Chemo-enzymic Synthesis of Guanosine 5'-Diphosphomannose (GDP-Mannose) and Selected Analogues

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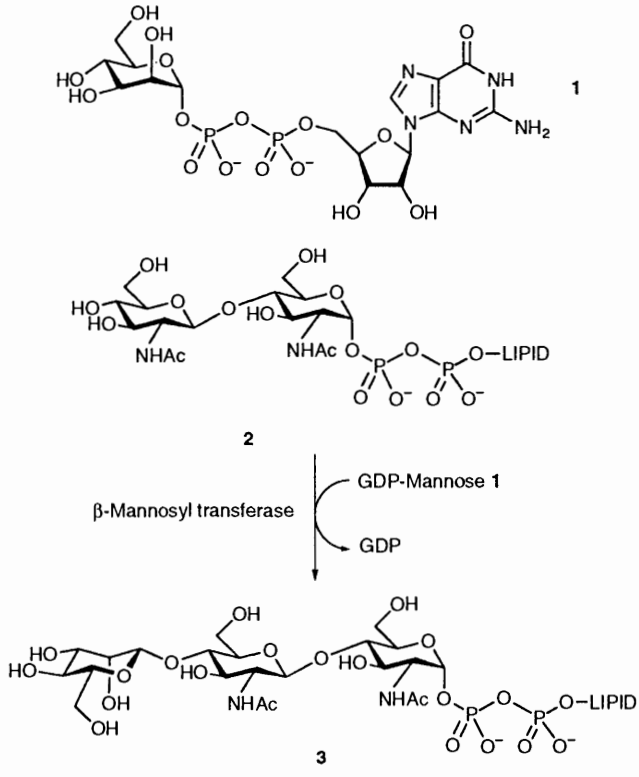
Guanosine 5'-diphosphomannose (GDP-mannose, **1**) has been prepared *via* two routes from α-D-mannose 1-phosphate **5**. The non-enzymic route involves coupling of the phosphate **5** to guanosine 5'-monophosphate dibutyl phosphinothioc anhydride (GMP-anhydride 11) to give compound **1** in 40% yield. The enzymic routes require coupling of compound **5** with guanosine 5'-triphosphate (GTP) catalysed by GDP-mannose pyrophosphorylase (37% yield).

Guanosine 5'-diphosphomannose (GDP-mannose **1**) is one of a group of nucleotide-activated carbohydrates that act as co-factors in the biosynthesis of oligosaccharides. These nucleotide-activated sugars are substrates for glycosyl transferases that catalyse the addition of individual carbohydrates to the non-reducing end of an oligosaccharide chain.[1] This process can be illustrated by the reaction shown in Scheme 1. D-Mannose is transferred stereospecifically from GDP-mannose **1** to the lipid-linked disaccharide **2**, catalysed by a β-mannosyl transferase to yield the trisaccharide **3** containing a β-D-mannosyl residue. Analogous reactions lead to the addition of other glycosyl residues by combination of the appropriate nucleotide sugar (*e.g.*, UDP-glucose) with the corresponding glycosyl transferase (*e.g.*, glucosyl transferase). In this way a diverse range of complex oligosaccharides can be synthesized.[2]

