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Synthesis of 3-*O*-β-D-Xylopyranosyl-L-Serine (Xylosylserine) and *O*-β-D-Galactopyranosyl-(1-4)-*O*-β-D-Xylopyranosyl-L-Serine (Galactosylxylosylserine) and Use of the Synthetic Products for Detection of Galactosyltransferase I Activity in Rat Liver

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3-*O*-β-D-Xylopyranosyl-L-serine (xylosylserine) was synthesized by the following three-step procedure: 1) 2,3,4-tri-*O*-benzoyl-α-D-xylopyranosyl bromide (benzobromoxylose) was condensed with *N*-carbobenzoxy-L-serine benzyl ester using the silver triflate-collidine complex as promoter; 2) the *N*-carbobenzoxy and benzyl ester groups in the resultant glycoside were cleaved by transfer hydrogenation with palladium black as catalyst and ammonium formate as hydrogen donor; and 3) the benzoyl groups were removed with methanolic ammonia. Xylosylserine was obtained in an overall yield of 70%.

***O*-β-D-Galactopyranosyl-(1-4)-*O*-β-D-xylopyranosyl-(1-3)-L-serine (galactosylxylosylserine) was also synthesized by this methodology and was characterized by 2-dimensional (2D) NMR spectroscopy techniques. The two serine glycosides (xylosylserine and galactosylxylosylserine) were used in detection and partial purification of galactosyltransferase I (UDP-D-galactose:D-xylose galactosyltransferase) from adult rat liver.**

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In most mammalian proteoglycans, the polysaccharide chains are linked to the core proteins of these substances via an *O*-glycosidic linkage between xylose and the hydroxyl group of a serine residue [1]. Following its isolation from a hydrolysate of heparin [2], the linkage region fragment, xylosylserine (3-*O*-β-D-xylopyranosyl-L-serine), was first synthesized by Lindberg and Silvander [3], and improved methods were subsequently developed by Kum and Roseman [4] and by Brendel and Davidson [5]. Since these preparations were described, substantial progress has been made in the area of glycoside synthesis, and silver triflate-promoted coupling has been particularly valuable in this regard [6, 7]. Also, trifluoromethanesulfonic (triflic) anhydride has been used in the coupling of *O*-benzylated sugars, with only OH-1 free, to serine and threonine derivatives [8]. However, loss of stereochemical control (no participating group in position 2 of the carbohydrate moiety) and the strongly acidic conditions led to mixtures of α- and β-anomers, with the α-anomer predominating in each case. Xylosylserine prepared by this method was obtained in 82% yield, with an α/β ratio of 1.22, and the effective yield of the naturally occurring β-anomer was thus only 37%. In the present study, we have developed a facile three-step procedure for the synthesis of the β-anomer of xylosylserine based on triflate-promoted coupling, and additional simplification has been introduced by using catalytic transfer hydrogenation [9-11]. The latter procedure was also applied in an improved synthesis of *O*-β-D-galactopyranosyl-(1-4)-*O*-β-D-xylopyranosyl-(1-3)-*O*-L-serine (galactosylxylosylserine) along with modifications which eliminated the difficulties encountered by other authors [12, 13] in the de-*O*-acylation with methanolic ammonia.

Xylosylserine has been useful not only as a reference compound in structural studies but also as a substrate for galactosyltransferase I (UDP-D-galactose:D-xylose galactosyltransferase), which catalyze the second glycosyl transfer step in the assembly of the xylose-containing connective tissue polysaccharides [1, 14]. As part of the characterization of the xylosylserine prepared in this study, it was shown that the synthetic compound was indeed a substrate for galactosyltransferase I in an established system, where a microsomal fraction from embryonic chick cartilage was used as the enzyme source. Galactosyltransferase I was also detected in rat liver and was partially purified from this source.

In addition to the characterization of xylosylserine and galactosylxylosylserine by chromatographic, electrophoretic and enzymatic methods, analysis by NMR spectroscopy was also carried out, and some of the results of this study are reported in this communication.

