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Synthesis and photolytic activation of 6″-*O*-2-nitrobenzyl uridine-5′-diphosphogalactose: a 'caged' UDP-Gal derivative

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Abstract—Placing an 2-nitrobenzyl group on *O*-6 of the galactosyl residue in uridine-5′-diphosphogalactose (UDP-Gal) gives 6″-*O*-2-nitrobenzyl-UDP-Gal that is shown to be inactive as a donor substrate for β-(1→4)-galactosyltransferase (GalT). On irradiation at 365 nm, the nitrobenzyl group is completely removed yielding native UDP-Gal that then transfers normally to produce the expected βGal-(1→4)-βGlcNAc disaccharidic linkage. 6″-*O*-2-Nitrobenzyl-UDP-Gal thus fulfils the minimum requirements of a 'caged' UDP-Gal for application in time-resolved crystallographic studies of β-(1→4)-GalT.

Keywords: Galactosyltransferases; Sugar nucleotide analogue; Caged compound; Enzyme structure and mechanism

1. Introduction

Mammalian glycosyltransferases catalyze the transfer of glycosyl-residues from their activated glycosyl-donors, usually sugar nucleotide diphosphates, to OH-groups on acceptor molecules. Hundreds of glycosyltransferases are known to be involved in the biosynthesis of the oligosaccharide chains of mammalian glycoproteins and glycolipids.[1] These enzymes are broadly classified into two main families, termed either 'retaining' or 'inverting', depending on whether the stereochemistry of the glycosidic bond in the donor is retained or inverted in the product glycoside. The extensively studied β-(1→4)-galactosyltransferase (β-(1→4)-GalT),[2] for example, is an inverting enzyme transferring galactose from α-linked UDP-galactose (**1**, UDP-Gal) to OH-4 of *N*-acetylglucosamine (GlcNAc) residues (**2**) to give *N*-acetyllactosamine terminated structures (**3**) (Chart 1). This enzyme has been cloned and extensively studied kinetically, through mutagenesis experiments and structurally by crystallography.[2] Comparable systematic investigations have been performed on several retaining galactosyltransferases, most notably the β-(1→3)-galactosyltransferase[3] and the human blood-group B galactosyltransferase.[4]

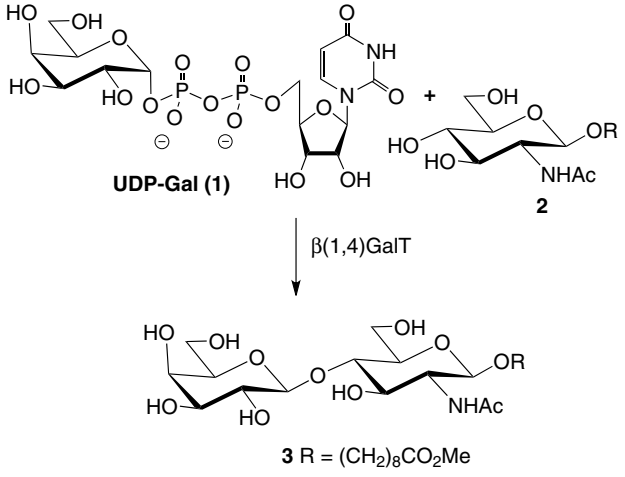

Chart 1. The reaction catalyzed by β-(1→4)-GalT, an inverting enzyme.

The structures of the galactosyltransferases noted above have been studied by X-ray crystallography as the native enzymes, the native enzymes with either the donor or acceptor in the active sites or with analogues of the donor and/or the acceptor. Complexes of mutant

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enzymes have also been solved. The general mechanistic picture that has emerged[5] is that after binding the donor, one or more flexible peptide loops undergo conformational changes from open to closed forms creating acceptor binding sites thus enabling the transfer reaction. It has, of course, not been possible to get crystal structures of the native enzymes in the presence of both the acceptor and the donor as these react to give the product glycoside. We report here the initiation of a new research project aimed at providing 'caged' substrate analogues for galactosyltransferases that we hope will enable time-resolved crystallographic experiments[6] to yield atomic details of the native transfer reaction.

