Synthesis of Fluorophore-Tagged Xylosides That Prime Glycosaminoglycan Chains
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Supporting Information

ABSTRACT: Biosynthesis and functions of glycosaminoglycan (GAG) chains are complex and remain elusive. To better understand the factors that regulate the biosynthesis and functions, fluorophore-tagged xylosides carrying two different linkages between fluorophore and xylose residue were synthesized and evaluated for their ability to prime GAG chains such as heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) in various cell lines. These in vitro studies resulted in the identification of fluorophore-tagged xylosides that prime high molecular weight GAG chains. Primed GAG chains carrying a fluorophore group has several advantages for studying the factors that regulate the biosynthesis, analyzing intact fine structures at low detection limits, and setting the stage for studying structure–function relations of GAG chains of cellular origin.

INTRODUCTION
Proteoglycans modulate numerous pathophysiological functions such as development, angiogenesis, axonal growth, anticoagulation, cancer progression, microbial pathogenesis, and so forth.¹–⁴ The quantity and quality of GAG structures, made by various cells, are dynamically regulated in a spatiotemporal manner during the development of an organism and during the normal aging of an organism, as well as during the progression of several pathological conditions.⁵,⁶ Profiling and deciphering dynamic changes in GAG structures will provide new avenues to diagnose disease states and may thwart those conditions with novel therapeutic strategies.⁷ Most of these structural changes have been deduced using radiolabeled monosaccharides and sulfate as biosynthetic precursors in various cellular systems. However, these radiolabeled precursors cannot be used in organisms as they pose toxicity and other challenges. Several β-xyloside derivatives have been shown to act as acceptors and substitute for core proteins in vivo as well as in vitro in the production of core protein free GAG chains.⁸–¹⁴ Fluorophore-tagged xylosides that are able to prime GAG chains will be an excellent tool to study the structure–function relationship in vivo. Commercial 4-methylumbelliferyl-β-D-xyloside (UMB-β-D-xyloside) has been shown until now to function as acceptor for the elongation of GAG chains; however, UMB-β-D-xylosides prime mostly CS chains or small oligosaccharides.¹⁵–¹⁷ Earlier studies examined several other fluorophore-tagged xylosides for studying the mechanism of GAG biosynthesis and GAG priming activity.¹⁸ These studies have shown successful internalization of fluorophore-conjugated xylosides into the para- and perinuclear regions of the cells. However, these molecules were unfortunately not found to initiate GAG biosynthesis suggesting that either these fluorophore xylosides failed to reach GAGOSOMES where GAG biosynthetic enzymes reside within the complex Golgi apparatus or biosynthetic enzymes could not recognize these fluorophore-conjugated xylosides after these molecules reach GAGOSOMES. It has been known that both the structure of the (fluorophore) aglycone and the type of linkage between the (fluorophore) aglycone and xylose can affect the priming activity of xylosides.⁹,¹⁰,¹⁸ Our efforts are, therefore, focused on the synthesis of expanded repertoire of fluorophore-tagged xylosides, based on developments in our lab and other laboratories, and screening of these novel xylosides for their ability to prime GAG chains in a given cellular system and provides novel avenues to profile and elucidate cellular GAG signatures in a robust manner, and assist in establishing cell-specific GAG–protein interactions.

EXPERIMENTAL SECTION

General Synthetic Procedures. All chemical reactions were carried out under a nitrogen atmosphere in oven-dried glassware using standard techniques.¹⁹ ¹H and ¹³C NMR spectra were obtained on a Bruker 400-MHZ spectrometer. Chemical shifts are relative to the deuterated solvent peak or the tetramethylsilane (TMS) peak at (δ 0.00) and are in parts per million (ppm). High-resolution mass spectrometry (HRMS) was performed using a Finnigan LCQ mass spectrometer in either positive or negative ion mode. Thin layer chromatography (TLC) was done on 0.25-mm-thick precoated silica gel aluminum sheets. Chromatograms were observed under short...
and long wavelength UV light, and were visualized by heating plates that were dipped in a solution of Von’s reagent containing ammonium (VI) molybdate tetrahydrate (12.5 g) and cerium (IV) sulfate tetrahydrate (5.0 g) in 10% aqueous sulphuric acid (500 mL). Flash column chromatography was performed using silica gel 60 (230–400 mesh) and employed a stepwise solvent polarity gradient, correlated with TLC mobility, and were run under pressure of 5–7 psi. HPLC was used to purify final products using C18 column (VYDAC 2.2 cm × 25 cm) with solvent A (25 mM formic acid) and solvent B (95% acetonitrile) at a flow rate of 5 mL/min in a linear gradient over 120 min starting with 0% B.

