Chemoenzymatic synthesis of thio-nod factor intermediates — Enzymatic transfer of glucosamine on thiochitobiose derivatives[1]

Latino Loureiro Morais, Hideya Yuasa, Khalil Bennis, Isabelle Ripoche, and France-Isabelle Auzanneau

Abstract: The chemoenzymatic syntheses of thioanalogues of nodulation factors in which the nonreducing end glucosamine residue is available for the introduction of the fatty acid moiety at the free NH_2 group are reported. We are describing the chemical synthesis of UDP-GlcNH_2 and its use in the enzymatic transfer of GlcNH_2 by the bovine galactosyltransferase (EC 2.4.1.90) onto O-4 of the nonreducing end *N*-acetylglucosamine residues of chitobiose, thiochitobiose, and allyl thiochitobioside. The enzymatic reactions on chitobiose and thiochitobiose were followed by TLC and MALDI MS and showed about 50% conversion of the disaccharides to the desired products. However, these reducing trisaccharides could not be obtained totally free of salts and degraded on ion exchange chromatography. Thus, we investigated the enzymatic transfer on the nonreducing allyl thiochitobioside analogue. We describe here the chemical synthesis of this thiodisaccharide and the enzymatic transfer of GlcNH_2 at O-4 of its nonreducing end glucosamine residue to give the desired allyl thiotrisaccharide. This thiotrisaccharide was obtained pure in 41% yield and was characterized by ^{1}H NMR (HSQC) and HRMS.

Key words: nodulation factors, synthesis, enzymatic transfer, thiooligosaccharides, UDP-glucosamine.

Résumé : On a réalisé la synthèse chimioenzymatique de thioanalogues de facteurs de nodulation dans lesquels le résidu glucosamine de l'extrémité non réductrice est disponible pour l'introduction de portions d'acides gras au niveau du groupe NH_2. On décrit la synthèse chimique de UDP-GlcNH_2 ainsi que son utilisation dans le transfert enzymatique du GlcNH_2 par la galactosyltransférase bovine (EC 2.4.1.90) sur le O-4 des résidus *N*-acétylglucosamine non réducteurs du chitobiose, du thiochitobiose et du thiochitobioside d'allyle. Les réactions enzymatiques sur le chitobiose et le thiochitobiose ont été suivies par chromatographie sur couche mince et par spectrométrie de masse MALDI et elles montrent qu'il se produit une conversion d'environ 50 % des disaccharides en produits désirés. Toutefois, on n'a pas pu éliminer complètement le sel de ces trisaccharides réducteurs et ils se dégradent lors de la chromatographie par échange d'ions. On a donc étudié le transfert enzymatique sur l'analogue thiochitobioside d'allyle non réducteur. On décrit les synthèses chimiques de ce thiodisaccharide et le transfert enzymatique du GlcNH_2 vers le O-4 du résidu glucosamine de l'extrémité non réductrice pour obtenir le thiotrisaccharide d'allyle désiré. Ce thiotrisaccharide a été obtenu à l'état pur avec un rendement de 41 % et on l'a caractérisé par RMN du ^{1}H (HSQC) et par spectrométrie de masse à haute résolution.

Mots clés : facteurs de nodulation, synthèse, transfert enzymatique, thiooligosaccharides, UDP-glucosamine.

[Traduit par la Rédaction]

Introduction

Symbiotic association (1) of leguminous plants with soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* leads to the formation of nodules on the plant roots. Within these nodules the bacteria can fix and metabolize atmospheric nitrogen into ammonia and alanine and release it for use by the host plant, which in turn supplies the

Received 6 September 2005. Published on the NRC Research Press Web site at http://canjchem.nrc.ca on 5 May 2006.

This paper is dedicated to Professor W. Szarek on his 65th birthday.

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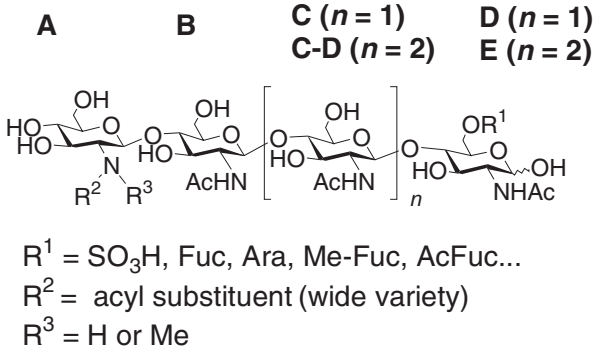
 doi:10.1139/V06-043

bacteria with various nutrients. Nodulation, the key step leading to this symbiotic association, is induced by the interaction with specific plant receptors of nodulation factors (Nod factors) produced by the bacteria (2). These lipooligosaccharides (3) are comprised of a tetra- or penta-oligomer of chitin with substitutions that determine the host specificity of the bacteria (Fig. 1, ref. 3). Although only very small concentrations (10^{-6} to 10^{-12} mol/L) of natural Nod factors are required to initiate nodulation, their activity is limited by the action of chitinases that cleave the glycosidic bond between the sugar units B and C (4). Therefore, we have developed a program (5) aimed at preparing thioanalogues of Nod factors in which a sulphur atom replaces one or more interglycosidic oxygen atoms, thus increasing the overall resistance of the lipooligosaccharide to chitinases (6). The synthesis of such oligosaccharide analogues with well-defined glycosidic linkages remains a challenge for carbohydrate chemists despite the advances made over the past decades. Therefore, we are investigating here the possibility of introducing a glucosamine unit at the nonreducing end of thioanalogues of Nod factors by enzymatic transfer (7). We report the use of the well-studied β-(1,4)-galactosyltransferase (7) isolated from bovine milk and involved in the biochemical synthesis of lactose and *N*-acetyllactosamine. The transfer of a glucosamine unit to the nonreducing end of chitotriose using the commercially available bovine galactosyltransferase (UDP-D-galactose:2-acetamido-2-deoxy-D-glucose 4-β-D-galactosyl tranferase; EC 2.4.1.90) has already been described (8). The work described here is, to our knowledge, the first enzymatic transfer to chitobiose derivatives that contain a sulphur atom in the glycosidic linkage and thus constitutes the first enzymatic synthesis of thiochitotriose derivatives. We describe herein the efficient chemical synthesis of UDP-glucosamine as well as the chemoenzymatic synthesis of new thiotrisaccharides precursors of thioanalogues of nodulation factors using this nucleotide donor and the bovine galactosyltranferase EC 2.4.1.90.

Results and discussion

As an alternative to the known (9) enzymatic preparation of UDP-$GlcNH_2$, the nucleotide donor **6** was synthesized chemically (Scheme 1) from the trifluoroacetamido derivative (**1**) obtained as described by Wolform and Conigliaro (10) from commercially available crystalline glucosamine hydrochloride. The synthetic strategy followed to prepare the key intermediate analogue (**5**) was similar to that employed by Sala et al. (11). Acetylation in pyridine – acetic anhydride gave the crude peracetate (**2**) as an anomeric mixture that contained the α anomer as the major compound. The crude peracetate was treated with hydrazine acetate to give the known (10, 11) α-hemiacetal (**3**) with an improved yield of 74% from **1** upon purification on silica gel. The hemiacetal **3** was treated with butyllithium to give the anomeric alkoxide that was reacted with dibenzyl phosphoryl chloride (12) in THF to give the anomeric dibenzylphosphate (**4**) in 54% yield, a yield similar to that described by Sala et al. (11) for the synthesis of the analogous anomeric diphenylphosphate (52%). The benzyl groups were removed by hydrogenolysis and the crude triacetylated anomeric phosphate (**5**) was activated with 1,1′-carbonyldiimidazole

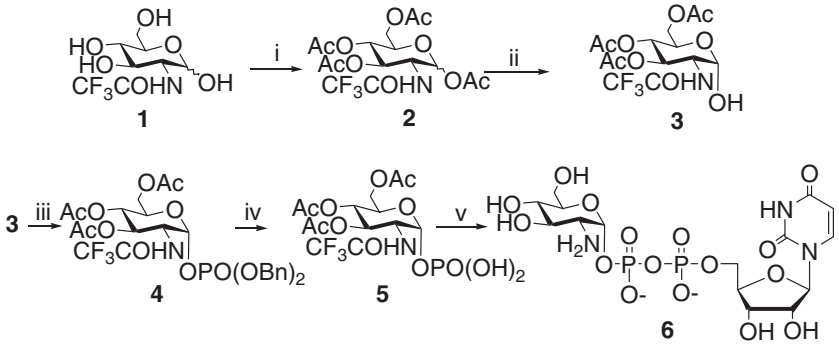
Fig. 1. Schematic representation of nodulation factors.



in acetone and coupled with the bistrioctylammonium salt of uridine 5′-monophophate dissolved in DMF. The crude adduct was in turn treated with ammonium hydroxide in methanol to remove the *O*-acetyl and *N*-trifluoroacetyl groups and the desired UDP-$GlcNH_2$ (**6**) was isolated pure in 51% yield (from **4**) following ion exchange chromatography.

