Supporting Information

for

Synthesis of C6-modified mannose 1-phosphates and evaluation of derived sugar nucleotides against GDP-mannose dehydrogenase

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Detailed experimental protocols and characterisation data; spectral NMR data (\(^1\)H, \(^{13}\)C and \(^{31}\)P NMR for compounds 10–17 and 19)
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S1. General experimental

All reagents and solvents which were available commercially were purchased from Acros, Alfa Aesar, Fisher Scientific, Sigma Aldrich or TCI. All reactions in non-aqueous solvents were conducted using oven-dried glassware with a magnetic stirring device under an inert atmosphere of nitrogen passed through a drying column using a vacuum manifold. Solvents were purified by passing through activated alumina columns and used directly from a Pure Solv-MD solvent purification system and were transferred under nitrogen unless otherwise stated. Reactions were followed by thin layer chromatography (TLC) using Merck silica gel 60 F$_{254}$ analytical plates (aluminium support) and were developed using short wave UV radiation (245 nm) and/or 10% sulfuric acid in methanol/$\Delta$. Purification via flash column chromatography was conducted manually using Sigma Aldrich silica gel 60 (0.040–0.063 mm) under a positive pressure of compressed air or via automation using a Büchi Reveleris X2 or a Büchi Pure C-815 Flash with pre-packed silica cartridges. Purification via strong ion exchange (SAX) chromatography was conducted using a Thermo Scientific™ HyperSep™ SAX 500 mg cartridge (column volume = 5 mL) with deionized water followed by aqueous NH$_4$HCO$_3$ (1.0 M). Purification via reversed phase separation was conducted using a Thermo Scientific™ HyperSep™ C18 cartridge (column volume = 5 mL) with deionized water followed by EtOAc and MeCN. Optical activities were recorded on an automatic Rudolph Autopol I or Bellingham and Stanley ADP430 polarimeter (concentration in g/100mL). $^1$H NMR spectra were recorded at 400 MHz, $^{13}$C NMR spectra at 100 MHz, and $^{31}$P NMR spectra at 161 MHz respectively using Bruker Magnet system 400’54 Ascend. $^1$H NMR resonances were assigned with the aid of
gDQCOSY. $^{13}$C NMR resonances were assigned with the aid of gHSQCAD. Coupling constants are reported in hertz. Chemical shifts (δ, in ppm) are standardized against the deuterated solvent peak. NMR data were analyzed using Mestrenova. $^1$H NMR splitting patterns were assigned as follows: br. s (broad singlet), s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), app. t (apparent triplet), t (triplet), quartet (q) or m (multiplet and/or multiple resonances). HRMS (ESI) were obtained on Agilent 6530 Q-TOF, LQT Orbitrap XL1 or Waters (Xevo, G2-TOF or G2-S ASAP) Micromass LCT spectrometers using a methanol mobile phase in positive/negative ionization modes, as appropriate.

S2. Experimental procedures for compounds 10-17 and 19

Synthesis of 6-amino-6-deoxy-α-D-mannose 1-phosphate 13

2,3,4-Tri-O-benzyl-6-bromo-6-deoxy-1-thio-α-D-mannopyranoside

To a solution of phenyl 2,3,4-tri-O-benzyl-1-thio-α-D-mannopyranoside¹ (10, 500 mg, 0.92 mmol, 1.0 equiv) in DCM (9 mL) at 0 °C was added successively Ph3P (410 mg, 1.56 mmol, 1.7 equiv) and CBr₄ (520 mg, 1.56 mmol, 1.7 equiv), before warming to rt. After stirring for 16 h, the reaction mixture was poured onto H₂O (30 mL) and diluted with DCM (30 mL). The organic layer was washed with H₂O (2 × 30 mL), brine (30 mL), dried over MgSO₄, filtered, and concentrated in vacuo. Purification by silica gel column chromatography, eluting with pet. ether/EtOAc 6:1 afforded the title compound as a yellow oil (420 mg, 0.69 mmol, 75 %). Rf (Pet. Ether:EtOAc, 3:1) = 0.90; [α]$^D$ +51.4 (c = 0.70 M, CHCl₃); $^1$H NMR (400 MHz, CDCl₃) δ 7.48-7.45 (m, 2H, Ar-H), 7.40-7.27 (m, 18H, Ar-H), 5.59 (d, $^3$J₁₁-H₂ = 1.5 Hz, 1H, H-1), 5.01 (d, $^2$J₁₁-CH = 10.9 Hz, 1H, CH₂Ph), 4.76-4.60 (m, 5H, CH₂Ph), 4.31-4.25 (m, 1H, H-5), 4.06-4.00 (m, 2H, H-2, H-4), 3.87 (dd, $^3$J₁₃-H₄ = 9.3 Hz, $^3$J₉-H₂ = 3.0 Hz, 1H, H-3), 3.69-3.66 (m, 2H, H-6a, H-6b); $^{13}$C NMR (100 MHz, CDCl₃) δ 138.3 (Ar-C), 138.1 (Ar-C), 137.9 (Ar-C), 134.2 (Ar-C), 131.8 (Ar-C), 129.2 (Ar-C), 128.6 (2C, Ar-C), 128.5 (Ar-C), 128.2 (Ar-C), 128.0 (3C, Ar-C), 127.9 (Ar-C), 127.7 (Ar-C), 86.0 (C-1), 80.1 (C-3), 76.9 (C-4), 76.3 (C-2), 75.6 (CH₂Ph), 72.3 (C-5), 72.2 (CH₂Ph), 72.1 (CH₂Ph), 33.4 (C-6); HRMS m/z (ESI) found: (M+Na)$^+$ 628.1184, C₃₃H₃₃BrO₄S requires 628.1180.

