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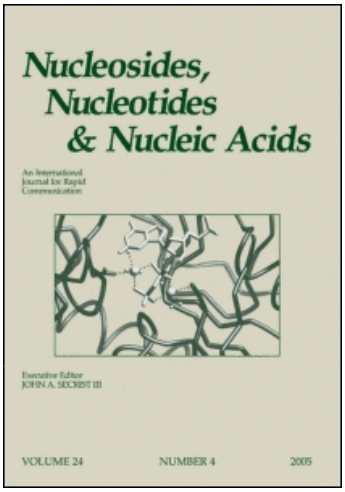

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Synthesis of Uridine 5'-[2-*S*-Pyridyl-3-thio-α-d-galactopyranosyl Diphosphate]: Precursors of UDP-Thiogal Sugar Nucleotide Donor Substrate for β-1,4-Galactosyltransferase

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Synthesis of Uridine 5′-[2-*S*-Pyridyl-3-thio-α-D-galactopyranosyl Diphosphate]: Precursor of UDP-Thiogal Sugar Nucleotide Donor Substrate for β-1,4-Galactosyltransferase†

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ABSTRACT

The syntheses of a novel uridine diphosphate galactose (UDP-Gal) analog, (UDP-2,4,6-tri-*O*-acetyl-3-*S*-acetyl-3-thio-α-D-galactopyranose) (**11**) and the thiolpyridine protected (Uridine 5′-[3-*S*-(2-*S*-pyridyl)-3-thio-α-D-galactopyranosyl diphosphate) analog (**12**) are described. The reported synthesis relies on the novel use of thiolpyridine to generate **12** which is a suitably protected intermediate for generating a UDP-thioGal derivative by reduction prior to enzyme transfer via β-1,4-galactosyltransferase.

Key Words: Sugar nucleotides; β-1,4-galactosyltransferase; Thio sugars; Dithiodipyridine; Thiopyridine derivatives.

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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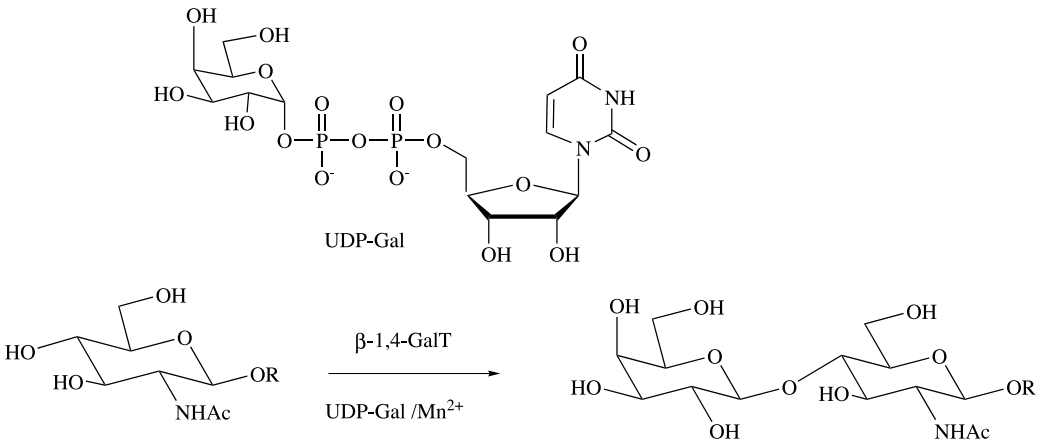
INTRODUCTION

β-1,4-galactosyltransferase (β-1,4-GalT, EC 2.4.1.90) is one of the late acting enzymes of the N-glycan biosynthetic pathway.[1] The enzyme utilizes uridine diphosphate galactose (UDP-Gal) as a donor substrate to transfer a galactopyranose moiety to an acceptor possessing a terminal 2-deoxy-2-*N*-acetyl-α/β-glucopyranose (GlcNAc) residue as shown in Scheme 1. The donor and acceptor specificity of β-1,4 GalT has been studied extensively and recently the crystal structure of the enzyme has been elucidated.[2,3]

Several studies have substituted unnatural substituents into UDP-Gal to probe the active site of β-1,4 GalT. The 6-fluoro analog of UDP-Gal (UDP-6-deoxy-6-fluoro-α-D-Gal) proved to be a substrate that transferred at a relative rate of 0.2%[4] as opposed to UDP-2-fluoro-2-deoxy-α-D-Gal which was a competitive inhibitor. The carbocyclic analog of UDP-Gal, UDP-5a-carba-α-D-Gal,[5] was also a competitive inhibitor whereas a derivative containing a 5 thiol (UDP-5-thio-α-Gal)[6] was transferred at a relative rate of 5% compared to UDP-Gal. In general, *O*-methylated analogs of UDP-Gal were not substrates for the enzyme, the exception being the 2-*O*-methylated analog[7] which was transferred at a rate of 0.20% compared to UDP-Gal. Of the deoxy analogs, only the UDP-6-deoxy-α-D-Gal and 3-deoxy were utilized as donor substrates by the enzyme, the latter of which represents the only example of an unnatural UDP-Gal analog modified at the 3 position that is utilized by the enzyme.