The enzymatic transfer of glucosamine by the β-1,4-galactosyltransferase isolated from bovine milk (13) was first attempted on the reducing disaccharides chitobiose (**7**) and thiochitobiose (**8**) (Scheme 2) Although the products obtained in these reactions could not be purified (see the following), these reactions were used to determine the best reaction and purification conditions for the enzymatic transfer and more importantly to establish MALDI-MS conditions that can be used to follow such transfers. While chitobiose is commercially available, the known (14) thiodisaccharide (**8**) was prepared in one step through the deacetylation of the previously reported (5*b*) peracetylated thiochitobiose. The disaccharide (**8**) obtained as an anomeric mixture could not be totally purified by conventional chromatography. However, since ^{1}H NMR (Fig. 2C and ref. 14*b*) showed a purity superior than 90% and HRMS confirmed its structure, the α,β-thiochitobiose (**8**) was submitted to enzymatic transfer. The reducing chitobiose (**7**) and thiochitobiose (**8**) were incubated with UDP-$GlcNH_2$ (**6**, 1.2 equiv.) and galactosyltransferase (5.6 U) at ambient temperature with occasional gentle mixing. TLC analysis of the reaction mixture showed in both cases the formation of a new ninhydrin positive compound that was more polar than the starting material. The formation of the trisaccharides **9** and **10** was also assessed by MALDI-MS of the reaction mixtures (Fig. 3). As can be seen in Fig. 3A, we observed for the enzymatic transfer on **7** (+Na, *m*/*z* 448 ± 1) the appearance of a signal at *m*/*z* 608 ± 1 (+Na) corresponding to the desired adduct **9**. Similarly, both the starting thiochitobiose (**8**) and the adduct **10** were observed at *m*/*z* 464 ± 1 (+Na) and *m*/*z* 625 ± 1 (+Na), respectively, by MALDI-MS for the enzymatic transfer on disaccharide **8**. After 2 weeks of incubation, the conversion evaluated by TLC had reached ~50% in both cases, and the reaction mixtures were applied to a gel permeation chromatography column of Bio-Gel eluted with a 0.25 mol/L ammonium acetate solution. While this was an efficient way to separate the unreacted disaccharides **7** and **8** from the desired trisaccharides **9** and **10**, respectively, the products could not be isolated totally free of ammonium acetate even after repeated freeze drying from water. Additional attempts to remove the excess acetate ions

Scheme 1. Reagents: (*i*) Ac_2O–pyridine (crude, α/β = 94:6); (*ii*) NH_2NH_2-AcOH, DMF (74% pure α over *i* and *ii*); (*iii*) a. BuLi, THF; b. $ClPO(OBn)_2$, THF (59%); (*iv*) H_2-Pd/C, MeOH (not isolated); (*v*) a. **5**, 1,1′-carbonyldiimidazole, acetone; b. uridine 5′-monophosphate bistrioctylammonium salt, DMF; c. MeOH–NH_4OH (51% over *iv* and *v*).



Scheme 2.

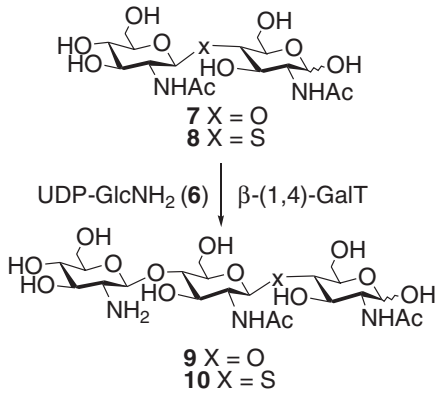
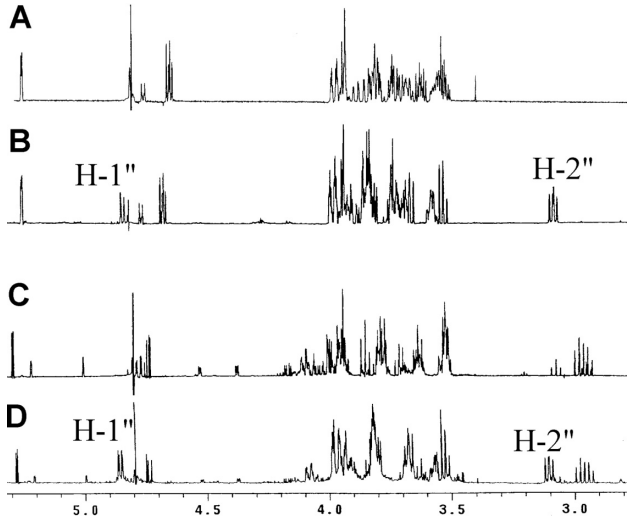


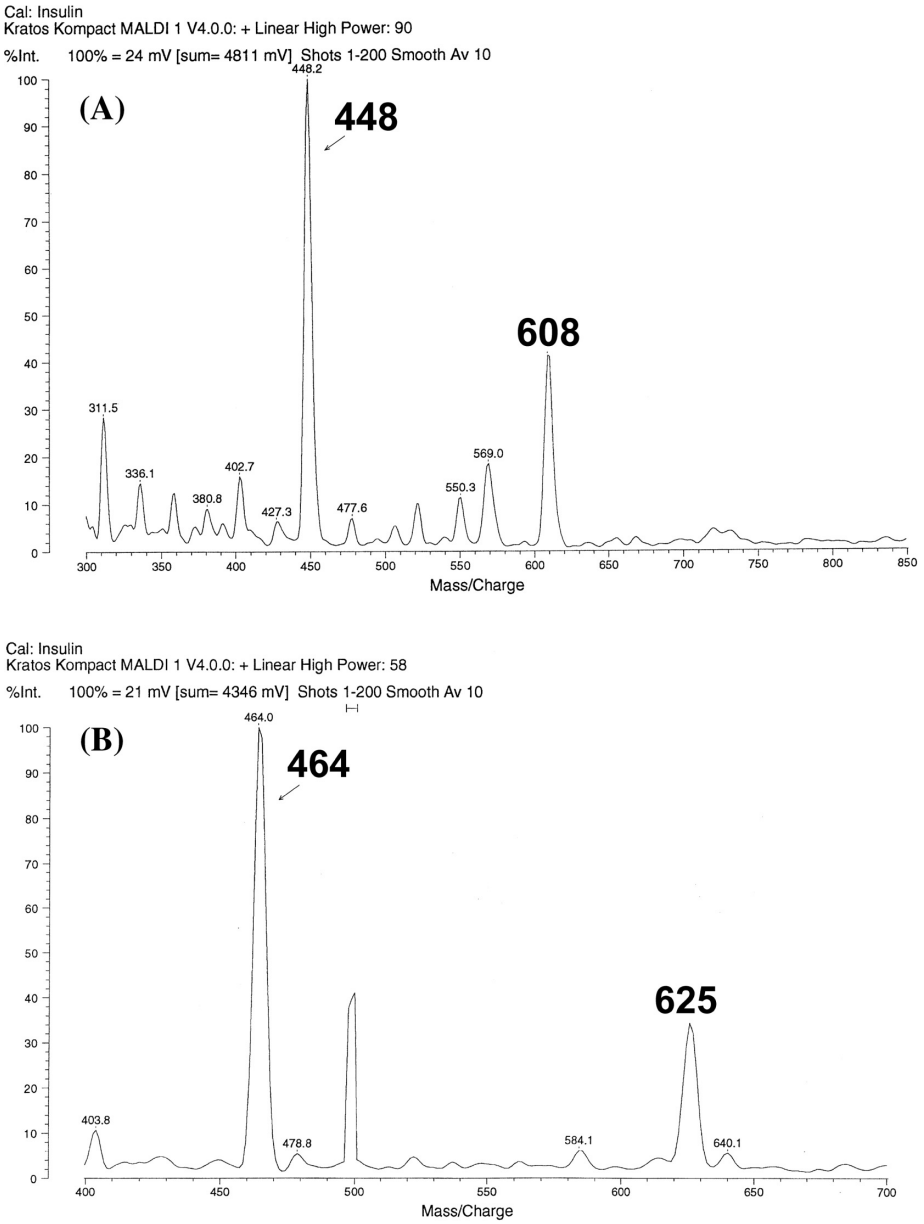
Fig. 2. ^1H NMR (600 MHz) of disaccharide acceptor (A) **7**, (B) semi-pure trisaccharide **9**, (C) disaccharide acceptor **8**, and (D) semi-pure trisaccharide **10**.