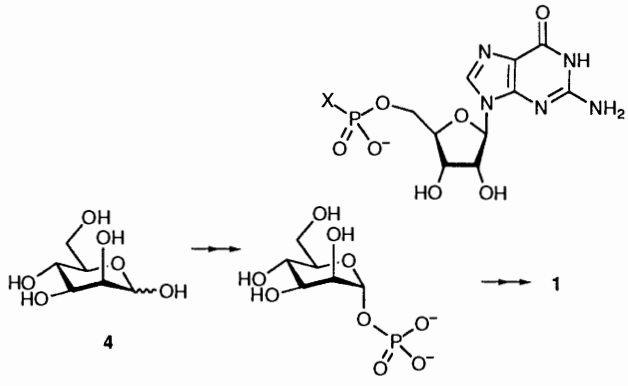


Scheme 1

Recently there has been much effort directed towards exploiting glycosyl transferases for the synthesis of oligosaccharides on a preparative scale. Important advances have been made in the availability of many glycosyl transferases (*e.g.*, sialyl transferases, fucosyl transferases) by the over-expression of the corresponding genes.[3] Equally important has been the need to develop both efficient syntheses and methods for *in situ* recycling of the co-factors.[4] As part of a programme aimed at exploring the synthetic utility and substrate specificity of mannosyl transferases[5-7] we required an efficient synthesis of GDP-mannose **1** and close structural analogues. Our aim was to explore both enzymic and non-enzymic routes to GDP-mannose **1**. Recent work by Whitesides and co-workers has been aimed at developing convenient syntheses of several nucleotide-linked sugars[8] (including GDP-mannose **1**) focussing particularly on enzymic processes. Herein we report methods that complement those reported by Whitesides and which allow the possibility of preparing not only GDP-mannose **1** itself but also its structural analogues.

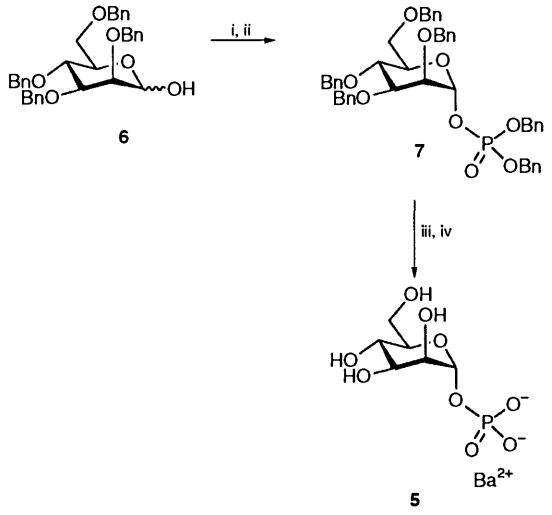
Results and Discussion

The synthesis of GDP-mannose **1** from D-mannose **4** can be broken down into two parts (Scheme 2) (i) the synthesis of



Scheme 2

α-D-mannose 1-phosphate (Man-1-P **5**) and (ii) coupling of Man-1-P **5** to a suitably activated guanosine 5'-monophosphate derivative. Initially we were able to prepare Man-1-P **5** in good overall yield from D-mannose based on procedures developed by Schmidt (Scheme 3). The fully benzyl-protected mannose phosphate **7** (prepared from tetrabenzylmannose **6**) had been previously reported by Schmidt[9] although conditions were not given for its deprotection to compound **5**. Attempts to apply the suggested debenzylation protocol (H_2/Pd; EtOAc–THF) to compound **7** led to only a modest yield of Man-1-P **5** (41%), the major side-product being mannose. The yield of compound **5** was improved considerably by the use of Na–liq. NH_3 which gave a product which could be isolated as its amorphous

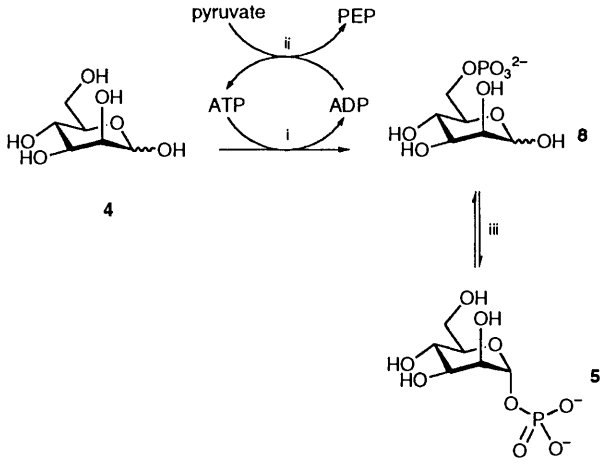


Scheme 3 *Reagents and conditions:* i, NaH, CCl_3CN, room temp., 4.5 h (81%); ii, $HO(O)P(OBn)_2$, room temp., 1 h (99%); iii, Na–liq. NH_3, −33 °C; iv, $BaCl_2 \cdot 6H_2O$ (60%)

barium salt by treatment with an aqueous solution of barium chloride followed by ethanol (60% yield). The barium salt is suitable for long-term storage and could be converted to other salts when required. The overall yield of Man-1-P **5** from tetrabenzylmannose **6** was 48%.

