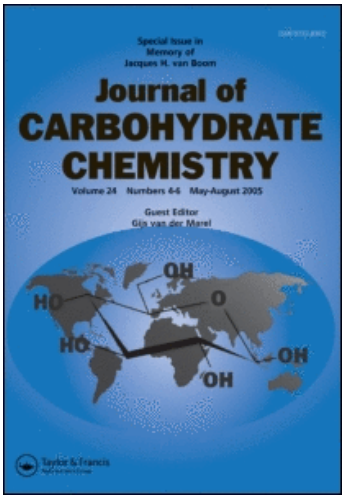

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Synthesis of O-β-D-Galactopyranosyl-(1→3)-O-β-D-Galactopyranosyl-(1→4)-O-β-D-Xylopyranosyl-(1→3)-L-Serine (Gal-Gal-Xyl-Ser)

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SYNTHESIS OF O-β-D-GALACTOPYRANOSYL-(1→3)-O-β-D-GALACTOPYRANOSYL-(1→4)-O-β-D-XYLOPYRANOSYL-(1→3)-L-SERINE (Gal-Gal-Xyl-Ser)

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ABSTRACT

O-β-D-Galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→3)-L-serine (Gal-Gal-Xyl-Ser) was prepared by a procedure involving the synthesis of a benzoylated galactobiosyl bromide and a xylosylserine derivative with an unsubstituted hydroxyl group at C-4 (3-O-(2,3-di-O-benzoyl-β-D-xylopyranosyl)-N-carbobenzoxy-L-serine benzyl ester), followed by condensation and deblocking. The structure of the product was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Upon incubation with an extract of embryonic chick cartilage, the synthetic Gal-Gal-Xyl-Ser served as an acceptor for enzymatic glucuronosyl transfer from UDP-D-glucuronic acid.

INTRODUCTION

In several mammalian proteoglycans, the carbohydrate-protein linkage is an O-glycosidic linkage between xylose and serine (Fig. 1).[1] The carbohydrate-protein linkage region also contains two galactose residues, which are followed by a glucuronic acid and an N-acetylhexosamine residue, constituting the first repeating disaccharide unit in the poly-

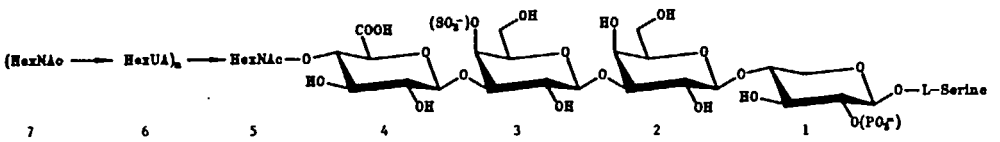


FIG. 1. The carbohydrate-protein linkage region of xylose-serine linked proteoglycans. In chondroitin 4- and 6-sulfate, HexUA is always β-D-GlcUA; in dermatan sulfate, heparin, and heparan sulfate, HexUA is either α-L-IdUA or β-D-GlcUA. HexNac represents β-D-GalNAc in dermatan sulfate and the chondroitin sulfates, and α-D-GlcNAc in heparin and heparan sulfate. Residues 1 and 3 may be substituted with a phosphate group at C-2 and a sulfate group at C-4, respectively.

saccharide chain. Two novel structural features in the carbohydrate-protein linkage region have recently been discovered, i.e. a 2-phosphate group on some of the xylose residues[2-6] and a 4-sulfate group on some of the glucuronic acid-bound galactose residues (unit 3 in Fig. 1).[7]

The biosynthesis of the polysaccharide chains of the xylose-containing proteoglycans is initiated by transfer of xylose from UDP-D-xylose to specific serine residues in the core protein, and residues 2-7 are then added stepwise in reactions catalyzed by specific glycosyltransferases. Currently available evidence indicates that the first repeating disaccharide unit (residues 4 and 5) is synthesized by two glycosyltransferases which are distinct from those involved in the formation of the rest of the chain.[1,8]

Because of the difficulties inherent in the preparation of native substrates for the several glycosyltransferases participating in proteoglycan biosynthesis, these enzymes are presently assayed with oligosaccharides of appropriate structure or with serine-linked saccharides such as xylosylserine or galactosylxylosylserine. The use of acceptors of the latter type affords practical advantages when many samples are to be assayed since the free amino group on the serine residue allows adsorption of the reaction product to a cation exchange resin, while the radioactive nucleotide sugar and its degradation products are not adsorbed. The reaction product is subsequently eluted with ammonium hydroxide, and its radioactivity is measured.

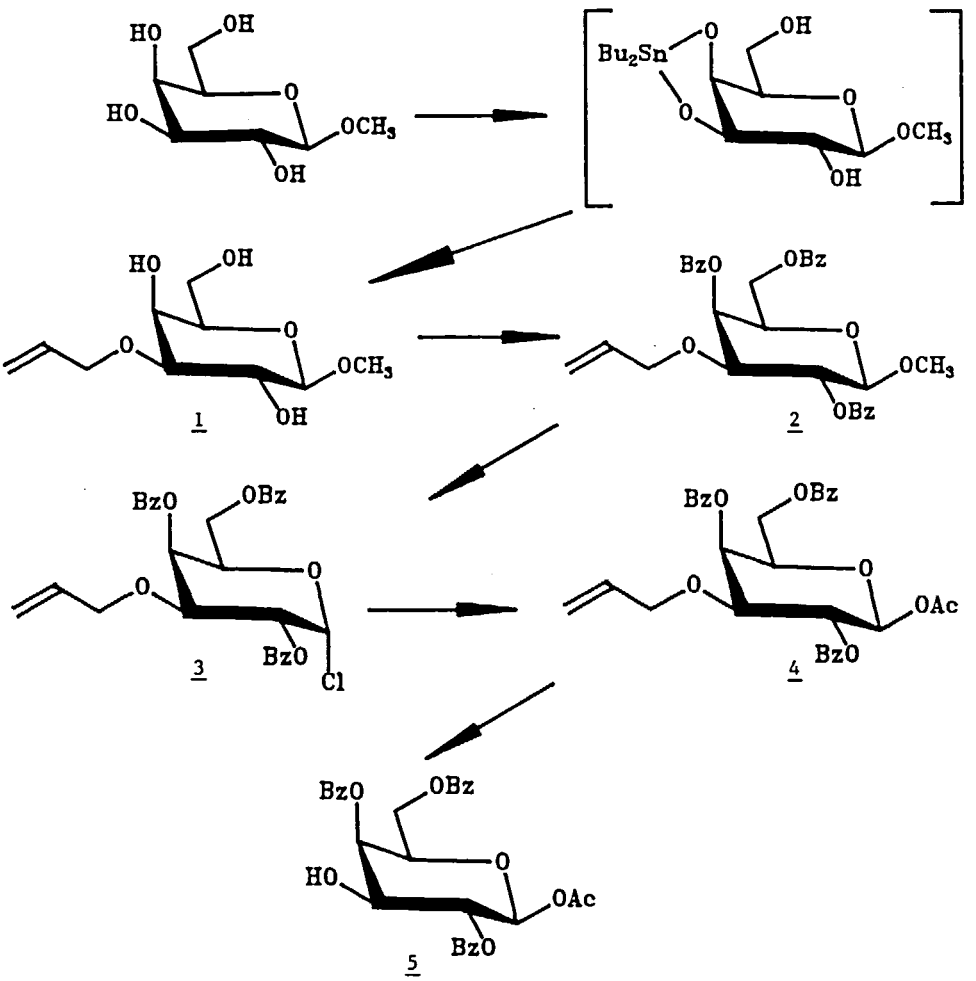
Several fragments from the carbohydrate-protein linkage region of the proteoglycans have been synthesized previously, including xylosylserine,[9-13] galactosylxylosylserine,[13-15] galactosylgalactosylxylosyl-

serine,[15] and the reducing oligosaccharides galactosylxylose, galactosylgalactose, galactosylgalactosylxylose,[16,17] and glucuronosylgalactose.[18] Some of these compounds were obtained in low overall yields due to inefficient glycosidation procedures or degradation during deblocking. Thus, there is a need for improved syntheses of some of the previously synthesized compounds as well as synthesis of linkage region fragments that have not yet been prepared synthetically. In this communication we report a substantially improved synthesis of O-β-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→3)-L-serine (Gal-Gal-Xyl-Ser).

RESULTS AND DISCUSSION

O-β-D-Galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→3)-L-serine (Gal-Gal-Xyl-Ser) was synthesized by a procedure encompassing the assembly of two blocks, a galactobiose derivative and a xylosylserine derivative, which were condensed and deprotected to yield the desired compound. The specific steps of this procedure are illustrated in Schemes 1-4.

