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Thiosugar nucleotide analogs: Synthesis of 5′-(2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl diphosphate)

Jordan Elhalabi, Kevin G. Rice*

Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065, USA

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

The synthesis of a novel analog of uridine diphosphate galactose (UDP-Gal) is described. A sulfur atom was inserted into the 6-position of galactose to give uridine 5′-(2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl diphosphate). This peracetylated thiol analogue of UDP-Gal has been synthesized in nine steps starting from methyl α-D-galactopyranoside in an overall yield of 3%.

Keywords: Thiosugars; UDP-galactose; Sugar nucleotides; β-(1→4)-Galactosyltransferase

1. Introduction

Glycosyltransferases are a group of enzymes that participate in the biosynthesis of oligosaccharides. Numerous glycosyltransferases have been cloned,[1] and very recently some of their crystal structures have been elucidated.[2–4] In addition to transferring natural substrates, the specificity of these enzymes is sufficiently flexible to allow the synthesis of unnatural or ''modified'' oligosaccharides. This has the advantage of being both regio- and stereoselective, thereby avoiding all the selective chemical protection and deprotection steps that otherwise would be needed.

One of the late-acting enzymes in *N*-glycan biosynthesis is β-(1→4)-galactosyltransferase (GalT, EC 2.4.1.90) that transfers a galactose moiety from the sugar nucleotide donor, uridine diphosphate galactose (UDP-Gal), to 2-acetamido-2-deoxy-D-glucose-terminating glycoconjugates to create a new β-(1→4)-glycosidic linkage (Fig. 1). Morrison and Ebner[5,6] have determined the kinetic mechanism to be ordered sequential. The donor substrate specificity of β-(1→4)-galactosyltransferase has been studied extensively. The 2-, 3-, 4-, and 6-deoxy-Gal analogs[7–10] have been shown to serve as substrates for the enzyme. Likewise, UDP-arabinose (UDP-Ara), which lacks the 6-CH_2OH arm,[11] as well as the 6-fluorogalactose derivative,[9] are used by the enzyme, while the 2-deoxy-2-fluoro derivative was found to be a competitive inhibitor.[12] Even some O-methylated galactose derivatives of UDP-Gal were used as substrates.[13] Several UDP-Gal analogs, where the ring oxygen is replaced by either sulfur or a carbon atom, have been prepared by different groups. Yuasa et al.[14] reported the synthesis of UDP-5S-Gal that was found to be transferred to a GlcNAc acceptor at 5% of the rate of the natural substrate. UDP-5S-GalNAc was also prepared and was transferred at a 0.23% rate.[15] However, the carbocyclic analog[16] of UDP-Gal was an inhibitor of β-(1→4)-GalT, which is consistent with the proposed mechanism of this enzyme that proceeds through an oxocarbonium ion-like transition state.

At least one of these analogs has been used to prepare unnatural oligosaccharides. Kajihara et al.[10]

* Corresponding author. Present address: Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA 52242, USA; tel.: +1-319-3359903; fax: +1-319-3358766

E-mail address: kevin-rice@uiowa.edu (K.G. Rice).

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transferred UDP-6-deoxy-Gal to asialo agalacto α_1-acid glycoprotein, showing the potential application of unnatural sugar nucleotides to remodel the *N*-glycans on a glycoprotein. The synthesis and utilization of different sugar nucleotide analogs by different glycosyltransferases has been extensively reviewed.[17]

To study further the functional and biological properties of remodeled *N*-glycans, we propose to introduce a thiol into the C-6 position of terminal Gal residues. This thiol moiety can then be used as a unique and flexible derivatization site. Toward this aim, the synthesis of a novel modified UDP-Gal possessing a thiol group at the 6-position of Gal is described.