6-Azido-2,3,4-tri-O-benzyl-6-deoxy-1-thio-α-D-mannopyranoside 11

To a solution of 2,3,4-tri-O-benzyl-6-bromo-6-deoxy-1-thio-α-D-mannopyranoside (360 mg, 0.59 mmol, 1.0 equiv) in DMF (4 mL) was added NaN₃ (77 mg, 1.18 mmol, 2.0 equiv). The
reaction mixture was heated to 75 °C and stirred for 18 h, before being cooled to rt, poured onto H₂O (15 mL), and extracted with EtOAc (30 mL). The organic layer was washed with saturated aqueous Na₂S₂O₃ solution (20 mL), H₂O (20 mL), brine (20 mL), dried over MgSO₄, filtered, and concentrated in vacuo. Purification by silica gel column chromatography, eluting with toluene/EtOAc (12:1, 9:1, 6:1) afforded 11 as a yellow oil (214 mg, 0.38 mmol, 64%). Rᵣ (Tol/EtOAc, 6:1) = 0.74; [α]²⁰⁻D +35.5 (c = 0.45 M, CHCl₃); ^1H NMR (400 MHz, CDCl₃) δ 7.46-7.28 (m, 20H, Ar-H), 5.58 (d, ^3JH₁-H₂ = 1.6 Hz, 1H, H-1), 5.00 (d, ^2JCH-CH = 11.0 Hz, 1H, CH₂Ph), 4.77-4.68 (m, 2H, CH₂Ph), 4.69-4.62 (m, 3H, CH₂Ph), 4.29-4.23 (1H, m, H-5), 4.03 (dd, ^3JH₂-H₃ = 2.9 Hz, ^3JH₂-H₁ = 1.6 Hz, 1H, H-2), 3.99 (app. t, ^3JH₄-H₃/HS = 9.4 Hz, 1H, H-4), 3.88 (dd, ^3JH₃-H₄ = 9.4 Hz, ^3JH₃-H₂ = 2.9 Hz, 1H, H-3), 3.48 (m, 2H, H-6a, H-6b); ^13C NMR (100 MHz, CDCl₃) δ 138.3 (Ar-C), 138.1 (Ar-C), 137.9 (Ar-C), 134.1 (Ar-C), 131.6 (Ar-C), 129.3 (Ar-C), 128.6 (2C, Ar-C), 128.2 (Ar-C), 128.1 (Ar-C), 128.0 (2C, Ar-C), 127.9 (Ar-C), 127.7 (Ar-C), 85.8 (C-1), 80.1 (C-3), 76.3 (C-2), 75.6 (CH₂Ph), 75.5 (C-4), 72.7 (C-5), 72.2 (2C, CH₂Ph), 51.6 (C-6); HRMS m/z (ESI⁻) found: (M+Na)⁻ 590.2113, C₃₃H₃₃N₄O₄S requires 590.2090.