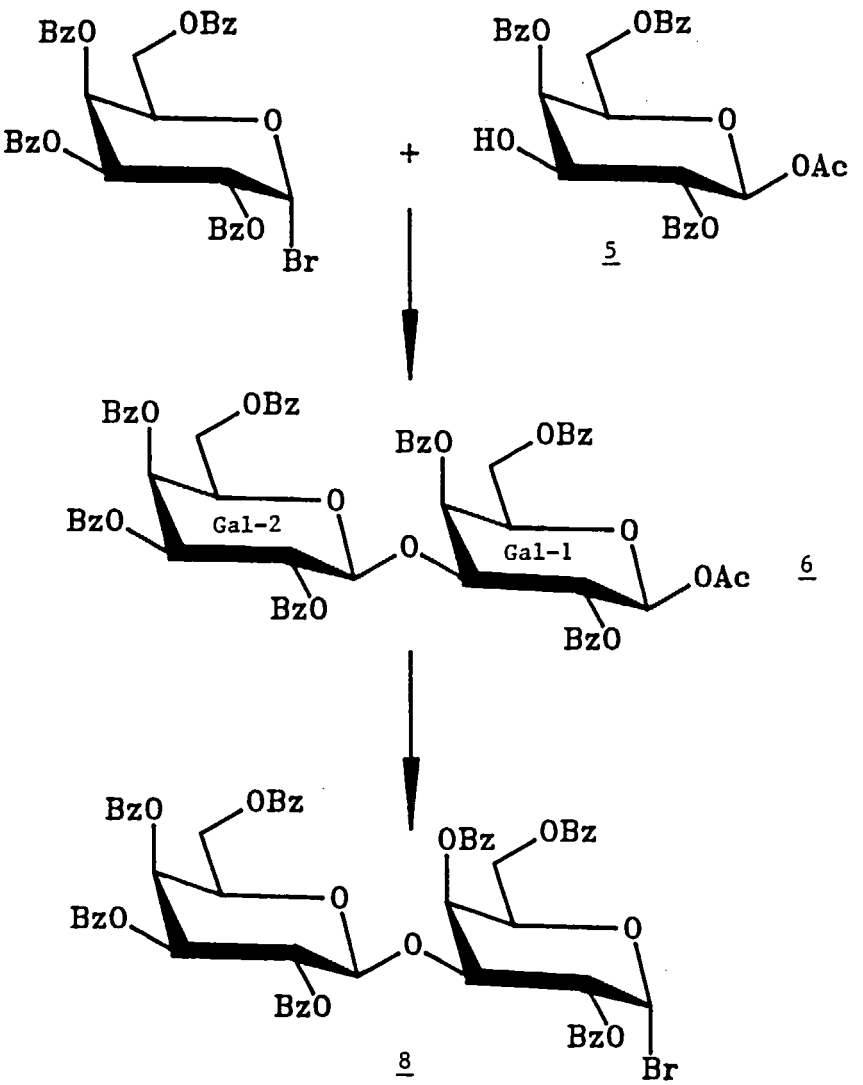
Synthesis of galactobiosyl bromide 8 (Schemes 1 and 2). A key intermediate in this synthesis was 1-O-acetyl-2,4,6-tri-O-benzoyl-β-D-galactopyranose (5),[24] in which OH-3 is unsubstituted and available for coupling with 2,3,4,6-tetra-O-benzoyl-α-D-galactopyranosyl bromide (benzobromogalactose). Alcohol 5 was synthesized as outlined in Scheme 1. Methyl β-D-galactopyranoside was treated with dibutyltin(II) oxide to give an intermediary dibutylstannylidene derivative,[19-23] which was treated with allyl bromide to give the 3-O-allyl ether 1 as the main product. After chromatography to remove organotin compounds and quaternary ammonium salts used as promoters, the crude 1 was benzoylated to give the desired methyl 3-O-allyl-2,4,6-tri-O-benzoyl-β-D-galactopyranoside (2) in approximately 60% yield from the starting material. Compound 2 was treated with dichloromethyl methyl ether (DCMME)[24-26] to give galactosyl chloride 3 in >90% yield. Chloride 3 was then converted to the β-1-O-acetate 4 by treatment with the silver triflate-collidine complex in glacial acetic acid. The $PdCl_2$-NaOAc-aq. HOAc reagent system[27] was used to remove the allyl group from 4 to afford the known[24] alcohol 5 in 76% yield. It should be noted that the allyl ether was



SCHEME 1. Synthesis of a galactose aglycon with OH-3 free

chosen as a temporary protecting group for position 3 on galactose because of its excellent stability, compatibility with other protecting groups, and ease of removal when necessary.

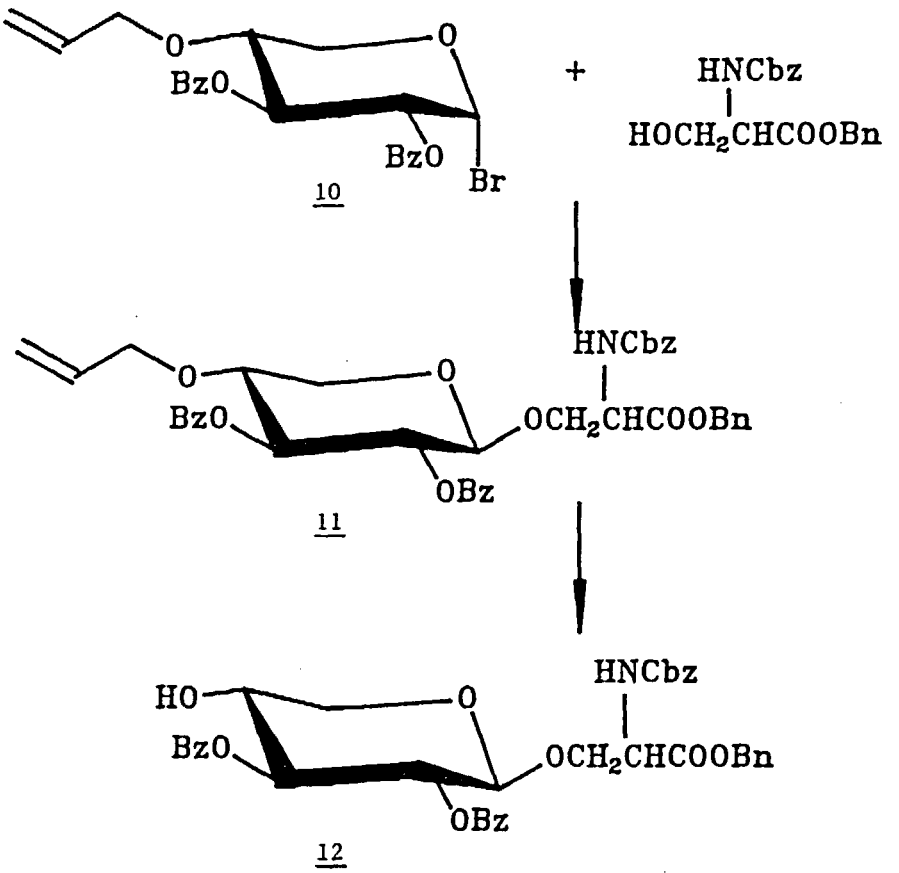
As shown in Scheme 2, alcohol 5 was condensed with benzobromogalactose, using the silver triflate-collidine complex in toluene as promoter,[24] to afford the desired β-1⟶3-linked galactobiose derivative 6 in 52% yield, along with the 28% of the corresponding α-1⟶3-linked product (7). The yields were similar in two separate experiments (β/α