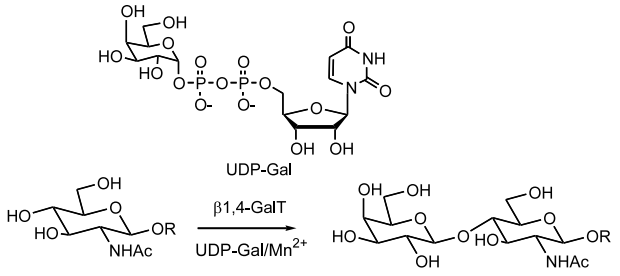
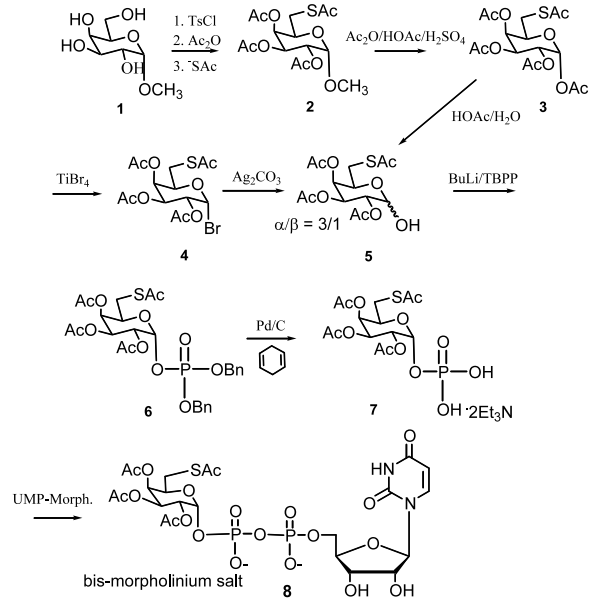
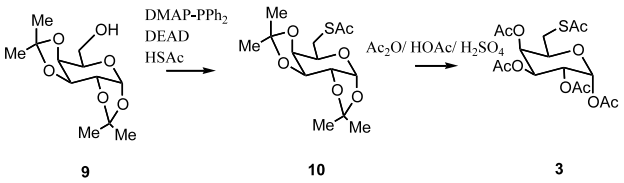


Fig. 1. Enzymatic synthesis of oligosaccharides by galactosyltransferase (β-(1→4)-GalT).



Scheme 1.



Scheme 2.

2. Discussion and results

The chemical synthesis of uridine 5′-(2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl diphosphate) (**8**) is shown in Scheme 1. Starting with the commercially available methyl α-D-galactopyranoside (**1**), selective primary hydroxyl group tosylation, acetylation and introduction of the thiol group at the 6-position with potassium thioacetate at reflux temperature gave methyl 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-galactopyranoside (**2**) in 53% yield. An attempt to replace the *O*-methyl group with a bromo group using dimethylboron bromide[18] was only partially successful. Instead, the *O*-methyl group was converted into an *O*-acetyl group using low temperature acetolysis to give 1,2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-galactopyranose (**3**) in 89% yield. A more efficient synthesis of **3** involved the use of the commercially available 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**9**) (Scheme 2). The thioacetate group was introduced using Mitsunobo conditions[19] to give 6-*S*-acetyl-1,2,3,4-di-*O*-isopropylidene-6-thio-α-D-galactopyranose (**10**), which in turn was converted to **3** in 47% overall yield using the same acetolysis conditions as shown in Scheme 2.

Hydrolysis of the anomeric acetate using benzylamine at room temperature produced a mixture of products. Alternatively, selective hydrolysis using glacial acetic acid–water[7] at 75 °C provided 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α/β-D-galactopyranose (2:1 α/β) (**5**), but in poor yield (26%). Instead, **3** was converted into 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl bromide (**4**) using $TiBr_4$. The glucosyl bromide was then hydrolyzed to give **5** as a (3:1) α/β mixture in 56% yield from **3**.

Phosphorylation of **5** was achieved using *n*-butyllithium and tetrabenzyl pyrophosphate (TBPP). This gave the unstable dibenzyl 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl phosphate (**6**) which was debenzylated by transfer hydrogenolysis using 1,4-cyclohexadiene and palladium-on-charcoal catalyst[20] to give 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl 1-phosphate mono-triethylammonium salt (**7**). The hydrogenolysis reaction was sluggish and required excess catalyst, presumably due to sulfur poisoning. Alternatively, the benzyl groups were removed using bromotrimethylsilane[21] in the presence of triethylamine, followed by water hydrolysis. Product **7** was in turn coupled to UMP-morpholidate[22] to produce uridine 5′-(2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranosyl diphosphate) (**8**). Purification of the final peracetylated product was achieved by preparative C_{18} RP-HPLC to give **8** as a bis-morpholinium salt. The final purification yielded micromole quantities of **8** and has the advantage of speed versus a lengthy low-pressure ion-exchange column, followed by repeated desalting using gel filtration.

