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Ruthenium(II)- and copper(I)- catalyzed synthesis of click-xylosides and assessment of their glycosaminoglycan priming activity

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ABSTRACT

Xylosides are small molecules that serve as primers of glycosaminoglycan biosynthesis. Xyloside mediated modulation of biological functions depends on the extent of priming activity and fine structures of primed GAG chains. In earlier studies, copper (Cu) catalyzed synthesis of click-xylosides and their priming activity were extensively documented. In the current study, ruthenium (Ru) mediated catalysis was employed to synthesize xylosides with a 1,5-linkage between the xylose and the triazole ring instead of a 1,4-linkage as found in Cu-catalyzed click-xyloside synthesis. Mono- and bis- click-xylosides were synthesized using each catalytic method and their glycosaminoglycan priming activity was assessed *in vitro* using a cellular system. Ru-catalyzed click-xylosides showed a higher priming activity as measured by incorporation of radioactive sulfate into primed glycosaminoglycan chains. This study demonstrates that altering the linkage of the aglycone to the triazole ring changes the priming activity. Computational modeling provides a molecular rationale for higher priming ability of Ru-mediated click-xylosides. Higher GAG priming activity is attributed to the formation of more stable interactions between the 1,5-linked xylosides and β-1,4-galactosyltransferase 7 (β4GalT7).

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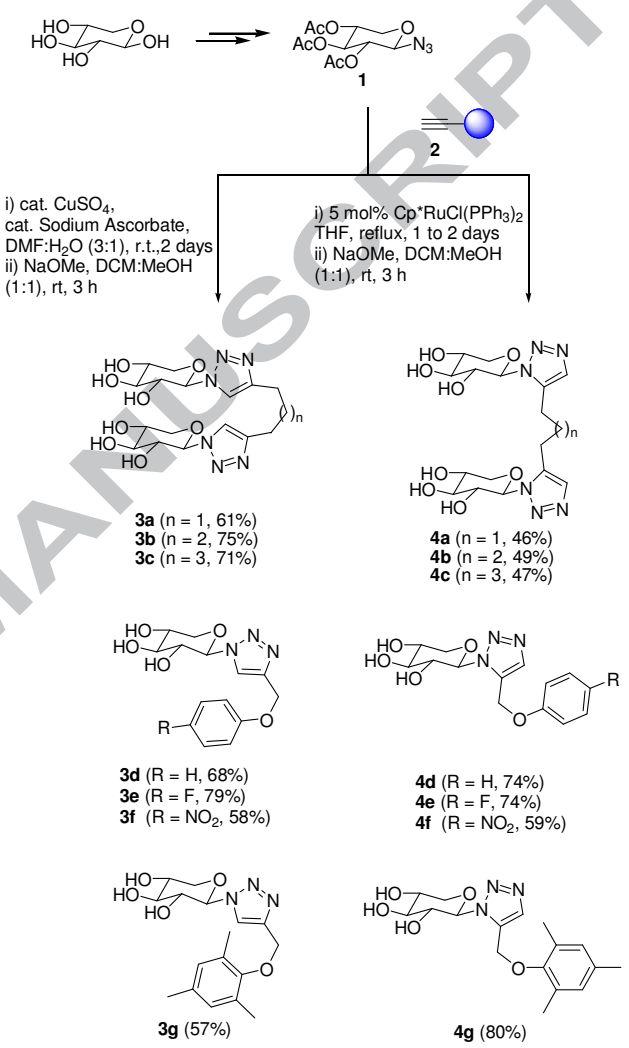
Proteoglycans (PGs) consist of a core protein and one or more glycosaminoglycan (GAG) side chains. Biosynthesis of PG occurs in the golgi and requires a number of enzymatic modifications. Xylosylation of certain serine residues is the first and essential step in the formation of GAG chains such as heparan sulfate and chondroitin sulfate onto PGs.[1,2] Previous studies have found that xylosides can induce the formation of GAG chains without the presence of a core protein.[3-9] Several monomeric and dimeric xylosides have been investigated in earlier studies.[9] Multimeric xylosides, which contain two or more xylose residues attached to the aglycone, prime multiple GAG chains on the same scaffold, closely mimic endogenous PGs. Endogenous PGs often have two or more GAG chains attached to the core protein and it is predicted that many of the biological interactions between PGs and other proteins or factors are due to the multivalent GAG chains. The nature of aglycone attached to the xylose can affect not only the priming activity but also the type, sulfation pattern and molecular weight of the primed GAGs.[4-6] Click-xylosides, a promising class of xylosides, were primarily synthesized using copper [Cu(I)] catalyzed click[10] reactions. The triazole linkage affords these molecules metabolic stability in culture and *in vivo*.[7-9] Earlier studies have examined how these click-xylosides induce GAG formation and how alteration of the aglycone can change the fine structures of primed GAG chains.[7-9]