Dibenzyl 6-azido-2,3,4-tri-O-benzyl-6-deoxy-α-d-mannopyranosyl phosphate 12
Thioglycoside 11 (196 mg, 0.26 mmol, 1.0 equiv) was dissolved in DCM (2.6 mL) and stirred with powdered 4 Å MS for 1 h. DBP (108 mg, 0.39 mmol, 1.5 equiv) was added and the reaction mixture stirred for a further 30 min before being cooled to -30 °C. NIS (88 mg, 0.39 mmol, 1.5 equiv) and AgOTf (20 mg, 78 μmol, 0.3 equiv) were added successively and the reaction mixture was stirred until TLC analysis indicated the reaction was complete (45 min). The reaction was quenched with Et₃N, filtered over Celite™ and diluted with DCM (20 mL). The organic layer was washed with saturated aqueous Na₂S₂O₃ solution (20 mL), saturated aqueous NaHCO₃ solution (20 mL), H₂O (20 mL), brine (20 mL), dried over MgSO₄, filtered, and concentrated in vacuo. Purification by silica gel column chromatography, eluting with pet. ether/EtOAc (5:1, 3:1, 2:1) afforded 12 as a yellow oil (124 mg, 0.16 mmol, 65%). Rᵣ (Tol:EtOAc, 6:1) = 0.44; [α]²⁰⁻D +53.6 (c = 0.2 M, CHCl₃); ^1H NMR (400 MHz, CDCl₃) δ 7.34-7.28 (m, 23H, Ar-H), 7.26-7.24 (m, 2H, Ar-H), 5.70 (dd, ^3JH₁-P = 6.1 Hz, ^3JH₁-H₂ = 1.9 Hz, 1H, H-1), 5.08-4.89 (m, 5H, CH₂Ph), 4.63 (s, 2H, CH₂Ph), 4.56 (d, ^2JCH-CH = 11.0 Hz, 1H, CH₂Ph), 4.48 (s, 2H, CH₂Ph), 3.95 (app. t, ^3JH₄-H₃/5 = 9.5 Hz, 1H, H-4), 3.83 (ddd, ^3JH₅-H₄ = 9.5 Hz, ^3JH₅-H₆a = 4.5 Hz, ^3JH₅-H₆b = 2.7 Hz, 1H, H-5), 3.78 (dd, ^3JH₃-H₄ = 9.5 Hz, ^3JH₃-H₂ = 3.0 Hz, 1H, H-3), 3.70-3.68 (m, 1H, H-2), 3.35-3.25 (m, 2H, H-6a, H-6b); ^31P NMR (100 MHz, CDCl₃) δ 138.1 (Ar-C), 138.0 (Ar-C),
137.7 (Ar-C), 135.6 (2C), 135.5 (2C, Ar-C), 128.7 (3C, Ar-C), 128.4 (3C, Ar-C), 128.1 (Ar-C), 128.0 (Ar-C), 127.9 (2C, Ar-C), 127.8 (2C, Ar-C), 127.7 (Ar-C), 96.0 (C-1), 78.6 (C-3), 75.3 (CH2Ph), 74.3 (C-4), 74.2 (C-2), 73.3 (C-5), 72.8 (CH2Ph), 72.1 (CH2Ph), 69.6 (CH2Ph), 69.5 (CH2Ph), 51.0 (C-6); 31P NMR (161 MHz, CDCl3) δ -2.80 (d, 3JH1-P = 6.1 Hz, 1P); HRMS m/z (ESI+) found (M+Na)+ 758.2647 C41H42N3O8P requires 758.2608.

6-Amino-6-deoxy-α-d-mannopyranosyl phosphate (disodium salt) 13

A suspension of 12 (48 mg, 65 μmol, 1.0 equiv), Pd/C (10% loading, 11 mg, 11 μmol, 0.03 equiv per benzyl) and Pd(OH)2/C (20% loading, 8 mg, 11 μmol, 0.03 equiv per benzyl) in EtOH/THF 2:1 (0.9/0.4 mL) and 0.1 M HCl (0.76 mL, 76 μmol, 1.18 equiv) were stirred vigorously under an atmosphere of H2 for 24 h. The reaction mixture was filtered over Celite™ and washed with MeOH/water 2:1 then 1:1 (20 mL total) then passed through Dowex® 50W-X8 resin (Na+ form) before being concentrated under reduced pressure. The resultant residue was re-suspended in D2O and lyophilized to afford 13 as a white solid (15 mg, 58 μmol, 90 %). Rf (MeCN:H2O (3:1 plus 3 drops AcOH) = 0.07; [α]26D +24.0 (c = 0.46 M, H2O); 1H NMR (400 MHz, D2O) δ 5.42 (d, 3JH1-P = 5.6 Hz, 1H, H-1), 3.99 (brs, 2H, H-2), 3.89 (app. d, 3J = 8.3 Hz, 1H, H-3), 3.58 (app. t, 3JH4-H3/H5 = 9.5 Hz, 1H, H-4), 3.46 (d, 3JH6a-H6b = 12.9 Hz, 1H, H-6a), 3.19-3.06 (m, 1H, H-6b); 13C[31P] NMR (100 MHz, D2O) δ 95.8 (C-1), 70.3 (C-2 or C-3), 70.2 (C-2 or C-3), 69.2 (C-4), 68.1 (C-5), 40.5 (C-6); 31P NMR (161 MHz, D2O) δ -2.00 (d, 3JH1-P = 5.6 Hz, 1P); HRMS m/z (ESI) found: (M-H)− 258.0388 C6H13NO6P requires 258.0378.

