out in MeOH:H₂O (8:1 or 1:1) at a carboxylic acid concentration of 100 mM. Propargylamine was added at a concentration from 120 - 150 mM, followed by incubation for 30 min at room temperature before addition of 120 - 150 mM DMT-MM. Reactions were incubated at room temperature with mixing for up to 24 h.

DMT-MM mediated amidation at the C-1 position was carried out in MeOH:H₂O (1:1) at a carboxylic acid concentration of 100 mM. Azidopropylamine was added at an amine : acid ratio of 4, followed by incubation for 30 min at room temperature. The reaction temperature was

increased to 50°C and 2 eq DMT-MM were added each hour for 3h, followed by an additional hour of incubation before evaporating the reaction mixture under a stream of air and freezing at - 20°C.

CuAAC click reaction

Click reactions were performed in milliQ water or 1:1 H_2O :*tert*-butanol using equimolar amounts of alkyne and azide groups. Reactions were initiated by addition of 2 mol% CuSO₄ and 10 mol% sodium ascorbate, and mixed at room temperature for up to 24 h.

High resolution mass spectrometry (HR-MS) analysis

Solutions of U^{4m2}XX and derivatives were prepared in MeOH:H₂O (1:1) and injected using a nano-ESI source equipped with a disposable pico-emitter on a Q-Exactive mass spectrometer (Thermo-Fisher, USA), or injected using a micro-ESI source on an Agilent 6538 Q-TOF spectrometer (Agilent Technologies, USA).

Samples run on Q-Exactive were injected at a spray voltage of -1.5 kV or +2.5 kV, capillary temperature of 250 °C, AGC target 1x10⁶, injection time of 100 ms, and resolution of 70,000 at 1 Hz. Data was analyzed using Qual Browser in Thermo Xcalibur (v3.0) software (Thermo-Fisher, USA).

NMR analysis

¹H NMR spectra, COSY and HSQC experiments were performed in D₂O using an Agilent DD2 700 MHz spectrometer equipped with an HFX probe, and data was processed using MestReNova (v11.0.2) software.

Results and Discussion

Production of acidic xylo-oligosaccharide fragments (U^{4m2}XX)

To generate uniform $U^{4m^2}XX$ (2) fragments, solubilized beechwood xylan was digested using a β -xylanase GH10 from *C. japonicus*, which cleaves the xylan backbone at the non-reducing side of MeGlc*p*A-substituted xylose units.³⁷ During digestion, the release of several soluble carbohydrate compounds was observed after only 2 h of reaction time, with minimal additional increase in concentration over 72 h (Figure S1).

Following GH10 digestion, acidic oligosaccharides were collected using Dowex 1X8 anion exchange resin and concentrated prior to purification by size exclusion chromatography. SEC fractions were analyzed by TLC to determine purity, and at least two larger acidic fragments were observed in addition to $U^{4m2}XX$ (Figure S2). These larger fragments were likely a result of incomplete digestion by GH10 β -xylanase, which could result in additional xylose units on either side of the $U^{4m2}XX$ structure (e.g. $XU^{4m2}XX$ or $U^{4m2}XXX$).³⁸ While the pathway presented here would theoretically accommodate a range of acidic xylo-oligosaccharide structures, a pure starting material was preferred for simplified product analysis and proof of concept. Therefore, SEC fractions containing only $U^{4m2}XX$ were pooled and lyophilized, yielding a light, white powder, which was confirmed by TLC and mass spectrometry (Figure 1).



Figure 1. Mass spectrometry of purified $U^{4m^2}XX$ (centre). TLC comparison of prepared $U^{4m^2}XX$ against $U^{4m^2}XX$ standard, Glc*p*A, and xylotriose (X₃, right) (mobile phase = ethyl acetate/acetic acid/isopropanol/formic acid/water (25:10:5:1:15)).

Amidation of U^{4m2}XX C-6 position using EDAC

To generate bifunctional monomers capable of polymerization via CuAAC click chemistry, terminal azide and alkyne groups were added to U^{4m2}XX sequentially. This was performed by coupling the primary amines, propargylamine and azidopropylamine, to the C-6 and C-1 carboxylic acids of U^{4m2}XX, respectively. These click functionalities were linked to the oligosaccharide structure via amide linkages using the two water-soluble coupling reagents, EDAC and DMT-MM.