by applying the impure products to ion exchange columns (AG1X-8/OH^-) resulted in the degradation or adsorption and subsequent loss of the reducing sugars. Although the products could not be obtained free of salts, NMR confirmed that the products isolated were the desired trisaccharides. ^1H NMR of **9** (Fig. 2B) clearly showed signals that could be assigned to the newly introduced glucosamine residue at 4.82 ppm for the anomeric H-1″ and at 3.06 ppm for H-2″. The ^1H NMR spectrum obtained for **10** (Fig. 2D) showed the presence of the same contaminant already observed in **8**, which we concluded was also a substrate for the galactosyltransferase. However, it also clearly demonstrated that a glucosamine unit giving typical signals at 4.83 and 3.09 ppm for H-1″ and H-2″, respectively, had been transferred onto the thiochitobiose. Finally, the structures of **9** and **10** were unambiguously confirmed by HRMS that gave protonated molecular ions at *m/z* 586.2463 for **9** (calcd. [M + H] 586.2459) and *m/z* 602.2237 for **10** (calcd. [M + H] 602.2231). Thus, these results showed that the transfer of a glucosamine residue to thiochitobiose using UDP-$GlNH_2$ and bovine β-(1,4)-galactosyltransferase can be used for the preparation of thionodulation factor intermediate trisaccharides. However, because trisaccharide **10** could not be isolated pure and free of salt without degradation, we focused our synthetic effort on the preparation of the nonreducing allyl thiochitobioside derivative (**20**) (Scheme 3).

The allyl group was chosen as a versatile aglycon that is not only easily introduced and removed at the anomeric position, but can also be used to immobilize the oligosaccharides on solid support (15) to further attempt to isolate the specific plant receptor. Nucleophilic displacement of the known (5*c*) 1,6-anhydro triflate **12** with the anomeric thiolate obtained by treatment of the thiol **11** (5*b*) with sodium hydride, afforded the thiodisaccharide **13** in excellent yield (90%). The 1,6-anhydro ring in disaccharide **13** was opened with a mixture of Ac_2O and TFA to give the anomeric mixture of **14** (α/β, 65:35) in high yield (96%). Considering the low stability of the allyl group in debenzylation conditions, we replaced the benzyl group at the C-3 hydroxyl group of the reducing end glucosamine with an acetyl group prior to introducing the allyl aglycon. Thus, the benzyl group in compound **14** was removed using iron trichloride (16) in dichloromethane and the crude intermediate alcohol was acetylated to give the peracetate **15** as an anomeric mixture (Scheme 3). The thiodisaccharide **15** was subsequently converted to the hemiacetal **16** by selective deacetylation of the anomeric hydroxyl group and the hemiacetal **16** was treated with DBU and trichloroacetonitrile to give trichloroacetimidate **17** as an anomeric mixture in favor of the α anomer (α/β, 85:15). Glycosylation of allyl alcohol with the donor **17** was performed under $BF_3{\cdot}Et_2O$ catalysis in dichloromethane and gave the desired thiodisaccharide **18**

Fig. 3. MALDI-MS (accuracy *m*/*z*: ±1) of enzymatic transfer on (A) chitobiose (**7**) and (B) thiochitobiose (**8**).



in good yield (83%) and high selectivity in favor of the β anomer (α/β, 1:9). The β anomer **18**β was isolated anomerically pure by chromatography on alumina and converted to the *N*-acetate **19** in moderate yield through the reduction of the azido group (Zn-AcOH, ref. 17) followed by in situ N-acetylation. Finally, Zemplèn deacetylation of disaccharide **19** gave the acceptor **20** in good yield.

Enzymatic transfer of the glucosamine residue (Scheme 4) was accomplished using the same conditions as those described for the preparation of trisaccharides **9** and **10**. Thus, thiodisaccharide **20** was incubated for 2 weeks at ambient temperature with UDP-GlcNH$_2$ **6** (1.2 equiv.) and the bovine galactosyltransferase (5.6 U). The thiotrisaccharide **21** was obtained pure in 41% yield after chromatography on Bio-Gel P-2 ($AcONH_4$, 0.25 mol/L), followed by desalting on a small column of AG MP-1 (OH^-) eluted with water. The pure compound **21** was fully characterized by ^{1}H NMR spectroscopy, COSY, and HSQC, and its structure was confirmed by HR-ESI MS.

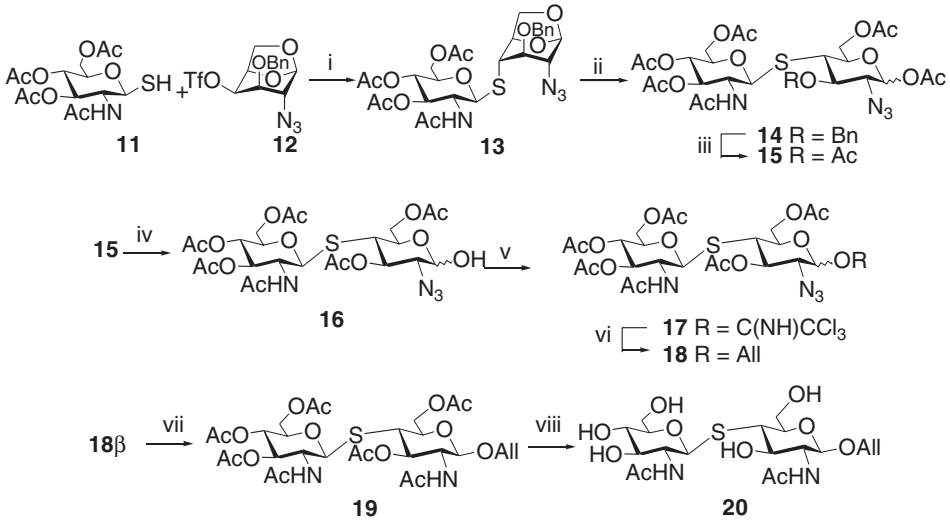
The results reported herein show that the transfer of a glucosamine unit to a thiochitobiose derivative by enzymatic catalysis using the well-known β-(1,4)-galactosyltransferase is applicable to the preparation of thiooligosaccharides. Further reactions on the thiotrisaccharide **21**, including the introduction of the fatty acid moiety, should provide access to a large series of thioanalogues of nodulation factors.

Experimental section

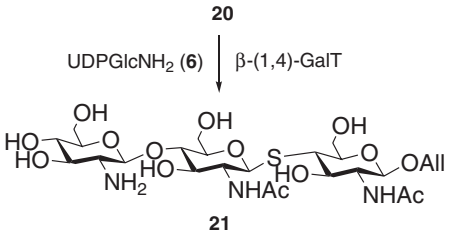
General procedure for the enzymatic reactions

The acceptors **7**, **8**, and **20** (~5 mg, ~11 μmol) and UDP-glucosamine (~9 mg, 1.2 equiv.) were dissolved in 1 mL of a pH 7.4 buffer (80 mmol/L sodium cacodylate, 5 mmol/L $MgCl_2$, 0.1 mmol/L DTT) containing β-1,4-galactosyl-

Scheme 3. Reagents: (*i*) a. **11**, NaH, DMF, 0 °C; b. **12**, 20 °C (90%); (*ii*) 9:1 Ac_2O–TFA (96%, α/β = 65:35); (*iii*) a. $FeCl_3$, CH_2Cl_2; b. Ac_2O, pyridine (62%, α/β = 8:2); (*iv*) H_2NNH_2–AcOH, DMF (75%, α/β = 7:3); (*v*) Cl_3CCN, DBU, CH_2Cl_2 (95%, α/β = 85:15); (*vi*) AllOH, $BF_3{\cdot}Et_2O$, CH_2Cl_2 (83%, α/β = 1:9); (*vii*) a. Zn, AcOH, THF; b. Ac_2O (45% from **18**β); (*viii*) MeONa, MeOH (72%).



