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Received Date : 30-Nov-2015

Revised Date : 27-Apr-2016

Accepted Date : 02-Jun-2016

Article type : Research Article

"CLICK" XYLOSIDES AS INITIATORS OF THE BIOSYNTHESIS OF GLYCOSAMINOGLYCANS: COMPARISON OF MONO-XYLOSIDES TO XYLOBIOSIDES

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Abstract

Different mono-xylosides and their corresponding xylobiosides obtained by a chemo-enzymatic approach featuring various substituents attached to a triazole ring were probed as priming agents for glycosaminoglycan (GAG) biosynthesis in the xylosyltransferase-deficient pgsA-745 Chinese hamster ovary cell line. Xylosides containing a hydrophobic aglycone moiety were the most efficient priming agents. Mono-xylosides induced higher GAG biosynthesis in comparison to their

corresponding xylobiosides. The influence of the degree of polymerization of the carbohydrate part on the priming activity was investigated through different experiments. We demonstrated that in case of mono-xylosides, the cellular uptake as well as the affinity and the catalytic efficiency of β-1,4-galactosyltransferase 7 were higher than for xylobiosides. Altogether, these results indicate that hydrophobicity of the aglycone and degree of polymerization of glycone moiety were critical factors for an optimal priming activity for GAG biosynthesis.

Keywords: xylosides, xylobiosides, glycosaminoglycans, click chemistry, enzymatic transglycosylation

Abbreviations:
4-MUX: 4-methylumbelliferyl-β-D-xylopyranoside
CHO: Chinese Hamster Ovary
CPC: cetylpyridinium chloride
CS: chondroitin sulfate
DS: dermatan sulfate
ECM: extracellular matrix
GAG: glycosaminoglycans
Gal*N*Ac: *N*-acetylgalactosamine
Glc*N*Ac: *N*-acetylglucosamine
HS: heparan sulfate
PG: proteoglycan

INTRODUCTION

Sulfated glycosaminoglycans (GAG) are linear heteropolysaccharide chains found in the extracellular matrix (ECM). Due to their high structural diversity and their interactions with the ECM components and cell-surface proteins, GAG critically modulate a large array of cell functions, including cell differentiation,[1, 2] proliferation,[3] migration, angiogenesis[4,5] and ECM homeostasis.[6] Sulfated GAG are covalently attached to protein core which vectorize the fore mention actions and additionally influence their functions.[7] The GAG biosynthesis process is dynamically regulated during development[8,9] and aging, in both physiological and pathological conditions.[10,11]

The initial step in the CS/HS GAG biosynthesis is the transfer of D-xylose from UDP-xylose to a serine amino acid of a core protein by the β-D-xylosyltransferase I or II.[12] This xylosylation stage takes place in the endoplasmic reticulum and/or in the Golgi apparatus. The β-1,4-galactosyltransferase 7 (β4GalT7) (also named galactosyltransferase I) catalyzes the first galactosylation step on the xylose residue using UDP-Gal as donor substrate and

initiates the formation of a tetrasaccharide region GlcA(1-3)Galβ(1-3)Galβ(1-4)Xylβ1-*O*. Then, the elongation of the chain occurs by the addition of the repeating disaccharide units composed of a *N*-acetylated hexosamine (Glc*N*Ac or Gal*N*Ac) and a glucuronic acid (GlcA), leading to the formation of a specific GAG chain, *i.e.* chondroitin/dermatan sulfate (CS/DS, respectively) or heparan sulfate (HS) chains. The linkage association between a core protein and at least one chain of GAG leads to the formation of a proteoglycan (PG) mostly attached to the cell plasma membrane or secreted into the ECM.[13]

Okayama and colleagues[14] identified a xyloside derivative, the *p*-nitrophenyl β-D-xylopyranoside, for its ability to initiate the synthesis of CS chains. Since this work, several libraries of xylosides carrying various aglycone moieties have been studied for their priming activity in the GAG biosynthesis. These compounds act as acceptors of the β4GalT7, leading to an exogenous biosynthesis of free GAG chains, independently of a core protein.[14-16] The abundance and the composition of the GAG chains depend on the structure and the hydrophobicity of the aglycone moiety[15,17] but also on the nature of the glycosidic linkage, the distance between the xylose and the aglycone, and the nature of the spacer.[18] In most cases, priming of CS/DS dominates while synthesis of HS remains low.[17,19] However, xylosides featuring polyaromatic structures such as hydroxynaphthyl derivatives were described to produce increased yields of HS leading to interesting biological properties.[20-24] Finally, these artificial GAGs are core protein-free, secreted by cells into the ECM and able to mimic some properties of the natural PGs. The main effects observed were the growth inhibition of tumor cells,[20,22-27] the activation of fibroblast growth factors[28] and anti-thrombotic effects.[29]

