Biosynthesis of Heparan Sulfate on \( \beta \)-d-Xylosides Depends on Aglycone Structure*

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We have reported that 3-estradiol-\( \beta \)-d-xyloside primes heparan sulfate synthesis in Chinese hamster ovary cells and that the proportion of heparan sulfate made rises with increasing concentration of xyloside (Lugemwa, F. N. and Esko, J. D. (1991) J. Biol. Chem. 266, 6674–6677). Using estradiol as a guide, we varied the structure of the aglycone and showed that \( \beta \)-d-xylosides containing two fused aromatic rings efficiently prime heparan sulfate. Thus, 2-naphthol-\( \beta \)-d-xyloside primed heparan sulfate at low dose (\(<1\) μm) and the proportion of heparan sulfate increased with concentration marked (adv to 50% of total glycosaminoglycan). Various ring additions and heterocyclic ring substitutions altered the efficiency of heparan sulfate priming, but had no effect on the overall level of glycosaminoglycan synthesis. Replacement of the bridging oxygen with sulfur (2-naphthalenethiol-\( \beta \)-d-xyloside) increased the efficiency of heparan sulfate priming. Priming of heparan sulfate correlated with hydrophobicity of the xyloside, but several exceptions suggested that the chemical structure of the aglycone played an equally important role. Interestingly, the heparan sulfate chains generated on 2-naphthol-\( \beta \)-d-xyloside showed a 2-fold decrease in the proportion of disaccharides containing 6-O-sulfate groups and a striking diminution in non-sulfated iduronic acid content. Efficiency of heparan sulfate priming, but had no effect on the overall level of glycosaminoglycan synthesis. The fine structure of the chains produced on the primers and on endogenous core proteins differ, suggesting that the aglycone on which the chains assemble affects their composition and overall structure.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phenol, 5,6,7,8-tetrahydro-2-naphthol, cis/trans-decahydro-2-naphthol, 2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, 9-phenanthroline, 5-hydroxyindole, 2,6-dihydroxyxynaphthalene, 2-bromothanol, bromomethane, 1-bromobutane, 1,4-dibromobutane, 1-octanol, potassium carbonate, phosphorous tribromide, 1,4-dioxane, phosphorous pentoxide, 4-A molecular sieves, Drierite and silica gel (60 Å, 200–400 mesh) from Aldrich. Acetic anhydride was from EM Science (Gibbstown, NJ). 4-n-Butylphenol was from MPM Research Chemicals (Windham, NH). 6-Hydroxyquinoline was from ICN Biochemicals (Costa Mesa, CA). 3-d-Equilenin was from Research Plus (Bayonne, NJ). \( \beta \)-Xylene, anhydrous calcium chloride and diazotizable earth were from Sigma. Anhydrous Na₂SO₄ and Na₂S₂O₃ were from Curtin Matheson Scientific (Atlanta, GA). Sodium hydride was from Alfa Inorganics (Beverly, MA). All solvents were American Chemical Society grade.

Acetonitrilum-o-xylopyranoside was prepared from o-xylopyranose, acetic anhydride, phosphorous tribromide, and perchloric acid as described by Hellrich and Ost (1982). The silver silicate catalyst was prepared as described by Paulson and Lockhoff (1981). Silver carbonate was prepared as described by Wolfrom and Lineback (1963). All xylosides were from Sigma, except 2-chloro-2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, and 9-phenanthroline, which were from Aldrich. All solvents were American Chemical Society grade. 2-Chloro-2-naphthol, 1-naphthol, and 4-phenylphenol were prepared as described by Wolfrom and Lineback (1963). All xylosides were from Sigma, except 2-chloro-2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, and 9-phenanthroline, which were from Aldrich. All solvents were American Chemical Society grade. 2-Chloro-2-naphthol, 1-naphthol, and 4-phenylphenol were prepared as described by Wolfrom and Lineback (1963). All xylosides were from Sigma, except 2-chloro-2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, and 9-phenanthroline, which were from Aldrich. All solvents were American Chemical Society grade. 2-Chloro-2-naphthol, 1-naphthol, and 4-phenylphenol were prepared as described by Wolfrom and Lineback (1963). All xylosides were from Sigma, except 2-chloro-2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, and 9-phenanthroline, which were from Aldrich. All solvents were American Chemical Society grade. 2-Chloro-2-naphthol, 1-naphthol, and 4-phenylphenol were prepared as described by Wolfrom and Lineback (1963). All xylosides were from Sigma, except 2-chloro-2-naphthol, 1-naphthol, 4-phenylphenol, 2-naphthalenemethiol, and 9-phenanthroline, which were from Aldrich. All solvents were American Chemical Society grade.
The abbreviations used are: NC\textsubscript{X}, 2-(2-naphthoxy)-1-ethyl-\(\beta\)-o-xylopyranoside; NC\textsubscript{X}, 6-ethoxy-2-naphtho-\(\beta\)-o-xylopyranoside; C\textsubscript{NX}, 6-butoxy-2-naphtho-\(\beta\)-o-xylopyranoside; PX, phenyl-\(\beta\)-o-xylopyranoside; BPX, 4-n-butyphenyl-\(\beta\)-o-xylopyranoside; THXX, 5,6,7,8-tetrahydro-2-naphtho-\(\beta\)-o-xylopyranoside; 2-NX, 2-naphthyl-\(\beta\)-o-xylopyranoside; IX, 5-hydroxyindole-\(\beta\)-o-xylopyranoside; QX, 5-hydroxyindole-6-ethoxy-\(\beta\)-o-xylopyranoside; PPX, 4-phenylphenol-\(\beta\)-o-xylopyranoside; PX, 9-phenyl-\(\beta\)-o-xylopyranoside; EQX, 3,4-diethelino-\(\beta\)-o-xylopyranoside; NSX, 2-naphtholenethanol-\(\beta\)-o-xylopyranoside; DX, cis/trans-decalyl-2-naphtho-\(\beta\)-o-xylopyranoside.