Synthesis of 6-chloro-6-deoxy-α-d-mannose 1-phosphate 17

2,3,4-Tri-O-acetyl-6-chloro-6-deoxy-α/β-d-mannopyranose 15

NH4OAc (1.01 g, 13.1 mmol, 4.0 equiv) was added to a solution of 1,2,3,4-tetra-O-acetyl-6-chloro-6-deoxy-β-D-mannopyranose2 (14, 1.20 g, 3.28 mmol, 1.0 equiv) in DMF (3 mL). The mixture was stirred for 42 h at rt. When the reaction was complete, as indicated by TLC (lower Rf spot), the remaining NH4OAc was filtered off and the filtrate concentrated to dryness in vacuo. To remove residual DMF, the crude material was suspended in LiCl solution for 18 h and then extracted with EtOAc (5 × 20 mL). The combined organic layers were washed again with LiCl solution (5 × 20 mL), dried over MgSO4, filtered and concentrated under reduced pressure to afford 15 (960 mg, 2.66 mmol, 80%) which was used without further purification. Rf (Hexane:EtOAc, 1:1) = 0.55; 1H NMR (400 MHz, DMSO) δ 7.40 (br.s 1H, OH), 5.20 (dd, 3JH3-
Diphenyl 2,3,4-tri-O-acetyl-6-chloro-6-deoxy-α-D-mannopyranosyl phosphate 16

n-BuLi (0.61 mL, 0.95 mmol, 1.59 M, 1.2 equiv) was added dropwise to a solution of 15 (261 mg, 0.80 mmol, 1.0 equiv) in THF (5 mL) at −78 °C. After stirring for 15 minutes, diphenyl phosphoryl chloride (0.20 ml, 0.95 mmol, 1.2 equiv) was added dropwise and the reaction mixture stirred for another 35 min at the same temperature. When TLC analysis indicated the complete consumption of the starting material (to a lower Rf spot), the reaction was gradually warmed to rt, quenched with saturated aqueous NH₄Cl solution (2 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with saturated aqueous NaCl solution (20 mL), dried over MgSO₄, filtered, and concentrated in vacuo. The residue was purified using silica gel via flash chromatography, eluting with DCM/Et₂O 1:0, 99:1, 98:2, 97:3 to afford 16 as a solid. This material was crystallized using a minimum amount of hot EtOH to further afford 16 as white crystals (260 mg, 0.47 mmol, 58%). Rf (DCM:Et₂O, 95:5) = 0.75; [α]D +53.7 (c = 0.50, DCM); ^1H NMR (400 MHz, DMSO) δ 7.47-7.43 (m, 4H, Ar), 7.31-7.27 (m, 6H, Ar), 6.01 (dd, 3JH1-p = 6.7 Hz, 3JH1-H2 = 1.8 Hz, 1H, H-1), 5.27-5.25 (m, 1H, H-2), 5.23-5.17 (m, 2H, H-3, H-4), 4.11-4.07 (m, 1H, H-5), 3.72 (dd, 1H, 2JH6a-H6b = 12.5 Hz, 3JH6a-H6b = 4.8 Hz, H-6a), 3.63 (dd, 1H, 2JH6a-H6b = 12.5 Hz, 3JH6b-H6s = 2.6 Hz, H-6b), 2.10 (s, 3H, CH₃COO), 2.05 (s, 3H, CH₃COO), 1.96 (s, 3H, CH₃COO); ^13C NMR (100 MHz, DMSO) δ 170.1 (C=O), 169.8 (C=O), 169.6 (C=O), 150.2 (CIV), 150.1 (CV), 130.7 (CAr), 126.4 (CAr), 126.4 (CAr), 126.3 (CAr), 120.5 (CAr), 120.4 (CAr), 120.4 (CAr), 96.1 (d, 3JH1-p = 5.9 Hz, C-1), 71.8 (C-5), 68.3 (d, 3JH1-p = 10.7 Hz, C-2), 68.1 (C-3), 65.5 (C-4), 43.6 (C-6), 21.0 (CH₃), 20.9 (CH₃), 20.8 (CH₃); ^31P NMR (162 MHz, DMSO) δ -14.34 (d, 3Jp-H1 = 6.6 Hz); HRMS m/z (NSI⁺) found: (M+NH₄)⁺ 574.1240, C₂₄H₂₆ClO₅NH₄ requires 574.1240.
6-Chloro-6-deoxy-α-D-mannopyranose 1-phosphate (sodium triethylammonium salt) 17