To further probe the active site of β-1,4-GalT, we have initiated the synthesis of thiol containing analogs of UDP-Gal that would not only provide information about the specificity of the enzyme but could also be used to generate unnatural complex oligosaccharide processing thiol groups. This thiol modification could be useful in probing the binding of complex oligosaccharides to lectins or to further functionalize oligosaccharides into biological probes. Recently we reported the synthesis of uridine 5′-(2,3,6-tri-*O*-acetyl-4-*S*-acetyl-4-thio-α-D-galactopyranosyl diphosphate)[8] and uridine



Scheme 1. Transfer of Gal from UDP-Gal to terminal GlcNAc by galactosyltranferase.

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5′-(2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl diphosphate).[9] With the exception of these examples, no other chemical synthesis of thiol sugar nucleotide analogs has been reported. In this report, we describe the synthesis of uridine 5′-(2,4, 6-tri-*O*-acetyl-3-*S*-acetyl-3-thio-α-D-galactopyranosyl diphosphate) (**11**) and its conversion into the corresponding thiopyridinylated form (**12**).

RESULTS AND DISCUSSION

The synthesis of **11** was carried out using a modification of the approach previously used for the synthesis of its 4-*S*-acetyl[8] and 6-*S*-acetyl[9] regioisomers. The introduction of the thiol group at the 3-position of a galactopyranose ring was based on Lemieux and Stick furanose methodology.[10] Nucleophilic displacement of a suitably protected gulopyranose triflate was not successful and it only led to elimination side products.

As shown in Scheme 2, 1,2:5,6-diisopropylidene-D-glucose (diacetone-D-glucose, DAG) (**1**) was oxidized using PDC/Ac_2O in CH_2Cl_2[11] to obtain 1,2 :5,6-di-*O*-isopropylidene-α-ribo-hexofuranos-3-ulose (**2**). Although different approaches have been reported for the synthesis of **2**,[12,13] the DMSO-based oxidation was not suitable in this case since traces of DMSO would inhibit a subsequent hydrogenation reaction.[14] Following Lemieux's procedure, **2** was enolized using pyridine-Ac_2O with heating to give the enol acetate, 3-*O*-acetyl-1,2 :5,6-di-*O*-isopropylidene-α-D-erythro-hex-3-enofuranose (**3**). The enol acetate was converted to 3-*O*-acetyl-1,2 :5,6-di-*O*-isopropylidene-α-D-gulofuranose (**4**) by hydrogenation. Following de-*O*-acetylation and triflation of the 3-OH, the thiol was introduced using potassium thioacetate in a biphasic mixture using phase transfer conditions according to the procedure of Turnbull and Field[15] to give 3-*S*-acetyl-3-thio-1,2 :5,6-di-*O*-isopropylidene-α-D-galactofuranose (**6**). Amberlite IR-20 (H^+) treatment of an aqueous suspension of **6**, followed by acetylation yielded 1,2,4,6-tetra-*O*-acetyl-4-*S*-acetyl-4-thio-α/β-galactopyranoside (**7**) in α/β ratio of 1:1. This in turn was treated with $TiBr_4$ to give the galactosyl bromide (**8**) which was hydrolyzed to give 2,4,6-tri-*O*-acetyl-3-*S*-acetyl-3-thio-α/β-galactopyranose (**9**) in α/β ratio of 2:1. Intermediate **9** was phosphorylated using tetrabenzylpyrophosphate (TBPP) and n-butyllithium to give dibenzyl 2,4,6-tri-*O*-acetyl-3-*S*-acetyl-3-thio-α-galactopyranosyl phosphate (**10**). The benzyl groups were removed by transfer hydrogenolysis and final coupling to UMP-morpholidate gave the desired pyrophosphate, uridine 5′-(2,4,6-tri-*O*-acetyl-3-*S*-acetyl-3-thio-α-galactopyranosyl diphosphate) (**11**) which was purified using preparative RP-HPLC.

Attempted deacetylation of **11** using a combination of diethylamine (DEA)-H_2O-MeOH led to decomposition of the pyrophosphate linkage as indicated by a change in retention time from 10 to 2 min on strong anion exchange (SAX)-HPLC. Further evidence of decomposition was provided by ^{31}P NMR analysis of the deacetylated product which showed a signal at 11.5 and 5 ppm corresponding to phosphomonoesters. Alternatively, deacetylation of peracetylated UDP-Gal under the same conditions preserved the pyrophosphate linkage using the criteria described above.

Instead of direct deacetylation, the thiol group was trapped during the deacetylation reaction with a temporary protecting group (2-thiopyridine) that can be removed during a subsequent reaction under neutral conditions. Scheme 3 illustrates the deacetylation