Experimental

Materials

Chemicals were purchased as follows: *N*-carbobenzoxy-L-serine benzyl ester from Bachem Inc. (Torrance, CA, USA), silver trifluoromethanesulfonate (silver triflate) from Aldrich Chemical Co. (Milwaukee, WI, USA), UDP-galactose from Sigma Chemical Co. (St. Louis, MD, USA), UDP-[^{14}C]galactose (337 mCi/mmol), UDP-[1-^{3}H]galactose (11.6 Ci/mmol), and UDP-[4,5-^{3}H]galactose (40.3 Ci/mmol) from New England Nuclear (Boston, MA, USA), Nonidet P-40 (Shell™) from Bethesda Research Laboratories (Gaithersburg, MD, USA), and DEAE-cellulose (DE-52) from Whatman Ltd. Silica Gel 60

(particle size 40-63 μm, 230-400 mesh) was obtained from E. Merck (Darmstadt, W. Germany), as were TLC glass plates precoated with Silica Gel 60 F_{254} (0.25 mm layer). AG 50W-X4 ion-exchange resin was obtained from Bio-Rad Laboratories (Richmond, CA, USA).

General Methods

Optical rotations were measured in a Perkin-Elmer 141 polarimeter. NMR experiments were performed on a Bruker WH-400 Spectrometer equipped with an Aspect-2000 computer. The spectra were obtained at 25°C. Standard 2D NMR pulse sequences were used to obtain 2D-COSY and ^{1}H-^{13}C chemical shift correlated spectra. The $HO^{2}H$ resonance signal was set to 4.8 ppm as the ^{1}H chemical shift reference. Internal dioxane (67.4 ppm) was used as the ^{13}C chemical shift reference. TLC was carried out in toluene/ethyl acetate, 8/1 by vol (solvent A), or in ethyl acetate/propan-2-ol/water, 6/2/1 by vol (solvent B). 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 min at 50 V/cm in 1.6 M acetic acid, which had been adjusted to pH 2 with formic acid. The 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 to 100°C. Radioactivity was measured in a Packard liquid scintillation counter, using ScintiVerse E (Fisher, Fairlawn, NJ, USA) as counting cocktail. Melting points are uncorrected.

Synthesis of Xylosylserine

*3-O(2,3,4-Tri-O-benzoyl-β-D-xylopyranosyl)-N-carbobenzoxy-L-serine benzyl ester (**1**).* 2,3,4-Tri-*O*-benzoyl-α-D-xylopyranosyl bromide (benzobromoxylose) was synthesized as described by Fletcher and Hudson [15] and was isolated from the reaction mixture as described for the corresponding mannose derivative [16]. *N*-Carbobenzoxy-L-serine benzyl ester (9.8 g, 29.8 mmol) and benzobromoxylose (18.0 g, 34.3 mmol) were dissolved in anhydrous toluene/nitromethane, 1/1 by vol (120 ml), and the solution was cooled to -15°C while being kept under nitrogen to ensure anhydrous conditions. A solution of silver triflate (9.9 g, 38.5 mmol) and 2,4,6-collidine (3.5 ml, 26.5 mmol) in anhydrous toluene/nitromethane, 1/1 by vol (100 ml), was added dropwise with stirring over a 5 min period. After stirring for an additional 15 min at -15°C, examination by TLC in solvent A showed one major product, with an R_F value of 0.47, and only small amounts of other products, faster as well as slower moving. The mixture was neutralized by addition of pyridine (1.5 ml), filtered through Celite, and diluted with toluene (500 ml). After washing with water (3 × 300 ml), it was dried with anhydrous sodium sulfate and concentrated to a syrup. Purification in two portions by chromatography on a column (5 × 52 cm) of silica gel, which was eluted with solvent A, yielded 21.7 g (94%) of chromatographically homogeneous title compound. After crystallization of a small sample from methanol, the m.p. was 83-85°C and $[\alpha]_D$ -36.5° (c 2.1, $CHCl_3$). Lit. [7] m.p. 106-108°C and $[\alpha]_D$ -36° ($CHCl_3$).

*3-O-β-D-Xylopyranosyl-L-serine (**3**).* Compound **1** (21.7 g, 28.0 mmol) was dissolved in methanol (1000 ml) containing 4.5% ammonium formate, with warming to 35°C. Freshly prepared palladium black from 3 g of $PdCl_2$ was added, and the mixture was stirred gently (magnetic spin-bar) for 2 h at 35°C. At this time, TLC in solvent B showed only one