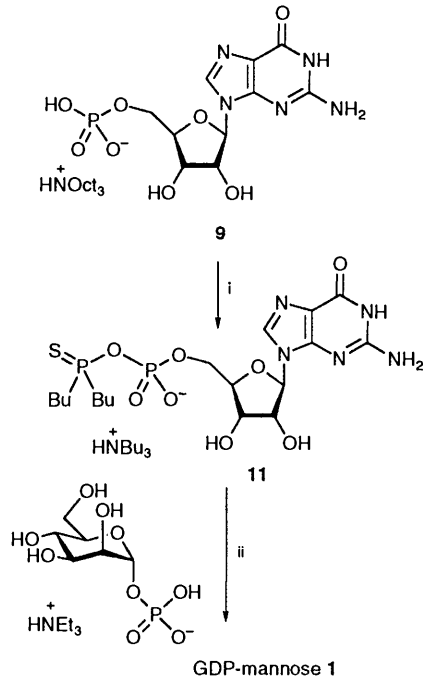
By way of comparison we next investigated the enzymic synthesis of Man-1-P from mannose (Scheme 4). D-Mannose **4** was easily converted into Man-6-P **8** by using hexokinase with *in situ* recycling of ATP *via* the phosphoenolpyruvate (PEP)/pyruvate kinase system.[10,11] The phosphate **8** was isolated as its barium salt in 83% yield. Unfortunately the subsequent conversion of Man-6-P **8** into Man-1-P **5** proved far more difficult to achieve. The first approach involved the use of phosphoglucomutase (commercially available) which naturally converts glucose-6-phosphate into glucose-1-phosphate. With Man-6-P the reaction was extremely slow even in the presence of large quantities of enzyme.* A second option was to employ the enzyme phosphomannomutase which is biosynthetically responsible for the conversion of Man-6-P **8** into Man-1-P **5**. This enzyme is not commercially available but has been shown to be present in a number of sources including yeast (*Saccharomyces cerevisiae*). However attempts to obtain partially purified samples of phosphomannomutase from *S. cerevisiae* following literature protocols[13] were unsuccessful and hence this approach was abandoned.

We now turned our attention to the second stage of the synthesis of GDP-mannose **1**, namely the coupling of Man-1-P **5** with a suitably activated guanosine 5′-monophosphate derivative. Several methods have been reported for the non-enzymic coupling of glycosyl 1-phosphates to give sugar nucleotides. In these methods the nucleoside phosphate is activated as its morpholidate,[14] 2-hydroxypyridyl ester,[15] mixed phosphate anhydride,[16] or phosphinothioic anhydride.[17] All of these coupling methods, with the exception of the phosphinothioic anhydride method, are very sensitive to traces of moisture and hence extremely rigorous drying protocols must be employed. Attempts to repeat Khorana's morpholidate methodology[14] for the synthesis of GDP-mannose **1** met with mixed results. Although preparation of GMP-morpholidate was straightforward and high yielding, subsequent coupling to the bis(trioctylammonium) salt of Man-1-P **5** gave GDP-mannose **1** in only 10% yield after 4 days. Changing the solvent from pyridine to DMF: pyridine (1 : 1) as reported by Heinz *et al.*[18] had little effect on the yield.