Xyloside derivatives can be obtained either by classical chemical strategies (Fischer glycosylation, Koenigs-Knorr reaction…)[30,31] or by enzymatic reactions using glycosidases.[31,32] We recently developed a two-step chemo-enzymatic synthesis of a series of xylosides and xylobiosides from xylans as raw material.[33] In a first step, an enzymatic synthesis of propargyl xyloside **1** and propargyl xylobioside **2** was carried out from beechwood xylans and propargyl alcohol using a commercially available xylanase. In a second step, a copper-catalyzed azide-alkyne cycloaddition (CuAAC) or "click" reaction was achieved to afford a series of xylosides **3** and xylobiosides **4** featuring a triazole heterocycle with various polar, apolar or xylose-derived substituents (Scheme 1, Table 1).

Some "click"-xylosides, which possess a *N*-linkage with the aglycone part, have been already studied for their high stability in *in vitro* and *in vivo* models.[18,34] The aglycone groups added on the "click"-xylosides were described for their modulation in the priming activity of GAG biosynthesis.[18,33] Different scaffolds of xylosides were used (bis, tris or tetrakis-xyloside

derivatives)[28,35] but to date, none of these studies compared the effect of the degree of polymerisation of the glycosidic moiety.

Xylobiosides, difficult to obtain by classical chemical synthesis but easily produced by our chemo-enzymatic approach, represent interesting molecules as they present various hydrophilic properties compared to xylosides which could be valuable for their further formulation. In contrast to xylosides, xylobiosides have never been studied for their ability to initiate GAG biosynthesis. However, Sarkar and colleagues[35,36] reported that a disaccharide β-D-Gal(1-4)β-D-Xyl-1-*O*-(2-naphthyl) can act as a primer of exogenous biosynthesis of GAGs. Moreover, Tsutsui *et al.* showed that xylobiose fits into the acceptor binding pocket of a β4GalT7.[37] Our study aimed to highlight the role of both aglycone and glycone parts of xylosides and xylobiosides for GAG biosynthesis. We hypothesize that "click" xylosides **3** and xylobiosides **4** featuring various aglycone moieties but also a different glycone part, could provide relevant information on the structure-activity relationships which govern GAG chain priming.

MATERIALS AND METHODS

Chemicals and reagents

CPC, bovine serum albumin (BSA), sodium chloride (NaCl), sodium sulfate (Na_2SO_4), formaldehyde, triton X-100 and 4-MUX were purchased from Sigma Aldrich (St-Quentin Fallavier, France). The fluorophore Alexa488 with an azide group was obtained from LifeTechnologies (St Aubin, France).

CHO pgsA-745 cells were obtained from the American Type Culture Collection (CRL-2242™). Dulbecco's modified Eagle's medium with nutrient F12 (DMEM:F12), Dulbecco's phosphate buffer saline (D-PBS) and antibiotics (penicillin-streptomycin) were obtained from Fisher Scientific (Illkirch, France) whereas Fetal Calf Serum (FCS) and Whatman® 3MM paper were purchased from Dominique Dutscher (Brumath, France). For radioactive experiments, DMEM without sulfate was from the Institute of Experimental Immunology PAN (Wrocław, Poland). Radioisotope carrier-free ^{35}S-sulfate and scintillation fluid Instagel® were provided by Perkin Elmer (Courtaboeuf, France).

General procedure for the synthesis of "click"-xylosides 3a-g and xylobiosides 4a-g.

Enzymatic synthesis of propargyl xyloside **1** and propargyl xylobioside **2** by a xylanase-catalyzed transglycosylation reaction was already described.[33] "Click" reactions for the preparation of xylosides **3a-g** and xylobiosides **4a-g** featuring a triazole heterocycle were also reported.[33] The chemical stability of all synthesized compounds was confirmed with an HPLC assay after a 24 h-incubation in the cell culture medium at 37 °C (data not shown).