Scheme 4.



transferase (5.6 U). Solutions of $MnCl_2$ (1 mol/L, 25 μL, 5 mmol), α-lactalbumin (25 mg/mL, 45 μL), and alkaline phosphatase (1 U) were added to the mixture, which was then left at room temperature for 2 weeks. TLC monitoring of the reactions (*i*-PrOH–NH_4OH–H_2O, 7:2:1 or $CHCl_3$–MeOH–H_2O, 65:35:2) showed, in all three cases, the formation of new compounds more polar than the starting materials in ~50% yield estimated from the TLC. The reaction mixtures were then applied directly to a Bio-Gel P-2 column (100 cm × 1 cm) eluted with a 0.25 mol/L $NH_4^+AcO^-$ solution. The fractions containing the new compounds were combined and freeze dried repeatedly from MQ water. The allyl thiotrisaccharide **21** was additionally deionized on a column of AG MP-1(OH^-) eluted with H_2O.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranose (3)

2-Deoxy-2-trifluoroacetamido-D-glucopyranose (4.72 g, 17.1 mmol) was prepared as described by Wolform and Conigliaro (10) from commercially available crystalline D-glucosamine hydrochloride and was dissolved in pyridine (32 mL) and acetic anhydride (18 mL). The reaction mixture was kept at room temperature for 16 h and poured into ice water and stirred for 1 h. The mixture was extracted with dichloromethane and the organic layer was washed with aq. $NaHCO_3$ and dried on Na_2SO_4. The solution was concentrated to give a crude syrup (8.56 g) containing the peracetylated 2-deoxy-2-trifluoroacetamido-α/β-D-glucopyranose (**2**). ^{1}H NMR analysis showed that the α/β ratio was largely in favor of the desired α anomer (94:6). Therefore, the crude material was dissolved in DMF (50 mL) and the solution was warmed to 50 °C. Hydrazine acetate (1.74 g, 18.9 mmol, 1.05 equiv.) was added to the solution and stirring at 50 °C was maintained for 25 min. The reaction mixture was diluted with EtOAc and washed three times with brine. The organic layer was dried over $MgSO_4$ and concentrated to give a syrup (8.55 g). Chromatography on silica gel (hexane–EtOAc, 3:2) gave the title compound **3** (5.09 g, 74%) as a pure white powder. Compound **3** had characteristics in excellent agreement with those described (10, 11). $[\alpha]_D$ +21° (*c* 1.6, $CHCl_3$) (lit. value (10) +25° (*c* 1, $CHCl_3$), lit. value (11) +20° (*c* 0.5, $CHCl_3$)). ^{1}H NMR (360 MHz, $CDCl_3$) δ: 6.75 (d, 1H, $J_{NH,2}$ = 9.7 Hz, NH), 5.37 (dd, 1H, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 5.37–5.34 (m, 1H, H-1), 5.27 (t, 1H, $J_{4,5}$ = 9.5 Hz, H-4), 4.32 (dt, 1H, $J_{1,2}$ = 3.5 Hz, H-2), 4.27–4.08 (m, 3H, H-5, H-6a, H-6b), 3.78 (bs, 1H, OH), 2.11, 2.06, and 2.04 (3 s, 9H, 3 × CH_3CO). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.4, 171.0, 169.4 (*C*OCH_3), 157.3 (q, $J_{C,F}$ = 37.4 Hz, *C*OCF_3), 115.5 (q, $J_{C\text{-}F}$ = 290 Hz, *C*F_3), 91.0 (C-1), 70.5, 67.9 (C-3, C-4, C-5), 61.9 (C-6), 52.8 (C-2), 20.8, 20.6, 20.5 (*C*H_3CO).

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl dibenzyl phosphate (4)

A solution of butyllithium (1.6 mol/L, 8.4 mL, 13.4 mmol) in hexane was added to a solution of hemiacetal **3** (5.31 g, 13.2 mmol) in THF (160 mL) stirred at –70 °C. After 2 min, a solution of dibenzyl chlorophosphate (12) (5.5 mL, 29.3 mmol) in THF (200 mL) was added slowly at –70 °C under stirring and the mixture was stirred for a further 30 min while the temperature was kept between –60 and –50 °C. Triethylamine was added to pH 8 and the solvent was evaporated. The residue was partitioned between chloroform and aqueous sodium chloride. The organic layer was dried ($MgSO_4$), concentrated, and chromatography on silica gel (hexane–EtOAc, 3:2) gave the pure dibenzylphosphate **4** (5.17 g, 59%) as a colorless glass. $[\alpha]_D$ +55° (*c* 1.2, $CHCl_3$). ^{1}H NMR (360 MHz, $CDCl_3$) δ: 8.2 (d, 1H, $J_{NH,2}$ = 9.0 Hz,

NH), 7.40–7.20 (m, 10H, H-aromatics), 5.80 (dd, 1H, $J_{1,2}$ = 3.2 Hz, $J_{1,P}$ = 6.7 Hz, H-1), 5.35 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 10.0 Hz, H-3), 5.16 (t, 1H, $J_{4,5}$ = 10.0 Hz, H-4), 5.28–4.98 (m, 4H, 2 × CH_2Ph), 4.41 (ddd, 1H, $J_{2,P}$ = 3.2 Hz, H-2), 4.25–3.98 (m, 3H, H-5, H-6a, H-6b), 2.03, 2.02, 1.99 (3s, 9H, 3 × CH_3CO). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.0, 170.5, 169.1 (*C*OCH_3), 157. 6 (q, $J_{C,F}$ = 37.7 Hz, *C*OCF_3), 135.2, 135.0, 129.0,128.8, 128.2 (C-aromatics), 115.5 (q, J_{C-F} = 286.8 Hz, *C*F_3), 95.1 (d, J_{C-P} = 5.7 Hz, C-1), 70.3, 70.2, 69.8, 67.2 (C-3, C-4, C-5, 2 × *C*H_2Ph), 61.1 (C-6), 52.6 (d, $J_{2,P}$ = 7.5 Hz, C-2), 20.6, 20.5, 20.4 (*C*H_3CO). Anal. calcd. for $C_{28}H_{31}NO_{12}PF_3$: C 50.84, H 4.72, N 2.12; found: C 50.90, H 4.67, N 1.97.

Uridine 5′-(2-amino-2-deoxy-α-D-glucopyranosyl)-diphosphate (6)

A solution of dibenzylphosphate **4** (5.17 g, 7.82 mmol) in MeOH (400 mL) containing 5% Pd/C (2.14 g) was stirred under hydrogen for 2.5 h. The catalyst was filtered off and the filtrate concentrated to dryness to give 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl phosphate (**5**) as a white solid. R_f 0.28 (60:40:3, CH_2Cl_2–MeOH–H_2O). ^{1}H NMR (360 MHz, $CDCl_3$–CD_3OD, 2:1) δ: 5.64 (dd, 1H, $J_{1,2}$ = 3.0 Hz, $J_{1,P}$ = 6.0 Hz, H-1), 5.42 (dd, 1H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 5.17 (t, 1H, $J_{4,5}$ = 10.0 Hz, H-4), 4.42 (dt, 1H, $J_{2,P}$ = 3.0 Hz, H-2), 4.33–4.10 (m, 3H, H-5, H-6a, H-6b), 2.11, 2.06, 2.02 (3s, 9H, 3 × CH_3CO). Crude **5** was dissolved in acetone (130 mL) and 80% 1,1′-carbonyldiimidazole (3.3 g, 16.3 mmol) was added and the mixture was stirred for 15 h at room temperature. After completion of the reaction ($CHCl_3$–MeOH, 3:1, R_f 0.28), methanol (170 μL) was added and the mixture was stirred for another 0.5 h. The reaction mixture was concentrated and dried under reduced pressure over P_2O_5 for 6 h. While the crude imidazolate was drying, uridine 5′-monophosphate disodium salt (3.5 g, 9.51 mmol) was passed slowly through a column of Dowex 50-5X8 (H^+) resin and the column was thoroughly washed with water. The eluate was concentrated and trioctylamine (8.8 mL, 20 mmol) was added. The mixture was coevaporated three times with DMF and a solution of the aforementioned dry imidazolate in DMF (100 mL) was added. The mixture was stirred for 24 h at room temperature and the formation of the adduct was followed by TLC (CH_2Cl_2–MeOH–H_2O, 60:40:3, R_f 0.35). The solvent was evaporated and the dry residue was dissolved in methanol (150 mL) and ammonium hydroxide (60 mL). The solution was stirred at room temperature for 24 h and concentrated. The crude deacylated product was dissolved in 40 mmol/L aqueous ammonium bicarbonate and slowly passed through a Dowex 1X2-200 (HCO_2^-) column (3 cm × 30 cm). The column was kept at 23 °C and was first washed with 40 mmol/L aq. NH_4HCO_2, then it was eluted (1.6 mL/min) with a linear gradient of ammonium bicarbonate (mixing vessel 550 mL of 40 mmol/L NH_4HCO_2; reservoir 550 mL of 700 mmol/L NH_4HCO_2). The UV absorption of the eluate at 280 nm was monitored and 20 mL fractions were collected. Those fractions containing the product were pooled and concentrated and the residue dissolved in water was treated with Dowex 50-X8 (H^+) until the pH was below 7 and CO_2 gas had evolved. The resin was filtered off and the pH of the filtrate was adjusted to 8 by adding aqueous sodium hydroxide. The solution was passed through a Millex SR filter unit and the filtrate was lyophilized to give pure UDP-GlcNH_2 (**6**) as a white solid (2.43 g, 51%). ^{1}H NMR (400 MHz, D_2O) δ: 7.96 (d, 1H, $J_{5,6}$ = 8.0 Hz, H-6), 5.99 (d, 1H, $J_{1′,2′}$ = 4.4 Hz, H-1′), 5.96 (d, 1H, H-5), 5.75 (dd, 1H, $J_{1″,2″}$ = 3.3 Hz, $J_{1″\text{-}P}$ = 6.8 Hz, H-1″), 4.36 (m, 2H, H-5a′, H-5b′), 4.31–4.16 (m, 3H, H-2′, H-3′, H-4′), 3.78–3.96 (m, 4H, H-3″, H-5″, H-6a″, H-6b″), 3.53 (t, 1H, $J_{3″,4″}$ = $J_{4″,5″}$ = 9.6 Hz, H-4″), 3.16 (dd, 1H, $J_{2″,3″}$ = 10.4 Hz, $J_{2″,P}$ = 3.5 Hz, H-2″). ^{13}C NMR (75 MHz, D_2O) δ: 167.1 (C-4), 153.7 (C-2), 142.5 (C-6), 103.5 (C-5), 93.7 (d, $J_{C,P}$ = 5.3 Hz, C-1″), 89.5 (C-1′), 83.9 (d, $J_{C,P}$ = 8.7 Hz, C-4′), 74.6, 74.1, 70.8, 70.4, 69.9 (C-2′, C-3′, C-3″, C-4″, C-5″), 65.9 (d, $J_{C,P}$ = 5.3 Hz, C-5′), 60.9 (C-6″), 55.0 (d, $J_{C,P}$ = 9.3 Hz, C-2″). ^{31}P NMR (145 MHz, D_2O) δ: –10.9 (d, $J_{P,P}$ = 22.0 Hz, P-O-uridine), –13.5 (d, P-O-Glc). HRMS calcd. for $C_{15}H_{23}O_{16}N_3Na_2P_2S$ *m*/*z*: 632.0247 [M + Na]; found: 632.0245.