Scheme 4 *Enzymes:* i, hexokinase; ii, pyruvate kinase; iii, phosphomannomutase

It was therefore decided to investigate the phosphinothioic anhydride methodology of Furusawa *et al.*,[17] which had not previously been applied to the synthesis of GDP-mannose **1** (Scheme 5). The mono(trioctylammonium) salt of GMP **9**



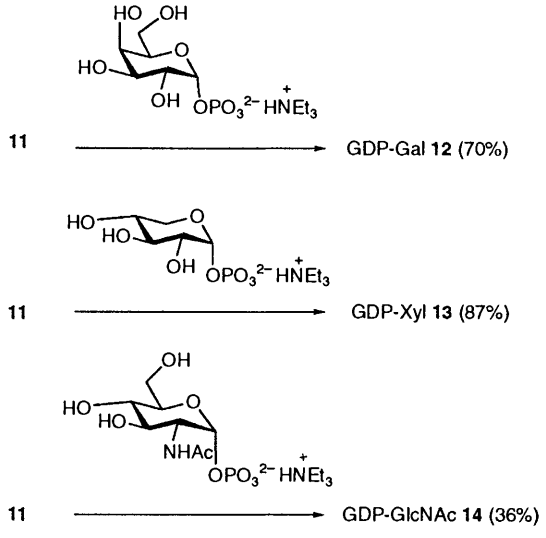
Scheme 5 *Reagents:* i, $Bu_2P(S)Br$ **10**, NBu_3, THF (61%); ii, AgOAc, pyridine (40%)

(1 mol. equiv.) was treated with dibutylthiophosphoryl bromide **10** (2.4 mol equiv.) in the presence of tributylamine (4 mol equiv.) to yield GMP-anhydride **11** in low yield. Modification of this protocol by reducing the amount of thiophosphoryl bromide **10** (1.1 mol equiv.) and changing the solvent from pyridine to tetrahydrofuran (THF) yielded compound **11** as its tributylammonium salt in 61% yield as fine needle-like crystals after recrystallisation from hot methanol. Thereafter GMP anhydride **11** (2 mol equiv.) was coupled with the mono-(triethylammonium) salt of Man-1-P **5** in the presence of silver acetate (4 mol equiv.) to give compound **1**.† To facilitate the

* The rate of isomerisation of Man-6-P to Man-1-P is reported to be 50 000-times slower than that of Glc-6-P to Glc-1-P.[12]

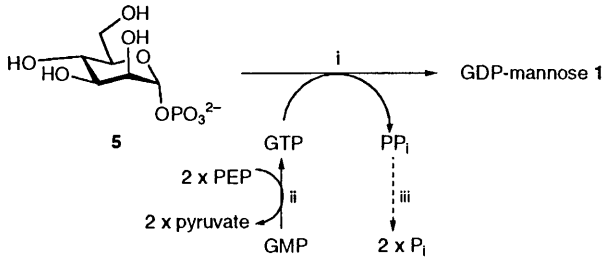
† The use of the mono-triethylammonium salt has been reported to be more effective than the mono-tributylammonium salt.[19]

isolation of compound **1**, the crude reaction mixture was dissolved in water and was then treated with alkaline phosphatase at pH 7.5. This procedure greatly simplified the subsequent purification by dephosphorylating any terminal phosphates (*e.g.*, Man-1-P, GMP) that were present in the reaction mixture. The enzyme was precipitated by heating the mixture at 100 °C for 45 s followed by centrifugation. The resulting solution containing compound **1** was purified by ion-exchange chromatography on AG1X-8-200 (HCO_3^- form) with stepwise elution with increasing concentrations of aq. triethylammonium hydrogen carbonate. Subsequent ion exchange yielded compound **1** as its disodium salt in 40% yield representing an efficient overall conversion from Man-1-P **5**. The above procedure was then applied to the synthesis of other GDP-sugars, *i.e.* GDP-galactose **12** (70%), GDP-xylose **13** (87%), and GDP-*N*-acetylglucosamine **14** (36%) (Scheme 6). Compounds **12–14** were each purified directly by reversed-phase HPLC, thereby obviating the need for ion-exchange chromatography.*



Scheme 6

Finally we investigated the possibility of preparing GDP-Man **1** *via* a direct enzymic conversion from Man-1-P **5** (Scheme 7). In this approach Man-1-P **5** is coupled with GTP