The NMR data for xylsides are presented in Table I. The migration of the compounds on thin layer C\(\textsubscript{18}\) reverse-phase plates (E. Merck, methanol/H\(\textsubscript{2}O\), 7:3, \(v/v\)) or on Silica Gel 60-A plates (Whatman) is also presented.

### Table I: \(R_f\) values and \(\textsuperscript{1}H\) NMR data for xylsides

| Xylside                  | \(R_f\) \(^a\) | H-1 (ppm) | J(\text{Hz}) | Other protons (ppm) |
|--------------------------|----------------|-----------|--------------|---------------------|
| NC\textsubscript{X}      | 0.39           | 4.24      | 7.56         |                     |
| NC\textsubscript{X}      | 0.24           | 4.26      | 7.59         |                     |
| C\textsubscript{NX}      | 0.41           | 4.96      | 7.23         |                     |
| C\textsubscript{NX}      | 0.20           | 4.96      | 7.10         |                     |
| PX                       | 0.67           | 4.85      | 7.48         |                     |
| BPX                      | 0.25           | 4.79      | 7.40         |                     |
| THXX                     | 0.36           | 4.75      | 7.37         |                     |
| 2-NX                     | 0.48           | 5.05      | 7.19         |                     |
| 1-NX                     | 0.47           | 5.02      | 7.57         |                     |
| IX                       | 0.67           | 4.72      | 7.36         |                     |
| QX                       | 0.62           | 5.06      | 7.14         |                     |
| PPX                      | 0.36           | 4.91      | 7.35         |                     |
| PNX                      | 0.28           | 5.15      | 7.50         |                     |
| EQX                      | 0.34           | 5.05      | 7.16         |                     |
| NSX                      | 0.45           | 4.77      | 9.14         |                     |
| DX                       | 0.21           | 3.33      | 9.30         |                     |
| 6-Ethoxy-2-naphthol      | 0.24           |           |              |                     |

\(a\) Relative mobility of each compound on C\(\textsubscript{18}\) reverse-phase \((R_f\,1)\) or Silica Gel 60-A thin layer chromatography plates \((R_f\,2)\) (*Experimental Procedures*).

\(b\) Both the anomeric proton and a methylene group gave peaks in the range of 4.13-4.08 ppm and gave an integration of 3H. A coupling constant could not be assigned.

\(c\) Due to the mixture of isomers, a multiplet in the range of 4.19-4.11 ppm occurred and could not be assigned a coupling constant.

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*agnostic protons are included in Table I. The migration of the compounds on thin layer C\(\textsubscript{18}\) reverse-phase plates (E. Merck, methanol/H\(\textsubscript{2}O\), 7:3, \(v/v\)) or on Silica Gel 60-A plates (Whatman) is also presented.

**Synthesis of Aglycoones.—2-(2-Naphthoxy)ethanol was prepared by stirring 2-naphthol (7.2 g, 50 mmol) and \(\text{K}_2\text{CO}_3\) (6.9 g, 50 mmol) in 10 ml of acetone for 30 min at room temperature. 2-Bromoethanol (6.2 g, 50 mmol) was added, and the mixture was gently refluxed for 7 h. The reaction was then distilled with water, and the organic layer was repeatedly extracted with 2 ml of NaOH. The NaOH washings were extracted with diethyl ether, and the ether extracts were added to the organic layer. The organic layer was dried with CaCO\(\textsubscript{3}\), concentrated, and then deacetylated overnight at 0-5 °C. The amorphous product that formed was collected and placed at -20 °C. The amorphous product that formed was collected and placed at -20 °C. The amorphous product that formed was collected and placed at -20 °C. The amorphous product that formed was collected and placed at -20 °C. The amorphous product that formed was collected and placed at -20 °C.

