19-aldehyde **22** (12 mg, 0.0310 mmol, 97% yield). Compound **22** had identical TLC and very similar ^{1}H NMR data compared to those of the unlabeled compound **8**. Mass spectroscopic analysis for deuterium in **22** (M - THP$^+$) revealed that d_0, 8.6%; d_1, 89.3%; d_2, 2.1%.

[1α-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)-19-oxoandrosta-2,4-diene-3,17β-diol (23). The labeled enone **22** (10 mg, 0.0258 mmol) in benzene (0.8 mL) was reacted with collidine and TBDMS triflate as described previously. Workup and purification were performed analogously to the reaction with unlabeled material to afford pure labeled dienol ether **23** (9.0 mg, 0.018 mmol, 70% yield). Compound **23** had identical TLC properties and very similar ^{1}H NMR data compared to those of the unlabeled compound **7**. From the 400-MHz ^{1}H NMR spectrum of **23**, it was determined that compound **23** contained 93% 1α-^{2}H with no detectable 1β-^{2}H (1β-H appeared as a broad singlet, residual 1α-H appeared as a symmetrical doublet). Mass spectroscopic analysis (M$^+$) for deuterium indicated d_0, 8.6%; d_1, 90.0%; d_2, 1.1%.

Transformation of [1α-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)-19-oxoandrosta-2,4-diene-3,17β-diol (23) into [1-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)estradiol (24). To a solution of labeled dienol ether **23** (7.5 mg, 0.015 mmol) in 20:1 MeOH-CH_2Cl_2 (1.05 mL) containing anhydrous $NaHCO_3$ (ca. 3 mg) was added aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol). The vortexed reaction mixture was allowed to stand at 4 °C for 3.5 days, after which time workup and purification were performed as for the reaction with the unlabeled material. The labeled estrogen derivative **24** (4.5 mg, 0.00955 mmol, 64% yield) was furnished as a white solid. The TLC properties of compound **24** were identical with those of the unlabeled estrogen derivative **14**. The ^{1}H NMR (400 MHz) spectrum of **24** was similar to that of **14** except that the 1-H signal was greatly reduced (*d*, 86%) and the 2-H signal appeared largely as a broad singlet (*d*, 0%). Mass spectroscopic analysis of **24** (M$^+$) for deuterium indicated d_0, 18.3%; d_1, 81.4%; d_2, 0.3%.

Transformation of Labeled Dienol Ether 25 to 25a and Estrogen Derivative 26. The labeled dienol ether **25**[29] (4.0 mg, 0.00798 mmol; 26% deuterium at the 1β-position, less than 3% deuterium in the 1α-position) was reacted with HOOH as previously described for the unlabeled compound **7**, except that, after 6 h, the reaction was quenched with aqueous saturated $Na_2S_2O_3$ (5 mL). The resultant mixture was partitioned between aqueous half-saturated $NaHCO_3$ (40 mL) and CH_2Cl_2 (40 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (20 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. One-fourth of the crude residue was set aside for a ^{1}H NMR spectrum, which indicated that the ratio of dienol ether **25a** to labeled estrogen derivative **26** was 79:21. The remaining three-fourths of the crude material was purified by flash chromatography (0.5 g of SiO_2, 4% EtOAc-hexanes containing 1 drop of Et_3N/10 mL) to afford **25a** (ca. 1.5 mg) and **26** (ca. 0.5 mg). ^{1}H NMR analysis of compound **25a** indicated that there was about 24% deuterium in the 1β-position with negligible deuterium in the 1α-position. ^{1}H NMR analysis of compound **26** indicated that there was about 4% deuterium at the 1-position.

Model Reaction Kinetic Studies, General Procedure. Test tubes containing 3 mg of anhydrous $NaHCO_3$ were charged with 1:50 CH_2Cl_2-MeOH (0.9 mL) solution containing dienol ether **7** (171 μg, 0.342 μmol) and placed in a Dubnoff shaker bath at 37 °C for ca. 2 min. The reactions were initiated with 0.1 mL of aqueous HOOH solution (2.2, 4.4, or 8.8 M HOOH). After vortexing for several seconds, the reactions were incubated for varying time lengths, e.g., 0, 5, 10, 20, 40, and 60 min for 0.88 M HOOH assays. Assays run with EDTA contained 10 μL of an aqueous 167 mM EDTA solution (pH 7.2). Assays run with BHT contained 2 mg (9 μmol) of BHT. Assay reactions were quenched with aqueous saturated sodium thiosulfate (4 mL), and then immediately partitioned between aqueous one-third saturated NH_4Cl (60 mL) and CH_2Cl_2 (20 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (20 mL), and the combined organic phases were dried (Na_2SO_4), concentrated in vacuo, analyzed for product **14** levels with reverse-phase HPLC (Altex ultrasphere octyl analytical column, eluent 100% MeOH, flow rate 0.7 mL/min, UV detection at 280 nm, retention time 10 min). The zero time point levels showed in all cases less than 1% product **14** formation. All assays were performed in duplicate, and the results were averaged. The data was analyzed by using the Enz-Fit first-order nonlinear regression program, and replots of ln ($P_\infty - P_0$) versus time gave straight lines with no systematic deviation observed. Errors in the rate constants were estimated to be ±15%.

[^{18}O]-*p*-Bromophenacyl Formate. The dienol ether **7** (14.0 mg, 0.028 mmol) was reacted with randomly labeled aqueous 20% [50%-^{18}O]-HOOH solution analogously to the reaction with unlabeled hydrogen peroxide, and the sodium formate product was converted to the labeled bromophenacyl formate derivative (1.5 mg, 0.0061 mmol, 22% yield) as previously described. The TLC and ^{1}H NMR data of the labeled derivative were identical with those of the unlabeled compound. Mass spectroscopic analysis of the labeled formate derivative for ^{18}O indicated (M) $^{18}O_0$, 49.8%; $^{18}O_1$, 50.2%. The fragment (M - $C_2H_3O_2$) possessed no detectable ^{18}O.

Acknowledgment. We are grateful to Dr. Joseph Kachinski for obtaining the mass spectra and Syntex Co. for a generous gift of 19-hydroxydehydroepiandrosterone. We thank the Department of Chemistry for access to their 400-MHz NMR spectrometer. This work was supported by the National Institutes of Health (Grant HD-11840). We thank the Medical Scientist Training Program for a Grant to P.A.C. (GM-07309).

Probing the Acceptor Specificity of β-1,4-Galactosyltransferase for the Development of Enzymatic Synthesis of Novel Oligosaccharides

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Abstract: β-1,4-Galactosyltransferase has been investigated with regard to its acceptor specificity and used in the synthesis of galactosides with 5-thioglucose, glucal, deoxynojirimycin, modified *N*-acetylglucosamine, and glucose derivatives as acceptors. The galactoside products are potentially useful as endoglycosidase or glycosyltransferase inhibitors or as intermediates for the synthesis of complex oligosaccharides. The conformation of each enzyme product has been investigated with NMR; all are shown to possess similar glycosidic torsional angles based on a significant NOE between H-1 of Gal and H-4 of the acceptor. Comparison of the transferase reactions with the β-1,4-galactosidase-catalyzed galactosyl transfer reactions indicates that the transferase reactions provide exclusively a β-1,4-glycosidic linkage while the galactosidase reactions predominantly form a β-1,6-glycosidic linkage.

The stereocontrolled synthesis of oligosaccharides based on sophisticated protection/deprotection, activation, and coupling strategies has been well established.[2,3] Enzymatic oligosaccharide synthesis based on glycosyltransferases[4] is a useful alternative to

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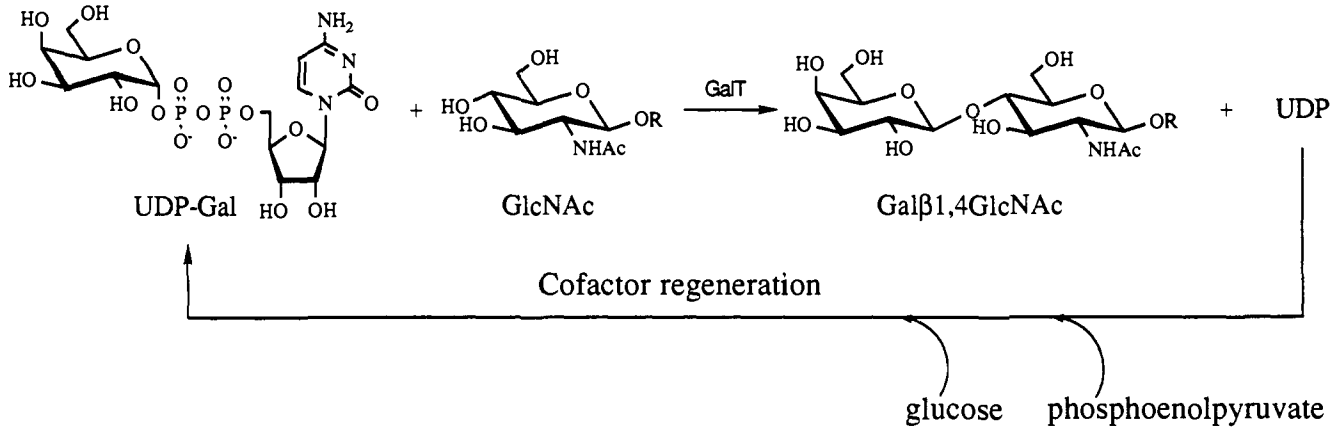
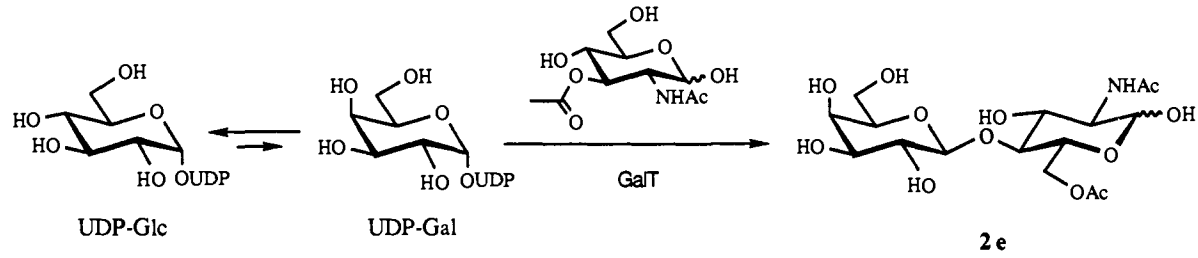
Scheme I



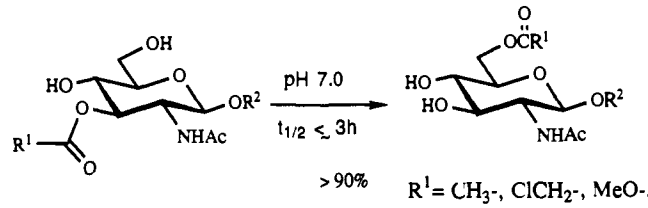
Scheme II



the chemical synthesis. Glycosyltransferases are highly specific with regard to the formation of glycosidic linkages, and no protection/deprotection steps are required. Procedures for the regeneration of sugar nucleotides are also available for large-scale processes.[5,6] As part of our interests in the development of enzymatic procedures for the synthesis of oligosaccharides and their derivatives, we report here our study on the acceptor specificity of β-1,4-galactosyltransferase from bovine milk (GalT, EC 2.4.1.22) and its use in the synthesis of galactosides.