Compound **8** is the acetate-protected form of the sugar nucleotide donor analog UDP-6S-Gal. Removal of the acetyl groups of **8** under basic conditions led to decomposition due to hydrolysis of the phosphodiester linkage. Alternatively, the thiol group could be trapped during the O,S-deacetylation using 2,2′-dithioldipyridine, resulting in a stable, reducible intermediate that is readily deprotected by reduction prior to enzyme transfer. The full details of this procedure will be reported separately along with the results of chemoenyzymatic synthesis using this and related analogues.

3. Experimental

General methods.—^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker NMR (ADVANCE DRX 500 or ADVANCE DPX 300). Samples in $CDCl_3$ used 1% TMS as an internal standard, whereas samples prepared in MeOH or D_2O used acetone as the 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 using a magnetic sector (Micromass, model 70-250S) by chemical ionization with ammonia. 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 to standard procedures. Organic solvents were removed on a rotary evaporator under water aspiration vacuum with bath temperature of 40 °C unless specified otherwise. 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 Chemical Co. with the exception of the following: methyl α-D-galactopyranoside and uridine 5-monophosphomorpholidate were purchased from Sigma Chemical Co. Thioacetic acid was purchased from Lancaster Synthesis, Inc. Analytical RP-HPLC experiments were carried out using a C_{18} column (Microsorb-MW, 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 C_{18} column (Alltech, 25 cm × 22 mm) on a Isco model 2350 dual pump system.

Methyl 2,3,4-tri-O-*acetyl-6*-S-*acetyl-6-thio-α*-D-*galactopyranoside* (**2**).—Methyl α-D-galactopyranoside (**1**) (1.0 g, 5.5 mmol) was dissolved in anhyd pyridine (50 mL) and reacted with stirring with one equiv of *p*-toluenesulfonyl chloride (TsCl, 1.05 g) at rt. The reaction was monitored by TLC using acetone–toluene as the mobile phase, which resolved the starting material **1** (R_f 0) from the tosylated Gal derivative (R_f 0.1). After 24 h, an additional 0.1 equiv (0.17 g) of TsCl was added and allowed to react with stirring for 2 h. Ac_2O (10 mL) was then added, and the reaction was stirred at rt for 4 h.

Et_2O (100 mL) and water (50 mL) were added to the reaction in an ice-bath, and the organic layer was separated and washed twice with 1 N HCl (50 mL), satd aq $NaHCO_3$ (2 × 30 mL) and water (2 × 30 mL). The organic layer was dried over $MgSO_4$ and, after filtration, was concentrated to dryness using a water aspirator for 12 h.

The oily residue was dissolved in CH_3CN (50 mL). KSAc (2.98 g, 4.75 equiv) was added, and the mixture was refluxed at 70 °C for 5 h while monitoring the reaction progress on TLC using 2:1 hexane–EtOAc. Product **2** migrated with R_f 0.6 relative to the acetylated, tosylated derivative with R_f 0.4. The reaction was diluted with $CHCl_3$ (70 mL), filtered, washed with satd aq NaCl (3 × 100 mL) and water (3 × 100 mL), and the organic extract was then concentrated. The dark oily residue was applied to a silica gel column (4 × 20 cm) using 3:1 hexane–EtOAc as the mobile phase. The fractions corresponding to the product were collected, and the solvent was evaporated to yield **2** as a brown solid (1.75 g, 53%). ^{1}H NMR ($CDCl_3$, 1% TMS 300 MHz): δ 5.48 (dd, 1 H, H-4), 5.32 (dd, 1 H, H-3), 5.13 (dd, 1 H, H-2), 4.98 (d, 1 H, H-1), 3.99 (dt, 1 H, H-5), 3.43 (s, 3 H, OMe), 2.91–3.10 (m, 2 H, H-6, H-6′), 2.36 (s, 3 H, SAc), 2.18 (s, 3 H, OAc), 2.10 (s, 3 H, OAc), 1.99 (s, 3 H, OAc). ^{13}C NMR ($CDCl_3$, ext. TMS, 120 MHz): δ 194.62 (SAc), 170.45, 170.34, 169.93 (OAc), 98.48, 69.01, 68.04, 67.71, 67.45, 55.42, 30.48 (OMe), 28.63 (SAc), 20.86, 20.72, 20.66 (OAc). HRMS: Calcd for $C_{15}H_{22}O_9S$, 378.0985. Found, m/z 378.0985.

