

## Xylosides As Artificial Primers Of Glycosaminoglycan Biosynthesis From Peruvian Rain Forest Plants.

Jain Amit Kumar

UMS Raghapur, Revelganj, Saran, Bihar India – 841305

Email [ID-amitjainmjr@gmail.com](mailto:ID-amitjainmjr@gmail.com)

### ABSTRACT

We have developed a rapid, high throughput screening assay for compounds that alter the assembly of glycosaminoglycan chains in Chinese hamster ovary cells. The assay uses autoradiography to measure the binding of newly synthesized proteoglycans and glycosaminoglycans to a positively charged membrane. Screening over 1000 extracts from a random plant collection obtained from the Amazon rain forest yielded five plants that stimulated glycosaminoglycan assembly in both wild-type cells and a mutant cell line defective in xylosyltransferase (the first committed enzyme involved in glycosaminoglycan biosynthesis). Fractionation of an extract of *Maieta guianensis* by silica gel and reversephase chromatography yielded two pure compounds with stimulatory activity. Spectroscopic analysis by NMR and mass spectrometry revealed that the active principles were xylosides of dimethylated ellagic acid. One of the compounds also contained a galloyl group at C-3 of the xylose moiety. These findings suggest that plants and other natural products may be a source of agents that can potentially alter glycosaminoglycan and proteoglycan formation in animal cells. Glycosylation inhibitors provide powerful tools for understanding the biosynthesis and biological functions of glycoconjugates in animal cells. Toward that end, a number of substrate analogs for the glycosyltransferases have been synthesized and shown to act as specific inhibitors in vitro. Some of these compounds act competitively with respect to the acceptor substrates and have  $K_i$  values in the low micromolar range. Unfortunately, most of these compounds tend to lack activity in live cells, presumably because their hydrophilicity limits their access to the Golgi compartments where the glycosyltransferases reside. A second class of inhibitors, plant alkaloids, act both in vitro and in vivo to inhibit the processing glycosidases required for Asn-linked oligosaccharide formation on glycoproteins. Analogs of the alkaloids also can inactivate glycosyltransferases. Inhibition blocks the formation of complex N-linked oligosaccharides, leading to various alterations in glycoprotein secretion and function. One of the alkaloids, swainsonine, and its carbonoxyloxy analogs have potent antitumor activity in mice and humans, suggesting that these inhibitors may provide novel chemotherapeutic approaches for treating cancer. A third class of inhibitors act by diverting the synthesis of oligosaccharides from endogenous glycoconjugates to artificial acceptors (primers). Examples include  $\beta$ -D-xylosides, which prime glycosaminoglycan chains found on proteoglycans (chondroitin sulfate and heparan sulfate) and oligosaccharides found on glycolipids and glycoproteins. Other types of glycoside primers include  $\alpha$ -N-acetylgalactosaminides which target the O-linked pathways of glycoprotein formation,  $\beta$ -glucosides,  $\beta$ -galactosides,  $\beta$ -N-acetylglucosaminides, and even disaccharides. These simple compounds resemble natural biosynthetic intermediates and therefore trick cells into assembling oligosaccharide chains on the exogenous primer instead of on endogenous substrates. Glycoside-treated cells secrete large amounts of primed oligosaccharides, and accumulate glycoproteins and proteoglycans with truncated glycans. Primers also serve as starting points for making analogs that might have inhibitory activity without acting as a primer.

