Articles

Synthesis of *N*-(Substituted-phenyl)-D-xylopyranosylamines as Potential Modifiers of the Formation of Glycosaminoglycans†

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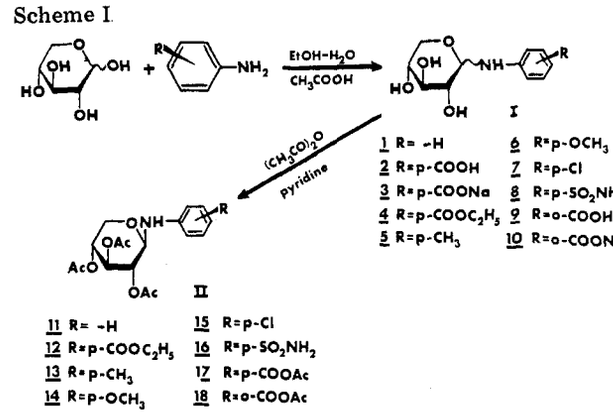
N-(Substituted-phenyl)-D-xylopyranosylamines and their *O*-peracetyl derivatives have been synthesized and tested for their ability (a) to inhibit the replication of cultured B16 melanoma cells and (b) to modify the synthesis of glycosaminoglycans by these neoplastic cells. The most cytotoxic compound synthesized was *N*-(*p*-methoxyphenyl)-D-xylopyranosylamine (**6**), which produced 50% inhibition of cellular proliferation at a concentration of 2 μM; a number of other compounds were relatively cytotoxic, causing 50% inhibition of cell replication at levels of 12 to 25 μM. Several of the synthesized xylosides appeared to be capable of serving as artificial initiators of glycosaminoglycan synthesis, with the most active agents causing approximately 2- to 4-fold increases in the incorporation of [^{35}S]sulfate into the glycosaminoglycans of B16 melanoma cells excreted into the culture medium.

Glycosaminoglycans (GAGS) are constituents of the extracellular matrix that have been implicated in having a role in cellular adhesion[1] and metastasis.[2] Certain classes of GAGS are covalently bound to protein by means of a galactosyl–galactosyl–xylosyl link, with the xylose moiety being attached to the hydroxy group of a seryl residue in the polypeptide.[3] A variety of alkyl β-D-xylopyranosides have been shown to be artificial initiators of GAG synthesis, both in the presence and absence of core acceptor protein.[4-6] Since the GAGS formed with an artificial initiator are of low molecular weight, it is conceivable that alkyl β-D-xylopyranosides might possibly modify the growth and metastatic potential of malignant neoplasms. For this reason, this laboratory synthesized a variety of haloacetamidoalkyl β-D-xylopyranosides[7] and demonstrated that two of these agents, 2-(chloroacetamido)- and 2-(bromoacetamido)ethyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside, were capable of modifying the biosynthesis of GAGS in B16 melanoma cells.[8,9]

Kariya et al.[10] recently reported that the sodium salt of *N*-(carboxyphenyl)-D-xylopyranosylamine when used in combination with a polysaccharide preparation was effective as an inhibitor of the growth of several malignant animal tumors, such as the Yoshida sarcoma, the P388 leukemia, and the Lewis lung carcinoma. This new antineoplastic agent has been shown to alter tumor cell architecture by reducing surface fine villi,[11] a phenomenon that may be associated with the ability of this xyloside to serve as an artificial acceptor for GAG chain initiation.[12] To determine the structural parameters necessary for the cytotoxic and GAG initiating properties of this agent, we have synthesized a series of *N*-(substituted-phenyl)-D-xylopyranosylamines (**1–10**), and their *O*-acetyl derivatives (**11–18**), and have measured the capacity of these agents (a) to inhibit the replication of cultured B16 melanoma cells and (b) to modify the biosynthesis of GAGS by these neoplastic cells.