**Preparation of \(\beta\)-o-xylopyranosides.—2-(2-Naphthoxy)-1-ethyl-\(\beta\)-o-xylopyranoside was prepared by the Koenigs-Knorr reaction with 2,6-dihydroxynaphthalene and NaH in dimethylformamide containing NaH (0.48 g, 20 mmol). Bromoethane (2.2 g, 20 mmol) was added, and the mixture was stirred at 50-55 °C for 18 h. Dimethylformamide was removed by coevaporation with toluene, and the resulting solution was diluted with ethyl acetate. Several volumes of 0.1-0.2 x NaOH were added, and the ethyl acetate layer was removed. The aqueous layer was extracted with ethyl acetate, and the organic extracts were combined with the original ethyl acetate phase, dried with Na\(\text{SO}_4\), and concentrated. The solution was diluted with acetone, and the precipitate that formed was filtered. The filtrate was concentrated to dryness, dissolved in acetonitrile, and insoluble material was again removed by filtration. The filtrate was concentrated, diluted with a minimal volume of toluene and product was purified by silica gel chromatography (20 g) by washing with hexanes and increasing the proportion of ethyl acetate.

6-Butoxy-2-naphthol was similarly prepared from 1-bromobutane (0.68 g, 5 mmol), 2,6-dihydroxynaphthalene (3.2 g, 20 mmol), and NaH (0.48 g, 20 mmol) in 30 ml of dimethylformamide. The reaction, however, was carried out at 90-95 °C for 3.5 h. Product was purified as for 6-ethoxy-2-naphthol on a 25-g silica gel column.

Preparation of \(\beta\)-o-xylopyranosides.—2-(2-Naphthoxy)-1-ethyl-\(\beta\)-o-xylopyranoside was prepared by the Koenigs-Knorr reaction (1901) with a modified version of the method described by VanAken et al. (1986). 2-(2-Naphthoxy)ethanol (0.377 g, 2 mmol) was dissolved in 10 ml of dichloromethane containing 1 g of 4Å molecular sieves. Silver silicate (1.5 g, 5.2 mmol) was added in the dark with stirring for 10 min, and the solution was cooled to 20 °C. Acetobromoo-o-xylopyranoside (0.85 g, 2.5 mmol) was added. After 10 min, the reaction was filtered through diatomaceous earth, concentrated, and then deacetylated overnight at 23 °C in 8 ml of methanol/water/triethylamine (2:1:1, \(v/v\)). Product was purified by silica gel chromatography (15 g) first washing with hexanes and then eluting with ethyl acetate/hexanes/acetic acid (90:10:1, \(v/v\)).

6-(2-Naphthoxy)-1-bromobutanol was prepared as described by VanAken et al. (1986). 6-(2-Naphthoxy)-1-butoxy-\(\beta\)-o-xylopyranoside was prepared by the Koenigs-Knorr reaction with 2,6-dihydroxynaphthalene (1 g, 5 mmol), Ag\(\text{CO}_3\) (0.5 g, 1.8 mmol), and 0.7 g of Drierite were stirred in 10 ml of dichloromethane for 2 h at 23 °C in the dark. The reaction was completed as described by VanAken et al. (1986). The 6-(2-Naphthoxy)-1-butoxy-\(\beta\)-o-xylopyranoside was prepared by the Koenigs-Knorr reaction with 2,6-dihydroxynaphthalene (1 g, 5 mmol), Ag\(\text{CO}_3\) (0.5 g, 1.8 mmol), and 0.7 g of Drierite were stirred in 10 ml of dichloromethane for 2 h at 23 °C in the dark. The reaction was completed as described by VanAken et al. (1986). The 6-(2-Naphthoxy)-1-butoxy-\(\beta\)-o-xylopyranoside was prepared by the Koenigs-Knorr reaction with 2,6-dihydroxynaphthalene (1 g, 5 mmol), Ag\(\text{CO}_3\) (0.5 g, 1.8 mmol), and 0.7 g of Drierite were stirred in 10 ml of dichloromethane for 2 h at 23 °C in the dark.
filtered through diatomaceous earth and washed with 5% Na2S2O3 (w/v). The mixture was concentrated and dissolved in 90% aqueous acetone containing 5 mM H2SO4 to dry hydrolyze orthoesters. After 30 min at 23 °C, the solution was neutralized with pyridine, concentrated, and deacetylated as described above. Product was purified by silica gel chromatography (15 g) in ethyl acetate/toluene/acetic acid (5:5:0.1, v/v) and eluting by increasing the proportion of ethyl acetate.

6-Ethoxy-2-naphthyl-β-D-xylopyranoside was similarly prepared from 6-ethoxy-2-naphthol (0.34 g, 1.6 mmol), NaH (48 mg, 2 mmol), and aceto-β-D-xylopyranoside (1.2 g, 3.5 mmol). However, after the initial reaction, additional NaH (0.125 g, 5.2 mmol) and aceto-β-D-xylopyranoside (1.5 g, 4.4 mmol) were added and allowed to react overnight at 23 °C. The product was purified using methods described for 6-ethoxy-2-naphthyl-β-D-glucopyranoside.

Benzyl 2-naphthol (15 mg, 3.5 mol%), 6,6,7,8-tetrahydro-2-naphthol, 2-naphthol, 1-naphthol, 6-hydroxynaphthalene, and 6-hydroxyquinoline-β-D-xylopyranosides were prepared by the Koenigs-Knorr reaction (1801) with a modified version of the method described by Conchie and Levy (1963). The corresponding chlorophenols (25 mmol) were dissolved in 2.1 ml of 1 M NaOH, and enough aceton was added to completely solubilize the sample. Acetobromo-β-D-xylopyranoside (1-3 mmol) was dissolved in aceton and added to the reaction. The final volume of aceton never exceeded 3.1 ml he action. After stirring the reactions for 5-24 h at 23 °C, the solution was neutralized with pyridine, concentrated, and deacetylated as described above. After deacetylation, the samples were concentrated to about 0.5 ml of acetone.