### \*Corresponding author

Jain Amit Kumar

Science Teacher, UMS Raghapur, Revelganj, Saran, Bihar India – 841305

Email [ID-amitjainmjr@gmail.com](mailto:ID-amitjainmjr@gmail.com)

## **INTRODUCTION**

Analogs of simple monosaccharides have been made and tested in cells with some success, but their mode of inhibition is unclear. The derivation of primers and inhibitors by directed synthesis is tedious and requires a certain amount of serendipity to find active compounds. An alternative discovery strategy consists of screening random chemical libraries. The success of this approach depends on rapid, high throughput screening assays to detect potential inhibitors in crude mixtures from microbial or plant sources or from large combinatorial libraries of synthetic compounds. In the studies reported here, we took advantage of the enormous diversity of compounds elaborated by plants to find compounds that alter glycosaminoglycan synthesis in cultured animal cells. Five different plants from a random plant collection were discovered to prime glycosaminoglycan biosynthesis. The active principles have been purified and characterized as xylosides of methylated ellagic acid. The discovery of primers in plants suggests possible roles for these compounds in chemical defense and ethnobotany, and that large scale screenings might yield other kinds of primers that affect glycosylation.

## **EXPERIMENTAL**

**1. Cell Culture**—Chinese hamster ovary cells (CHO-K1),<sup>1</sup> were obtained from the American Type Culture Collection (CCL-61; ATCC, Rockville, MD). Wild-type CHO cells and the xylosyltransferase-deficient mutant pgsA-745 (50) were maintained in Ham's F-12 medium supplemented with 7.5% fetal bovine serum (HyClone), penicillin G (100 units/ml), and streptomycin sulfate (100 mg/ml) at 37 °C under an atmosphere of 5% CO<sub>2</sub> in air and 100% relative humidity. The cells were passaged every 3–4 days with 0.125% (w/v) trypsin, and after 10–15 cycles, fresh cells were revived from stocks stored under liquid nitrogen. Low sulfate medium was prepared from individual components by substituting chloride salts for sulfate and omitting streptomycin sulfate. This medium was supplemented with fetal bovine serum that had been dialyzed 106-fold against phosphate-buffered saline.

**2. Plant Preparation**—Approximately 250 plants were collected from the Amazon rain forest near Iquitos, Peru, as part of a general collection from the area in June, 1993. Voucher specimens have been deposited in the herbarium at the Institute for Botanical Exploration at Mississippi State University. Plant parts (bark, twigs, leaves, flowers, or combinations thereof) were air-dried (40–50 °C) and extracted in the following manner. The dried plant material was dampened with methanol/water (3:2, v/v) and stirred for 3 h at 37–40 °C with enough hexane to achieve a thorough suspension. After vacuum filtration, the residue was sequentially extracted in the same manner with hexane/ethyl acetate (1:1, v/v), ethyl acetate, and 95% ethanol. Each filtrate was dried by rotary evaporation and lyophilization, and test samples were dissolved at ;100 mg/ml (w/v) in dimethyl

sulfoxide. An aliquot of each extract was added to low sulfate growth medium to a final concentration of ;1 mg/ml (w/v, final Me<sub>2</sub>SO concentration, 1%, v/v). A serial dilution series (1:3, v/v) was then prepared down the rows of a 96-well plate using low sulfate growth medium as diluent.

**3. Microtiter Screening of Plant Extracts**—To screen for inhibitors, microtiter plates (96-well) were seeded with  $1.3 \times 10^4$  wild-type cells/well in 0.2 ml of growth medium and incubated at 37 °C. The next day, the medium was aspirated and 0.1 ml from each well of the 96-well dilution tray prepared above was transferred to the corresponding wells in the plate with cells. Two days later, 20 ml of low sulfate medium containing 5 mCi of <sup>35</sup>S<sub>4</sub>O<sub>4</sub> was added to each well in order to measure glycosaminoglycan biosynthesis. After 5 h, the contents of each well were adjusted to 0.1 M NaOH and 100 ml of a stopping solution containing 0.4 M acetic acid, 0.4% Zwittergent 3–12, 20 mM sodium sulfate, and 50 mg/ml chondroitin sulfate was added. To screen for primers, plates were seeded with mutant pgsA-745 cells (deficient in xylosyltransferase, the first enzyme involved in glycosaminoglycan biosynthesis) and incubated until the wells were nearly confluent. Extracts and <sup>35</sup>S<sub>4</sub>O<sub>4</sub> were added, and 18 h later the wells were treated with NaOH and the stopping solution described above. GeneScreen Plus (NEN Life Science Products) membranes were cut to fit a 96-well vacuum Minifold apparatus (Schleicher & Schuell). The membranes were soaked sequentially in 1 M acetic acid, 1 M sodium acetate, and water before use, and placed wet on top of a supporting piece of Whatman 1Chr paper in the apparatus. Samples of solubilized cells and medium were applied under slight vacuum, and the samples were washed with a solution containing 0.2% Zwittergent 3–12, 0.2 M acetic acid, and 10 mM sodium sulfate. The membrane was removed from the Minifold and swirled for 5 min in fresh solution, and then in water were destained in fresh solvent, rinsed with water, and air-dried. Inhibition of cell growth was judged visually by the staining intensity.