PtO₂ (16 mg, 0.07 mmol, 1.0 equiv) was added to a solution of 16 (130 mg, 0.23 mmol, 1.0 equiv) in EtOH (3 mL) and sodium bicarbonate (39 mg, 0.47 mmol, 2.0 equiv). The resulting mixture was stirred overnight at rt under an atmosphere of H₂ (1 atm, balloon). The reaction was monitored by TLC (hexane:EtOAc 1:1, Rf = 0.00 and MeCN:H₂O:NH₄OH 9:1:0.1, Rf = 0.55) and upon completion was filtered through Celite® and concentrated in vacuo. The crude product was subjected to NMR to protecting group removal. ¹H NMR (400 MHz, MeOD) δ 5.50 (dd, ³J_H-H₂ = 7.6 Hz, ³J_H₁-H₂ = 1.3 Hz, 1H, H-1), 5.42-5.41 (m, 2H, H-3, H-4), 5.33 (app. s, 1H, H-2), 4.39 (dt, ³J_H₅-H₆ = 8.8 Hz, ³J_H₅-H₆ = 3.1 Hz, 1H, H-5), 3.81 (dd, ³J_H₆b-H₆a = 12.3 Hz, ³J_H₆a-H₅ = 2.8 Hz, 1H, H-6a), 3.79 (dd, ³J_H₆b-H₆a = 12.3 Hz, ³J_H₆b-H₅ = 3.2 Hz, 1H, H-6b), 2.14 (s, 3H, CH₃COO), 2.05 (s, 3H, CH₃COO); ¹³C NMR (100 MHz, MeOD) δ 171.7 (C=O), 171.6 (C=O), 171.4 (C=O), 94.7 (d, ³J_H₁-H₂ = 4.6 Hz, C-1), 71.4, 70.8, 68.0, 58.3, 44.6 (C-6), 20.6 (CH₃), 20.6 (CH₃), 18.4 (CH₃); ³¹P NMR (162 MHz, MeOD) δ -0.50 (d, ³J_P-H₁ = 7.1 Hz). Et₃N (1 mL) was added to the above crude in MeOH (2 mL), and the solvent removed in vacuo. The residue was dissolved in Et₃N:H₂O:MeOH 1:3:7 (v/v/v, 5 mL) and stirred for 26 h at rt. TLC analysis (MeCN:H₂O:NH₄OH 9:1:0.1, Rf = 0.00) showed complete conversion of starting material and the mixture was concentrated in vacuo. The crude was dissolved in water (2 mL), stirred for 1 h at rt with ion exchange resin (Amberlite® IR120 Na⁺ form), filtered and the filtrate freeze dried to afford crude 17 as a white powder. This material was purified using a RP-C18 column, eluting with H₂O (2CV), EtOAc (2CV) and MeCN (2CV). The product containing fractions were collected and freeze dried to afford 17 as a white powder (91 mg, 0.23 mmol, 99 %). [α]²⁴⁺D +20.7 (c = 0.45, H₂O); ¹H NMR (400 MHz, D₂O) δ 5.28 (d, ³J_H₁-H₂ = 7.3 Hz, 1H, H-1), 4.00 (dt, ³J_H₅-H₆ = 9.6 Hz, ³J_H₅-H₆ = 2.9 Hz, 1H, H-5), 3.91 (br.s, 1H, H-2), 3.88-3.85 (m, 3H, H-3, H-6a, H-6b), 3.76 (t, ³J_H₄-H₃ = 9.5 Hz, 1H, H-4), 3.13 (q, 6H, ³J_CH₂-CH₃ = 6.5 Hz, CH₂-NEt₃), 1.21 (t, ³J_CH₃-CH₂ = 6.9 Hz, 9H, CH₃-NEt₃); ¹³C NMR (100 MHz, D₂O) δ 95.3 (d, ³J_C-P = 4.7 Hz, C-1), 71.5 (C-5), 70.8 (d, ³J_C-P = 7.4 Hz, C-2), 68.8 (C-3), 67.1 (C-4), 46.6 (CH₂-NEt₃), 44.6 (C-6), 8.2 (CH₃-NEt₃); ¹³C-GATED (101 MHz; D₂O): δ 98.5 (¹J_C₁-H₁ = 170.0 Hz, C-1α); ³¹P NMR (162 MHz, D₂O) δ 0.79 (br.s). HRMS m/z (NSI) found (M-H) 276.9887, C₆H₁₁ClO₆P requires 276.9886.
S3 Enzymatic synthesis of sugar nucleotides

Expression and purification of GDP-mannose-pyrophosphorylase (GDP-Man-PP)

The transformant was grown according to the literature. Briefly, 1 L of transformant in LB medium containing appropriate antibiotic (kanamycin, 25 µg/mL) was incubated at 37 °C with gentle shaking until an OD600 of about 0.6. Heterologous protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.5 mM final concentration, followed by incubation at 18 °C overnight at 180 rpm. Afterwards, cells were harvested by centrifugation (4000 × g, 4 °C, 20 min) and stored at −80 °C until use. Frozen cells were thawed in 50 mM Tris-Cl pH 8.0, 500 mM NaCl, 20 mM imidazole supplemented with DNase (10 µg/mL, Sigma) and proteinase inhibitor cocktail (Roche), then lysed by sonication in ice. After centrifugation (20,000 × g, 4 °C, 20 min) to remove the cell debris, the crude protein solution was purified at 4 °C using an ÄKTA pure FPLC system (GE Healthcare). The supernatant was passed through a HisTrapTM HP column (5 mL, GE healthcare), pre-equilibrated with buffer A (50 mM Tris-Cl pH 8.0, 500 mM NaCl, 20 mM imidazole). Unbound proteins were washed with five column volumes of buffer A, followed by elution with buffer B (50 mM Tris-Cl pH 8, 500 mM NaCl, 500 mM imidazole). GDP-man-PP comprising fractions were pooled together and concentrated to ≈7 mg/mL (concentration determined by Pierce™ BCA assay, ThermoFisher or Bradford assay, Sigma). Concentrated GDP-man-PP was then divided into aliquots and stored at −80 °C until required.
**Evaluation of C6-modified glycosyl 1-phosphates**

