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In the biosynthesis of N-glycans in connective tissue of the snail *Lymnaea stagnalis* of incorporation GlcNAc by β2GlcNAc-transferase I is an essential prerequisite for the action of β2GlcNAc-transferase II and β2Xyl-transferase

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Using a series of relevant substrates, connective tissue of the snail *Lymnaea stagnalis* was shown to contain β1-2 xylosyltransferase (β2Xyl-T), β1-2 *N*-acetylglucosaminyltransferase I (β2GlcNAc-T I), and β1-2 *N*-acetylglucosaminyltransferase II (β2GlcNAc-T II) activities. These enzymes are probably involved in the biosynthesis of the N-linked carbohydrate chains, like those present in hemocyanin. The products formed by incubation of GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-R [where R = -4GlcNAcβ1-4GlcNAc or O-$(CH_2)_7CH_3$] with UDP-Xyl and connective tissue microsomes have been purified and characterized by ^{1}H-NMR spectroscopy in conjunction with methylation analysis to be GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-R. Substrate specificity studies focused on connective tissue β2Xyl-T show that the minimal structure requirements are fulfilled in GlcNAcβ1-2Manα1-3Manβ1-O-$(CH_2)_7CH_3$. The enzyme activity can therefore be characterized as UDP-Xyl:GlcNAcβ1-2Manα1-3Manβ-R (Xyl to Manβ) β1-2 xylosyltransferase. In substrate-specificity studies directed to connective tissue β2GlcNAc-T I, it could be demonstrated that the enzyme is active towards acceptors having at the minimum a Manα1-3Manβ-R sequence, and that introduction of a βXyl residue at C2 of βMan totally abolishes the enzyme activity. Xylose-containing oligosaccharides are not acceptors for β2GlcNAc-T I. In combination with the substrate specificity of β2Xyl-T, this shows that in snail connective tissue β2GlcNAc-T I must act before β2Xyl-T. The connective tissue β2GlcNAc-T II activity follows the earlier established biosynthetic routes. Based on the substrate specificities of the various connective tissue glycosyltransferases known so far, and the structures isolated from *L. stagnalis* hemocyanin, a partial biosynthetic scheme for N-glycosylation in snail connective tissue is proposed.

Keywords: β1-2 xylosyltransferase; β1-2 *N*-acetylglucosaminyltransferase I; β1-2 *N*-acetylglucosaminyltransferase II; *Lymnaea stagnalis*.

Glycoproteins containing the Asn-linked Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc oligosaccharide element have been found in plants and in animals [1]. The only two examples of xylose-containing N-glycans in animals are the hemocyanins of the snails *Helix pomatia* [2, 3] and *Lymnaea stagnalis* [4, 5]. In both cases typical extensions at the αMan residues with (MeO-3)Galβ1-3GalNAcβ1-4GlcNAcβ1-2 fragments do occur. In the framework of our interest in the structural analysis, organic synthesis, conformational analysis, and biosynthesis of xylose-containing N-linked carbohydrate chains, reports on novel UDP-Gal:GalNAcβ1-4GlcNAc-R β1-3 galactosyltransferase [6] and UDP-GalNAc:GlcNAcβ-R β1-4 *N*-acetylgalactosaminyltransferase [7] activities in connective tissue of the snail *L. stagnalis* have appeared. Since hemocyanin is biosynthesized in connective tissue pore cells, the choice for connective tissue as glycosyltransferase source is obvious.

The biosynthesis of N-linked oligosaccharides on glycoproteins starts with the synthesis of a dolichol-linked precursor structure $Glc_3Man_9GlcNAc_2$, which is transferred to an Asn residue of the nascent protein, and then trimmed to $Man_5GlcNAc_2Asn$~ by the action of several glycosidases [8]. The conversion of $Man_5GlcNAc_2AsnX$ into *N*-acetyllactosamine and hybrid-type N-glycans is controlled by UDP-GlcNAc:α-3-D-mannoside β1-2 *N*-acetylglucosaminyltransferase I (β2GlcNAc-T I) [9]. This enzyme is specific for the Manα1-3 arm of the $Man_3GlcNAc_2$ core structure, and is essential for the subsequent action of several enzymes including β1-2 *N*-acetylglucosaminyltransferase II (β2GlcNAc-T II) [9] and β1-2 xylosyltransfer-

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Abbreviations. β2GlcNAc-T, β1-2 *N*-acetylglucosaminyltransferase; HOHAHA, homonuclear Hartmann-Hahn; HPAEC, high-pH anion-exchange chromatography; MLEV, M. Levitt; PAD, pulsed amperometric detection; WEFT, water-eliminated Fourier-transform; β2Xyl-T, β1-2 xylosyltransferase; Np-β-D-GlcNAc, *p*-nitrophenyl glycoside of β-D-*N*-acetylglucosamine.

Enzymes. β-D-Galactoside galactohydrolase (EC 3.2.1.23); acylneuraminyl hydrolase, sialidase (EC 3.2.1.18); *N*-acetyl-β-D-glucosaminide *N*-acetylglucosaminohydrolase (EC 3.2.1.52); *N*-acetylglucosaminyltransferase I (EC 2.4.1.101); *N*-acetylglucosaminyltransferase II (EC 2.4.1.143).

Table 1. Acceptor specificity of *L. stagnalis* connective tissue β2Xyl-T with various acceptors at concentrations of 1 mM. 100% activity for **2** corresponds to 1.4 mU/ml.

Acceptor	Code	β2Xyl-T activity
		%
GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-Asn GlcNAcβ1-2Manα1-3	**1**	81
GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3	**2**	100
GlcNAcβ1-2Manα1-6 Manβ1-O-$(CH_2)_7CH_3$ GlcNAcβ1-2Manα1-3	**3**	52
Manα1-6 Manβ1-O-$(CH_2)_7CH_3$ GlcNAcβ1-2Manα1-3	**4**	62
Manβ1-O$(CH_2)_7CH_3$ GlcNAcβ1-2Manα1-3	**5**	43
Manα1-6 Manβ1-O-$(CH_2)_7CH_3$ Manα1-3	**6**	0
GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manα1-3	**7**	14
GlcNAcβ1-2Manα1-6 GlcNAcβ1-4-Man GlcNAcβ1-2Manα1-3	**8**	0.8
Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	**9**	0

ase (β2Xyl-T) (so far only discussed for plant glycoproteins) [10]. β2GlcNAc-T I has been purified from several sources [11] (and references cited therein), and the chicken [12], human [13, 14], mouse [15], rabbit [16], and rat [17] genes have been cloned. Furthermore, the substrate specificity of the rat liver and bovine colostrum β2GlcNAc-T I has been extensively studied [18, 19]. Under specific substrate conditions, β2GlcNAc-T II is responsible for the introduction of a GlcNAc residue in β1-2-linkage on the Manα1-6 arm of the $Man_3GlcNAc_2$ core structure. This enzyme has been purified and characterized from rat liver [20, 21], insect cells [22], and plants [23]. The genes for human [24] and rat [25] β2GlcNAc-T II have been cloned.