3,5,6,7,8-Tetrahydro-2-naphthol, 5,6,7,8-tetrahydro-2-naphthol, 6-hydroxynaphthalene, and 6-hydroxyquinoline-β-D-xylopyranosides were prepared by the Koenigs-Knorr reaction (1801) with a modified version of the method described by Conchie and Levy (1963). The corresponding chlorophenols (25 mmol) were dissolved in 2.1 ml of 1 M NaOH, and enough aceton was added to completely solubilize the sample. Acetobromo-β-D-xylopyranoside (1-3 mmol) was dissolved in aceton and added to the reaction. The final volume of aceton never exceeded 3.1 ml (v/v). After stirring the reactions for 5-24 h at 23 °C, the solution was neutralized with pyridine, concentrated, and deacetylated as described above. After deacetylation, the samples were concentrated to about 0.5 ml of acetone.

O-xylopyranoside (1.5 g, 4.4 mmol) were added and allowed to react overnight at 23 °C. The product was purified using methods described by Shively and Conrad (1976). The pellets were dissolved in 2.5 ml of 1 M NaClN in 20 mM sodium acetate buffer (pH 6.0). GAGs were precipitated by adding 10 ml of cold 95% ethanol and incubating the samples at 4 °C for 2 h. The samples were centrifuged for 10 min, the supernatant was aspirated, and the pellets were resuspended in 1 ml of 0.5 M sodium acetate in water (1/8, v/v) and redissolved with 4 ml of ethanol. The final pellets were dried under vacuum and resuspended in 0.2 ml of 20 mM sodium acetate (pH 6.0).

Aliquots (45 µl) of the resuspended pellets were treated with low pH nitrous acid (Shively and Conrad, 1976), which cleaves heparan sulfate at N-sulfated glucosamine residues and generates a series of short oligosaccharides. The remaining chondroitin sulfate was isolated by anion-exchange chromatography as described above, and the amount of [35S]Heparan sulfate was determined as the difference between the total [35S]GAG and the remaining [35S]Chondroitin sulfate.

In each experiment a pair of wells (unlabeled) was washed three times with 1 ml of cold phosphate-buffered saline and solubilized in 1 M NaOH, and enough aceton was added to completely solubilize the sample. Acetobromo-β-D-xylopyranoside (1-3 mmol) was dissolved in aceton and added to the reaction. The final volume of aceton never exceeded 3.1 ml (v/v). After stirring the reactions for 5-24 h at 23 °C, the solution was neutralized with pyridine, concentrated, and deacetylated as described above. After deacetylation, the samples were concentrated to about 0.5 ml of acetone.
purified by gel filtration chromatography (1 × 100-cm Bio-Gel P2 column, Bio-Rad) in the same buffer. Over 90% of the material eluted as disaccharides. The fractions were pooled, lyophilized, dissolved in water, and desalted by descending paper chromatography on Whatman 3MM paper in butanol/ethanol/water (52:32:16, v/v). Disaccharides were eluted from the paper with water/ethanol (9:1, v/v).

The disaccharides were separated by reverse-phase ion-pairing chromatography essentially as described by Guo and Conrad (1988) using a Hi-Chrom S5 ODS C18 column (4.6 × 50 mm, Regis Chemical Co.) at a flow rate of 1 ml/min. Starting buffer contained 38 mM NH₄H₂PO₄, 2 mM H₃PO₄, and 1 mM tetrabutylammonium phosphate in water (pH 3.6). Disaccharides were eluted with a gradient of water/acetonitrile (70:30, v/v) containing 38 mM NH₄H₂PO₄, 2 mM H₃PO₄, and 1 mM tetrabutylammonium phosphate (pH 4.9). Their elution was monitored with a Radiomatic FLO-ONE Beta in-line detector. Radioactivity was measured at 6-s intervals, and the data were averaged over 0.5-min time frames. Individual peaks were identified by comparison with heparin disaccharides and published results (Guo and Conrad, 1988). Heparin disaccharides were prepared as described and labeled by reduction with NaBH₄ (500 Cimol, Amersham Corp.) as described by Guo and Conrad (1988). Non-sulfated disaccharides from the reverse-phase ion-pairing column were pooled and analyzed by descending paper chromatography on Whatman 1MM paper in ethyl acetate/acetic acid/H₂O (3:1:1, v/v) as described by Bame et al. (1991). Radiolabeled GlcA-AmanR used as a standard for paper chromatography was prepared as Escherichia coli K-12 N-acetylhyaluronan by hydrazinolysis and pH 4.5 nitrous acid cleavage as described by Lindahl et al. (1973). The disaccharides were radioabeled by reduction with NaBH₄ as described by Guo and Conrad (1988).