Cell culture and screening of "click"-xyloside priming activity in CHO pgsA-745 cell line.

To determine whether the original xylosides were able to prime the GAG biosynthesis, CHO pgsA-745 cells, which are deficient for the xylosyltransferase I and therefore do not produce any sulfated GAGs, were used. They require exogenous xyloside supply to restore the GAG biosynthesis.[38] This cell line constitutes a convenient cell model to determine the priming activity of GAG biosynthesis induced by xyloside derivatives. Cells were maintained in DMEM:F-12, supplemented with 10% FCS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere.

Cells were seeded on a 24 well-plate (300,000 cells/well). The cell monolayers were washed after 24 h twice with D-PBS and treated with different concentrations (1, 10 or 100 µM) of mono-xylosides **3** or xylobiosides **4** featuring a triazole heterocycle, with DMEM depleted for sulfate and in the presence of ^{35}S-sulfate (10 µCi/mL). To ensure the GAG biosynthesis, a commercial xyloside 4-MUX was used, as a positive control. The cells were incubated at 37 °C for 24 h.

The GAG fraction secreted to the cell culture medium was spotted on Whatman® 3MM paper and precipitated with quaternary amine CPC (1% CPC, 1% NaCl, 0.5% Na_2SO_4; w/v) as previously described.[39] Samples were prepared for scintillation counting (Hidex 300SL) with the addition of 2 mL of scintillation fluid (Instagel®).

***In vitro* click-labeling of propargyl xyloside and xylobioside.**

CHO pgsA-745 cells (30,000 cells/well) were seeded on coverslips 24 h prior to experiment. To determine the uptake of propargyl xyloside **1** or xylobioside **2**, cells were treated with 100 µM of appropriate molecules for 1 h at 37 °C. Then, cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in D-PBS (v/v) for 20 min and washed 3 times with a 3% BSA/D-PBS solution (m/v). Fluorescent Alexa488 (15 µM) carrying an azide moiety were added to the cells, at room temperature for 45 min, to

catalyze the "click" reaction in presence of 2 mM of copper I ($CuSO_4$/sodium ascorbate). Coverslips were then washed 3 times again with the 3% BSA/D-PBS solution and nuclei were counterstained with DAPI, which is a fluorescent probe strongly bound to the DNA.[40]

Determination of the kinetic parameters of β4GalT7.

The catalytic domain of the human recombinant β4GalT7 (corresponding to the protein lacking 60 N-terminal amino acids) was expressed as a fusion protein with glutathion-S-transferase (GST) appended at its *N*-terminal end with a 6His-tag in *Escherichia coli* (*E. coli*), as previously described[41] with slight modifications. The bacterial cells transformed with the corresponding plasmid (pETM-30-6His-GST-β4GalT7ΔNter-60) were cultivated at 37 °C in a Luria Bertani (LB) broth containing 50 µg/mL kanamycin until the A_{600} value reached 0.6. The expression of the recombinant protein was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the cellular suspension, and bacteria were incubated overnight at 25 °C under continuous shaking (200 rpm). The bacterial cells were then harvested by centrifugation at 7,000 × g for 10 min at 4 °C, resuspended in lysis buffer (50 mM sodium phosphate, 1 mM EDTA, 300 mM NaCl, 10 mM DTT, 0.5% Triton X-100 and 5% (v/v) glycerol, pH 7.4) supplemented with Pierce Universal Nuclease (250 U / 10 mL, Fisher Scientific). The suspended cells were lysed using a Constant Systems cell disrupter at 20,000 psi. Soluble proteins were collected from the supernatant after centrifugation for 20 min at 12,000 × g and clarification by filtration (0.45 µm Supor® Membrane; PALL-Life Science). Clarified extracts (20 mL) were applied onto a 5 mL Glutathione Sepharose High Capacity column (GSTrap 4B; GE Healthcare) connected to an AKTA prime plus instrument (GE Healthcare). Proteins were eluted as 1 mL fractions with a 300 mM NaCl, 50 mM Tris-HCl, pH 8.0 buffer containing 10 mM reduced glutathione. Protein purity was evaluated by SDS-PAGE analysis, followed by Coomassie Brilliant Blue staining. Kinetic analyses were performed on a batch containing approximately 1 mg/mL pure protein.