So far, the specific β1-2 xylosyltransferase involved in the biosynthesis of N-linked oligosaccharides has not been described in animals; in plants, two xylosyltransferase activities have been reported [10, 26]. Other known xylosyltransferases are an animal xylosyltransferase, which initiates the biosynthesis of cartilage chondroitin sulfate proteoglycans by transferring a xylose residue to serine on the core protein [27], and a plant xylosyltransferase, involved in the xylosylation of the 3-position of 3,5,7,4′-tetrahydroxyflavone (kaempferol) in young leaves of *Euonymus alatus f. ciliato-dentarus* [28]. Recently, a novel xylosyltransferase has been discovered in the soluble fraction of rat kidney homogenate, and it was tentatively concluded that this xylosyltransferase and its acceptor are the renal form of glycogenin [29].

Here, connective tissue β2Xyl-T, β2GlcNAc-T I, and β2GlcNAc-T II activities are described, which are probably involved in the biosynthesis of the inner part of the hemocyanin glycans of *L. stagnalis*. The β2Xyl-T transfers Xyl from UDP-

Table 2. Acceptor specificities of *L. stagnalis* connective tissue β2GlcNAc-T I and β2GlcNAc-T II with various acceptors at concentrations of 1 mM (or 0.5 mM for 10). A dash (−) means incubation has not been performed. 100% activity for **9** and β2GlcNAc-T I corresponds to 0.04 mU/ml; 100% activity for **4** and β2GlcNAc-T II corresponds to 0.01 mU/ml.

Acceptors	Code	Activity	
		β2GlcNAc-T I	β2GlcNAc-T II
		%	
Manα1-6 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 Asn	**10**	130	–
Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3	**11**	–	165
Manα1-6 Manβ1-O-$(CH_2)_7CH_3$ Manα1-3	**6**	80	–
Manα1-6 Manβ1-O-$(CH_2)_7CH_3$ GlcNAcβ1-2Manα1-3	**4**	–	100
Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	**9**	100	–
Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 Xylβ1-2	**12**	0	–

Xyl in β1-2-linkage to the βMan residue of the trimannosyl core of N-glycans and has been called UDP-Xyl:GlcNAcβ1-2Manα1-3Manβ-R (Xyl to Manβ) β1-2-xylosyltransferase. Taking into account the earlier data on the galactosyl- and *N*-acetylgalactosaminyltransferases [6, 7], a biosynthetic scheme will be presented for the biosynthesis of N-linked oligosaccharide chains in this particular hemocyanin.

EXPERIMENTAL PROCEDURES

Materials. Connective tissue from mantle edges was dissected from laboratory bred, adult specimens of *Lymnaea stagnalis*, fed lettuce *ad libitum*, and kept at 20°C under carefully controlled conditions of photoperiod (12-h light/12-h dark). UDP-D-[U-^{14}C]Xyl (specific activity 258 Ci/mol) and UDP-D-[U-^{14}C]GlcNAc (specific activity 200 Ci/mol) were obtained from New England Nuclear, and diluted as needed by non-radioactive UDP-D-Xyl (Sigma) and UDP-D-GlcNAc (Sigma), respectively. *p*-Nitrophenyl glycoside of β-D-*N*-acetylglucosamine (Np-β-D-GlcNAc) was obtained from Sigma.

Oligosaccharides and glycoproteins. The structures of the acceptors tested with β2Xyl-T are summarized in Table 1, and those of the acceptors tested with β2GlcNAc-T I or β2GlcNAc-T II in Table 2. Compound **1** was prepared by desialylation (*Vibrio cholerae* sialidase, Boehringer Mannheim) and subsequent degalactosylation (Jack bean β-galactosidase, Oxford Glycosystems) of human serotransferrin. Compound **2** was prepared from bovine fibrinogen as described [30]. Compounds **3**–**6** were gifts of Prof. Dr H. Paulsen (University of Hamburg), compound **7** was a gift of Dr G. Strecker (Université des Sciences et Techniques de Lille Flandres-Artois, Laboratoire de Chimie Biologique, Villeneuve d'Ascq), compound **8** was a gift of Dr J. Lönngren (Stockholm University) and compound **9** was a gift of Dr K. Hård (Bijvoet Center, Department of Bio-Organic Chemistry). The compounds **10** and **11** were isolated as described [11]. Compound **12** was a gift of Dr Ir J. P. M. Lommerse (Bijvoet Center, Department of Bio-Organic Chemistry). The purity of the various acceptors was checked by 300-MHz ^{1}H-NMR spectroscopy. The concentrations of stock solutions of listed acceptors were determined by monosaccharide analysis.

Protein determination. Protein was determined according to [31] using bovine serum albumin as a standard.

Monosaccharide analysis. Monosaccharides were analyzed by GLC on a capillary SE-30 fused-silica column

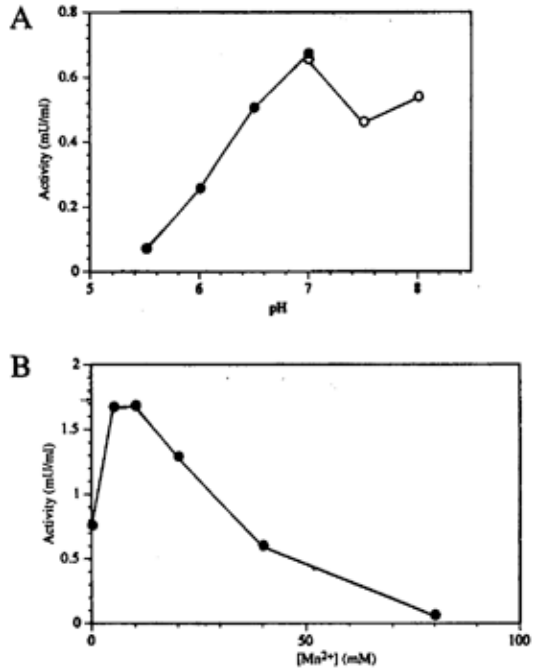


Fig. 1. Optimization of snail connective tissue xylosyltransferase activity using compound 2 as acceptor and UDP-[^{14}C]Xyl as donor with respect to (A) pH and (B) $MnCl_2$ concentration. In the pH optimization assay 0.5 M Mes (●) or 0.5 M Hepes (○) was added, depending on the pH tested.

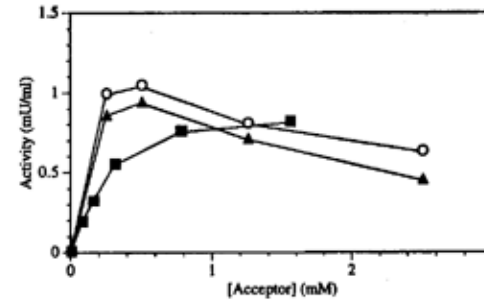


Fig. 2. Xylosyltransferase activity using compounds 2–4 as acceptor and UDP-[^{14}C]Xyl as donor in the presence of connective tissue microsomal suspension. Different concentrations of acceptor were added to the standard assay mixture. Compounds: **2** (■), 3 (○) and 4 (▲).

(25 m×0.32 mm, Pierce) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis, *N*-(re)acetylation, and trimethylsilylation [32].

Methylation analysis. Oligosaccharides were permethylated [33] and converted into a mixture of partially methylated alditols by hydrolysis with 2 M trifluoroacetic acid (1 h, 120°C), followed by reduction with NaB^2H_4. After removal of boric acid by co-evaporation with acidified methanol, the alditol derivatives were acetylated with acetic anhydride (3 h, 120°C), and analyzed by GLC/MS [34], using a Fisons MD800 mass spectrometer (electron energy, 70 eV), coupled with a Fisons GC8060 gas chromatograph, equipped with a DB-1 capillary column (J+T Scientific).