RESULTS

Heparan Sulfate Priming on 2-Naphthol-β-D-xyloside—We recently showed that estradiol-β-D-xyloside primes over 50% heparan sulfate in CHO cells, whereas other hydrophobic xylosides prime only chondroitin sulfate (Lugemwa and Esko, 1991). To test if smaller, less complex aglycones than estradiol might facilitate biosynthesis of heparan sulfate, a series of β-D-xylosides were synthesized and fed to a mutant CHO cell line that lacks xylosyltransferase (pgsA-745). The absence of GAG synthesis on endogenous proteoglycans in mutant pgsA-745 (Lidholt et al., 1992) makes the cells a convenient system for studying priming of GAGs by exogenous β-D-xylosides.

Beginning with the phenolic A ring of estradiol, xylosides attached to aglycones of increasing complexity were tested (Fig. 1). Each of the xylosides at 50 μM primed comparable levels of [35S]GAG (0.8 ± 0.2 × 10⁶ cpm/well). However, the relative amount of [35S]heparan sulfate varied dramatically. Xylosides containing phenyl, 4-n-butylphenyl, or cis/trans-decahydro-2-naphthol failed to prime heparan sulfate, whereas 5,6,7,8-tetrahydro-2-naphthol-β-D-xyloside, which contains the A and B rings of estradiol, showed some priming of heparan sulfate (20%). When both of the rings were aromatic (2-naphthol-β-D-xyloside) priming of heparan sulfate increased to a level comparable to that stimulated by 3-estradiol-β-D-xyloside (52% of the total [35S]GAG) versus 48%, respectively. Thus, of the compounds tested 2-naphthol-β-D-xyloside directed heparan sulfate synthesis as efficiently as 3-estradiol-β-D-xyloside.

Priming of Heparan Sulfate by 2-Naphthol-β-D-xyloside Depends on Concentration—When the concentration of 2-naphthol-β-D-xyloside was varied from 1–100 μM, priming of [35S]heparan sulfate increased and then gradually declined (Fig. 2A). At low dose (1–5 μM) only [35S]chondroitin sulfate was made. [35S]Heparan sulfate synthesis occurred at 3 μM, increased up to 30 μM, and then remained nearly constant. Priming of total [35S]GAG, [35S]chondroitin sulfate, and [35S]heparan sulfate by 3-estradiol-β-D-xyloside behaved in a similar fashion except that the curves were shifted to higher concentrations (Fig 2B). However, above 30 μM a pronounced decrease in both chondroitin sulfate and heparan sulfate occurred, probably due to the detergent properties of estradiol-β-D-xyloside. High concentrations of 2-naphthol-β-D-xyloside did not cause a decline in priming.

Nearly all of the xylosides tested to date prime GAGs in a dose-dependent manner. Since the midpoint for maximal priming of heparan sulfate for 2-naphthol-β-D-xyloside was about 10 μM (Fig. 2), we used this concentration to evaluate the relative efficacy of other xylosides. At this concentration, 2-naphthol-β-D-xyloside primed heparan sulfate better than 3-estradiol-β-D-xyloside (32% of total GAG versus 10%, respectively).

Ring Modifications and Additions—We examined whether adding alkoxy “spacers” that increase the distance between naphthol and xylose would affect priming (Fig. 3). Since the addition of the spacers would also change the hydrophobicity of the aglycone, we tested a set of related compounds containing similar “tails” attached to the 6-position of 2-naphthol-β-D-xyloside. The addition of a 2-carbon spacer (2-(2-naphthoxy)-1-ethyl-β-D-xyloside) had no effect on priming, nor did the addition of a 2-carbon tail (6-ethoxy-2-naphthol-β-D-xyloside). In contrast, the addition of a 4-carbon spacer (4-(2-naphthoxy)-1-buty1-β-D-xyloside) or a 4-carbon tail (6-butoxy-2-naphthol-β-D-xyloside) decreased priming of heparan sulfate. Neither modification affected to a significant degree the overall extent of GAG priming. The corresponding 8-carbon adducts also were tested, but the compounds exhibited strong detergent properties and solubilized the cells (data not shown).

To examine whether heteroatom substitutions might influence heparan sulfate priming, 6-hydroxyquinoline-β-D-xyloside, 5-hydroxyindole-β-D-xyloside, 2-naphthalenethiol-β-D-xyloside, and 2-naphthol-β-D-xyloside were compared (Fig. 4). At 10 μM, both 6-hydroxyquinoline-β-D-xyloside and 5-hydroxy-

| Xyloside (50 μΜ) | Total [35S]GAG (cpmwell × 10⁶) | Heparan Sulfate (%) |
|------------------|-----------------------------|-------------------|
| phenyl-β-D-xyloside | 1.0 | 0 |
| 4-n-butylphenyl-β-D-xyloside | 0.8 | 0 |
| 5,6,7,8-tetrahydro-2-naphthol-β-D-xyloside | 0.7 | 20 |
| cis/trans-decahydro-2-naphthol-β-D-xyloside | 0.9 | 0 |
| 2-naphthol-β-D-xyloside | 0.7 | 52 |
| 3-estradiol-β-D-xyloside | 0.6 | 48 |

FIG. 1. Aglycone structure of β-D-xylosides affects heparan sulfate biosynthesis. CHO pgS-745 cells were labeled with [35S]SO₄ for 4 h in 24-well culture dishes in the presence of 50 μM of the indicated xyloside. GAGs were prepared and analyzed as described under “Experimental Procedures.”
dole-β-D-xyloside primed heparan sulfate less efficiently compared to 2-naphthol-β-D-xyloside (9 and 14% versus 32%, respectively) but the total amount of GAG primed by each was comparable. Interestingly, replacement of the bridging oxygen with sulfur (2-naphthalenethiol-β-D-xyloside) increased the efficiency of heparan sulfate priming. Nearly 50% of the GAG primed by 2-naphthalenethiol-β-D-xyloside at 10 μM was heparan sulfate.

