Chemical and enzymatic synthesis of the alginate sugar nucleotide building block: GDP-D-mannuronic acid

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Keywords: sugar nucleotide, glycosyl-1-phosphate, mannuronate, pyrophosphorylation, alginate

1. Introduction

Alginate is a heterogenous polysaccharide composed of β-1,4-linked D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G) (*Figure 1a*). Within alginate sub-structure the relative proportions of M and G units, their homo- or heteropolymeric block-groupings and the possibility for acetylation at the C2 and/or C3 positions of M residues produces a structurally diverse biopolymer. This structural microheterogeneity varies depending on the alginate source and the biopolymer is produced by both plants and bacteria. The study of alginate biochemistry and biosynthesis has largely focused on the bacterial genera *Pseudomonas*, owing to the prevalence of the opportunistic human pathogen *Pseudomonas aeruginosa*, which causes chronic infections in cystic fibrosis patients, contributing to a reduction in lung function and increased mortality rates.[1] Alginate is also an important industrial biomaterial, currently sourced from marine algae and utilised as a stabiliser, viscosifier and gelling agent in the food, beverage, paper and pharmaceutical industries.[2]

Alginate biosynthesis utilises the sugar nucleotide GDP-D-ManA, **1** (*Figure 1b*), which is sourced from the cytosolic metabolic pool through a series of enzymatic transformations starting from fructose 6-phosphate and ultimately obtained *via* oxidation of GDP-D-Man to the uronate by GDP-mannose dehydrogenase (GMD).[3] Following this, an intricate, multi-enzyme mediated polymerisation process assembles the β-D-mannuronate polymer, which is then further modified by epimerisation, acetylation and truncation before export.

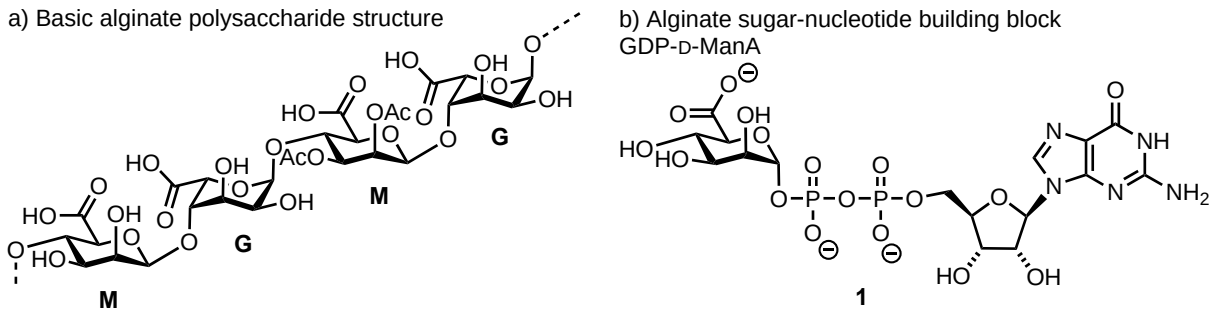


Figure 1. a) Chemical structure of alginate showing constituent M/G residues and C2/C3 acetylation for one M residue, b) GDP-D-ManA **1**, the sugar nucleotide building block of alginate.

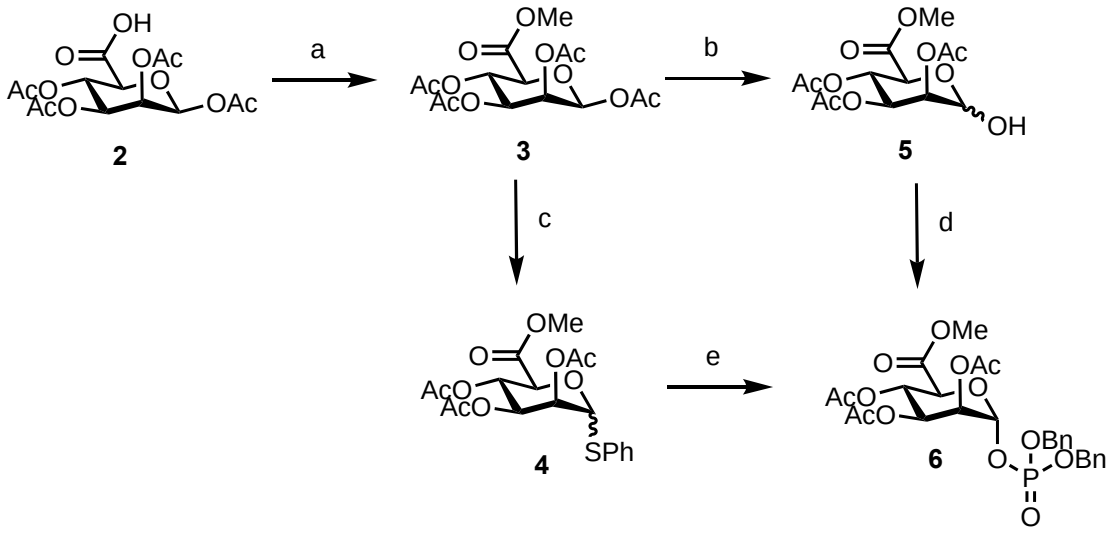
As part of a program to investigate the enzymes involved in the biosynthesis of alginate,[4] were interested to chemically synthesise **1** and deliver an enabling sugar nucleotide tool to support elucidation of the alginate polymerisation process. A chemical synthesis of **1** was recently completed by Codée *et al*[5] using P^{III}-amidite-P^{V} chemistry to accomplish the key pyrophosporylation step in forming **1**. Herein we report our approach to **1**, instead using a P^{V}-P^{V} pyrophosphorylation and present the results of evaluating two differentially protected D-ManA 1-phosphates for coupling. Alongside this we evaluated an enzymatic approach to **1** from GDP-D-Man using recombinant GMD from *P. aeruginosa*.

2. Results and Discussion

2.1. Synthesis of D-ManA 1-phosphates

Enzymatic and chemical approaches to synthesise uronic acid 1-phosphates have been explored.[6,7] From a chemical perspective, the inclusion of an acidic, charged functional group in pyrophosphorylative coupling is challenging and efforts to circumvent this have involved completing late-stage (post-diphosphate formation) oxidation to the uronate[8] and protecting the carboxylate.[9] We first sought to synthesise two differentially protected D-ManA 1-phosphates, **7** and **8**, as we wanted to examine the effect of retaining a protected carboxylate group (against the free acid form) when completing chemical pyrophosphorylation. Previously it was noted by Linhardt[9] that retaining a methyl ester protecting group (for their synthesis of UDP-L-IdoA) avoided problems during the pyrophosphorylation coupling reaction (65% reported yield). However, the synthesis of the related UDP-D-GlcA reported by Khorana[10] using free D-GlcA 1-phosphate indicated an equally successful approach (66% yield).