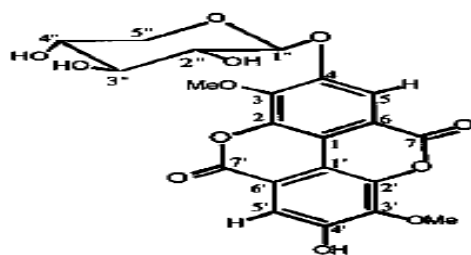
**4. Purification of Active Compounds**—The whole plant of *Maieta guianensis* was ground (2.6 kg), moistened with 60% (v/v) methanol in water (1 liter) for 1 h, and then extracted at 40 °C in turn with hexane (4 liter), 50% (v/v) hexane-ethyl acetate (4 liter), ethyl acetate (4 liter), and ethanol (4 liter) for 4 h each. After removal of solvents by rotary evaporation at 40 °C, the residues were tested for priming activity.

## RESULTS

To identify novel compounds that modulate proteoglycan biosynthesis, we developed a rapid, high throughput assay to screen large collections of natural and synthetic compounds. Many methods for measuring proteoglycans and glycosaminoglycans chemically and radiochemically have been described based on binding to anion exchange resins or on precipitation with cetylpyridinium chloride or ethanol. These techniques depend on the high negative charge of the glycosaminoglycan chains (sulfate and carboxyl groups) or their relative insolubility in an organic solvent. In general,

the application of these techniques to multiple samples is rather tedious. To simplify the processing of many samples, we took advantage of the binding properties of proteoglycans and glycosaminoglycans to cationic membranes routinely used for Southern and Northern blotting of nucleic acid. Briefly, the screening method involves culturing Chinese hamster ovary cells in 96-well dishes, labeling newly made proteoglycans with  $^{35}\text{S}$  in the presence of potential agonists or antagonists, and collection of  $^{35}\text{S}$ -labeled proteoglycans on cationic membranes. A vacuum manifold set-up facilitated the collection process, and autoradiography allowed semiquantitative detection of  $^{35}\text{S}$  incorporated into the highly charged proteoglycans (see “Experimental Procedures”). Pilot experiments comparing a mutant CHO line defective in glycosaminoglycan biosynthesis (pgsA-745) to the wild-type showed that over 90% of the bound radioactive material consisted of proteoglycans or glycosaminoglycans (compare samples in the bottom row of Fig. 1).