1,2,3,4-Tetra-O-*acetyl-6*-S-*acetyl-6-thio-α*-D-*galactopyranose* (**3**).—Methyl 2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranoside (**2**) (1.50 g, 3.96 mmol) was dissolved in Ac_2O (70 mL) and HOAc (70 mL glacial) at 0 °C. Concentrated H_2SO_4 (1.5 mL) was added dropwise while stirring for 30 min at 0 °C. The reaction proceeded for 20 h during which time it was allowed to warm to rt. TLC (2:1 hexane–EtOAc) showed the complete conversion of **2** (R_f 0.6) to product **3** (R_f 0.55). The reaction was poured over ice-water and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with ice-cold water (50 mL) and satd aq $NaHCO_3$ (50 mL), and the solvent was evaporated to yield 1.44 g of **3** (89%). ^{1}H NMR ($CDCl_3$, 1% TMS 300 MHz): δ 6.36 (d, 1 H, H-1, $J_{1,2}$ 3.7 Hz), 5.52 (pseudo d, 1 H, H-4), 5.30–5.35 (m, 2 H, H-2, H-3), 4.14 (pseudo t, 1 H, H-5), 3.06 (dd, 1 H, H-6), 2.98 (dd, 1 H, H-6′), 2.34 (s, 3 H, SAc), 2.19, 2.16, 2.03, 2.01

(each s, each 3 H, each OAc). ^{13}C NMR, $CDCl_3$, (120 MHz): δ 194.39 (SAc), 170.24, 170.11, 169.91, 168.99, 89.72 (C-1), 70.17, 68.10, 67.62, 66.35, 30.45, 28.17, 20.88, 20.67, 20.63, 20.54. HRMS: Calcd for $C_{16}H_{22}O_{10}S$, 406.0934. Found, m/z 406.0922.

2,3,4-Tri-O-*acetyl-6*-S-*acetyl-6-thio-α,β*-D-*galactopyranose* (**5**).—1,2,3,4-Tetra-*O*-acetyl-6-*S*-acetyl-6-thio-α-D-galactopyranose (**3**) (1.00 g, 2.46 mmol) was dissolved in a mixture of 1:1 AcOH–water (40 mL) and heated at 75 °C for 18 h. The reaction mixture was then evaporated to dryness using a vacuum pump, and the crude product was purified by flash chromatography on silica gel (4 × 10 cm) eluting with 2:1 hexane–EtOAc to give 240 mg of **5** in 26% yield (2:1 α/β ratio).