It is known that GalT from bovine milk accepts *N*-acetylglucosamine (GlcNAc) and its glycosides (β is better than α-glycoside) as substrates (Scheme I).[7] Glucose and its α- and β-glucosides are also acceptable; however, lactalbumin is required for α-glucosides. When the enzyme forms a complex with lactalbumin, the specificity changes.[7] In the absence of lactalbumin, the K_m values and relative rates (%) compared to *N*-acetylglucosamine (GlcNAc) for glucose (Glc) and GlcNAc, for example, are 1400 (<0.1%) and 8.3 mM (100%), respectively. In the presence of lactalbumin, the numbers change to 5 (93%) and 1.7 mM (17%).[7a] Some 6-*O*-glycosyl derivatives[8a] (e.g., Fucα1,6GlcNAcβOR, NeuAc(OMe)α2,6GlcNAcβOR), 3-*O*-methyl GlcNAc,[8a] and some unusual monosaccharide derivatives[8b] were reported to be weak substrates for the enzyme. Several UDP derivatives of galactose analogues (e.g., glucose, 4-deoxyglucose, arabinose, *N*-acetylgalactosamine, and glucosamine) were also acceptable as donors with a rate ~ <5% that of GlcNAc.[9]

Scheme III



Results and Discussion

Acceptor Specificity. Our study on the acceptor specificity of GalT (Table I) indicates that the enzyme is highly specific for GlcNAc and glucose and their related glycosides. A substantial

(1) Supported by NIH (GM44154). G.C.L. acknowledges the support of an American Cancer Society Postdoctoral Fellowship (PF-3525).

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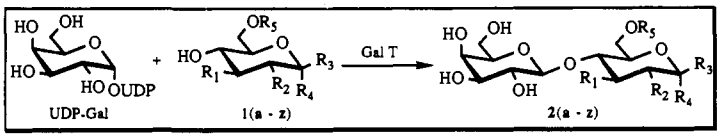
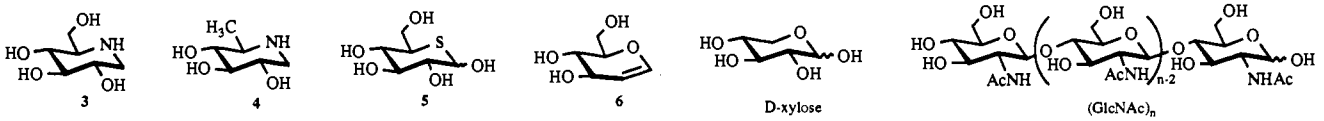
Table I[a]





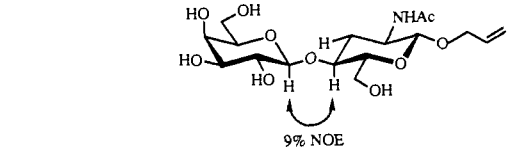
compd	R_1	R_2	R_3	R_4	R_5	rel rate (%)
1a	OH	AcNH	(H,	OH)	H	100
1b	OH	AcNH	H	CH_3O–	H	75
1c	OH	AcNH	H	CH_2=$CHCH_2O$–	H	25
1d	$CH_3CHO(-)CO_2^-$	AcNH	(H	OH)	H	0.6
1e	OH	AcNH	(H,	OH)	CH_3CO–	4
1f	AcO–	AcNH	(H,	OH)	H	0.4
1g	CH_2=$CHCH_2O$–	AcNH	H	OMe	H	0
1h	$CH_3(CH_2)_2O$–	AcNH	(H,	OH)	H	0.5
1i	H	AcNH	CH_2=$CHCH_2O$–	H	H	1.0
1j	CH_2=$CHCH_2O$–	AcNH	$CH_3(CH_2)_3O$–	H	H	0.3
1k	MeOCOO–	AcNH	CH_2=$CHCH_2O$–	H	H	*b*
1l	AllylOCOO–	AcNH	CH_2=$CHCH_2O$–	H	H	*b*
1m	$MeOCH_2O$–	AcNH	CH_2=$CHCH_2O$–	H	H	2.0
1n	O	AcNH	CH_3O–	H	H	2.0
1o	epi-OH	AcNH	(H,	OH)	H	0.04
1p	OH	AcNH	-SPh	H	H	0
1q	O	AcNH	H	OMe	H	0.1
1r	OH	phthalimido	H	SPh	H	0
1s	epi-OH	AcNH	H	OMe	H	0
1t	OH	OH	(OH,	H)	H	100
1u	CH_2=$CHCH_2O$–	OH	(OH,	H)	H	0
1v	MeO–	OH	(OH,	H)	H	10
1w	OH	OH	SPh	H	H	0.1
1x	OH	OH	SPh	H	CH_2Ph	0.04
1y	OH	H	(OH,	H)	H	60
1z	OH	AcNH	CH_3O–	H	CH_3–	20
3						3
4						0.1
5						70
6						0.4
D-xylose						90[c]
$(GlcNAc)_n$						
$n = 2$						500[d]
$n = 3$						60[d]
$n = 4$						70[d]

[a] Condition: UDP-Gal (0.2 mM), acceptor (25 mM), lactalbumin (0.1 mg/mL for Glc derivatives, **1t–1y**, **3–6**, and D-xylose), $MnCl_2$ (5 mM), phosphoenolpyruvate (1 mM), NADH (0.3 mM), pyruvate kinase (10 U), and L-lactate dehydrogenase (10 U) in 1 mL of cacodylate (0.05 M), pH 7.0, was added to 40 mU of Gal T. (Fitzgerald, D. K.; Colvin, B.; Maval, R.; Ebner, K. E. *Anal. Biochem.* **1970**, *36*, 43). The rates for Glc derivatives are compared to that of Glc, and the rates for GlcNAc derivatives are compared to GlcNAc. [b] Poor substrate. Acyl migration occurs. [c] See ref 7b. [d] See ref 7a.

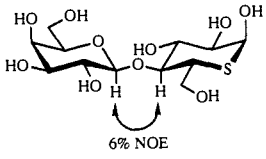
decrease in activity was observed for GlcNAc derivatives with modification at position 3. While some weak activities were observed for 3-substituted β-glycosides, no activity was observed for the corresponding α-glycosides (e.g., **1g**). Glucose and derivatives are acceptable in the presence of lactalbumin. The same trend was observed with 3-substituted glucose derivatives. The 3-oxo-(**1n**) and 3-epimeric (**1o**) derivatives of GlcNAc are acceptable at 2% and 0.04% rate, respectively. 6-*O*-Acetyl-GlcNAc is also a reasonable substrate (4%). 2-Deoxyglucose, 5-thioglucose (**5**), and D-xylose are good substrates each with a relative rate >60% that of glucose. Disaccharide $(GlcNAc)_2$ is a better substrate than GlcNAc, and tri- and tetrasaccharide $(GlcNAc)_{3-4}$ are also good substrates. Azasugars **3** and **4** and glucal **6**, however, are weak substrates.

Synthesis of Galactosides. Despite the weak activity with several unnatural substrates, GalT was used as a catalyst in small-scale synthesis. In a representative procedure for the synthesis of **7**, 5-thioglucose (100 mg, 500 μmol), GalT (5 U), UDP-glucose (350 mg, 500 μmol), α-lactalbumin (0.1 mg/mL), and UDP-glucose epimerase (10 U)[10] were dissolved in 10 mL of 50 mM sodium cacodylate (pH 7.0) containing 5 mM of $MnCl_2$. The reaction mixture was incubated at 37 °C for 2 days. The product was isolated via a Dowex 1 formate column followed by gel filtration (Bio Gel P-2) to give 90 mg of **7** in 50% yield. A similar procedure was applied to the synthesis of **2i**, **8**, and **10** with longer reaction times (4 days), and each was obtained with the yield in the range of 20–40%. It is worth noting that no byproducts were observed; the low yields were due to incomplete reactions. Both reaction rate and yield can be improved by converting the released UDP to UTP catalyzed by pyruvate kinase in the presence of phosphoenolpyruvate. When 3-*O*-acetyl-*N*-acetylglucosamine was used as a substrate (1 equiv), the product obtained, however, was compound **2e** (43% yield based on consumed UDP-sugar) with the acetyl group migrated to position 6 of the *N*-acetylglucosamine moiety (Scheme II). No other byproduct was obtained. To further study this unexpected acyl migration, 3-*O*-acetyl-*N*-acetylglucosamine was incubated at pH 7.0 in the absence of the

(10) This enzyme catalyzes the epimerization of UDP-Glc to UDP-Gal. UDP-Glc is less expensive than UDP-Gal. See Wong et al. in ref 5.



2i



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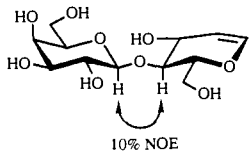

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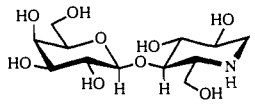
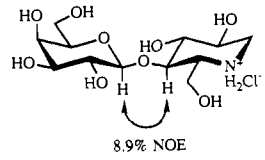


10a



10b

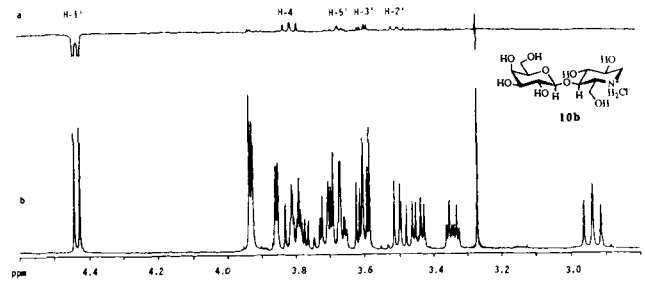
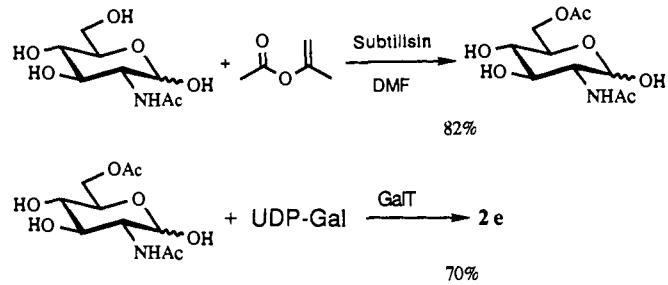


Figure 1. NOE experiment for **10b**. Saturation of H-1′ gave 8.9% NOE on H-4.