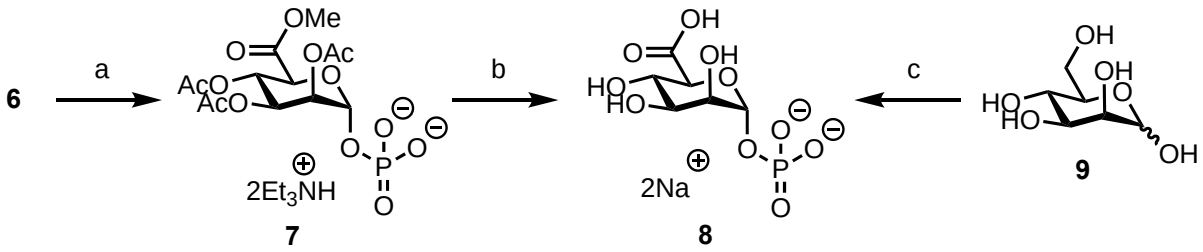
Our synthetic route began from mannuronic acid derivative **2**, for which we recently reported a multi-gram scale synthesis.[11] Methylation of **2** was achieved using iodomethane and K_2CO_3 to give ester **3** in good yield (77%, *Scheme 1*). We next evaluated MacDonald's conditions ($H_3PO_{4(s)}$, high vacuum and 60 °C)[12] to directly form glycosyl 1-phosphates from **2** and **3**. Unfortunately, in our hands, **2** and **3** largely decomposed under the reaction conditions or formed significant amounts of the C4-C5 elimination product and we instead attempted to access thioglycoside donor **4**, as a means to provide protected mannuronate 1-phosphate **6**. We found the reaction to form **4** from **3** to be sluggish and low-yielding, with significant amounts of orthoester formation observed. This was attributed to the disarming nature of the uronate and use of acetate protecting groups.[13] We were able to optimise this reaction using TMSOTf as a Lewis acid ($BF_3{\cdot}Et_2O$ showed no reaction) to a yield of 63% (5:1 α/β ratio of **4** with 4:1 α/orthoester, as judged by ^{1}H NMR) using a reaction temperature of -15 °C for 6 h. Raising the temperature to 0 °C and extending the reaction time to 32 h caused a significant reduction in yield (17%, with 20% returned **3**), but did reduce orthoester formation (6:1 α/β ratio of **4** with 33:1 α/orthoester). With amounts of pure **4** in hand, following silica gel chromatography, we next converted to the protected 1-phosphate **6** using dibenzyl phosphate (DBP) under standard thioglycoside activation conditions. This afforded **6**, albeit in low yield (23%), but with the expected ^{31}P NMR resonance for the anomeric phosphate (-3.24 ppm) and the characteristic doublet of doublets for H_1 ($^3J_{H1\text{-}31P}$ = 6.4 Hz, $^3J_{H1\text{-}H2}$ = 1.9 Hz).



Scheme 1. *Synthesis of protected D-ManA 1-phosphate* ***6****. a) MeI, K_2CO_3, DMF, 77% b) NH_2-NH_2·AcOH, DMF, 68% c) TMSOTf, HSPh, DCM, 63% d) i) Cl_3CCN, K_2CO_3, DCM, 88% ii) HO-P(O)$(OBn)_2$, TMSOTf, DCM, 49% e) HO-P(O)$(OBn)_2$, NIS, AgOTf, DCM, 23%.*

Owing to the problems we encountered in accessing **6** *via* **4** (15%, 2 steps), we explored an alternative route, firstly removing the anomeric acetate from **3** to give hemi-acetal **5**, in 68% yield, followed by conversion to a trichloroacetimidate donor (88% yield) and immediate reaction with DBP using TMSOTf as promoter. Although successful, our attempts at optimisation did not deliver **6** in a yield greater than 49% (from **5**), but did afford the material in 30% overall yield from **3**, double that observed for the route from **4**.

We next undertook a two-stage deprotection of **6** to deliver D-ManA 1-phosphates **7** and **8** (*Scheme 2*). We removed the phosphate benzyl protecting groups using hydrogenolysis, followed by conversion to a bis-triethylammonium salt, delivering semi-protected phosphate **7** in very good yield (71%). The acetate protecting groups of **7** were then cleaved to give D-ManA 1-phosphate **8**. At this juncture we re-visited the Macdonald phosphorylation and were able to establish conditions to afford **8** directly from D-mannose **9** (*Scheme 2*). Following per-acetylation and anomeric phosphorylation,[14] the crude 1-phosphate could be conveniently oxidised using TEMPO/BAIB or TEMPO/NaOCl[15] to deliver **8** in 15% yield over three steps. This compares to an overall yield of 15% over 5 steps for the route to **8** from **2**, which whilst longer, did afford access to the partially protected 1-phosphate **7**. With differentially protected D-ManA 1-phosphates, **7** and **8**, in hand we next evaluated their pyrophosphorylative coupling (with GMP-morpholidate) to deliver **1**.

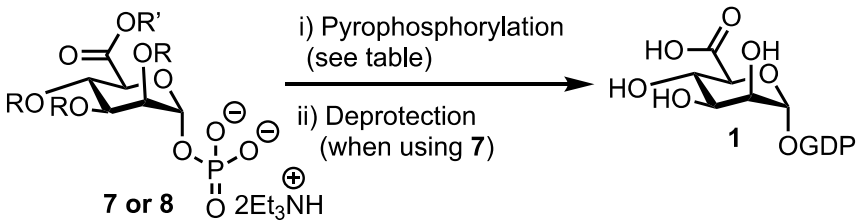


Scheme 2. *Synthesis of semi-protected and free D-ManA 1-phosphates* ***7*** *and* ***8****. a) H_2, Pd/C, MeOH, Et_3N, 71%; b) Et_3N/MeOH/H_2O, 2:2:1, IR120 Na^+ resin, 96%; c) i) Ac_2O, pyridine, DMAP, 93% ii) H_3PO_4, then LiOH, 56% iii) TEMPO, BAIB, H_2O/MeCN, 30% or TEMPO, NaOCl, NaOH, H_2O/MeCN, 21%.*

2.2. Chemical Synthesis of GDP-D-ManA

In recent years, chemical approaches to synthesise sugar nucleotides have favoured P^V-P^V and P^V-P^{III} pyrophosphorylation methods, removing any anomeric integrity consequences of glycosylating a nucleoside diphosphate.[16,17] We selected a P^V-P^V approach using GMP-morpholidate as the coupling partner for **7** or **8** and trialled different activators, solvents and durations, the results of which are summarised in Table 1.