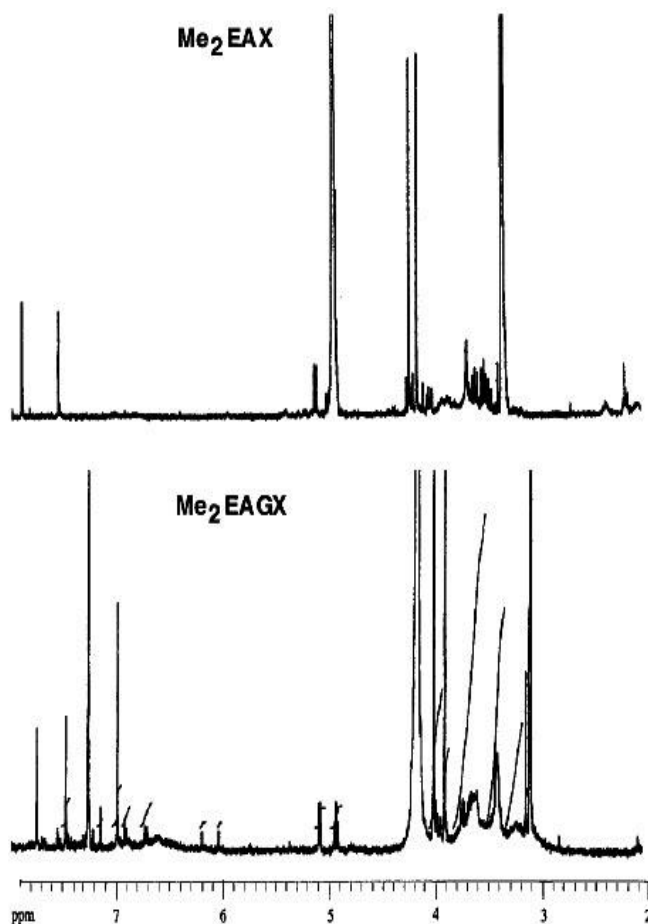
**Screening for Compounds That Alter Proteoglycan Assembly**—To find compounds that modulate proteoglycan formation, wild-type CHO cells were grown in 96-well dishes in the presence of crude extracts from terrestrial plants. Our initial studies focused on plant-derived compounds, as plants are known to produce a variety of organic compounds as part of chemical defense against insects and herbivores. All of the plants were collected from flowering species in the Amazon rain forest near Iquitos, Peru. In general, several kilograms of roots, bark, stems, or leaves were collected, dried, and small samples (<50 mg) were sequentially extracted with solvents of varying polarity (see “Experimental Procedures”). The individual extracts were dried, dissolved in Me<sub>2</sub>SO and tested for activity (see “Experimental Procedures”). Some extracts caused a decrease in  $^{35}\text{S}$  incorporation, as measured by loss of signal on the autoradiogram compared with wild-type cells incubated in the absence of extract (compare extract 12.4 to the control in Fig. 1). A comparison of the autoradiogram and the staining intensity of a duplicate plate treated with Coomassie Blue showed that the loss of signal was generally associated with a decrease in cell number, indicating that the extract was cytotoxic. To date, all potential “inhibitors” discovered in this way exhibited cytotoxicity.<sup>2</sup> Occasionally, we found extracts that appeared to stimulate the incorporation of  $^{35}\text{S}$



1 3,3'-Di-O-methyl ellagic acid 4-O- $\beta$ -D-xylopyranoside ( $\text{Me}_2\text{EAX}$ )

2  
gu

FIG. 3  $^1\text{H}$  NMR spectra for compounds 1 (dimethyl ellagic acid xyloside) and 2 (dimethyl ellagic acid) from *M. guianensis*. In the top panel, the peaks at 4.82 and 3.30 are due to water and MeOH in the  $\text{CD}_3\text{OD}$ , respectively. In the bottom panel, the peaks at 7.26, 4.20, and 3.30 are due to  $\text{CHCl}_3$ , water, and MeOH in  $\text{CD}_3\text{OD}$ , respectively. The other peaks are diagnostic for the compounds (see text and "Experimental Procedures").



Interestingly, many of these extracts also restored  $^{35}\text{SO}_4$  incorporation in a proteoglycan-deficient CHO cell mutant, designated pgsA-745 (50) (Fig. 1). This mutant cannot transfer xylose from UDPxylose to serines on proteoglycan core proteins due to a lesion in xylosyltransferase, the enzyme that initiates glycosaminoglycan chain synthesis. Thus, sample 13.2 bypasses the mutation and restores glycosaminoglycan biosynthesis. To date, five plants have been discovered that contain bypassing activity (*Psittacanthus cucullaris*, *M. guianensis*, *Alchornea triplinervia*, *Miconia myriantha*, and *Vismia angusta*). The priming activity of four solvent fractions from three of these plants is shown in Fig. 2. In general, the ability of the extracts to restore sulfate incorporation in the mutant was dose-dependent. The ethyl acetate fractions gave the highest activity in all five active plant species, which indicated that the active compounds were rather polar. In *P. cucullaris*, priming activity was seen in the hexanes/ethyl acetate, ethyl acetate, and ethanol fractions, suggesting that a