Scheme 7 *Reagents and conditions:* i, GDP-mannose pyrophosphorylase, Mg^{2+}, pH 7.5; ii, guanylate kinase, pyruvate kinase; iii, pyrophosphatase

to give GDP-Man **1** and pyrophosphate. The reaction is catlysed by the enzyme GDP-mannose pyrophorylase E.C. 2.7.7.13) which is well documented and has been previously used by others to prepare compound **1**.[8,20–22] Additionally, in this synthesis, the GTP is prepared enzymically from the more readily available GMP *via* a two-step process involving initial conversion of GMP into GDP, followed by further phosphorylation to GTP. Initially we envisaged using adenylate kinase for the conversion of GMP into GDP, but this enzyme was unable to accept GMP as an analogue of AMP. It was therefore necessary to resort to the more expensive guanylate kinase/ATP combination which, in combination with ATP/pyruvate kinase, resulted in an 88% conversion of GMP into GTP. As before, ATP was used in catalytic amounts and could be recycled *in situ* by the PEP/pyruvate kinase-linked system. A similar method for preparing GTP from GMP has been reported by two other groups.[8,23]

Since GDP-mannose pyrophorylase is not commercially available it was necessary to isolate it in partially purified form from natural sources. An initial screen was set up using the direct condensation of Man-1-P with GTP to form GDP-Man **1** as an assay. The formation of GDP-Man could be easily followed by TLC. Several yeast extracts were prepared by autolysis of the cells in phosphate buffer overnight followed by centrifugation to remove particulate matter, leaving a clear supernatant. This initial screen for GDP-mannose pyrophosphorylase activity revealed the commercially available brewers' yeast from the Boot's Company to be the best source. This yeast was then used to compare three methods of autolysis of the cells in order to obtain maximum release of active GDP-mannose pyrophosphorylase. Thus the yeast was either stirred in phosphate buffer, milled with glass beads, or sonicated. Both cell milling and sonication gave good activity and therefore sonication was eventually selected, being simple to perform and easy to scale up. The supernatant from the sonication of the yeast cells was then used for a preparative-scale experiment, resulting in a 37% yield of GDP-Man **1** after purification by ion-exchange chromatography.

To investigate the substrate specificity GDP-mannose pyrophosphorylase, Man-1-P was replaced by glucose-1-P and xylose-1-P. However, incubation with GTP using the method described above failed to produce the corresponding GDP-sugars.

Conclusions.—Two routes to GDP-mannose **1** have been investigated, namely an enzymic route from D-mannose (Schemes 4 and 7) and a non-enzymic route (Schemes 3 and 5). The non-enzymic route furnished target compound **1** in good overall yield and was found to be applicable to the synthesis of other GDP-sugars by simple variation of the sugar 1-phosphate. The enzymic route, on the other hand, despite being shorter, suffers from the fact that two of the crucial enzymes involved (phosphomannomutase and GDP-mannose pyrophosphorylase) must be isolated from natural sources. Of these two, phosphomannomutase is relatively poorly described in the literature and to our knowledge has not been used for preparative biotransformations before. The enzyme-catalysed preparation of GTP from GMP is straightforward.

Experimental

General Experimental Details.—Et_3N and pyridine were distilled from KOH, and stored over 4 Å sieves under nitrogen. Tributylamine and trioctylamine were dried over activated 4 Å sieves, distilled under reduced pressure, and stored over freshly activated sieves. All liquid amines were stored in amber bottles. Aq. triethylammonium hydrogen carbonate (TEAB) (1.0 mol dm^{-3}) was prepared by suspending freshly distilled Et_3N (101.19 g, 139.4 cm^3, 1.0 mol) in double-deionised water (750 cm^3) and bubbling CO_2 gas through the vigorously stirred biphasic mixture at 0–4 °C *via* a sparger, until a clear solution had formed with pH ~ 7.50. The solution was made up to 1000 cm^3 with more water and stored in an amber bottle at 0–4 °C.

Enzymes were purchased from Sigma Chemicals Ltd., Poole,

* Attempts to obtain either satisfactory microanalyses or high resolution mass spectra for compounds **12**, **13** and **14** proved unsuccessful. However, all other spectroscopic data were in accord with their structures.