The term 'caged' compound is used to refer to a biologically unreactive molecule that can be released from its 'cage' at the will of the experimenter to instantaneously produce the biologically-active compound.[7] Most commonly, a caged compound is blocked with a photolabile group so that irradiation can cleave this group. The ATP derivative **4**, functionalized with an 2-nitrobenzyl group, was the first reported caged compound that was cleaved to release free ATP on irradiation at 340 nm (Chart 2).[8]

To extend this concept to study galactosyltransferases, we needed to establish a system where the enzyme could bind to the acceptor and caged-UDP-Gal donor without any reaction occurring, and to demonstrate the reaction after cleavage of the caging group. We chose the readily available β-(1→4)-GalT, the inverting enzyme catalysing the reaction shown in Chart 1. β-(1→4)-GalT has been shown to have a relaxed specificity towards the sugar being transferred from the UDP-sugar with measurable rates for glucose, 2-deoxy-galactose, some 6-O-substituted galactose derivatives and even GalNAc.[9] Nishimura's group,[10] however, has shown that O-6 of UDP-Gal can be functionalized with a 10-atom long PEG-based tether terminating in a naphtyl group and that resulting molecule is a potent inhibitor of β-(1→4)-GalT. In the same report, they demonstrated

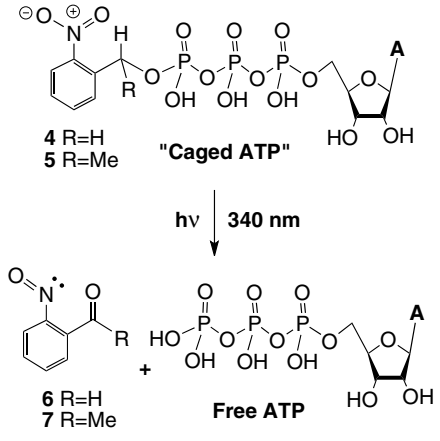


Chart 2. Photolytic removal of nitrobenzyl groups from caged ATP (Ref. 8).

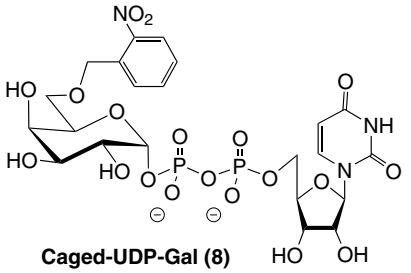


Chart 3. Structure of the target caged UDP-Gal derivative **8**.

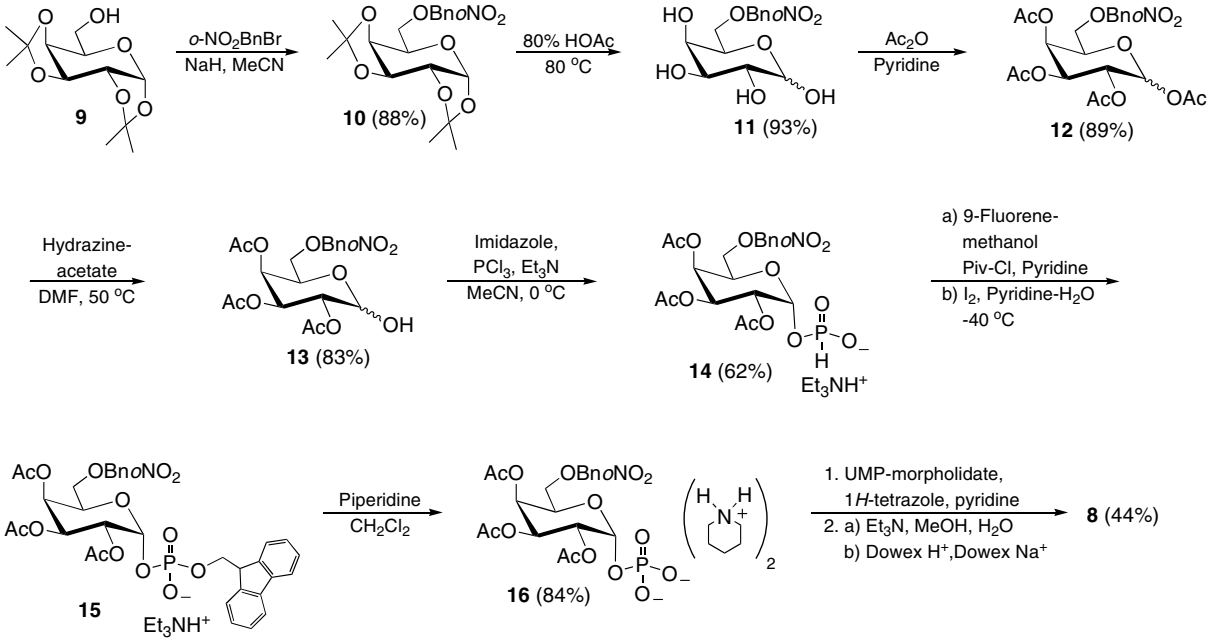
that a bromomethyl-naphthyl group (instead of a naphthyl group) at the end of the tether successfully affinity-labelled a tryptophan residue in the crystallographically-flexible loop, suggesting that this position would be ideal for installing a photocleavable caging group.