SCHEME 2. Synthesis of the galactobiose glycosyl donor

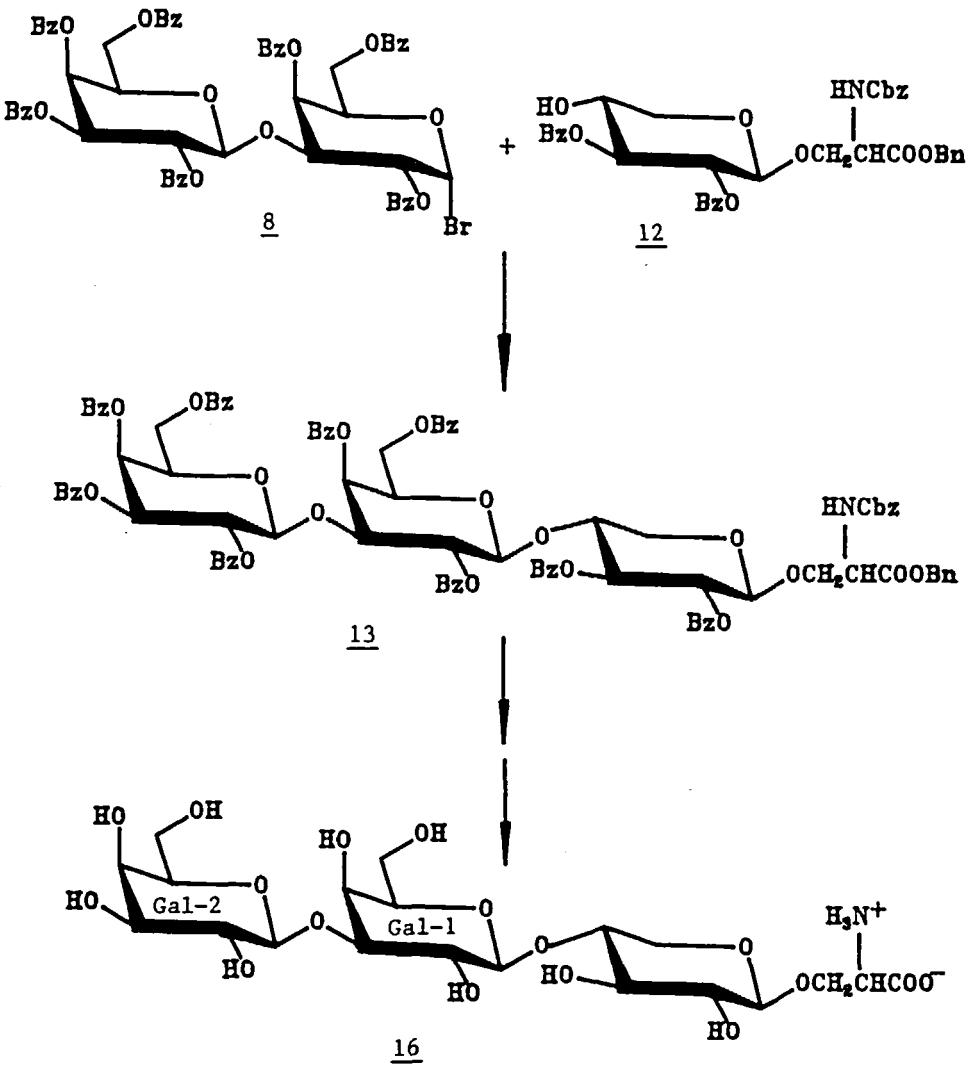
ratio 1.85:1), in contrast to a reported yield of 72% of 6,[24] with no mention of any α-1—>3-linked product. Treatment of disaccharide 6 with hydrogen bromide in glacial acetic acid afforded the known galactobiosyl bromide 8.[15]

Synthesis of xylosylserine aglycon 12 (Scheme 3). In the synthesis of the xylosylserine glycosyl acceptor, the allyl group was again used



SCHEME 3. Synthesis of the xylosylserine aglycon

for temporary protection, now on position 4 of xylose, and was introduced in >95% yield on the starting material, benzyl 2,3-anhydro-β-D-ribopyranoside, under phase-transfer catalysis conditions.[28-30] Although isolation of the product only requires washing of the organic layer with water and concentration to dryness, complete removal of the quaternary ammonium salts used as promoters was ensured by treatment with a mixed-bed ion-exchange resin. An economical advantage of this procedure is that the use of expensive silver salts is avoided. Conversion of the resulting 4-O-allyl ether 9 into 4-O-allyl-2,3-di-O-benzoyl-α-D-xylopyranosyl bromide (10; a 4-step procedure) was performed as described previously.[15] Xylosyl bromide 10 was condensed with N-carbobenzoxy-L-serine benzyl ester, using the silver triflate-collidine complex as promoter, to give the fully protected xylosylserine derivative



SCHEME 4. Synthesis of Gal-Gal-Xyl-Ser

11 (known, see ref. 15) in 80% yield. The allyl derivative (11) was isomerized to the corresponding propen-1-yl ether, using Wilkinson's catalyst.[31] It was observed that a prolonged reaction time - reflux overnight - was necessary for complete reaction. The propen-1-yl ether was cleaved by treatment with mercury(II) acetate in 90% aqueous acetone to give the desired partially protected xylosylserine derivative 12 (known, see ref. 15) in almost 80% yield.

Condensation of galactobiosyl and xylosylserine derivatives 8 and 12 (Scheme 4). With the silver triflate-collidine complex as pro-

moter, galactobiosyl bromide 8 was condensed with xylosylserine aglycon 12 to give the fully protected Gal-Gal-Xyl-Ser derivative 13 with a β-1—>4 Gal-Xyl linkage, in 72% yield, along with 15% of the isomer with an α-1—>4 Gal-Xyl linkage (14). The combined yield of 87% was noticeably higher than that previously reported,[15] with a similar α/β ratio. Catalytic transfer hydrogenation[32,33] removed the benzyl ester and carbobenzoxy groups to give the amino acid deblocked derivative 15 in 90% yield. Treatment of 15 with methanolic ammonia then afforded the pure title compound (16) in quantitative yield. No signal splitting in the ^{13}C-NMR spectrum, diagnostic of racemization, could be observed, nor was there any indication of β-elimination products. The product was homogeneous on high-voltage electrophoresis, with a $R_{xylosylserine}$ of 0.69.

The ^{13}C-NMR spectrum of 16 showed signals in the anomeric region at δ 104.21 assigned to C-1 Gal-2, at 102.48 assigned to C-1 Xyl, and at 101.26 assigned to C-1 Gal-1 (see Schemes 2 and 4 for numbering of galactose residues). Other signals were observed inter alia at δ 81.90 for C-3 Gal-1, at 76.29 for C-4 Xyl, at 74.98 and 74.83 for the two C-5 Gal (1 and 2), at 70.95 for C-2 Gal-2, and at 69.73 for C-2 Gal-1. At δ 61.05 and 60.91 signals for the two C-6 Gal (1 and 2) were observed, and a signal for C-5 Xyl was observed at δ 62.92. These data compare well with the ^{13}C-NMR spectrum reported for Gal-Xyl-ser.[13,14] The observed ^{1}H-NMR spectrum of 16 was virtually identical to that observed by Van Halbeek et al.[34] However, we assigned the signal at δ 4.25 ($J_{\alpha,\beta}$ 5.6 Hz, $J_{\beta,\beta'}$ -11.1 Hz) to one of the protons of the methylene group on serine, in accord with our observations on Gal-Xyl-Ser.[13] Our assignments were confirmed by COSY and ^{1}H-^{13}C correlation spectroscopy. These data and the specific optical rotation ($[\alpha]_D$ -3.5°) conclusively demonstrate that the assigned structure of 16 is that of the title compound.

Transfer of [^{14}C]glucuronic acid from UDP-D-[^{14}C]glucuronic acid to synthetic Gal-Gal-Xyl-Ser. The ability of the synthetic Gal-Gal-Xyl-Ser to serve as an acceptor in the reaction catalyzed by glucuronosyltransferase I was tested in experiments similar to those described previously for the assay of this enzyme.[35,36] A crude enzyme preparation from embryonic chick cartilage was incubated with UDP-D-[^{14}C]glucuronic acid in the presence or absence of added Gal-Gal-Xyl-Ser, and formation of the expected reaction product was assessed as described in Experimental. In the presence of Gal-Gal-Xyl-Ser, a product was formed which, on high voltage electrophoresis, migrated as an anion at pH 5.3 and as a cation