Preparation of connective tissue microsomal suspension. All procedures were performed at 4°C. Dissected connective tissue (5 mg) was homogenized in 10 ml 10 mM sodium cacodylate pH 7.0 using a Polytron apparatus, and the homogenate was centrifuged for 60 min at 100000×*g*. The pellet was resuspended in 5 ml 0.25 M sucrose with a Potter-Elvehjem homogenizer, and centrifuged again. The resulting pellet was resuspended in 2.5 ml 0.25 M sucrose and stored at 4°C until use.

Xylosyltransferase assay. The standard incubation mixture contained, in a total volume of 20 µl, 0.2 mM compound **2**, 0.25 mM UDP-[^{14}C]Xyl (5000−10000 dpm/nmol), 0.125 M Mes pH 7.0, 0.5% (by vol.) Triton X-100, 10 mM $MnCl_2$, 5 mM ATP, and connective tissue microsomal suspension [7 µU, 10 µg protein; one unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the transfer of 1 µmol Xyl/min using the standard incubation system and compound **2** as an acceptor at a concentration of 1 mM]. Incubations were conducted for 60−120 min at 37°C, and reactions were terminated by adding 0.5 ml 20 mM ice-cold sodium borate containing 2 mM EDTA. The samples were then loaded onto Pasteur pipette columns containing 0.5 ml AG 1-X8 (acetate form, 100−200 mesh; Bio-Rad), and neutral carbohydrates (radioactive Xyl, radioactive product, substrate) were eluted with 0.5 ml water. Solutions were mixed with 4 ml Emulsifier Safe (Packard 6013389), and analyzed for radioactivity. Control incubations in the absence of exogenous acceptor were performed routinely. All assays were at least duplicated, and corrected for incorporation and degradation in the absence of exogenous acceptor.

***N*-Acetylglucosaminyltransferase assay.** *N*-Acetylglucosaminyltransferase was assayed as described for the xylosyltransferase assay, except that the standard incubation mixture contained, in a total volume of 20 µl, 1 mM compound **6** (β2GlcNAc-T I) or 1 mM compound **4** (β2GlcNAc-T II), 0.25 mM UDP-[^{14}C]GlcNAc (5000−10000 dpm/nmol), 0.125 M sodium cacodylate pH 7.5, 0.4% (by vol.) Triton X-100, 80 mM $MnCl_2$, 5 mM ATP, and connective tissue microsomal suspension [0.2 µU, 10 µg protein; one unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the transfer of 1 µmol GlcNAc/min using the standard incubation system and either compound **6** or compound **4** as an acceptor at a concentration of 1 mM].

***N*-Acetylhexosaminidase assay.** The *N*-acetylhexosaminidase activity was measured in an assay system optimized for the *N*-acetylglucosaminyltransferase reaction but containing, in a total volume of 20 µl, 5 mM Np-β-D-GlcNAc, 0.25 mM UDP-Xyl, 0.125 M sodium cacodylate pH 7.5, 0.4% (by vol.) Triton X-100, 80 mM $MnCl_2$, 5 mM ATP, and connective tissue microsomal suspension (7 µU, 10 µg protein). The mixture was incubated for 1 h at 37°C and stopped by adding 50 µl 1 M sodium carbonate. The amount of liberated *p*-nitrophenol was determined colorimetrically at 400 nm.

High-pH anion-exchange chromatography. High-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was performed on a Dionex LC system consisting of a Dionex Bio LC quaternary gradient module and a PAD 2 detector, using a CarboPac PA1 column (25 cm×4 mm, Dionex). Elutions were performed with a concentration gradient of 30−150 mM NaOAc in 0.1 M NaOH in 25 min, at a flow rate of 4 ml/min. Detection was done with a gold electrode and triple-pulsed amperometry, comprising the following pulse potentials and durations: $E_1 = 0.05$ V (300 ms); $E_2 = 0.65$ V (60 ms); $E_3 = -0.95$ V (180 ms). The column effluent of preparative runs was passed through an anion micromembrane suppressor unit (Dionex AMMS) for on-line desalting [35]. The regenerant solution for the AMMS unit was 50 mM H_2SO_4 at a flow rate of 6.5 ml/min.

^{1}H-NMR spectroscopy. Prior to ^{1}H-NMR analysis, carbohydrates were exchanged twice in ^2H_2O (99.9 atom % ^{2}H, MSD