The target for the synthesis was therefore UDP-Gal with a photolabile group protecting O-6″, namely **8** (Chart 3). While many caging groups are known, we elected to first make the simplest one: the 2-nitrobenzyl ether. This group is not ideal, particularly as the photolytic product 2-nitrobenzaldehyde (**6**, Chart 2) has been reported to undergo chemical side-reactions with proteins.[7] For this reason, the 2-nitrophenylethyl caging group shown in **5** is often used, yielding a more stable ketone product (**7**). We therefore view **8** as a prototype caged UDP-Gal designed to test the feasibility of this approach, with superior photolabile groups to be selected later if required.

2. Results and discussion

The synthesis of **8** (Scheme 1) started from commercially available 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose (**9**), which was 2-nitrobenzylated using NaH and 2-nitrobenzyl bromide in MeCN, assisted by microwave heating at 180 °C for 4 min,[11] to give **10** in 88% yield. Removal of the isopropylidene protecting groups with 80% HOAc in 80 °C gave **11** (93%) which, on acetylation, gave **12** (89%). Selective anomeric deacetylation using hydrazine acetate in DMF afforded **13** in 83% yield.

The phosphate group was introduced by treatment of **13** with PCl_3–imidazole–Et_3N[12] to give the α-anomeric H-phosphonate **14** (62%) on hydrolytic work-up. The H-phosphonate was converted into the fluorenyl-9-methyl phosphodiester[13] with 9-fluorenemethanol and pivaloyl chloride in pyridine, followed by the oxidation using iodine in pyridine–water to give **15**. The fluorene-9-methyl protecting group was cleaved with piperidine in CH_2Cl_2 to give phosphomonoester **16** in 84% yield (from **14**). Coupling of **16** with UMP-morpholidate in the presence of 1*H*-tetrazole in pyridine,[14] followed by deacetylation using Et_3N in MeOH–H_2O provided target compound **8** in 44% yield.



Scheme 1. Synthesis of **8**.

The photolability of the 2-nitrobenzyl group in **8** was confirmed by irradiation of an aqueous solution of **8** (0.5 mg/0.5 mL) at 365 nm with a 400 W Hg(Xe) high-pressure mercury lamp, while monitoring the disappearance of **8** (*m*/*z* 700.1) and the appearance of UDP-Gal (**1**) with *m*/*z* 564.8 in the negative-ion-mode electrospray mass spectrum (Fig. 1). Irradiation for sequential periods of 30 s showed that a total of 2 min of irradiation were required to complete the conversion of **8** to **1** under these irradiation conditions. This uncaging reaction is summarized in Scheme 2, Eq 1.

The results of the preliminary evaluation of **8** as a caged donor for β-(1→4)-GalT are summarized in Scheme 2. 8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**), a known acceptor for β-(1→4)-(GalT),[15] was incubated in parallel with UDP-Gal (**1**) and caged-UDP-gal (**8**) in the presence of the enzyme. The progress of the reaction was monitored by mass-spectrometry where the known disaccharide product **3**[15] could be seen at *m*/*z* 554 $[M+H]^+$ and *m*/*z* 576 $[M+Na]^+$ (see Supplementary data). Acceptor **2** was completely converted to **3** within 2 h in the presence of UDP-Gal (Eq

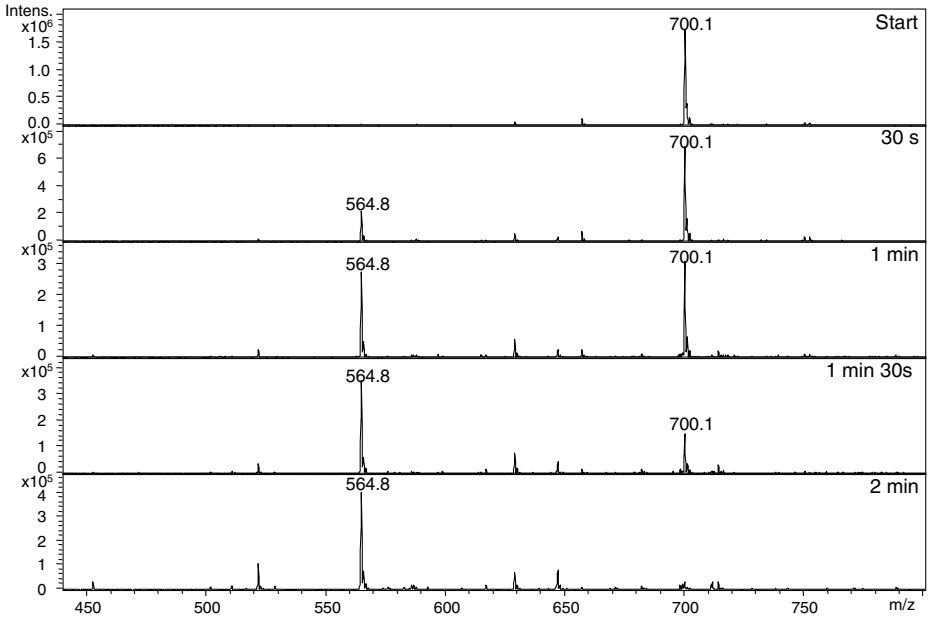
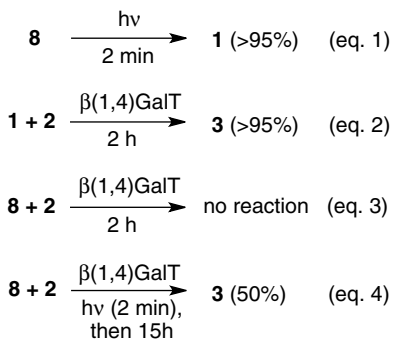