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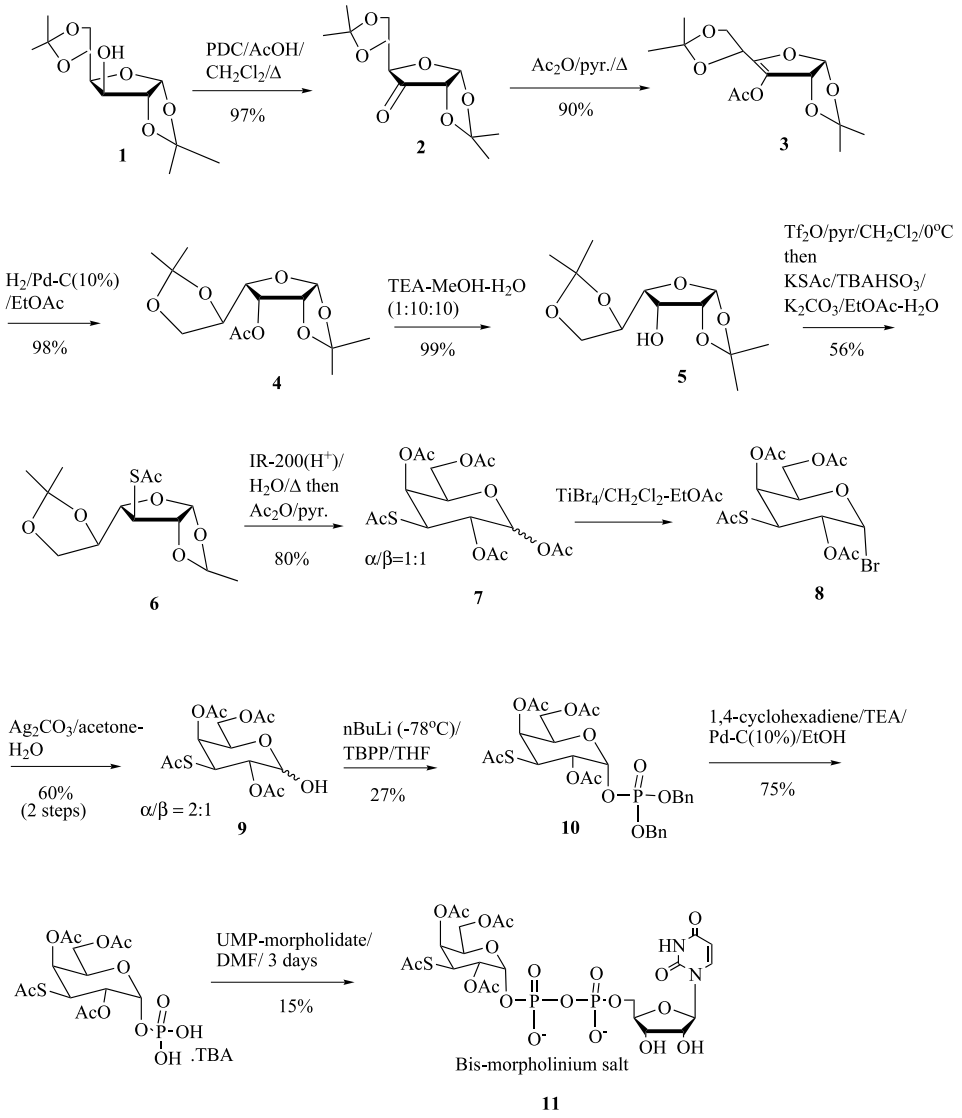

Scheme 2. Synthesis of acetylated UDP-3-thiogalactose.

thiopyridinylation of **11** which worked most efficiently with 50 mol excess of DTDP in MeOH-H_2O-DEA.

Following purification of **12**, the removal of the 2-*S*-pyridyl group was accomplished under neutral conditions using TCEP (Scheme 3). SAX-HPLC analysis of the fully deprotected product verified the stability of the pyrophosphate linkage as did ^{31}P NMR analysis. ESI-MS analysis of **13** established the presence of a major peak at 582.2 m/z indicating the formation of the desired UDP-*S*-Gal derivative and a peak at m/z 403.0 corresponding to the UDP fragment.

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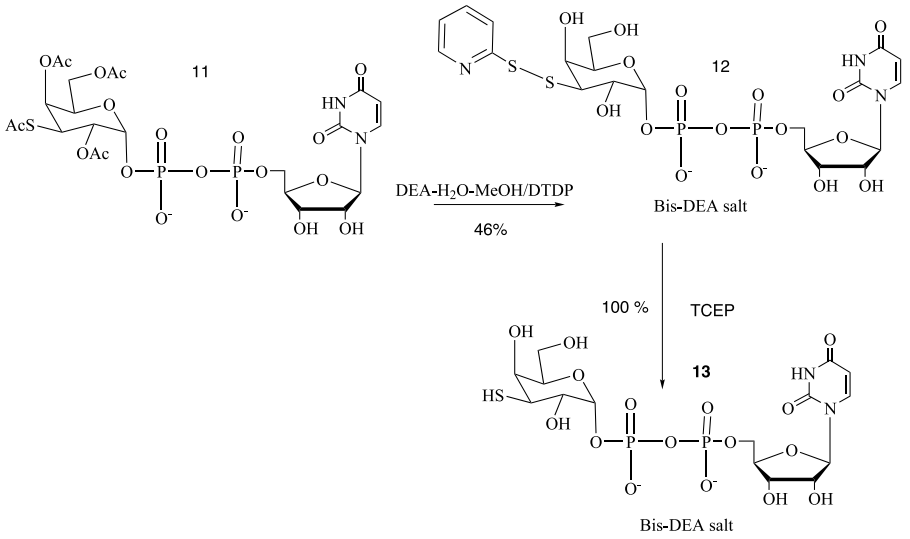

Scheme 3. Final deprotection of UDP-3-thiogalactose.

CONCLUSION

The synthesis of a novel 3-thiol sugar nucleotide analog of UDP-Gal was accomplished by introducing the thiol stereoselectively into the 3 position of a suitable protected gulofuranose derivative followed by manipulation of the anomeric position to give the desired sugar nucleotide pyrophosphate. The thiol analog was found to be unstable during removal of acetyl groups under basic conditions. This problem was solved by deacetylation in the presence of DTDP to form a thiolpyridine intermediate that allowed conversion to the corresponding UDP-thioGal analog by reduction with TCEP under neutral conditions.