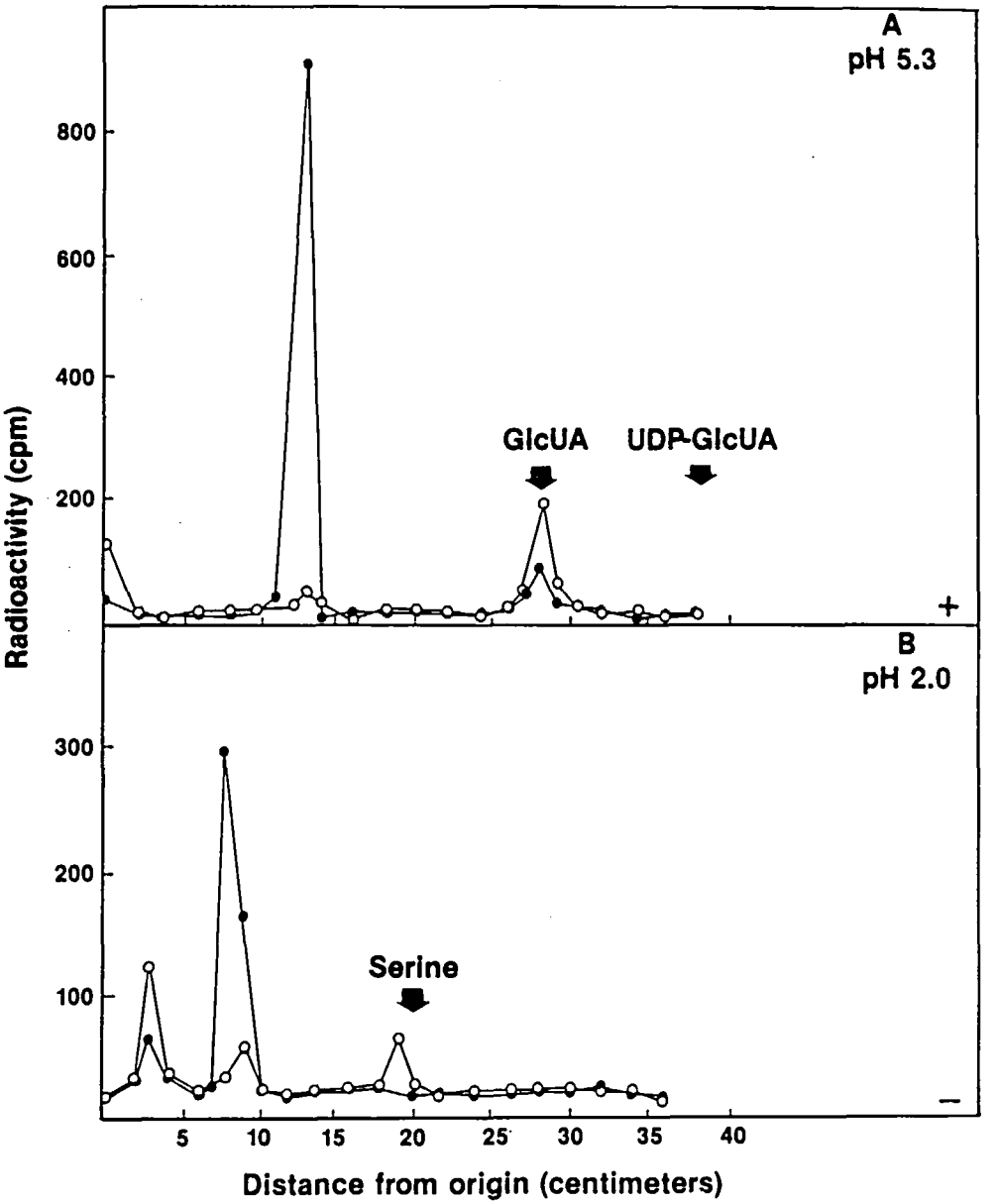


FIG. 2. High voltage paper electrophoresis at pH 5.3(A) or 2.0(B) of the products of transfer of glucuronic acid to Gal-Gal-Xyl-Ser.

○——○, reaction mixtures without Gal-Gal-Xyl-Ser;

●——●, complete reaction mixtures.

at pH 2. Relative to standards (glucuronic acid, serine, UDP-D-glucuronic acid), its mobility was as expected for the product of glucuronosyl transfer to Gal-Gal-Xyl-Ser. No product with the same mobility was found in reaction mixtures without added Gal-Gal-Xyl-Ser. It was thus apparent that the synthetic compound was a substrate for glucuronosyltransferase I.

EXPERIMENTAL

Materials. TLC plates precoated with silica gel F_{254} (0.25 mm layer) and Silica Gel 60 (particle size 40-63 μm, 230-400 mesh) for column chromatography were from E. Merck, Darmstadt, W. Germany. Dowex 50W X4 (H^+, 200-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA. Polydat P-40 was purchased from Polysciences, Warrington, PA. D-Glucuronic acid, L-serine and UDP-D-glucuronic acid were obtained from Sigma Chemical Co., St. Louis, MO. Scintiverse E and chromatography solvents were from Fisher Scientific Co., Fairlawn, NJ. UDP-D-[^{14}C]-glucuronic acid (sp. act. 304.3 mCi/mmol) was from NEN Research Products, Boston, MA. Other chemicals were of reagent grade or better and were obtained from Aldrich, Milwaukee, WI.

General methods. Microanalyses were performed by Atlantic Microlab, Atlanta, GA. Optical rotations were measured in a Perkin-Elmer 141 polarimeter. NMR experiments were performed at 25 °C on a Bruker WH-400 spectrometer equipped with an Aspect-3000 computer or on a Bruker AM 600 spectrophotometer, also equipped with an Aspect-3000 computer. The HOD resonance signal was set to 4.8 ppm as the ^{1}H chemical shift reference in D_2O. Internal dioxane (67.4 ppm) was used as the ^{13}C chemical shift reference in D_2O. Compounds on TLC plates were detected either by spraying with 5% (w/v) ammonium sulfate in 50% aqueous methanol, followed by charring at 120 °C, or, for ninhydrin-positive compounds, by spraying with 0.1% (w/v) ninhydrin in n-butanol containing 1% glacial acetic acid and heating at 100 °C. High voltage electrophoresis was carried out on Whatman No. 3MM paper for 60-90 min at 50 V/cm in 1.6 M acetic acid, which had been adjusted to pH 2.0 with formic acid, or in pyridine/acetic acid, pH 5.3.[37] Ninhydrin-positive compounds were detected by dipping the dried papers in a solution of 0.2% (w/v) ninhydrin in 95% aqueous acetone containing 1% pyridine, followed by heating at 100 °C. D-Glucuronic acid (reference standard) was detected by silver

staining.[38] Column loads for chromatography on silica gel were typically in the range of 0.5-2 g per 100 g of silica gel. Melting points are uncorrected.

Methyl 3-O-Allyl-2,4,6-tri-O-benzoyl-β-D-galactopyranoside (2). Finely powdered methyl β-D-galactopyranoside (1.94 g, 10 mmol) and dibutyltin(II) oxide (2.50 g, 10 mmol) were stirred under reflux in benzene (30 mL) for 48 h with continuous removal of water by passing the condensate through a Soxhlet extractor filled with molecular sieves (3A; 25-mL bed). The temperature was lowered to 60 °C, and allyl bromide (3.70 mL, 44 mmol) and tetrabutylammonium iodide (3.70 g, 10 mmol) were added. An inert atmosphere was maintained by keeping the reaction mixture under nitrogen at all times. After stirring at 60 °C for 12 h, examination by TLC ($CHCl_3$-MeOH 9:1) showed a major product at R_F 0.25 and two minor, faster moving products. After cooling, the reaction mixture was concentrated, and the residue was chromatographed on a silica gel column ($CHCl_3$-MeOH 9:1) to give 3.24 g of crude methyl 3-O-allyl-β-D-galactopyranoside (1). This material was not further purified, but was benzoylated directly with an excess of benzoyl chloride in pyridine. The usual work-up, chromatography on a silica gel column (toluene-EtOAc 16:1), yielded 3.30 g (60%) of chromatographically pure 2, which crystallized on standing. After recrystallization from methanol, 2 had m.p. 140-142 °C and $[\alpha]_D$ +46° (c 1.34, $CHCl_3$).

^{1}H-NMR spectroscopy (400 MHz, $CDCl_3$) showed: δ 8.20-7.35 (m, 15 H, aromatic H), 5.87 (d, 1 H, $J_{3,4}$ 3.4 Hz, H-4), 5.66 (m, 1 H, H-2 allyl), 5.55 (dd, 1 H, $J_{1,2}$ 8.0 Hz, $J_{2,3}$ 10.0 Hz, H-2), 5.16 (dd, 1 H, $J_{H,H}$ 1.4 Hz, $J_{H,H}$ 17.3 Hz, H-1 allyl), 5.03 (dd, 1 H, $J_{H,H}$ 0.87 Hz, $J_{H,H}$ 10.4 Hz, H-1' allyl), 4.65 (dd, 1 H, $J_{5,6}$ 7.0 Hz, $J_{6,6'}$ -11.4 Hz, H-6), 4.63 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.45 (dd, 1 H, $J_{5,6'}$ 7.0 Hz, $J_{6,6'}$ -11.4 Hz, H-6'), 4.17 (dd, 1 H, $J_{5,6}$ 6.1 Hz, $J_{5,6'}$ 7.0 Hz, H-5), 4.16 (dd, 1 H, $J_{2,3}$ 5.6 Hz, $J_{3,3'}$ -13.2 Hz, H-3 allyl), 3.99 (dd, 1 H, $J_{2,3'}$ 6.3 Hz, $J_{3,3'}$ -13.2 Hz, H-3 allyl), 3.87 (dd, 1 H, $J_{3,4}$ 3.4 Hz, $J_{2,3}$ 10.0 Hz, H-3), and 3.54 (s, 3 H, OCH_3).

^{13}C-NMR spectroscopy (100 MHz, $CDCl_3$) showed inter alia: δ 102.35 (C-1), 71.24 (C-5 and C-3 allyl), 70.69 (C-2), 66.94 (C-4), 62.50 (C-6), and 56.86 (OCH_3). The signal for C-3 (Gal) is hidden under one of the strong $CDCl_3$ signals.