Scheme IV



enzyme and a ^{1}H NMR spectrum was taken. It was observed that the intensity of a new peak at 1.82 ppm increased while the signal at 1.90 ppm (CH_3CONH–) decreased, and a downfield shift of H-6 and upfield shift of H-3 were observed at the same time. After 24 h, 90% of the original compound was converted to the new product which was identical with an authentic 6-*O*-(methoxycarbonyl)-*N*-acetylglucosamine prepared separately (Scheme III). The identity was further confirmed by the high-resolution mass spectroscopy analysis.

6-*O*-Acetyl-*N*-acetylglucosamine was then tested as a substrate for GalT, and it was found to be about 10 times as effective as the 3-*O*-acyl isomer under the same condition. A separate synthesis of the 6-*O*-acetyl disaccharide was then accomplished in 70% isolated yield with the use of 6-*O*-acetyl-*N*-acetylglucosamine as a substrate (Scheme IV). It is worth noting that 6-*O*-acetyl-*N*-acetylglucosamine was easily prepared in 82% yield from GlcNAc and isopropenyl acetate in anhydrous dimethylformamide catalyzed by subtilisin.

Although the problem of acyl migration prohibits the preparation of 3-*O*-acyl disaccharides, it provides a new route to 6-*O*-acylated disaccharides. In addition to acetyl group, methoxycarbonyl, chloroacetyl, and allyloxycarbonyl groups also showed 3→6 *O*-acyl migration. The half-life for each of these migration was about 3 h for acetyl, methoxycarbonyl, and allyloxycarbonyl group, and <3 h for chloroacetyl group at room temperature and pH 7.0 as measured by NMR. The synthesis of 3-*O*-acyl-GlcNAc is straightforward. Starting from the readily available 4,6-*O*-benzylidene derivative, various acyl groups can be introduced to the 3-*O* position.

These syntheses demonstrate that GalT can be used as catalyst for the preparation of galactosides on milligram scales with weak acceptor substrates. Given that the enzyme is relatively stable,[5] GalT (and perhaps other glycosyltransferases) seems amenable for the small-scale synthesis of a number of unusual oligosaccharides.

β-Galactosidase Reactions. Since β-galactosidase can also be used in the synthesis of β-galactosides,[11] the enzyme was investigated for the synthesis of GalT products. 5-Thioglucose (**5**) was reacted with *p*-nitrophenyl β-galactoside in the presence of β-1,4-galactosidase (EC 3.2.1.23 from *Escherichia coli*). The product obtained, however, possesses a β-1,6-linkage (**9**). When compound **3** or **4** was used as a substrate, no product was obtained. Neither azasugar was an inhibitor since the substrate *p*-nitrophenyl β-galactoside was hydrolyzed by the enzyme at the same rate as that in the absence of the azasugars. It appears that a glycosidase inhibitor (e.g., **5** is an inhibitor of α-glucosidase) can be used as a substrate for another glycosidase (e.g., β-galactosidase in this case).

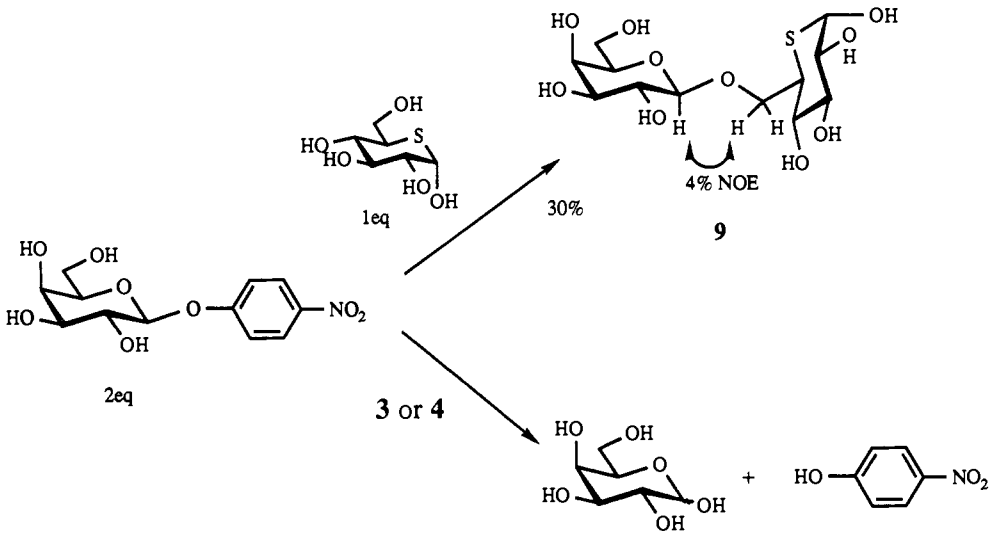
NMR Determination of Glycosidic Linkage and Conformation. The assignment of the disaccharide linkage was based upon ^{1}H and ^{13}C NMR data (at 300 or 500 and 125 MHz, respectively). The ^{1}H NMR chemical shift assignment for each proton was established by extensive decoupling experiments. Significantly, a large downfield shift (0.1–0.3 ppm) for the protons attached to the carbon in a glycosidic linkage relative to the starting monosaccharide was observed. Correspondingly, the other protons experienced little shifts. ^{1}H nuclear Overhauser effect (NOE) experiments were used to further confirm the linkage and conformation. For example, **9** displayed a 4% enhancement of one of the H-6 resonances of the 5-thioglucose when the H-1 of galactose was irradiated, indicative of the close proximity of the H-6 of the 5-thioglucose to the H-1 of the galactose moiety. Additionally, in the ^{13}C NMR spectra, a downfield shift of the C-6 resonance was observed, along with no significant shifts for the other carbon signals. The information obtained allowed us to assign the glycosidic linkage as 1 → 6.

Further evidence for the assignment of the regiochemistry of the disaccharide linkage was provided by the analysis of the ^{1}H spectra of the peracetylated disaccharide. Thus, the H-1 to H-4 resonances of the 5-thio-D-glucose moiety experienced larger downfield shifts of 1.3 to 1.8 ppm (relative to the free disaccharide) upon acetylation. Correspondingly, the H-5 and H-6 resonances experienced only minor shift (−0.33 to +0.38 ppm) upon acetylation. A similar approach was applied to the other disaccharides. Compounds **2e**, **2i**, **7**, **8**, **10a**, and **10b** prepared in this study are presented as their stable conformations in aqueous solution. A representative NOE study of **10b** is shown in Figure 1. All the disaccharides in this study possess similar glycosidic torsional angle as evidenced by the significant NOEs (6–10%) between H-1′ and H-4. This observation is consistent with that reported by Lemieux[12] in that the glycosidic torsional angle is mainly determined by the exoanomeric effect.

(11) Nilson, K. G. I. *Carbohydr. Res.* **1988**, *180*, 53.

(12) Lemieux, R. U.; Bock, K.; Delbaere, L. T. J.; Koto, S.; Rao, V. S. *Can. J. Chem.* **1980**, *58*, 631. Lemieux, R. U.; Koto, S. *Tetrahedron* **1974**, *30*, 1933. Lemieux, R. U.; Koto, S.; Voisin, D. In *Anomeric Effect, Origin and Consequences*; ACS Symposium Series, No. 87, Szarek, W. A.; Horton, D., Eds.; American Chemical Society: Washington, DC, 1979; p 17.

Scheme V



Since **3**, **4**, **5**, and **6** are potent inhibitors of exoglucosidases (e.g., β-glucosidase),[13] their glycosides may be potent inhibitors of sequence-specific glucosidases, as several natural and synthetic products of this type are potent endoglycosidase inhibitors.[14] Compounds **2i** and **2o** are expected to be inhibitors of α-1,3-fucosyltransferase[15] due to the lack of an appropriately oriented 3-OH group. Compound **2j** is useful for the synthesis of Le^x (Galβ1,4(Fucα1,3)GlcNAc)[16] and sialyl Le^x (Neuα2,3Galβ1,4Fuc(α1,3)GlcNAc).[17]

Expression of β-1,4-Galactosyltransferase in *E. coli*. Galactosyltransferase is, like many other glycosyltransferases,[18] primarily present in the golgi apparatus in a membrane-bound form, which, after proteolysis, generates a soluble active form, the so-called "catalytic domain", in body fluids such as milk and serum. The catalytic domain of bovine GalT is composed of 324 amino acids corresponding to the C-terminal sequence of the 402-amino acid membrane-bound enzyme. The active domain of human GalT is very similar to bovine GalT both in sequence (>90% homology) and substrate specificity (Figure 2) and has been cloned into an *E. coli* expression system pIN-GT (Figure 3). This system incorporates a fusion of the catalytic domain of GalT with the signal sequence of omp A, the major lipoprotein of prokaryotes, so that the enzyme is translocated to the periplasmic space where it is released from the signal sequence by action of signal peptidase to give enzymatically active GalT.[19] The enzyme produced in this system contains an additional tripeptide Ala-Glu-Leu attached to the N-terminal Thr residue of the soluble GalT. To improve

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  1 TGGARPPPPLGASSQPRPGGDSS....PVVDSGPGPASNLTSVPVPHTTA 46
    |. |||||..||.||. :.|.    . .:.||||:|||||.||| ||.
  1 LRGVAPPPPLQNSSKPRSRAPSNLDAYSHPGPGPGPGSNLTSAPVPSTTT 50

 47 LSLPACPEESPLLVGPMLIEFNMPVDLELVAKQNPNVKMGGRYAPRDCVS 96
    ||.||||||||||||||||||:||||.||..|||.||:||||.|.||:|
 51 RSLTACPEESPLLVGPMLIEFNIPVDLKLVEQQNPKVKLGGRYTPMDCIS 100

 97 PHKVAIIIPFRNRQEHLKYWLYYLHPVLQRQQLDYGIYVINQAGDTIFNR 146
    ||||||||||||||||||||||||:|||||||||||||||||:.:|||
101 PHKVAIIIPFRNRQEHLKYWLYYLHPILQRQQLDYGIYVINQAGESMFNR 150

147 AKLLNVGFQEALKDYDYTCFVFSDVDLIPMNDHNAYRCFSQPRHISVAMD 196
    |||||||.|||||||.||||||||||||||.|||||||||||||||
151 AKLLNVGFKEALKDYDYNCFVFSDVDLIPMNDHNTYRCFSQPRHISVAMD 200

197 KFGFSLPYVQYFGGVSALSKQQFLTINGFPNNYWGWGGEDDDIFNRLVFR 246
    |||:||||||||||||||||||||.|||||||||||||||||:|||.||
201 KFGLSLPYVQYFGGVSALSKQQFLSINGFPNNYWGWGGEDDDIYNRLAFR 250

247 GMSISRPNAVVGRCRMIRHSRDKKNEPNPQRFDRIAHTKETMLSDGLNSL 296
    |||:|||||||:|:||||||||||||||||||||||||||||||||||||
251 GMSVSRPNAVIGKCRMIRHSRDKKNEPNPQRFDRIAHTKETMLSDGLNSL 300

297 TYQVLDVQRYPLYTQITVDIGTPS 331
    || ||:|||||||.|||||||||
301 TYMVLEVQRYPLYTKITVDIGTPS 324
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Figure 2. Sequence comparison of the active domains of human (top) and bovine (bottom) β-1,4-galactosyltransferase showed 90% homology.

the expression level, the plasmid pIN-GT in *E. coli* SB221 was isolated and transformed into JM109, an *E. coli* with a damaged cell wall.[20] Approximately 2×10^{-3} U of GalT can be obtained from a 150-mL fermentation, corresponding to a 35-fold increase of activities compared to the previous expression in SB221.[19] The enzyme appeared primarily in the chloroform extracted periplasmic fraction as no significant difference in activity excreted into the media was observed. This system is now available for the preparation of native soluble and site-directed mutant GalT. Our initial study indicates that GalT from human and bovine has the same substrate specificity. A detailed specificity study will be published separately.