N-(β-D-xylopyranosyl) Azide (5). 2,3,4-Tri-O-acetyl-β-D-xylopyranosyl azide S1 (0.1 mmol) was taken in dry methanol and was treated with freshly prepared 0.5 M solution of CH3ONa (0.1 mL) in dry methanol at room temperature for 3 h. Neutralization with H+ resin followed by concentration at reduced pressure gave a syrupy liquid, which was purified by silica flash column chromatography to give the title compound 5.

Propargyl UMB Derivative (11). To the solution (10 mL) of UMB derivative (10a and 10b) (1 mmol) in acetonitrile was added potassium carbonate (3 mmol). The reaction mixture was stirred for 30 min at room temperature. Propargyl bromide (3 mmol) was then added and the mixture was stirred overnight. The reaction mixture was concentrated. The resulting crude material was dissolved in ethyl acetate, washed with water and saturated sodium chloride solution, dried over Na2SO4 and rotary evaporated under reduced pressure. The residue was purified by column chromatography to give the compound 11.

Synthesis of Fluoroaphore-Tagged Xylosides with Amide Linkages (2, 3, 4, 7, 8, and 9). N-(β-D-xylopyranosyl) aminocacetamide 1 or N-(β-D-xylopyranosyl) amine 6 (0.1 mmol) was dissolved in dry DMF (10 mL). Diisopropylamlylene (0.1 mmol) was added. The whole mixture was stirred for 30 min before adding the commercially available activated fluorescent reagents (Dansyl chloride, FITC and N-hydroxysuccinimidyl-1-pyrene butyrate) (1 mmol). The reaction mixture was stirred for 4 h and purified by C18-HPLC column.

Synthesis of Fluoroaphore-Tagged Xylosides with Click Linkages (13a and 13b). To a solution of alkyne (1 mmol) and azide (1 mmol) in DMF and water (4:1.3) solvent mixture were added sodium ascorbate (0.8 mmol) followed by Cu2SO4·SH2O (0.4 mmol) at room temperature, and the mixture was stirred for 12 h or until disappearance of one of the starting materials as indicated by TLC. At the end of the reaction as confirmed by TLC analysis, the solvent of the reaction mixture was evaporated using rotary-evaporator under reduced pressure. The reaction mixture was purified by flash chromatography columns as described above. The purified acetylated product (0.1 mmol) was taken in dry methanol and was treated with freshly prepared 0.5 M solution of CH3ONa (0.1 mL) in dry methanol at room temperature for 3 h. Neutralization with H+ resin followed by concentration at reduced pressure gave a syrupy liquid, which was purified by HPLC using C18 column to give the desired deprotected xyloside derivatives. All xylosides were characterized using Bruker 400 MHz NMR spectrometer, and structural data are furnished below:

**Compound 2.** 1H NMR (CD3OD): δ 8.57 (d, J = 8.6 Hz, 1H), 8.34 (d, J = 9.0 Hz, 1H), 8.20 (d, J = 7.45 Hz, 1H), 7.60 (t, J = 8.0 Hz, 1H), 7.57 (t, J = 8.0 Hz, 1H), 7.28 (d, J = 7.8 Hz, 1H), 4.70 (d, J = 9.0 Hz, 1H), 3.75 (dd, J = 5.1, 11.3 Hz, 1H), 3.56 (d, J = 3.9 Hz, 2H), 3.44–3.37 (m, 1H), 3.20–3.05 (m, 3H), 3.89 (s, 6H). Mass (ESI): calcd for C19H26N2O6S [M+H]+ 548.159, found 548.151.
the incubator for 24 h before the addition of 6× Pronase solution (100 μL) followed by incubation at 37 °C overnight.