product, with an R_F value of 0.35, and no remaining starting material could be detected. The catalyst was removed by decantation, and the supernatant was concentrated to a solid. Salts were removed by trituration with several washes of water to give 3-*O*-(2,3,4-tri-*O*-benzoyl-β-D-xylopyranosyl)-L-serine (**2**) as a crystalline solid (14.5 g, 97%), pure enough to be used directly in the next step. Compound **2** (14.4 g, 26.9 mmol) was suspended in methanol (250 ml), the mixture was cooled at 0°C in an ice-water bath, and methanolic ammonia (250 ml, saturated at 0°C, kept at -15°C) was added. The flask was tightly stoppered, and the mixture was stirred at room temperature overnight. TLC in solvent B showed complete disappearance of the starting material and formation of product(s) which remained at the origin. The clear solution was concentrated to a residue, which was partitioned between water (400 ml) and ethyl acetate/light petroleum, 3/1 by vol (2 × 200 ml). The aqueous layer was concentrated to a crystalline residue (6.35 g), which yielded pure xylosylserine (**3**, 4.90 g, 77%) on recrystallization from ethanol-water. The pure material had m.p. 245-250°C (dec.) and $[\alpha]_D$ -54° (c 1.38, H_2O). Lit. [4] m.p. 224-227°C (dec.) and $[\alpha]_D$ -47.4° (c 2.2, H_2O). High voltage electrophoresis at pH 2 showed this product to be indistinguishable from an authentic sample, which had an R_{serine} value of 0.6. ^{13}C-NMR spectroscopy (100 MHz, ^2H_2O with internal dioxane at 67.4 ppm as reference) gave the following chemical shift data: δ 172.45 (**C**=O serine), 103.58 (C-1), 76.28 (C-3), 73.60 (C-2), 69.97 (C-4), 68.66 (C-β serine), 65.99 (C-6), and 55.36 (C-α serine).

*Synthesis of O-β-D-Galactopyranosyl-(1-4)-O-β-D-xylopyranosyl-(1-3)-L-serine (**6**)*

O-(2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-*O*-(2,3-di-*O*-benzoyl-β-D-xylopyranosyl)-(1-3)-*N*-carbobenzoxy-L-serine benzyl ester (**4**, prepared as described in [12]; 2.58 g, 2.07 mmol) was dissolved in a mixture of methanol (200 ml), ethyl acetate (50 ml), and water (10 ml), which also contained ammonium formate (2.50 g). Freshly prepared palladium black from 2.5 g of $PdCl_2$ was added as a slurry in methanol (50 ml), and the mixture was stirred gently at room temperature for 1.5 h, when examination by TLC in solvent B showed a major product with R_F 0.45 and only minor amounts of faster and slower moving impurities. The catalyst was removed by decantation, and the supernatant was concentrated to dryness. The residue was dissolved in methanol (100ml and diluted with water (900 ml). The resulting suspension was extracted with ethyl acetate (2 × 500 ml), the combined extracts were washed with water (1000 ml), and the aqueous phase was then back-extracted with ethyl acetate (500 ml). The combined ethyl acetate extracts were concentrated to give crude *O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-*O*-(2,3-di-*O*-benzoyl-β-D-xylopyranosyl)-(1-3)-L-serine (**5**, 2.08 g). After purification on a silica gel column (solvent B), 1.79 g (86%) of chromatographically homogeneous **5** was obtained as an amorphous solid. Compound **5** was not further characterized but was used directly in the next step (debenzoylation).

A solution of **5** (1.66 g, 1.64 mmol) in methanol (100 ml) was cooled to 0°C in an ice-water bath, and an equal volume of cold (-15°C) methanolic ammonia (saturated at 0°C) was added. The flask was tightly stoppered and allowed to warm to room temperature overnight. After four days at room temperature, the reaction was complete, as indicated by TLC in solvent B, which showed only material at the origin; no free galactosylxylose could be detected. The reaction mixture was concentrated to a residue, which was dissolved in water (100 ml) and washed with ethyl acetate (3 × 50 ml) to remove methyl benzoate and benzamide. The aqueous layer was concentrated to give 0.70 g (quan-