EXPERIMENTAL SECTION

General Methods for All Synthetic Schemes and Reactions Described. ^{1}H- and ^{13}C-NMR spectra were recorded at ambient temperature on a Bruker NMR (AVANCE DRX500 or AVANCE DPX300). Samples in $CDCl_3$ used 1% TMS as an internal standard whereas samples prepared in MeOH or D_2O used acetone as an internal standard. ^{31}P-NMR spectra were recorded using neat H_3PO_4 as an external standard. Chemical shifts are expressed in δ (ppm) and coupling constants (*J*) in Hz. High-resolution mass spectra (HRMS) were recorded on a magnetic sector (Micromass, model 70-250S) by chemical ionization with ammonia, sodium, methane or hydrogen. All reactions were monitored by TLC on aluminum sheets precoated with Silica Gel 60 F_{254} (Alltech) (0.2 mm thickness) visualized by UV light or by charring with 10% H_2SO_4 in MeOH. Flash column chromatography was carried out using silica gel (40 μm) (Scientific Adsorbents). All reaction solvents were dried prior to use according

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to standard procedures. Organic solvents were removed on a rotary evaporator under water aspiration vacuum with bath temperatures of 35–40°C. Whenever anhydrous conditions were required, the reactions were conducted under dry nitrogen and reagent transfer was performed using hypodermic syringes. Silica gel chromatography solvents were of HPLC grade. All reagents were purchased from Aldrich with the exception of the following. Uridine 5′-monophosphomorpholidate was purchased from Sigma. Analytical RP-HPLC experiments were carried out using a C18 column (Microsorb-MW, 25 cm × 4.6 mm or Prosphere-C18, 25 cm × 4.6 mm) on an Isco model 2350 pump equipped with a 2360 gradient programmer while preparative RP-HPLC purification steps were carried out using a Prosphere C18 column (Alltech, 25 cm × 22 mm) on an Isco model 2350 dual pump HPLC. Analytical SAX-HPLC experiments were carried out using a Spherisorb strong anion exchange column (25 cm × 4.5 mm). RP-HPLC and SAX-HPLC separations were monitored at 262 nm for the uridine ring and in some instances at 343 nm for the 2-pyridylthione. As described below, RP-HPLC mobile phase consisted of a gradient of 0 to 35% acetonitrile over 30 min in 10 mM ammonium acetate (pH 5.0) while SAX-HPLC mobile phase consisted of a gradient of 0 to 20% sodium phosphate (200 mM, pH 7.5) over 30 min.

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1,2:5,6 Di-*O*-isopropylidene-α-D-ribohexofuranos-3-ulose (2): DAG (**1**) (4.00 g, 15.3 mmol) was dissolved in CH_2Cl_2 (40 mL) along with Ac_2O (4 mL). PDC (7.0 gm) was added to the reaction which was refluxed for 2 h. TLC analysis using toluene:MeOH (19:1, v/v) showed complete conversion of the starting material (R_f 0.2) to **2** (R_f 0.3). The solvent was evaporated using a water aspirator and then further dried using a vacuum pump, redissolved in EtOAc (10 mL) and purified on a short silica gel column (5 cm) eluted with 100% EtOAc. The fractions corresponding to the product were collected and dried to give 3.85 g of **2** (14.8 mmol, 97%). NMR data ($CDCl_3$, TMS): ^{1}H (300 MHz) δ 6.15 (d, 1 H, H-1, $J_{1,2}$ 4.5), 4.34–4.42 (m, 3 H, H-2, H-4, H-5), 4.02–4.06 (m, 2 H, H-6, H-6′), 1.46, 1.42 (each s, each 3 H, each Me), 1.34 (s, 6 H, 2 × Me).

3-*O*-Acetyl-1,2:5,6-di-*O*-isopropylidene-α-D-erthyrohex-3-enofuranose (3): 1,2:5,6-Di-*O*-isopropylidene-α-D-ribohexofuranos-3-ulose (**2**) (3.50 g, 13.5 mmol) was dissolved in pyridine (20 mL) and Ac_2O (20 mL) and refluxed gently for 10 h. TLC analysis revealed disappearance of the starting material and the appearance of single new spot (toluene:MeOH, 19:1, v/v, R_f 0.8). The reaction was thoroughly dried using a vacuum pump. This yielded 3.66 g of **3** (12.2 mmol, 90%). NMR data ($CDCl_3$, TMS): ^{1}H (300 MHz) δ 6.04 (d, 1 H, H-1, $J_{1,2}$ 3.8), 5.41 (d, 1 H, H-2, $J_{1,2}$ 3.8), 4.71 (pseudo t, 1 H, H-5), 4.07 (m, 2 H, H-6, H-6′), 2.21 (s, 3 H, OAc), 1.54, 1.48, 1.45, 1.38 (each s, each 3 H, each Me). ^{13} C (75 MHz) δ 169.36, 145.68, 113.85, 110.82, 104.42, 68.99, 66.31, 28.30, 28.25, 26.27, 26.02, 20.93. HRMS for $C_{14}H_{20}O_7$: 300.1205 observed, 300.1209 calculated.