Table 1. *Evaluation of pyrophosphorylation conditions to synthesise **1**.*



Entry	1-phosphate	Additive (equiv.)[£]	Reaction Time (h)	Solvent (conc.)	Yield (%)	Notes
1	**7**	*N*-MIC (2.9)	60	DMF (0.06)	0	No rxn.
2	**7**	DCI (4.0)	56	DMF (0.05)	<5[$]	DCI contamination
3	**7**	DCI (1.0)	108	DMF (0.05)	<5[$]	Reduced DCI
4	**7**	None then DCI (1.0)	144[^]	Pyr. (0.06)	22[$]	Reduced DCI
5	**7**	DCI (1.0)	120	Pyr. (0.06)	46[$]	Reduced DCI
6	**8**	DCI (1.0)	40	DMF (0.07)	0	No rxn.
7	**8**	None	144	Pyr. (0.04)	0	No rxn.

[£]along with 1.5 equiv. GMP-morpholidate and 1.0 equiv. of 1-phosphate.
[$]following deprotection of the crude coupling reaction (Et_3N, MeOH, H_2O).
[^] DCI was added after 48 h, as no reaction was indicated to have taken place by TLC.
R = Ac, R' = Me.

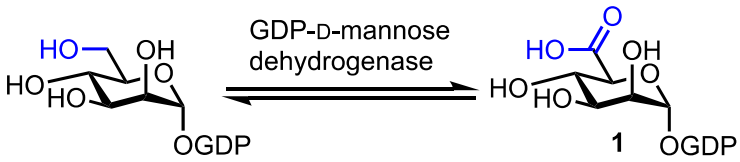
N-Methylimidazole hydrochloride (*N*-MIC[18], Table 1, entry 1) has been reported as a superior pyrophosphorylative catalyst to the traditional use of 1-*H*-tetrazole. Utilising it here, we were unable to detect the formation of **1** by TLC (isopropyl alcohol/ammonium hydroxide/water, 6:3:1) and observed baseline material after 60 h. Repeating the reaction (including several co-evaporations with toluene under N_2 prior to reaction) led to similar outcomes and we thus switched to using 4,5-dicyanoimidazole (DCI, Table 1, entry 2). The reaction proceeded smoothly over 56 h with TLC analysis indicating significant consumption of **7** and crude ^{31}P NMR confirming nucleoside diphosphate formation (δ_P -11.4, -14.5 ppm). The crude material isolated was immediately subjected to pyranoside deprotection using Et_3N/MeOH/H_2O followed by strong-anion exchange (SAX) purification which delivered **1** but only in very poor yields (<5%). We encountered problems here during SAX purification, namely that the large amount of DCI used (4.0 equiv.) co-eluted with **1**, thus requiring additional C18 reverse phase purification to remove this impurity which reduced the overall yield. In order to solve these problematic final purification(s) we investigated reducing the equivalents of DCI alongside changing the reaction solvent to pyridine (Table 1, entries 3 and

4). Pleasingly, we were able to improve the yield of **1** to 46% using 1.0 equiv. of DCI in pyridine (Table 1 entry 5). We also observed that the uncatalysed reaction was very slow (no reaction after 48 h), but did not investigate reducing the amount of DCI further. Using only 1.0 equivalent of DCI we were also able to return to using DMF as solvent, which improved solubility of the reagents slightly, obtaining similar results to those using pyridine.

For ManA 1-phosphate **8** we observed no indicative conversion to **1** by TLC (Table 1, entry 6) and we surmised that poor solubility of the components was hindering the reaction in DMF. Changing solvent to pyridine (Table 1, entry 7) unfortunately had no positive effect on the reaction outcome and we concluded that the material was not reacting under the conditions tried (GMP-morpholidate could still be observed by crude ^{31}P NMR). In summary, we observed that successful pyrophosphorylative coupling to form **1** could best be achieved using carboxylate protected mannuronate 1-phosphate **7**. The chemical synthesis route developed here delivers multi-milligram access to **1** in five steps and 8% overall yield from **2**. Whilst more involved than the direct enzymatic option considered below, this methodology will be underpinning to the development of analogues syntheses derived from **1**, which is essential to the continued study of sugar-nucleotide-mediated alginate biosynthesis.

2.3. Enzymatic Synthesis of GDP-D-ManA

Within alginate biosynthesis, **1** is produced by dehydrogenative oxidation of GDP-D-Man by GMD. In order to investigate enzymatic production of **1** we incubated GDP-D-Man with recombinant GMD from *P. aeruginosa* in the presence of NAD^+ at room temperature with gentle shaking. The reaction was monitored by strong anion exchange chromatography at different time points. After 21 h, the conversion of GDP-D-Man to **1** reached 70% using 2 equivalents of NAD^+ and enabled the isolation of mg quantities of the desired material (*Scheme 3*). After 72 h, complete consumption of the starting material was evident, following the addition of four further equivalents of NAD^+ (see SI).



Scheme 3. *Enzymatic synthesis of **1** from GDP-D-Man. a) NAD^+, DTT, $MgCl_2$, pH 7.4, 70%.*

3. Conclusion

We have established chemical (P^V-P^V) and enzymatic routes to the alginate sugar nucleotide feedstock GDP-D-ManA. Synthetic access to partially protected and fully deprotected anomeric 1-phosphates of D-mannuronic acid enabled their evaluation in pyrophosphorylative coupling to the target nucleoside diphosphate. Only the partially protected glycosyl 1-phosphate was effective for this reaction under the conditions examined. This procedure is complimented by an enzymatic approach to the same sugar nucleotide using the GDP-D-mannose dehydrogenase from *P. aeruginosa*.

4. Experimental section

4.1. General Methods and Materials

All reagents and solvents which were available commercially were purchased from Acros, Alfa Aesar, Fisher Scientific, or Sigma Aldrich. All reactions in non-aqueous solvents were conducted in oven dried glassware under a nitrogen atmosphere with a magnetic stirring device. 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. Reactions requiring low temperatures used the following cooling baths: -78 °C (dry ice/acetone), -30 °C (dry ice/acetone), -15 °C (NaCl/ice/water) and 0 °C (ice/ water). Infra-red spectra were recorded neat on a Perkin Elmer Spectrum 100 FT-IR spectrometer; selected absorbtion frequencies (νmax) are reported in cm^{-1}. ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C spectra at 100 MHz respectively using a Bruker AVIII400 spectrometer. ^{1}H NMR signals were assigned with the aid of gDQCOSY. ^{13}C NMR signals were assigned with the aid of gHSQCAD. Coupling constants are reported in Hertz. Chemical shifts (δ, in ppm) are standardised against the deuterated solvent peak. NMR data were analysed using Nucleomatica iNMR or Mestrenova software. ^{1}H NMR splitting patterns were assigned as follows: br s (broad singlet), s (singlet), d (doublet), app. t (apparent triplet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), or m (multiplet and/or multiple resonances). Reactions were followed by thin layer chromatography (TLC) using Merck silica gel 60F254 analytical plates (aluminium support) and were developed using standard visualising agents: short wave UV radiation (245 nm) and 5% sulfuric acid in methanol/Δ. Purification *via* flash column chromatography was conducted using silica gel 60 (0.043-0.063 mm). Melting points were recorded using open glass capillaries on a Gallenkamp melting point apparatus and are uncorrected. Optical activities were recorded on automatic polarimeter Rudolph autopol I or Bellingham and Stanley ADP430 (concentration in g/100mL). pH measurements were recorded using a Hanna® pH 20 meter. MS and HRMS (ESI) were obtained on Waters (Xevo, G2-XS TOF) or Waters Micromass LCT spectrometers using a methanol mobile phase. High resolution (ESI) spectra were obtained on a Xevo, G2-XS TOF mass spectrometer. HRMS was obtained using a lock-mass to adjust the calibrated mass. HPLC was performed on an Agilent Technologies 1200 series machine, using a Waters Bridge Reversed-phase prep-C18 column (5 μm OBD, 19 × 100 mm). MeCN:H2O, 60:40→100% was used as a mobile phase and the product was detected using UV at 254 nm. Purification by C18 chromatography was conducted using a Thermoscientific X30 SPE column (HyperSep C18, 6 mL) eluting with H_2O. Purification *via* ion exchange chromatography was conducted on Bio-Rad Biologic LP system using a Bio-Scale Mini UNOsphere Q (strong anion exchange) cartridge (5 mL): flow rate (3.0 mL/min), 0 → 100% 1.0 M $(NH_4)HCO_3$ over 33 min or strong anion-exchange (SAX) HPLC on Poros HQ 50 was performed as published earlier.[19]