A variety of other ring modifications and additions to the aglycone were tested (Fig. 5). Changing the aglycone to 1-naphthol or 9-phenanthrol had little effect on heparan sulfate priming relative to 2-naphthol (20% for both compounds versus 26%, respectively). Separation of the aromatic rings in 4-phenylphenol-β-D-xylose somewhat reduced priming of heparan sulfate (15%), suggesting that the planar structure afforded by the fused aromatic rings in naphthol may be important. The xyloside of 3-d-equilenin was found to be more potent than 2-naphthol-β-D-xyloside (55% heparan sulfate at 10 μM), and the dose response showed a clear shift to lower concentration (data not shown). 3-d-Equilenin resembles 3-estradiol, but the aromatic A and B rings correspond to those of 2-naphthol. These results point to the importance of two fused aromatic rings and suggest that certain adducts might further enhance priming of heparan sulfate.

The various modifications to 2-naphthol-β-D-xyloside shown in Figs. 3–5 would affect aqueous solubility of the xylosides and possibly their transfer across cell membranes. As shown in Table II, the extent of heparan sulfate priming seemed to correlate with the partitioning of xyloside into the organic phase of an octanol-water mixture. However, the correlation did not hold for all compounds since 9-phenanthrol-β-D-xyloside primed heparan sulfate to a similar extent as 2-naphthol-β-D-xyloside, but was more soluble in the octanol phase. 4-n-Butylphenol-β-D-xyloside also preferentially partitioned into octanol but primed heparan sulfate poorly.

The Fine Structure of Heparan Sulfate Primed by 2-Naphthol-β-D-xyloside—Priming of chondroitin sulfate and dermatan sulfate on xylosides can result in undersulfation of the chains relative to those produced on endogenous core proteins (Gibson et al., 1977; Cöster et al., 1991). To test if the heparan sulfate chains built on xylosides might be undersulfated, cells were incubated with 10 μM 2-naphthol-β-D-xyloside and various concentrations of [35S]SO₄ at constant radiospecific activity (Fig. 6A). Incorporation of [35S]SO₄ increased as the concentration of [35S]SO₄ in the medium was increased and remained nearly constant above 75 μM. This finding suggested that adding ≥75 μM sulfate would ensure full sulfation of GAGs.

To test if the extent of sulfation would depend on the dose of β-D-xyloside, cells were labeled with [3H]glucosamine and [35S]SO₄ in the presence or absence of inorganic sulfate and increasing concentrations of 2-naphthol-β-D-xyloside (Fig. 6B). In the absence of added inorganic sulfate, the ratio of incorporated [3H] and [35S] remained essentially constant at all concentrations of 2-naphthol-β-D-xyloside up to 300 μM, the highest concentration tested. Thus, the inclusion of 100
ride composition of the heparan sulfate primed on 2-naphthol-β-D-xyloside by analyzing the disaccharide composition of the chains. The composition of heparan sulfate primed on 2-naphthol-β-D-xyloside did not vary by more than 5% from the mean.

| Xyloside (10 μM) | Total 35S GAG (cpm/μg x 10^4) | Heparan Sulfate (%) |
|------------------|---------------------------------|---------------------|
| 2-naphthol-β-D-xyloside | 2.8 | 32 |
| 3-hydroxyindole-β-D-xyloside | 2.2 | 14 |
| 6-hydroxyquinoline-β-D-xyloside | 2.6 | 9 |
| 2-naphthalenethiol-β-D-xyloside | 2.7 | 50 |

![Image](image.png)

**Fig. 4. Heterorotom substitutions.** CHO pgsA-745 cells were labeled with 35SO4 in the presence of 10 μM of the indicated xylosides. GAGs were analyzed as described under "Experimental Procedures." Data are expressed as 35S cpm/μg cell protein and are the mean of duplicate determinations which did not vary by more than 5% from the mean.

| Xyloside (10 μM) | Total 35S GAG (cpm/μg x 10^4) | Heparan Sulfate (%) |
|------------------|---------------------------------|---------------------|
| 2-naphthol-β-D-xyloside | 4.2 | 26 |
| 3-naphthol-β-D-xyloside | 4.5 | 20 |
| 4-phenylphenol-β-D-xyloside | 4.1 | 15 |
| 9-phenanthrol-β-D-xyloside | 4.4 | 20 |
| 3-o-quinoid-β-D-xyloside | 3.8 | 55 |

![Image](image.png)

**Fig. 5. Aromatic rings.** CHO pgsA-745 cells were labeled with 35SO4 in the presence of 10 μM of the indicated xylosides. GAGs were analyzed as described under "Experimental Procedures." Data are expressed as 35S cpm/μg cell protein and are the mean of duplicate determinations which did not vary by more than 5% from the mean.