Figure 1. ESIMS analysis of the time course of irradiation of **8** (*m*/*z* 700.1) to give **1** (*m*/*z* 564.8).



Scheme 2. Evaluation of **8** as a caged donor for β-(1→4)-GalT.

2), but no disaccharide product (either with or without a nitrobenzyl group) could be detected in the corresponding incubation with **8** (Eq 3). The latter solution was then irradiated at 365 nm for 2 min and incubated overnight after which the mass-spectrum suggested an approximately 50% yield of product **3** (Eq 4). The presence of the hydrophobic aglycone in **2** and **3** allowed their isolation from the reaction mixture by reverse-phase extraction cartridges. The ^{1}H NMR spectrum of this retrieved material confirmed the presence of **2** and **3** in a ratio of near 1:1 (see Supplementary data).

The above results confirmed our hope that **8** would not be a substrate for the enzyme, but it remained to demonstrate that it did in fact bind to the enzyme which is a requirement for its use as a caged donor in time-resolved protein crystallography. To demonstrate this, **8** was evaluated as an inhibitor for the transfer reaction shown in Chart 1 and in Eq 2 (Scheme 2) using a reported radioactive assay,[16] where it could be compared to the known inhibitor uridine 5′-diphosphate (UDP). Assuming **8** to be a competitive inhibitor allowed the estimation of the K_i-values for **8** (42 μM) and UDP (29 μM) using the equation $i = [I]/([I] + K_i\{1 + [S]/K_m\})$,[17] where i is the fractional inhibition, [I] the inhibitor concentration and [S] the substrate concentration. The K_m-value for UDP-Gal was set to 43 μM.[18] Compound **8** was thereby shown to be an inhibitor of β-(1→4)-GalT and only slightly less potent than the known inhibitor UDP.

It was disappointing to see that the enzyme looses substantial activity as a result of the irradiation. This is most likely due to the reaction of the released aldehyde **6** with the enzyme, as discussed above. Further evidence for this proposal comes from the observation that addition of a pre-irradiated solution of **8** to the enzyme and acceptor **2** led to the same reduced yield of disaccharide formation.

Whether or not this inactivation side-reaction will turn out to be detrimental in a short-duration (millisecond) crystallographic experiment using a rapid pulsed-laser remains to be seen. Should that turn out to be the case, the addition of aldehyde-sequestering agents like hydroxylamine or hydrazides may suppress this side-reaction. Alternatively, the use of the methylbenzyl group (shown in **5**) or even more promising new-generation photolabile groups[19] will be explored.

In summary, we synthesized the caged-UDP-Gal compound **8** and confirmed that it is not a substrate for β-(1→4)-GalT. After brief irradiation, however, **8** becomes uncaged to give UDP-Gal (**1**) and the enzymatic reaction proceeds to produce disaccharide **3**. This system therefore fulfills the minimum requirements for time-resolved crystallography experiments.

3. Experimental

3.1. General methods

Organic solvents were concentrated under diminished pressure at <40 °C (bath temperature). TLC was carried out on silica plates (Merck 60 F_{254} aluminium plates) with detection by UV-light or 8% sulfuric acid. Column chromatography was performed on silica gel (Merck, 230–400, 60 Å) and reversed phase chromatography was performed on silica gel (Bondapak C18 125 Å 37–55 μm, Waters). Photolysis reactions were carried out under a 400 W Hg(Xe) high-pressure mercury lamp.[20] ESIMS was recorded on a Bruker Esquire 3000-Plus Ion Trap instrument with samples injected as solutions in 1:1 MeCN–water mixtures. HRESIMS was recorded on a Q-Tof Ultima instrument from Micromass. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 20 °C on a Bruker DPX 250 MHz instrument, Varian Unity 500 MHz (compounds **3** and **8**) or Bruker Avance 600 MHz instrument (compound **16**) using $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 for ^{13}C), D_2O (4.79 ppm for ^{1}H and acetone 31 ppm for ^{13}C) or CD_3OD (3.31 ppm for ^{1}H and 49.0 ppm for ^{13}C) as the internal standards. ^{31}P NMR spectra were recorded in $CDCl_3$, CD_3OD or D_2O using 2% H_3PO_4 in D_2O as the external standard.