**Purification and Quantification of GAGs.** The entire contents of the wells were transferred to a microcentrifuge tube and subjected to centrifugation at 16 000×g for 5 min. The supernatant was transferred to a fresh tube and half-a-volume of 0.016% Triton X-100 was added. The diluted supernatant was loaded on to a DEAE-sepharose column (0.2 mL) pre-equilibrated with 10 column volumes of wash buffer (20 mM NaOAc buffer (pH 6.0) containing 0.1 M NaCl and 0.01% Triton X-100) and the column was washed with 20 column volumes of wash buffer. The bound HS/CS was eluted using 6 column volumes elution buffer (20 mM NaOAc (pH 6.0) containing 1 M NaCl). The amount of GAG primed by various xylosides was determined by quantifying the 3H-radioactivity incorporated in the purified HS/CS eluate. 50 μL of the various eluates was diluted with 5 mL of scintillation cocktail and triplicate samples were measured using a scintillation counter for total radioactivity.

**Analysis of Primed GAG Chains.** The chain length of the primed GAG was determined by measuring the migration time on two tandem G2000SWXL (Tosoh, 7.8 mm × 30 cm) size exclusion columns using the HPLC Hitachi system with an inline radiodetector or fluorescent detector. The solvent containing phosphate (100 mM KH₂PO₄, 100 mM NaCl, pH 6) was used as an eluent. The average molecular weight was determined by measuring the migration time of GAG chains in comparison to those of polystyrene sulfonate standards examined under similar conditions.

The HS/CS composition of the primed GAG chains was determined by digesting the GAG chains with heparitinase I/II/III or chondroitinase ABC enzymes. The solution containing GAGs was diluted to 0.2 M NaCl, followed by the addition of heparitinase or chondroitinase ABC buffer and 5 mU of heparitinase I/II/III or chondroitinase ABC enzyme. The
reaction mixture was incubated at 37 °C for 2 h, the solution was then loaded on to two tandem G2000 SWXL columns (7.8 mm × 30 cm) and analyzed with the aid of an inline radiometric detector using phosphate buffer (100 mM KH₂PO₄, 100 mM NaCl, pH 6) as an eluent. The percentage of HS/CS was determined based on the percentage area of undigested and digested GAG peaks.

### RESULTS AND DISCUSSION

Several fluorophore-tagged xylosides were synthesized and examined to determine whether these fluorophore-tagged xylosides can elongate GAG chains. These fluorophore-tagged xylosides offer prospects to further our understanding of factors that regulate GAG biosynthesis as well as new knowledge on the role of GAG chains in various signaling events associated with pathophysiological processes.

**Synthesis of Fluorophore-Tagged Xylosides.** Several studies proved that stimulation of GAG chains is affected not only by hydrophobic aglycones of xylosides, but also by their glycosidic linkages. Therefore, several fluorophore-tagged xylosides with amide and triazole in the glycosidic linkage were synthesized in this study. N-(2,3,4-Trihydroxy-β-xlylopyranosyl) acetamide was synthesized from xylosyl azide as outlined in Supporting Information (Scheme S1). Fluorophore-tagged xylosides (2, 3, and 4) were synthesized from corresponding commercially available activated fluorescent reagents (dansyl chloride, FITC, and N-hydroxysuccinimidyl-1-pyrene butyrate) by reacting with the xyloside 1 that contains the reactive amine group (Scheme 1).

It is known that the linker between xylene and the aglycone moiety (fluorophore-tag in this case) may dramatically influence the priming activity. Therefore, a second strategy was devised in which xylene was differentially attached to fluorescent tags by reacting xylosyl azide with triple bond containing amine groups using click chemistry as shown in Scheme 2. Xyloside 6 with a triazole linkage is prepared, which contains the reactive amine group for conjugating with activated fluorescent reagents to obtain the fluorophore-tagged xylosides with triazoyl linkages (7, 8, and 9).

The commercial, well-known UMB-O-xyloside primes mostly short chains of GAG chains or oligosaccharides in various cell types. Click chemistry was used to conjugate fluorescent UMB derivatives 10a and 10b to the xylene unit. Fully acetylated xylene was reacted with UMB derivatives containing a triple bond and these UMB-click-xylosides were deprotected under Zemplen condition to obtain the final products 13a and 13b, as outlined in Scheme 3. All final products were purified on a reverse phase C18 column using HPLC as described in the Experimental Section, followed by structural analysis using NMR and MS.