μm inorganic sulfate was sufficient to permit full sulfation of GAGs.

We next examined the fine structure of the heparan sulfate primed on 2-naphthol-β-D-xyloside by analyzing the disaccharide composition of the chains. The composition of heparan sulfate primed on 2-naphthol-β-D-xyloside in pgsA-745 cells was qualitatively similar (Fig. 7). However, the amount of 6-O-sulfated disaccharides declined about 2-fold (ISMS, derived mostly from GlcA-GlcNS0360S03 units). The amount of 6-O-sulfated disaccharides declined about 2-fold (ISMS, derived mostly from GlcA-GlcNS0360S03 units), and GMS, derived mostly from IdoA-GlcNS0360S03 units, was resolved by paper chromatography, a striking decrease in IdoA-αManR was found (Table III). The changes in disaccharide composition were also seen in a separate experiment in which the cells were incubated with only 10 μM 2-naphthol-β-D-xyloside.

### Table II

| Xyloside | K<sub>ow</sub> = C<sub>C</sub>/C<sub>W</sub> | Heparan Sulfate (%) |
|----------|----------------------|---------------------|
| Phenyl-β-D-xyloside | 0.89 | ≤5 |
| 6-Hydroxyquinoline-β-D-xyloside | 1.2 | 5 |
| 5-Hydroxyindole-β-D-xyloside | 4.8 | 14 |
| 5,6,7,8-Tetrahydro-2-naphthol-β-D-xyloside | 9.2 | 20 |
| 2-Naphthol-β-D-xyloside | 17.6 | 32 |
| 2-Naphthalenethiol-β-D-xyloside | 55.4 | 50 |
| 4-n-Butylphenyl-β-D-xyloside | >100 | >5 |
| 9-Phenanthrol-β-D-xyloside | >100 | 20 |

Some ISMS, IMS, and ISM may have originated from the corresponding N-acetylated disaccharide units. However, the modified disaccharides tend to predominate in sections of the chain that have undergone extensive GlcN S-sulfation.
What is the mechanism that controls the addition of GlcNAc and GalNAc and therefore the type of GAG chain assembled on particular xylosides? One possibility is that chondroitin sulfate and heparan sulfate synthesis occurs in different subcellular compartments. Selective entry of xylosides according to the structure or hydrophobicity of the aglycone could explain the observed differences in heparan sulfate priming. Although some work has been done to localize various enzymes involved in chondroitin sulfate (Sugumaran et al., 1992; Spiro et al., 1991) and heparan sulfate biosynthesis (Uhlin-Hansen and Yanagishita, 1993), definitive evidence for their location is lacking (Kjellén and Lindahl, 1991). The endoplasmic reticulum, Golgi, and plasma membranes differ in protein and lipid composition (Daum, 1985), suggesting the possibility that xylosides of different hydrophobicity or chemical properties might partition preferentially into particular compartments. Molecules such as benzo(a)pyrene have been shown to localize to distinct sites within cells (Plant et al., 1985) and exogenous membrane lipids sort to different subcellular locations (Pagano, 1990). Perhaps xylosides with different aglycones follow distinct pathways after they enter cells as well.

Another explanation for the differential priming ability of various xylosides should be considered. Free xylose and xylosides containing simple aglycones (e.g. methanol) prime chondroitin sulfate readily, suggesting that chondroitin sulfate assembly probably occurs by default. In contrast, heparan sulfate synthesis on xylosides requires a more complex aglycone. One can speculate that the aromatic rings of naphthol mimic a

**Table III**

**Disaccharide composition of heparan sulfate chains**

Heparan sulfate chains produced on 2-naphthol-β-D-xyloside or endogenous core proteins were depolymerized to disaccharides by hydrazinolysis and nitrous acid cleavage. The disaccharides were reduced and analyzed by reverse phase ion pairing chromatography as described under "Experimental Procedures." The amount of radioactivity in each of the disaccharides was used to determine the relative abundance of each unit. The abbreviations are the same as in Fig. 7.

| Cells                      | GM | IM | ISM | GMS and GSM | IMS | GMS₂ | ISMS | GSMS |
|----------------------------|----|----|-----|-------------|-----|------|------|------|
| Wild-type pgsA-745         | 30 | 13 | 19  | 6           | 6   | 0    | 22   | 0    |
| Mutant pgsA-745 + 50 μM 2-naphthol-β-D-xyloside | 57 | 0  | 21  | 3           | 3   | 0    | 12   | 0    |

An alternative explanation for the differential priming ability of various xylosides should be considered. Free xylose and xylosides containing simple aglycones (e.g. methanol) prime chondroitin sulfate readily, suggesting that chondroitin sulfate assembly probably occurs by default. In contrast, heparan sulfate synthesis on xylosides requires a more complex aglycone. One can speculate that the aromatic rings of naphthol mimic a