Dorset, and from Boehringer Mannheim UK, Lewes, East Sussex. Ion-exchange resins and columns were obtained from commercial suppliers, and were converted into different ionic forms as described by Bio-Rad Laboratories Ltd. Water for all enzymic procedures was double-deionised using the Milli-Q®-(Millipore) system. All enzymes were assayed prior to use, and all enzymic assays were performed as described by Bergmeyer.[24] Yeasts and reagents for yeast culture were obtained from commercial sources.

TLC was performed on Merck 60F-254 (0.25 mm, Art. 5715) glass-backed silica gel plates. Visualisation was achieved by UV fluorescence, and by heating after treatment with an acidic solution of ethanolic anisaldehyde or with an acidic solution of naphthoresorcinol (naphthalene-1,3-diol) and diphenylamine in EtOH. Flash chromatography was performed on silica gel 60H (Merck 7385). Ion-exchange chromatography was performed using Dowex 50W-X8 (H^+ form), which was easily converted into the pyridinium and sodium forms; Dowex 1X8–200 (Cl^- form) or Bio-Rad AG® 1-X8 100–200 (AcO^- form) were converted into (OH^-), (HCO_2^-), and (HCO_3^-) forms in a similar fashion.

Measurements of pH were made using an Alpha 500 Laboratory pH/mV meter. Centrifugation was performed using a Hi-Spin 21 centrifuge (MSE, Fisons). Sonication was achieved using a Soniprep® sonicator. Optical-rotation measurements were made on a Thorn NPL 243 automatic polarimeter, with $[\alpha]_D$-values given in units of 10^{-1} deg cm^2 g^{-1}. M.p.s. were obtained using an Electrothermal instrument and are uncorrected. HPLC (Gilson) and FPLC® (Pharmacia) were performed on appropriate sets of equipment, as described in the text. UV-visible spectroscopy was performed using an LKB Ultrospec II 4050 UV-visible spectrophotometer. IR spectra were recorded as wavenumbers relative to a polystyrene standard, using a Perkin-Elmer 398 IR spectrophotometer. Mass spectra were initially performed on a VG MM-16F mass spectrometer; later spectra (including accurate mass determinations) were recorded at the SERC mass spectrometry centre, Swansea, using a VG ZAB-E high-resolution instrument.

60 MHz ^{1}H NMR spectra were recorded on an R-24B Hitachi Perkin-Elmer high-resolution instrument. 100 MHz ^{1}H and 25 MHz ^{13}C NMR spectra were obtained using a JEOL FX-100 spectrophotometer. 250 MHz ^{1}H, 101 MHz ^{31}P, and 62.9 MHz ^{13}C NMR spectra were recorded on a Brucker AM-250 spectrometer. *J* Values are given in Hz.

α-D-*Mannose 1-Phosphate* (*Man-1-P*) **5**.—The protected phosphate **7**[9] (950 mg, 1.19 mmol) was dissolved in dry THF (30 cm^3) at −78 °C and ammonia (30 cm^3) was condensed into the solution. Sodium (~250 mg, ~11 mmol) was added as freshly cut slivers (20 mg) over a period of 2.25 h, and extra ammonia was condensed into the mixture as required, to maintain the correct volume. When the dark blue colour persisted, dry freshly powdered NH_4Cl (150 mg) was added in 3 × 50 mg portions, with stirring of the mixture between additions, until no blue colour remained. The cold finger was removed and solvents were driven off under a stream of nitrogen. The gummy residue was dissolved in water (15 cm^3) and $BaCl_2 \cdot 2H_2O$ (340 mg, 1.44 mmol) was added to the stirred mixture. Absolute EtOH (15 cm^3) was added, and the mixture was kept at 0–4 °C overnight. The precipitate was collected by filtration, and the mother liquor was reserved. The precipitate was washed successively with ice-cold portions of water (25 cm^3), water–ethanol (1:1) (25 cm^3) and absolute EtOH (25 cm^3), then was freeze dried overnight to give the barium salt of Man-1-P **5** as an off-white, amorphous solid (373 mg, 60%) (assuming the heptahydrate). A portion of the barium salt (250 mg) was exchanged to the disodium form by using Dowex 50W-X8 (Na^+ form) to give the disodium salt of compound **5** as an off-white solid (141 mg, 52% from the protected phosphate). R_f [acetonitrile–0.1 mol dm^{-3} NH_4Cl (7:3)] 0.12; δ_H (250 MHz; D_2O) 3.65–3.90 (m) and 3.90–4.10 (m) (6 H, non-anomeric 4 × CH, CH_2) and 5.50 (1 H, dd, $J_{H,H}$ 1.5, $J_{H,P}$ 8.6, 1-H).

