Primers of Glycosaminoglycan Biosynthesis from Peruvian Rain Forest Plants*

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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 [35S]glycosaminoglycans (and [3H]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 reverse-phase 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 (1–7). 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 (8, 9). Analogs of the alkaloids also can inactive glycosyltransferases (10–12). 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 carbonoyloxy analogs have potent antitumor activity in mice and humans (13–23), 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 β-d-xylosides, which prime glycosaminoglycan chains found on proteoglycans (chondroitin sulfate and heparan sulfate) (24–32) and oligosaccharides found on glycolipids (33) and glycoproteins (34–38). Other types of glycoside primers include α-N-acetylgalactosaminides which target the O-linked pathways of glycoprotein formation (39, 40), β-glucosides (41), β-galactosides (42), β-N-acetylglucosaminides (43), and even disaccharides (44–46). 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. Analogs of simple monosaccharides have been made and tested in cells with some success, but their mode of inhibition is unclear (47–49).

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 PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1), were obtained from the American Type Culture Collection (CCL-61; ATCC,

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‡ The abbreviations used are: CHO, Chinese hamster ovary; FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high performance liquid chromatography; H-H COSY, homonuclear proton-proton correlation spectroscopy.
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 μg/ml) at 37 °C under an atmosphere of 5% CO2 in air and 100% relative humidity. The cells were passaged every 3–4 days with 0.125% (w/v) trypsin, and after 10–15 passages, cells and medium were applied under slight vacuum, and the samples were decanted and the wells were washed twice with phosphate-buffered saline (52). This medium was supplemented with fetal bovine serum that had been dialyzed 106-fold against phosphate-buffered saline (52). 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 35SO4 were added, and 18 h later the wells were treated with NaOH and the stopping solution described above. To screen for inhibitors, microtiter plates (96-well) were seeded with 1 × 105 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. To test if the extracts inhibited cell growth, a duplicate plate was seeded with wild-type cells in the absence of extract; lane 2, wild-type cells incubated with extract 12.4; lane 3, wild-type cells incubated with extract 13.2; lane 4, pgsA-745 cells incubated with extract 13.2. Each sample was assayed neat (−1 mg/ml, v/v) and at serial 1:3 dilutions.

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 (1:1, v/v),air-dried, mounted on paper, and exposed to γ-rays of 1 M acetic acid, 1 M sodium sulfate. The membrane was removed or substituting Triton X-100 or Tween 20 for Zwittergent 3–12 did not change the staining intensity.

### Purification of Active Compounds

To test if the extracts inhibited cell growth, a duplicate plate was seeded with cells and incubated for 2 days with plant samples. The structures of dimethylallylglycine, quercetin, and ellagic acid 4-O-β-d-xyloside-3,3′-dimethyl ether were determined by comparison with known compounds (53–55). A portion of compound 2 (ellagic acid-4-O-β-d-xyloside-3,3′-dimethyl ether-3′-O-gallate, 4 mg) was acetylated with acetic anhydride/pyridine (0.2 ml/0.5 ml, 24 h, room temperature) and the reaction mixture was worked up as usual. The product was purified by hexane/ethyl acetate (1:4, v/v) to a colorless white residue (3 mg); mass spectrum: m/z 889 (M + Na), m/z 867 (M + H) calculated for C34H34O23 13C NMR (125 MHz, CDCl3): δ 6 169.74, 169.37, 167.71, 166.39, 163.04, 158.0, 157.75, 155.0, 152.0, 149.4, 144.75, 143.6, 139.69, 126.93, 122.74, 120.29, 117.36, 116.08, 113.24, 112.28, 98.23, 69.42, 68.79, 67.23, 62.18, 62.00, 61.13, 21.00, 20.84, 20.73, 20.59, and 20.21.

### FIG. 1. Example of a screening assay for glycosaminoglycan formation.

Plant samples were analyzed for their ability to inhibit or activate 35SO4 incorporation into proteoglycans and glycosaminoglycans (see “Experimental Procedures”). Lane 1, wild-type cells in the absence of extract; lane 2, wild-type cells incubated with extract 12.4; lane 3, wild-type cells incubated with extract 13.2; lane 4, pgsA-745 cells incubated with extract 13.2. Each sample was assayed neat (−1 mg/ml, v/v) and at serial 1:3 dilutions.
cells were seeded into 24-well plates in growth medium supplemented with plant extract or purified compound. After 2 days, 50 μCi/ml of $^{35}$SO$_4$ (25–40 Ci/mg, NEN Life Science Products) was added. Sixteen hours later, the $[^{35}S]$glycosaminoglycans were isolated from cells and spent medium by anion-exchange chromatography as described previously (56). Samples were precipitated from ethanol, and the final pellets were dried by lyophilization, resuspended, and counted by liquid scintillation spectrometry. Portions were analyzed by anion exchange HPLC and by enzymatic digestion with chondroitinase ABC (56).