titative yield) of the title compound (**6**). When this product was examined by high voltage electrophoresis and NMR spectroscopy (^{1}H and ^{13}C), no racemization or β-elimination products could be detected. Compound **6** had $[\alpha]_D$ -21° (c 0.65, H_2O). Lit. [12] $[\alpha]_D$ -28° (c 0.5, H_2O). High voltage electrophoresis at pH 2 showed this product to be homogeneous, with an $R_{xylosylserine}$ of 0.84. Lit. [17] 0.84. ^{1}H-NMR spectroscopy (400 MHz, ^2H_2O at 25°C, HO^2H at 4.8 ppm as internal standard) showed *inter alia*: δ 4.43 and 4.42 (two d partially overlapping, 1 H each, $J_{1,2}$ 7.9 Hz and $J_{1,2}$ 7.7 Hz, H-1 Gal and H-1 Xyl), 4.22 (dd, 1 H, $J\alpha,\beta$ 5.4 Hz, $J\beta,\beta'$ -11 Hz, H-β serine), 4.08 (dd, 1 H, $J_{4,5e}$ 5.3 Hz, $J_{5e,5a}$ 11.8 Hz, H-5e Xyl), 3.97 (dd, $J\alpha,\beta'$ 3.4 Hz, $J\beta,\beta'$ -11 Hz, H-β' serine), 3.92((dd, 1 H, $J\alpha,\beta'$ 3.4 Hz, $J\alpha,\beta$ 5.4 Hz, H-α serine), 3.88 (d, 1 H, $J_{3,4}$ 3.3 Hz, H-4 Gal), 3.61 (dd, 1 H, $J_{3,5}$ 3.3 Hz, $J_{2,3}$ 9.9 Hz, H-3 Gal), 3.57 (dd, 1 H, $J_{2,3}$ and $J_{3,4}$ 9.2 Hz, H-3 Xyl), 3.47 (dd, 1 H, $J_{1,2}$ 7.9 Hz, $J_{2,3}$ 9.9 Hz, H-2 Gal).

^{13}C-NMR spectroscopy (100 MHz, ^2H_2O at 25°C, internal dioxane at 67.40 ppm as reference) showed: δ 103.40 (C-1 Xyl), 102.58 (C-1 Gal), 77.28 (C-4 Xyl), 76.13 (C-5 Gal), 74.52 C-3 Xyl), 73.40 (2C, C-3 Gal and C-2 Xyl), 71.44 (C-2 Gal), 69.42 (C-4 Gal), 68.67 (C-β Ser), 63.78 (C-5 Xyl), 61.94 (C-6 Gal), and 55.35 (C-α Ser).

Galactosyl Transfer to Xylosylserine

Enzyme Preparation. Livers from adult Sprague-Dawley rats were excised and stored frozen until used. The thawed tissue (13 g) was homogenized in three volumes of 50 mM MES, pH 6.5, containing 50 mM KCl, 12 mM $MgCl_2$, and 6 mM $MnCl_2$. The homogenate was centrifuged at 1000 × *g* for 15 min at 4°C, and the supernatant was then centrifuged at 10 000 × *g* for 20 min. After centrifugation of the supernatant fluid at 100 000 × *g* for 60 min, the resulting pellet was resuspended in 50 mM MES, pH 5.5 (27.5 ml), containing 0.2 M KCl, and Nonidet P-40% and solid KCl were added to final concentrations of 0.5% and 0.5 M, respectively [14, 18]. The mixture was incubated at 4°C for 30 min and then centrifuged at 100 000 × *g* for 60 min. A 100 000 × *g* pellet from embryonic chick cartilage was solubilized by the same procedure, essentially as described previously [14, 18]. The resulting supernatants were dialyzed overnight against 50 mM MES/0.2 M KCl, pH 5.5

Enzyme Assay. Galactosyltransferase I activity was assayed essentially as described previously [14, 18]. Reaction mixtures contained, in a total volume of 75μl: 1% xylosylserine, 10 μl; 0.2 M $MnCl_2$, 5 μl; either UDP-[1-^{3}H]galactose or UDP-[4,5-^{3}H]galactose (both adjusted to 110 μCi/μmol by addition of unlabeled UDP-D-galactose; 30 μCi/ml) in 50 mM Tris-acetate, 50 mM KCl, pH 7.5, containing 37.5 mM 2,3-dimercaptopropanol and 0.75 mM ATP, 10 μl; and enzyme (50-100 μg of protein) in 50 mM MES/0.2 M KCl, pH 5.5, 50 μl. After incubation at 37°C for 1 h, 50 μl of 1% bovine serum albumin and 375 μl of absolute ethanol were added, and the mixtures were cooled to -20°C and centrifuged. The resulting supernatants were concentrated to dryness under a stream of air and were then dissolved in 0.5 ml of 0.01 M HCl and applied to columns (0.7 ml bed volume) of AG 50W-X4 (H^+, 200-400 mesh). The columns were washed with 6 ml of 0.01 M HCl, and the products were eluted with 2 ml of 2 M ammonium hydroxide. Portions (0.25 ml) of the eluates were neutralized with 11 μl of glacial acetic acid, mixed with 0.25 ml of water, and radioactivity was measured by liquid scintillation counting after addition of 4.5 ml of ScintiVerse E.

