Glycoside Primers of *Psittacanthus cucullaris*

Anjana Sinha,[†] William H. Taylor,[†] Ikhlas H. Khan,[‡] Sidney T. McDaniel,[§] and Jeffrey D. Esko*,[†]

Division of Cellular and Molecular Medicine, Glycobiology Program, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0687, National Center for the Development of Natural Products, Research Institute of Pharmaceutical Sciences, The University of Mississippi, University, Mississippi 38677, and Institute for Botanical Exploration and Department of Biological Sciences, Mississippi State University, Mississippi State, Mississippi 39762

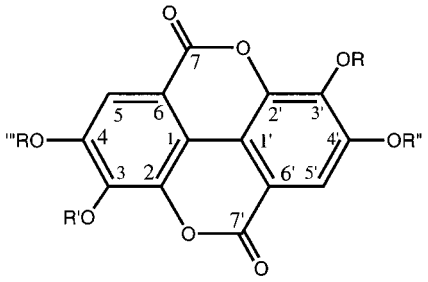
Received November 30, 1998

Bioassay-directed chromatographic separation of the ethyl acetate extract of the whole plant of *Psittacanthus cucullaris* afforded a new phenolic xyloside, ellagic acid-4-*O*-*β*-xyloside-3,3′,4′-trimethyl ether (**1**) together with four known compounds, ellagic acid-4-*O*-*β*-xyloside-3,3′-dimethyl ether (**2**), gallic acid, *β*-sitosterol, and *β*-sitosterol *β*-D-glucoside. The structure of the new compound was determined by spectroscopic methods. Like other *β*-D-xylosides, compounds **1** and **2** stimulated the formation of glycosaminoglycan chains when fed to the cultured Chinese hamster ovary cells.

A proteoglycan consists of one or more glycosaminoglycan chains covalently attached to a protein core. The synthesis of the glycans proceeds stepwise from the reducing end, initiated by the transfer of xylose from the high energy nucleotide sugar donor, UDP-xylose, to specific serine residues in the protein. The transfer of xylose appears to be rate-limiting for glycosaminoglycan synthesis because the addition of *β*-D-xylosides to cells and tissues greatly stimulates the formation of free chains on the exogenous primer.[1] The structure of the aglycon attached to the xyloside affects the type of glycosaminoglycan chain produced (e.g., heparan sulfate or chondroitin sulfate), suggesting that the aglycon mimics a structural element of the protein core that interacts with a key enzyme in the pathway.[2] The primed chains made on xylosides can have interesting biological properties, such as activation of fibroblast growth-factor binding to tyrosine kinase receptors[3] and an antithrombotic effect in animals.[4] These findings led us to look for natural primers of glycosaminoglycan chains, and plant extracts were selected as a potential library for discovering novel compounds inasmuch as xylose is an abundant sugar in many plants. After screening more than 1000 extracts, five plants showed priming activity. *Psittacanthus cucullaris* (Lam.) Bl. (Loranthaceae), a parasitic shrub native to Peru,[5] was the most active.[6]

Plants of the Loranthaceae family are mostly parasitic shrubs and are widely distributed throughout temperate and tropical regions of both hemispheres. No phytochemical investigation has been carried out on *P. cucullaris*. An activity guided systematic fractionation of an ethyl acetate extract of *P. cucullaris* led to the isolation of a new phenolic xyloside, ellagic acid-4-*O*-*β*-xyloside-3,3′,4′-trimethyl ether (**1**), in addition to four known compounds, ellagic acid-4-*O*-*β*-xyloside-3,3′-dimethyl ether (**2**), gallic acid, *β*-sitosterol, and *β*-sitosterol *β*-D-glucoside. The new and known compounds were characterized by detailed spectroscopic analysis (NMR and MS) and comparison of their spectral data with reported values.[7,8]

The whole plant of *P. cucullaris* was moistened with MeOH−H_2O (3:2 v/v) and then successively extracted with hexane, 50% hexane−EtOAc, EtOAc, and EtOH at 40 °C. The ethyl acetate extract showed GAG priming activity in a xylosyltransferase mutant cell line, pgsA-745. Column chromatography of the ethyl acetate extract on Si gel in combination with repeated Sep-Pak C_{18} cartridges and preparative TLC over Si gel led to the isolation of new compound **1** and known compound **2**.