3.2. 1,2:3,4-Di-*O*-isopropylidene-6-*O*-2-nitrobenzyl-α-D-galactopyranose (10)

2-Nitrobenzyl bromide (83 mg, 0.384 mmol) and NaH (9 mg, 0.384 mmol) were added to a soln of 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose (**9**) (50 mg, 0.192 mmol) in MeCN (1 mL) and irradiated (Biotage Initiator microwave synthesizer) in a closed vessel for 4 min at 180 °C. The reaction mixture was quenched with MeOH, concentrated and purified by silica gel chromatography (10:1 toluene–EtOAc) to give **10** (67 mg, 0.169 mmol, 88%). ^{1}H NMR ($CDCl_3$, δ ppm): 1.33 (s, 6H, CCH_3), 1.43 (s, 3H, CCH_3), 1.55 (s, 3H, CCH_3), 3.75 (m, 2H, H-6a, H-6b), 4.07 (m, 1H, $J_{4,5}$ 1.8 Hz, H-5), 4.28 (dd, 1H, $J_{4,5}$ 1.9 Hz, $J_{4,3}$ 7.9 Hz, H-4), 4.32 (dd, 1H, $J_{2,3}$ 2.4 Hz, H-2), 4.62 (dd, 1H, $J_{2,3}$

2.4 Hz, $J_{3,4}$ 7.9 Hz, H-3), 4.95 (s, 2H, $ArCH_2$), 5.54 (d, 1H, $J_{1,2}$ 5.0 Hz, H-1), 7.41 (dt, 1H, *J* 1.4 Hz, *J* 8.2 Hz, Ar-H), 7.62 (dt, 1H, *J* 1.2 Hz, *J* 7.6 Hz, Ar-H), 7.82 (d, 1H, *J* 7.8 Hz, Ar-H), 8.03 (dd, 1H, *J* 1.2 Hz, 8.2 Hz, Ar-H); ^{13}C NMR ($CDCl_3$, δ ppm): 24.6, 25.0, 26.1, 26.2, 66.8, 69.9, 70.0, 70.7, 70.8, 71.3, 96.5, 108.7, 109.5, 124.7, 127.9, 128.9, 129.2, 133.7; HRESIMS: $[M+H]^+$ calcd for $C_{19}H_{25}NO_8$, 396.1658; found, 396.1656.

3.3. 1,2,3,4-Tetra-*O*-acetyl-6-*O*-2-nitrobenzyl-α-D-galacto-pyranose (12)