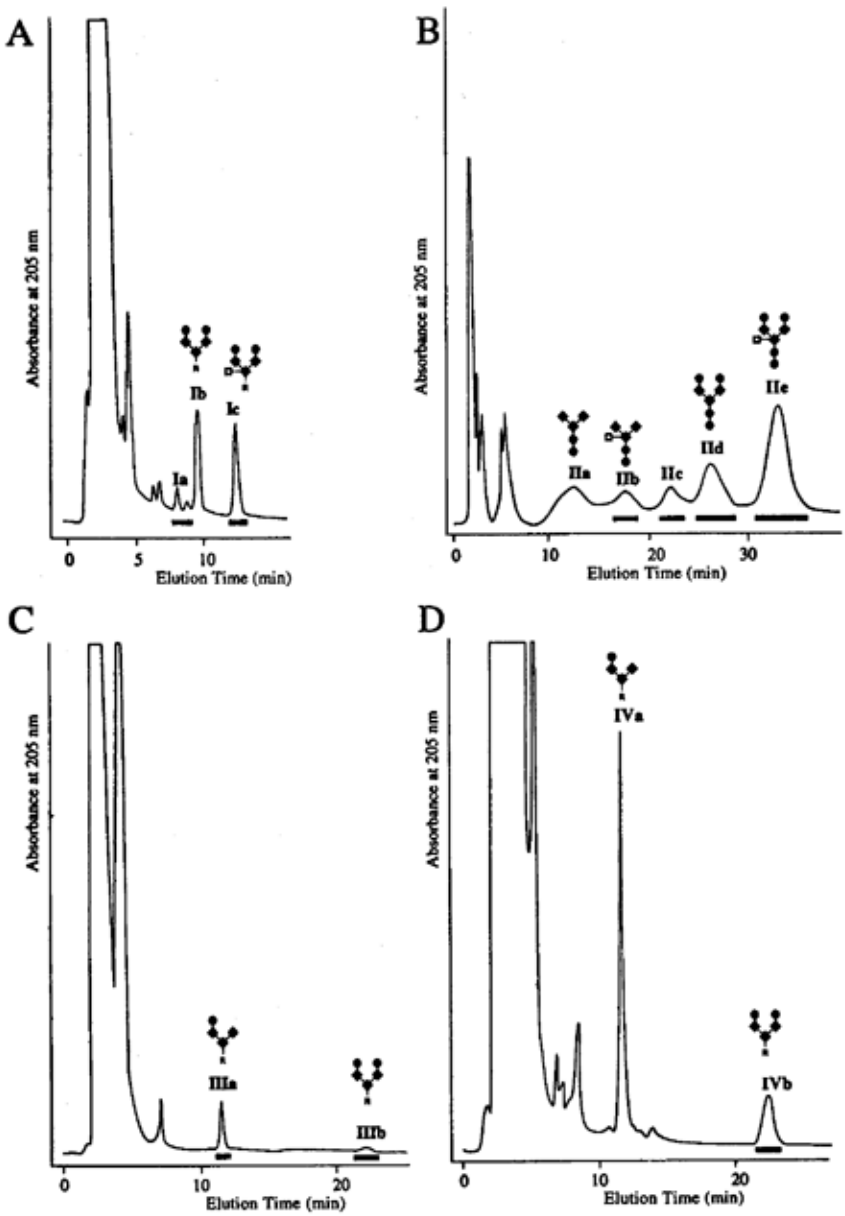


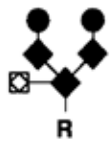
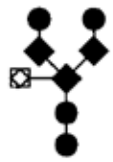
Fig. 3. HPLC of the mixtures of oligosaccharides obtained by incubation of different acceptors with UDP-[^{14}C]Xyl and connective tissue microsomes (A and B) or with UDP-[^{14}C]GlcNAc and connective tissue microsomes (C and D) on Lichrosorb-NH$_2$. (A) 24-h incubation of acceptor **3**, elution with water/acetonitrile (25/75, by vol.) at a flow rate of 1 ml/min; (B) 24-h incubation of acceptor **2**, elution conditions as for A, but at a flow rate of 2 ml/min; (C) 24-h incubation of acceptor **6**, elution with water/acetonitrile (20/80, by vol.) at a flow rate of 1 ml/min; (D) 24-h incubation of acceptor **4**, elution conditions as for C. The bars indicate radioactive fractions. R = O-$(CH_2)_7CH_3$.

Isotopes) with intermediate lyophilization. Finally, samples were dissolved in 99.96% ^2H_2O (MSD Isotopes). ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University) or at 500 MHz on a AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University), at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225) [36]. Typically, one-dimensional (1D) spectra were recorded with a spectral width of 5000 Hz at 500 MHz, collecting 16–500 free induction decays of 8 K or 16 K complex data points. Suppression of HO^{2}H was achieved by applying the water-eliminated Fourier-transform (WEFT) pulse sequence as described [37]. The resolution of the 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomal function when necessary.

Two-dimensional homonuclear Hartmann-Hahn (2D HOHAHA) measurements were obtained using a MLEV-17 mixing sequence of 100 ms, and 388 measurements of 2 K data points were recorded. The spectral width was 4717 Hz in each dimension. The HOHAHA experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation. The HO^{2}H signal was suppressed by presaturation during 1.0 s. 2D NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software (Bijvoet Center, Department of NMR Spectroscopy).

Large-scale incubations with connective tissue β2Xyl-T. *GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-O-$(CH_2)_7$-CH_3 (3) as substrate.* The incubation mixture contained in a volume of 400 µl: 40 mM $MnCl_2$, 0.5% (by vol.) Triton X-100, 125 mM Mes pH 7.0, 5 mM ATP, 1 mM UDP-[^{14}C]Xyl (500 dpm/nmol), connective tissue microsomal suspension

Table 3. Relevant ^{1}H-NMR chemical shifts of structural-reporter groups of the constituent monosaccharides for the components Ib and Ic of the incubation mixture of compound 3/UDP-[^{14}C]Xyl/snail connective tissue enzyme, and of constituent monosaccharides for the components 2 and IIe′ of the incubation mixture of compound 2/UDP-[^{14}C]Xyl/snail connective tissue enzyme. Chemical shifts are at 27°C and were measured in ^2H_2O relative to internal acetone (δ 2.225). Compounds are represented by short-hand symbolic notation: ●, D-GlcNAc; ◆, D-Man; ⊡, D-Xyl. R = -O-$(CH_2)_7CH_3$. n.d. not determined. For numbering of the residues, see Fig. 5.

Residue	Reporter group	Chemical shift in			
		3/Ib 	Ic 	2 	IIe′ 
		ppm			
GlcNAc-1	H1α	–	–	5.187	5.192
	H1β	–	–	4.697	4.698
	NAc	–	–	2.037	2.039
GlcNAc-2	H1$_\alpha$	–	–	4.613	4.609
	H1$_\beta$	–	–	4.604	4.601
	NAc	–	–	2.078	2.077
Man-3	H1	4.669	4.842	n.d.	4.872
	H2	4.13	4.182	4.249	4.255
Man-4	H1	5.126	5.165	5.118	5.143
	H2	4.185	4.155	4.188	4.154
Man-4′	H1	4.915	4.895	4.918	4.902
	H2	4.13	4.132	4.107	4.107
GlcNAc-5	H1	4.553	4.519	4.556	4.517
	NAc	2.050	2.046	2.051	2.047
GlcNAc-5′	H1	4.581	4.577	4.556	4.553
	NAc	2.058	2.058	2.051	2.051
Xyl-X	H1	–	4.504	–	4.438
	H5$_{ax}$	–	3.253	–	3.248

(1.4 mU, 0.2 mg protein), and 0.5 mM compound **3**. The mixture was kept for 24 h at 37°C, and after adding of 2 ml ice-cold water, the sample was loaded on a freshly conditioned Sep-Pak C_{18} column [38]. After washing with five 5-ml portions of water, the column was eluted with two 5-ml portions of methanol. The first 5-ml methanol fraction, containing radioactivity, was concentrated using a stream of nitrogen, and the residue was fractionated by HPLC on a 10-µm Lichrosorb-NH_2 column (25 cm×4.6 mm, Chrompack) using water/acetonitrile (25/75, by vol.) as eluent. The flow rate was 1 ml/min. The elution pattern was monitored at 205 nm and aliquots of the 1-ml fractions were analyzed by scintillation counting.

GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (2) as substrate. The incubation mixture contained in a volume of 400 µl: 10 mM $MnCl_2$, 0.5% (by vol.) Triton X-100, 125 mM Mes pH 7.0, 5 mM ATP, 4 mM UDP-[^{14}C]Xyl (500 dpm/nmol), connective tissue microsomal suspension (1.4 mU, 0.2 mg protein), and 0.5 mM compound **2**. The mixture was kept for 24 h at 37°C, and after freezing and thawing, passed over a 10-ml column of AG 1-X8 (acetate form, 100–200 mesh). The resin was washed with 20 ml water and the total eluate was lyophilized. The residue was taken up in water, and the solution was desalted on a column (50×1 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad) using water as eluent. To remove interfering Triton X-100, the pooled radioactive fractions were rinsed over a freshly conditioned Sep-Pak C_{18} column, and after washing with 5 ml water, the total eluate was lyophilized. The residue was taken up in water and fractionated by HPLC as described above, at a flow rate of 2 ml/min.

Large-scale incubation with connective tissue β2GlcNAc-T I. *Manα1-6(Manα1-3)Manβ1-O-$(CH_2)_7CH_3$ (6) as substrate.* The incubation mixture contained in a volume of 200 µl: 80 mM $MnCl_2$, 0.4% (by vol.) Triton X-100, 125 mM sodium cacodylate pH 7.5, 5 mM ATP, 4 mM UDP-[^{14}C]GlcNAc (500 dpm/nmol), connective tissue microsomal suspension (2 mU, 0.2 mg protein), and 1 mM compound **6**. The mixture was kept for 24 h at 37°C, and after adding of 2 ml ice-cold water, the sample was loaded on a freshly conditioned Sep-Pak C_{18} column [38]. In a similar way as described above for the octyl derivative **3**, a radioactive 5-ml methanol fraction was isolated, concentrated, and fractionated by HPLC using water/acetonitrile (20/80, by vol.) as eluent. The flow rate was 1 ml/min.