Conclusion

In summary, we have investigated the acceptor specificity of β-1,4-galactosyltransferase and found that the enzyme accepts several unnatural substrates stereospecifically to form β-1,4-galactosides. The activities of many substrates, however, are very low and only milligram quantities of products have been prepared. Coupling with the cofactor regeneration system to drive the re-

(13) Kajimoto, T.; Liu, K. K.-C.; Pederson, R. L.; Zhong, Z.; Ichikawa, Y.; Porco, J. A.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6187.
(14) Liotta, L. J.; Bernotas, R. C.; Wilson, D. B.; Ganem, B. *J. Am. Chem. Soc.* **1989**, *111*, 783.
(15) Lowe, J. B.; Stockman, L. M.; Nair, R. P.; Larsen, R. D.; Berhend, T. L.; Marks, R. M. *Cell* **1990**, *63*, 475.
(16) Nicolaou, K. C.; Caufield, T. J.; Kataoka, H.; Stylianides, N. A. *J. Am. Chem. Soc.* **1990**, *112*, 3694. Sato, S.; Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1988**, *29*, 5267. Sato, S.; Ito, Y.; Nukada, T.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1987**, *167*, 197.
(17) Sialyl-Le^x is a ligand of endothelial leukoxyte adhesion molecule (Phillips, M. L.; Nudelman, E.; Gaeta, F. C. A.; Perez, M.; Singhal, A. K.; Hakomori, S.; Paulson, J. C. *Science* **1990**, *250*, 1130). For the total synthesis, see: Kameyama, A.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1991**, *209*, C1. Nicolaou, K. C.; Hummel, C. W.; Bockovich, N. J.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 870. A chemical-enzymatic synthesis of sialyl-Le^x can be carried out with peracetylation of **2j** followed by selective deprotection of the 3-o-allyl group with a Ru catalyst (Corey, E. J.; Suggs, J. W. *J. Org. Chem.* **1973**, *38*, 3224) to liberate the 3-OH group for fucosylation.[15] For a recent enzymatic synthesis, see: Dumas, D.; Ichikawa, Y.; Wong, C.-H.; Lowe, J. B.; Nair, R. P. *Biomed. Lett.*, in press.
(18) Paulson, J. C.; Colley, K. J. *J. Biol. Chem.* **1989**, *264*, 17615.
(19) Aoki, D.; Appert, H. E.; Johnson, D.; Wong, S. S.; Fukuda, M. N. *EMBO* **1990**, *9*, 3171.
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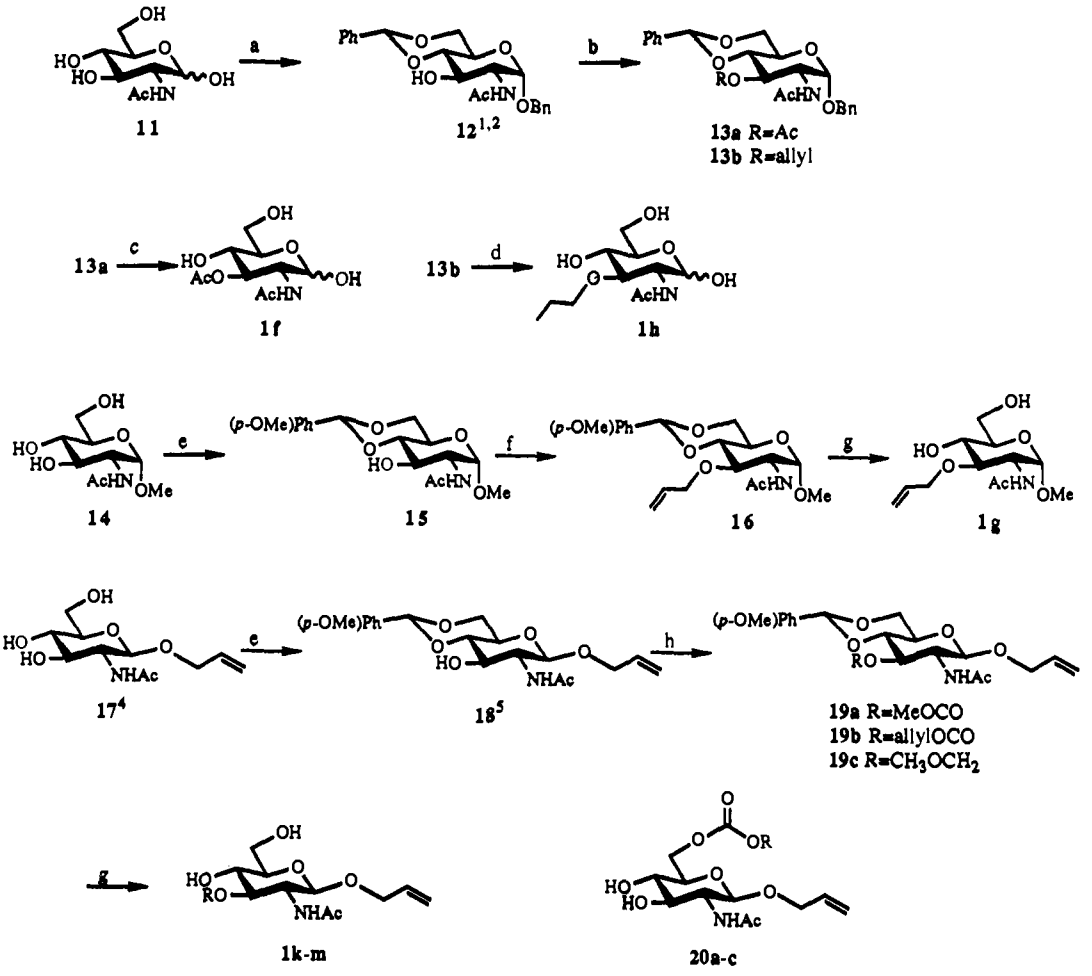
Scheme VI[a]



[a] Conditions: (a) (i) BnOH, H^+; (ii) $ZnCl_2$, PhCHO; (b) for **13a**: Ac_2O, pyridine, 65%; for **13b**: NaH, allyl bromide, 60%; (c) H_2, 5% Pd–C, EtOH–AcOH, 67%; (d) PdO, cyclohexene, EtOH, 46%; (e) $ZnCl_2$, *p*-methoxy benzaldehyde (ref 5); (f) NaH, allyl bromide, THF; (g) AcOH, 80 °C; (h) for **19a**: methyl chloroformate, pyridine, 87%; for **19b**: allyl chloroformate, pyridine, 71%; for **19c**: NaH, chloromethyl ether, THF, 64%. (1) Florent, J.-C.; Monneret, C. *Synthesis* **1982**, *29*. (2) Kuhn, R.; Baer, H. H.; Selinger, A. *Justus Liebigs Ann. Chem.* **1958**, *611*, 236. (3) Warren, C. D.; Nasir, D.; Jeanloz, R. W. *Carbohydr. Res.* **1974**, *37*, 193. (4) Lee, R. T.; Lee, Y. C. *Carbohydr. Res.* **1974**, *37*, 193. (5) Pozsgay, V.; Brisson, J.-R.; Jenning, H. J. *Carbohydr. Res.* **1990**, *205*, 1335.

action and to lessen the problem of product inhibition, gram quantities of products may be obtained. Work is in progress to explore the substrate specificity of other glycosyltransferases.

Experimental Section

N-Acetylglucosamine β-1,4-galactosyltransferase from bovine milk was purchased from Sigma. The enzyme had a specific activity of 4–7 U/mg (1 U = 1 μmol of UDP-Gal transfered per min). The purified enzyme had a reported specific activity of 15 U/mg with GlcNAc as an acceptor.[7e] The K_m value for UDP-Gal is 0.5 mM.[7f]

Expression of GalT in *E. coli* JM109. The plasmid pIN-GT in *E. coli* SB221 was isolated and transformed into JM109 with standard protocol.[21] The transformants were introduced directly into 150 mL of LB media and grown without induction overnight at 37 °C. Cells were harvested by centrifugation at 4000 × g at 4 °C. The cells were resuspended in 3 mL of LB media and 1 mL of chloroform was added. The mixture was allowed to stand at room temperature for 15 min before addition of 30 mL of 50 mM HEPES buffer, pH 7.4. Similarly, SB221 was freshly transformed and grown as a control. The enzyme was isolated, and the activity was determined according to the reported procedures.[7]