Scheme I



Chemistry. *N*-(Substituted-phenyl)-D-xylopyranosylamines (I) were synthesized as shown in Scheme I by condensation of molar equivalent amounts of D-xylose and various monosubstituted anilines in the presence of glacial acetic acid as a catalyst.[13] One of these amine derivatives,

† This paper has been presented in part; see Lin Wang, Charles A. Maniglia, Sharon L. Mella, and Alan C. Sartorelli, in "Abstracts of Papers", 182nd National Meeting of the American Chemical Society, New York, NY, Aug 23–28, 1981, American Chemical Society, Washington, DC, 1981, Abstr CARB 23.

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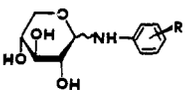
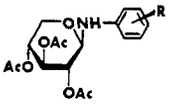
Table I. Properties of *N*-(Substituted-phenyl)-D-xylopyranosylamines



no.	R	mp, °C	recrystn solvent	yield,[a] %	$[\alpha]^{20}_D$,[b] deg	formula	anal.[c]
1	H[d]	140-142	EtOH	28	−50.4[e]	$C_{11}H_{15}NO_4$	C, H, N
2	*p*-COOH[f]	167-168 dec	H_2O	66	−52.2	$C_{12}H_{15}NO_6$	C, H, N
3	*p*-COONa	150-160 dec	H_2O/EtOH	56	+119.6[e]	$C_{12}H_{14}NO_6Na$	C, H, N
4	*p*-$COOC_2H_5$[g]	116-117	EtOH	42	−4.7	$C_{14}H_{19}NO_6$	C, H, N
5	*p*-CH_3	115-116 dec	EtOH/H_2O	13	−72.9	$C_{12}H_{17}NO_4$	C, H, N
6	*p*-OCH_3	122-124	EtOH	29	+1.5[h]	$C_{12}H_{17}NO_5$	C, H, N
7	*p*-Cl[f]	122-124	EtOH	60	−74.4[h]	$C_{11}H_{14}ClNO_4$	C, H, N
8	*p*-SO_2NH_2[i]	167-170 dec	EtOH/H_2O	51	−60.2[e]	$C_{11}H_{10}N_2O_6S$	C, H, N
9	*o*-COOH[j]	169-175 dec		43	−33.8	$C_{12}H_{15}NO_6$	C, H, N
10	*o*-COONa	136-138 dec	EtOH/H_2O	27	−26.3[e]	$C_{12}H_{14}NO_6Na \cdot H_2O$	C, H, N

[a] Yields were based on the final recrystallized products obtained, except for **9**. [b] Measured as 1% solutions in pyridine unless otherwise indicated. [c] The analytical results obtained for elements were within ±0.4% of the theoretical value unless otherwise indicated. All compounds had IR spectra compatible with expected structures. [d] Literature[24] mp 142 °C, $[\alpha]_D$ −87°. [e] Measured as 1% solution in H_2O. [f] Literature[13] **2**: mp 155-157 °C, $[\alpha]_D$ −59.0° (pyridine); **7**: mp 125-126 °C, $[\alpha]_D$ −61.5° (EtOH). [g] Literature[25] mp 116-117 °C, $[\alpha]_D$ −28.0° (EtOH). [h] Measured as 1% solution in EtOH. [i] Literature[26] mp 157 °C, $[\alpha]_D$ −48° (EtOH). [j] Literature[27] mp 170 °C, $[\alpha]_D$ −23° (EtOH).