Alternatively, 1.3 g (3.2 mmol) of **3** was dissolved in CH_2Cl_2 (20 mL) and EtOAc (2 mL). Titanium tetrabromide[23] (3.12 g, 2.7 equiv) was added, and the reaction was stirred at rt under nitrogen for 16 h. TLC 2:1 (hexane–EtOAc) demonstrated the complete conversion of the α-acetate **3** (R_f 0.55) into **4** (R_f 0.8). NaOAc (1.2 g) was added and stirred for 15 min, then filtered through Celite, and the Celite pad was washed with CH_2Cl_2 (10 mL). The organic filtrate was washed with cold water (100 mL) and evaporated to dryness. The crude mixture was dissolved in acetone (30 mL) containing water (1 mL). Ag_2CO_3 (2.0 g) was added, and the mixture was stirred for 12 h at rt in the dark, after which time TLC (2:1 hexane–EtOAc) identified the major product **5** (R_f 0.2). The reaction was filtered, the filter paper was washed with acetone (15 mL), and the solvent was evaporated to dryness using a water aspirator. A final purification was performed by flash chromatography on silica gel (4 × 10 cm) using 2:1 hexane–EtOAc to give of **5** (650 mg, 56%) as an α/β (3:1) mixture. ^{1}H NMR ($CDCl_3$, 1% TMS, 1 drop of D_2O, 300 MHz): δ 5.48–5.52 (m, 2 H, H-1 (α), H-4 (α)), 5.44 (dd, 0.5 H, H-4 (β)), 5.40 (dd, 1 H, H-3 (α)), 5.16 (dd, 1 H, H-2 (α), $J_{2,3}$ 10.5, $J_{1,2}$ 3.7 Hz), 5.02–5.08 (m, 1 H, H-2 (β), H-3 (β)), 4.68 (d, 0.5 H, H-1 (β)), 4.27 (pseudo t, 1 H, H-5 (α)), 3.75 (pseudo t, 0.5 H, H-5 (β)), 2.95–3.17 (m, 3 H, H-6 (α/β), H-6′(α/β)), 2.36 (s, 4.5 H, SAc), 2.20, 2.12, 2.01 (each s, each 1.5 H, each OAc (β)), 2.19, 2.11, 2.00 (each s, each 3 H, each OAc (α)). ^{13}C NMR ($CDCl_3$, 1 drop D_2O, 120 MHz): δ (α-anomer only) 195.04, 170.77, 170.76, 170.65, 91.14, 71.43, 69.31, 68.60, 68.05, 67.85, 30.91, 28.87, 21.28, 21.15, 21.09. HRMS: Calcd for $C_{14}H_{20}O_9S$, 364.0828. Found, m/z 364.0836.

1,2,3,4-Tetra-O-*acetyl-6*-S-*acetyl-6-thio-α*-D-*galactopyranose* (**3**) *from* **9**.—1:2,3:4-Di-*O*-isopropylidene-α-D-galactopyranose (**9**) (5.00 g, 19.2 mmol) was dissolved along with (*p*-dimethylamino)phenyl diphenylphosphine (5.0 g, 1.2 equiv) in THF (50 mL) with stirring at −10 °C under nitrogen according to the established procedure.[21] Diethyl azodicarboxylate (DEAD, 3.45 mL, 17.5 mmol) was added, followed by of thioacetic acid (1.1 mL), and the reaction was carried out at rt for 6 h while monitoring by TLC (3:1 hexane–EtOAc) (R_f 0.8). At reaction completion, the solvent was evaporated, and the residue was dissolved in Et_2O (150 mL), cooled to −10 °C and filtered. The cold filtrate was washed with cold dilute M HCl (50 mL), satd aq $NaHCO_3$ (50 mL) and water (50 mL). The organic extract was concentrated to dryness, and the crude product was then flash chromatographed on silica gel (4 × 10 cm) using 4:1 hexane–EtOAc to give **10** (4.11 g, 67%) that was converted to **3** in 70% yield following the procedure described above for **2**→**3**. Product **10**: ^{1}H NMR ($CDCl_3$, 1% TMS 300 MHz): δ 5.51 (d, 1 H, H-1), 4.61 (dd, 1 H, H-3). 4.24–4.31(m, 2 H, H-2, H-4), 3.85 (pseudo t, 1 H, H-5), 3.17 (dd, 1 H, H-6), 3.03 (dd, 1 H, H-6′), 2.34 (s, 3 H, SAc), 1.48, 1.46, 1.35, 1.32 (each s, each 3 H, each –CH_3). ^{13}C NMR ($CDCl_3$) (120 MHz): δ 196.26 (SAc), 109.86, 109.19, 96.92, 72.42, 71.31, 70.88, 67.19, 30.95, 30.08, 26.34, 25.39, 24.82. HRMS: Calcd for $C_{14}H_{12}O_6S$, 318.1137. Found, m/z 318.1122.