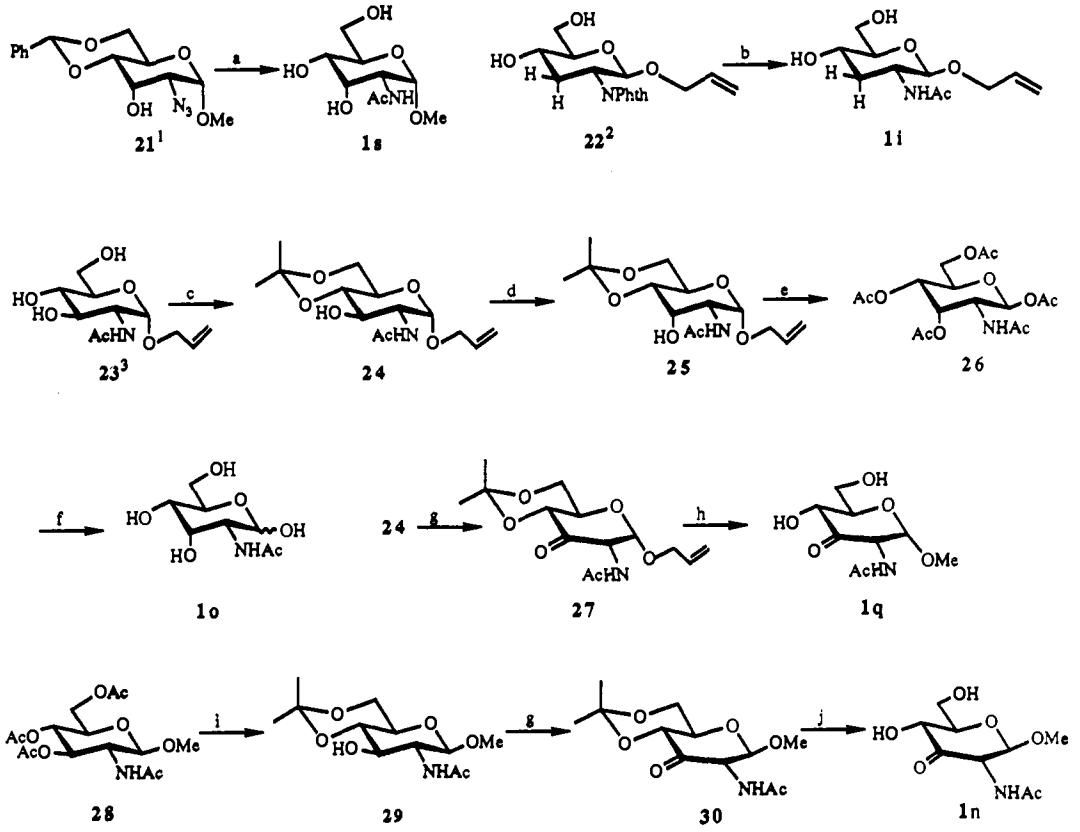
Benzyl 2-Acetamido-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, 13a. Compound **12** (2 g, 5 mmol) was dissolved in 20 mL of dry pyridine and treated with 4 equiv (2.6 g) of acetic anhydride. The mixture was refluxed for 10 h, then quenched with ice, and extracted with 2 N HCl (2 × 100 mL), water (50 mL), and brine (50 mL). After being dried over $MgSO_4$ and evaporation, the product was crystallized from ethyl acetate to yield 1.43 g (65%): ^1H NMR (acetone-d_6) δ 1.93 (s, 3 H, NHAc), 2.06 (s, 3 H, $COCH_3$), 3.72–4.28 (m, 5 H, H-2, 4, 5, 6a, 6b), 4.44, 4.68 (2d, J = 14 Hz, 2 H, CH_2Phe), 4.79 (d, J = 4 Hz, 1 H, H-1), 5.08 (s, 1 H, benzylic), 5.27 (dd, J = 10 Hz, J = 9.5 Hz, 1 H, H-3), 7.25–7.40 (m, 5 H, Ar), 8.50 (d, J = 9 Hz, 1 H, NH).

Benzyl 2-Acetamido-3-*O*-allyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, 13b. Compound **12** (2 g, 5 mmol) was dissolved in 30 mL of THF. NaH (240 mg, 605 in oil, 1.2 equiv) was added at 0 °C and subsequently followed by 0.52 mL of allyl bromide (1.2 equiv). The mixture was heated to reflux for 12 h and then quenched with ice and NH_4Cl solution. After extraction with water (100 mL) and brine (50 mL) the organic solution was dried over $MgSO_4$ and evaporated. Recrystallization from ethyl acetate/hexane gave 1.32 g of **13b** (60%): ^1H NMR (acetone-d_6) 1.85 (s, 3 H, NHAc), 3.60–4.30 (m, 8 H, H-2, 3, 4, 5, 6a, 6b, CH_2 allyl), 4.50, 4.72 (2d, J = 15 Hz, CH_2Ph), 4.81 (d, 1 H, J = 4 Hz, 1H, H-1), 5.03–5.25 (m, 2 H, CH_2=C), 5.13 (s, 1 H, benzylic), 5.75–5.96 (m, 1 H, CH=), 7.30–7.50 (m, 5 H, Ar), 8.12 (d, J = 9 Hz, 1 H, NH).

2-Acetamido-3-*O*-acetyl-2-deoxy-D-glucopyranose, 1f. Compound **13a** (800 mg, 2 mmol) was dissolved in 100 mL of ethanol and 20 mL of acetic acid, and the mixture was hydrogenated under 50 psi at room temperature with 250 mg of 5% Pd/C. After filtration through Celite, evaporation, and column chromatography, **1f** (350 mg, 67%) was obtained and further crystallized from methanol/ethyl ether: ^1H NMR (D_2O) δ 1.94 (s, 3 H, OAc), 2.06 (s, 3 H, NAc), 3.36 (ddd, J = 9, 5, and 2.5 Hz, H-5β), 3.51 (t, J = 9.5 Hz, H-4β), 3.56 (t, J = 9,5 Hz, H-4α), 3.72 (dd, J = 12, 5 Hz, H-6$_a$α), 3.78 (dd, J = 12, 2.5 Hz, H-6$_b$α), 3.87 (ddd, J = 9.5, 5 and 2.5 Hz, H-5α), 4.05 (dd, J = 10.5, 3.5 Hz, H-2α),

(21) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Ford, N., ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989.

Scheme VII[a]



[a] Conditions: (a) (i) PPh_3, $CH_2Cl_2H_2$ (ref 4); (ii) Ac_2O, MeOH; (iii) 80% AcOH, 78% overall yield; (b) (i) $BuNH_2$, MeOH (ref 5); (ii) Ac_2O, MeOH, 54% overall yield; (c) 2,2-dimethoxypropane, *p*-TsOH, acetone, 66%; (d) (i) DMSO, Ac_2O; (ii) $NaBH_4$, CH_2Cl_2–EtOH–H_2O (ref 1), 61% overall yield; (e) (i) $PdCl_2$, NaOAc, 95% AcOH (ref 6); (ii) Ac_2O, pyridine, 23% overall yield; (f) (i) NaOMe/MeOH; (ii) Dowex 50W-X8 [H^+], 90% overall yield; (g) DMSO, Ac_2O (ref 1); (h) (i) $PdCl_2$, NaOAc, 95% AcOH; (ii) SiO_2 chromatography, $CHCl_3$–EtOAc–MeOH, 44% overall yield; (i) (i) NaOMe, MeOH; (ii) Dowex 50W-X8 [M^+]; (iii) 2,2-dimethoxypropane, *p*-TsOH, 1,4-dioxane, 66% overall yield; (j) 60% CF_3CO_2H, 91%. (1) Ali, Y.; Richardson, A. C. *Carbohydr. Res.* **1967**, *5*, 441. (2) Lee, R. T.; Lee, Y. C. *Carbohydr. Res.* **1974**, *37*, 193. (3) Lee, R. T.; Ichikawa, Y.; Allen, H. J.; Lee, Y. C. *J. Biol. Chem.* **1990**, *265*, 7864. (4) Iwabuchi, J.; Kurokawa, T.; Gou, Z.; Hasegawa, A. *J. Carbohydr. Chem.* **1988**, *7*, 605. (5) Durette, P. L.; Meitzner, E. P.; Shen, T. Y. *Tetrahedron Lett.* **1979**, 4013. (6) Ogawa, T.; Nakabayashi, S. *Carbohydr. Res.* **1981**, *93*.

4.72 (d, J = 9 Hz, H-1β), 4.95 (dd, J = 10.5, 9 Hz, H-3β), 5.07 (d, J = 3.5 Hz, H-1α), 5.18 (dd, J = 10.5, 9 Hz, H-3α); ^{13}C NMR (D_2O) 21.3, 22.7 ($2CH_3$), 53.2 (C-2), 61.3 (C-6), 68.7, 72.3, 74.8, 76.7 (C-3, C-4, C-5), 91.9, 95.9 (C-1), 174.8, 175.3 (2CO).

2-Acetamido-2-deoxy-3-*O*-propyl-D-glucopyranose, 1h. Compound **13b** (440 mg) was dissolved in 10 mL of ethanol and 5 mL of cyclohexane. One hundred milligrams of PdO was added, and the mixture was refluxed for 16 h. The catalyst was removed by filtration, and the filtrate was evaporated. After column chromatography with silica gel ($CHCl_3$/methanol/hexane 6:2:1), **1h** was obtained (120 mg, 46%): ^1H NMR (D_2O) 0.92 (t, J = 7 Hz, 3 H, CH_3), 1.56 (m, 2 H, H-2′$_2$), 1.96 (s, 3 H, NHAc), 3.23–3.85 (m, 6 H, H-3, 4, 5, 6, 1′), 3.93 (dd, J = 9 Hz, J = 4 Hz, H-2α), 4.58 (d, J = 8 Hz, H-1β), 5.02 (d, J = 4 Hz, H-1α).

Methyl 2-Acetamido-3-*O*-allyl-2-deoxy-α-D-glucopyranoside, 1g. Compound **16** (500 mg, 1.15 mmol) was dissolved in 4 mL of concentrated acetic acid. The solution was stirred for 12 h at 80 °C. After evaporation of the acetic acid the residue was purified by column chromatography (ethyl acetate/methanol 20:1) on silica gel to yield 157 mg (50%) of **1g**: ^1H NMR (pyridine-d_5) 2.16 (s, 3 H, NHAc), 3.32 (s, 3 H, Ar-OMe), 3.66 (s, 3 H, C-1-OMe), 3.80–4.08 (m, 5 H, 3-H, 4-H, 5-H, 6-H_2), 4.35 (dd, J = 9.5 Hz, J = 4.5 Hz, 1 H, 2-H), 4.16–4.60 (m, 2 H, CH_2-allyl), 4.78–4.90 (m, 1 H, H-1), 5.04–5.40 (m, 2 H, CH_2=C), 5.74 (s, 1 H, OCHO), 5.90–6.10 (m, 1 H, CH=C), 7.00–7.70 (m, 4 H, Ar), 8.95 (d, J = 9 Hz, NH).

Allyl 2-Acetamido-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside, 18. *p*-Methoxy benzaldehyde (30 mL) was treated with 5 g of dry $ZnCl_2$. After stirring for 1 h, **17** (4 g, 15 mmol) was added to the mixture at room temperature. The reaction mixture was stirred for 16 h and then treated with 100 mL of $CHCl_3$ and 100 mL of water. The combined organic solutions were washed with 100 mL of brine and dried over $MgSO_4$. The solvent was removed in vacuo. The product was recrystallized from ethyl acetate/hexane to yield 4.2 g (74%) of **18**: ^1H NMR (pyridine-d_5) δ 2.08 (s, 3 H, NHAc), 3.67 (s, 3 H, OCH_3), 3.70 (ddd, J = 9 Hz, J = 4 Hz, J = 2 Hz, 1 H, 5-H), 3.98–4.03 (m, 2 H, 6-H_2), 4.27 (ddd, J = 12.5 Hz, J = 5 Hz, J = 1 Hz, 1 H, $CH_{2a}C$=C), 4.46 (dd, J = 12 Hz, J = 4 Hz, 1 H, $CH_{2b}C$=C), 4.52–4.70 (m, 3 H, 2-H, 3-H, 4-H), 5.13 (d, J = 8.5 Hz, 1 H, 1-H), 5.18 (dd, J = 9 Hz, J = 1 Hz, 1 H, CH_{2a}=C), 5.48 (dd, J = 17 Hz, J = 2 Hz, 1 H, CH_{2b}=C), 5.77 (s, 1 H, OCHO), 5.98–6.14 (m, 1 H, CH=C), 6.95–7.60 (m, 4 H, CH, Ar), 9.10 (d, J = 8 Hz, 1 H, NH).