4-*S*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-4-thio-α,β-D-glucopyranose (8)

The peracetylated thiochitobiose (5*b*) (37.2 mg, 0.040 mmol) was dissolved in anhydr. MeOH (4 mL) and a solution of sodium methoxide in MeOH (1 mol/L, 40 μL) was added. The reaction mixture was stirred for 3 days at room temperature and deionized with Amberlite® IR120 resin. The resin was filtered off, rinsed with MeOH, and the combined filtrate and washings were concentrated to dryness. The anomeric mixture of thiochitobiose (**8**) was obtained as a white precipitate (11.2 mg) from a mixture of MeOH and acetone. ^{1}H NMR was reported previously (14*b*) for the two anomers and showed the presence of less than 10% of an impurity (Fig. 2C) that could not be removed nor identified. Nevertheless, HRMS confirmed that the wanted anomeric mixture of thiochitobiose was the main product isolated. HRMS calcd. for $C_{16}H_{28}O_{10}N_2S$ *m*/*z*: 463.1362 [M + Na]; found: 463.1364.

Enzymatic transfer on chitobiose (7) and thiochitobiose (8)

MALDI-MS conditions (Kratos Kompact MALDI-TOF, external calibration on insulin): 10 μL of the reaction mixture were diluted with 90 μL of water or 0.25 mol/L $AcONH_4$ and 0.5 μL of these solutions were deposited on the target with 0.5 μL of THAP as the matrix. The trisaccharides **9** (5.7 mg) and **10** (10 mg) were isolated as amorphous white powders showing residual acetate salts by NMR (Supplementary material).[5] Compound **10** also showed the impurity seen in **8**. Nevertheless, HRMS and ^{1}H NMR showed that these compounds were the desired trisaccharides. HRMS for **9** calcd. for $C_{22}H_{39}O_{15}N_3$ *m*/*z*: 586.2459 [M + H]; found: 586.2463. HRMS for **10** calcd. for $C_{22}H_{39}O_{14}N_3S$ *m*/*z*: 602.2231 [M + H]; found: 602.2237.

[5] Supplementary data for this article are available on the journal Web site (http://canjchem.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0R6, Canada. DUD 5022. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

4-*S*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,6-anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-thio-β-D-glucopyranose (13)

A solution of thiol **11** (5*b*) (17.6 g, 43 mmol) in DMF (300 mL) was added to a suspension of NaH (55% in oil, 2.33 g, 53 mmol, 1.2 equiv.) in DMF (10 mL) cooled to 0 °C. The mixture was stirred under Ar for 10 min at 0 °C, 10 min at 20 °C and was, in turn, added to triflate **12** (5*c*) (18.6 g, 51 mmol, 1.2 equiv.). The reaction mixture was stirred for 1 h at 20 °C and the reaction was quenched by addition of AcOH (20 mL), followed by stirring for a further 30 min at room temperature. The solvent was removed in vacuo and the residue was purified by chromatography on silica gel (1:0 to 1:4 toluene–EtOAc) to give thiodisaccharide **13** (24.5 g, 90%) as a white solid; mp 183 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.36 (m, 5H, H-aromatics), 5.54 (d, 1H, $J_{NH,2}$ = 9.1 Hz, NH), 5.44 (s, 1H, H-1), 5.26 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3′), 5.09 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.8 Hz, H-4′), 4.93 (d, 1H, $J_{1',2'}$ = 10.4 Hz, H-1′), 4.76–4.70 (m, 2H, C*H*Ph, H-5), 4.67 (d, 1H, *J* = 11.9 Hz, C*H*Ph), 4.27 (d, 1H, $J_{6a,6b}$ = 7.4 Hz, H-6a), 4.19 (dd, 1H, $J_{6a',6b'}$ = 12.4 Hz, $J_{5',6a'}$ = 4.7 Hz, H-6a′), 4.14 (dd, 1H, $J_{5',6b'}$ = 2.3 Hz, H-6b′), 4.00 (td, 1H, $J_{2',3'}$ = 10.4 Hz, H-2′), 3.82 (m, 1H, H-4), 3.77 (dd, 1H, $J_{5,6b}$ = 5.6 Hz, H-6b), 3.68 (m, 1H, H-5′), 3.52 (s, 1H, H-3), 3.23 (s, 1H, H-2), 2.06, 2.05, 2.03, 1.95 (4s, 12H, 4 × CH_3CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.9, 170.6, 170.2, 169.4 (*C*OCH_3), 137.5 (C-ipso), 128.6, 128.0, 127.7 (C-aromatics), 100.1 (C-1), 83.4 (C-1′), 78.6 (C-4), 76.1 (C-5′ and C-5), 73.3 (C-3′), 72.3 (*C*H_2Ph), 68.3 (C-4′), 67.8 (C-6), 62.0 (C-6′), 60.2 (C-3), 53.8 (C-2′), 44.0 (C-2), 23.3, 20.7, 20.6 (*C*H_3CO). HRMS calcd. for $C_{27}H_{34}N_4O_{11}S$ *m/z*: 645.1843 [M + Na]; found: 645.1845.

1,6-Di-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-azido-3-*O*-benzyl-2-deoxy-4-thio-α,β-D-glucopyranose (14)

Thiodisaccharide **13** (24.5 g, 39 mmol) was dissolved in Ac_2O–TFA (9:1, 166 mL), and the solution was stirred for 6.5 h at 40 °C. Solvents were evaporated under reduced pressure and residual Ac_2O was coevaporated with toluene. The residue was submitted to chromatography (3:2 to 0:1 toluene–EtOAc) to furnish compound **14** (23 g, 96%). ^{1}H NMR spectroscopy showed an α/β ratio of 65:35. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.50–7.30 (m, 5Hα and 5Hβ, H-aromatics), 6.29 (d, 1Hα, $J_{1,2}$ = 3.2 Hz, H-1α), 5.58 (d, 1Hα, $J_{1',2'}$ = 8.6 Hz, H-1′α), 5.47 (m, 2Hβ, H-1β and H-1′β), 5.30 (t, 1Hα and 1Hβ, $J_{2',3'+3',4'}$ = 19.7 Hz, H-3′α and H-3′β), 5.08–4.95 (m, 2Hα and 2Hβ, C*H*Phα, C*H*Phβ, H-4′α and H-4′β), 4.95–4.85 (m, 2Hα and 2Hβ, C*H*Phα, C*H*Phβ, NHα and NHβ), 4.57–4.0 (m, 2Hα and 2Hβ, H-6aα, H-6aβ, H-6bα and H-6bβ), 4.19 (dd, 1Hα, $J_{6a',6b'}$ = 12.5 Hz, $J_{5',6a'}$ = 4.9 Hz, H-6a′α), 4.11–3.96 (m, 2Hα and 2Hβ, H-5α, H-6b′α, H-6b′β and H-6a′β), 3.89–3.77 (m, 1Hα and 2Hβ, H-2′α, H-2β and H-5β), 3.73 (t, 1Hα, *J* = 10.1 Hz, H-3α), 3.69–3.63 (m, 2Hα and 1Hβ, H-5′α, H-5′β and H-2α), 3.60 (t, 1Hβ, $J_{1,2} = J_{2,3}$ = 8.9 Hz, H-2β), 3.41 (t, 1Hβ, $J_{3,4}$ = 10.0 Hz, H-3β), 2.98 (t, 1Hα, $J_{3,4} = J_{4,5}$ = 10.5 Hz, H-4α), 2.93 (t, 1Hβ, $J_{4,5}$ = 10.5 Hz, H-4β), 2.20–2.17 (2s, 3Hα and 3Hβ, $CH_3COα$ and $CH_3COβ$), 2.12 (s, 3Hα, $CH_3COα$), 2.09 (s, 3Hα, $CH_3COα$), 2.05–2.00 (m, 6Hα and 12Hβ, 2 × $CH_3COα$ and 4 × $CH_3COβ$), 1.71 (s, 3Hα, C*H*$_3$CONHα), 1.68 (s, 3Hβ, $CH_3CONHβ$). ^{13}C NMR (100 MHz, $CDCl_3$) for the α anomer δ: 170.5, 168.7 (*C*OCH_3), 128.6, 128.2, 127.6, 127.5 (C-aromatics), 90.4 (C-1), 83.6 (C-1′), 77.0 (C-3), 75.6 (*C*H_2Ph), 75.2 (C-5′), 72.8 (C-3′), 72.5 (C-5), 68.2 (C-4′), 63.9 (C-2), 63.2 (C-6), 62.1 (C-6′), 54.5 (C-2′), 46.8 (C-4), 23.1, 21.0, 20.6 (*C*H_3CO). HRMS calcd. for $C_{31}H_{40}N_4O_{14}S$ *m/z*: 747.2159 [M + Na]; found: 747.2158.