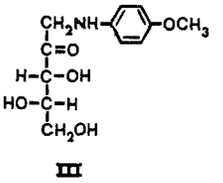
Table II. Properties of *N*-(Substituted-phenyl)-tri-*O*-acetyl-β-D-xylopyranosylamines



no.	R	mp, °C	recrystn solvent	yield,[a] %	$[\alpha]^{20}_D$,[b] deg	formula	anal.[c]
11	H[d]	151-153	EtOH	61	−14.0	$C_{17}H_{21}NO_7$	C, H; N[e]
12	*p*-$COOCH_2CH_3$	169-171	EtOH	74	−16.0[f]	$C_{20}H_{25}NO_9$	C, H, N
13	*p*-CH_3	140-142	EtOH	75	−11.2	$C_{18}H_{23}NO_7$	H, N; C[g]
14	*p*-OCH_3	139-141	EtOH	44	−10.5	$C_{18}H_{23}NO_8$	C, H, N
15	*p*-Cl	130-132	EtOH	48	−19.6	$C_{17}H_{20}ClNO_7$	C, H, N
16	*p*-SO_2NH_2	205-207		62	−5.8[f]	$C_{17}H_{22}N_2O_9S$	C, H, N
17	*p*-$COOCOCH_3$	163-164	EtOH	83	+14.2[f]	$C_{20}H_{23}NO_{10}$	C, H, N
18	*o*-$COOCOCH_3$	134-135	EtOH	21	−11.8	$C_{20}H_{23}NO_{10}$	C, H, N

[a] Yields were based on the final recrystallized products obtained. [b] Measured as 1% solutions in chloroform unless otherwise indicated. [c] The analytical results obtained for elements were within ±0.4% of the theoretical value unless otherwise indicated. All compounds had IR and NMR spectra compatible with expected structures. [d] Literature[24] mp 151 °C. [e] N: calcd, 3.98; found, 3.36. [f] Measured as 1% solution in pyridine. [g] C: calcd, 59.16; found, 58.70.

N-(*p*-methoxyphenyl)-D-xylopyranosylamine (**6**) could not be obtained by this methodology; instead, a white powder (III), which melted at 158–161 °C, was isolated. This



III

product (III) exhibited sharp characteristic carbonyl (1704), hydroxy (3300), and phenyl (1558, 1513 cm^{-1}) IR absorption bands. Its elemental analysis was consistant with the formula $C_{12}H_{17}NO_5$. The structure of III was further confirmed by mass spectral analysis. The molecular ion peak was not detected; however, $M^+ + 1$ and $M^+ + 2$ peaks with *m/e* of 256.1 and 257.1 were observed. In addition, three major fragments with *m/e* of 134, 123, and 108 were also present. It appears that the strongly electron-donating methoxy substituent in the benzene ring of **6** caused an Amadori rearrangement[14] that resulted in the formation of III. Since it is known that trace amounts of acid can catalyze this type of rearrangement,[15] the reaction was modified so that acetic acid was not empolyed as a catalyst and the reaction time was shortened to 1 h. Under these conditions, the desired compound **6** was obtained. The properties of the *N*-(substituted-phenyl)-D-xylopyranosylamines **1**–**10** are summarized in Table I.

Amines (I) were acetylated[16] with excess acetic anhydride in pyridine at 5 °C to give *O*-peracetyl derivatives (II).[17] Acetylation of **2** and **9** under these conditions, however, gave the corresponding mixed acetic anhydride derivatives, **17** and **18**, in which both the hydroxy and carboxy groups were acetylated. These structures were confirmed by NMR spectroscopy. The acetoxy methyl signals that appeared at relatively low field (δ 2.35 for **17** and δ 2.36 for **18**) indicated the existence of a $COOCOCH_3$ group. The *O*-acetyl signals were observed at higher field[18–20] with δ 2.08 (3 H) and 2.07 (6 H) in **17** and δ 2.13,

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Table III. Effect of N-(Substituted-phenyl)-D-xylopyranosylamines on the Growth of B16 Melanoma Cells in Tissue Culture

no.	ID_{50},[a] μM
1	20
2	100
3	1000
4	23
5	20
6	2
7	16
8	20
9	700
10	15
11	20
12	12
13	23
14	20
15	15
16	20
17	200
18	25

[a] Each compound was tested at concentrations of 0.1 μM to 0.1 mM in at least two replicate experiments, and the 50% inhibitory level (ID_{50}) was determined graphically.