Coupling of propargylamine to $U^{4m^2}XX$ using EDAC was performed in aqueous MES buffer (pH 4.5), producing a small amount of propargyl- $U^{4m^2}XX$ (2, <25% yield) together with two neutral side products after 3 hours of reaction time (Figure 2A). During activation of carboxylic acids with EDAC, an active *O*-isoacylurea intermediate is formed which undergoes

nucleophilic attack by the amine. The formation of the active *O*-isoacylurea intermediate occurs most rapidly at pH 4.5; ³⁹ unfortunately, this intermediate is susceptible to hydrolysis and has a half-life of only 4 h at pH 5.0.⁴⁰ In addition, the amine must exist in the deprotonated form for nucleophilic attack, meaning the pH must be elevated to a level near the pKa of the amine after the activation step. These requirements complicate the efficient synthesis of amides using EDAC, and improper control of pH or extended exposure of the activated acid to low pH can significantly decrease yields.

A commonly reported technique for avoiding premature hydrolysis of the O-isoacylurea intermediate is the addition of N-hydroxy(sulfo)succinimide (sulfo-NHS), which forms a more stable NHS-ester intermediate following activation with EDAC.³⁰ After attempting to improve yields using a variety of NHS:EDAC ratios, the results showed no significant improvements and the best yields were still obtained using EDAC alone. As can be seen from the TLC results in Figure 2A, the reaction produced three distinct carbohydrate compounds, which were visualized using a diphenylamine stain (Figure S3). After passing the crude reaction mixture through an anion-exchange column, analysis of the flow through showed that all three products were neutral, suggesting the acid had been modified in each case. It has been shown that the unstable Oisoacylurea intermediate can rearrange to form a stable N-acylurea side product (Scheme 3) if the acid is hindered or if a nucleophile is not present.²⁹ This could account for one of the two neutral side products observed after the reaction, and would render the U^{4m2}XX unavailable for further modification. In addition, the presence of the *N*-acylurea side product together with 2 would pose significant purification challenges, since both compounds are uncharged and have similar molecular weights.

Scheme 3. EDAC intermediate and side product.



Unstable O-isoacylurea intermediate (left) rearranges to stable N-acylurea side product (right).

The three observed neutral reaction products were tested for the presence of alkyne functionality by performing a CuAAC click reaction on the mixture, using azidopropylamine as the azide donor, and monitoring subsequent changes in TLC profile (Figure 2A). The loss of compound **2** in the TLC profile after CuAAC treatment suggested the formation of a triazole product between **2** and azidopropylamine, whereas the remaining neutral side products did not appear to be modified, indicating they did not contain any alkyne functionality.



Figure 2. TLC chromatograms of propargyl- $U^{4m^2}XX$ (2) synthesis (mobile phase = ethyl acetate/acetic acid/isopropanol/formic acid/water (25:10:5:1:15)). A: Crude reaction mixture of EDAC mediated coupling between 1 and propargylamine after 3h (top), neutral

fraction of reaction products after anion exchange chromatography (middle), and neutral reaction products after CuAAC treatment with azidopropylamine (bottom). B: Timecourse of DMT-MM mediated coupling between 1 and propargylamine. U^{4m2}XX (1) was mixed with 1.2 eq propargylamine in MeOH:H₂O (9:2) for 30 minutes prior to initiating the reaction by adding 1.3 eq DMT-MM. Raw TLC data were converted to chromatogram format using ImageJ software.

Ultimately, although **2** was successfully produced using EDAC, the degree of variability in yield and side product formation was deemed too great for reliable and effective use in the synthetic pathway presented here. It is noteworthy that EDAC is most often applied in the modification of polymeric substrates, perhaps indicating that this reagent is not well suited to small molecule coupling.

Amidation of U^{4m2}XX C-6 position using DMT-MM

An alternative synthetic route to propargyl- $U^{4m^2}XX$ (2) was performed using the coupling reagent DMT-MM, resulting in good substrate conversion in less than 1 h of reaction time at 25 °C (Figure 2B, 81% yield). At least two minor side products were also generated during the coupling reaction, the more prominent of which was attributed to methyl ester formation.⁴¹ Best results were achieved in MeOH:H₂O (9:2) with 100 mM U^{4m2}XX and a slight excess of DMT-MM and propargylamine, however comparable results were obtained using a 1:1 ratio of MeOH:H₂O which improved substrate solubility. Since the activated triazine intermediate is less sensitive to pH than the EDAC intermediate, DMT-MM reactions were performed without the use of a buffer, with the final pH of the solution remaining constant after addition of propargylamine at around pH 8.5. Although reactions in water alone were more successful than EDAC coupling, it was found that some amount of organic modifier (MeOH) was necessary to prevent precipitation of the coupling reagent at concentrations above \sim 50 mM.

GOOX oxidation of propargyl-U^{4m2}XX (2)

Prior to C-1 amidation at the reducing end of $U^{4m^2}XX$, a reactive carboxylic acid group was introduced through GOOX oxidation. Since the activity of GOOX has been established on a wide range of oligo-pyranose sugars, the enzyme was expected to show good activity on xylooligosaccharides modified at the non-reducing end C-6 position. In the case of $U^{4m^2}XX$, any modification would be sufficiently far from the reducing end unit to be unlikely to interfere with substrate binding or catalysis. In fact, GOOX activity for propargyl- $U^{4m^2}XX$ was found to be over twice that of $U^{4m^2}XX$, at 56% and 25% activity relative to xylotriose, respectively (Figure 3).