**Fig. 6.** Addition of inorganic sulfate enures the normal suflafa- tion of GAGs primed by xylosides. A, CHO pgsA-745 cells were incubated with 10 μM 2-naphthol-β-D-xyloside and the indicated amount of Na₂SO₄ at constant radioactivity. B, one set of cells were incubated in medium containing 1 mM glucose, 100 μM Na₂SO₄, 100 μM [6-3H]-glucosamine, and 50 μCi/ml Na₂SO₄ (○). A second set of cells were incubated in medium containing 100 μM [6-3H]-glucosamine, 8 μCi/ml Na₂SO₄, and no added Na₂SO₄ (□). The concentration of 2-naphthol-β-D-xyloside was varied, and the radioactive GAG chains were counted by liquid scintillation spectrometry using dual channels to determine the amount of [3H] and [35S] counts in the GAG chains. All data are presented as total incorporation of [3H] and [35S] cpm/well whereas data in panel B are presented as the ratio [3H]/[35S] in the GAG chains. All data are the mean of duplicate determinations which varied by less than 10% from the mean.

**Fig. 7.** Disaccharide composition of heparan sulfate primed on 2-naphthol-β-D-xyloside and endogenous core proteins. Wild-type (A) and pgsA-745 cells (B) were labeled for 36 h with [6-3H]-glucosamine, and heparan sulfate disaccharides were isolated and analyzed as described under "Experimental Procedures." The elution positions of the non sulfated disaccharides, GlcA-Man₉ (GM) and IdooMan₉ (AM), 2,5-anhydrodextran (AM), the monosulfated disaccharides, IdooSO₃-Man₉ (ISM), GlcA-Man₉(6OSO₃) (GSM), GlcA₂SO₃-Man₉ (GMS), and IdooMan₉(6OSO₃) (IMS), and the disulfated disaccharides, IdooSO₃-2-Man₉(6OSO₃) (ISMS), GlcA₂SO₃-Man₉(6OSO₃) (GMS₂) and GlcA-Man₉(6OSO₃) (GM₉) are indicated by the arrows.

![Fig. 6](image1.png)

![Fig. 7](image2.png)
hydrophobic determinant on proteoglycan core proteins that interacts with the transferase catalyzing the addition of the GlcNAcα1,4 to the linkage tetrasaccharide. In this model, core proteins that do not normally carry heparan sulfate chains would lack this determinant.

If the latter hypothesis is correct, tetrasaccharide intermediates containing naphthol may preferentially bind the transferase that catalyzes the addition of GlcNAcα1-4 to the terminal GlcA unit. This enzyme may differ from the GlcNAcα1-4 transferase involved in chain polymerization. A previous study of the substrate specificity of GalNAcβ1,4 addition suggests that two different enzymes catalyze chondroitin sulfate initiation and polymerization (Rohrmann et al., 1985).

The model predicts that GlcNAcα1-4 transferase recognizes the aglycone as well as the terminal GlcA of the linkage tetrasaccharide. Freeze et al. (1993) showed that Gal transferase II, which transfers Galβ1,3 to the intermediate Galβ1,4Xylβ1 during GAG synthesis, recognizes both the anomereric configuration at the reducing end of this disaccharide as well as the terminal galactose. Gal addition to xylene catalyzed by Gal transferase I occurred to both p-nitrophenyl-α- and p-nitrophenyl-β-xylene in cells. The addition of the second Gal residue, however, did not occur to the α-linked compound, suggesting that Gal transferase II distinguishes between α and β configuration of the terminal xylose. The α-2-3 sialyltransferase that generates NeuAcα2-3Galβ1-4xylsodides exhibits similar anomer-ic selectivity (Freeze et al., 1993).

Based on these observations, we predicted that the proximity of xylene to the hydrophobic aglycone might affect priming of heparan sulfate chains. However, the results were inconclusive since placing an ethoxy or butoxyl group between xylene and naphthol (2-(2-naphthoxy)-1-ethyl-β-n-xylolypirosidone and 4-(2-naphthoxy)-1-butyl-β-n-xylolypirosidone, respectively) had the same effect as adding the same group as a tail to 2-naphthol-β-o-xylolypirosidone (6-ethoxy-2-naphthol-β-o-xylolypirosidone and 6-butoxy-2-naphthol-β-o-xylolypirosidone, respectively). Current experiments focus on the synthesis and uptake of other glycosides derived from the linkage region (e.g. naphthol-β-o-galactosoide).

Examination of disaccharides generated from heparan sulfate chains made on 2-naphthol-β-o-xylolysodides revealed by β-n-xylolysodides can differ from that synthesized on core proteins (Coster et al., 1990; Fransson et al., 1991). Chain length, extent of epimerization of GlcA to IdoA, degree of sulfation, and the frequency of periodic repeats of clustered GlcA-GalNAC declined, depending on the concentration of xylolysodide (Fransson et al., 1992). In our study, the amount of non-sulfated IdoA-containing disaccharides in heparan sulfate declined dramatically (Table III), but when the enhanced rate of synthesis of GAG chains was taken into account, the total number of IdoA units actually increased.

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