Anal. Calcd. for $C_{31}H_{30}O_9$: C 68.12; H 5.53. Found: C 68.19; H 5.54.

3-O-Allyl-2,4,6-tri-O-benzoyl-α-D-galactopyranosyl Chloride (3). A solution of 2 (0.45 g, 0.82 mmol) in 1:2 chloroform-dichloromethyl methyl ether (DCMME; 6 mL) was heated at 75 °C under nitrogen in the presence of anhydrous zinc chloride (25 mg). After 8 h at 75 °C, examination by TLC (toluene-EtOAc 16:1) showed a major product with R_F 0.46 and no remaining starting material. A minor product with R_F 0.35 was presumably the α-methyl glycoside formed by anomerization of the starting material; traces of slower moving degradation products were also observed. The reaction mixture was concentrated after removal of the insoluble zinc chloride, and the residue was purified on a silica gel column (toluene-EtOAc 16:1) to give 3 (0.42 g, 92%) as a chromatographically pure amorphous solid. Compound 3 showed $[\alpha]_D$ +143° (c 1.74, $CHCl_3$).

^{1}H-NMR spectroscopy (400 MHz, $CDCl_3$) showed: δ 8.20-7.35 (m, 15 H, aromatic H), 6.63 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1), 6.00 (d, 1 H, $J_{3,4}$ 3.3 Hz, H-4), 5.80 (m, 1 H, H-2 allyl), 5.61 (dd, 1 H, $J_{1,2}$ 3.9 Hz, $J_{2,3}$ 10.2 Hz, H-2), 5.26 (dd, 1 H, $J_{H,H}$ 1.4 Hz, $J_{H,H}$ 17.2 Hz, H-1 allyl), 5.13 (dd, 1 H, $J_{H,H}$ 1.05 Hz, $J_{H,H}$ 10.5 Hz, H-1' allyl), 4.78 (dd, 1 H, $J_{5,6}$ 6.9 Hz, $J_{5,6'}$ 5.5 Hz, H-5), 4.58 (dd, 1 H, $J_{5,6}$ 5.5 Hz, $J_{6,6'}$ -11.6 Hz, H-6), 4.46 (dd, 1 H, $J_{5,6'}$ 5.5 Hz, $J_{6,6'}$ -11.6 Hz, H-6'), 4.29 (dd, 1 H, $J_{3,4}$ 3.3 Hz, $J_{2,3}$ 10.2 Hz, H-3), 4.23 (dd, 1 H, $J_{2,3}$ 5.2 Hz, $J_{3,3'}$ -13.0 Hz, H-3 allyl), and 4.11 (dd, 1 H, $J_{2,3'}$ 6.0 Hz, $J_{3,3'}$ -13.0 Hz, H-3' allyl).

1-O-Acetyl-3-O-allyl-2,4,6-tri-O-benzoyl-β-D-galactopyranose (4). A solution of chloride 3 (12.98 g, 23.6 mmol) in glacial acetic acid (75 mL) was added to a stirred mixture of silver triflate (7.28 g, 28.3 mmol) and sym-collidine (3.74 mL, 28.3 mmol) in glacial acetic acid (100 mL). After stirring for 1 h at room temperature, examination by TLC (toluene-EtOAc 16:1) showed a single major product (R_F 0.30), which migrated more slowly than the starting material. The reaction mixture was filtered through Celite; the combined filtrate and washings were reduced to approximately 100 mL on a rotary evaporator, diluted with toluene (400 mL), washed with water (2 x 200 mL), 0.5 M $Na_2S_2O_3$ (4 x 100 mL) and finally with water (100 mL), and then dried and concentrated to a residue (13.25 g). Chromatography of this material on a silica gel

column (toluene-EtOAc 16:1) yielded 12.45 g (92%) of chromatographically homogeneous title compound (4) as an amorphous solid. $[\alpha]_D$ +70° (c 1.13, $CHCl_3$).

^{1}H-NMR spectroscopy (400 MHz, $CDCl_3$) of 4 showed: δ 8.20-7.35 (m, 15 H, aromatic H), 6.00 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 5.92 (d, 1 H, $J_{3,4}$ 3.4 Hz, H-4), 5.70 (dd, 1 H, $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 9.9 Hz, H-2), 5.66 (m, 1 H, H-2 allyl), 5.16 (dd, 1 H, $J_{H,H}$ 1.5 Hz, $J_{H,H}$ 17.2 Hz, H-1 allyl), 5.04 (dd, 1 H, $J_{H,H}$ 0.92 Hz, $J_{H,H}$ 10.4 Hz, H-1' allyl), 4.60 (dd, 1 H, $J_{5,6}$ 6.8 Hz, $J_{6,6'}$ -11.5 Hz, H-6), 4.45 (dd, 1 H, $J_{5,6'}$ 5.8 Hz, $J_{6,6'}$ -11.5 Hz, H-6'), 4.32 (dd, 1 H, $J_{5,6'}$ 5.8 Hz, $J_{5,6}$ 6.8 Hz, H-5), 4.17 (dd 1 H, $J_{2,3}$ 4.9 Hz, $J_{3,3'}$ -13.1 Hz, H-3 allyl), 4.00 (dd, 1 H, $J_{2,3'}$ 6.3 Hz, $J_{3,3'}$ -13.1 Hz, H-3' allyl), 3.96 (dd, 1 H, $J_{3,4}$ 3.4 Hz, $J_{2,3}$ 9.9 Hz, H-3) and 2.05 (s, 3 H, $OCOCH_3$).

^{13}C-NMR spectroscopy (100 MHz, $CDCl_3$) showed *inter alia*: δ 92.34 (C-1), 72.39 (C-5), 70.75 (C-3 allyl), 70.09 (C-2), 66.75 (C-4), 62.83 (C-6), and 20.83 ($OCOCH_3$). The signal for C-3 Gal is hidden under one of the strong $CDCl_3$ signals.

1-O-Acetyl-2,4,6-tri-O-benzoyl-β-D-galactopyranose (5). To a solution of 4 (6.50 g, 11.3 mmol) in 90% aqueous acetic acid (200 mL), sodium acetate (4 g) and palladium(II)chloride (0.40 g, 2.25 mmol) were added. After stirring overnight at room temperature, examination by TLC (toluene-EtOAc 8:1) showed only partial cleavage of the allyl group. More $PdCl_2$ (0.40 g, 2.25 mmol) was added, and stirring was continued for 72 h at room temperature. TLC now showed only traces of remaining starting material and a major product at R_F 0.18. The reaction mixture was filtered through Celite to remove precipitated palladium, and the combined filtrate and washings were reduced to approximately 100 mL on a rotary evaporator. After addition of toluene (500 mL), the solution was washed with water 4 x 300 mL), dried over Na_2SO_4, and concentrated to dryness. The residue (5.80 g) was purified by chromatography on a silica gel column (toluene-EtOAc 8:1) to give 4.60 g (76%) of chromatographically pure 5, crystalline on standing. After recrystallization from ethanol, 5 had m.p. 165-166 °C; $[\alpha]_D$ +35° (c 1.69, $CHCl_3$). Lit:[24] m.p. 166-166.5 °C; $[\alpha]_D$ +27.7° (c 0.72, $CHCl_3$).

^{1}H and ^{13}C NMR spectra for 5 compared well with published data.[24]

O-(2,3,4,6-Tetra-O-benzoyl-β-D-galactopyranosyl)-(1→3)-1-O-acetyl-2,4,6-tri-O-benzoyl-β-D-galactopyranose (6) and O-(2,3,4,6-Tetra-O-

benzoyl-α-D-galactopyranosyl)-(1→3)-1-O-acetyl-2,4,6-tri-O-benzoyl-β-D-galactopyranose (7). A solution of alcohol 5 (3.88 g, 7.26 mmol), benzobromogalactose (5.75 g, 8.71 mmol), and sym-collidine (0.86 mL, 6.53 mmol) in toluene (50 mL) was added dropwise over a 20 min period to a stirred suspension of silver triflate (2.61 g, 10.16 mmol) in toluene (40 mL) at -20 °C to -23 °C. An atmosphere of dry nitrogen was maintained to ensure anhydrous conditions. After stirring for an additional 30 min at -20 °C to -23 °C, TLC (toluene-EtOAc 8:1) showed only a small amount of aglycon remaining and two major products at R_F 0.23 (larger) and R_F 0.45 (smaller). After neutralization with pyridine (1 mL) the reaction mixture was filtered through Celite, diluted with toluene (200 mL), and washed with water (2 x 100 mL), 0.5 M $Na_2S_2O_3$ (2 x 100 mL), 2 M H_2SO_4 (100 mL), saturated aq. $NaHCO_3$ (100 mL), and water again (100 mL). The resultant solution was dried over Na_2SO_4 and concentrated to dryness. Chromatography of the residue on a silica gel column (toluene-EtOAc 8:1) first yielded the faster moving 7, (2.28 g, 28%), followed by 6 (4.25 g, 53%), both obtained as amorphous solids after evaporation of the solvent. Optical rotations were 6, $[\alpha]_D$ +113° (c 1.37, $CHCl_3$); lit. value for 6[24] $[\alpha]_D$ +104° (c 0.72, $CHCl_3$). 7, $[\alpha]_D$ +178° (c 1.51, $CHCl_3$).