Inhibition studies with connective tissue β2Xyl-T. Compound **2** was used as acceptor at a concentration of 0.5 mM in the standard xylosyltransferase assay in the absence or presence of either **3** or **6** at a concentration of 2 mM. Each mixture was passed over a column of AG 1-X8 (see above), and the eluting fraction was either used directly for radioactivity measurement by scintillation counting or, after lyophilization, refractionated on a freshly conditioned Sep-Pak C_{18} column. In the latter case a separation between the radioactive product from **2** (washing with 20 ml water) and the radioactive product from either **3** or

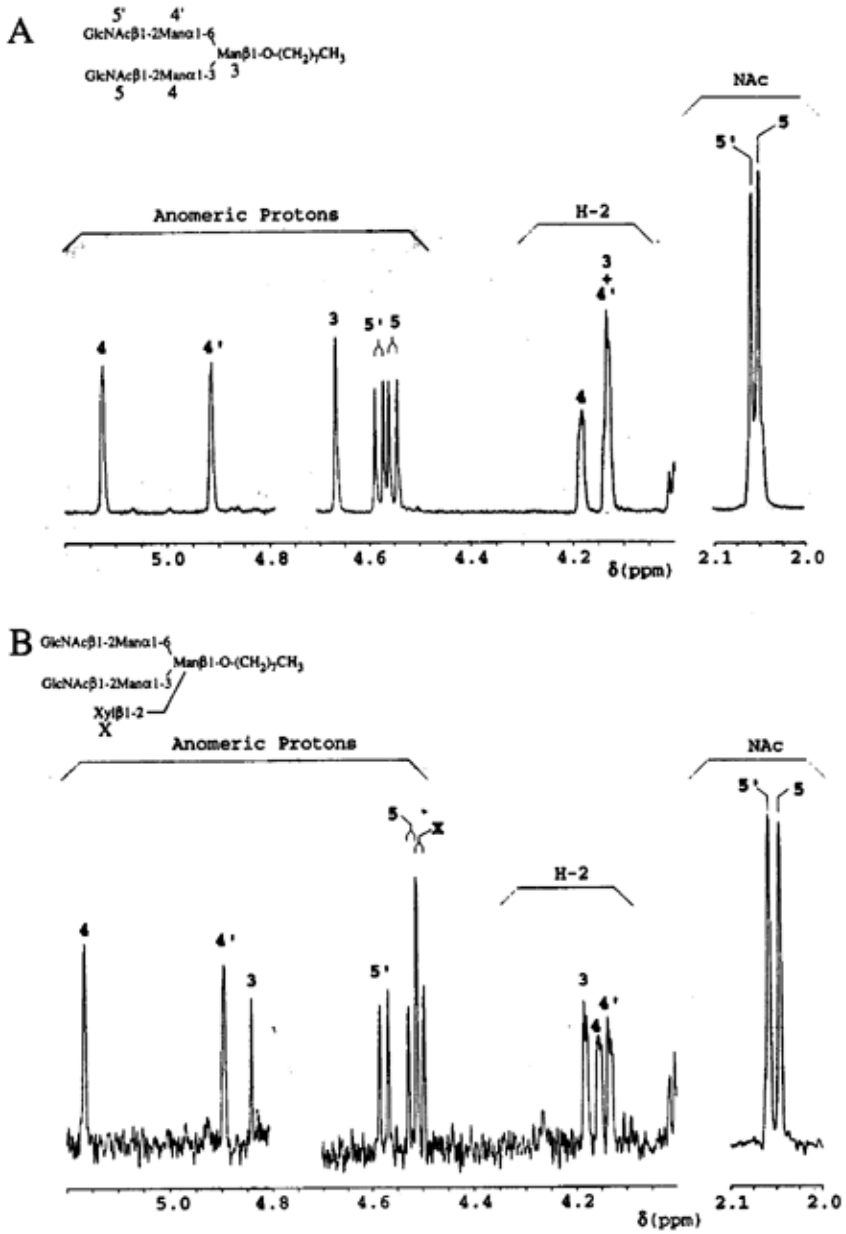


Fig. 4. Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of (A) compound 3 (Ib), and (B) GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-O-$(CH_2)_7CH_3$ (Ic) in ^2H_2O at 27°C. The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum.

6 (elution with 10 ml methanol) can be reached. Fractions were analyzed by scintillation counting.

RESULTS

Properties of connective tissue β2Xyl-T. The connective tissue β2Xyl-T activity was studied using UDP-[^{14}C]Xyl as donor and compound **2** as acceptor with *L. stagnalis* connective tissue microsomes as enzyme source. Radioactive product formation was proportional to time over a period of 10 h and to an enzyme concentration up to at least 5 mg connective tissue protein/ml (data not shown). The pH optimum for the β2Xyl-T activity was around 7.0 (Fig. 1 A), and the enzyme was stimulated by Mn^{2+} with an optimum concentration between 6.25−12.5 mM (Fig. 1 B). The β2Xyl-T activity was independent of the Triton X-100 concentration in the range of 0.25−2.5%, and no activation effect was found (data not shown).

The substrate specificity of connective tissue β2Xyl-T is summarized in Table 1. All effective acceptors have the structural element GlcNAcβ1-2Manα1-3Manβ-R. Compounds lacking the β1-2-linked GlcNAc residue at the Manα1-3-arm (**6** and **9**) were not effective acceptors. Compound **1** showed a K_m of 1.3 mM and a V_{max} of 0.9 mU/ml, whereas compound **2** showed a K_m of 0.4 mM and a V_{max} of 1.7 mU/ml. The determination of kinetic parameters for **3** and **4**, both having an octyl aglycon at the βMan residue, failed, because enzyme inhibition occurred at higher concentrations (Fig. 2). A discussion on this aspect will be presented below.

Product identification using connective tissue β2Xyl-T. *GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-O-$(CH_2)_7CH_3$ (3) as substrate.* Compound **3** was incubated with UDP-[^{14}C]Xyl and connective tissue microsomes and, after workup, the remaining mixture of compounds was fractionated by HPLC