Table IV. Effect of N-(Substituted-phenyl)-D-xylopyranosylamines on [^{35}S]Sulfate Incorporation into Glycosaminoglycans of B16 Melanoma Cells Excreted into the Medium[a]

no.	% stimulation of GAG synth
4	197
5	277
8	270
9	228
11	180
12	199
13	274
14	427
15	205
16	370

[a] B16/F10 cells were incubated in culture with xylosides at a concentration of 10 μM. Twenty-four hours later, 10 μCi/mL of [^{35}S]sulfate was added, and incorporation was permitted for 24 h. Radioactivity in GAGS present in the medium was measured as described in the text. The data represent the mean of results from six flasks, with less than 20% variation between flasks. All synthesized xylosides were tested, and only those agents that increased the synthesis of GAGS over that of untreated control cells are listed.

2.09, and 2.07 (each of 3 H) in 18. The properties of the N-(substituted-phenyl)-tri-O-acetyl-β-D-xylopyranosylamines 11–18 are listed in Table II.

The configuration and conformation of the N-(substituted-phenyl)-O-peracetyl-D-xylopyranosylamines 11–18 were determined by 270-MHz NMR spectroscopy. These studies showed that the xylopyranoside sugar ring existed in the C1 conformation, and the anomeric carbon atom had the β configuration. The NMR identification of the structures of compounds 11–18 has been presented in part[21] and will be reported in detail elsewhere.

Biological Evaluation. The growth inhibitory activity of N-(substituted-phenyl)-D-xylopyranosylamines and their peracetylated derivatives 1–18 was evaluated against B16/F10 melanoma cells in culture. Most of these agents exhibited significant inhibition of cellular proliferation in vitro (Table III). Compounds **1**, **4**, **5**, **7**, **8**, and **10** and their peracetylated derivatives **11–16** and **18** exhibited ID_{50} values in the range of 12 to 25 μM. Several compounds in this series (**2**, **3**, **9**, and **17**), however, demonstrated significantly less cytotoxicity, having ID_{50} values of 100 to 1000 μM. The greatest cytotoxicity was produced by compound **6**, which had an ID_{50} value of 2 μM.

This laboratory has previously described the synthesis of several β-D-xylopyranosides[7] that have been shown to function as artificial initiators of GAG synthesis.[8,9] We have extended these studies to examine the effects of the xylopyranosylamines synthesized in this report on B16/F10 melanoma GAG production. Although these newly fabricated compounds did not significantly alter the degree of incorporation of [^{35}S]sulfate into the cetylpyridinium chloride precipitable GAGS associated with the B16 melanoma cells, significant stimulation of GAG release into the medium was produced by several compounds (Table IV). Acetylation of the xylose moiety rendered some of the compounds more effective than the free sugar derivatives in stimulating GAG synthesis. Thus, while compound **1** did not increase GAG synthesis, its acetylated derivative **11** stimulated the formation of GAGS approximately 2-fold. This relationship also existed for both the p-chloro and p-methoxy compounds; the most dramatic increase was observed with the peracetylated methoxy derivative **14**, which stimulated GAG synthesis 4-fold. However, acetylation of the p-$COOCH_2CH_3$-substituted derivative did not increase GAG synthesis over that of the nonacetylated parent compound; thus, both **4** and **12** increased GAG synthesis about 2-fold. A similar inability of acetylation of the xylopyranosylamine to further increase GAG synthesis over that produced by the free sugar derivative was also seen for compounds **5** and **13**. Some potentiation of the increase in GAG synthesis produced by the p-SO_2NH_2-substituted compound **8** occurred following acetylation to compound **16**. The increase in [^{35}S]sulfate incorporation into GAGS produced by some of the peracetylated derivatives may be the result of increased uptake by passive diffusion of the more hydrophobic acetylated form of the sugars, resulting in high intracellular levels of an artificial initiator of GAG synthesis.