**Figure S1**: TLC of GDP-man-PP catalyzed guanylyltransfer of GMP from GTP to 6Cl-Man-1P **17**, 6SH-Man-1P **18**, and 6NH₂-Man-1P **13** after 16 h at 37 °C. No reaction is observed for 6-thio **18** or 6-amino **13**. Lane 1: GDP-Man (authentic); Lane 2: GTP; Lane 3: Man-1P; Lane 4: Man-1P guanylyltransfer reference reaction; Lane 5: 6Cl-Man-1P **17**; Lane 6: 6Cl-Man-1P guanylyltransfer reaction; Lane 7: 6NH₂-Man-1P **13**; Lane 8: 6NH₂-Man-1P guanylyltransfer reaction; Lane 9: 6SH-Man-1P **18**; Lane 10: 6SH-Man-1P guanylyltransfer reaction.

No reaction was observed for 6-thio Man-1P **18** or 6-amino Man-1P **13**. The 6-thio substrate was found to form a disulfide in solution; increasing the concentration of reducing agent within the reaction from 1 mM to 20 mM had no effect on reaction progression. The addition of solid supported PPh₃ to stabilize the reduced form or the addition of 20 mM DTT also had no effect on reaction progression.

**Guanosine diphosphate-6-chloro-6-deoxy-α-D-mannose 19**

The enzymatic synthesis of sugar-nucleotides by GDP-Man-PP was completed as follows: The buffer was Tris-HCl (pH 8.0, 40 mM) containing MgCl₂ (8 mM) and DTT (1 mM). The final concentrations were as follows: glycosyl 1-phosphate **17** (7.5 mg, 18.7 µmol, 1.0 equiv, 6.0 mM) and GTP (10.59 mg, 20.2 µmol, 1.68 equiv). The enzyme concentrations were as follows: GDP-Man-PP (0.6 mg/mL) and inorganic pyrophosphatase (iPPase, Sigma, 2.70 U/mL). The reaction was incubated with shaking at 37 °C until formation of an NDP-sugar was observed by TLC (IPA/NH₄OH/H₂O 6:3:1). MeOH (213 µL) was added and the mixture was
centrifuged (9300 rpm) for 2 min to remove insoluble protein, passed through a syringe filter (0.4 μM, PTFE) and purified by SAX chromatography ThermoFisher Dionex UltiMate 3000 HPLC system using a Poros HQ 50 SAX column (5 mL), flow rate (7.0 mL/min), 5 → 250 mM NH₄HCO₃ over 15 min with in-line UV detector to monitor at 265 nm, to afford 19 as a white solid (6.9 mg, 11.0 µmol, 59%).

¹H NMR (500 MHz, D₂O) δ 8.12 (s, 1H, H-8’), 5.93 (d, 3J_H1'-H2' = 6.1 Hz, 1H, H-1’), 5.49 (dd, 3J_H1-P = 7.6 Hz, 3J_H1-H2 = 1.2 Hz, 1H, H-1), 4.77 (s, hidden, H-2’), 4.51 (dd, 3J_H1'-H2' = 5.1 Hz, 3J_H3'-H4' = 3.5 Hz, 1H, H-3’), 4.35 (dd, 3J_H4'-H3' = 3.1 Hz, 3J_H4'-H5' = 1.8 Hz, 1H, H-4’), 4.21 (dd, J = 5.2 Hz, J = 3.8 Hz, 2H, H-5’), 4.08 – 4.04 (m, 2H, H-4, H-2), 3.94 (dd, 3J_H3-H4 = 9.9 Hz, 3J_H3-H2 = 3.4 Hz, 1H, H-3), 3.91-3.85 (m, 2H, H-5, H-6a), 3.84-3.80 (m, 1H, H-6b); ¹³C NMR (125 MHz, D₂O) δ 96.5 (C-1), 86.8 (C-1’), 83.7 (C-4’), 73.5 (C-2’), 72.1 (C-4), 70.3 (C-3’), 70.1 (C-2), 69.5 (C-3), 66.6 (C-5), 62.6 (C-5’), 44.2 (C-6); ³¹P{¹H} NMR (200 MHz, D₂O) δ −11.50 (d, 2J_CP = 24.0 Hz), −14.01 (d, 2J_CP = 25.1 Hz); HRMS m/z (NSI-) found (M-H) 622.0335, C₁₆H₂₃ClN₅O₁₅P₂ requires 622.0360.