*4.2. Methyl (1,2,3,4-tetra-O-acetyl-β-D-mannopyranosyluronate (**3**)*

To a stirred solution of 1,2,3,4-tetra-*O*-acetyl-β-D-mannuronic acid **2**[11] (600 mg, 1.70 mmol, 1.0 equiv.) in anhydrous dimethylformamide (8 mL) was added methyl iodide (250 μL, 4.02 mmol, 2.4 equiv.) and K_2CO_3 (156 mg, 1.13 mmol, 1.5 equiv.). The solution was stirred at room temperature for 72 h, whereupon TLC analysis (hexane/ethyl acetate, 3/1) indicated complete conversion of starting material to a higher R_f spot. The reaction was quenched with methanol (5 mL), ethyl acetate (25 mL) was added and the solution washed with H_2O (15 mL). The aqueous layer was extracted with ethyl acetate (25 mL), the combined organic layers washed with water (15 mL) and brine (15 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The resultant yellow solid was triturated with methanol to afford **2** as a white solid (480 mg, 1.3 mmol, 77%). R_f 0.23 (hexane/ethyl acetate, 3/1); $[\alpha]_D^{26}$ = -16.0 (*c* = 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) $\boldsymbol{\delta}_H$ 5.91 (1H, d, *J* = 1.3 Hz, H_1), 5.50 (1H, dd, *J* = 3.5, 1.2 Hz, H_2), 5.42 (1H, t, *J* = 9.4 Hz, H_4), 5.19 (1H, dd, *J* = 9.6, 3.2 Hz, H_3), 4.15 (1H, d, *J* = 9.4 Hz, H_5), 3.74 (3H, s, C(O)OCH_3), 2.21 (3H, s, C(O)CH_3), 2.12 (3H, s, C(O)CH_3), 2.07 (3H, s, C(O)CH_3), 2.03 (3H, s, C(O)CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) $\boldsymbol{\delta}_C$ 170.6 (C=O), 170.0 (2 x C=O), 168.8 (C=O), 167.2 (C=O), 90.1 (C_1), 73.6 (C_5), 70.0 (C_3), 67.8 (C_2), 66.7

(C_4), 53.3 (CO_2CH_3), 21.1 (C(O)CH_3), 21.1 (C(O)CH_3), 21.0 (C(O)CH_3), 20.9 (C(O)CH_3); HRMS $[M+NH_4]^+$ calculated for $C_{15}H_{24}O_{11}N$: 394.1344; found: 394.1337.

4.3 Methyl (phenyl-2,3,4-tri-O-acetyl-1-thio-α/β-D-mannopyranosyl)uronate ***(4)***
Uronate **3** (200 mg, 500 µmol, 1.0 equiv.) and powdered 4Å molecular sieves were dissolved in anhydrous dichloromethane (3 mL) and stirred under N_2 atmosphere for 12 h. Thiophenol (82 µL, 820 µmol. 1.5 equiv.) was added, the solution cooled to − 15 °C and TMSOTf (0.30 mL, 1.62 mmol, 3 equiv.) was added dropwise. The reaction mixture was stirred at − 15 °C for 6 h, whereupon TLC analysis (hexane/ethyl acetate 3/1) showed complete conversion of the starting material to a higher R_f spot. The yellow reaction mixture was quenched through the addition of triethylamine until pH = 7, filtered over Celite™ and diluted with dichloromethane (25 mL). The organic layer was washed with distilled water (15 mL) and brine (15 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford a pale-yellow oil. Purification by silica column chromatography, eluting with hexane/ethyl acetate (1/0, 3/1) afforded **4** as an opaque, colourless oil (134 mg, 315 µmol, 63%). R_f 0.30 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, $CDCl_3$) $\boldsymbol{\delta}_H$ α-anomer 7.51 (2H, d, *J* = 6.6 Hz, Ar-*H*), 7.30 (3H, m, Ar-*H*), 5.59 (1H, d, *J* = 3.8 Hz, H_1), 5.48-5.40 (2H, m, H_2, H_4), 5.33 (1H, dd, *J* = 8.4, 3.1 Hz, H_3), 4.79 (1H, d, *J* = 7.8 Hz, H_5), 3.76 (3H, s, C(O)OCH_3), 2.11 (3H, s, C(O)CH_3), 2.08 (3H, s, C(O)CH_3), 2.02 (3H, s, C(O)CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) $\boldsymbol{\delta}_C$ 169.7 (C=O), 169.5 (C=O), 169.3 (C=O), 167.8 (C=O), 132.3 (Ar-*C*), 131.7 (Ar-*C*), 129.1 (Ar-*C*), 128.0 (Ar-*C*), 84.4 (C_1), 71.0 (C_5), 69.2 (C_2), 68.3 (C_3), 67.4 (C_4), 52.6 (*C*(O)OCH_3), 20.7 (C(O)CH_3), 20.6 (C(O)CH_3), (C(O)CH_3) 20.5 (C(O)CH_3); HRMS $[M+NH_4]^+$ calculated for $C_{19}H_{26}O_9SN$: 444.1323; found: 444.1323.