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 (57). 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 acids (58, 59). Briefly, the screening method involves culturing Chinese hamster ovary cells in 96-well dishes, labeling newly made proteoglycans with $^{35}$SO$_4$ in the presence of potential agonists or antagonists, and collection of $^{35}$S-labeled proteoglycans on cationic membranes. A vacuum manifold set-up facilitated the collection process, and autoradiography allowed semiquantitative detection of $^{35}$SO$_4$ 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 g) were sequentially extracted with solvents of varying polarity (see “Experimental Procedures”). The individual extracts were dried, dissolved in Me$_2$SO and tested for activity (see “Experimental Procedures”). Some extracts caused a decrease in $^{35}$SO$_4$ 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 plant extract was cytotoxic. To date, all potential “inhibitors” discovered in this way exhibited cytotoxicity.$^2$

$^2$ Under normal conditions, CHO cells produce a mixture of heparan
Occasionally, we found extracts that appeared to stimulate the incorporation of \(^{35}\)SO\(_4\) (Fig. 1, sample 13.2). Interestingly, many of these extracts also restored \(^{35}\)SO\(_4\) incorporation in a proteoglycan-deficient CHO cell mutant, designated pgsA-745 (50) (Fig. 1). This mutant cannot transfer xylose from UDP-xylose 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 Fig. 2. A second collection of each plant, made at a different time of the year, yielded nearly identical results (data not shown).

**Identification of Bypassing 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, 1 and 2 (Fig. 3). The structure of 1 was found to be dimethyl ellagic acid xyloside by comparison to previously published spectroscopic data (53–55). Compound 2 was the galloylated dimethyl ellagic acid xyloside on the basis of following analyses. Its molecular formula was \(C_{28}H_{22}O_{16}\) based on its molecular ion at \(m/z\) 613 (M – H\(^{+}\)) in its negative FAB-MS spectrum. The \(^1\)H NMR spectrum showed the presence of one galloyl group by two proton singlets at \(\delta\) 6.98, an ellagic acid moiety by the signals at \(\delta\) 7.74 and 7.45 (s, 1H each), and six hydrogens of the xylose at \(\delta\) 5.10 (d, \(J\) 5.8 Hz, 1H anomeric), 4.94 (t, \(J\) 7.3 Hz, 1H), 3.98 (dd, \(J\) 11 and 5 Hz, 1H), and 3.75–3.40 (m, 3H). The sugar was identified as \(\beta\)-D-xylose from the coupling constant of the anomeric hydrogen and by Dionex chromatography of the acid hydrolyzed product. The \(^1\)H NMR of 2 also revealed the presence of two aromatic methoxy group at \(\delta\) 4.04 and 3.92 (s, 3H each) (Fig. 4). The \(^1\)H NMR spectrum of the peracetylated form of compound 2 showed signals for six acetoxyethyl groups at \(\delta\) 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 \(\delta\) 7.97 (s, 1H) and 7.89 (s, 3H). The assignment of six sugar hydrogens were made by \(^1\)H-\(^1\)H COSY experiment. They appear at \(\delta\) 5.50 (1H, d, \(J\) 5.0 Hz H-1, anomic), 5.48 (1H, t, \(J\) 8.0 Hz, H-3\(^{-}\)), 5.32 (1H, m, H-2\(^{-}\)), 5.30 (1H, m, H-4\(^{-}\)), 4.30 (1H, dd, \(J\) = 12.5 and 4.0 Hz, H-5\(^{-}\)), and 3.75 (1H, dd, \(J\) =
12.5 and 5.4 Hz, H-5 (Fig. 5). The downfield shift of H-3 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 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 (29, 32). Analysis of the 35S-labeled material generated in the presence of different amounts of the samples showed that priming of radioactive chains was dose-dependent, with ED50 values of approximately 0.1 mM (Fig. 6). This behavior resembles the activity of many synthetic β-D-xylosides, although the dose range is somewhat higher (29). 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 (58, 59), we were able to develop a facile, high throughput screening method. The technique has several use-
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ful features. (i) 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 multwell 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. (ii) 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. (iii) 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. (iv) It should be possible to quantify 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. (v) It should be possible to target other glycosylation pathways by using other metabolic precursors (e.g. [3H]mannose for N-linked oligosaccharides) or affinity matrices with selective binding properties (e.g. immobilized lectins or antibodies).

Our initial screenings focused on plant extracts, since compounds that alter glycosylation have already been identified from this source (e.g. the alkaloids castanospermine, swainsonine, nojirimycin, and the calystegines; Refs. 8, 9, and 60–62). Other examples of plant xylosides have been reported (see, e.g. Refs. 74–79), but their ability to prime glycosaminoglycan biosynthesis as well. Xylosides are known here, some terrestrial plants contain compounds that stimulate glycosaminoglycan biosynthesis. Xylosides are also known to have antifungal (e.g. 45). Apparently, these enzymes also can remove more complex glycogen chains has not yet been tested. Plants may yield other types of compounds that might prove useful for studying proteoglycans, including primers that contain a sugar other than xylose, two or more sugar residues in covalent linkage, or sugar mimetics (e.g. polyhydroxylated aromatics). These compounds would provide novel entities for the generation of enzyme-directed inhibitors, which would extend our current repertoire of agents for studying proteoglycans in cells. An appealing aspect of this approach is the possibility of identifying carbohydrate based agents with useful medicinal value. Although no ethnobotanical use for M. guianensis has been documented as yet (80, 81), some of the other active plants have been used in local populations for treating maladies in which proteoglycans may play a role (e.g. the latex of V. angusta is used for treating wounds and herpes infections) (81). Further studies are needed to determine if xylosides or other glycosides are active principles in these plants.

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