Characterization of Reaction Products. The ammonia eluates (see above) were concentrated to dryness, resuspended in a small volume of 50% ethanol, and subjected to high

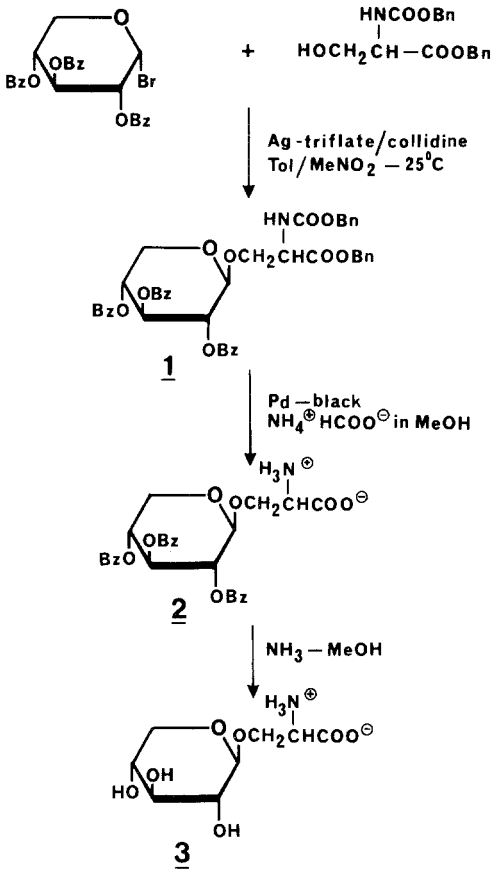


Figure 1. Synthesis of xylosylserine.

voltage electrophoresis at pH 2. Xylosylserine and galactosylxylosylserine (10-20 μg) were electrophoresed in parallel and then detected by staining with ninhydrin. The lanes with radioactive samples were cut into 1 cm strips, which were placed in scintillation vials and eluted with 0.5 ml H_2O, and the radioactivity was measured by liquid scintillation counting. In some experiments, the ammonia eluates were neutralized with acetic acid, and the ammonium acetate was removed by repeated lyophilizations.

Digestion with β-galactosidase was carried out as follows. Putative [^{3}H]-galactosylxylosylserine (1500 cpm), synthesized by the rat liver enzyme, was eluted from electrophoresis strips, concentrated to dryness, and the residue was suspended in 0.1 ml of 50 mM Tris-HCl, pH 7.4, containing 50 units of β-galactosidase from *E. coli* (Sigma no. G-5635, Grade VIII). After 20 h at room temperature, the reaction mixture was boiled for 2 min, centrifuged, and the supernatant was examined by descending paper chromatography on Whatman No. 3MM paper in ethyl acetate/pyridine/H_2O, 8/2/1 by vol. The radioactivity was located by counting 1 cm wide strips of the lane containing radioactive material, as described for electrophoretograms. Galactose, which was run in parellel, was located with alkaline silver nitrate [19].

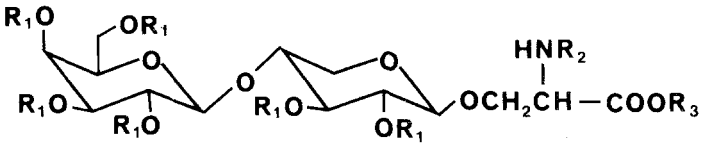
DEAE-Cellulose Chromatography of Rat Liver Galactosyltransferase I. A rat liver microsome preparation containing 40 mg protein/ml was obtained as previously described [20]. The transferase was solubilized by adding 2.5 ml of 2.5% (v/v) Nonidet P-40 to 4 ml of the microsomal suspension and gently mixing for 1 min on a vortex mixer. After standing for 10 min, the mixture was diluted to 25.5 ml by the addition of a solution containing water (14 ml), glycerol (5 ml), and mercaptoethanol (0.05 ml). The mixture was vortexed for 0.5 min and centrifuged at 100 000 × *g* for 1 h. The resulting supernatant was applied to a column (2.5 × 13.5 cm) of DEAE-cellulose, equilibrated in 0.01 M Tris-acetate, pH 7.0, containing 10% glycerol, 0.1% Nonidet P-40, and 0.5 mM dithiothreitol. The column was washed with the equilibration buffer (0.50 ml) and then eluted with a linear gradient of 0-0.5 M NaCl (250 ml) in 0.05 M Tris-acetate, pH 7.0, containing 10% glycerol, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 1 mM EDTA. Fractions (4 ml) were collected from the beginning of the gradient, and aliquots (0.05 ml) of selected fractions were assayed for galactosyltransferase activity (see legend to Fig. 4 for details). Fractions 22-33 were pooled (48 ml, 58 mg protein) and dialyzed overnight against 0.05 M MES/KOH, pH 6.0, containing 10% glycerol, 0.1% Nonidet P-40 and 1 mM EDTA. The dialyzed enzyme was clarified by centrifugation for 5 min at 10 000 rpm. This preparation contained 0.6 mg protein/ml and was stable for at least two weeks when stored frozen at -20°C.