*4.4. Methyl 2,3,4-tri-O-acetyl-α-D-mannopyranosyluronate (**5**)*
To a stirred solution of **3** (100 mg, 0.27 mmol, 1.0 equiv.) in anhydrous DMF (2 mL) was added hydrazine acetate (38 mg, 0.41 mmol, 1.5 equiv.). The solution was stirred at room temperature for 3 h, whereupon TLC analysis (hexane/ethyl acetate, 1/1) indicated complete conversion of starting material to a lower R_f spot. The solvent was removed *in vacuo* and the residue dissolved in ethyl acetate (10 mL). The organic layer was washed with distilled water (10 mL) and the aqueous layer re-extracted with ethyl acetate (10 mL). The combined organic layers were washed with distilled water (20 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by silica gel column chromatography eluting with hexane/ethyl acetate (3/1, 1/1) afforded **5** as a colourless oil (95% α-anomer, 58 mg, 0.17 mmol, 64%). R_f 0.47 (hexane/ethyl acetate, 1/1); ^{1}H NMR (400 MHz, $CDCl_3$) $\boldsymbol{\delta}_H$ α-anomer 5.45 (1H, d, *J* = 3.2 Hz, H_3), 5.41 (1H, d, *J* = 9.0 Hz, H_4), 5.34 (1H, d, *J* = 2.5 Hz, H_1), 5.26-5.25 (1H, m, H_2), 4.58 (1H, d, *J* = 8.9 Hz, H_5), 2.15 (3H, s, C(O)OCH_3), 2.07 (3H, s, C(O)OCH_3), 2.02 (3H, s, C(O)OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) $\boldsymbol{\delta}_C$ 170.2 (C=O), 169.7 (C=O), 168.6 (C=O), 162.8 (C=O), 92.14 (C_1), 69.7 (C_2), 69.6 (C_5), 68.3 (C_3), 67.2 (C_4), 54.7 ($C(O)_2CH_3$), 20.9 (C(O)CH_3), 20.7 (C(O)CH_3), 20.6 (C(O)CH_3); HRMS $[M+NH_4]^+$ calculated $C_{13}H_{22}O_{10}N$: 352.1283; found: 352.1246.

*4.5. Methyl 2,3,4-tri-O-acetyl-α-D-mannopyranosyluronate dibenzyl 1-phosphate (**6**)*
From **5**: To a stirred solution of **5** (550 mg, 1.65 mmol, 1.0 equiv.) and oven dried anhydrous K_2CO_3 (360 mg, 2.64 mmol, 1.6 equiv.) in anhydrous dichloromethane (5.5 mL) was added trichloroacetonitrile (0.37 mL, 4.62 mmol, 2.8 equiv.). The solution was stirred at room temperature for 24 h, whereupon TLC analysis (hexane/ethyl acetate, 1/1) indicated conversion of the starting material to a higher R_f spot. The dark brown solution was filtered through Celite®, washing with dichloromethane and concentrated *in vacuo* to afford methyl (2,3,4,-tri-*O*-acetyl-β-D-mannopyranose) uronate trichloroacetimidate as a pale brown oil

(774 mg, 1.62 mmol, 88%). This crude material (774 mg, 1.62 mmol, 1.0 equiv.) was dissolved in anhydrous dichloromethane (15 mL), powdered 4 Å molecular sieves were added and the suspension stirred for 2 h. Dibenzyl phosphate (770 mg, 2.75 mmol, 1.7 equiv.) was then added and stirring continued for 30 min. The solution was then cooled to -10 °C and TMSOTf (0.15 mL, 810 µmol, 0.5 equiv.) added dropwise. The solution was warmed slowly to room temperature over 1 h. TLC analysis (hexane/ethyl acetate, 1/1) indicated complete conversion of starting material to a lower R_f spot. The light orange reaction mixture was quenched by addition of Et_3N (until pH = 7) and filtered over Celite™ washing with dichloromethane (20 mL). The organic layer was then washed with saturated aqueous $NaHCO_3$ solution (25 mL), distilled water (25 mL), brine (25 mL), dried ($MgSO_4$), concentrated *in vacuo* to give a yellow oil. This crude material was purified by silica gel column chromatography eluting with toluene/acetone (10/1, 7/1, 3/1) to afford **6** as a colourless oil (223 mg, 0.38 mmol, 34%). R_f 0.30 (hexane/ethyl acetate, 1/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.35-7.34 (8H, m, Ar*H*), 7.19-7.17 (2H, m, Ar*H*), 5.70 (1H, dd, *J* = 6.4, 1.9 Hz, H_1), 5.41-5.31 (2H, m, H_3, H_4), 5.24 (1H, d, *J* = 2.1 Hz, H_2), 5.09 (4H, m, CH_2Ph), 4.39 (1H, d, *J* = 8.8 Hz, H_5), 3.69 (3H, s, C(O)OCH_3), 2.13 (3H, s, C(O)CH_3), 2.05 (3H, s, C(O)CH_3), 2.01 (3H, s, C(O)CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 169.6 (C=O), 169.5 (C=O), 169.5 (C=O), 167.2 (C=O), 129.1 (Ar-*C*), 128.7 (Ar-*C*), 128.7 (Ar-*C*), 128.2 (Ar-*C*), 128.1 (Ar-*C*), 94.7 (C_1), 70.8 (C_5), 70.1 (*C*H_2Ph), 69.9 (*C*H_2Ph), 68.2 (C_2), 67.6 (C_3), 66.4 (C_4), 52.8 (C(O)$_2$*C*H_3), 21.5 (C(O)*C*H_3), 20.7 (C(O)*C*H_3), 20.6 (C(O)*C*H_3); ^{31}P NMR (161 MHz, $CDCl_3$) δ_P -3.20 (1P, s); HRMS $[M+H]^+$ calculated for $C_{27}H_{32}O_{13}P$: 595.5158; found: 595.1586. These data were in good agreement with literature values.[5]

From **4**: Uronate **4** (180 mg, 0.4 mmol, 1.0 equiv.) and powdered 4Å molecular sieves were dissolved in anhydrous dichloromethane (5 mL) and stirred under a N_2 atmosphere at RT for 12 h. Dibenzyl phosphate (198 mg, 0.7 mmol, 1.7 equiv.) was added and stirred for 30 min. *N*-iodosuccinimide (0.14 g, 0.6 mmol, 1.5 equiv.) and silver trifluoromethanesulfonate (33 mg, 0.1 mmol, 0.3 equiv.) were added at −30 °C and the temperature was raised to –10 °C over 40 min. TLC analysis (hexane/ethyl acetate, 1/1) indicated complete conversion of the starting material to a higher R_f spot. The dark red reaction mixture was quenched through the addition of triethylamine until pH = 7, filtered through Celite™ and diluted with dichloromethane (25 mL). The organic layer was washed with saturated aqueous $Na_2S_2O_3$ solution (15 mL), saturated aqueous sodium hydrogen carbonate solution (15 mL), distilled water (15 mL) and brine (15 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford a dark orange oil. Purification by silica gel column chromatography, eluting with hexane/ethyl acetate (3/1, 2/1, 1/1), afforded **6** as a colourless oil (61 mg, 100 µmol, 23%).