Neither the p- nor o-($COOCOCH_3$)-substituted compounds **17** and **18** affected GAG synthesis. The para and ortho sodium salts of **3** and **10** and the free carboxylic acid derivative **2** were also ineffective at increasing GAG synthesis, while the ortho-substituted carboxylic acid derivative **9** stimulated GAG synthesis 2-fold. No simple structural relationship that explained the inactivity of certain derivatives was evident from these investigations.

Experimental Section

Chemistry. All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. NMR spectra were obtained with a Bruker 270 HX spectrometer at 270 MHz with Me_4Si as an internal standard. IR absorption spectra were obtained with a Perkin-Elmer 15 spectrometer using KBr pellets of solids. The mass spectral data were obtained with a hp 5985B GC/MS mass spectrometer. All analytical samples were homogeneous by thin-layer chromatography, which was performed on EM silica gel 60 F_{254} sheets (0.2 mm) with $C_6H_6/C_2H_5OH/H_2O$ (1:3:1, v/v) as the developing solvent; spots were detected with iodine vapor. Elemental analyses and optical rotations were performed by the Baron Consulting Co., Orange, CT.

General Procedure for the Preparation of N-(Substituted-phenyl)-D-xylopyranosylamines (1–9). N-(Substituted-phenyl)-D-xylopyranosylamines (1–9) were synthesized by condensation of 0.5 g (3.3 mmol) of D(+)-xylose dissolved in 2 mL

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of H_2O with a molar equivalent amount of a monosubstituted aniline in 6 to 10 mL of EtOH, depending upon the degree of solubility. Three drops of glacial acetic acid were added to catalyze the reaction, except for compounds **3** and **6**. The resulting solutions were stirred for 2 to 3 h at room temperature and concentrated under reduced pressure below 30 °C, except for compounds **2** and **10**, which separated during stirring. The residues obtained were treated with diethyl ether and EtOH to give the desired products. Recrystallization solvents, yields, and melting points are given in Table I.

***N*-(Carboxyphenyl)-D-xylopyranosylamine Sodium Salt (10).** A solution of 0.068 g (0.25 mmol) of compound **9** in 20 mL of 50% EtOH was carefully neutralized to pH 7 with a 1% sodium carbonate solution while stirring. The resulting solution was concentrated under reduced pressure at 30 °C. The residue obtained was dissolved in EtOH and diethyl ether and refrigerated to give 0.02 g (27%) of a precipitate of **10**.

General Procedure for the Preparation of the Acetates of *N*-(Substituted-phenyl)-β-D-xylopyranosylamines (11–18). A solution of 5 mL of acetic anhydride in 5 mL of pyridine was cooled to 5 °C, 1 g of an *N*-(substituted-phenyl)-D-xylopyranosylamine (**1–9**) was added, and each mixture was stirred at 5 °C until solubilization was complete. Solutions were maintained at 5 °C for 48 h and then poured with stirring into 100 mL of ice–water. Resulting syrups were stirred vigorously with succesive quantities of ice and water to give white solids, which were collected, washed with water at 0 °C, and finally recrystallized from EtOH to yield the desired acetyl derivatives, which are listed in Table II.

1-Deoxy-1-*p*-anisidino-2,2-didehydro-D-xylose (III). A solution of 0.41 g (3.3 mmol) of *p*-anisidine in 6 mL of EtOH was mixed with 0.5 g (3.3 mmol) of D(+)-xylose in 2 mL of H_2O, and 3 drops of glacial acetic acid were added. The resulting solution was stirred at room temperature for 4 h and kept at 5 °C for several days. Crystals that separated were collected by filtration and washed with diethyl ether to give 0.06 g of III: mp 158–161 °C; IR (KBr) 3300 (OH), 1704 (C=O, unconj), 1558, 1513 (phenyl) cm^{-1}; mass spectrum, *m/e* (relative intensity), 256 ($M^+ + 1$, 77.5), 257 ($M^+ + 1$, 12.5), 134 [$CH_2OH(CH_2OH)_2COCH_2^+ + H^+$, 10], 123 (*p*-$CH_3OC_6H_4NH + H^+$, 100), 108 (*p*-$CH_3OC_6H_4 + H^+$, 63). Anal. ($C_{12}H_{17}NO_5$) C, H, N. The filtrate was kept at 5 °C for several days to obtain an additional crop of III (0.07 g), mp 150–153 °C.