3-*O*-Acetyl-1,2:5,6-di-*O*-isopropylidene-α-D-gulofuranose(4): The 3-enofuranose **3** (3.43 g, 11.4 mmol) was dissolved in ethyl acetate (50 mL) and hydrogenated at room temperature (35 psi) over 10% Pd–C (500 mg). The reaction was monitored by TLC using hexane:EtOAc (2:1, v/v) where it showed the disappearance of the starting material (R_f 0.8) and the formation of the gulofuranose derivative **4** (R_f 0.6). After 20 h, the catalyst was removed by filtration and the solvent was removed by evaporation.

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This yielded 3.39 g of **4** (11.2 mmol, 98%). NMR data ($CDCl_3$, TMS): ^1H (300 MHz) δ 5.81 (d, 1 H, H-1, $J_{1,2}$ 4.0), 5.08 (dd, 1 H, H-3, $J_{2,3}$ 5.6, $J_{3,4}$ 6.7), 4.82 (dd, 1 H, H-2, $J_{1,2}$ 4.0, $J_{2,3}$ 5.6), 4.62 (ddd, 1 H, H-5), 4.08–4.12 (m, 2 H, H-6, H-6′), 3.53 (dd, 1 H, H-4), 2.14 (s, 3 H, OAc), 1.59, 1.45, 1.39, 1.37 (each s, each 3 H, each Me). ^{13}C (75 MHz) δ 169.64, 111.48, 109.26, 105.01, 81.31, 78.49, 75.16, 71.76, 66.35, 26.76, 26.67, 25.25, 21.05, 20.62. HRMS for $C_{14}H_{22}O_7$: 302.1368 observed, 302.1366 calcd.

1,2:5,6-Di-*O*-isopropylidene-α-D-gulofuranose (5): The gulofuranose 3-*O*-acetate **4** (3.25 g, 10.8 mmol) was dissolved in MeOH (20 mL), H_2O (20 mL) and triethylamine (4 mL). The mixture was stirred at room temperature for 3 h and monitored by TLC using hexane:EtOAc (2:1, v/v) as the mobile phase. The solvent was evaporated and the residue further dried by repeated coevaporation with toluene. This yielded 2.77 g of **5** (10.65 mmol, 99%). NMR data ($CDCl_3$, TMS). ^1H (300 MHz) δ 5.82 (d, 1 H, H-1, $J_{1,2}$ 4.1), 4.67 (dd, 1 H, H-2, $J_{1,2}$ 4.1, $J_{2,3}$ 5.7), 4.48 (dd, 1 H, H-5), 4.22 (m, 2 H, H-6, H-6′), 3.88 (dd, 1 H, H-3, $J_{2,3}$ 5.7, $J_{3,4}$ 7.9), 3.72 (dd, 1 H, H-4, $J_{4,5}$ 7.4, $J_{3,4}$ 7.9), 2.67 (broad s, 1 H, 3-OH), 1.63, 1.46, 1.43, 1.38 (each s, each 3 H, each Me). ^{13}C (75 MHz) δ 105.33, 84.35, 79.89, 75.59, 69.67, 66.38, 27.18, 27.13, 26.71, 25.21. HRMS for $C_{12}H_{20}O_6$: 260.1252 observed, 260.1260 calcd.

1,2:5,6-Di-*O*-isopropylidene-3-S-acetyl-3-thio-α-D-galactofuranose (6): Trifluoromethanesulfonic acid anhydride (5 mL) was dissolved in dry CH_2Cl_2 (5 mL) and dry pyridine (2 mL) previously cooled at −10°C. The alcohol **5** (1.0 g, 3.8 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and added dropwise to the triflic anhydride solution at a constant −10°C. Additional CH_2Cl_2 was added to prevent the mixture from freezing. TLC analysis using hexane:EtOAc (2:1, v/v) revealed the formation of a new product at R_f 0.85. After 1 h, the reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with ice-cold 1 N HCl (2 × 20 mL), ice-cold 10 % NH_4Cl (2 × 20 mL) and water (2 × 20 mL). The organic solution was dried over Mg_2SO_4, filtered and concentrated to an orange oily residue which was immediately dissolved in 10 mL EtOAc. K_2CO_3 (1.05 g), tetrabutylammonium hydrogen sulfate (2.58 g) were dissolved in water (10 mL) and added to the gulose-3-O-triflate solution. KSAc (1.74 g) was finally added and the biphasic mixture was stirred at room temperature for 4 h. TLC analysis using hexane:EtOAc (4:1, v/v) revealed the formation of a new product at R_f 0.45 and the complete disappearance of the triflate. The organic layer was collected, washed with water (2 × 10 mL) and purified using flash chromatography using hexane:EtOAc (4:1, v/v) as the mobile phase. The fractions corresponding to the product were collected and dried. This yielded 690 mg of **6** as a brown solid (2.2 mmol, 56%). NMR data ($CDCl_3$, TMS): ^1H (300 MHz) δ 5.92 (d, 1 H, H-1, $J_{1,2}$ 3.7), 4.62 (dd, 1 H, H-2, $J_{2,3}$ 1.5, $J_{1,2}$ 3.7), 4.43 (dd, 1 H, H-5), 4.10 (dd, 1 H, H-6), 3.99 (dd, 1 H, H-4, $J_{4,5}$ 7.2, $J_{3,4}$ 4.7), 3.86 (dd, 1 H, H-6′), 3.70 (dd, 1 H, H-3, $J_{3,4}$ 4.7, $J_{2,3}$ 1.5), 2.39 (s, 3 H, SAc), 1.62, 1.45, 1.38, 1.35 (each s, each 3 H, each Me). ^{13}C (75 MHz) δ 194.40, 114.25, 110.39, 106.20, 87.13, 85.54, 76.57, 66.23, 47.80, 31.97 (SAc), 27.75, 27.02, 25.76, 23.05. HRMS for $C_{13}H_{20}O_6S$: 304.1147 observed, 304.1137 calcd.