In this report, Ru-mediated[11] 1,5-linked click-xylosides were prepared for the first time and their priming activity was analyzed in comparison to corresponding 1,4-linked click-xylosides prepared by Cu-mediated catalysis.[12-14] Specifically, this work investigates the impact of xyloside linkage size and orientation as it relates to GAG priming activity.

D-Xylose served as a starting material to prepare β-D-xylopyranosyl azide **1** in two consecutive steps.[7-9] The treatment of xylopyranosylazide **1** with 1,6-heptadiyne in the presence of cat. $CuSO_4$ and sodium ascorbate in DMF: H_2O (3:1) followed by deprotection of acetate groups using sodium methoxide readily afforded dimeric xyloside **3a** in overall 61% yield (Scheme 1). The structure of the compound **3a** was confirmed by ^{1}H-, ^{13}C-NMR and mass spectrometry. Under the similar Cu-catalyzed reactions conditions, 1,7-octadiyne and 1,8-nonadiyene were reacted with **1** to prepare corresponding dimeric xylosides **3b** and **3c** in 75% and 71% yields, respectively (**Scheme 1**). Next, the monomeric xylosides **3d-3g** were synthesized under similar reaction conditions in good yields (**Scheme 1**) for the comparative GAG priming activity studies.

Next, efforts were directed toward preparing 1,5- linked xylosides. In this case, the reaction of xylopyranosylazide **1** with 1,6-heptadiyne in the presence of 5 mol% of $(Cp^*RuCl(PPh_3)_2$ in THF at reflux followed by sodium methoxide mediated deprotection of acetate groups readily afforded the dimeric xyloside **4a** in overall 46% yield (**Scheme 1**). The structure of the compound **4a** was confirmed by the spectral data. Similarly, the Ru-catalyzed reactions of **1** with a number of alkynes followed by deprotection of acetate groups afforded corresponding 1,5-bis- and mono- xylosides **4b-4g** in good yields (**Scheme 1**).

Four sets of monomeric (**3d-3g**, **4d-4g**) and three sets of dimeric xylosides (**3a-3c**, **4a-4c**) were then assessed for their GAG priming activity *in vitro* using pgsA-745 CHO cells[6], which are unable to synthesize GAG chains as they lack xylosyltransferase, at a concentration of 100 µM, using previously described methods.[7,8] As determined by ^{35}S-sulfate incorporation into primed GAG chains, both Cu- and Ru- click-xylosides initiated GAG production but there were distinct differences in priming ability between 1,4- and 1,5-xylosides (**Fig. 1**). 1,5-Xylosides **4a-4g**, synthesized using Ru-click chemistry, showed higher priming activity than the corresponding 1,4-xylosides **3a-3g**, synthesized using Cu-click chemistry. Also in comparing the dimeric xylosides, it should be noted that the length of the linker between two xylose residues dramatically affects the priming activity.



Scheme 1. Synthesis of xylosides via Cu- or Ru-click chemistry.