range of active compounds varying in polarity may exist. Extraction of fresh plant material yielded similar results to that shown in A second collection of each plant, made at a different time of the year, yielded nearly identical results. Identification of By passing Activities from *M. guianensis*—The first sample submitted for further fractionation and chemical analysis was the ethyl acetate fraction of *M. guianensis*. A large scale extraction of material was done (;2.6 kg dry weight of stems, leaves, and roots), and bioassay-directed, gross fractionation over silica gel followed by reversed phase chromatography afforded two compounds, fig (1 and 2). The structure of 1 was found to be dimethyl ellagic acid xyloside by comparison to previously published spectroscopic data. Compound 2 was the galloylated dimethyl ellagic acid xyloside on the basis of following analyses. Its molecular formula was C<sub>28</sub>H<sub>22</sub>O<sub>16</sub> based on its molecular ion at m/z 613 (M - 2 H)<sup>+</sup> its negative FAB-MS spectrum. The <sup>1</sup>H NMR spectrum showed the presence of one galloyl group by two proton singlets at δ 6.98, an ellagic acid moiety by the signals at δ 7.74 and 7.45 (s, 1H each), and six hydrogens of the xylose at δ 5.10 (d, J 5 5.8 Hz, 1H anomeric), 4.94 (t, J 5 7.3 Hz, 1H), 3.98 (dd, J 5 11 and 5 Hz, 1H) and 3.75–3.40 (m, 3H). The sugar was identified as β-D-xylose from the coupling constant of the anomeric hydrogen and by Dionex chromatography of the acid hydrolyzed product. The <sup>1</sup>H NMR of 2 also revealed the presence of two aromatic methoxy group at δ 4.04 and 3.92 (s, 3H each) (Fig. 4). The <sup>1</sup>H NMR spectrum of the peracetylated form of compound 2 showed signals for six acetoxymethyl groups at δ 2.39, 2.31, 2.12, 2.09, and 2.34 (s, 3H each and s, 6H), which indicated the presence of six free hydroxyl groups in the parent compound. The four aromatic hydrogens now appeared as a set of singlets at δ 7.97 (s, 1H) and 7.89 (s, 3H). The assignment of six sugar hydrogens were made by 1H-1H COSY experiment. They appear at δ 5.50 (1H, d, J 5 5.0 Hz H<sub>0</sub>-1, anomeric), 5.48 (1H, t, J 5 8.0 Hz, H-3<sub>0</sub>), 5.32 (1H, m, H-2<sub>0</sub>), 5.30 (1H, m, H-4<sub>0</sub>), 4.30 (1H, dd, J 5 12.5 and 4.0 Hz, H-5<sub>0</sub>), and 3.75 (1H, dd, J 5 FIG. 6. Priming activity of ellagic acid xylosides. Various concentrations of dimethyl ellagic acid xyloside and dimethyl ellagic acid were added to pgsA-745 cells with <sup>35</sup>S<sup>35</sup>SO<sub>4</sub>. After 18 h, the radioactive glycosaminoglycans in the cells and medium were collected and analyzed by DEAE chromatography and liquid scintillation chromatography (see “Experimental Procedures”). 12.5 and 5.4 Hz, H-5<sub>0</sub>). The downfield shift of H-3<sub>0</sub> hydrogen of the xylose indicates the location of the galloyl group, which was further substantiated by the heteronuclear multiple bond correlation spectrum showing the H-3<sub>0</sub> hydrogen of the xylose (δ 5.48) is connected to the carbonyl carbon of the galloyl group at δ 163.04. In summary, both compounds contained a D-xylose residue in β-linkage to ellagic acid. In addition, one of the compounds contained a galloyl moiety in ester linkage at the hydroxyl attached to C-3 of the sugar. These findings suggested that the bypassing activity consisted of complex xylosides, which by analogy to more simple synthetic derivatives can substitute for xylosylated core proteins. Analysis of the <sup>35</sup>S-