The kinetic parameters towards 4-MUX and other tested xylosides (**3d** and **4d**) were determined as previously described[41,43] with following modifications. Briefly, 0.1 µg of purified recombinant enzyme were incubated in a 50 mM Bis-Tris buffer pH 6.7, 10 mM $MnCl_2$ with concentrations from 0 to 5 mM of xyloside in the presence of a fixed 1 mM UDP-Gal concentration, for 30 min at 37 °C. For each set of experiments, control assays in which the acceptor substrate was omitted were systematically run under the same conditions. The incubation reaction was stopped by addition of 5 µL HCl 6 N on ice.

The supernatant was analyzed by high performance liquid chromatography (HPLC) with a reverse phase C18 column (xBridge, 4.6 x 150 mm, 5 µm, Waters) using a Waters equipment (Alliance Waters e2695) coupled to a UV detector (Waters 486). Reaction product formation was followed at a detection wavelength of 320 nm for 4-MUX and 250 nm for compounds **3d** and **4d**. The mobile phase was composed of 13% (v/v) acetonitrile (ACN) and 0.02% (v/v) trifluoroacetic acid (TFA) in water for 4-MUX, **3d** and **4d** and run at a flow rate of 1 mL/min. Quantitation of the reaction product was performed with calibration curves drawn with increasing concentrations of compounds **3d** and **4d** (0.25-50 nmoles) and analyzed under similar chromatographic conditions than assays. A typical chromatogram corresponding to the galactosyltransferase assay towards **3d** and **4d** is shown as supplementary data (Fig. S1).

For 4-MUX and **4d,** apparent kinetic parameters k_{cat} and K_M were determined by nonlinear least squares regression analysis of the data fitted the Michaelis-Menten rate equation, $v=V_{max}$ [S]/(K_M+[S]), where v, V_{max}, S and K_M correspond to the initial velocity, substrate concentration, maximal velocity and Michaelis constant, respectively. For compound **3d,** kinetic parameters were determined using nonlinear least squares regression analysis fitting the experimental data to substrate inhibition kinetics, $v=V_{max}[S]/(K_m + [S](1+[S]/K_i)$, where K_i corresponds to the inhibition constant, using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Each data point represents the mean value ± SEM of three independent experiments performed in duplicate.

RESULTS AND DISCUSSION

Screening the priming activity of "click"-xylosides.

The series of "click" mono-xylosides **3** and xylobiosides **4** were screened to determine whether these compounds were able to prime the GAG production. We took advantage of the mutant CHO pgsA-745 cells, which do not produce sulfated GAGs.[38] Cells were incubated with 14 previously prepared xylosides or with 4-methylumbelliferyl-β-D-xylopyranoside (4-MUX), known to be the best chemical primer used for GAG production,[42.] and the neo-synthesized GAGs were labeled with ^{35}S-sulfate for 24 h. Cell media were harvested and the GAG fraction was isolated by cetylpyridinium chloride (CPC) precipitation on a Whatman paper.[39] The results of ^{35}S-sulfate incorporation are shown in Figure 1.

As expected, CHO pgsA-745 cells did not incorporate ^{35}S-sulfate to the CPC precipitated fraction of culture media. However, the presence of exogenous mono-xylosides **3** or xylobiosides **4** restored the GAG biosynthesis. Mono-xylosides **3a**, **3d**, **3e** carrying a

hydrophobic aglycone moiety were able to prime the GAG production with a comparable efficiency to 4-MUX (from 92% to 100%, Fig. 1A) whereas more polar molecules (**3b**, **3c**) were less efficient (3.2 to 23-fold, respectively). These results are in accordance with previous studies, which stressed the importance of a hydrophobic aglycone moiety in the GAG biosynthesis initiation,[34] and the presence of a tryptophan and three tyrosine residues creating a hydrophobic environment in the active site of β4GalT7.[41] Among these hydrophobic xylosides, compounds **3d** and **3e**, which possess aromatic moieties, were as efficient as the butyl xyloside **3a** to prime the GAG biosynthesis (Fig. 1A). Similar results were previously highlighted in the literature with other xyloside derivatives carrying an aromatic moiety.[17]