Allyl 2-Acetamido-2-deoxy-3-*O*-(methoxycarbonyl)-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside, 19a. Compound **18** (400 mg, 1.06 mmol) was dissolved in 3 mL of pyridine. At 0 °C, 380 mg (4 equiv) of methyl chloroformate were added. After stirring for 14 h, the reaction was quenched with ice water. The precipitate was filtered off and washed extensively with water and ether. The remaining solid was dried and used without further purification: yield, 395 mg (86.5%); ^1H NMR (pyridine-d_5) δ 2.12 (s, 3 H, $NCOCH_3$), 3.60 (s, 3 H, $COOCH_3$), 3.66–3.80 (m, 1 H, 5-H), 3.85–4.08 (m, 2 H, 6-H_2), 4.23–4.47 (m, 2 H, CH_2C=C), 4.45–4.55 (m, 1 H, 4-H), 4.62 (ddd, J = 9 Hz, J = 8 Hz, 1 H, 1-H), 5.15–5.50 (m, 2 H, CH_2=C), 5.20 (d, J = 8 Hz, 1 H, 1-H), 5.70 (s, 1 H, OCHO), 5.84 (dd, J = 9.5 Hz, J = 9.5 Hz, 1 H, 3-H), 5.95 (m, 1 H, CH=C), 6.90–7.6 (m, 4 H, CH, Ar), 9.40 (d, J = 8 Hz, 1 H, NH).

Allyl 2-Acetamido-3-*O*-(allyloxycarbonyl)-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside, 19b. Starting with 400 mg of **18** and 480 mg of allyl chloroformate, **19b** was obtained: yield, 347 mg, 71%; ^1H NMR (pyridine-d_5) δ 2.16 (s, 3 H, $COCH_3$), 3.65 (s, 3 H, OCH_3), 3.65–3.75 (m, 1 H, 5-H), 3.84–3.94 (m, 2 H, 6-H_2), 4.20–4.65 (m, 6 H, 2-H, 4-H, $2CH_2C$=C), 4.95–5.49 (m, 5 H, $2CH_2$=C, 3-H), 5.22 (d, J = 8 Hz, 1 H, 1-H), 5.72 (s, 1 H, OCHO), 5.95–6.13 (m, 2 H, 2CH=C), 7.00–7.70 (m, 4 H, CH, Ar), 9.20 (d, J = 8 Hz, 1 H, NH).

Allyl 2-Acetamido-2-deoxy-3-*O*-(methoxymethyl)-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside, 19c. To a solution of compound **18** (400 mg, 1.05 mmol) in 10 mL of THF was added 50 mg of NaH (60% in oil) at 0 °C. After stirring for 1 h, the reaction mixture was treated with 170 mg of chloromethyl ether. The reaction was complete after 16 h and was quenched with water. The precipitate was filtered

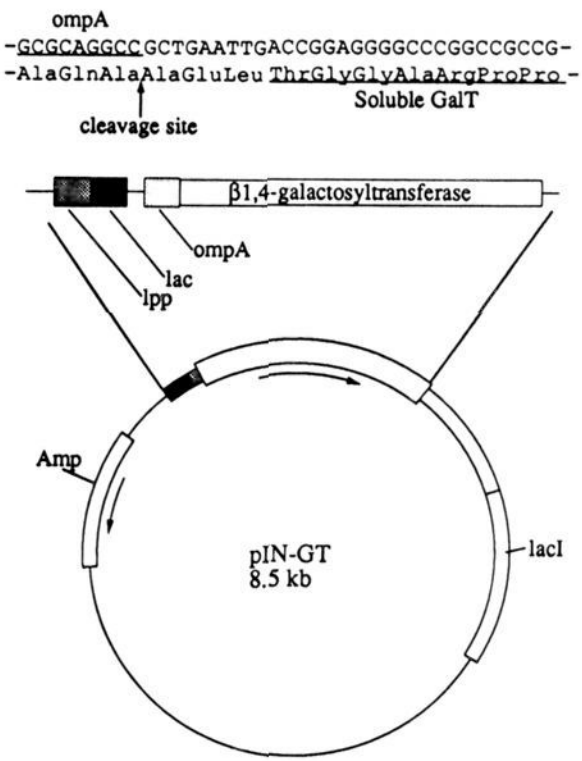


Figure 3. The plasmid pIN-GT containing the active domain sequence of β-1,4-galactosyltransferase fused with the signal sequence of ompA was transformed into *E. coli* JM109 for expression of the enzyme.

off and washed with water and ether and was used without further purification: yield, 285 mg, 64%; ^{1}H NMR (pyridine-d_5) δ 2.16 (s, 3 H, NHAc), 3.45 (s, 3 H, OCH_3), 3.65 (s, 3 H, Ar-C-OCH_3), 3.55–3.70 (m, 1 H, 5-H), 3.80–3.94 (m, 2 H, $6H_2$), 4.18–4.55 (m, 4 H, 2-H, 4-H, CH_2C=C), 4.80–5.45 (m, 6 H, CH_2=C, OCH_2O, 1-H, 3-H), 5.66 (s, 1 H, OCHO), 5.92–6.10 (m, 1 H, CH=C), 6.90–7.65 (m, 4 H, CH, Ar), 9.25 (d, J = 8 Hz, 1 H, NH).

General Procedure for the Hydrolysis of *p*-Methoxybenzylidene Acetals. An acetal derivative (0.5 mmol) was treated with 2 mL of glacial acetic acid and stirred for 5 h at 60 °C. The acetic acid was removed in vacuo, and the crude product was purified by column chromatography (silica gel, chloroform/MeOH/hexane = 6:1:1).

Allyl 2-Acetamido-2-deoxy-3-*O*-(methoxycarbonyl)-β-D-glucopyranoside, 1k. ^{1}H NMR (D_2O) δ 1.82 (s, 3 H, NHAc), 3.38 (ddd, J = 10 Hz, J = 4.5 Hz, J = 2 Hz, 1 H, 5-H), 3.53 (dd, J = 9.5 Hz, J = 9 Hz, 1 H, 2-H), 3.56–3.82 (m, 3 H, 4-H, $6H_2$), 4.02 (dd, J = 13 Hz, J = 6 Hz, 1 H, $CH_{2a}C$=C), 4.20 (dd, J = 13 Hz, J = 5 Hz, 1 H, $CH_{2b}C$=C), 4.57 (d, J = 8 Hz, 1 H, 1-H), 4.66 (dd, J = 10 Hz, J = 9.5 Hz, 1 H, 3-H), 5.07–5.22 (m, 2 H, CH_2=C), 5.62–5.83 (m, 1 H, CH=C); yield, 112 mg, 70%.

Allyl 2-Acetamido-3-*O*-(allyloxycarbonyl)-2-deoxy-β-D-glucopyranoside, 1l. ^{1}H NMR (D_2O) δ 1.76 (s, 3 H, NHAc), 3.28–3.38 (m, 1 H, 5-H), 3.42–3.76 (m, 4 H, 2-H, 4-H, $6H_2$), 3.96 (dd, J = 13 Hz, J = 6 Hz, 1 H, $CH_{2a}C$=C), 4.14 (dd, J = 13 Hz, J = 5 Hz, 1 H, $CH_{2b}C$=C), 4.50 (d, J = 8 Hz, 1 H, 1-H), 4.35–4.70 (m, 2 H, CH_2-C=C, alloc), 5.0–5.18 (m, 5 H, $2CH_2$=C, 3-H), 5.60–5.84 (m, 2 H, CH=C); yield, 108 mg, 66%.

Allyl 2-Acetamido-2-deoxy-3-*O*-(methoxymethyl)-β-D-glucopyranoside, 1m. ^{1}H NMR (D_2O) δ 1.80 (s, 3 H, NHAc), 3.14 (s, 3 H, OCH_3), 3.20–3.70 (m, 4 H, 3-H, 4-H, $6H_2$), 3.92 (dd, J = 13 Hz, J = 6 Hz, 1 H, $CH_{2a}C$=C), 4.10 (dd, J = 13 Hz, J = 5 Hz, 1 H, $CH_{2b}C$=C), 4.35 (d, J = 8 Hz, 1 H, 1-H), 4.50 (d, J = 7.5 Hz, 1 H, $OCH_{2a}O$), 4.60 (d, J = 7.5 Hz, 1 H, $OCH_{2b}O$), 4.98–5.14 (m, 2 H, CH_2=C), 5.57–5.74 (m, 1 H, CH=C); yield; 110 mg, 72%.

Allyl 2-Acetamido-2-deoxy-3-*O*-(monochloroacetyl)-α-D-glucopyranoside. ^{1}H NMR (D_2O) δ 1.81 (s, 3 H, NHAc), 4.08–4.10 (m, 2 H, $COCH_2Cl$), 4.78 (d, 1 H, J = 3.60 Hz, H-1), 5.08 (dd, 1 H, J = 9.12 and 10.32 Hz, H-3).

Allyl 2-Acetamido-2-deoxy-6-*O*-(monochloroacetyl)-α-D-glucopyranoside. ^{1}H NMR (D_2O) δ 1.88 (s, 3 H, NHAc), 3.38 (dd, 1 H, J = 9.07 and 9.56 Hz, H-4), 3.59 (dd, 1 H, J = 9.07 and 10.34 Hz, H-3), 3.78 (dd, 1 H, J = 3.78 and 10.34 Hz, H-2), 3.75–3.83 (m, 1 H, H-5), 4.26 (dd, 1 H, J = 5.04 and 12.10 Hz, H-6a), 4.37 (dd, 1 H, J = 2.52 and 12.10 Hz, H-6b), 4.62 (s, 2 H, $COCH_2Cl$), 4.74 (d, 1 H, J = 3.58 Hz, H-1).

Allyl 2-Acetamido-6-*O*-(methoxycarbonyl)-2-deoxy-β-D-glucopyranoside, 20a. ^{1}H NMR (D_2O) δ 1.87 (s, 3 H, NHAc), 3.30–3.42 (m, 2 H, 2-H, 3-H), 3.50 (dd, J = 10 Hz, J = 9 Hz, 1 H, 4-H), 3.66 (s, 3 H, OCH_3), 3.97 (dd, J = 12.5 Hz, J = 6 Hz, 1 H, $CH_{2a}C$=C), 4.13 (dd, J = 12.5 Hz, J = 5 Hz, 1 H, $CH_{2b}C$=C), 4.20 (dd, J = 12 Hz, J = 5 Hz, 1 H, $6H_{2a}$), 4.35 (dd, J = 12 Hz, J = 2.5 Hz, 1 H, $6H_{2b}$), 4.42 (d, J = 8.5 Hz, 1 H, 1-H), 5.08 (dd, J = 6.5 Hz, J = 1.5 Hz, 1 H, CH_{2a}=C), 5.14 (dd, J = 14 Hz, J = 1.5 Hz, 1 H, CH_{2b}=C), 5.55–5.71 (m, 1 H, CH=C).