**Screening of Fluorophore-Tagged Xylosides.** The priming activity of these novel xylosides may perhaps be attributed to the presence of a very hydrophobic fluorescent group, helping their transport across the cell surface and Golgi membranes. At the beginning, the priming ability of fluorophore-tagged xylosides are investigated using a mutant Chinese hamster ovary (CHO) cell line, pgsA-745, which lacks active xylosyltransferase enzyme. This cell line does not make HS, CS, or DS chains, as the assembly of these GAG chain types requires the xylosylation of core proteins by xylosyltransferase. It requires the exogenous supply of β-xlyosides to produce GAG chains such as HS, CS, and DS, and is thus a convenient cellular system to ascertain the quantity of the primed GAG chains by exogenously supplied fluorophore-tagged xylosides. Neither dansyl group attached xylosides (2 and 7) nor fluorescein attached xylosides (3 and 8) primed any detectable GAG chains. It may perhaps be due to the presence of charged amine (in dansyl moiety) and carboxyl (in fluorescein moiety) groups preventing the uptake of xylosides across the cell membrane. This is in accordance with Johnson et al. who found out that the dansyl group attached xylosides were unable to prime any detectable amount of GAG chains. Fluorophore-tagged xylosides without a charged group are next chosen to be synthesized. Fluorophore-tagged xylosides (4, 9, 13a, and 13b), in which xylene residue is attached to 1-pyrene butyrate and UMB derivatives, were synthesized. Pyrene containing xylene with amide linkages 4 was not able to prime GAG chains, while the pyrene containing xylene with triazoyl linkage 9 was able to prime at various concentrations. It is interesting to observe that the pyrene containing xylene with triazoyl linkage 9 can prime GAG chains, but the pyrene containing xylene with amide linkage 4 cannot prime GAG chains. It is predicted that the triazoyl ring may increase the diffusion rate and direct the primer to Golgi compartments.

Next, UMB-click-xylosides 13a and 13b were compared to the commercial UMB-O-xylosides that prime mostly GAG chains with a short chain length. The priming activities of the UMB-click-xylosides 13a and 13b and the commercial xyloside were compared at various concentration (50 μM, 100 μM, 300 μM, 600 μM, and 1 mM) (Figure 1). It is interesting to note that priming activity of the UMB-click-xylosides 13a and 13b was concentration-dependent but the UMB-O-xyloside was not. We also tested the priming activity of UMB-click-xylosides 13a and 13b in endothelial cells (BLMVEC). Both UMB-click-

![Scheme 3. Synthesis of Umbelliferyl Xylosides with Triazoyl Linkages](image-url)
xyllosides primed GAG chains very well in BLMVEC cells at various concentrations.

**Structural Analysis of Primed GAG Chains in CHO Cells.** The GAG chains primed by these fluorophore-tagged xylosides 9, 13a, and 13b were further analyzed for their molecular weights using size exclusion columns, as outlined in the Experimental Section. The chain length of GAG chains primed by fluorophore-tagged xylloside 9 in CHO cells was determined by measuring the migration time of GAG chains in comparison to those of polystyrene sulfonate standards performed under similar conditions on the size exclusion column and suggests that GAG chains, primed by fluorophore-tagged xylloside 9 in CHO cells, have 27 KDa at 50 μM and 20 KDa at 100 μM (Figure S1). However, the fluorophore-tagged xylloside 9 was not sensitive enough; therefore, the structural analysis of GAG chains primed by fluorophore-tagged xylloside 9 could not be studied using fluorescent detectors and relied instead on radiometric detector.

The UMB-O-xylloside has been shown to function as acceptor for the elongation of GAG chains by several groups. However, this well-known fluorophore-tagged xylloside mostly prime CS with low MW chains. By changing the O linkage of the fluorophore-tagged xylloside to click linkage, the fluorophore-tagged click-xylosides were predicted to prime both HS and CS with higher MW chains as many other click-xylosides. The results from priming activity analysis suggest that optimized priming concentration of UMB-click-xylosides 13a and 13b is 300 μM. Therefore, the GAG chains primed in CHO cells by these xylosides at 300 μM were further analyzed for their molecular weight using the size exclusion column and HS/CS composition using heparitinase I, II, and III. It is interesting to note that both fluorophore-tagged click-xylosides 13a and 13b primed GAG chains whose average molecular weight (42 kDa) is higher than those primed by commercial UMB-O-xylloside (4 kDa) (Figure 2). Furthermore, it is surprising to note that fluorophore-tagged click-xylosides 13a and 13b primed about 30% HS chains, whereas commercial UMB-O-xyllosides primed less than 5% HS chains (Figure 3). Some minor peaks appeared beyond Vt in the size exclusion column, and appearance of these late peaks may be attributed to the interactions of unknown small molecules carrying hydrophobic fluorophore with the column.