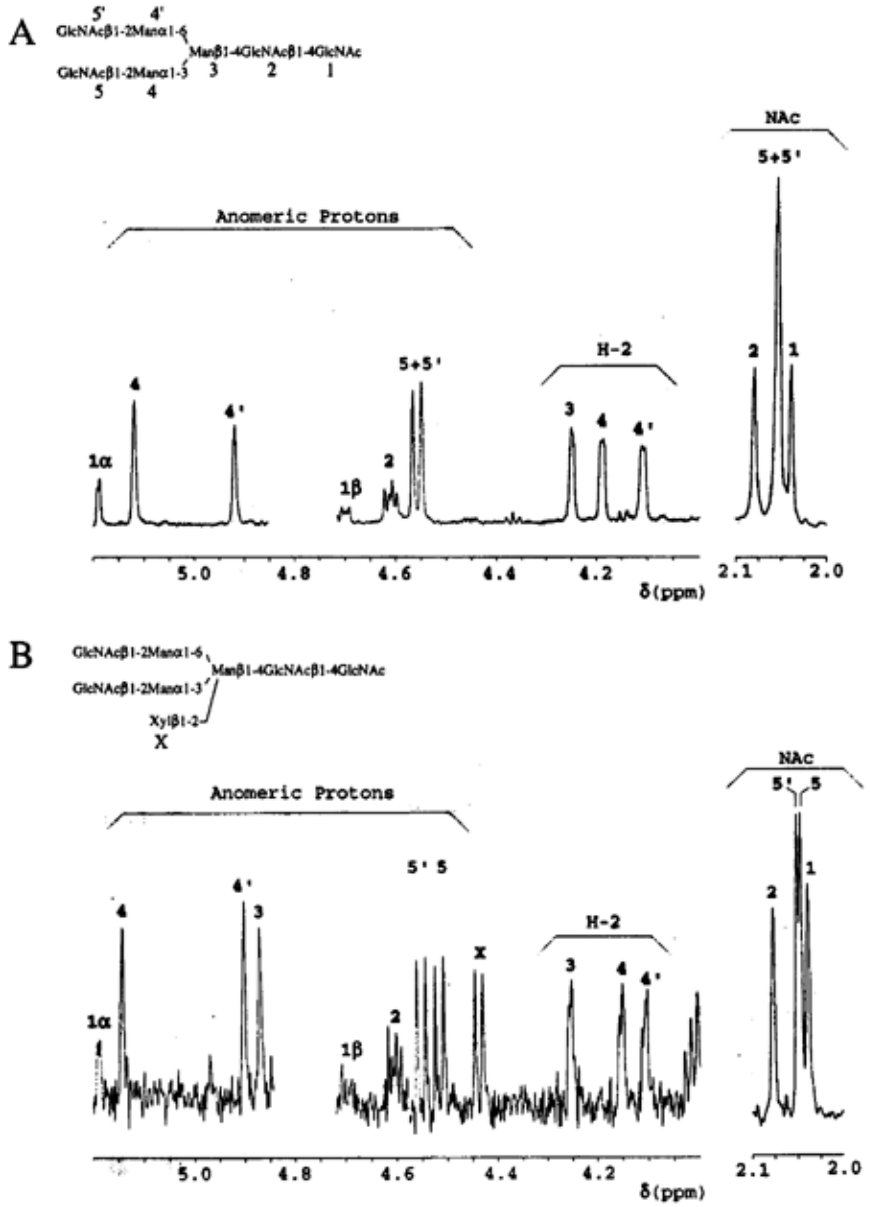


Fig. 5. Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of (A) compound 2 and (B) GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc (IIe′) in ^2H_2O at 27 °C. The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum.

on Lichrosorb-NH_2 yielding a non-radioactive fraction **Ib**, coeluting with acceptor **3**, and two radioactive fractions **Ia** and **Ic** (Fig. 3 A). The isolated amounts of fraction **Ia** were too low for detailed structural studies, but the elution position of this radioactive fraction suggests that it probably represents a degradation product of **Ic**, formed by the action of a connective tissue β-*N*-acetylhexosaminidase. The presence of this glycosidase activity in connective tissue microsomes could be confirmed by incubation with Np-β-D-GlcNAc using the standard assay conditions. The relevant ^{1}H-NMR data of **Ib** and **Ic** are given in Table 3. The assignments of the structural-reporter-group signals of **Ib** (≡ **3**) are based on the ^{1}H-NMR data reported for compounds **4** [19] and **6** [39]. Comparison of the ^{1}H-NMR spectra of **Ib** and **Ic** (Fig. 4) shows the effect of the introduction of one β-linked Xyl residue (H1, δ 4.504, $J_{1,2}$ 7.2 Hz; $H5_{ax}$, δ 3.253). The presence of the Xyl residue has a severe influence on the structural-reporter groups of the other monosaccharide constituents, and 2D HOHAHA experiments were performed to assign the Man H2 and Xyl H1 signals. The attachment site of the Xyl residue was deduced from methylation analysis studies on **Ic**. GLC/MS of the mixture of partially methylated alditol acetates indicated the presence of terminal Glc*p*NAc, terminal Xyl*p*, 2-substituted Man*p*, and 2,3,6-trisubstituted Man*p*. Based on the various analytical data, **Ic** is identified as GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-O-$(CH_2)_7CH_3$.

GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (2) as substrate. Compound **2** was incubated with UDP-[^{14}C]Xyl and connective tissue microsomes, and after workup, a fractionation of the remaining mixture of compounds by HPLC on Lichrosorb-NH_2 yielded five fractions, denoted **IIa**–**e** (Fig. 3 B). The isolated amounts of fractions **IIa–d** were too low for ^{1}H-NMR analysis, but some information could be deduced from the HPLC elution positions. Non-radioactive fraction **IIa** coeluted with compound **9**, whereas radioactive fraction

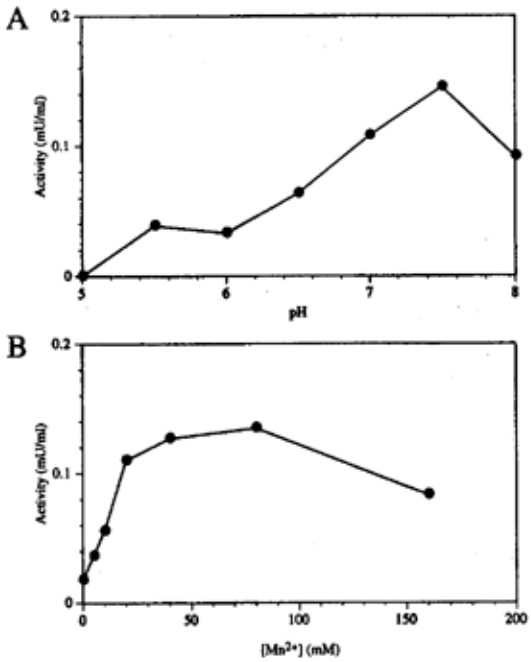


Fig. 6. Optimization of snail connective tissue β2GlcNAc-T I activity using compound 6 as acceptor and UDP-[^{14}C]GlcNAc as donor with respect to (A) pH and (B) $MnCl_2$ concentration. In the pH optimization assay 0.5 M sodium cacodylate buffer (●) was added.

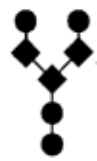
IIb coeluted with compound **12**. Radioactive fraction **IIc** did not coelute with any of the available standards, but the presence of radioactivity shows that Xyl has been incorporated. Radioactive fraction **IId** coeluted with acceptor **2**, and probably contains a mixture of compound **2** and a radioactive Xyl-T product. The major radioactive fraction **IIe** was subfractionated by HPAEC on CarboPac PA1 yielding one major fraction, denoted **IIe′** (data not shown). The relevant ^{1}H-NMR data of **2** and **IIe′** are summarized in Table 3. The ^{1}H-NMR spectrum (Fig. 5B) of fraction **IIe′** shows structural-reporter-group signals characteristic of the presence of a β-linked Xyl residue (H1, δ 4.438, $J_{1,2}$ 7.7 Hz; $H5_{ax}$, δ 3.248). These chemical shifts are identical to those found for oligosaccharide-alditols containing GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAc as internal element, derived from hemocyanin (H1, δ 4.436; $H5_{ax}$, δ 3.246–3.248) [5]. Also the assignment of the structural reporters of the relevant monosaccharide constituents fits the data reported for the internal element of the hemocyanin glycans [5]. Therefore, the ^{1}H-NMR data show that fraction **IIe′** is GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc. The finding of **IIa** (from **2**) and of radioactive compounds **IIb**, **IIc**, and **IId** (from **IIe′**) are probably the result of connective tissue β-*N*-acetylhexosaminidase action on compounds **2** and **IIe′**, respectively (see above).