Figure S2: HPLC Purification of 6-Cl GDP-Man 19 after 16 hours using ThermoFisher Dionex UltiMate 3000 HPLC system using a Poros HQ 50 SAX column (5 mL), flow rate (7.0 mL/min), 5 → 250 mM NH₄HCO₃ over 15 min. HPLC after 16 hours reaction indicated presence of GTP (9.377 min), presence of GDP (7.800 min) and presence of the desired nucleotide 9 (6.124 min).
S4. Evaluation of sugar nucleotide probes with GMD

Expression and purification of GMD from *P. aeruginosa*

The recombinant plasmid (pET-3a) containing the *algD* gene encoding for GDP-mannose dehydrogenase (GMD) from *P. aeruginosa* was kindly donated by P. Tipton. The plasmid was transformed into *E. coli* solubleBL21(DE3) chemically competent cells and the transformant grown according to the literature. Briefly, 1 L of the transformant in LB medium containing the appropriate antibiotic (carbenicillin, 100 µg/mL) was incubated at 37 °C with gentle shaking in baffled flasks until an OD$_{600}$ of 0.6–0.8 was reached. Heterologous protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM, followed by incubation at 37 °C for 4 hours at 180 rpm. Afterwards the cells were harvested by centrifugation (4000 x g, 4 °C, 20 min) and stored at −80 °C until use. Frozen cells were thawed in 20 mM HEPES (pH 7.5), 150 mM NaCl supplemented with DNase A (10 µg/mL, Sigma) and proteinase inhibitor cocktail (Roche), then lysed by sonication on ice. The supernatant was recovered by centrifugation (20,000 x g, 4 °C, 20 min) and nucleic acid precipitated through the addition of protamine sulfate (5 mg per gram wet cell pellet) and incubated on ice for 30 min. Precipitated nucleic acid removed by centrifugation (20,000 x g, 4 °C, 20 min), the crude protein solution was fractionated with ammonium sulfate, with GMD precipitating between 45 and 60% saturation. Protein pellets were redissolved in 20 mM HEPES (pH 7.5), 150 mM NaCl and purified using an ÄKTA pure FPLC system (GE Healthcare) by gel filtration chromatography using a Superdex S200 16/600 column (GE Healthcare). Proteins were eluted with 20 mM HEPES (pH 7.5) and 150 mM NaCl at the flow rate of 1 mL/min. GMD containing fractions were combined and concentrated to ≈4.5 mg/mL (concentration determined by Pierce™ BCA assay, ThermoFisher or Bradfords Assay, Sigma). Concentrated GMD was then divided into aliquots and stored at −80°C until required in 10% glycerol.

GMD Inhibition assay

Assay protocol

The assay was performed in 96-well flat bottomed, non-binding, polystyrene microtiter plates (Grenier 655906). NAD$^+$ (200 µM), 19 (50 µM) and GMD (25 or 50 µg/mL) were prepared in 50 mM sodium phosphate (pH 7.4) containing 0.5 mM MgCl$_2$ and 1 mM DTT. A solution of
GDP-Man (final: 10 µM) was added to the plate and the fluorescence was measured at 25 °C for 65 minutes using a BMG labtech FLUOStar Omega microplate reader (excitation 355 nm; emission 460 nm). The limits of detection were analyzed by control samples as followed: positive control contained no inhibitor; negative control contained no inhibitor or GMD.

**Figure S3:** GMD function with probe 19 (50 µM) over 65 minutes. GMD (50 µg/mL, unless stated), GDP-Man 1 (50 µM), NAD⁺ (200 µM). Negative control experiment was run with no GMD.

**GMD alkylation by iodoacetamide**

![Deconvoluted protein LC–MS of GMD following overnight incubation with iodoacetamide (10 equiv) showing multiple surface-exposed alkylation sites.](image)

**Figure S4:** Deconvoluted protein LC–MS of GMD following overnight incubation with iodoacetamide (10 equiv) showing multiple surface-exposed alkylation sites.
S5. X-Ray crystallography data