There appears to be a negative correlation between the length of the linker and the priming activity of the bis-xyloside. In both Cu-click and Ru-click bis-xylosides, the compound with the three and four carbon linker (**3a**, **4a**, **3b**, **4b**) show similar priming activity whereas bis-xylosides (**3c**,**4c**) containing the five carbon linker, -$[CH_2]_5$- have lower priming activity. The length of the carbon chain linker in dimeric xylosides influences priming in both the 1,4- bis-xylosides and 1,5- bis-xylosides. However, even in the situations of lower priming ability, the data clearly shows that 1,5-linked xylosides have higher level of GAG priming activity as measured by radioactive sulfate incorporation.

After assessing the priming activity of the xylosides, the primed GAGs were analyzed for their chain length, sulfation density and disaccharide composition. We found that all xylosides primed primarily highly sulfated GAG chains. These primed GAGs elute at similar retention times in weak-anion exchange DEAE HPLC (**S1.1**), regardless of 1,5- and 1,4- linked xyloside primers. It is interesting to note that all dimeric xylosides produced a narrow peak within the HPLC profile while the monomeric xylosides produced a much broader peak. After determining sulfation density, the size of the primed GAGs was assessed. The size differences of primed GAGs between 1,4- and 1,5- linked xylosides were found to be less significant.

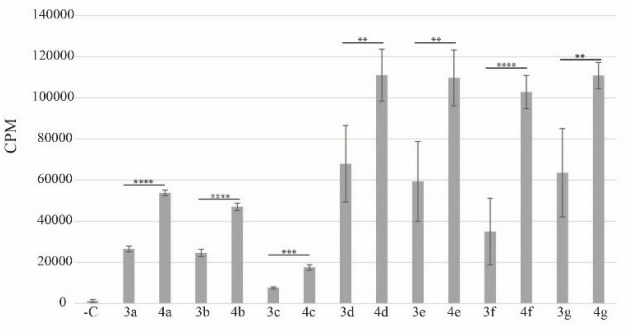


Figure 1. Comparison of the incorporation of radioactive sulfate in GAG chains primed by Cu-xylosides (**3a-g**) with Ru-xylosides (**4a-g**) as measured by ^{35}S scintillation count from three independent experiments (n=3, mean±SD,**<0.01, *** <0.001, ****<0.0001) .

Finally, the disaccharide compositional analysis of the primed GAGs was undertaken. All xylosides produced primarily chondroitin sulfate (CS) type GAG chains. After digestion of CS chains with chondroitinase ABC, the disaccharide analyses showed that the major disaccharide was found to be a monosulfated disaccharide (**S1.2**). These results showed that while the priming activity was dependent on the 1,5- *versus* 1,4-linkage of the xylose to the triazole ring, the structural characteristics of the primed GAGs did not change significantly in terms of disaccharide composition and chain length.

To further understand the differences observed in the priming ability of these synthetic click-xylosides, we conducted computational docking experiments using a molecular modeling software, Sybyl-X 2.1.[15] We investigated the potential binding position and orientation of low energy poses of all the monomeric xylosides (**3d-3g**, **4d-4g**) and their interaction with *β*-1,4-galactosyltransferase 7 (*β*4GalT7), an enzyme involved in GAG biosynthesis. The binding of 1,4-xylosides (**3d-3g**) and 1,5-xylosides (**4d-4g**) showed distinct binding modes within the *β*4GalT7 pocket (PDB ID: 4IRQ[16]). The binding mode of the phenoxymethyl moiety of 1,4-linked click-xylosides is much more non-predictive due to less sterically spaced aryl aglycone substitutions, causing two different modes whereas the substituted phenoxymethyl moiety of 1,5-xylosides exhibits predictive single binding mode due to rigidity at the 5th position. The key features of 1,5- xylosides responsible for higher priming is attributed to the π-stacking interaction either through edge-face and sandwich stacking interactions (Tyr194, Tyr196, Tyr199, Trp224) as shown in **Figure 2a**. Therefore, by shifting the aglycone from 1,4- (**Figure 2b**) to 1,5- linkage, the aromatic ring was positioned to interact directly with aromatic amino acid residues of *β*4GalT7 enzyme. These docking experiments suggest that higher priming activity of 1,5-xylosides is attributed to the mode of binding of the phenoxymethyl moiety, its rigidity at position 5, and stable π-stacking interactions with Tyr194, Tyr196, Tyr199, and Trp224 residues. Our modeling studies and docking results are consistent with earlier mutagenesis studies in which Tyr196 was found to directly interact with aromatic aglycone moiety,[17] and that a highly hydrophobic environment, consisting of Tyr194, Tyr196, and Tyr199, was critical for binding of the xyloside substrates.[18]