No difference in priming the GAG biosynthesis was evidenced between xylosides with peracetylated β-D-xylopyranosyl or β-D-xylopyranosyl grafted on a triazole ring (compounds **3f** and **3g** respectively, Fig. 1A). Nguyen and co-workers[28] demonstrated that cluster xyloside derivatives, carrying several xylosides on the same scaffold, were able to prime two or three GAG chains. Although the ability of compounds **3f** and **3g** to restore the GAG production was reduced compared to 4-MUX (about 3.2-fold lower, Fig. 1A), the priming of two GAG chains cannot be excluded. These results could be attributed to an intracellular deacetylation of xylose residues as previously described.[35]

Given that compounds **3a**, **3d** and **3e** (butyl, phenyl and benzyl residues, respectively) were efficient in initiating the GAG biosynthesis at 100 µM, we performed the same experiment with lower concentrations. At 10 µM, compounds **3a** and **3e** initiated a limited production of GAGs, compared with 4-MUX and **3d** which were able to initiate the GAG biosynthesis with a similar efficacy at 100 µM and 10 µM (Fig. 1B). Interestingly, mono-xyloside **3d** was more efficient (1.7 fold) than 4-MUX to prime the GAG biosynthesis at 1 µM (Fig. 1C). This result suggests that the compounds 4-MUX and **3d** already reached their maximal activity to initiate the GAG biosynthesis at 10 µM. 4-MUX has been recognized for several years as a xyloside derivative that efficiently stimulates CS synthesis in chondrocytes and cancer cell lines.[44] To our knowledge, these compounds were not compared to 4-MUX in term of GAG priming activity. The capacity of 4-MUX to act as an efficient primer for GAG synthesis could be attributed at least in part to its ability to strongly bind the human β4GalT7 active site through a bond between the *N*-backbone of histidine residue and the carbonyl group of 4-MU.[42]

Very few disaccharides featuring a β-xylopyranoside moiety have been reported as GAG chain primers. However, a naphthyl xylopyranoside attached to a galactopyranose previously

described as a promoter of GAG production.[36] In our study, xylobiosides **4a-g** were not able to induce a GAG production as extensively as the corresponding mono-xylosides **3a-g** (Fig. 1A). While mono-xylosides **3a**, **3d** and **3e** are good GAG chain initiators in comparison to 4-MUX, this effect is not found for the xylobiosides carrying the same aglycone moiety (**4a**, **4d** and **4e**; about 5-fold less than the corresponding mono-xyloside). Concerning the less efficient mono-xylosides **3b**, **3c**, **3f** and **3g**, the counterpart xylobiosides were also weak initiators (from 1.2 to 3.9-fold less than the corresponding mono-xyloside).

These differences in the production of GAG chains between mono-xylosides and the corresponding xylobiosides could be attributed to two main factors: a less efficient diffusion through the plasma and Golgi membranes and/or substrates kinetic parameters for the β4GalT7. We have undertaken some experiments to evaluate the impact of these two factors on GAG chains biosynthesis.

Assessment of cellular uptake of xylosides.

The major limitations for the priming of GAG biosynthesis for therapeutic use are the cellular uptake of exogenous xylosides[36] and their cytotoxicity, which could impact the general metabolism. None of the compounds **3** and **4** affected the viability of CHO pgsA-745 cells (data not shown). To determine whether mono-xylosides **3** or xylobiosides **4** equally penetrate through the plasma membrane, we took advantage of the ability of propargyl β-D-xyloside **1** or propargyl β-D-xylobioside **2** to perform a "click"-reaction, after their penetration in the cells, with a fluorescent azide, allowing the detection of the corresponding fluorescent-labeled triazoles. Previously, we ascertained that compounds **1** and **2** were able to induce a GAG production at 100 µM (114% and 11% respectively compared with 4-MUX, data not shown). The cells were seeded on glass coverslips and incubated with **1** or **2** at 100 µM during 1 h. At the end of the stimulation, the cells were washed, fixed and a "click"-reaction was performed using the fluorescent probe Alexa488 carrying an azide group. The microscopic analysis of staining demonstrated that the uptake was more efficient when cells were treated with the propargyl β-D-xyloside **1** (Fig. 2A, pictures d-f) than with the propargyl β-D-xylobioside **2** (Fig. 2A, pictures g-i). The cellular uptake was also quantified using fluorescence units. The results indicated that the staining of propargyl β-D-xyloside **1** was significantly 4-fold higher than the staining of propargyl β-D-xylobioside **2** ($p < 0.05$, Fig. 2B). Our observations are in accordance with a previous study, which stressed the fact that the cellular uptake of a mono-xyloside derivative, the 2-naphthyl β-D-xyloside, occurred at 100 µM by diffusion but disaccharides featuring the same aglycone part penetrated less