^{13}C and ^1H NMR data for 6 were almost identical to those published.[24]

^1H-NMR spectroscopy (400 MHz, $CDCl_3$) of 7 showed: δ 8.25-6.95 (m, 35 H, aromatic H), 6.00 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1 Gal-1), 5.90 (dd, 1 H, $J_{1,2}$ 8.3 Hz, $J_{2,3}$ 10.0 Hz, H-2 Gal-1), 5.84 (d, 1 H, $J_{3,4}$ 3.4 Hz, H-4 Gal-2), 5.82 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1 Gal-2), 5.76 (dd, 1 H, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 10.7 Hz, H-2 Gal-2), 5.52 (dd, 1 H, $J_{3,4}$ 3.4 Hz, $J_{2,3}$ 10.7 Hz, H-3 Gal-2), 5.30 (d, 1 H, $J_{3,4}$ 3.4 Hz, H-4 Gal-1), 4.54 (dd, 1 H, $J_{5,6}$ 8.1 Hz, $J_{6,6'}$ -11.5 Hz, H-6 Gal-2), 4.51 (dd, 1 H, $J_{5,6}$ 5.9 Hz, $J_{6,6'}$ -12.0 Hz, H-6 Gal-1), 4.41 (m, 2 H, H-6' Gal-1 and H-5 Gal-2), 4.25 (dd, 1 H, $J_{5,6'}$ 6.4 Hz, $J_{6,6'}$ -11.5 Hz, H-6' Gal-2), 4.03 (m, 2 H, H-5 Gal-1 and H-3 Gal-1), and 2.11 (s, 3 H, $OCOCH_3$).

Examination of 7 by ^{13}C-NMR spectroscopy (100 MHz, $CDCl_3$) showed inter alia: δ 92.73 and 92.34 (2 x C-1), 72.73 and 72.43 (C-5 Gal-1 and C-3 Gal-1), 69.33 (C-2 Gal-1), 68.74 (C-4 Gal-1), 67.83, 67.54, and 67.43 (C-2, C-3, C-4, all Gal-2), 64.91 (C-5 Gal-2), 62.73 (C-6 Gal-1), and 61.81 (C-6 Gal-2).

Data published in references 23 and 24 were used for comparative purposes when making these assignments.

O-(2,3,4,6-Tetra-O-benzoyl-β-D-galactopyranosyl)-(1→3)-2,4,6-tri-O-benzoyl-α-D-galactopyranosyl Bromide (8). Bromide 8 was obtained from the β-1-O-acetate 6 exactly as described previously (see reference 15) for the anomeric 1-O-benzoates. However, due to the higher reactivity of the 1-O-acetyl group, the reaction time could be shortened to 15 min. The product was obtained in quantitative yield and was used directly in glycosidations.

Benzyl 4-O-Allyl-2,3-anhydro-β-D-ribopyranoside (9). A mixture containing benzyl 2,3-anhydro-β-D-ribopyranoside (5.64 g, 25.4 mmol), tetrabutylammonium bromide (1.60 g, 5 mmol), tetrabutylammonium iodide (0.75 g, 1.75 mmol), allyl bromide (7.5 mL, 86 mmol), methylene chloride (100 mL), and 10% aqueous sodium hydroxide (80 mL) was stirred vigorously for five days at room temperature. At this time, examination by TLC (toluene-EtOAc 8:1) showed only traces of starting material and a single, faster moving product (R_F 0.60). The reaction mixture was transferred to a separatory funnel, and methylene chloride (200 mL) and water (100 mL) were added. After shaking, the aqueous layer was discarded, and the organic layer was washed with water (4 x 150 mL), dried with Na_2SO_4, and concentrated. The oily residue was dissolved in methanol (200 mL) and treated with a mixed bed ion-exchange resin (Bio-Rad RG 501-X8, 20-50 mesh, 75 mL) to remove residual quaternary ammonium salts. After concentration of the methanolic solution, 6.50 g (97%) of the title compound (9) was obtained. This preparation was sufficiently pure to be used directly in the conversion of 9 into 4-O-allyl-2,3-di-O-benzoyl-α-D-xylopyranosyl bromide (compound 10), which was performed exactly as described in reference 15 (a 4-step procedure).

3-O-(4-O-Allyl-2,3-di-O-benzoyl-β-D-xylopyranosyl)-N-carbobenzoxy-L-serine Benzyl Ester (11). To a stirred solution of bromide 10 (4.02 g, 8.7 mmol, prepared from 9 as described in reference 15), and N-carbobenzoxy-L-serine benzyl ester (2.60 g, 7.92 mmol) in anhydrous 1:1 toluene-nitromethane (30 mL) at -20 °C was added, over a 10 min period, a solution of silver triflate (2.90 g, 11.3 mmol) and sym-collidine (0.98 mL, 7.40 mmol) in anhydrous 1:1 toluene-nitromethane (10 mL). After an additional 15 min at -20 °C, TLC (toluene-EtOAc 8:1) showed a major product at R_F 0.22 and some minor, slower moving by-products. The reac-

tion mixture was worked up exactly as described above for the synthesis of disaccharide 6, and the crude reaction product was chromatographed on a silica gel column (toluene-EtOAc 8:1) to give the chromatographically homogeneous title compound (11) (4.49 g, 80%; amorphous). $[\alpha]_D$ +40° (c 1.39, $CHCl_3$). Lit:[15] $[\alpha]_D$ +35° (c 0.5, $CHCl_3$).

In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$ + 10% CD_3OD) 11 showed: δ 8.10-7.15 (m, 20 H, aromatic H), 5.77 (m, 1 H, H-2 allyl), 5.50 (dd, 1 H, $J_{2,3}$=$J_{3,4}$ 7.7 Hz, H-3 Xyl), 5.23-4.98 (m, 7 H, H-2 Xyl, 4 x benzyl H, H-1 and H-1' allyl), 4.65 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1 Xyl), 4.53 (dd, 1 H, $J_{\alpha,\beta}$ 2.6 Hz, $J_{\alpha,\beta'}$ 3.5 Hz, H_α Ser), 4.32 (dd, 1 H, $J_{\alpha,\beta}$ 2.6 Hz, $J_{\beta,\beta'}$ -10.1 Hz, H_β Ser), 4.06 (m, 2 H, 2 x H-3 allyl), 3.96 (dd, 1 H, $J_{4,5}$ 4.5 Hz, $J_{5e,5a}$ -12.0 Hz, H-5e Xyl), 3.82 (dd, 1 H, $J_{\alpha,\beta'}$ 3.5 Hz, $J_{\beta,\beta'}$ -10.1 Hz, $H_{\beta'}$ Ser), 3.64 (ddd, 1 H, $J_{3,4}$ 7.7 Hz, $J_{4,5e}$ 4.5 Hz, $J_{4,5a}$ 8.0 Hz, H-4 Xyl), and 3.37 (dd, 1 H, $J_{4,5a}$ 8.0 Hz, $J_{5e,5a}$ -12.0 Hz, H-5a Xyl).

^{13}C-NMR spectroscopy (100 MHz, $CDCl_3$ + 10% CD_3OD) of 11 showed inter alia: δ 100.34 (C-1 Xyl), 73.65 (C-4 Xyl), 71.72 (C-3 allyl), 71.37 (C-3 Xyl), 70.61 (C-2 Xyl), 68.66 (C_β Ser), 67.01 and 66.67 (2 x benzyl C), 62.15 (C-5 Xyl), and 53.90 (C_α Ser).

3-O-(2,3-di-O-benzoyl-β-D-xylopyranosyl)-N-carbobenzoxy-L-serine Benzyl Ester (12). A solution of 11 (4.00 g, 5.63 mmol) in a mixture of ethanol (100 mL), benzene (50 mL), and water (12.5 mL) was refluxed with tris[triphenylphosphine]rhodium(I) chloride (0.30 g) and 1,4-diazabicyclo[2.2.2]octane (70 mg) overnight under a nitrogen atmosphere. Examination by TLC (toluene-EtOAc 4:1) showed complete conversion of the starting material (R_F 0.47) into a faster moving product (R_F 0.62), presumed to be the propen-1-yl ether. After cooling, the reaction mixture was reduced to 50 mL in a rotary evaporator, diluted with EtOAc (300 mL), washed with water (3 x 150 mL), dried over Na_2SO_4, and concentrated to dryness. The residue was dissolved in 90% aqueous acetone and stirred for 3 h with mercury(II) acetate (1.30 g), at which time a single, slow moving product (R_F 0.11) was observed on TLC (toluene-EtOAc 4:1). The reaction mixture was concentrated to approximately 100 mL, diluted with EtOAc (500 mL), washed with water (2 x 300 mL) and 1 M KBr (2 x 150 mL), dried over Na_2SO_4, and concentrated to dryness. Chromatography on a silica gel column (toluene-EtOAc 2:1) afforded 3.01 g (79%) of the chromatographically homogeneous title compound (12). $[\alpha]_D$ +31° (c 0.76, $CHCl_3$). Lit:[15] $[\alpha]_D$ + 32° (c 0.5, $CHCl_3$).