Crystal and refinement parameters are given in Table S1. All data were collected on a Bruker D8 Quest ECO diffractometer using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) and a Photon II-C14 CPAD detector. Crystals were mounted on Mitegen micromounts in NVH immersion oil, and all collections were carried out at 150 K using an Oxford cryostream. Data collections were carried out using φ and ω scans, with collections and data reductions carried out in the Bruker APEX-3 suite of programs. Multi-scan absorption corrections were applied for all datasets using SADABS unless otherwise stated. The data were solved with the intrinsic phasing routine in SHELXT, and all data were refined on F² with full-matrix least squares procedures in SHELXL, operating within the OLEX-2 GUI. All non-hydrogen atoms were refined with anisotropic displacement parameters. Carbon-bound hydrogen atoms were placed in riding positions and refined with isotropic displacement parameters equal to 1.2 or 1.5 times the isotropic equivalent of their carrier atom. Crystals of 16 exhibited unavoidable non-merohedral twinning related by a 180 degree rotation which could not be mechanically separated. The two domains were indexed and their contributions to each reflection were separated using TWINABS, and the final refinement was performed on the HKLF5 file with a batch scale factor of 0.45. A global RIGU restraint and localised ISOR restraints were necessary to avoid non-positive definite ADPs in the final refinement caused by the substantial overlap of the two lattices and resulting impact on the data quality. CCDC 2165925

Table S1 Crystal data and structure refinement for 16

| Identification code | 16                |
|---------------------|-------------------|
| Empirical formula   | C_{24}H_{26}ClO_{11}P |
| Formula weight      | 556.87            |
| Temperature/K       | 150.0             |
| Crystal system      | monoclinic        |
| Space group         | P2_1              |
| a/Å                 | 11.9261(4)        |
| b/Å                 | 8.1862(3)         |
| c/Å                 | 14.2882(5)        |
| **Parameter**                  | **Value**       |
|-------------------------------|-----------------|
| $\alpha/^{\circ}$            | 90              |
| $\beta/^{\circ}$             | 109.912(2)      |
| $\gamma/^{\circ}$            | 90              |
| Volume/Å³                     | 1311.55(8)      |
| $Z$                           | 2               |
| $\rho_{\text{calc}}$/g/cm³   | 1.410           |
| $\mu$/mm⁻¹                   | 0.265           |
| F(000)                        | 580.0           |
| Crystal size/mm³              | 0.19 $\times$ 0.07 $\times$ 0.03 |
| Radiation                     | MoKα ($\lambda = 0.71073$) |
| 2$\Theta$ range for data collection/° | 5.468 to 50.992 |
| Index ranges                  | -14 $\leq$ h $\leq$ 14, -9 $\leq$ k $\leq$ 9, -17 $\leq$ l $\leq$ 17 |
| Reflections collected         | 43101 [17377 with $I$ $\geq$ 2$\sigma$ ($I$)] |
| Independent reflections       | 4867 [$R_{\text{int}}(\text{HKLF4}) = 0.1235$, $R_{\text{sigma}} = 0.1014$] |
| Data/restraints/parameters    | 4867/329/364    |
| Goodness-of-fit on $F^2$      | 1.216           |
| Final R indexes [$I$ $\geq$ 2$\sigma$ ($I$)] | $R_1 = 0.0919$, $wR_2 = 0.1285$ |
| Final R indexes [all data]    | $R_1 = 0.1091$, $wR_2 = 0.1342$ |
| Largest diff. peak/hole / e Å⁻³ | 0.69/-0.76     |
| Flack parameter               | -0.04(5)        |
| CCDC Number                   | 2165925         |
S6. References

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S7. Spectral Data: $^1$H, $^{13}$C and $^{31}$P NMR for compounds 10-17 and 19

2,3,4-Tri-O-benzyl-6-bromo-6-deoxy-1-thio-α-D-mannopyranoside

Molecular Weight: 605.59
6-Azido-6-deoxy-2,3,4-tri-O-benzyl-1-thio-α-D-mannopyranoside 11

Molecular Weight: 567.70
Dibenzyl 6-azido-6-deoxy-2,3,4-tri-O-benzyl-α-D-mannopyranosyl phosphate 12

Molecular Weight: 735.77
6-Amino-6-deoxy-α-D-mannopyranose 1-phosphate (disodium salt) 13

Molecular Weight: 303.11
2,3,4-Tri-O-Acetyl 6-chloro-6-deoxy-D-mannopyranose 15

Molecular Weight: 324.71
Diphenyl 6-chloro-6-deoxy-2,3,4-tri-O-acetyl-α-D-mannopyranosyl phosphate 16

Molecular Weight: 556.88
1,2,3-Tri-O-acetyl-6-chloro-6-deoxy-α-D-mannopyranose-1-phosphate

Molecular Weight: 402.67
6-Chloro-6-deoxy-α-D-mannopyranose 1-phosphate (sodium triethylamine salt)
Guanosine diphosphate 6-chloro-6-deoxy-α-D-mannose 19

Molecular Weight: 623.7855
HRMS (ESI negative mode) of 6-Cl GDP-Man 19. (\textsuperscript{35}Cl: M–H)\textsuperscript{−} = 622.0335 [\Delta = \text{−}4.0 \text{ ppm}]; (\textsuperscript{37}Cl: M–H)\textsuperscript{−} = 624.0313 [\Delta = \text{−}2.8 \text{ ppm}].