1,3,6-Tri-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-azido-2-deoxy-4-thio-α,β-D-glucopyranose (15)

Anhydrous $FeCl_3$ (20.4 g, 126 mmol, 4 equiv.) was added to a solution of benzyl **14** (22.7 g, 31 mmol) in freshly distilled anhydr. CH_2Cl_2 (220 mL) and the mixture was stirred for 1.5 h under Ar at 20 °C. The solvent was evaporated then pyridine (210 mL) and Ac_2O (105 mL) were added. The reaction mixture was stirred for 1.5 h at 20 °C and concd. EtOAc (350 mL) was added to the residue and the resulting solution was washed successively with 1 mol/L HCl (250 mL), satd. $NaHCO_3$ (250 mL), and brine (250 mL). The aqueous layers were reextracted with EtOAc (2 × 250 mL) and the combined organic layers were dried and concentrated. The crude product was filtered off from EtOH and further purified by chromatography on silica gel (0:1 to 9:1 EtOAc–cyclohexane) to afford acetate **15** (13.1 g, 62%) as a white powder. ^{1}H NMR spectra gave an α/β ratio of 8:2. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.33 (d, 1Hα, $J_{1,2}$ = 3.3 Hz, H-1α), 5.77 (d, 1Hα, $J_{NH,2}$ = 9.8 Hz, NHα), 5.69 (d, 1Hβ, $J_{NH,2}$ = 10.1 Hz, NHβ), 5.49 (d, 1Hβ, $J_{1,2}$ = 8.6 Hz, H-1β), 5.43 (t, 1Hα, $J_{2,3} = J_{3,4}$ = 10.5 Hz, H-3α), 5.11 (t, 1Hα, $J_{2',3'} = J_{3',4'}$ = 9.7 Hz, H-3′α), 5.08–4.91 (m, 1Hα and 3Hβ, H-4′α, H-4′β, H-3′β and H-3β), 4.74 (d, 1Hα and 1Hβ, $J_{1',2'}$ = 10.4 Hz, H-1′α and H-1′β), 4.60 (dd, 1Hα, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 3.9 Hz, H-6aα), 4.55 (dd, 1Hβ, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.6 Hz, H-6aβ), 4.46 (m, 1Hβ, H-6bβ), 4.40–4.29 (m, 2Hα, H-5α and H-6bα), 4.28–4.13 (m, 2Hα and 2Hβ, H-2′α, H-2′β, H-6a′α and H-6a′β), 4.10–4.00 (m, 1Hα and 1Hβ, H-6b′α and H-5β), 3.95 (dd, 1Hβ, $J_{6a',6b'}$ = 12.1 Hz, $J_{5',6b'}$ = 7.9 Hz, H-6b′β), 3.76–3.63 (m, 2Hα and 2Hβ, H-2α, H-2β, H-5′α and H-5′β), 2.95 (t, 1Hα, *J* = 11.2 Hz, H-4α), 2.86 (t, 1Hβ, $J_{3,4} = J_{4,5}$ = 10.5 Hz, H-4β), 2.22 (s, 3Hα, $CH_3COα$), 2.19 (s, 3Hα, $CH_3COα$), 2.19–2.18 (2s, 6Hβ, 2 × $CH_3COβ$), 2.13 (s, 3Hβ, $CH_3COβ$), 2.07 (s, 3Hα, $CH_3COα$), 2.06–2.01 (m, 9Hα and 9Hβ, 3 × $CH_3COα$ and 3 × $CH_3COβ$), 1.95 (s, 3Hα, $CH_3CONHα$) and 1.92 (s, 3Hβ, $CH_3CONHβ$). ^{13}C NMR (100 MHz, $CDCl_3$) for the α anomer δ: 171.3, 170.6, 170.3 (*C*OCH_3), 90.3 (C-1), 82.3 (C-1′), 75.9 (C-5′), 74.2 (C-3′), 71.1 (C-5), 68.5 (C-4′), 67.8 (C-3), 63.0, 62.9 (C-6 and C-6′), 61.8 (C-2), 51.9 (C-2′), 45.8 (C-4), 23.0, 20.9, 20.7, 20.6 (*C*H_3CO). HRMS calcd. for $C_{26}H_{36}N_4O_{15}S$ *m/z*: 699.1796 [M + Na]: found: 699.1796.

3,6-Di-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-azido-2-deoxy-4-thio-α,β-D-glucopyranose (16)

Hydrazine acetate (2.1 g, 23 mmol, 1.2 equiv.) was added to a solution of compound **15** (12.8 g, 19 mmol) in DMF (110 mL). The mixture was stirred for 1 h under Ar at room temperature and the solvent was removed under reduced

pressure. Residual traces of DMF were coevaporated with toluene and the residue was dissolved in EtOAc (200 mL) and washed with water (150 mL) and brine (150 mL). The aqueous layers were reextracted with EtOAc (2 × 250 mL) and the combined organic solvents were dried and concentrated. The crude product was purified by chromatography on silica gel (1:0 to 95:5 $CHCl_3$–MeOH) to yield hemiacetal **16** (8.9 g, 75%). The ^{1}H NMR spectra showed an α/β ratio of 7:3. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.90 (d, 1Hα, $J_{1',2'}$ = 9.9 Hz, H-1′α), 5.81 (d, 1Hβ, $J_{1',2'}$ = 9.9 Hz, H-1′β), 5.55 (t, 1Hα, $J_{2,3}$ = $J_{3,4}$ = 10.5 Hz, H-3α), 5.40 (d, 1Hα, $J_{1,2}$ = 3.1 Hz, H-1α), 5.09 (t, 1Hα, $J_{2',3'}$ = $J_{3',4'}$ = 9.5 Hz, H-3′α), 5.08–4.97 (m, 1Hα, 3Hβ, H-3′β, H-3β, H-4′α and H-4′β), 4.82 (d, 1Hα, $J_{NH,2}$ = 10.4 Hz, NHα), 4.78 (d, 1Hβ, $J_{NH,2}$ = 10.5 Hz, NHβ), 4.69 (d, 1Hβ, $J_{1,2}$ = 7.9 Hz, H-1β), 4.57 (dd, 1Hα, $J_{6a,6b}$ = 11.8 Hz, $J_{5,6a}$ = 3.7 Hz, H-6aα), 4.55 (m, 1Hα and 2Hβ, H-5α, H-6aβ and H-6bβ), 4.44 (dd, 1Hα, $J_{5,6b}$ = 1.6 Hz, H-6bα), 4.26–4.16 (m, 2Hα and 2Hβ, H-2′α, H-2′β, H-6a′α and H-6a′β), 4.01 (dd, 1Hα and 1Hβ, $J_{6a',6b'}$ = 12.3 Hz, $J_{5',6b'}$ = 6.0 Hz, H-6b′α and H-6b′β), 3.92 (m, 1Hβ, H-5β), 3.73 (m, 1Hα and 1Hβ, H-5′α and H-5′β), 3.50 (m, 1Hα and 1Hβ, H-2α and H-2β), 2.91–2.82 (m, 1Hα and 1Hβ, H-4α and H-4β), 2.17 (s, 3Hα and 3Hβ, $CH_3COα$ and $CH_3COβ$), 2.10 (s, 3Hα, $CH_3COα$), 2.09 (s, 3Hβ, $CH_3COβ$), 2.07 (s, 3Hα, $CH_3COα$), 2.06 (s, 3Hβ, $CH_3COβ$), 2.03 (s, 3Hα and 3Hβ, $CH_3COα$ and $CH_3COβ$), 2.01 (s, 3Hα and 3Hβ, $CH_3COα$ and $CH_3COβ$), 1.94 (s, 3Hα, $CH_3COα$) and 1.92 (s, 3Hβ, $CH_3COβ$). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.8, 170.7, 170.1, 169.4 (*C*OCH_3), 96.1 (C-1β), 91.8 (C-1α), 81.9 (C-1′α), 81.7 (C-1′β), 75.5 (C-5′α), 75.4 (C-5′β), 74.3 (C-3′α), 74.1 (C-3′β), 73.6 (C-5β), 70.1 (C-3β), 68.6 (C-5α), 68.4 (C-4′α), 68.3 (C-4′β), 67.9 (C-3α), 66.2 (C-2β), 63.5 (C-6α and C-6β), 63.2 (C-2α), 62.5 (C-6′α and C-6′β), 52.0 (C-2′α and C-2′β), 46.3 (C-4α), 46.1 (C-4β), 22.9, 21.1, 20.9, 20.7, 20.6 (*C*H_3CO). HRMS calcd. for $C_{24}H_{34}N_4O_{14}S$ *m/z*: 657.1690 [M + Na]; found: 657.1714.