2,3,4-Tri-O-*acetyl-6*-S-*acetyl-6-thio-α*-D-*galactopyranosyl-phosphate mono-triethylammonium salt* (**7**).—Tetrabenzylpyrophospahte (TBPP) was prepared[22] by dissolving dibenzyl phosphate (2.24 g, 8.05 mmol) with *N*,*N*-dicyclohexylcarbodiimide (DCC, 830 mg 4.02 mmol) in toluene with stirring at rt for 5 h. The *N*,*N*-dicyclohexylurea was removed by filtration. The toluene was evaporated using a vacuum pump to give an oily residue that solidified as a white powder upon freezing to yield TBPP (2.05 g, 95%), which was used immediately in the next step without further purification.

2,3,4-tri-*O*-acetyl-6-*S*-acetyl-6-thio-α,β-D-galactopyranose (**5**) (550 mg, 1.51 mmol) was dissolved in dry THF (10 mL) at −78 °C. Butyllithium (1.0 mL, 1.6 mmol, in hexane) was added to **5** at −78 °C. The reaction was stirred for 5 min, after which time, TBPP (1.63 g, 3.02 mmol) in dry THF (3 mL) was added dropwise over 5 min. The reaction mixture was brought to −60 °C and allowed to react with stirring for 30 min. The reaction mixture was brought to rt, diluted with 30 mL of Et_2O, washed with satd aq $NaHCO_3$ (2 × 10 mL) and water (2 × 10 mL) and evaporated to dryness. The residue was then applied to a silica gel column (4 × 10 cm) using 2:1 hexane–EtOAc, containing 1% Et_3N as the mobile phase. The fractions corresponding to the product (monitored by both TLC charring and UV detection) were collected, and the solvent was evaporated to give **6** (290 mg, 31% yield). ^{1}H NMR ($CDCl_3$, TMS 300 MHz): δ 7.34–7.40 (m, 10 H, Ar), 5.95 (dd, 1 H, H-1, $J_{1,2}$ 3.6, $J_{1,P}$ 7.7 Hz), 5.48 (d, 1 H, H-4), 5.30 (dd, 1 H, H-3), 5.20 (dt, 1 H, H-2), 5.10 (pseudo d, 4 H, benzylic), 4.13 (pseudo t, 1 H, H-5), 2.97 (m, 2 H, H-6, H-6′), 2.23, 2.17, 2.00, 1.90 (each s, each 3 H, 3 × OAc, SAc). ^{13}C NMR ($CDCl_3$, TMS,

(120 MHz): δ 194.40 (SAc), 170.59, 170.49, 170.30 (3 OAc), 128.22–129.12, 94.95 (d, C1), 70.26, 70.06 (d, benzylic), 69.95 (d, benzylic), 68.45, 67.56, 67.67, 67.32, 30.75, 28.44, 21.09, 21.04, 20.87. ^{31}P NMR (120 MHz): δ −1.52. HRMS: Calcd for $C_{28}H_{33}O_{12}PS$, 624.1430. Found, 624.1419. Compound **6** (290 mg) was debenzylated in EtOH (10 mL) with 150 mg Pd-on-charcoal (10% 150 mg), Et_3N (160 μL) and 1,4-cyclohexadiene (0.2 mL). The reaction was followed by TLC using 1:1 EtOAc–hexane to monitor the removal of the first benzyl group and using 60:35:4, $CHCl_3$–MeOH–water to monitor the removal of the second benzyl group with the addition of catalyst (40–60 mg) every 2 h until the reaction was complete. The mixture was filtered, the filter paper was washed with EtOH (5 mL), and the solvent was evaporated to give **7** (185 mg, 67% yield).