Results and Discussion

Synthesis of Xylosylserine

Xylosylserine was synthesized by a three-step procedure which is illustrated in Fig. 1. The first step, condensation of 2,3,4-tri-*O*-benzoyl-α-D-xylopyranosyl bromide (benzobromoxylose) with *N*-carbobenzoxy-L-serine benzyl ester, using the silver triflate-collidine complex as promoter [7], afforded the glycoside **1** in yields which ranged between 93 and 97% for several preparations. Apart from the higher yields, the triflate procedure also had the advantage over the classical Koenigs-Knorr method used in previous syntheses [3-5] that the reaction was completed in much shorter time (less than 15 min). In the second step, the *N*-carbobenzoxy and benzyl groups were removed by catalytic transfer hydrogenation [9-11]. This procedure is faster, safer, and simpler than the more common hydrogenation methods in that handling of hydrogen gas and the need for special equipment have been eliminated. In the present synthesis, the hydrogenation was completed in less than 2 h and yielded a product (**2**), which could be used directly without further purification. In the last step, the benzoyl groups were removed by treatment with methanolic ammonia, to give xylosylserine (**3**) in 70% overall yield, after recrystallization. On examination of the crude **3** by ^{13}C-NMR spectroscopy before recrystallization, no racemization or β-elimination products could be detected. The observed spectrum was in good agreement with previously published data for xylosylserine [12, 21].

The identity of the synthetic product was further established by the following findings: (a) on high-voltage electrophoresis at pH 2, the synthetic material had the same mobility as an authentic sample of xylosylserine which had been prepared as described previously [22]; and (b) the synthetic product was an acceptor for galactosyl transfer by galactosyltransferase I from cartilage, as described below.



4 R_1 = Bz, R_2 = COOBn, R_3 = Bn

5 R_1 = Bz, R_2 = R_3 = H

6 R_1 = R_2 = R_3 = H

Figure 2. Intermediates in the preparation of galactosylxylosylserine.

Preparation of Galactosylxylosylserine

O-(2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-*O*-(2,3-di-*O*-benzoyl-β-D-xylopyranosyl)-(1-3)-*N*-carbobenzoxy-L-serine benzyl ester (**4**) (Fig. 2) was prepared as described previously [12]. In the deblocking procedure, benzyl and carbobenzoxy groups were removed by catalytic transfer hydrogenation [9-11] to give compound **5** in 86% yield after purification on a silica gel column. Debenzoylation of **5** by treatment with methanolic ammonia gave galactosylxylosylserine (**6**) in quantitative yield. On examination of this product by ^{1}H and ^{13}C NMR spectroscopy, no racemization or β-elimination products could be detected. The ^{13}C NMR spectrum was in excellent agreement with the published spectrum [12]; no signal splitting could be observed. The identity of **6** was further established as follows: (a) on high-voltage electrophoresis at pH 2, **6** had the same $R_{xylosylserine}$ as that previously published for galactosylxylosylserine [17]; and (b) the assigned structure of **6** was corroborated by its ^{1}H-NMR spectrum.

The ^{1}H and ^{13}C NMR spectra were analyzed on the basis of chemical shifts of reference compounds, and by 2D-NMR experiments. The chemical shifts for the ^{1}H and ^{13}C nuclei from the two sugars and the serine residue are given in the experimental section. The ^{1}H resonance signals were assigned by sequential connection through the COSY cross peaks. The resonance signals at 4.22, 3.97 and 3.92 ppm were assigned to the β, β′, and α protons of the serine residue, respectively. These assignments, which were different from those previously reported by van Halbeek *et al.* [23], were further supported by the ^{1}H-^{13}C chemical shift correlated data. The ^{13}C resonances of galactosylxylosylserine were assigned from the ^{1}H-^{13}C chemical shift correlated spectrum. The ^{13}C resonance signal at 68.7 ppm showed two cross peaks at 4.22 and 3.97 ppm of proton chemical shift, and the ^{13}C signal at 55.9 ppm had a cross peak at 3.92 ppm. Thus the resonance at 68.7 ppm must be from a methylene carbon and was assigned to the Cβ of the serine residue, while the resonance signal at 55.4 ppm was assigned to the Cα of serine. These results confirmed our proton resonance assignments for the serine.