3,6-Di-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-azido-3-*O*-benzyl-2-deoxy-4-thio-α,β-D-glucopyranosyl trichloroacetimidate (17)

Trichloroacetonitrile (27.5 mL, 275 mmol, 19 equiv.) and DBU (532 µL, 3.6 mmol, 0.26 equiv.) were added to a solution of hemiacetal **16** (8.74 g, 14 mmol) in anhydr. CH_2Cl_2 (130 mL). The reaction mixture was stirred under Ar at room temperature for 45 min. The reaction mixture was concentrated and the residue submitted to chromatography on silica gel (silica gel was previously washed with a 5% solution of Et_3N in EtOAc) (1:0 to 95:5 $CHCl_3$–MeOH) to give the unstable imidate **17** (10.2 g, 95%) as a glass. ^{1}H NMR spectra showed an α/β ratio of 85:15. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.81 (s, 1Hα, $NHCCl_3α$), 8.73 (s, 1Hβ, $NHCCl_3β$), 6.52 (d, 1Hα, $J_{1,2}$ = 3.4 Hz, H-1α), 5.78 (d, 1Hα, $J_{NH,2}$ = 9.9 Hz, NHα), 5.70 (d, 1Hβ, $J_{NH,2}$ = 10.2 Hz, NHβ), 5.66 (d, 1Hβ, $J_{1,2}$ = 8.5 Hz, H-1β), 5.52 (t, 1Hα, $J_{2,3}$ = $J_{3,4}$ = 10.5 Hz, H-3α), 5.11–5.00 (m, 2Hα and 2Hβ, H-3′α, H-3′β, H-3β and H-4′α), 4.95 (t, 1Hβ, $J_{3',4'}$ = $J_{4',5'}$ = 9.6 Hz, H-4′β), 4.78–4.68 (m, 1Hα and 1Hβ, H-1′β and H-1′α), 4.66–4.55 (m, 1Hα and 1Hβ, H-6aα and H-6aβ), 4.50–4.38 (m, 2Hα and 1Hβ, H-5α, H-6bα and H-6bβ), 4.28–4.17 (m, 1Hα and 2Hβ, H-2′α, H-2′β and H-6a′β), 4.09 (dd, 1Hα, $J_{6a',6b'}$ = 12.4 Hz, $J_{5',6a'}$ = 2.8 Hz, H-6a′α), 4.06–3.98 (m, 1Hα and 1Hβ, H-6b′α and H-5β), 3.94 (dd, 1Hβ, $J_{6a',6b'}$ = 12.0 Hz, $J_{5',6b'}$ = 8.4 Hz, H-6b′β), 3.88–3.78 (m, 1Hα and 1Hβ, H-2α and H-2β), 3.74 (m, 1Hβ, H-5′β), 3.54 (m, 1Hα, H-5′α), 3.01 (t, 1Hα, $J_{4,5}$ = 11.0 Hz, H-4α), 2.93 (t, 1Hβ, $J_{3,4}$ = $J_{4,5}$ = 11.0 Hz, H-4β), 2.20 (s, 3Hβ, $CH_3COβ$), 2.19 (s, 3Hα, $CH_3COα$), 2.14 (s, 3Hβ, $CH_3COβ$), 2.06 (s, 3Hα, $CH_3COα$), 2.05–2.03 (m, 6Hα and 6Hβ, 2 × $CH_3COα$ and 2 × $CH_3COβ$), 2.02 (s, 3Hα and 3Hβ, $CH_3COα$ and $CH_3COβ$), 1.95 (s, 3Hα, $CH_3COα$), 1.93 (s, 3Hβ, $CH_3COβ$).

Allyl 3,6-di-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-azido-2-deoxy-4-thio-β-D-glucopyranoside (18β)

A suspension of imidate **17** (8.87 g, 11 mmol) in anhydr. CH_2Cl_2 (140 mL) containing 4 Å activated molecular sieves (8.0 g) was stirred under Ar at room temperature for 1 h and was then cooled down to – 45 °C. Allyl alcohol (1.55 mL, 23 mmol, 2 equiv.) and $BF_3·Et_2O$ (920 µL, 3.4 mmol, 3.2 equiv.) were added to the suspension and the mixture was allowed to reach –10 °C over 2 h. Triethylamine (200 µL) was added and after additional stirring (20 min) at room temperature, the molecular sieves were filtered off. The filtrate was diluted with CH_2Cl_2 (250 mL) and washed with brine (250 mL). The aqueous layer was reextracted with CH_2Cl_2 (3 × 100 mL) and the combined organic solutions were dried and concentrated. The residue was submitted to chromatography on neutral alumina (1:2, 1:1, 3:1, 5:1, then 1:0, EtOAc–cyclohexane) to give the thiodisaccharide **18**β (5.71 g, 74%) as a white powder; mp 187 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.94 (m, 1H, $OCH_2CH_cCH_2$), 5.70 (d, 1H, $J_{NH,2}$ = 9.7 Hz, NH), 5.34 (dd, 1H, $J_{c,e}$ = 17.2 Hz, $J_{d,e}$ = 1.5 Hz, $OCH_2CHCH_dH_e$), 5.26 (dd, 1H, $J_{c,d}$ = 10.4 Hz, $OCH_2CHCH_dH_e$), 5.08–4.96 (m, 2H, H-3′ and H-4′), 4.93 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 11.0 Hz, H-3), 4.72 (d, 1H, $J_{1',2'}$ = 10.7 Hz, H-1′), 4.54–4.49 (m, 2H, H-6a and H-6b), 4.43–4.35 (m, 2H, H-1 and $OCH_aH_bCHCH_2$), 4.25–4.09 (m, 3H, H-2′, H-6a′ and $OCH_aH_bCHCH_2$), 4.01 (dd, 1H, $J_{6a',6b'}$ = 12.3 Hz, $J_{5',6b'}$ = 6.4 Hz, H-6b′), 3.84 (m, 1H, H-5), 3.69 (m, 1H, H-5′), 3.52 (dd, 1H, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 9.7 Hz, H-2), 2.83 (t, 1H, $J_{4,5}$ = 10.9 Hz, H-4), 2.17, 2.09, 2.07, 2.04, 2.02, 1.92 (6s, 6 × CH_3CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.3, 170.9, 170.5, 170.2, 169.7, 169.3 (*CO*CH_3), 133.0 ($OCH_2CH=CH_2$), 118.1 ($OCH_2CH=CH_2$), 100.8 (C-1), 81.7 (C-1′), 75.7 (C-5′), 74.1 (C-3′), 73.5 (C-5), 70.3 (C-6), 69.8 (C-3), 68.3 (C-4′), 65.0 (C-2), 63.5 ($OCH_2CH=CH_2$), 62.7 (C-6′), 51.9 (C-2′), 46.2 (C-4), 23.0, 21.1, 20.8, 20.6 (*C*H_3CO). HRMS calcd. for $C_{27}H_{38}N_4O_{14}S$ *m/z*: 697.2003 [M + Na]; found: 697.1996.