Alternatively, compound **6** (40 mg, 0.064 mmol) was dissolved in CH_2Cl_2 (5 mL) at 0 °C with Et_3N (9 μL, 65 μmol) and bromotrimethylsilane (52 μL, 0.38 mmol). The reaction was stirred at rt for 6 h after which time water (200 μL) was added, and the mixture was stirred vigorously for an additional 20 min. The mixture was dried using a vacuum pump, and the crude product was then loaded on a silica gel column (5 × 0.5 cm) and eluted with EtOAc (100 mL), followed by MeOH (50 mL), to remove side products. The methanolic fraction was collected and dried to give **7** (25 mg, 72% yield). ^{1}H NMR (D_2O, acetone as int. std., 300 MHz): δ 5.58 (dd, 1 H, H-1, $J_{1,2}$ 3.6, $J_{1,P}$ 7.8 Hz), 5.40 (d, 1 H, H-4), 5.24 (dd, 1 H, H-3), 5.07 (dt, 1 H, H-2), 4.32 (pseudo t, 1 H, H-5), 3.06 (m, 2 H, H-6, H-6′), 2.24 (s, 3 H, SAc), 2.12, 2.01, 1.91 (each s, each 3 H, 3 × OAc). ^{31}P (120 MHz): δ −0.52 ppm. ESIMS (negative-ion mode): Calcd for $C_{14}H_{21}O_{12}PS$, 444.05. Found, m/z 443.1.

Uridine 5′-(2,3,4-tri-O-*acetyl-6*-S-*acetyl-6-thio-α*-D-*galactopyranosyl diphosphate)* (**8**).—Uridine 5′-monophosphomorpholidate (4-morpholine-*N*′,*N*-dicyclohexylcarboxamidine salt) (100 mg, 0.14 mmol) was coevaporated with dry DMF (3 × 3 mL) and added to a solution of **7** (10 mg, 18.3 μmol) in dry DMF (3 mL), which was previously coevaporated with dry DMF (3 × 3 mL). The coupling was performed at rt under nitrogen atmosphere for 10 h. The reaction was monitored by injecting 100 μL (1 μL reaction diluted into 1 mL of water) onto analytical C_{18} RP-HPLC while eluting at 1 mL/min using a mobile phase of 10 mM NH_4OAc (pH 5.0) and a gradient of MeCN (0–35% over 30 min) while monitoring Abs_{262nm}. The reaction mixture was dried under vacuum, dissolved in water (3 mL) and injected (500 μL, 3 μmol) onto a C_{18} RP-HPLC (2 × 25 cm) column eluted at 10 mL/min with 10 mM NH_4OAc and a gradient of 0–35% MeCN over 30 min. The peak detected by Abs_{262nm} eluting at 19 min was collected and concentrated repeatedly under vacuum at 30 °C. Final traces of NH_4OAc were removed by repeated freeze-drying. Re-chromatography of the purified product on analytical RP-HPLC revealed a single peak eluting at 19 min. The final coupling and purification gave 4.5 μmol of **8** (3.36 mg, 24.6% yield) as the bis-morpholinium salt based on the absorbance spectrum (ε_{262nm} of 9000 M^{-1} cm^{-1} for uridine). The product was stable when stored frozen at −30 °C. ^{1}H NMR (D_2O, acetone internal std, 500 MHz): δ 7.90 (d, 1 H, H-6 of Ur), 5.85–5.91 (not resolved) (m, 2 H, H-5 of Ur, H-1 ribose), 5.69 (dd, 1 H, H-1 pyranose), 5.38 (d, 1 H, H-4 pyranose), 5.23 (dd, 1 H, H-3 pyranose), 5.15 (dt, 1 H, H-2 pyranose), 4.35 (pseudo t, 1 H, H-5 pyranose), 4.25 (m, 2 H, H-2, H-3 furanose), 4.18 (m, 2 H, H-5, H-5′furanose), 4.10 (m, 1 H, H-4 furanose), 3.05 (m, 2 H, H-6, H-6′ pyranose), 2.24 (s, 3 H, SAc), 2.12, 2.04, 1.90 (each s, each 3 H, 3 × OAc), morpholine signals at 3.69, 3.34 (each t, each 8 H). ^{31}P NMR (120 MHz): δ −10.18, −12.27 (each d, J 20.0 Hz). ESIMS (negative-ion): Calcd for $C_{23}H_{30}N_2O_{20}P_2S$ 749.0, $[M+H]^-$. Found, m/z 749.2.

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