Enzymatic Transfer of Galactose to Xylosylserine

Incubation of a crude preparation of galactosyltransferase I from embryonic chick cartilage with the synthetic xylosylserine and either UDP-[1-^{3}H]galactose or UDP-[4,5-^{3}H]-galactose resulted in the incorporation of label into a product which was soluble in

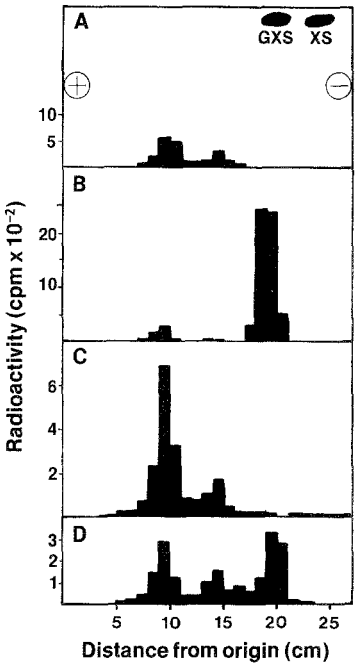


Figure 3. Paper electrophoresis of ammonia eluates from galactosyltransferase reaction mixtures. Electrophoresis was performed at pH 2.0 on 70 cm strips of Whatman No. 3MM paper at 50 V/cm for 105 min. Incubation mixtures contained: (A) chick cartilage enzyme without added xylosylserine, (B) chick cartilage enzyme with 2.0 μmol of xylosylserine; (C) rat liver enzyme without added xylosylserine; (D) rat liver enzyme with 2.0 μmol of xylosylserine. Unlabeled xylosylserine (XS) and galactosylxylosylserine (GXS) were electrophoresed in parallel and detected with ninhydrin (see panel A).

ethanol and was adsorbed to AG 50W under acidic conditions. In one such experiment (using 62 μg of enzyme protein), the material eluted from the AG 50W column with 2 M ammonium hydroxide contained 16 540 cpm, while a control without xylosylserine contained 1840 cpm. The electrophoretic mobility of the major reaction product was the same as that of galactosylxylosylserine (Fig. 3B). This product was not formed, when xylosylserine was omitted from the reaction mixtures (Fig. 3A), and it was therefore concluded that the synthetic xylosylserine was indeed a substrate for chick cartilage galactosyltransferase I.

The synthetic xylosylserine was subsequently used to detect galactosyltransferase I activity in a crude extract of adult rat liver. In the initial experiments, product formation was measured by the AG 50W assay and was approximately the same whether or not xylosylserine was included in the reaction mixtures. Thus, reaction mixtures (100 μg of enzyme protein) with 0.4 and 2.0 μmol of xylosylserine yielded 4384 and 3608 cpm, respectively, while a control without xylosylserine incorporated 3656 cpm. On electrophoresis of the ammonia eluates from reaction mixtures with added xylosylserine, a major reaction product with the same mobility as galactosylxylosylserine was observed (Fig. 3D). This product was not formed when xylosylserine was omitted from the

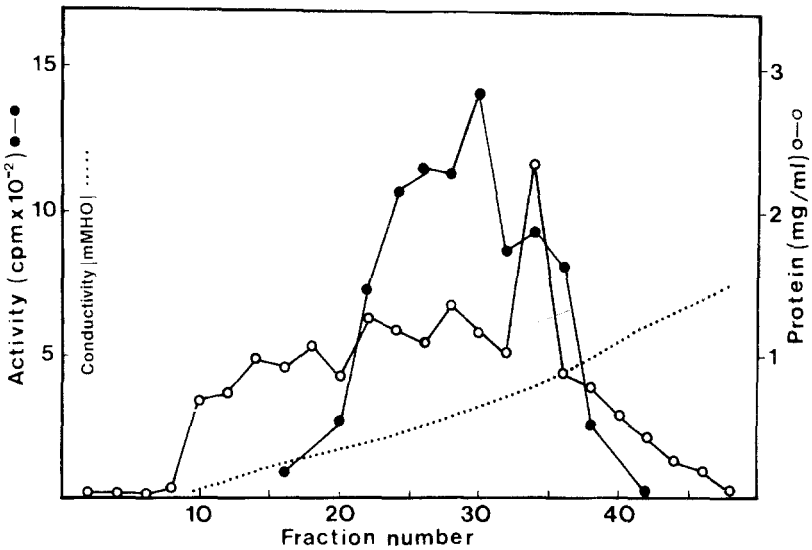


Figure 4. DEAE-Cellulose chromatography of solubilized rat liver galactosyltransferase I. For experimental details, see text. Selected fractions were assayed for galactosyltransferase I activity. Reaction mixtures had a total volume of 75 μl and contained enzyme (50 μl), 1% xylosylserine (10 μl), 0.1 M $MnCl_2$ (10 μl), and UDP-[^{14}C]galactose (337 μCi/μmol; 10 μCi/ml; 5 μl). After incubation for 1 h at 37°C, reaction products were isolated by the AG 50W procedure [18].