Allyl 3,6-di-*O*-acetyl-4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-gluco-pyranosyl)-2-acetamido-2-deoxy-4-thio-β-D-glucopyranoside (19)

Zinc (52.4 g) and AcOH (23 mL) were added to a solution of compound **18**β (5.4 g, 8 mmol) in anhydr. THF (260 mL). The mixture was stirred under Ar at room temperature for 1.5 h and Ac_2O (140 mL) was added. The reaction mixture was stirred for 1.5 h at room temperature and filtered through Celite. The filtrate was concentrated to dryness and the residue dissolved in EtOAc (300 mL), which was washed successively with water (200 mL), satd. $NaHCO_3$ (200 mL),

and brine (200 mL). The aqueous layers were reextracted with EtOAc (6 × 100 mL) and the combined organic solutions were dried and concentrated. The crude product was submitted to chromatography on silica gel (1:0 and 95:5 $CHCl_3$–MeOH) to give the title compound **19** (2.5 g, 45%) as a white powder; mp 238 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.87 (m, 1H, $OCH_2CH_cCH_2$), 5.78 (d, 1H, $J_{NH,2}$ = 9.4 Hz, NH′), 5.74–5.65 (m, 2H, H-3 and NH), 5.27 (dd, 1H, $J_{c,e}$ = 17.2 Hz, $J_{d,e}$ = 1.6 Hz, $OCH_2CHCH_dH_e$), 5.21 (dd, 1H, $J_{c,d}$ = 10.3 Hz, $OCH_2CHCH_dH_e$), 5.12–4.98 (m, 2H, H-3′ and H-4′), 4.91 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 4.74 (d, 1H, $J_{1',2'}$ = 10.5 Hz, H-1′), 4.53 (m, 2H, H-6a and H-6b), 4.32 (m, 1H, $OCH_aH_bCHCH_2$), 4.23–4.12 (m, 2H, H-2′ and H-6a′), 4.10–4.02 (m, 2H, H-6b′ and $OCH_aH_bCHCH_2$), 3.90 (m, 1H, H-5), 3.74 (m, 1H, H-5′), 3.46 (m, 1H, H-2), 2.83 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 10.8 Hz, H-4), 2.11, 2.10, 2.07, 2.04, 2.02, 1.99, 1.92 (7s, 7 × 3H, CH_3CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.7, 169.8 ($COCH_3$), 133.6 ($OCH_2CH=CH_2$), 117.8 ($OCH_2CH=CH_2$), 98.8 (C-1), 82.2 (C-1′), 75.7 (C-5′), 74.3 (C-3′), 73.4 (C-5), 70.0 ($OCH_2CH=CH_2$), 68.9 (C-3), 68.2 (C-4′), 63.6 (C-6), 62.6 (C-6′), 57.3 (C-2), 52.2 (C-2′), 46.7 (C-4), 23.5, 23.0, 21.0, 20.9, 20.6 (CH_3CO). HRMS calcd. for $C_{27}H_{38}N_4O_{14}S$ *m/z*: 713.2204 [M + Na]; found: 713.2200.

Allyl 4-*S*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-4-thio-β-D-glucopyranoside (20)

MeONa (75 mg, 1.4 mmol) was added to a solution of allyl glycoside **19** (300 mg, 434 µmol) in anhydr. MeOH (3 mL). The mixture was stirred for 4 h under Ar at room temperature and then diluted with water. The pH was adjusted to 7 by adding Amberlite® IR120 resin, the resin was filtered off, and the filtrate was concentrated to dryness. The residue was precipitated from a mixture of acetone–MeOH (2:1) to afford the pure thiodisaccharide **20** (150 mg, 72%) as a white powder. ^{1}H NMR (400 MHz, D_2O) δ: 5.92 (m, 1H, $OCH_2CH_cCH_2$), 5.40–5.22 (m, 2H, $OCH_2CHCH_dH_e$ and $OCH_2CHCH_dH_e$), 4.74 (d, 1H, $J_{1',2'}$ = 10.4 Hz, H-1′), 4.55 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 4.35 (dd, 1H, $J_{a,b}$ = 12.9 Hz, $J_{a,c}$ = 4.5 Hz, $OCH_aH_bCHCH_2$), 4.17 (dd, 1H, $J_{b,c}$ = 6.0 Hz, $OCH_aH_bCHCH_2$), 4.09 (d, 1H, $J_{6a,6b}$ = 11.8 Hz, H-6a), 3.98–3.85 (m, 2H, H-6a′ and H-6b), 3.82–3.54 (m, 6H, H-2′, H-6b′, H-2, H-5, H-3, and H-3′), 3.54–3.40 (m, 2H, H-4′ and H-5′), 2.89 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 10.5 Hz, H-4), 2.04 (s, 6H, 2 × CH_3CO). ^{13}C NMR (100 MHz, D_2O) δ: 172.6, 172.5 ($COCH_3$), 131.3 ($OCH_2CH=CH_2$), 116.2 ($OCH_2CH=CH_2$), 97.7 (C-1), 81.8 (C-1′), 77.8 (C-4′ or C-5′), 74.4 (C-5), 72.9 (C-3′), 69.4 (C-3), 68.4 ($OCH_2CH=CH_2$), 67.6 (C-5′ or C-4′), 59.4 (C-6), 58.7 (C-6′), 54.8 (C-2), 52.9 (C-2′), 46.1 (C-4), 20.1 (CH_3CO). HRMS calcd. for $C_{19}H_{32}N_2O_{10}S$ *m/z*: 503.1675 [M + Na]; found: 503.1672.

Allyl 4-*S*-(2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-4-thio-β-D-glucopyranoside (21)

Thiodisaccharide **20** (5.45 mg, 11.35 µmol) was incubated for 14 days with UDP-$GlcNH_2$ (**6**) (8.9 mg, 13.7 µmol, 1.2 equiv.) and β-1,4-galactosyltransferase (5.6 U) as described in the general method for the enzymatic reactions. The pure trisaccharide **21** (3 mg, 41%) was isolated as a glass after freeze-drying from water. ^{1}H NMR (400 MHz, D_2O) δ: 5.88 (m, 1H, $OCH_2CH_cCH_2$), 5.35–5.19 (m, 2H, $OCH_2CHCH_dH_e$ and $OCH_2CHCH_dH_e$), 4.71 (d, 1H, $J_{1',2'}$ = 10.1 Hz, H-1′), 4.52 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1), 4.46 (d, 1H, $J_{1'',2''}$ = 8.0 Hz, H-1″), 4.30 (dd, 1H, $J_{a,b}$ = 13.1 Hz, $J_{a,c}$ = 6.3 Hz, $OCH_aH_bCHCH_2$), 4.12 (dd, 1H, $J_{b,c}$ = 6.4 Hz, $OCH_aH_bCHCH_2$), 4.05 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 1.4 Hz, H-6a), 3.96–3.82 (m, 3H, H-6a′, H-6b and H-3′ or H-4′ or H-5′), 3.82–3.64 (m, 6H, H-2, H-2′, H-6b′, H-6a″, H-6b″ and H-3′ or H-4′ or H-5′), 3.63–3.52 (m, 3H, H-5, H-3 and H-3′ or H-4′ or H-5′), 3.46 (m, 1H, H-4″ or H-5″), 3.42–3.32 (m, 2H, H-3″ and H-4″ or H-5″), 2.85 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 10.8 Hz, H-4), 2.66 (m, 1H, H-2″), 2.03, 2.02 (2s, 2 × 3H, CH_3CO). ^{13}C NMR (D_2O, measured from HSQC at 400 MHz) δ: 132.0 ($CH_2CH=CH_2$), 117.0 ($CH_2CH=CH_2$), 102.0 (C-1″), 99.0 (C-1′), 83.0 (C-1), 78.0, 77.2, 76.1, 76.0, 75.5, 72.8, 71.0, 68.9 (not assigned), 69.0 ($CH_2CH=CH_2$), 61.0 (C-6), 59.7, 59.0, 56.5 (not assigned), 56.0 (C-2″), 54.0 (not assigned), 47.3 (C-4), 21.8 (CH_3CO). HRMS calcd.for $C_{25}H_{43}N_3O_{14}S$ *m/z*: 642.2544 [M + H]; found: 642.2563.

Acknowledgements

L. Loureiro Morais was supported by a grant from the French Ministry in charge of higher education. The authors are thankful for a grant from the France–Canada Research Foundation to support the collaborative exchange between the French and Canadian (F-IA) groups. LLM, KB, IR, and F-IA are thankful to Professor J. Gelas (École Nationale Supérieure de Chimie de Clermont-Ferrand (ENSCCF)) for his support of this work. UDP-$GlcNH_2$ was synthesized by HY in Professor O. Hindsgaul's group (University of Alberta, Edmonton, Alberta) and the isolation of the galactosyltransferase, as well as the transfers on **7** and **8,** were carried out by F-IA in Professor M. Palcic's group (University of Alberta). The authors are grateful to both O. Hindgsaul and M. Palcic (now at the Carlsberg Laboratories, Valby, Denmark) for their support of this work.

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