Cell Culture. B16/F10 melanoma cells were maintained in monolayer culture at 37 °C in a 5% humidified atmosphere by twice weekly passage of 3×10^6 cells/flask in Eagle's minimal essential medium with Hanks' salts supplemented with 10% fetal calf serum (Flow Laboratories), nonessential amino acids, sodium pyruvate, streptomycin (110 μg/mL), penicillin (100 units/mL), minimal essential medium, vitamins, and L-glutamine. Exponentially growing B16/F10 melanoma cells were seeded at 1.5 to 3×10^5 cells/25-cm^2 flask. The appropriate xylosides **1–18** were added to each flask at various concentrations at the time of cell seeding and, at the end of a 72-h incubation period, cells were removed from the tissue culture flask by treatment with 0.25% trypsin–EDTA, and the cell number was determined with a Coulter Model ZBI electronic particle counter. The 50% inhibitory concentration for each agent was determined graphically.

Analysis of Glycosaminoglycans. B16/F10 melanoma cells were seeded at a level of 3×10^5 cells/25-cm^2 flask in the presence of xylosides at a concentration of 10^{-5} M. Cultures were incubated at 37 °C; after 24 h, 10 μCi/mL of [^{35}S]sulfate (1 Ci/mmol, New England Nuclear Corp.) was added, and the cells were incubated for another 24 h. At the end of this period, cells were washed with phosphate-buffered saline (pH 7.2) and treated with 0.25% trypsin–EDTA for approximately 1 to 2 min, and an equal volume of complete medium was added. The cell number was determined as described above and radiolabeled GAGS were isolated by a modification of the method of Cohn et al.[22] Cellular and medium samples were treated with proteinase K (0.1 mg/flask) at 37 °C, and 24 h later, DNAse (0.08 mg/mL) was added, and the samples were incubated at 37 °C for an additional 24 h. Samples were then stored frozen. After thawing, radiolabeled GAGS were assayed by cetylpyridinium chloride (CPC) precipitation according to the method of Glimelius et al.[23] Each aliquot was incubated in 1 mL of 2% CPC and 40 mM Na_2SO_4 at 45 °C. One hour later, samples were collected on 0.45-μm HA filters (Millipore Corp.), washed with 15 mL of 1% CPC and 40 mM Na_2SO_4, and dried, and radioactivity was measured in Biofluor (New England Nuclear Corp.) by using a Beckman 7500 scintillation spectrometer.

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Registry No. 1, 84894-09-7; **2**, 10396-71-1; **3**, 74488-14-5; **4**, 84851-64-9; **5**, 3054-60-2; **6**, 2774-41-6; **7**, 2776-65-0; **8**, 10396-72-2; **9**, 72921-42-7; **10**, 72921-43-8; 11, 53131-05-8; **12**, 84851-65-0; **13**, 2776-69-4; **14**, 2776-68-3; **15**, 2776-85-4; **16**, 84851-66-1; **17**, 84851-67-2; **18**, 84851-68-3; III, 84851-69-4; D-xylose, 58-86-6; aniline, 58-86-6; 4-aminobenzoic acid, 62-53-3; ethyl 4-aminobenzoate, 150-13-0; *p*-toluidine, 106-49-0; *p*-anisidine, 104-94-9; *p*-chloroaniline, 106-47-8; *p*-aminobenzenesulfonamide, 63-74-1; *o*-aminobenzoic acid, 118-92-3.

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