Compound **10** (85 mg, 0.215 mmol) was dissolved in 80% HOAc, heated to 80 °C overnight, concentrated to give **11** (63 mg, 200 mmol, 93%). ^1H NMR (D_2O, δ ppm): 3.45 (dd, 0.6H, H-2β), 3.60 (dd, 0.6H, $J_{3,2}$ 3.4 Hz, $J_{3,4}$ 9.9 Hz, H-3β), 3.70–3.93 (m, 4.4H, H-2α, H-3α, H-4α, H-4β, H-5β, H-6a, H-6b), 4.21 (t, 0.4H, $J_{4,5}$ 6.0 Hz, H-5α), 4.54 (d, 0.6H, $J_{1,2}$ 7.8 Hz, H-1β), 4.90 (s, 2H, $ArCH_2$), 5.22 (d, 0.4H, $J_{1,2}$ 3.3 Hz, H-1α), 7.53 (m, 1H, ArH), 7.69 (m, 1H, ArH), 8.03 (d, 1H, *J* 8.2 Hz, ArH); ^{13}C NMR (D_2O, δ ppm): 68.7, 69.1, 69.4, 69.5, 70.0, 70.2, 70.3, 70.5, 70.7, 72.2, 73.1, 73.8, 92.7, 96.8, 125.4, 129.6, 130.4, 134.6; ESIMS: $[M+Na]^+$ calcd for $C_{13}H_{17}NO_8$, 338.1; found, 338.0. The residue was acetylated using Ac_2O (1 mL) and pyridine (2 mL) for 1 h. The reaction mixture was concentrated and purified by silica gel chromatography (5:1 toluene–EtOAc) to give **12** (86 mg, 0.178 mmol, 89%). ^1H NMR ($CDCl_3$, δ ppm): 1.96–2.15 (m, 12H, CCH_3), 3.53–3.75 (m, 2H, H-6a, H-6b), 4.05 (t, 0.6H, $J_{4,5}$ 6.4 Hz, H-5β), 4.35 (t, 0.4H, *J* 6.2 Hz, H-5α), 4.85 (m, 2H, $ArCH_2$), 5.09 (dd, 0.6H, H-3β), 5.32 (m, 2H, H-3α, H-2α, H-2β), 5.51 (d, 0.6H, $J_{3,4}$ 3.3 Hz, H-4β), 5.57 (s, 0.4H, H-4α), 5.69 (d, 0.6H, $J_{1,2}$ 8.3 Hz, H-1β), 6.36 (d, 0.4H, $J_{1,2}$ 2.6 Hz, H-1α), 7.40 (t, 0.6H, *J* 7.3 Hz, Ar-H), 7.65 (m, 2H, Ar-H), 8.03 (d, 1H, *J* 8.1 Hz, Ar-H); ^{13}C NMR ($CDCl_3$, δ ppm): 20.6, 20.7, 20.9, 21.0, 66.7, 67.4, 67.7, 68.1, 68.6, 68.9, 70.0, 70.1, 71.1, 73.0, 89.9, 92.3, 124.8, 128.2, 128.2, 128.7, 128.8, 133.9, 134.0, 169.1, 169.5, 170.0, 170.2; HRESIMS: $[M+Na]^+$ calcd for $C_{21}H_{25}NO_{12}$, 506.1274; found, 506.1268.

3.4. 2,3,4-Tri-*O*-acetyl-6-*O*-2-nitrobenzyl-D-galacto-pyranose (13)

Hydrazine acetate (14 mg, 0.155 mmol) was added to compound **12** (50 mg, 0.103 mmol) in DMF and heated to 50 °C for 30 min. The reaction mixture was diluted with EtOAc and washed with NaCl (satd, aq), dried (Na_2SO_4), filtered, concentrated and purified by silica gel chromatography (5:1 toluene–EtOAc) to give **13** (38 mg, 0.086 mmol, 83%). ^1H NMR ($CDCl_3$, δ ppm): 1.98–2.16 (m, 9H, CCH_3), 3.64 (m, 2H, H-6a, H-6b), 3.97 (t, 0.25H, $J_{4,5}$ 6.3 Hz, H-5β), 4.51 (t, 0.75H, $J_{4,5}$ 6.3 Hz, H-5α), 4.72 (br s, 0.25H, H-1β), 4.88 (m, 2H, $ArCH_2$), 5.09 (d, 0.5H, H-2β, H-3β), 5.16 (dd, 0.75H, $J_{2,3}$ 10.8 Hz, $J_{3,4}$ 3.6 Hz, H-2α), 5.43 (dd, 0.75H, $J_{2,3}$ 10.7 Hz, $J_{3,4}$ 3.2 Hz, H-3α), 5.51 (m, 1.75H, H-4α, H-4β, H-1α), 7.44 (m, 1H, Ar-H), 7.67 (m, 2H, Ar-H), 8.07 (m, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, δ ppm): 20.8, 20.8, 21.0, 67.5, 67.6, 67.8, 68.6, 69.0, 69.2, 69.0, 69.6, 70.1, 70.2, 70.7, 71.3, 73.2, 90.9, 96.2, 124.8, 124.9, 128.3, 128.9, 128.9, 133.9, 134.0, 170.2, 170.4, 170.5; HRESIMS: $[M+Na]^+$ calcd for $C_{19}H_{23}NO_{11}$, 464.1169; found, 464.1168.

3.5. 2,3,4-Tri-*O*-acetyl-6-*O*-2-nitrobenzyl-α-D-galacto-pyranosyl hydrogen-phosphonate triethylammonium salt (14)