1: R= R'=R"= Me; R"'=β-xyl

2: R=R'=Me; R"=H; R"'=β-xyl

The molecular formula of **1** was determined as $C_{22}H_{20}O_{12}$ by the presence of a molecular ion peak at *m*/*z* 477 $(MH)^+$ in its positive FABMS. The ^{1}H NMR spectrum of **1** showed the presence of two protons as singlets at *δ* 7.76 and 7.62 assigned to the ellagic acid moiety; six hydrogens of the xylose at *δ* 5.17 (1H, d, J = 7.24 Hz), 3.80 (1H, m), 3.39−3.33 (3H, m), and three broad signals at *δ* 5.58, 5.34, and 5.18 exchangeable with D_2O, assigned to three hydroxyl groups. The ^{1}H NMR spectrum of **1** also revealed the presence of three aromatic methoxy groups at *δ* 4.07, 4.03, and 3.99 (3H, s each). The sugar was identified as *β*-D-xylose from the coupling constant of the anomeric hydrogen and Dionex chromatography of the acid hydrolyzed products of **1**.

The position of the glycosidic linkage to the aglycon was confirmed on the basis of a NOESY experiment. The NOESY spectrum of **1** showed the anomeric proton of xylose (*δ* 5.17) correlated with H-5 (*δ* 7.76) of ellagic acid. This interaction is only possible when the sugar residue is glycosidically linked at C-4. Thus, the structure of **1** is

* To whom correspondence should be addressed. Tel.: (619) 822-1100. Fax: (619) 534-5611. E-mail: jesko@ucsd.edu.
[†] University of California, San Diego.
[‡] The University of Mississippi.
[§] Mississippi State University.

10.1021/np980543a CCC: $18.00
Published on Web 06/22/1999

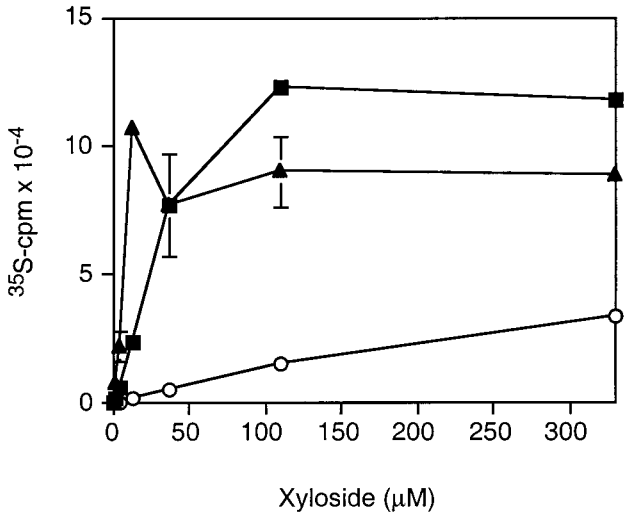


Figure 1. Priming activity of ellagic acid xylosides (**1** and **2**) and naphthol xyloside. Various amounts of dimethyl ellagic acid xyloside (○), trimethyl ellagic acid xyloside (▲), and naphthol xyloside (■) were added to pgsA-745 cells with $^{35}SO_4$. After 16 h, the radioactive glycosaminoglycans in the cells and medium were collected and analyzed by DEAE chromatography and liquid scintillation chromatography (see Experimental Procedures).

ellagic acid-4-*O*-*β*-xyloside-3,3′,4′-trimethyl ether. Dimethyl ellagic xyloside (**2**) and gallic acid were also isolated from this plant.

Priming activity of the two ellagic acid xylosides (**1** and **2**) was tested in mutant pgsA-745 cells.[1] This mutant does not produce any glycosaminoglycan chains on core proteins due to a deficiency in xylosyltransferase. However, addition of synthetic *β*-D-xylosides to the cells bypasses the defect, allowing glycosaminoglycan synthesis to occur on the xyloside and secretion of the free chains from the cells. Both compounds **1** and **2** were effective primers, with **1** having greater potency than **2** or synthetic naphthol xyloside (Figure 1). The large difference in the dose curve for **1** and **2** is consistent with a previous finding, indicating that the extra hydroxyl group on **2** most likely interferes with uptake rather than priming per se.[1,9]

Only limited ethnobotanical studies of Loranthaceous plants have been reported to date. The Karijones Indians of Peru believe that bathing in a warm decoction of the leaves of *P. cucullaris* helps keep their skin free of wrinkles.[2] Glycosaminoglycans are extracellular matrix molecules and alterations in their synthesis can lead to dramatic changes in skin architecture and resiliency. Thus, it is conceivable that topically applied (or ingested) xylosides could affect skin extracellular matrix by altering the composition or content of the glycosaminoglycans. Further studies are needed to establish if xylosides such as the ones described herein can have can this effect in vivo.