Methyl 2-Acetamido-2-deoxy-α-D-allopyranoside, 1s. A solution of methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (200 mg, 0.65 mmol) and PPh_3 (208 mg, 0.79 mmol) in CH_2Cl_2 (10 mL) and water (0.5 mL) was stirred for 4 h at room temperature, and the mixture was concentrated in vacuo. Ac_2O (2 mL) was added to a solution of the residue in MeOH (10 mL) at 0–5 °C, and the mixture was stirred for 2 h at 0–5 °C. After the mixture was concentrated, the residue was chromatographed on silica gel, with toluene–EtOAc (1:2), to give 4,6-*O*-benzylidene derivative of **21** (110 mg, 72%), which was treated with 80% AcOH (10 mL) for 3 h at 80 °C. After the mixture was concentrated and dissolved in Et_2O and water, the aqueous layer was washed with Et_2O, and the aqueous layer was concentrated. The residue was triturated with MeOH and Et_2O to give **1s** (50 mg, 45%): $[\alpha]_D$ +77.9° (*c* 0.68, H_2O); ^{1}H NMR (D_2O) δ 2.00 (3 H, s, NHAc), 3.33 (3 H, s, OMe), 3.62 (1 H, dd, J = 2.64, 9.39 Hz, H-4), 3.69–3.89 (3 H, m, H-5, 6a, 6b), 3.96–4.02 (2 H, m, H-2, 3), 4.71 (1 H, d, J = 3.76 Hz, H-1); ^{13}C NMR (D_2O) δ 22.22, 50.29, 55.84, 61.19, 66.44, 67.33, 69.63, 98.20, 174.19.

Allyl 2-Acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-glucopyranoside, 24. A solution of allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**23**) (2.95 g, 11.3 mmol), 2,2-dimethoxypropane (2.35 g, 22.6 mmol, 2.78 mL), and *p*-toluenesulfonic acid monohydrate (172 mg, 0.90 mmol) in acetone (80 mL) was stirred for 2 days at room temperature. During this time, another 2,2-dimethoxypropane (2.35 g, 22.6 mmol, 2.78 mL) was added to the mixture. After the addition of Et_3N (1 mL), the mixture was concentrated in vacuo. The residue was chromatographed on silica gel, with toluene–EtOAc (1:2∼1:3) to give **24** (2.26 g, 66%): mp 108.5–109.0 °C (from EtOAc–hexane); ^{1}H NMR ($CDCl_3$) δ 1.44, 1.53 (3 H, s, CH_3), 2.04 (3 H, s, NHAc), 4.84 (1 H, d, J = 3.78 Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 19.01, 23.24, 29.01, 54.00, 62.14, 63.44, 68.34, 70.56, 74.62, 96.94, 99.87, 118.05, 133.29, 171.37; HRMS calcd for $C_{14}H_{23}NO_6$ (M^+) 434.0580, found 434.0600.

Allyl 2-Acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-allopyranoside, 25. A mixture of **24** (1.0 g, 3.32 mmol) and Ac_2O (5 mL) in DMSO (10 mL) was stirred for 10 h at room temperature and poured into ice-cold aqueous NaOAc. The mixture was stirred for 3 h and extracted with $CHCl_3$. The extracts were successively washed with aqueous $NaHCO_3$ and water, dried over anhydrous $MgSO_4$, and concentrated. $NaBH_4$ (380 mg, 10.1 mmol) was added to a cooled solution of the residue in CH_2Cl_2 (10 mL), EtOH (10 mL), and water (2 mL) at 0–5 °C, and the mixture was stirred for 20 min at 0–5 °C. To the mixture were added acetone (5 mL) and saturated NH_4Cl (5 mL), and the mixture was stirred for 10 min. The mixture was concentrated, the residue was dissolved in $CHCl_3$ and water, and the aqueous layer was extracted with $CHCl_3$. The extracts were washed with water, dried over anhydrous $MgSO_4$, and concentrated. The residue was chromatographed on silica gel, with toluene–EtOAc (1:3), to give **25** (612 mg, 61%): mp 113.5–114.5 °C (from EtOAc–hexane); ^{1}H NMR ($CDCl_3$) δ 1.45, 1.52 (3 H, s, CH_3), 2.04 (3 H, s, NHAc), 2.78 (1 H, d, J = 6.78 Hz, OH), 3.68 (1 H, dd, J = 2.77, 9.70 Hz, H-6a), 3.73–3.84 (1 H, m), 3.90–4.04 (4 H, m, H-3, 4, 6b, allylic), 4.24 (1 H, br dt, J = 3.52, 8.97 Hz, H-2), 4.86 (1 H, d, J = 3.97 Hz, H-1), 5.21–5.34 (2 H, m, vinylic of allyl), 5.81–5.87 (1 H, m, vinylic of allyl), 6.38 (1 H, d, J = 9.13 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 19.01, 23.16, 28.95, 49.42, 58.36, 62.33, 68.38, 69.06, 71.06, 97.15, 99.62, 118.32, 133.17, 169.66; HRMS calcd for $C_{14}H_{23}NO_6Cs$ (M + Cs^+) 434.0580, found 434.0551.

2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-allopyranose, 26. A mixture of **25** (489 mg, 1.62 mmol), $PdCl_2$ (317 mg, 1.79 mmol), and NaOAc (320 mg, 3.90 mmol) in AcOH (10 mL) and water (0.5 mL) was heated at 80 °C for 10 h. After cooling, the mixture was filtrated through a Celite pad, and the filtrate was concentrated. The residue was chromatographed on silica gel, with $CHCl_3$–EtOAc–MeOH (5:2:1), to give the main product, which was acetylated with Ac_2O (5 mL) and pyridine (5 mL) to afford **26** (142 mg, 23%) after recrystallization from EtOAc–hexane: mp 170.5–171.0 °C; ^{1}H NMR ($CDCl_3$) δ 1.96, 1.98, 2.08, 2.13, 2.18 (3 H, s, 4 × OAc, NHAc), 4.08–4.27 (3 H, m, H-5, 6a, 6b), 4.48 (1 H, dt, J = 2.98, 9.20 Hz, H-2), 4.98 (1 H, dd, J = 2.81, 9.87 Hz, H-4), 5.57 (1 H, br t, J = 2.93 Hz, H-3), 5.56–5.61 (1 H, br s, NH), 5.89 (1 H, d, J = 8.72 Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 20.42, 20.71,

20.93, 23.01, 49.39, 61,94, 66.18, 69.62, 70,96, 91.35, 169.04, 169.51, 169.71, 169.98, 170.68; HRMS calcd for $C_{16}H_{23}NOCs$ (M + Cs^+) 522.0376, found 522.0376.

2-Acetamido-2-deoxy-D-allopyranose, 1o. A mixture of **26** (77 mg, 0.20 mmol) and methanolic NaOMe (1 mL, M solution) in MeOH (10 mL) was stirred for 3 h at room temperature and was neutralized with Dowex 50W-X8 [H^+] resin. After the resin was removed by filtration, the filtrate was concentrated. The residue was triturated with MeOH and Et_2O to give **1o** (40 mg, 90%) as a fluffy solid (α/β = 1:2.8): ^1H NMR (D_2O) δ 2.00 (s, NHAc of β-isomer), 2.02 (s, NHAc of α-isomer), 4.91 (d, J = 8.72 Hz, H-1 of β-isomer), 5.09 (d, J = 3.50 Hz, H-1 of α-isomer); ^{13}C NMR (D_2O) δ (β-isomer) 54.7, 61.6, 66.9, 70.2, 74.2, 92.8, 171.5; HRMS calcd for $C_8H_{15}NO_6Cs$ (M + Cs^+) 353.9954, found 353.9975.

Allyl 2-Acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-glucopyran-3-uloside, 27. A mixture of **24** (690 mg, 2.3 mmol) and Ac_2O (5 mL) in DMSO (10 mL) was stirred for 10 h at room temperature and poured into ice-cold aqueous NaOAc. The mixture was stirred for 3 h at room temperature and extracted with $CHCl_3$. The extracts were successively washed with aqueous $NaHCO_3$ and water, dried over anhydrous $MgSO_4$, and concentrated. The residue was chromatographed on silica gel, with toluene–EtOAc (1:4), to give the product, which was crystallized from EtOAc–hexane, to give **27** (343 mg, 50%): mp 156.0–156.5 °C; ^1H NMR ($CDCl_3$) δ 1.51, 1.53 (3 H, s, CH_3), 2.07 (3 H, s, NHAc), 3.92–4.03 (4 H, H-5, 6a, 6b, allylic), 4.11–4.20 (1 H, m, allylic), 4.39–4.51 (1 H, m, H-4), 4.95 (1 H, ddd, J = 1.05, 4.25, 7.98 Hz, H-2), 5.18–5.34 (2 H, m, vinylic of allyl), 5.32 (1 H, d, J = 4.25 Hz, H-1), 5.73–5.91 (1 H, m, vinylic of allyl), 6.34 (1 H, d, J = 7.98 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 18.72, 22.98, 28.77, 58.72, 62.65, 66.94, 68.80, 76.15, 100.15, 100.46, 118.32, 132.73, 170.04, 196.30; HRMS calcd for $C_{14}H_{21}NO_6Cs$ (M + Cs^+): 432.0423, found 432.0438.

Methyl 2-Acetamido-2-deoxy-D-glucopyran-3-uloside, 1q. A mixture of **27** (170 mg, 0.57 mmol), $PdCl_2$ (121 mg, 0.68 mmol), NaOAc (112 mg, 1.36 mmol) in acetic acid (10 mL), and water (0.5 mL) was heated at 80 °C for 10 h. After cooling, the mixture was filtered through a Celite pad, and the filtrate was concentrated. The residue was chromatographed on silica gel, with $CHCl_3$–EtOAc–MeOH (5:2:1), during the column chromatography methyl glycoside was formed, to give the product, which was dissolved in water and lyophilized to give **1q** (55 mg, 44%): ^1H NMR (D_2O) δ 2.042 (3 H, s, NHAc), 3.36 (3 H, s, OMe), 3.76–3.94 (3 H, m, H-5, 6a, 6b), 4.43 (1 H, dd, J = 0.95, 9.58 Hz, H-4), 4.90 (1 H, dd, J = 0.98, 4.12 Hz, H-2), 5.16 (1 H, d, J = 4.08 Hz, H-1); ^{13}C NMR (D_2O) δ 22.00, 55.68, 59.53, 61.00, 72.49, 75.08, 100.85, 174.83, 204.65; HRMS calcd for $C_9H_{15}NO_6Cs$ (M + Cs^+) 365.9954, found 365.9960.