^{1}H-NMR spectroscopy (400 MHz, $CDCl_3$ + 20% CD_3OD) showed: δ 8.05-7.20 (m, 20 H, aromatic H), 5.41 (dd, 1 H, $J_{2,3}$=$J_{3,4}$ 8.6 Hz, H-3 Xyl), 5.22 (dd, 1 H, $J_{1,2}$ 6.9 Hz, $J_{2,3}$ 8.6 Hz, H-2 Xyl), 5.16 and 5.11 (two d, 1 H each, AB-spectrum, $J_{H,H}$ -12.3 Hz, 2 x benzyl H), 5.04 and 4.96 (two d, 1 H each, AB-spectrum, $J_{H,H}$ -12.2 Hz, 2 x benzyl H), 4.67 (d, 1 H, $J_{1,2}$ 6.9 Hz, H-1 Xyl), 4.50 (dd, 1 H, $J_{\alpha,\beta}$ 4.3 Hz, $J_{\alpha,\beta'}$ 3.6 Hz, H_α Ser), 4.31 (dd, 1 H, $J_{\alpha,\beta}$ 4.3 Hz, $J_{\beta,\beta'}$ -10.2 Hz, H_β Ser), 4.00 (dd, 1 H, $J_{4,5e}$ 5.0 Hz, $J_{5e,5a}$ -11.6 Hz, H-5e Xyl), 3.92 (ddd, 1 H, $J_{3,4}$ 8.6 Hz, $J_{4,5e}$ 5.0 Hz, $J_{4,5a}$ 9.0 Hz, H-4 Xyl), 3.85 (dd, 1 H, $J_{\alpha,\beta'}$ 3.6 Hz, $J_{\beta,\beta'}$ -10.2 Hz, $H_{\beta'}$ Ser), and 3.41 (dd, 1 H, $J_{4,5a}$ 9.0 Hz, $J_{5e,5a}$ -11.6 Hz, H-5a Xyl).

In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$ + 20% CD_3OD) 12 showed *inter alia*: δ 100.90 (C-1 Xyl), 74.63 (C-3 Xyl), 71.09 (C-2 Xyl), 68.78 (C_β Ser), 67.53 (C-4 Xyl), 67.15 and 66.77 (2 x benzyl C), 64.75 (C-5 Xyl), and 53.99 (C_α Ser).

(O-(2,3,4,6-Tetra-O-benzoyl-β-D-galactopyranosyl)-(1→3)-O-(2,4,6-tri-O-benzoyl-β-D-galactopyranosyl)-(1→4)-O-(2,3-di-O-benzoyl-β-D-xylopyranosyl)-(1→3)-N-carbobenzoxy-L-serine Benzyl Ester (13). Disaccharide bromide 8 (4.99 g, from 4.82 g (4.33 mmol) of β-1-O-acetate 6) and alcohol 12 (2.61 g, 3.90 mmol) were dissolved in anhydrous 1:1 toluene-nitromethane (20 mL) and cooled to -20 °C to -30 °C. A solution of silver triflate (1.44 g, 5.60 mmol) and sym-collidine (0.44 g, 3.32 mmol) in anhydrous 1:1 toluene-nitromethane (10 mL) was added dropwise with stirring over a 5 min period. After stirring for an additional 1 h at -25 °C to -30 °C, examination by TLC showed only traces of remaining nucleophile and a major product at R_F 0.36; slightly ahead, a minor product at R_F 0.46 was also observed, along with some by-products, both faster and slower moving. The reaction mixture was processed exactly as described in the synthesis of disaccharide 6, and the reaction product was chromatographed on a silica gel column (toluene-EtOAc 6:1). The α-1→4 (Gal-Xyl) linked trisaccharide 14 (1.04 g, 15%) was eluted first, followed by the title compound (13; 4.85g, 72%). The specific optical rotations were: 13, $[\alpha]_D$ +44° (c 1.68, $CHCl_3$); Lit. value[17] for 13 $[\alpha]_D$ +37° (c 0.5, $CHCl_3$); 14, $[\alpha]_D$ +90 (c 1.36, $CHCl_3$); lit. value[15] for 14 $[\alpha]_D$ +82° (c 0.5, $CHCl_3$).

^{1}H-NMR spectroscopy (400 MHz, $CDCl_3$ + 10% CD_3OD) of 13 showed *inter alia*: δ 5.86 (m, 2 H, H-4 Gal-1 and H-4 Gal-2), 5.44 (dd, 1 H, $J_{3,4}$ 3.3

Hz, $J_{2,3}$ 10.5 Hz, H-3 Gal-2), 4.81 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1 Gal-1), 4.66 (dd, 1 H, $J_{5,6}$ 5.2 Hz, $J_{6,6'}$ -10.1 Hz, H-6 Gal-2), 4.62 (d, 1 H, $J_{1,2}$ 4.3 Hz, H-1 Xyl) 4.50 (dd, 1 H, $J_{\alpha,\beta}$ 2.7 Hz, $J_{\alpha,\beta'}$ 5.5 Hz, H_{α} Ser), 4.18 (dd, 1 H, $J_{3,4}$ 2.7 Hz, $J_{2,3'}$ 10.4 Hz, H-3 Gal-1), 4.09 (dd, 1 H, $J_{5,6}$ 4.3 Hz, $J_{5,6'}$ 7.3 Hz, H-5 Gal-1), 3.47 (dd, 1 H, $J_{\alpha,\beta'}$ 3.2 Hz, $J_{\beta,\beta'}$ -12.4 Hz, $H_{\beta'}$ Ser), and 3.02 (dd, 1 H, $J_{4,5a}$ 5.7 Hz, $J_{5e,5a}$ -12.4 Hz, H-5a Xyl).

In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$ + 10% CD_3OD) 13 showed *inter alia*: δ 101.83 (C-1 Gal-1), 101.33 (C-1 Gal-2), 99.02 (C-1 Xyl), 77.74 (C-3 Gal-1), 74.88 (C-4 Xyl), 71.78 (C-5 Gal-1), 71.30 (C-3 Xyl and C-3 Gal-2), 71.12 (C-5 Gal-2), 70.82 (C-2 Gal-1), 70.15 (C-2 Xyl), 69.29 and 69.06 (C-4 Gal-1 and C-2 Gal-2), 68.32 (C_{β} Ser), 67.48 (C-4, Gal-2), 66.93 and 66.81 (2 x benzyl C), 62.68 (C-5 Xyl), 61.51 (C-6 Gal-1), 60.22 (C-6 Gal-2), and 53.78 C_{α} Ser).

In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$ + 10% CD_3OD) 14 showed *inter alia*: δ 6.03 (d, 1 H, $J_{3,4}$ 3.1 Hz, H-4 Gal-2), 5.92 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-4 Gal-1), 4.94 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1 Xyl), 4.82 (dd, 1 H, $J_{5,6}$ 4.8 Hz, $J_{6,6'}$ -9.6 Hz, H-6 Gal-2), 4.56 (m, 2 H, 2 x H-6 Gal-1), 3.65 (dd, 1 H, $J_{\alpha,\beta'}$ 3.3 Hz, $J_{\beta,\beta'}$ -9.9 Hz, $H_{\beta'}$, Ser), and 3.43 (dd, 1 H, $J_{4,5a}$ 8.5 Hz, $J_{5e,5a}$ -11.8 Hz, H-5a Xyl).

^{13}C-NMR spectroscopy (100 MHz, $CDCl_3$ + 10% CD_3OD) of 14 showed *inter alia*: δ 101.03 (C-1 Gal-2), 100.18 (C-1 Xyl), 97.81 (C-1 Gal-1), 74.70 (C-4 Xyl), 73.05 (C-3 Gal-1), 71.83 (C-5 Gal-2), 71.36 (C-4 Gal-1), 71.12 (C-3 Xyl and C-3 Gal-2), 70.87 (C-2 Xyl), 69.60 (C-2 Gal-2), 69.30 (C-2 Gal-1), 68.32 (C_{β} Ser), 68.02 (C-4 Gal-2), 67.58 (C-5 Gal-1), 67.11 and 66.75 (2 x benzyl C), 63.53 (C-5 Xyl), 62.70 (C-6 Gal-1), 61.59 (C-6 Gal), and 53.89 (C_{α} Ser).