labeled material generated in the presence of different amounts of the samples showed that priming of radioactive chains was dose dependent, with ED<sub>50</sub> values of approximately 0.1 mM. This behavior resembles the activity of many synthetic b-Dxylosides, although the dose range is somewhat higher. Fractionation of the primed oligosaccharides by DEAE chromatography and digestion with glycosaminoglycan degrading enzymes showed that their products consisted of both chondroitin sulfate and heparan sulfate chains.

## **DISCUSSION**

Inhibitors of oligosaccharide biosynthesis provide a powerful method for studying the biology of glycoconjugates in cells, tissues and organisms. Therefore, we set out to discover if modulatory activities exist in crude extracts from plants, focusing on the assembly of glycosaminoglycans in our initial studies. By taking advantage of the affinity of the anionic glycosaminoglycan chains found on proteoglycans for cationic membranes, we were able to develop a facile, high throughput screening method. The technique has several useful features. It has high capacity. Since one individual can easily manipulate 10 or more 96-well plates in a few hours, over 1,000 samples can be analyzed per day. Higher capacity plates coupled with automated methods for manipulating multiwell dishes would increase capacity easily by 1 or 2 orders of magnitude. The increase makes it possible to analyze very large libraries of compounds at a single concentration as well as chromatography fractions at various dilutions. Prior separation of samples or partial purification of proteoglycans and glycosaminoglycans is not needed. Serum proteins and other cell constituents do not appear to interfere with binding, and the membranes have a high capacity for polyanions. The method allows detection of compounds that either inhibit or stimulate proteoglycan biosynthesis. By simultaneously measuring cytotoxicity (e.g. through dye binding or uptake methods), it should be possible to eliminate the need for an additional plate to assess cell growth. It should be possible to quantitate the amount of proteoglycan bound to the membrane by using imaging technology. This would allow full automation of the assay and the sensitive detection of agents that only partially alter proteoglycan synthesis.

## **ACKNOWLEDGMENTS**

The author is grateful thank of P.G. Department of Chemistry Rajendra College, Chapra for providing laboratory facilities.

## **REFERENCES**

1. Hindsgaul, O., Kaur, K. J., Srivastava, G., Blaszczyk-Thurin, M., Crawley, S. C., Heerze, L. D., and Palcic, M. M. *J. Biol. Chem.* 1991; 266: 17858–17862
2. Khan, S. H., Crawley, S. C., Kanie, O., and Hindsgaul, O. *J. Biol. Chem.* 1993; 268: 2468–2473

3. Lowary, T. L., and Hindsgaul, O. *Carbohydr. Res.* 1993; 249: 163–195
  4. Lowary, T. L., Swiedler, S. J., and Hindsgaul, O. *Carbohydr. Res.* 1994; 256: 257–273
  5. Lowary, T. L., and Hindsgaul, O. *Carbohydr. Res.* 1994; 251: 33–67
  6. Zacharias, C., van Echten-Deckert, G., Plewe, M., Schmidt, R. R., and Sandhoff, K. *J. Biol. Chem.* 1994; 269: 13313–13317
  7. Paulsen, H., Springer, M., Reck, F., Brockhausen, I., and Schachter, H. *Carbohydr. Res.* 1995; 275: 403–411
  8. Elbein, A. D. *FASEB J.* 1991; 5: 3055–3063
  9. Winchester, B., and Fleet, G. W. *Glycobiology* 1992; 2: 199–210
  10. Platt, F. M., Neises, G. R., Dwek, R. A., and Butters, T. D. *J. Biol. Chem.* 1994; 269: 8362–8365
-