*4.6. Methyl 2,3,4-tri-O-acetyl-α-D-mannopyranosyluronate 1-phosphate (bis-triethylammonium salt) (**7**)*

A suspension of **6** (200 mg, 0.34 mmol, 1.0 equiv.) and 10% Pd/C (15 mg, 0.14 mmol, 0.2 eq. per Bn) in anhydrous methanol (5 mL) was stirred under an atmosphere of hydrogen (1 atm, balloon) at room temperature for 5 h. TLC analysis (hexane/ethyl acetate, 1/2) showed complete conversion of starting material to a lower R_f spot. The reaction mixture was filtered through Celite®, washing with methanol and the filtrate treated with Et_3N (95 µL, 0.68 mmol, 2.0 equiv.) followed by solvent removal *in vacuo* to afford methyl **7** as a white solid (148 mg, 0.24 mmol, 71%). R_f 0.45 (ethyl acetate/methanol/water, 5/3/1); $[\alpha]_D^{26}$ = + 16.05 (*c* = 0.3, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 5.62 (1H, d, *J* = 7.0 Hz, H_1), 5.47 (1H, dd, *J* = 9.9, 3.3 Hz, H_3), 5.37-5.30 (2H, m, H_2, H_4), 4.70 (1H, d, *J* = 10.0 Hz, H_5), 3.70 (3H, s, CO_2CH_3), 2.93 (12H, q, *J* = 6.6 Hz, $[CH_3CH_2]_3NH^+$), 2.13 (3H, s, C(O)CH_3), 2.03 (3H, s, C(O)CH_3), 1.96 (3H, s, C(O)CH_3), 1.25 (18H, t, *J* = 6.9 Hz, $[CH_3CH_2]_3NH^+$); ^{13}C NMR (100 MHz,

$CDCl_3$) δ_C 169.9 (2 x C=O), 169.6 (C=O), 168.6 (C=O), 93.6 (C_1), 69.6 (C_2), 69.5 (C_5), 68.6 (C_3), 67.1 (C_4), 52.5 (CO_2CH_3), 45.6 ($N(CH_2CH_3)_3$), 20.9 ($C(O)CH_3$), 20.7 ($C(O)CH_3$), 20.6 ($C(O)CH_3$), 9.2 ($N(CH_2CH_3)_3$); ^{31}P NMR (160 MHz, $CDCl_3$) δ_P -0.90 (1P, s); HRMS $[M+H]^+$ calculated for $C_{13}H_{19}O_{13}P$: 413.0951; found: 413.0945.

*4.7. α-D-mannopyranuronic acid 1-phosphate (disodium salt) (**8**)*

From 7: To a stirred solution of **7** (130 mg, 0.21 mmol, 1.0 equiv.) in methanol/water (3 mL/1.5 mL) was added triethylamine (3 mL). The solution was stirred for 18 h at room temperature, whereupon TLC analysis (acetonitrile/water with 4 drops of NH_4OH, 2/1) indicated conversion of starting material to a lower R_f spot. The solution was concentrated *in vacuo* (water bath temperature not exceeding 30 °C) to afford a yellow residue. This was passed down an ion-exchange column (Dowex® 50W-X4 Na^+ form, 200-400 mesh) eluting with water. The sugar containing fractions were pooled and freeze dried to afford **8** as a fluffy cream solid (53 mg, 0.19 mmol, 96%). R_f 0.33 (acetonitrile/water with 4 drops NH_4OH, 2/1); $[\alpha]_D^{26}$ = + 22.22 (*c* = 0.45, H_2O); ^{1}H NMR (400 MHz, D_2O) δ_H 5.28 (1H, d, $J_{H\text{-}P}$ = 8.6 Hz, H_1), 4.02 (1H, d, *J* = 10.0 Hz, H_4), 3.89-3.83 (2H, m, H_2, H_3), 3.75-3.65 (1H, m, H_5); ^{13}C NMR (100 MHz, D_2O) δ_C 177.4 (C=O), 95.2 (C_1), 72.9 (C_4), 71.0 (C_2), 70.0 (C_3), 69.0 (C_5); ^{31}P NMR (161 MHz, D_2O) δ_P 1.35 (1P, s); HRMS $[M-H]^-$ calculated for $C_6H_{11}O_{10}P$: 273.0012; found: 273.0013.

From 9: D-mannose (5.00 g, 30.0 mmol, 1.0 equiv.) and DMAP (61 mg, 0.5 mmol, 0.02 equiv.) were dissolved in anhydrous pyridine (70 mL) and cooled to 0 °C. Acetic anhydride (18.0 mL, 190 mmol, 6.8 equiv.) was added dropwise and reaction warmed to room temperature and stirred for 71 hours. After this time the solution was poured onto iced water (100 mL) and stirred vigorously for 1 h, whereupon the majority of the solvent was removed *in vacuo* and the water extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed with water (50 mL), saturated aqueous $NaHCO_3$ solution (3 x 50 mL), brine (2 x 50 mL), dried ($MgSO_4$), and concentrated *in vacuo* to give a yellow oil. This crude material was purified by silica gel column chromatography eluting with EtOAc/hexane (2/1) to afford (1,2,3,4,6)-*O*-acetyl-D-mannose as a colourless syrup (9.80 g, 25.1 mmol, 90%). This material (9.80 g, 25.1 mmol, 1.0 equiv.) and phosphoric acid (14.1 g, 144.0 mmol, 5.7 equiv.) were dissolved in anhydrous THF (20 mL) and the solvent removed *in vacuo*. The resulting syrup was stirred at room temperature under high vacuum for 1 hour (0.35 kPa). The temperature was ramped to 60 °C over a period of 30 minutes and stirred for a further 2 h under vacuum (0.35 kPa). The reaction was cooled to room temperature, THF (20 mL) was added and the solution further cooled to 0 °C. The reaction was then quenched using 25% NH_4OH solution (12 mL), the resulting precipitate filtered off and washed with ice-cold THF (10 mL). To the filtrate was added LiOH (3.08 g, 128 mmol, 5.1 equiv.) in H_2O (5 mL) and the solution stirred at room temperature overnight. The reaction was then neutralised using IR120-H^+ ion exchange resin and filtered through a Whatman® GF/A glass microfibre filter. The solvent was evaporated *in vacuo* and the residue treated with MeOH (30 mL). The resulting suspension was centrifuged at 4000 rpm for 5 mins, the supernatant removed, the pellets washed with ice cold MeOH and then dried *in vacuo* to give α-D-mannose-1-phosphate (3.74 g, 14.5 mmol, 56%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 5.23 (1H, d, *J* = 8.6 Hz, H_1), 3.82-3.87 (2H, m, H_2, H_3), 3.75-3.82 (2H, m, H_5, H_{6b}), 3.63 (1H, dd, *J* = 11.7, 6.1 Hz, H_{6a}), 3.50 (1H, apt, *J* = 9.7 Hz, H_4); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 94.1 (d, $J_{1,P}$ = 4.4 Hz, C_1), 72.0 (C_5), 70.2 (d, $J_{2,P}$ = 7.3 Hz, C_2), 69.3 (C_3), 66.2 (C_4), 60.4 (C_6); ^{31}P NMR (161 MHz, D_2O) δ_P 1.79 (d, $J_{P,1}$ = 7.8 Hz); HRMS $[M+Li]^+$ calculated for $C_6H_{13}O_9PLi$: 267.0457; found: 267.0469.