Experimental Section

General Experimental Procedures. FABMS were recorded in both the negative and positive modes with a VG ZAB-VSE instrument; ^{1}H and ^{13}C NMR were obtained with a Bruker 400 AMX spectrometer. The chemical shifts are reported in parts per million (δ) relative to that of tetramethylsilane as an internal standard ($\delta = 0$), and coupling constants are given in Hertz. Melting points were determined in a Fisher−Johns apparatus and are uncorrected. The following adsorbents were used for purification: column chromatography, Merck Kieselgel 60 Si gel (200−400 mesh); Waters Sep-Pak vac C_{18}-cartridges; Chromatotron radial TLC plates of Si gel 60 PF_{254}; Merck Kieselgel 60 F_{254} for analytical TLC. The TLC chromatograms were visualized at 254 and 366 nm and/or sprayed with anisaldehyde and then heated.

Plant Material. The whole plant of *Psittacanthus cucullaris* was collected in Peru, in June 1993. A voucher specimen (IBE 11964) is deposited in Mississippi (Herbarium of the Institute for Botanical Exploration at Mississippi State University).

Extraction and Isolation. The air-dried, ground whole plant of *P. cucullaris* (1.7 kg) was moistened for 1 h with 60% MeOH in H_2O (1 L) and then extracted at 40 °C in turn with hexane (4 L), 50% hexane−EtOAc (4 L), EtOAc (4 L), and EtOH (4 L) for 4 h each. After removal of solvents in vacuo at 40 °C, four residues were obtained and tested for priming activity. The active EtOAc extract (20 g) was fractionated over Si gel (100 g) using $CHCl_3$−MeOH (1:0→0:1) as a stepwise gradient. In all, eight fractions (0.3 L each) were collected. The two active fractions, fraction A [$CHCl_3$−MeOH (9:1), 6.6 g] and fraction B [$CHCl_3$−MeOH (8:2), 0.7 g], were subjected to further purification as follows: Fraction A was repeatedly passed through SiO_2 gel column using ($CHCl_3$−MeOH) as a gradient, and *β*-sitosterol was isolated from the earlier fractions of $CHCl_3$−MeOH (95:5). The active subfraction of $CHCl_3$−MeOH (9:1) elution (2.1 g) was passed through Sep-Pak C_{18}-cartridges (10 g) using H_2O−MeOH and $CHCl_3$ as a solvent. The MeOH- and $CHCl_3$-soluble part (0.2 g) was analyzed by Chromatotron radial TLC. Elution with $CHCl_3$−MeOH (97:3) yielded **1** (0.025 g) and *β*-sitosterol-*β*-D-glucoside (0.02 g). Fraction B was (0.7 g) was passed through Sep-Pak C_{18}-cartridges (5 g) and eluted with H_2O−MeOH (1:0→0:1). Elution with H_2O−MeOH (9:1) yielded gallic acid (0.1 g), and elution with H_2O−MeOH (7:3) yielded **2** (0.01 g).

Ellagic acid-4-*O*-*β*-xylopyranoside-3,3′4′-trimethyl ether (1): mp 198−200 °C; ^{13}C NMR [$(CD_3)_2SO$, 125 MHz]: δ 157.3, 157.2 (s, C-7 and C-7′), 153.3, 150.6 (s, C-4 and C-4′), 140.9, 140.2 (s, C-2 and C-2′), 140.1, 139.9 (s, C-3 and C-3′), 112.6, 111.70 (s, C-1 and C-1′), 111.5, 111.4 (s, C-6 and C-6′), 111.0, 106.5 (d, C-5 and C-5′), 100.8 (d, C-1″), 75.1 (d, C-3″), 72.0 (d, C-2″), 68.3 (d, C-4″), 65.8 (d, C-5″), 60.6 (q, −OMe at C-3), 60.3 (q, −OMe at C-3′) and 55.7 (q, −OMe at C-4′).

Ellagic acid-4-*O*-*β*-xylopyranoside-3,3′-dimethyl ether (2). MS, ^{1}H and ^{13}C NMR data were identical with published data.[5]

Gallic acid, *β*-Sitosterol, and *β*-sitosterol-*β*-D-glucoside. The MS and ^{1}H NMR were identical with an authentic samples of above compounds.