PCl_3 (127 μL, 1.45 mmol) was added to a soln of imidazole (125 mg, 1.84 mmol) and Et_3N (275 μL, 1.97 mmol) in MeCN at 0 °C and stirred for 30 min. Compound **13** (116 mg, 0.263 mmol) in MeCN was added during 30 min at 0 °C. The reaction mixture was stirred at rt for 15 min, quenched with TEAB (triethylammonium bicarbonate) (1 M) and concentrated. The residue was dissolved in $CHCl_3$, washed with TEAB (triethylammonium hydrogen carbonate buffer, 1 M), filtered through cotton wool, concentrated and purified by silica gel chromatography (1:0→9:1 $CHCl_3$–MeOH containing 1% Et_3N) to give **14** (98 mg, 0.162 mmol, 62%). ^1H NMR ($CDCl_3$, δ ppm): 1.93 (s, 3H, CCH_3), 2.00 (s, 3H, CCH_3), 2.07 (s, 3H, CCH_3), 3.57 (m, 2H, H-6a, H-6b), 4.50 (t, *J* 6.3 Hz, H-5), 4.80 (m, 2H, $ArCH_2$), 5.20 (m, 1H, H-2), 5.42 (dd, 1H, $J_{2,3}$ 10.7 Hz, $J_{3,4}$ 3.2 Hz, H-3), 5.51 (m, 1H, H-4), 5.81 (dd, 1H, $J_{1,P}$ 8.8 Hz, $J_{1,2}$ 3.4 Hz, H-1), 6.92 (d, 1H, $J_{P,H}$ 636 Hz, P–H), 7.38 (t, 1H, *J* 7.3 Hz, Ar-H), 7.63 (m, 2H, Ar-H), 7.99 (d, 1H, *J* 8.2 Hz, Ar-H); ^{13}C NMR ($CDCl_3$, δ ppm): 20.7, 20.8, 67.8, 68.0, 68.1, 69.1, 70.0, 91.9, 92.0, 124.6, 128.1, 128.8, 134.0, 170.0, 170.2, 170.3; ^{31}P NMR ($CDCl_3$, δ ppm): 1.81; HRESIMS: $[M+Na]^+$ calcd for $C_{19}H_{23}NO_{13}P$, 528.0883; found, 528.0864.

3.6. 2,3,4-Tri-*O*-acetyl-6-*O*-2-nitrobenzyl-α-D-galacto-pyranosyl phosphate bispiperidinium salt (16)

Pivaloyl chloride (24 μL, 0.191 mmol) was added to a soln of compound **14** (28 mg, 0.0462 mmol) and 9-fluorenemethanol (23 mg, 0.120 mmol) in pyridine. The reaction mixture was stirred at rt for 3 h and then cooled to −40 °C and a soln of I_2 (23 mg, 0.0924 mmol) in pyridine–water (49:1, 2 mL) was added. The oxidation was completed at 0 °C and the reaction was diluted with $CHCl_3$, washed with $Na_2S_2O_3$ (10%) and TEAB (1 M), filtered (cotton wool) and concentrated. The residue was dissolved in CH_2Cl_2 and piperidine (0.4 mL) was added and stirred at rt for 30 min,

concentrated and purified by reversed phase chromatography (1:0→9:1 water–MeOH) to give **16** (27 mg, 0.039 mmol, 84%). ^{1}H NMR (CD_3OD, δ ppm): 1.96 (s, 3H, CCH_3), 2.07 (s, 3H, CCH_3), 2.11 (s, 3H, CCH_3), 3.60 (t, 1H, H-6a), 3.70 (m, 1H, H-6b), 4.60 (t, 1H, $J_{4,5}$ 6.9 Hz, H-5), 4.90 (m, 2H, $ArCH_2$), 5.11 (m, 1H, H-2), 5.43 (dd, 1H, $J_{3,4}$ 3.3 Hz, $J_{2,3}$ 10.9 Hz, H-3), 5.58 (d, 1H, J 2.1 Hz, H-4), 5.74 (dd, 1H, $J_{1,P}$ 7.5 Hz, $J_{1,2}$ 3.3 Hz, H-1), 7.50 (t, 1H, J 7.9 Hz, ArH), 7.72 (m, 1H, ArH), 8.04 (d, 1H, J 8.0 Hz, ArH); ^{13}C NMR (CD_3OD, δ ppm): 20.6, 20.7, 20.9, 68.6, 69.2, 69.5, 69.9, 70.9, 93.4, 93.5, 125.6, 129.6, 130.4, 134.7, 171.7, 172.1, 172.2; ^{31}P NMR (CD_3OD, δ ppm): −0.93; ESIMS: $[M-H]^-$ calcd for $C_{19}H_{24}NO_{14}P$, 520.1; found, 520.1.