Oxidation:

Secondary Oxidant NaOCl.

α-D-mannose-1-phosphate (89 mg, 0.33 mmol, 1.0 equiv.) and TEMPO (9 mg, 0.06 mmol, 0.2 equiv.) were dissolved in a mixture of H_2O and MeCN (2 mL, 1:1, *v*/*v*) at 0 °C. To this solution, aqueous 1 M NaOH was added to pH 9. NaOCl solution (1 mL, available chlorine 10%) was then added slowly to the rapidly stirring solution. The pH was maintained at 9 by adding 1 M NaOH several times over the course of the reaction. After 2 hours, the reaction mixture was concentrated and MeOH (5 mL) was added to the resultant residue causing a precipitate to form. This suspension was centrifuged at 4000 rpm for 5 minutes, the supernatant removed, the pellet washed (once with MeOH and twice with MeCN) and dried *in vacuo* to give **8** (23 mg, 0.08 mmol, 21%) as a white amorphous solid.

Secondary Oxidant BAIB

α-D-mannose-1-phosphate (505 mg, 1.85 mmol, 1.0 equiv.), TEMPO (46 mg, 0.3 mmol, 0.15 equiv.) and bis(acetoxy)iodobenzene (1.29 g, 3.99 mmol, 2.2 equiv.) were dissolved in a mixture of H_2O/ MeCN (8 mL, 1:1, *v*/*v*). The reaction was then stirred at room temperature for 24 hours, concentrated *in vacuo* and MeOH (10 mL) added to the resultant residue, causing a precipitate to form. This suspension was centrifuged at 4000 rpm for 5 minutes, the supernatant removed, the pellet washed (once with MeOH and once with MeCN) and dried *in vacuo* to give crude **8**. This material was dissolved in H_2O and passed through a Sephadex® G-25 gel filtration column. The sugar containing fractions were pooled, treated with an NH_4^+ ion exchange resin, filtered and freeze-dried to yield **8** (182 mg, 0.67 mmol, 30%) as needle-like crystals. See above procedure for analytical data for **8**.

4.8. Guanosine-5'-phosphoromorpholidate

Method A (Khorana[20]): Dowex® 50W-X8 resin (H^+ form, 17 × 700 mm column) was exchanged to its morpholine form by passing a 10% aqueous morpholine solution through the column (200 mL). Exchange was indicated through a basic pH of the eluate (pH 10.87). Guanosine 5'-monophosphate disodium salt (Na_2GMP) (407 mg, 1.0 mmol, 1.0 equiv.) in distilled water (50 mL) was then applied to the column and eluates containing sodium morpholine-GMP were concentrated to a final volume of 10 mL. Morpholine (210 µL, 2.4 mmol, 2.4 equiv.) and *t*-butanol (10 mL) were added and the solution was heated to 100 °C at reflux, whilst a solution of dicyclohexylcarbodiimide (825 mg, 4.0 mmol, 4.0 equiv.) in *t*-butanol (15 mL) was added dropwise over 2 h. The solution was heated at reflux for a further 3 h, where TLC analysis (isopropyl alcohol/NH_4OH/water, 7/1/2) showed two new spots (R_f = 0.52 + 0.93).The yellow solution cooled to room temperature and was left for 72 h whereupon a white crystalline by-product (dicyclohexylurea) had formed. The suspension was filtered, concentrated *in vacuo* and the remaining aqueous phase extracted with diethyl ether (2 × 20 mL). The combined aqueous phases were concentrated *in vacuo* then purified by Sephadex® G-25 column chromatography, eluting with a linear gradient of triethylammonium bicarbonate (0.005 – 0.5 M). Fractions containing the product were collected and concentrated under reduced pressure. Residual bicarbonate was removed by sequential evaporations from methanol (2 × 25 mL). The residue was dissolved in methanol (10 mL), 4-morpholine- *N,N'*-dicyclohexylcarboxamidine (600 mg, 2.0 mmol, 2.0 equiv.) was added then the solution concentrated under reduced pressure. The residue was dissolved in methanol (5 mL) and diethyl ether (25 mL) was added to form a white precipitate. The liquid was decanted and the precipitate was washed with diethyl ether (2 × 10 mL), re-dissolved in water and lyophilized to afford the title compound as a cream solid (198 mg, 0.27 mmol, 27%). R_f 0.52 (isopropyl alcohol/ammonium hydroxide/water, 7/1/2); $^{31}P\{^1H\}$ (161 MHz, D_2O) $\boldsymbol{\delta_P}$ -7.41 (1P, s).

Method B (Mukaiyama[21]): Dowex® 50W-X8 resin (H^+ form, 17 × 700 mm column) was exchanged to its morpholine form by passing a 10% aqueous morpholine solution through the column (200 mL). Exchange was indicated through a basic pH of the eluate (pH 10.87). Guanosine 5'-monophosphate disodium salt (Na_2GMP) (500 mg, 1.23 mmol, 1.0 equiv.) in distilled water (50 mL) was applied to the column and eluates containing sodium morpholine-GMP were concentrated *in vacuo* to afford a cream solid. To a solution of morpholine-GMP in dimethyl sulfoxide (DMSO) (10 mL) was added morpholine (0.58 mL, 6.64 mmol, 5.4 equiv.) to form an opaque white solution. After stirring for 5 min at room temperature, dipyridyl disulfide (0.89 g, 4.06 mmol, 3.3 equiv.) was added slowly to the solution followed by triphenyl phosphine (1.06 g, 4.06 mmol, 3.3 equiv.). The resultant bright yellow solution was stirred for 4 h at room temperature and a solution of sodium iodide (0.1 M in acetone) was then added until a precipitate formed. The This was collected by filtration, dissolved in distilled water and purified by Sephadex® G-25 column chromatography, eluting with a linear gradient of triethylammonium bicarbonate (TEAB) (0.005 – 0.5 M). Fractions containing the product were collected and concentrated under reduced pressure. Residual TEAB was removed by sequential evaporations from methanol (2 × 25 mL) and the solid was lyophilized to afford the title compound as a white solid (161 mg, 0.37 mmol, 30%). R_f 0.52 (isopropyl alcohol/NH_4OH/water, 7/1/2); $[\alpha]_D^{26} = -16.0$ (*c* = 0.5, H_2O); ^1H NMR (400 MHz, D_2O) $\boldsymbol{\delta_H}$ 7.93 (1H, s, H_8), 5.79 (1H, d, *J* = 5.0 Hz, $H_{1'}$), 4.67 (1H, t, *J* = 5.1 Hz, $H_{2'}$), , 4.41 (1H, t, *J* = 4.8 Hz, $H_{3'}$), 4.20 (1H, bs, $H_{4'}$), 3.95-3.88 (2H, m, $H_{5'}$, $H_{5''}$), 3.47 (4H, t, *J* = 4.5 Hz, 2 × C*H*$_2$ morpholine), 2.85-2.82 (4H, m, 2 × C*H*$_2$ morpholine); ^{13}C NMR (101 MHz, D_2O) $\boldsymbol{\delta_C}$ 159.5 (guanine C), 154.5 (guanine C), 151.68 (guanine C), 137.25 (C_8), 116.4 (guanine C), 87.3 ($C_{1'}$), 83.7 ($C_{4'}$), 83.7 ($C_{1'}$), 73.7 ($C_{2'}$), 70.4 ($C_{3'}$), 66.9 (*C*H_2 morpholine), 64.1 ($C_{5'}$), 44.7 (*C*H_2 morpholine); $^{31}P\{^1H\}$ (161 MHz, D_2O) $\boldsymbol{\delta_P}$ -7.46 (1P, s); HRMS $[M-H]^-$ calculated for $C_4H_{20}N_6O_8P$: 431.1080; found: 431.1082.