Figure 3. Relative activity of GOOX on xylotriose, $U^{4m^2}XX$ (1), and propargyl- $U^{4m^2}XX$ (2). Activity assays were performed in triplicate at a substrate concentration of 1 mM and enzyme concentration of 16 nM, and measured by detecting H₂O₂ production via a colorimetric method.¹⁴

Although GOOX exhibits maximum activity on cellobiose, activity on xylotriose has been shown to be only slightly lower,¹⁶ and was therefore used to define 100% activity. The increased

activity on propargyl-U^{4m2}XX suggests that a charged side group on the xylan backbone has a more negative effect on enzyme activity compared to a relatively small neutral side group, which is consistent with previously published data.¹⁷

Oxidation of propargyl- $U^{4m^2}XX$ (2) was performed at a substrate concentration of 2 mM with 160 nM wGOOX, and reaction progress was monitored using TLC (Figure 4A). The crude reaction mixture was passed through an anion exchange column, and the acidic product (3) was eluted and lyophilized to a cloudy white solid (60% yield).

Although the enzymatic conversion of 2 appeared to be complete, yield was likely significantly reduced during the purification process of 3, which consisted of isolating the product by anion exchange chromatography followed by desalting and polishing using size exclusion chromatography.

Amidation of U^{4m2}XX C-1 position using DMT-MM

After enzymatic oxidation of the reducing end of propargyl-U^{4m2}XX, amidation of the resulting C-1 acid with azidopropylamine was performed using DMT-MM and reaction progress was monitored by TLC (Figure 4B).



Figure 4. TLC chromatograms of oxidized propargyl-U^{4m2}XX (**3**) production and subsequent conversion to (triazine)-propargyl-U^{4m2}XX-azide (**4**/**6**) (mobile phase = butanol/pyridine/boric acid/acetic acid (6:4:3:1)). A: Timecourse of GOOX oxidation of propargyl-U^{4m2}XX (**2**, 2 mM) performed in Tris buffer (5mM, pH 8.5) at 37°C with two equivalent additions of enzyme at 0h and 24h to a final concentration of 120 nM. B: Timecourse of DMT-MM mediated coupling of **3** and azidopropylamine. **3** was mixed with 3 eq azidopropylamine in MeOH:H₂O (8:1) for 30 min at 5° C before addition of 3 eq DMT-MM, the reaction was then slowly brought to room temperature and another 1 eq of azidopropylamine and DMT-MM were added after 165 minutes. After 230 minutes, the reaction was frozen at -80 °C prior to purification. Raw TLC data were converted to chromatogram format using ImageJ software.

The coupling reaction resulted in the production of at least three new carbohydrate compounds; a feature which was not observed during C-6 amidation. This observation was attributed to the formation of a C-2 triazine side product resulting from nucleophilic attack by the C-2 hydroxyl group on the active ester intermediate (Scheme 3). This unwanted side reaction resulted in excessive consumption of DMT-MM equivalents and reduced substrate amidation. This phenomenon has been previously observed (not published) in situations where a hydroxyl or amine group is present in the α -position to the acid. As shown in Scheme 3, the regenerated acid in the C-2-triazine side product (**5**) is capable of reacting with another molecule of DMT-MM to produce the intended amide linkage, in this case producing compound **6**.

Scheme 3. Formation of C-2 triazine side products during DMT-MM coupling.



Activated ester of oxidized propargyl- $U^{4m^2}XX$ (3, top) undergoes rearrangement to form a C-2 triazine acid (5, middle), which may then react with a 2^{nd} eq of DMT-MM yielding a C-2 triazine amide product (6, bottom).

Despite side product formation, reaction conditions were modified to promote amide formation, while minimizing any premature click reactions at the C-6 end of the oligosaccharide. To achieve this, excess equivalents of both azidopropylamine and DMT-MM were used, and the reaction was performed at reduced temperatures (5 - 25 °C) to avoid non-catalyzed cycloaddition between the azide and alkyne groups in solution. This coupling reaction resulted in nearly complete consumption of **3**, however a significant amount of material remained as the reaction intermediate **5**, causing a decrease in yield of the final products **4** and **6**, which were not well resolved by TLC. Following purification, the reaction products were analyzed by MS and compared with unmodified $U^{4m^2}XX$ (Figure 5).



Figure 5. HR-MS analysis of final bifunctional monomers. U4m2XX starting material (1, top) was compared with final purified mixture of bifunctional clickable monomers (4/6, bottom).