efficiently into the cells.[35] Moreover, it has been reported that clusters carrying several xylose residues may present reduced transport through the plasma membrane.[44] The degree of polymerisation of xylose moiety clearly impacts on the cellular uptake, likely contributing to the differences observed between mono-xylosides **3** and xylobiosides **4** on the GAG priming activity.

Comparison of the kinetic parameters of the human recombinant β4GalT7 towards mono-xyloside 3d and xylobioside 4d.

We next studied the capacity of mono-xyloside **3d** and xylobioside **4d** to be used as substrates by the human recombinant β4GalT7. To this aim, we set up an *in vitro* HPLC assay to compare the kinetic properties of this enzyme towards compound **3d**, chosen as the most potent initiator of GAG synthesis, and its xylobioside counterpart **4d**. The recombinant enzyme was expressed in *E. coli* as a fusion protein with GST. The galactosyltransferase activity towards **3d** and **4d** was compared to 4-MUX used as a reference substrate. Kinetic parameters of the recombinant β4GalT7 towards 4-MUX *i.e.* k_{cat}, K_M and k_{cat}/K_M were in the same range as previously published data.[41-43] The K_M and k_{cat} values of β4GalT7 towards **3d** were about 2- and 2.5-fold that found for 4-MUX, respectively, leading to a 23% higher efficacy for this compound compared to 4-MUX (Table II). Notheworthy, substrate inhibition occurred with **3d** but not with 4-MUX (Fig. S2). It can be hypothesized that this kinetic behavior may be due to the binding of two **3d** molecules in the enzyme active site, since this compound presents a less bulky aglycone moiety compared to 4-MUX.

However, it is interesting to note that the "click"-xyloside **3d** is taken up by β4GalT7 as efficiently as 4-MUX that is, until now, known as one of the best substrates of this enzyme. We recently identified an important bond between the *N*-backbone of the histidine residue 195 and the carbonyl group of 4-MUX accounting for its capacity to accommodate the acceptor binding site.[42] Altogether, our results suggest that the **3d** "click"-xyloside efficiently initiates GAG synthesis by its potent use as a substrate for β4GalT7. Further molecular studies are required to precisely define the influence of the triazole moiety on the position of compound **3d** into the enzyme active site.

Furthermore, we found that the catalytic efficiency of β4GalT7 towards the xylobioside **4d** was about 10-fold lower than towards 4-MUX and its xyloside analog **3d** (Table 2). Interestingly, this reduced catalytic efficiency was accounted for a significant decrease in affinity (8-16-fold increase in K_M value). This result suggests that the xylobioside bound less tightly to the active site, likely indicating that the proximate position to the acceptor xylose

molecule is preferentially either the core protein of a PG or a hydrophobic aglycone.[44,45] Indeed, we showed that three tyrosine residues represent key structural elements of the acceptor active site likely creating a hydrophobic environment for the aglycone moiety of exogenous xylosides.[42] Altogether, our results indicate that the mono-xyloside **3d** is taken up by β4GalT7 as efficiently as 4-MUX, though with different kinetic behavior, whereas the second xylose moiety present in the xylobioside **4d** molecule does not accommodate the hydrophobic environment of the enzyme active site.[46,47]

CONCLUSIONS

A library of original mono-xylosides and xylobiosides, prepared by a chemo-enzymatic approach, was screened for their biological properties. We showed that both « click » mono-xylosides and xylobiosides are able to prime exogenous biosynthesis of GAG in a cellular model lacking the endogenous production. We demonstrated that the priming activity of the GAG biosynthesis is dependent on the aglycone moiety. Hydrophobic substituents on the triazole ring led to an intense GAG production. Compound **3d**, carrying a phenyl substituent, was identified as a more powerful GAG chain initiator than the usually applied 4-MUX, especially at low concentration range. In addition, our study showed that "click" xylobiosides failed to initiate the GAG biosynthesis as efficiently as their homologous mono-xylosides. An original experiment, a “click”-reaction in fixed and permeabilized cells, was performed to demonstrate that the cellular uptake is different with mono-xyloside or xylobioside. Moreover, we showed that xylobiosides are poor substrates for the galactosylation step.