**Figure 1.** Priming activity of UMB-click-xylosides (13a, 13b) and UMB-O-xylosides (control) in pgsA-745 cell line. CHO cells were treated with fluorophore-tagged xylosides at 50 μM, 100 μM, 300 μM, 600 μM, and 1 mM in the presence of [H](100 μCi) as described in the Experimental Section. The GAG chains were purified by anion exchange chromatography and quantitated using a liquid scintillation counter. The results were the average of two independent experiments.

**Figure 2.** Size exclusion profiles of GAG chains primed by fluorophore-tagged xylosides (UMB-O-xyloside, 13a, and 13b) in pgsA-745 cells. GAG chains primed by fluorophore-tagged xylosides at 300 μM concentration in pgsA-745 cells for 24 h. The primed GAG chains were then purified and analyzed as described in the Experimental Section. The elution profile of the GAG chains primed by UMB-O-xyloside (gray trace), by fluorophore-tagged xylloside 13a (red trace), and by fluorophore-tagged xylloside 13b (black trace).

**Figure 3.** HS/CS compositions of GAG chains primed by fluorophore-tagged xylosides (UMB-O-xyloside, 13a, and 13b) in pgsA-745 cells. GAG chains primed by fluorophore-tagged xylosides at 300 μM concentration in pgsA-745 cells for 24 h. The HS/CS composition of the primed GAG chains was determined by digesting the GAG chains with heparitinase I, II, III. The purified GAG chains were analyzed by size exclusion chromatography. The elution profiles of the primed GAG chains without heparitinase I, II, III (gray trace). The elution profiles of the primed GAG chains with heparitinase I, II, III (black trace). (A) The elution profiles of GAG chains primed by UMB-O-xylloside. (B) The elution profile of GAG chains primed by fluorophore-tagged click-xylloside 13a. (C) The elution profiles of GAG chains primed by fluorophore-tagged click-xylloside 13b.

**Figure 4.** Structural Analysis of Primed GAG Chains in Endothelial Cells (BLMVEC). The fluorophore-tagged xylosides did prime GAG chains well in endothelial cells at various concentrations. At the optimal concentration (300 μM), molecular weight of GAG chains primed by fluorophore-tagged
xyloside 13b is much higher than those primed by commercial UMB-O-xyloside but the molecular weight of GAG chains primed by fluorophore-tagged xyloside 13a is not higher than those primed by commercial UMB-O-xyloside. The highly electron withdrawing substituent of −CF3 may result in longer GAG chains in BLMVEC cells. These GAG chains primed by fluorophore-tagged xylosides 13a and 13b and commercial UMB-O-xyloside were digested with heparitinase I/II/III to determine the percentage of HS. These fluorophore-tagged click-xylosides produced a significant amount of HS than the commercial xyloside as shown in Figure 4.

■ CONCLUSIONS

A small library of novel fluorophore-tagged xylosides was synthesized to evaluate their GAG-priming activity. Pyrene-click-xyloside 9 and UMB-click-xylosides 13a and 13b were able to participate in the stimulation of GAG biosynthesis. These fluorophore-tagged xylosides containing the triazol rings were more stable than commercially available xylosides and predictably have a longer in vivo half-life. Moreover, they were able to prime a significant amount of HS chains and higher MW than a commercial fluorophore-tagged O-xyloside. Therefore, these novel fluorophore-tagged click-xylosides have the potential to profile and elucidate cellular-specific GAG chains to define various dynamic interactions in the complex systems and to offer prospects to further our understanding of factors that regulate GAG biosynthesis as well as new knowledge on the role of GAG chains in various signaling events associated with pathophysiological processes.

■ ASSOCIATED CONTENT

* Supporting Information
Experimental data and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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