Allyl 2-Acetamido-2,3-dideoxy-β-D-glucopyranoside, 1i. A mixture of allyl 2,3-dideoxy-2-phthalimido-β-D-glucopyranoside **22** (100 mg, 0.30 mmol) and $BuNH_2$ (4 mL) in MeOH (20 mL) was refluxed for 10 h, then cooled, and evaporated. Ac_2O (2 mL) was added to a solution of the residue in MeOH (10 mL) at 0–5 °C, and the mixture was stirred for 3 h at 0–5 °C. The mixture was concentrated, and the residue was triturated in MeOH with Et_2O to give **1i** (40 mg, 54%): ^1H NMR (D_2O) δ 1.52 (1 H, q, J = 12.35 Hz, H-3ax), 1.98 (3 H, s, NHAc), 2.25 (1 H, dt, J = 4.76, 12.38 Hz, H-3eq), 3.39 (1 H, ddd, J = 2.30, 6.44, 9.45 Hz, H-5), 3.59 (1 H, dt, J = 4.79, 9.45 Hz, H-4), 3.65 (1 H, dd, J = 6.44, 12.30 Hz, H-6a), 3.73 (1 H, ddd, J = 4.76, 8.45, 12.90 Hz, H-2), 3.84 (1 H, dd, J = 2.30, 12.30 Hz, H-6b), 4.49 (1 H, J = 8.45 Hz, H-1); ^{13}C NMR (D_2O) δ 22.30, 36.52, 49.24, 61.22, 64.57, 80.09, 102.03, 118.54, 133.67, 174.03; HRMS calcd for $C_{11}H_{19}NO_5Cs$ (M + Cs^+) 378.0318, found 378.0318.

β-D-Galactopyranosyl-(1→4)-2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose, 2e. 3-*O*-AcetylGlcNAc (20 mg, 76 μmol) and 1 equiv of UDPGlc (50.5 mg) were dissolved in 1 mL of 0.05 M Na-cacodylate/HCl containing 0.05 mM NAD^+, 10 mM DTT, and 5 mM $MnCl_2$ pH 7.0. UDPGlc epimerase (1 U) and galactosyltransferase (2 U) were added. The mixture was shaken at 37 °C, and after 2 days another 2 U of galactosyltransferase was added. After 4 days the solution was lyophilized, and the residue was purified with a silica gel column chromatography to obtain 6 mg of **2e** (19%, 43% based on recovered starting material). Purification with Bio Gel P-2 column is sufficient for the other disaccharide derivatives: ^1H NMR (D_2O) δ 1.98 (s, 3 H, NAc), 2.08 (s, 3 H, OAc), 3.50–3.57 (m, 1 H, 2-H, (GlcNAc), 3.63–3.82 (m, 6 H, 2′-H (Gal), 3′-H, 4′-H, 5′-H, 3-H, 4-H), 3.85–3.97 (m, 2 H, 6′-H_2), 4.01 (ddd, J = 7 Hz, J = 4 Hz, J = 2.2 Hz, 5-Hα), 4.10–4.19 (m, 5-Hβ), 4.21 (dd, J = 12 Hz, J = 4 Hz, 6-H_aα), 4.22 (d, J = 7.8 Hz, 1-Hα), 4.23 (d, J = 7.8 Hz, 1-Hβ), 4.29 (dd, J = 2.2 Hz, J = 12 Hz, 6-H_bβ), 4.35–4.48 (m, 6-H_2β); ^{13}C NMR 21.0, 22.7 ($2CH_3$), 54.4, 56.8 (C-2′), 61.8, 63.8 (C-6, C-6′), 69.3, 70.1, 71.7, 73.3, 76.3 (C-2′, C-3′, C-4′, C-5′, C-3, C-4, C-5), 79.4 (C-4), 91.4, 96.1 (C-1), 104.0 (C-1′), 174.5, 175.0 (2CO); HRMS calcd for ($C_{12}H_{27}O_{12}N$ + Cs^+) 558.0588, found 558.0590.

Allyl β-D-Galactopyranosyl-(1→4)-2-acetamido-2,3-dideoxy-β-D-glucopyranoside, 2i. ^1H NMR (D_2O) δ 1.62 (1 H, q, J = 12.18 Hz, H-3ax), 1.94 (3 H, s, NHAc), 2.46 (1 H, br dt, J = 4.61, 12.57 Hz, H-3eq), 3.44 (1 H, dd, J = 8.04, 9.92 Hz, H-2′), 4.41 (1 H, d, J = 7.82 Hz, H-1′), 4.51 (1 H, d, J = 8.38 Hz, H-1); ^{13}C NMR (D_2O) δ 22.45, 35.78, 49.23, 61.03, 61.26, 68.89, 70.55, 71.26, 73.03, 74.19, 75.52, 78.85, 102.01, 104.21, 118.60, 133.81, 174.21; HRMS calcd for $C_{17}H_{29}NO_{10}Cs$ (M + Cs^+) 540.0846, found 540.0846.

β-D-Galactopyranosyl-(1→4)-5-thio-D-glucopyranose, 7. ^1H NMR, ^{13}C, and HRMS data are identical to those reported previously.[8b]

β-D-Galactopyranosyl-(1→4)-D-Glucal, 8. ^1H NMR, ^{13}C, and HRMS data are the same as reported previously.[8b]

β-D-Galactopyranosyl-(1→4)deoxynojirimycin, 10a. ^1H NMR, ^{13}C, and HRMS data are the same as reported previously.[8b]

β-D-Galactopyranosyl-(1→6)-5-thio-D-glucopyranose, 9. β-Galactosidase from *E. coli* (EC 3.2.1.23: 0.50 mg, 172 U) was added at 23 °C to a solution of 4-nitrophenyl β-D-galactopyranoside (150 mg, 0.50 mmol) and 5-thio-D-glucose (49 mg, 0.25 mmol) in $Na_2HPO_4/MgCl_2$ buffer (4 mL of a 0.10 M solution in Na_2HPO_4 and 10 mM in $MgCl_2$, pH 7.0) and Tris (1 mL of a 0.05 M solution, pH 7.3). The reaction was maintained at 23 °C with periodic monitoring by TLC. After 58 h, the reaction was terminated by heating at 100 °C for 30 min. The solution was filtered and lyophilized, and the residue was purified by column chromatography (silica gel, 3:2:1 ethyl acetate–acetic acid–water). The fraction containing the disaccharide was further purified by gel filtration chromatography with a Bio-Gel P-2 column (2 × 40 cm, 200–400 mesh) eluted with H_2O to afford the disaccharide **9** (26.4 mg, 29.5% based on 5-thio-D-glucose) as a white amorphous solid (R_f 0.44, silica gel, 3:2:1 EtOAc–HOAc–H_2O). The silica gel chromatography also afforded galactose and 5-thio-D-glucose. Analysis of the disaccharide indicated a mixture of α to β anomers in a ratio of 11:1: α anomer ("A" refers to the 5-thioglucose moiety while "B" refers to the galactose moiety). (The differences in coupling constants are due to roundoff error:) ^1H NMR (500 MHz, D_2O) δ 4.82 (d, J = 3.0 Hz, 1 H, H1-A), 4.22 (d, J = 8.0 Hz, 1 H, H1-B), 4.01 (dd, J = 2.5, 11.0 Hz, 1 H, H6-A), 3.82 (dd, J = 5.5, 11.0 Hz, 1 H, H6-A), 3.74 (d, J = 3.0 Hz, 1 H, H4-B), 3.63–3.54 (m, 3 H, H2-A, H3-A, H5-B), 3.50 (t, J = 10.5 Hz, 1 H, H4-A), 3.48–3.45 (m, 2 H, H6-B), 3.46 (dd, J = 3.5, 10.0 Hz, 1 H, H3-B), 3.36 (app t, J = 9.0 Hz, 1 H, H2-B), 3.20–3.14 (m, 1 H, H5-A); ^{13}C NMR (125 MHz, D_2O) δ 103.8, 75.6, 74.0, 73.7, 73.6, 73.1, 71.1, 69.1, 68.8 (CH_2), 61.4 (CH_2), 41.6; exact mass calcd for $C_{12}H_{22}O_{10}SCs$ (M + Cs^+) 490.9988, found 491.0013.

NOE Experiment: Irradiation of the H1-B resonance at δ 4.82 ppm afforded a 4% enhancement of the H6-A resonance at δ 3.82 ppm.

β-D-Galactopyranosyl-(1→6)-5-thio-D-glucopyranose Octaacetate. Pyridine (0.9 mL, 11.1 mmol), Ac_2O (0.14 mL, 1.48 mmol), and disaccharide **1** (33 mg, 0.09 mmol) were combined at 0 °C. The reaction mixture was allowed to warm to 23 °C, maintained for 23 h, and diluted with ethyl acetate (10 mL). The organic phase was rinsed with 1 N HCl (10 mL), and the acidic fraction was extracted with ethyl acetate (2 × 20 mL). The combined organic phases were rinsed with brine (10 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (silica gel, 3:1 EtOAc–toluene) to afford the peracetylated disaccharide **2** (24 mg, 93%) as a colorless glass (R_f 0.61, silica gel, 3:1 EtOAc–toluene). Analysis of the disaccharide indicated a mixture of α to β anomers in a ratio of 6:1. α Anomer: ^1H NMR (500 MHz, $CDCl_3$) δ 6.12 (d, J = 3.5 Hz, 1 H, H1-A), 5.42 (app t, J = 10.0 Hz, 1 H, H3-A), 5.38 (dd, J = 1.0, 3.5 Hz, 1 H, H4-B), 5.21 (dd, J = 3.0, 10.0 Hz, 1 H, H2-A), 5.19 (app t, J = 11.0 Hz, 1 H, H4-A), 5.18 (dd, J = 8.0, 10.5 Hz, 1 H, H2-B), 4.99 (dd, J = 3.5, 10.5 Hz, 1 H, H3-B), 4.40 (d, J = 9.0 Hz, 1 H, H1-B), 4.16 (dd, J = 6.5, 11.0 Hz, 1 H, H6-B), 4.10 (dd, J = 6.5, 11.0 Hz, 1 H, H6-B), 4.05 (dd, J = 3.5, 10.0 Hz, 1 H, H6-A), 3.88 (dt, J = 1.0, 7.0 Hz, 1 H, H5-B), 3.57 (ddd, J = 3.5, 7.0, 10.5 Hz, 1 H, H5-A), 3.49 (dd, J = 7.5, 10.5 Hz, 1 H, H6-A), 2.18 (s, 3 H, OAc), 2.14 (s, 3 H, OAc), 2.09 (s, 3 H, OAc), 2.052 (s, 3 H, OAc), 2.046 (s, 3 H, OAc), 2.01 (s, 3 H, OAc), 1.99 (s, 3 H, OAc), 1.98 (s, 3 H, OAc); ^{13}C NMR (62 MHz, $CDCl_3$) δ 170.3, 170.2, 170.1, 169.7, 169.6, 169.4, 169.1, 101.2, 73.1, 72.3, 70.8, 70.4, 68.1, 67.3 (CH_2), 66.8, 61.0 (CH_2), 40.8, 20.9, 20.8, 20.6, 20.5; exact mass calcd for $C_{28}H_{38}O_{18}SCs$ (M + Cs^+) 827.0833, found 827.0823.

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Supplementary Material Available: Assigned ^1H NMR spectra of **2e, 2i, 7, 8, 9,** and **10b** (6 pages). Ordering information is given on any current masthead page.