It is worth noting that this issue did not arise during DMT-MM mediated C-6 amidation, due to the lack of an α -hydroxyl group at the C-5 position. As potential options to avoid this issue during C-1 amidation, the C-2 hydroxyl group could theoretically be oxidized to the corresponding ketone using an enzyme such as pyranose dehydrogenase or pyranose-2-oxidase,⁴² or kept in the native acetylated form. This could enhance yields by reducing side product formation and prevent excessive consumption of DMT-MM, and should not dramatically affect the ability of the monomer to polymerize.

CuAAC polymerization of propargyl-U^{4m2}XX-azide monomers

After DMT-MM mediated functionalization of the C-1 position, any unreacted acidic compounds were removed using anion exchange resin and the final bifunctional monomers were purified as a mixture using size exclusion chromatography. The solid product was then dissolved in H2O:*tert*-butanol (1:1) at approximately 1.0 wt% and polymerization was initiated by addition of a CuSO₄/sodium ascorbate solution. The reaction was monitored by TLC (Figure 6A), which indicated the formation of a distribution of larger polysaccharides after 30 min. The TLC profile also revealed a small amount of residual side product in the final monomer which may have had an adverse effect on polymerization, however polymerization of the crude products prior to SEC purification appeared to proceed effectively, yielding a soft gel approximately 30 minutes after the addition of catalyst (Figure 6B).



Figure 6. Click polymerization of propargyl- $U^{4m^2}XX$ -azide monomers. A: TLC monitoring of click polymerization (mobile phase = butanol/pyridine/boric acid/acetic acid (6:4:3:1)). B: Image of gel pellet formed after polymerization of crude monomer mixture.

Polymerization was also performed in D_2O and reaction progress monitored using ¹H NMR (Figure 7). The NMR spectra showed a clear change in profile after 5 min of incubation with the Cu(I) catalyst. Most notably, the loss of the terminal alkyne proton (t, 2.69 ppm) and downfield

shifting of methylene protons adjacent to the appended azide group are consistent with the formation of a triazole product. ⁴³ The presence of a C-2 triazine adduct in compound **6** resulted in slightly shifted peak positions for the methylene protons at the C-1 position of the oligosaccharide (protons d-f) compared to those in compound **4** (protons a-c), with peak areas suggesting approximately equal concentrations of each compound in the final product mixture.



Figure 7. NMR analysis of click polymerization performed in D₂O at 40°C. Additional COSY and HSQC experiments were performed to confirm peaks assignments.

Generally, the aromatic triazole proton (g') is found at a chemical shift close to 8.0 ppm, but was not observed in this experiment. ⁴⁴ This absence could be attributed to the rapid relaxation time often associated with aromatic systems, which may have broadened the peak to a point at which it could not be observed. In addition, further broadening may have been caused by coordination of newly formed triazole rings with copper ions in solution and/or by aggregation between polymer chains.

Overall, polymerization of the monomers proceeded to near completion after approximately 1 hour following addition of the Cu(I) catalyst, suggesting that the presence of a C-2 triazine adduct did not have a significantly adverse effect on the assembly of oligosaccharide monomers.

Conclusion

In this study, a clickable oligosaccharide monomer was synthesized from beechwood xylan using both enzymatic and chemical transformations to introduce terminal azide and alkyne functionalities. Modification of C-6 acids was performed efficiently using the coupling reagent DMT-MM, however, amidation at the C-1 position was hindered by formation of a C-2 triazine side product. Further experiments in which the C-2 hydroxyl group is modified, for example through enzymatic oxidation or acetylation, are needed to resolve this issue. Despite side product formation, synthesized monomers were capable of polymerizing via CuAAC click chemistry, producing a soft gel material with potentially unique characteristics in areas such as cellulose binding and coordination of transition metals. With only minor modifications, this pathway can be designed to accept any sugar fragments containing at least 2 carboxylic acid groups, and could serve as a potential route to the valorization of both neutral and acidic hemicellulose fractions from biomass processing side streams.

Supporting Information

TLC analyses of beech wood xylan digestion with β -xylanase GH10; acidic xylo-oligosaccharides recovered by size exclusion chromatography following GH10 β -xylanase treatment; EDAC coupling of propargylamine and U^{4m2}XX; propargyl-U^{4m2}XX synthesis via DMT-MM; propargyl-U^{4m2}XX oxidation via GOOX; and DMT-MM mediated coupling between oxidized propargyl-U^{4m2}XX and azidopropylamine.

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ABBREVIATIONS

AEC, anion exchange chromatography; BWX, beechwood xylan; CuAAC, copper-catalyzed azide-alkyne cycloaddition; DP, degree of polymerization; GOOX, gluco-oligosaccharide oxidase; MW, molecular weight; SEC, size exclusion chromatography; X₃, xylotriose

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