1,2,4,6-Tetra-*O*-acetyl-3-S-acetyl-3-thio-(α/β)-D-galactopyranoside (7): To a suspension of **6** (650 mg, 2.0 mmol) in water (10 mL), 550 mg of IR-20 (H^+) was added. Under a continuous gentle stream of nitrogen, the mixture was heated at 70°C

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for 3 h during which the sugar became soluble in water. The mixture was then rapidly filtered and the water was evaporated using a vacuum pump to give a white powder with a strong mercaptan odor. Nitrogen was introduced to the reaction vessel which was in turn cooled in an ice-water bath. Pyridine (10 mL) and Ac_2O (5 mL) were added and the reaction was stirred for 2 h after which the solvent was evaporated and the residue dried by repeated coevaporation with ethanol and then by toluene. This yielded 660 mg of **7** (1.6 mmol, 80 %) as an oily residue in an α/β ratio of 1:1. NMR data ($CDCl_3$, TMS): ^{1}H (300 MHz) δ 6.33 (d, 1 H, H-1 α, $J_{1,2}$ 3.5), 5.77 (d, 1 H, H-1 β, $J_{1,2}$ 8.1), 5.4 (d, 1 H, H-4 α, $J_{3,4}$ 3.7), 5.15–5.34 (m, 3 H, H-4 β, H-2 α, H-2 β), 4.41 (pseudo t, 1 H, H-5 α), 4.27 (dd, 1 H, H-3 α, $J_{3,4}$ 3.7, $J_{2,3}$ 10.8), 3.98–4.16 (m, 6 H, H-3 β, H-5 β, H-6 α, H-6′ α, H-6 β, H-6′ β), 2.34 (SAc, α), 2.33 (SAc, β), 2.21, 2.16, 2.12, 2.10, 2.06 (OAc, α/β). ^{13}C (75 MHz) δ 192.66, 193.87 (Sac, α/β), 170.77, 170.17, 169.85, 169.38, 93.60 (C1 β), 89.60 (C1 α), 74.44, 69.82, 69.20, 68.76, 67.14, 65.75, 47.25, 43.35, 30.94 (SAc), 30.82 (SAc), 21.29, 21.14, 21.02, 20.89. HRMS for $C_{16}H_{22}O_{10}S$: 406.0926 observed, 406.0934 calcd.

2,4,6-Tri-*O*-acetyl-3-S-acetyl-3-thio-(α/β)-D-galactopyranoside (9): 1,2,4, 6-Tetra-*O*-acetyl-3-*S*-acetyl-3-thio-(α/β)-D-galactopyranoside (**7**) (520 mg, 1.28 mmol) was dissolved in CH_2Cl_2 (20 mL), EtOAc (2 mL) along with $TiBr_4$ (0.9 g, 2.5 mmol). The reaction was stirred at room temperature and monitored by TLC using hexane:EtOAc (2:1, v/v) as the mobile phase. After 7 h, 300 mg of sodium acetate was added to the reaction and further stirred for 30 min. The reaction was then diluted with CH_2Cl_2 (20 mL), filtered over a celite pad, washed with ice-cold water (2 × 40 mL) and concentrated using a water aspirator to give a crude mixture of the galactosyl bromide **8** that was immediately used in the next step.

The crude galactosyl bromide **8** was dissolved in acetone (20 mL) containing water (2 mL) along with Ag_2CO_3 (680 mg). The reaction was stirred at room temperature with the exclusion of light for 6 h during which the hydrolysis of **8** was monitored by TLC using the same mobile phase as above. The reaction was then filtered, concentrated and finally purified on silica gel using hexane:EtOAc (1:1, v/v) as the mobile phase. The fractions corresponding to the product were collected and concentrated to give 290 mg of **9** (0.8 mmol, 62 %) as an α/β mixture of 2:1. NMR data ($CDCl_3$, TMS, one drop of D_2O): ^{1}H (300 MHz) δ 5.46 (d, 1 H, H-1 α), 5.39 (d, 1 H, H-4 α), 5.32 (d, 0.5 H, H-4 β), 5.12 (dd, 1 H, H-2 α, $J_{1,2}$ 3.4, $J_{2,3}$ 12.2), 4.94 (dd, 1 H, H-2 β, $J_{1,2}$ 7.8, $J_{1,2}$ 11.7), 4.77 (pseudo t, 0.5 H, H-1 β), 4.55 (pseudo t, 1 H, H-5 α, $J_{3,4}$ 3.0), 4.39 (dd, 1 H, H-3 α, $J_{2,3}$ 12.2), 3.96–4.17 (m, 4 H, H-5 β, H-3 β, H-6 α, H-6′ α, H-6 β, H-6′ β), 2.34, 2.17, 2.11, 2.07. ^{13}C (75 MHz): 193.91, 170.97, 170.55, 170.26, 97.40 (C1 β), 90.66 (C1 α), 70.07, 69.20, 67.58, 62.71, 43.05, 30.95, 21.10, 20.94. HRMS for $C_{14}H_{20}O_9S$: 364.0823 observed, 364.0828 calcd.