The noticeable difference in priming activity indicates that triazole linkage modulates GAG biosynthesis. In comparison to the 1,4- linkage, the1,5- linkage may facilitate xyloside entry into the cell and golgi. Additionally, the length of the carbon linker in bis-xyloside substrates affects the priming capability of both 1,4- and 1,5-linked xylosides, indicating that for dimeric xylosides both the linkage and the linker length are important for priming activity. Computational modeling suggests that the ability of the Ru-click xylosides to form more stable interactions with *β*4GalT7 enzyme involved in GAG biosynthesis may provide insights into the determination of priming capabilities of xylosides with distinct aglycones. Further explorations of how modifications to the triazole alter priming activity are necessary to fully understand how the linkage between xylose and aglycone may alter the priming activity.

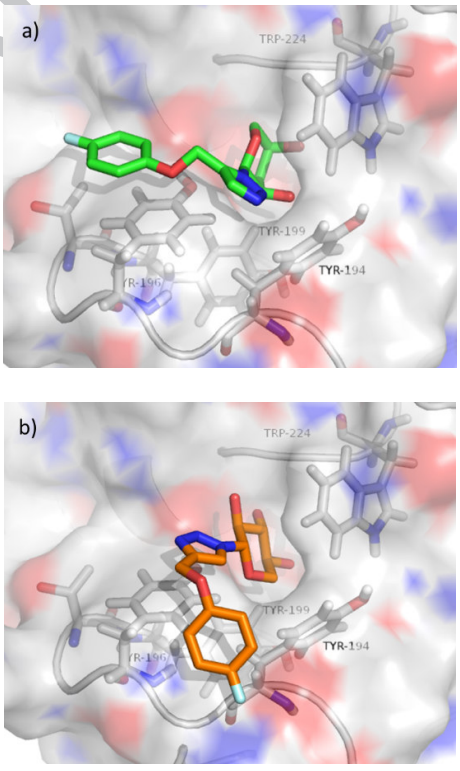


Figure 2. Docking results of **a)** 1,5-xylosides (ligand 4e shown in green stick) and **b)** 1,4-xylosides (ligand 3e shown in orange stick) on *β*4GalT7 binding pocket. While aglycone moiety adopts flexible orientation in 1,4-xylosides, the aglycone aromatic rings in 1,5-xylosides form consistent π-stacking interactions with Y194, Y196 and Y199.

Additionally, the ability to computationally predict the interaction between varying xyloside structures and GAG biosynthetic enzymes, especially those involved in the initiation of GAG biosynthesis, may lead to possible target molecules for more precise modulation of primed GAG chains. Examination of

different aglycone structures for Ru-click dimeric xylosides may also shed light on the prominence of aglycone in the regulation of GAG priming and may lead to the discovery of more potent xylosides. Though Cu-click chemistry has been an efficient route for synthesis of many xylosides, computational modeling shows that Cu-click chemistry may not provide the most desirable interaction between the enzyme and xyloside substrates. Coupled with the evidence of increased priming activity of the Ru-click xylosides as compared to their Cu-click counterparts, it becomes clear that other forms of chemistry should be explored to discover more potent xylosides carrying distinct aglycones to fine tune GAG priming activity and the resulting GAG fine structures.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/

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Graphical Abstract

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