3.7. Uridine 5′-(6-*O*-2-nitrobenzyl-α-D-galactopyranosyl) diphosphate disodium salt (8)

UMP-morpholidate (50 mg, 0.0726 mmol) and 1*H*-tetrazole (10 mg, 0.145 mmol) were added to a soln of **16** (22 mg, 0.0363 mmol) in pyridine. After stirring at rt for 2 days the reaction mixture was concentrated. The residue was dissolved in 7:1 MeOH–water and Et_3N (252 μL, 1.81 mmol) was added. The reaction mixture was stirred at room temperature for 48 h, concentrated and purified by DEAE-Sephacel ion-exchange column chromatography (Pharmacia, linear gradient from 30 mM to 400 mM NH_4HCO_3) and concentrated to about 10 mL. Dowex 50W X 8 (H^+, 200–400 mesh) was added slowly until the pH reached 6.5. The resin was removed by filtration and Dowex 50W X 8 (Na^+, 20–50 mesh) was added and stirred for a couple of minutes. The resin was removed by filtration and the filtrate lyophilized. The residue was passed through a gel filtration column (Sephadex G-10) eluting with water, followed by reversed phase chromatography eluting with water and lyophilization gave **8** (12 mg, 161 μmol, 44%). ^{1}H NMR (D_2O, δ ppm): 3.71–3.76 (m, 3H, H″-2, H″-6a, H″-6b), 3.88 (d, 1H, J 10.6 Hz, H″-3), 3.99 (s, 1H, H′-4), 4.11–4.18 (m, H′-2, H′-3, H′-4), 4.24 (m, H′-5a, H′-5b), 4.29 (t, 1H, $J_{4,5}$ 6.6 Hz, H″-5), 4.89 (q, 2H, J 14.4 Hz, J 6.7 Hz, $ArCH_2$), 5.58 (dd, 1H, $J_{1,P}$ 7.3 Hz, $J_{1,2}$ 3.4 Hz, H″-1), 5.84 (m, 2H, H-6, H′-1), 7.50 (m, 1H, H-5), 7.69 (m, 2H, ArH), 7.83 (d, 1H, ArH, J 8.2 Hz), 8.03 (d, 1H, ArH, J 8.1 Hz); ^{31}P NMR (D_2O, δ ppm): −11.2, −12.8; HRESIMS: $[M-H]^-$ calcd for $C_{22}H_{29}N_3O_{19}P_2$, 700.0792; found, 700.0795.

3.8. Photolysis of uridine 5′-(6-*O*-2-nitrobenzyl-α-D-galactopyranosyl) diphosphate disodium salt

Uridine 5′-(6-*O*-2-nitrobenzyl-α-D-galactopyranosyl) diphosphate disodium salt (2.5 mg, 3.35 μmol) and 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**) (0.7 mg, 1.79 μM) were dissolved in MOPS buffer (50 mM, 200 μL, pH 7.0). 400 mU β-(1→4)-galactosyltransferase (from bovine milk, Fluka, 1 U in 1 mL MOPS buffer pH 7.0, containing BSA 1 mg/mL and 5 mM $MnCl_2$) and alkaline phosphatase (Merck, 10 U) were added and the reaction mixture was left at rt. After 2 h the reaction was radiated with a 400 W Hg(Xe) high-pressure mercury lamp at 365 nm for 2 min and left at room temperature over night. The mixture was diluted with water and purified on SepPak C-18 cartridges, which were pre-washed with 15 mL MeOH and 50 mL water. The SepPak cartridges were washed with 50 mL water and the product was eluted with 10 mL MeOH to give disaccharide **3** (50%) and unreacted acceptor **2** (50%). The yields were determined from the ^{1}H NMR spectrum that was consistent with previously reported data.

3.9. Inhibition studies

Inhibition studies were carried out using a reported radiochemical assay.[16] Briefly, ^{3}H-labelled galactose is enzymatically transferred from labelled UDP-Gal to acceptor **2** giving the hydrophobic labelled product **3** (Chart 1) that can be isolated on a Sep-Pak C18 cartridge and eluted with MeOH for quantitation. The assays were performed at 37 °C in a total volume of 15 μL containing 50 mM MOPS buffer pH 7.0, 20 mM $MnCl_2$, 1 mg/mL bovine serum albumin, 50 μM UDP-Gal, UDP-[6-^{3}H]Gal (corresponding to 50,000 dpm) and 400 μM GlcNAc-$(CH_2)_8$COOMe (**2**). The concentration of the β-(1→4)-GalT was chosen so that not more than 10% of the donor was consumed in the reaction. The reaction mixtures were then transferred to pre-washed C18 Sep-Pak cartridges which were then washed with water (50 mL) and the radiolabelled product was eluted with MeOH (3.5 mL) and quantitated by liquid scintillation.[16] The K_i values for **8** and UDP were estimated from incubations performed at inhibitor concentrations of 25 μM, 50 μM and 100 μM.

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Supplementary data

^{1}H and ^{13}C NMR spectra of compounds **10**–**14** and **16**, ^{1}H NMR spectrum of **8** and the product described in Eq 4 (Scheme 2), ESIMS spectra of the products described

in Eqs 2–4 (Scheme 2). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.01.021.

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