Dibenzyl 2,4,6-tri-*O*-acetyl-3-S-acetyl-3-thio-α-galactopyranosyl phosphate (10): The hemiacetal **9** (210 mg, 0.58 mmol) was dissolved in THF (5 mL) and the mixture was cooled to −78°C using an acetone-dry ice bath. n-BuLi (360 μL, 0.6 mmol, from 1.6 M solution in hexane) was added and the reaction was stirred for 3 min after which a solution of THF (5 mL) containing previously prepared tetrabenzyl pyrophosphate (TBPP) (940 mg, 1.74 mmol) was added dropwise. The reaction was stirred for 15 min and then brought up to −45°C by the addition of EtOAc to the dry

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ice bath. The reaction was further stirred for 3 h after which it was diluted with diethyl ether (40 mL) and washed with 1 N cold NaHCO3 (2 × 20 mL) and ice-water (2 × 20 mL). Diethyl ether was evaporated using a water aspirator to give an oily residue which was finally purified by flash chromatography on silica gel using hexane:EtOAc (2:1, v/v, containing 1 % v/v triethylamine) as the mobile phase. The fractions were monitored by UV and TLC charring. Those fractions corresponding to product were collected and dried. This yielded 100 mg of **10** as an oily residue (27 %). NMR data ($CDCl_3$, TMS): ^{1}H (300 MHz) δ 7.36–7.40 (m, 10 H, aromatic), 5.90 (dd, 1 H, H-1, $J_{1,2}$ 3.2, $J_{1,P}$ 7.5), 5.36 (d, 1 H, H-4), 5.05–5.14 (m, 5 H, H-2, 2 × OCH_2Ph), 3.84–4.13 (m, 4 H, H-3, H-5, H-6, H-6′), 2.34 (SAc), 2.14, 1.91, 1.89. ^{13}C (75 MHz) δ 193.54, 170.62, 170.29, 170.11, 94.42 (d, C1), 70.05, 69.94, 69.92, 69.46, 69.27, 62.13, 42.84, 30.94, 20.88, 20.72. ^{31}P (121 MHz) δ − 1.39. HRMS for $C_{28}H_{33}O_{12}PS$: 624.1437 observed, 624.1430 calcd.

Uridine 5′-(2,4,6-tri-*O*-acetyl-3-S-acetyl-3-thio-α-D-galactopyranosyl diphosphate) (11): The sugar dibenzyl phosphate triester (**10**) (55 mg, 88 μmol) was dissolved in EtOH (3 mL) along with 10% Pd–C (50 mg), Bu_3N (20 μL) and 1, 4-cyclohexadiene (1 mL). The reaction was continuously monitored by TLC using EtOAc:hexane (1:1, v/v) and CH_2Cl_2:MeOH:H_2O (65:35:5, v/v/v). The first benzyl group was removed more rapidly than the second benzyl group which required the addition of more catalyst (20 mg) every 2 h to achieve complete hydrogenolysis. The mixture was filtered by gravity and the filter paper was washed with ethanol (5 mL) and the solution was dried under vacuum on a water aspirator to give 41 mg of tetra-*O*-acetylated 3-*S*-acetyl-3-thio-α-sugar 1-phosphate (66 μmol, 75%). NMR data (CD_3OD, 1% acetone) ^{1}H (300 MHz): δ 5.56 (dd, 1 H, H-1, $J_{1,2}$ 3.6, $J_{1,P}$ 7.9), 5.33 (dd, 1 H, H-4), 5.03 (dt, 1 H, H-2, $J_{2,P}$ 2.0, $J_{1,2}$ 3.6, $J_{2,3}$ 10.8), 4.51 (pseudo t, 1 H, H-5), 4.25 (dd, 1 H, H-3, $J_{2,3}$ 10.8, $J_{3,4}$ 3.0), 3.95–4.07 (m, 2 H, H-6, H-6′), 2.24 (SAc), 2.07, 1.98, 1.94 (3 × OAc) tributylamine: δ 3.03 (t, 6 H, $CH_3CH_2CH_2CH_2N$), 1.54 (m, 6 H, $CH_3CH_2CH_2CH_2N$), 1.23 (m, 6 H, $CH_3CH_2CH_2CH_2N$), 0.81 (t, 9 H, $CH_3CH_2CH_2CH_2N$). ^{31}P (120 MHz): δ − 0.43 ppm. ESIMS (negative mode) analysis for $C_{14}H_{21}O_{12}PS^{1-}$ showed a mass at 443.0, 443.0 calcd.