4.9. General procedure for sugar nucleotide synthesis:
Glycosyl 1-phosphate and GMP-morpholidate were exchanged to their *bis*-triethylammonium salt forms prior to the reaction and lyophilised. Glycosyl 1-phosphate (*bis*-triethylammonium salt, 1.0 equiv.), GMP-morpholidate (*bis*-triethylammonium salt, 1.5 equiv.) and activator were each co-evaporated with toluene or pyridine (3 × 2 mL) and then dissolved in DMF or pyridine, respectively. The reaction mixture was stirred at room temperature and conversion monitored by TLC analysis (isopropyl alcohol/NH_4OH/water, 6/3/1). The reaction mixture was concentrated under reduced pressure (water bath temperature not exceeding 30 °C) and dried under high vacuum before analysis by crude ^1H and ^{31}P NMR to confirm presence of a NDP-sugar.

4.10. General procedure for sugar nucleotide deprotection:
The crude reaction mixture was suspended in a mixture of MeOH and H_2O (1:1) then Et_3N was added until pH = 9. The reaction mixture was stirred for 24 h at room temperature or until TLC analysis (isopropyl alcohol/NH_4OH/water, 6/3/1) indicated conversion of starting material to a lower R_f value spot. The reaction mixture was concentrated under reduced pressure (water bath temperature not exceeding 30 °C) to give a dark yellow residue. The resultant residue was dissolved in H_2O and in entries using DCI, passed down a Thermoscientific X30 SPE column (HyperSep C18, 6 mL), eluting with H_2O to remove DCI. The resulting aliquots were purified by strong-anion exchange chromatography.

Table 1, Entry 5: **7** (22 mg, 53 µmol, 1.0 equiv.), GMP-morpholidate (36 mg, 84 µmol, 1.5 equiv.) and DCI (6 mg, 53 µmol, 1.0 equiv.) were dissolved in pyridine (1 mL) and stirred for

120 h. Following deprotection/purification as described in *4.9* & *4.10* this afforded **1** as a white powder (15 mg, 24 µmol, 46%).

Table 1, Entry 4: **7** (44 mg, 0.10 mmol, 1.0 equiv.) and GMP-morpholidate (73 mg, 0.17 mmol, 1.6 equiv.) were dissolved in pyridine (1.5 mL) and stirred for 48 h. DCI (11 mg, 93 µmol, 1.0 equiv.) was added and the reaction mixture was stirred for a further 96 h. Following deprotection/purification as described in *4.9* & *4.10* this afforded **1** as a white powder (14 mg, 22 µmol, 22%).

*4.11. Guanosine 5'-(α-D-mannopyranuronic diphosphate) (**1**)*
R_f 0.19 (isopropyl alcohol/NH_4OH/water, 6/3/1); **^{1}H NMR** (600 MHz; D_2O) δ 7.96 (1 H, s, $H_{8''}$), 5.79 (1 H, d, *J* = 6.1 Hz, $H_{1'}$), 5.39 (1 H, dd, *J* = 8.0, 1.7 Hz, H_1), 4.64 (1 H, hidden, $H_{2'}$), 4.35 (1 H, dd, *J* = 4.8, 3.3 Hz, $H_{3'}$), 4.34-4.29 (1 H, m, $H_{4'}$), 4.18 (2 H, dd, *J* = 5.2, 3.5 Hz, $H_{5'}$), 3.95 (1 H, d, *J* =10.0 Hz, H_5), 3.88 (1 H, dd, *J* = 2.2, 3.3 Hz, H_2), 3.78 (1 H, dd, *J* = 9.6, 3.3 Hz, H_3), 3.64 (1 H, t, *J* = 9.7 Hz, H_4); δ_P (101 MHz D_2O) δ -11.2, -13.7; HRMS [M-H]$^-$ calculated for $C_{16}H_{22}N_5O_{17}P_2$: 618.0491; found: 618.0484.

*4.12. Guanosine 5'-(α-D-mannopyranuronic diphosphate) (**1**)*
GDP-α-D-mannose (1.6 mg, 2.5 µmol) and NAD^+ (3.5 mg, 5.25 µmol) were dissolved in buffer (0.9 ml, 200 mM sodium phosphate, pH 7.4, 1 mM DTT, 0.5 mM $MgCl_2$) and GMD (0.8 mg/ml final concentration) was added to give total volume 1 ml. The mixture was incubated at room temperature with gentle shaking whilst being monitored by SAX on a Poros HQ 50 column. Samples (10 µl) were taken at time points, mixed with methanol (10 µl), vortexed and centrifuged to remove precipitated protein. The supernatant (10 µl) was analysed by SAX. After 21 hours the conversion of GDP-α-D-mannose into **1** reached 70%. The enzymatic transformation was stopped by addition of methanol (1 ml). The mixture was vortexed for 1 minute and centrifuged. The supernatant was filtered through a syringe disc filter (0.45 µm, PTFE) and the resulting crude product was purified by SAX. Fractions containing **1** were pooled and freeze dried to give the title compound as a bisammonium salt (1.2 mg, 2.0 µmol, 70%). See analytical data above for **1**.

Acknowledgements

Keele University are thanked for a PhD studentship to L.B. Work at the John Innes Centre is supported by the UK BBSRC Institute Strategic Program on Molecules from Nature - Products and Pathways [BBS/E/J/000PR9790] and the John Innes Foundation; and the InnovateUK IBCatalyst [BB/M02903411 and EP/N033167/10].We also thank the EPSRC UK National Mass Spectrometry Facility (NMSF) at Swansea University.

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