Cell Culture. The xylosyltransferase-deficient mutant pgsA-745[10] was 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% CO_2 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.[11] This medium was supplemented with fetal bovine serum that had been dialyzed 10^6-fold against phosphate-buffered saline.[12]

Priming Assay of Plant Extracts. To screen extracts and chromatography samples for primers, microtiter plates (96-well) were seeded with 1×10^4 pgsA-745 cells/well in 0.2 mL of growth medium and incubated at 37 °C for 2 days. Two days later, the medium was aspirated, and an aliquot of plant extracts (ca. 1 mg/mL w/v, 1% Me_2SO v/v) supplemented with 5 μCi of $^{35}SO_4$ in low sulfate growth medium (0.1 mL) was added to each well in order to measure glycosaminoglycan biosynthesis. The cells were incubated at 37 °C for 16 h. The content of each well was adjusted to 0.1M NaOH and 0.1 mL of a stopping solution containing 0.4 M HOAc, 0.4% Zwittergent 3−12, 20 mM Na_2SO_4, and 50 μg/mL chondroitin sulfate was added. GeneScreen Plus (NEN Life Science Products) membranes were cut out to fit a 96-well vacuum Minifold apparatus (Schleicher & Schuell). The membranes were soaked sequentially in 1 M HOAc, 1 M NaOAc, and H_2O before use, and placed wet on top of a supporting piece of Whatman 1

chromatography 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 HOAc, and 10 mM Na_2SO_4. The membrane was removed from the Minifold and swirled for 5 min in fresh solution, and then in H_2O. The membranes were air-dried, mounted on paper, and exposed to Kodak X-AR5 film for 1−2 h.[3]

Comparative Priming Activity of Compounds 1 and 2. Approximately 1×10^4 pgsA-745 cells were seeded into 24-well plates and incubated at 37 °C for 2 days. After 2 days, compounds **1** and **2** in low sulfate growth medium and 25 μCi/mL of ^{35}SO4 (25−40 Ci/mg, NEN Life Science Products) were added to separate wells. Sixteen hours later, the [^{35}S]glycosaminoglycans were isolated from cells and spent medium by anion-exchange chromatography as described previously.[13] Samples were precipitated from EtOH, and the final pellets were dried by lyophilization, resuspended, and counted by liquid scintillation spectrometry.

Acknowledgment. We thank Dr. S. C. Sinha (The Scripps Research Institute) for help in MS and NMR analyses, Juan Ruiz (Curator, Herbarium Amazonense, Universidad Nacional de la Amazonia Peruana in Iquitos, Peru) for collecting and processing plants from the rain forest, and James McChesney and Julie Mikell (Research Institute for Pharmaceutical Sciences at the University of Mississippi) for extracting the plant material. This work was supported by a grant the Mizutani Foundation (Japan) and grant CA46462 from the National Institute of Health to J. D. E.

References and Notes

(1) Fritz, T. A.; Lugemwa, F. N.; Sarkar, A. K.; Esko, J. D. *J. Biol. Chem.* **1994**, *269*, 300−307.
(2) Esko, J. D.; Zhang, L. *Curr. Opin. Struct. Biol.* **1996** *6*, 663−670.
(3) Hua-Quan, M.; Fritz, T. A.; Esko, J. D.; Yayon, A.; Vlodavsky, I. *J. Cell. Biochem.* **1995**, *57*, 173−184.
(4) Martin, N. B.; Masson, P.; Sepulchre, C.; Theveniaux, J.; Millet, J.; Bellamy, F. *Semin. Thromb. Hemostasis* **1996**, *22*, 247−254.
(5) Schultes, R. E.; Raffauf, R. F. *The Healing Forest*; Dioscorides Press: Portland, OR, 1990; pp 271−272.
(6) Taylor, W. H.; Sinha, A.; Khan, I. A.; McDaniel, S. T.; Esko, J. D. *J. Biol. Chem.* **1998**, *35*, 22260−22266.
(7) Castaneda, P.; Bahena, A.; Garcia, E.; Chavez, D.; Mata, R. *J. Nat. Prod.* **1993**, *56*, 1575−1579.
(8) Pakulski, G.; Budzianowski, J. *Phytochemistry* **1996**, *41*, 775−778.
(9) Sarkar, A. K.; Fritz, T. A.; Taylor, W. H.; Esko, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995** *92*, 3323−3327.
(10) Esko, J. D.; Stewart, T. E.; Taylor, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3197−3201
(11) Ham, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, 53, 288−293.
(12) Dulbecco, R.; Vogt, M. *J. Exp. Med.* **1954**, *99*, 167−182.
(13) Bame, K. J.; Esko, J. D. *J. Biol. Chem.* **1989**, *264*, 8059−8065.

NP980543A