In summary, we decipher in this report for the first time the role of the degree of polymerization of xylose derivatives in the initiation of GAG biosynthesis. Further investigations, fundamental to assess the potential to mimic the natural GAG functions, will be performed to determine the nature and the composition of the secreted GAG chains induced by a treatment with compound **3d**.

ACKNOWLEDGEMENTS

This work was supported by the "Région Champagne-Ardenne" (Xylocos Emergence program). The authors are grateful to the "Région Champagne-Ardenne" and FEDER for

financial support and for a doctoral (C. B.) and a post-doctoral (A. C.-C.) fellowships. This study was performed using facilities from “Glyco-Fluo” platform (UMR CNRS IMoPA, FR3209). This work was supported by Agence Nationale pour la Recherche (ANR) Meca-GT (Grant ANR-13-BSV8-0011-01) and grants from Région-Lorraine to N. R.

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FIGURE AND TABLE LEGEND

Scheme 1: Chemo-enzymatic route for the synthesis of xylosides 3 and xylobiosides 4 featuring a triazole ring in the aglycone moiety.

Table I: Nature of R substituent for triazole-linked *O*-xylosides 3 and triazole-linked *O*-xylobiosides 4. Click additions were carried out with both purified propargyl β-D-xyloside, propargyl β-D-xylobioside (compounds **1** and **2** on Scheme 1) and various azide moieties (R), as previously described.[33]

Figure 1: Priming activity of GAG biosynthesis induced by a series of mono-xylosides 3 or xylobiosides 4 featuring a triazole heterocycle. A. Relative ^{35}S-sulfate incorporation into the GAGs primed with xylosides carrying various aglycone moieties in comparison to 4-MUX at 100 µM. **B**. Comparison of mono-xylosides **3a**, **3d**, **3e** and 4-MUX at 10 µM. **C.** Comparison of mono-xyloside **3d** and 4-MUX at 1 µM. The results are expressed as the mean of four wells ± SEM.

Figure 2: Cellular uptakes of propargyl β-D-xyloside 1 and propargyl-β-D-xylobioside 2. **A**. Representative photographs of untreated cells (a-c), cells treated with **1** (d-f) or cells treated with **2** (g-i) (scale bar = 50 µm). **B**. Quantification of the integrated density of pixels in 10 statistically selected representative pictures with ImageJ software. The quantified intensities were analyzed with a Student's test and the results were considered as significant when $p < 0.05$ (*).

Table II: Kinetic parameters of the recombinant β4GalT7 towards 4-MUX, 3d and 4d. Kinetic parameters towards 4-MUX, compounds **3d** and **4d** as acceptor substrates were determined in the presence of 1 mM UDP-Gal as described under "Materials and Methods" section. The results are the mean values of three independent determinations on assays performed in duplicate. K_M and k_{cat} values were obtained by fitting the experimental data to the Michaelis-Menten equation for 4-MUX and **4d** and to an equation corresponding to a substrate inhibition mechanism for compound **3d**. The K_i inhibitory constant for compound **3d** is up to 3 mM. The results were analyzed with the Student's t test and considered as significant when $p < 0.05$ (*).

Table I:

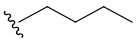
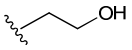
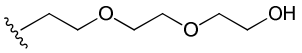
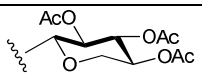
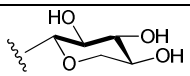
Entry	Compound	R
1	**3a, 4a**	
2	**3b, 4b**	
3	**3c, 4c**	
4	**3d, 4d**	
5	**3e, 4e**	
6	**3f, 4f**	
7	**4g, 4g**	

Table II:

Compound	k_{cat} (min^{-1})	K_M (mM)	k_{cat} / K_M ($min^{-1}.mM^{-1}$)
4-MUX	195.37 ± 14.97	0.34 ± 0.04	569.97
3d	494.17 ± 38.22*	0.70 ± 0.05*	702.07
4d	443.83 ± 2.00*	5.70 ± 0.26*	77.92

Scheme 1.