reaction mixtures (Fig. 3C). A second major product was also seen, which was present in all reaction mixtures and was located closer to the origin (Fig. 3C,D). When xylosylserine was omitted, there was a substantial increase in the amount of the slower migrating material. This product has not been examined further.

The putative [^{3}H]galactosylxylosylserine formed by the liver enzyme was isolated from a 6-fold scaled up reaction mixture by preparative paper electrophoresis, and samples (2000 cpm) were chromatographed on Whatman No. 3MM paper for 23-28 h in ethyl acetate/acetic acid/H_2O, 2/1/1 by vol, and *n*-butanol/ethanol/H_2O, 10/3/5 by vol. In both cases, all radioactivity migrated to the same position as unlabeled galactosylxylosylserine. After treatment of the putative [^{3}H]galactosylxylosylserine with β-galactosidase, all radioactivity migrated to the same position as galactose on paper chromatography in ethyl acetate/pyridine/H_2O, 8/2/1 by vol, indicating that a β-linked galactose residue was present in the product of transfer to xylosylserine.

Partial Purification of Galactosyltransferase I

Upon DEAE-cellulose chromatography of solubilized rat liver galactosyltransferase I, the enzyme was eluted broadly about midway through a 0-0.5 M NaCl gradient (Fig. 4). Although much of the applied protein was present in the enzyme-containing fractions, complete separation from the endogenous acceptors had occurred, since no product was formed in reaction mixtures without xylosylserine and only a single product was observed in the presence of added substrate, which had the same electrophoretic mobility as galactosylxylosylserine. The specific activity of galactosyltransferase I in the

pooled fractions from DEAE-cellulose chromatography was 27 picomol of galactose transferred per hour per mg of protein, and this value increased to 56 pmol/h/mg after dialysis (1 pmol of galactose corresponds to 666 cpm). The partially purified enzyme was stable on storage at -20°C and should therefore be amenable to further purification.

The experiments reported here indicate that rat liver contains an enzyme which catalyzes transfer of galactose from UDP-galactose to xylosylserine. This enzyme is likely to be galactosyltransferase I, which is involved in the formation of the carbohydrate-protein linkage regions of the xylose-linked connective tissue polysaccharides [1]. However, it should be pointed out that galactosyl transfer to xylose may also be effected by lactose synthetase [24] and that the product has been identified as 4-*O*-β-D-galactosyl-D-xylose (R.L. Hill and L. Rodén, unpublished results). Therefore, it remains to be established definitively whether galactosyltransferase I or lactose synthetase is responsible for the reaction reported here. In this regard, it should be noted that the addition of *N*-acetylglucosamine (2 μmol) to the liver enzyme reaction mixture did not inhibit the formation of the putative galactosylxylosylserine (data not shown), suggesting that this reaction is not mediated by lactose synthetase. While this work was in progress, the presence of galactosyltransferase I in subcellular fractions from rat liver was reported by Nuwayhid *et al.* [25], who used 4-methylumbelliferyl-β-D-xyloside as substrate and measured product formation by high performance liquid chromatography. Compared to rough and smooth endoplasmic reticulum, the Golgi fractions had 20- to 100-fold higher specific activity, indicating that the Golgi apparatus is the physiological site of the galactosyltransferase I reaction.

An important finding made in the course of the present study was the observation that the routinely used assay procedure, which involved adsorption of the reaction product to a cation exchange resin and elution with ammonia, lacked specificity when it was applied to crude enzyme preparations from liver. Due to the presence of substantial levels of endogenous acceptors for galactosyltransferase I or other galactosyltransferases, a large proportion of the incorporated radioactivity was located in products other than galactosylxylosylserine. Thus, whereas the results of the AG 50W assay truly reflect transfer of galactose to xylosylserine in extracts of embryonic chick cartilage, it is apparently necessary to validate the assay for each new tissue under investigation and to carry out further fractionation of the reaction products, when needed.

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