UMP-morpholidate (100 mg, 0.14 mmol) was coevaporated with dry DMF (3 × 3 mL) and this was added to a solution of sugar 1-phosphate (26 mg, 42 μmol) in dry DMF (2 mL). The sugar 1-phosphate had also been previously dried by co-evaporation with dry DMF (3 × 3 mL). The coupling was performed at room temperature with stirring under an atmosphere of nitrogen for 3 days and the reaction was monitored by injecting 100 μL (1 μL of reaction mixture diluted in 1 mL of water) onto an analytical C18 RP-HPLC column, eluted with a gradient of 0% to 35% MeCN in 10 mM ammonium acetate (pH 5.0) buffer over 30 min while monitoring Abs_{262nm} to detect the product **11** eluting at approximately 21 min. The reaction mixture was dried under vacuum using a vacuum pump and redissolved in 3 mL of distilled water. Purification was accomplished by injecting 500 μL of the aqueous mixture onto a preparative C18 RP-HPLC column. The column was eluted at 10 mL/min with the acetonitrile-ammonium acetate (pH 5.0) gradient described above as the mobile phase. Fractions corresponding to the product were pooled from multiple runs and repeatedly concentrated under vacuum using a vacuum pump while heating at 30°C. The residual ammonium acetate was removed by repeated freeze-drying. Analytical chromatographic

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analysis of the purified product produced a single peak eluting at 17 min using the gradient described above. The conjugation and purification yield was calculated using an ε_{262nm} of 9000 $M^{-1}cm^{-1}$ for uridine to give 6.1 μmols (5.6 mg) of **11** (15 % yield). NMR data (D_2O with acetone as internal standard): ^1H (500 MHz) δ 7.92 (d, 1 H, H-6 of uridine, $J_{5,6}$ 8.2), 5.87–5.90 (not resolved) (m, 2 H, H-5 of uridine, H-1 ribose, $J_{5,6}$ 8.2, $J_{1,2}$ 4.5), 5.60 (dd, 1 H, H-1 pyranose, $J_{1,2}$ 3.7, $J_{1,P}$ 7.7), 5.35 (d, 1 H, H-4 pyranose, $J_{3,4}$ 3.3), 5.03 (dt, 1 H, H-2, $J_{1,2}$ 3.7, $J_{2,3}$ 10.8, $J_{2,P}$ 2.0), (H-5 pyranose buried in water signal), 3.95–4.31 (m, 8 H, H-3 pyranose, H-2, H-3 furanose, H-5, H-5′ furanose, H-4 furanose, H-6, H-6′ pyranose), 2.29 (s, 3 H, SAc), 2.11, 1.99, 1.90 (each s, each 3 H, 3 × OAc), morpholine signals at 3.69 and 3.36 (each t, each 8 H). ^{31}P (200 MHz) δ − 10.29, − 12.28 (each d, *J* 19.6 Hz). ESIMS for $C_{23}H_{30}N_2O_{20}P_2S^{2-}$: calcd. 749.0 $[M + H]^-$, found 749.1.

Uridine 5′-[3-S-(2-S-pyridyl)-S-3-thio-α-D-galactopyranosyl diphosphate] 12. To a solution of **11** (1.3 mg, 1.2 μmols) in H_2O (350 μL), MeOH (300 μL) and DTDP (40 μmol dissolved in 60 μL MeOH) was added, followed by the addition of DEA (30 μL). The reaction was carried out at room temperature with constant monitoring by injecting 2 μL of the reaction mixture onto a C18 RP-HPLC column eluted with the same mobile phase and UV monitoring conditions used for the analysis of the formation of **11**. The starting material (17 min) was consumed after 4 hrs coincident with the appearance of a product peak at 10 min. This peak was purified by repeated injections of increasing amounts of the reaction mixture. The fractions corresponding to the product were pooled and freeze dried to give 550 nmol (0.4 mg) of 12 in a 46% yield. NMR data (D_2O, acetone): ^1H (500 MHz) δ 8.31 (dd, 1 H, H-6 pyridine), 7.79 (dd, 1 H, H-6 of uracil), 7.74 (m, 2 H, H-4 and H-5 of pyridine), 7.20 (dd, 1 H, H-3 of pyridine), 5.81 (d, 1 H, H-5 of uracil), 5.79 (d, 1 H, H-1 of furanose), 5.56 (dd, 1 H, H-1 pyranose), 4.20 (m, 2 H, H-2 and H-3 of furanose), 4.02–4.13 (m, 4 H, H-5 of pyranose, H-4, H-5 and H-5′ of furanose), 3.87 (dt, 1 H, H-2 of pyranose), 3.56–3.64 (m, 3 H, H-4, H-6 and H-6′ of pyranose), 3.35 (dd, 1 H, H-3 of pyranose), 2.97 (q, 8 H, CH_3CH_2N-), 1.17 (t, 12 H, CH_3CH_2N-). ^{31}P (200 MHz) δ − 9.8, − 11.7 (each d, J 19.3 Hz). ESIMS (negative mode) for $C_{20}H_{25}N_3O_{16}P_2S_2^{2-}$: calcd. 690.0 $[M + H^-]$, found 690.0.

Uridine 5′-[3-S-α-D-galactopyranosyl diphosphate] 13. To a solution of 100 nmols (790 μg) of 12 in 0.5 ml of water, 1 mmol of TCEP wa added and reacted for 1 hr at room temp. The progress of the reaction was moinored by injecting 1 nmol onto RP-HPLC eluted at 1 ml/min with 0.1 v/v % acetic acid and a gradient of 0–20% acetonitrile over 20 minutes while monitoring 262 nm. Compound 12 eluted at 16 min and the new product that formed eluted at 9 min. ESIMS confirmed the identity of the product (13). ESIMS (negative mode) for $C_{15}H_{22}N_2O_{16}P_2S^{2-}$: calcd. 581.3 $[M + H^-]$, found 582.0.

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