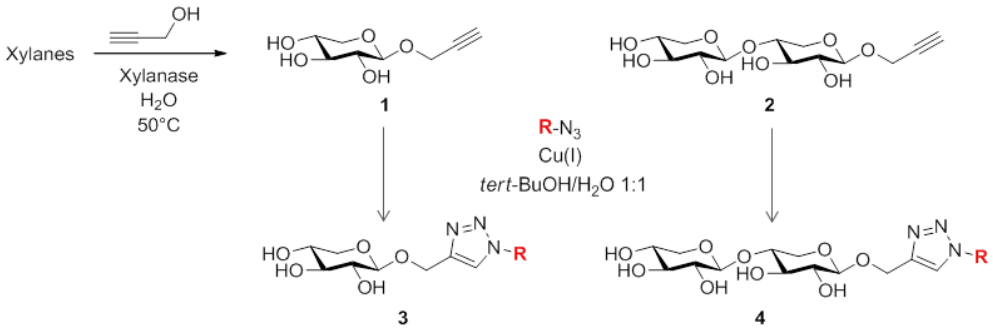


Figure 1

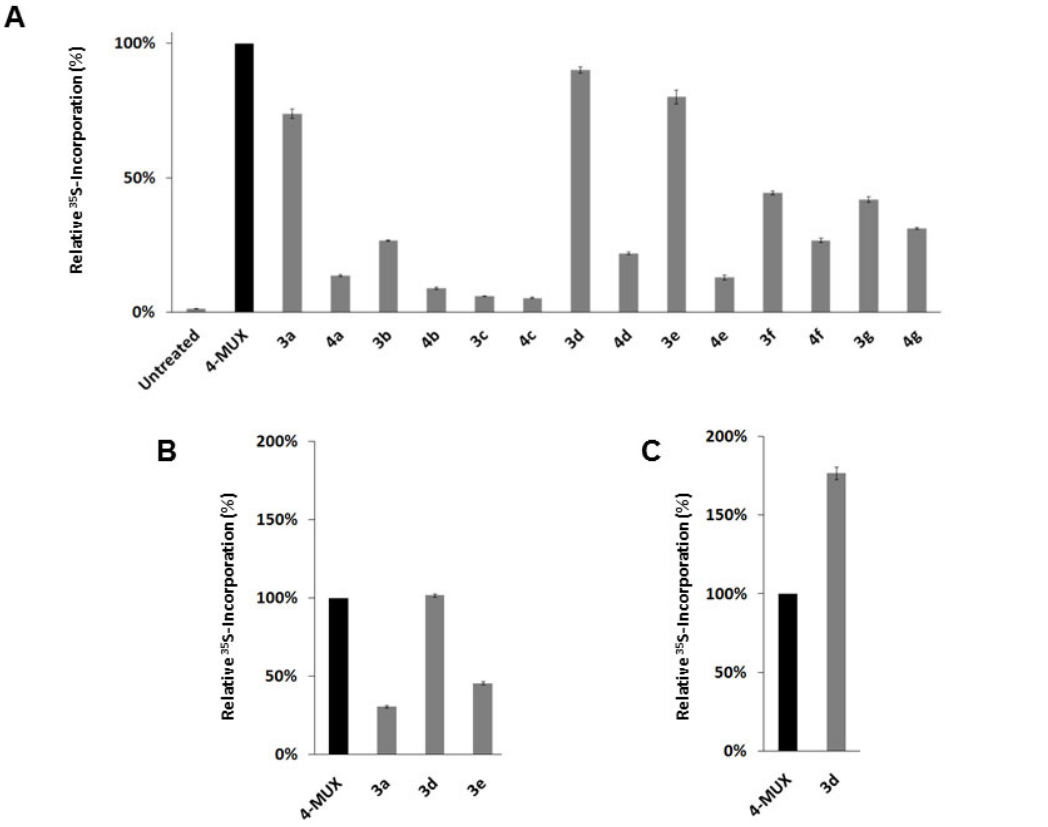


Figure 2