Properties of connective tissue β2GlcNAc-T I. The β2GlcNAc-T I activity was studied using UDP-[^{14}C]GlcNAc as donor, compound **6** as acceptor, and connective tissue as enzyme source. Radioactive product formation was proportional to time over a period of 24 h and to enzyme concentration up to at least 5 mg connective tissue protein/ml (data not shown). The pH optimum for β2GlcNAc-T I activity was around 7.5 (Fig. 6A), and the enzyme was stimulated by Mn^{2+} with an optimum concentration between 40–80 mM (Fig. 6B). The β2GlcNAc-T I activity was independent of the Triton X-100 concentration in the range of 0.25–2.0%, and no activation effect was found (data not shown).

Table 4. Relevant ^{1}H-NMR chemical shifts of structural-reporter groups of constituent monosaccharides for the components 6 and IIIa of the incubation mixture of compound 6/UDP-[^{14}C]GlcNAc/snail connective tissue enzyme. Chemical shifts are at 27°C and were measured in ^2H_2O relative to internal acetone (δ 2.225). Compounds are represented by short-hand symbolic notation: ●, D-GlcNAc; ◆, D-Man. R = O-$(CH_2)_7CH_3$. For numbering of the residues, see Fig. 5.

Residue	Reporter group	Chemical shift in	
		6 	**IIIa** 
		ppm	
Man-**3**	H1	4.672	4.671
	H2	4.133	4.13
Man-**4**	H1	5.104	5.129
	H2	4.065	4.18
Man-**4′**	H1	4.907	4.911
	H2	3.990	3.99
GlcNAc-**5**	H1	–	4.552
	NAc	–	2.051

Table 5. Inhibition studies using *L. stagnalis* connective tissue β2Xyl-T. Enzyme activities were determined, as described in the xylosyltransferase assay, using 0.5 mM **2**, and 2 mM of either **3** or **6**. The column AG 1-X8 represents enzyme activity determined by passage of the incubation mixture through AG 1-X8 and measures the sum of radioactive products due to **2** and either **3** or **6**. The columns water and methanol represent enzyme activities of experiments with the Sep-Pak C_{18} step; water = water fraction after Sep-Pak C_{18}, methanol = methanol fraction after Sep-Pak C_{18}. R = O-$(CH_2)_7CH_3$. n.a.d. no activity detected.

Incubation of			Activity		
2 	**6** 	**3** 	AG 1-X8	water	methanol
0.5 mM	2 mM	2 mM			
			mU/ml		
–	+	–	n.a.d.	n.a.d.	n.a.d.
–	–	+	0.50	n.a.d.	0.50
+	–	–	0.94	0.94	n.a.d.
+	+	–	1.18	0.84	n.a.d.
+	–	+	0.98	0.32	0.46

The substrate specificity of connective tissue β2GlcNAc-T I is summarized in Table 2. The effective acceptors (**6**, **9**, and **10**) have the structural element Manα1-3Manβ-R, as reported previously for β2GlcNAc-T I from other sources [18, 19]. The presence of a Xyl residue β1-2-linked to the βMan residue totally abolishes β2GlcNAc-T I activity (compare **9** and **12**). Kinetic parameters for β2GlcNAc-T I and **6** were K_m = 1.4 mM and V_{max} = 0.2 mU/ml. The K_m value is comparable with that reported for rat liver β2GlcNAc-T I [19].

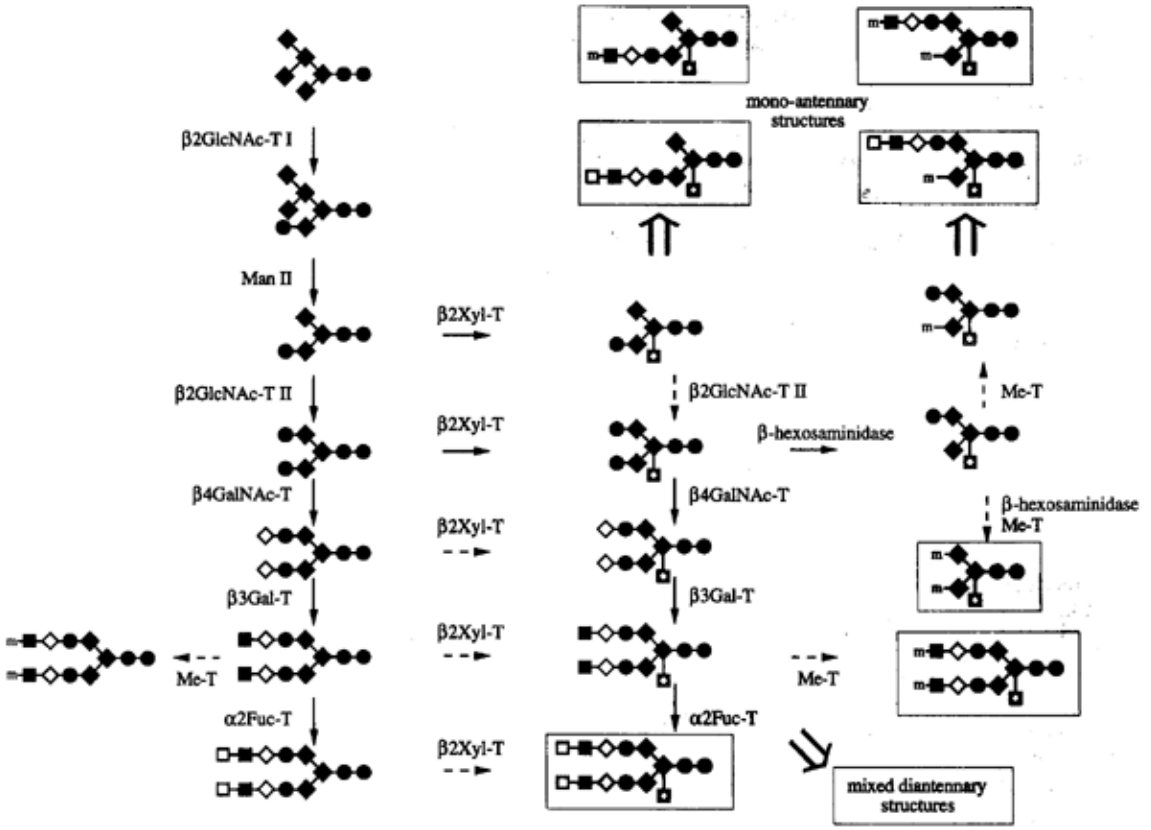


Fig. 7. Scheme for biosynthesis of N-linked oligosaccharides in connective tissue from *L. stagnalis*. Compounds are represented by short-hand symbolic notation: □, L-Fuc; ■, D-Gal; ◇, D-GalNAc; ●, D-GlcNAc; ◆, D-Man; ⊠, D-Xyl; m, methyl. Solid arrows indicate steps verified by experiments and dotted arrows indicate probable steps. Man II represents α-mannosidase II. Boxed structures have been isolated [4, 5].