O-β-D-Galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→3)-L-serine (16). Compound 13 (1.00 g, 0.58 mmol) was dissolved in a mixture of ethyl acetate (50 mL), methanol (50 mL), and water (20 mL) containing ammonium formate (1.00 g). To this solution, palladium black (freshly prepared from 0.5 g of $PdCl_2$) was added and the mixture was stirred gently for 1.5 h at room temperature. Examination by TLC ($CHCl_3$-MeOH-H_2O 90:15:1.5) showed a major, ninhydrin-positive product with R_F 0.41 and no remaining starting material. The catalyst was removed by decantation, and the supernatant was concentrated to a residue, which was redissolved in ethyl acetate and washed

with water (3 x 150 mL) to remove ammonium salts. The organic layer was concentrated, and the crude product was chromatographed on a silica gel column ($CHCl_3$-MeOH-H_2O 90:15:1.5) to give the amino acid deblocked product 15, 0.79 g (90%). Compound 15 was used directly in the next step (debenzoylation).

Compound 15 (0.78 g) was suspended in methanol (50 mL) and cooled to 0 °C in an ice-water bath. Cold (-15 °C) methanolic ammonia (50 mL, sat. at 0 °C) was added, and the stirred mixture was allowed to warm slowly to room temperature. After 5 days, examination by TLC ($CHCl_3$-MeOH-H_2O 90:15:1.5) indicated nearly complete debenzoylation. The reaction mixture was concentrated, and the residue was dissolved in water (150 mL) and washed with ethyl acetate (4 x 50 mL) to remove methyl benzoate and benzamide. The aqueous layer was concentrated, and the residue was examined by TLC, which indicated the presence of residual benzoyl groups. The treatment with methanolic ammonia was therefore repeated, with a reaction time of 4 days, and debenzoylation was now complete. The yield of the title compound (16) was 0.30 g (quant.). High-voltage electrophoresis showed a single, ninhydrin-positive spot at $R_{xylosylserine}$ 0.69. $[\alpha]_D$ -3.5° (c 1.15, H_2O). Lit.:[15] $[\alpha]_D$ -12° (c 0.3, H_2O).

Examination of 16 by ^{1}H-NMR spectroscopy (600 MHz, D_2O at 25 °C, HDO at 4.8 ppm as reference) showed inter alia: δ 4.60 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1 Gal-2), 4.52 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1 Gal-1), 4.46 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1 Xyl), 4.25 (dd, 1 H, $J_{\alpha,\beta}$ 5.6 Hz, $J_{\beta,\beta'}$ -11.1 Hz, H_β Ser), 4.18 (d, 1 H, $J_{3,4}$ 3.4 Hz, $J_{4,5}$ <1 Hz, H-4 Gal-1), 4.11 (dd, 1 H, $J_{4,5e}$ 5.3 Hz, $J_{5e,5a}$ -11.8 Hz, H-5e Xyl), 4.01 (dd, 1 H, $J_{\alpha,\beta'}$ 3.1 Hz, $J_{\beta,\beta'}$ -11.1 Hz, $H_{\beta'}$ Ser), 3,92 (dd, 1 H, $J_{\alpha,\beta'}$ 3.1 Hz, $J_{\alpha,\beta}$ 5.6 Hz), 3.91 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4.5}$ 1.2 Hz, H-4 Gal-2), 3.86 (ddd, 1 H, $J_{3,4}$ 9.2 Hz,, $J_{3,4}$ 5.3 Hz, $J_{4,5a}$ 10.2 Hz, H-4 Xyl), 3.81 (dd, 1 H, $J_{3,4}$ 3.4 Hz, $J_{2,3}$ 10.1 Hz, H-3 Gal-1), 3.79 (dd, 1 H, $J_{6,6'}$ -12.0 Hz, $J_{5,6}$ 7.4 Hz, H-6 Gal-2), 3.76 (dd, 1 H, $J_{6,6'}$ -12.0 Hz, $J_{5,6'}$ 4.7 Hz, H-6' Gal-2), 3.74 (dd, 1 H, $J_{6,6'}$ -11.4 H, $J_{5,6}$ 8.7 Hz, H-6 Gal-1), 3.73 (dd, 1 H, $J_{5,6}$ 8.7 Hz, $J_{5,6'}$ 5.1 Hz, $J_{4,5}$ <1 Hz, H-5 Gal-1), 3.71 (dd, 1 H, $J_{6,6'}$ -11.4 Hz, $J_{5,6'}$ 5.1 Hz, H-6' Gal-1), 3.68 (ddd, 1 H, $J_{5,6}$ 7.4 Hz, $J_{5,6'}$ 4.7 Hz, $J_{4,5}$ 1.2 Hz, H-5 Gal-2), 3.67 (dd, 1 H, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 10.1 Hz, H-2 Gal-1), 3.65 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{3,4}$ 3.5 Hz, H-3 Gal-2), 3.60 (dd, 1 H, $J_{3,4}$ 9.2 Hz, $J_{2,3}$ 9.3 Hz, H-3 Xyl), 3.59 (dd, 1

H, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 9.8 Hz, H-2 Gal-2), 3.39 (dd, 1 H, $J_{4,5a}$ 10.2 Hz, $J_{5e,5a}$ -11.8 Hz, H-5a Xyl), and 3.36 (dd, 1 H, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 9.3 Hz, H-2 Xyl).

In the ^{13}C-NMR spectrum (150 MHz, D_2O at 35 °C, dioxane at 67.4 ppm as reference) 16 showed: δ 104.21 (C-1 Gal-2), 102.48 (C-1 Xyl), 101.26 (C-1 Gal-1), 81.90 (C-3 Gal-1), 76.29 (C-4 Xyl), 74.98 (C-5 Gal-2), 74.83 (C-5 Gal-1), 73.61 (C-3 Xyl), 72.55 (C-2 Xyl), 72.47 (C-3 Gal-2), 70.95 (C-2 Gal-2), 69.73 (C-2 Gal-2), 67.89 (C_β Ser), 62.92 (C-5 Xyl), 61.05 (C-6 Gal-2), 60.91 (C-6 Gal-1), and 54.54 (C_α Ser).

Synthetic Gal-Gal-Xyl-Ser as Substrate for Glucuronosyltransferase I. A crude, soluble glucuronosyltransferase preparation was obtained from epiphyseal cartilage of 13-day-old chick embryos as described by Schwartz and Rodén,[36] except that Polydet P-40 was substituted for Nonidet P-40 and 0.5 M KCl included in the buffer rather than being added as a solid.

Glucuronosyltransferase I activity was assayed by a modification of the method of Helting and Rodén.[35] Reaction mixtures in 1.5-mL Eppendorf tubes contained 0.1 μCi UDP-D-[^{14}C]glucuronic acid (sp. act. 304.3 mCi/mmol), 5 mM Gal-Gal-Xyl-Ser, 20 mM $MnCl_2$, 50 mM Tris-acetate, pH 5.5, and cartilage enzyme (420 μg of protein) in a total volume of 275 μL. After incubation for 1 h at 37 °C, the reaction was stopped by addition of 1 mL of ethanol, the mixture was centrifuged for 10 min, and 1.1 mL of the supernatant was withdrawn and concentrated to dryness. The residue was dissolved in 3 mL of 0.01 M HCl and applied to a column (3-mL bed volume) of Dowex 50W-X4 (H^+; 200-400 mesh), which was rinsed with 5 mL of 0.01 M HCl and eluted with 4 mL of 2 M NH_4OH. Portions of the eluates were acidified with acetic acid and diluted to 0.5 mL with water. After addition of 4.5 mL of Scintiverse E, the radioactivity was measured in a Packard Model 2450 liquid scintillation spectrometer.

For characterization of the reaction products, the bulk of each ammonia eluate was concentrated to dryness and dissolved in 100 μL of water. A 25-μL sample was applied to Whatman No. 3 MM paper and subjected to high voltage electrophoresis at pH 5.3 or pH 2. The dried paper was cut into 1-cm strips, which were soaked in 0.5 mL of water for 3 h, and radioactivity was measured after addition of 4.5 mL of Scintiverse E. Reference compounds in the electrophoresis experiments were D-glucuronic acid, L-serine, UDP-D-glucuronic acid, and UDP-D-[^{14}C]gluc-

uronic acid. These were located on the paper by staining with silver (glucuronic acid) or ninhydrin (L-serine and Gal-Gal-Xyl-Ser), by measurement of radioactivity (UDP-D-[^{14}C]glucuronic acid), or by viewing under short-wave ultraviolet light (UDP-D-glucuronic acid).

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