Product identification using connective tissue β2GlcNAc-T I. *Manα1-6(Manα1-3)Manβ1-O-$(CH_2)_7CH_3$ (6) as substrate.* Compound **6** was incubated with UDP-[^{14}C]GlcNAc and connective tissue microsomal suspension and, after workup, a fractionation of the remaining mixture of compounds by HPLC on Lichrosorb-NH_2 yielded two radioactive fractions, **IIIa** and **IIIb** (Fig. 3C). The relevant ^{1}H-NMR data of fraction **IIIa** and compound **6** are summarized in Table 4. Radioactive fraction **IIIa** coeluted with compound **4**, and the ^{1}H-NMR spectra of both compounds are virtually identical, indicating the following structure for **IIIa**: Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-O-$(CH_2)_7CH_3$. The isolated amounts of **IIIb** were too low for ^{1}H-NMR studies, but **IIIb** coeluted with compound **3**, suggesting that **IIIb** is the product of β2GlcNAc-T II action on **IIIa** (see below).

Identification of connective tissue β2GlcNAc-T II activity. The presence of β2GlcNAc-T II activity was demonstrated by incubating Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-O-$(CH_2)_7CH_3$ (**4**), UDP-[^{14}C]GlcNAc, and connective tissue microsomal suspension as enzyme source. After workup, the mixture was fractionated by HPLC on Lichrosorb-NH_2 (Fig. 3D), and one non-radioactive fraction **IVa**, coeluting with acceptor **4**, and one radioactive fraction **IVb** were isolated. Radioactive fraction **IVb** coeluted with compound **3**, indicating that connective tissue contains β2GlcNAc-T II activity. When compound **3** was incubated with UDP-[^{14}C]GlcNAc and connective tissue microsomal suspension, no radioactive oligosaccharides could be traced, showing that snail connective tissue contains no GlcNAc-T III-VI enzyme activities [9], in accordance with the structural analysis studies on hemocyanin [4, 5]. The kinetic parameters for β2GlcNAc-T II and compound **4** are K_m = 0.3 mM and V_{max} = 0.04 mU/ml. The K_m value is comparable with that reported for rat liver β2GlcNAc-T II [21].

Inhibition of connective tissue β2Xyl-T. To study the substrate inhibition observed with acceptors **3** and **4** and connective tissue β2Xyl-T (Fig. 2), either **3** or **6** was added to the standard enzyme incubation using **2** as acceptor. Compound **6** (with an octyl aglycon) has no effect on the activity for **2** (which lacks an octyl aglycon), showing that the inhibition at high concentrations of **3** or **4** is probably not due to the octyl group *per se* (Table 5).

DISCUSSION

In this study, it has been shown that connective tissue of the snail *L. stagnalis* contains a xylosyltransferase capable of transferring xylose from UDP-Xyl to R″-GlcNAcβ1-2Manα1-3(R′-)Manβ1-R with R″ = H or Galβ1-4, R′ = H, Manα1-6, or GlcNAcβ1-2Manα1-6, and R = -4GlcNAc, -4GlcNAcβ1-4GlcNAc, -4GlcNAcβ1-4GlcNAcβ1-Asn, or O-$(CH_2)_7CH_3$. The minimal structure requirements are fulfilled in GlcNAcβ1-2Manα1-3Manβ1-O-$(CH_2)_7CH_3$ (**5**). The products formed by incubation of GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-R [where R = -4GlcNAcβ1-4GlcNAc or O-$(CH_2)_7CH_3$] have been purified and characterized by ^{1}H-NMR spectroscopy and methylation analysis, and were shown to be GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-R. The enzyme activity can therefore be characterized as UDP-Xyl:GlcNAcβ1-2Manα1-3Manβ-R (Xyl to Manβ) β1-2 xylosyltransferase.

Because compounds missing the GlcNAc residue on the Manα1-3 arm are not acceptors for β2Xyl-T, several enzymic steps are needed for the formation of structures like Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAc$_2$. First, β2GlcNAc-T I must introduce a GlcNAc residue, then β2Xyl-T can introduce a Xyl residue, and finally the GlcNAc residue must be removed by β-*N*-acetylhexosaminidase. During our studies on the β1-3 galactosyltransferase from connective tissue of *L. stagnalis* a hexosaminidase activity was traced acting on GalNAcβ1-4GlcNAc-R sequences [6], and in the present study a hexosaminidase activity has been found capable of removing GlcNAc residues from xylose-containing oligosaccharides.

The inhibition of β2Xyl-T observed at high concentrations of acceptors **3** and **4** is probably not due to the octyl aglycon, since the octyl-containing compound **6** showed no influence on the assay with acceptor **2**. It is possible that compounds **3** and **4** or their enzyme products or both bind to a second site on the enzyme at higher concentrations and thereby cause inhibition, but no evidence for this hypothesis was found.

This is the first report of an animal xylosyltransferase acting on N-linked oligosaccharide chains. N-glycans containing xylose are common in the plant kingdom and plant xylosyltransferases involved in the biosynthesis of these glycoproteins have been described for *Phaseolus vulgaris* cotyledons [10] and sycamore cells of *Acer pseudoplatanus* [26]. The bean cotyledon xylosyltransferase acts on glycopeptide acceptors having a terminal β1-2-linked GlcNAc residue on the Manα1-3-arm and it was suggested that xylose *in vivo* is introduced after the action of both β2GlcNAc-T I and α-mannosidase II since GlcNAc-Man$_5$GlcNAc$_2$Asn was a poor xylose acceptor [10]. For sycamore cell β2Xyl-T similar results have been reported, and this β2Xyl-T also required acceptors having the structural element GlcNAcβ1-2Manα1-3Manβ-R [26]. The substrate specificities of both of these plant β2Xyl-Ts are similar to that of the snail connective tissue β2Xyl-T. However, whereas Gal-terminated acceptors such as Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$Asn were inactive with the bean cotyledon β2Xyl-T, the Gal-containing compound **7** was an effective although relatively inefficient acceptor for the snail connective tissue β2Xyl-T.

The substrate specificity of connective tissue β2GlcNAc-T I shows that the enzyme is active towards acceptors having a Manα1-3Manβ-R sequence, but introduction of a Xyl residue at βMan totally abolished the β2GlcNAc-T I activity. Taking into account that xylose-containing oligosaccharides are not acceptors for β2GlcNAc-T I, and that β2Xyl-T is only active towards acceptors having the GlcNAcβ1-2Manα1-3Manβ-R sequence, it is clear that in snail connective tissue β2GlcNAc-T I must act before β2Xyl-T. This is in agreement with the enzyme activities reported in plants [10, 26].

With the identification of β2Xyl-T, β2GlcNAc-T I, β2GlcNAc-T II, and α1-2 fucosyltransferase (unpublished results) activity in snail connective tissue, the presence of all the glycosyltransferases necessary for the biosynthesis of the extensions of the N-linked oligosaccharides on hemocyanin of *L. stagnalis* have been demonstrated [6, 7]. The only enzymes involved in this biosynthesis not described so far are the methyltransferase(s) responsible for the formation of MeO-3Gal and MeO-3Man residues. Based on the substrate specificities of the described connective tissue glycosyltransferases and the structures isolated from hemocyanin, a biosynthetic scheme for the N-glycosylation starting with Man$_5$GlcNAc$_2$Asn~ in snail connective tissue can be proposed (Fig. 7).

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