A second crop of the barium salt was recovered by addition of more EtOH (15 cm^3) to the mother liquor, then was washed and collected as before. Yield of barium salt (122 mg, 19.6%). On conversion into the disodium form, the second crop was shown to be only slightly less pure than the first batch. Total yield of phosphate **5**, assuming the barium heptahydrate salt, was 495 mg (79%).

Guanosine 5′-Monophosphate Dibutylphosphinothioic Anhydride (*GMP-anhydride*) **11**.—GMP-anhydride **11** was prepared by a modification of the method reported by Furusawa *et al.*[17] GMP·H_2O **9** (96 mg, 0.25 mmol) was suspended in MeOH (5 cm^3) with dry distilled trioctylamine (89 mg, 110 mm^3, 0.25 mmol) and the suspension was stirred to give a colourless solution, which was concentrated to a glassy gum by rotary evaporation and was then rendered anhydrous by repeated evaporation with dry distilled pyridine (4 × 2 cm^3). Dry distilled THF (10 cm^3), thiophosphoryl bromide **10** (71 mg, 57 mm^3, 0.275 mmol), and dry distilled tributylamine (189 mg, 239 mm^3, 1 mmol) were added under nitrogen and the reaction mixture was stirred and gently warmed to give a clear, pale yellow solution, which was stirred for 21 h at 25–30 °C. The solvent was removed by rotary evaporation and the sticky brown residue was washed briefly with water (2 cm^3) which was decanted and reserved. The residue was triturated with Et_2O (10 cm^3), which precipitated the crude anhydride **11** as an off-white amorphous solid. The anhydride was collected by vacuum filtration, washed with Et_2O (2 × 2 cm^3), and dried under high vacuum. The aqueous washings were added to the crude solid and the mixture was recrystallised using the minimum amount of MeOH, to give the tributylammonium salt of phosphate **11** as fine needles (111 mg, 61%), m.p. 155.5–156.5 °C (lit.,[17] 154–155 °C); R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (7:3)] 0.68; ν_{max}(Nujol mull)/cm^{-1} 720–740 (P=S) 940 and 960 (POC), 1000 (POP), 1100, 1080, 1610, 1700, 2680, 3120 and 3300; δ_H(250 MHz; CD_3OD) 0.91 (6 H, overlapping t, $J_{H,H}$ 3.0, 2 × Me of diastereotopic Bu_2P=S), 1.00 [9 H, t, $J_{H,H}$ 7.3, 3 × Me of $HN(Bu)_3^+$], 1.40–1.70 (20 H, 2 m, 10 × CCH_2C), 2.15 (4 H, m, 2 × PCH_2), 3.10 (6 H, m, 3 × NCH_2), 4.30 (3 H, m), 4.42 (1 H, m) and 4.80 (1 H, t) (non-anomeric ribosyl 3 × CH, CH_2), 5.85 (1 H, d, $J_{H,H}$ 6.6, 1′-H) and 7.75 (1 H, s, 8-H); δ_C(62.9 MHz; CD_3OD) 13.9 and 14.0 (Me), 20.9 [CH_2Me of $HN(Bu)_3^+$], 24.6 (d, $J_{P,C}$ 68.5, CH_2Me of Bu_2P=S), 26.0 (d, $J_{P,C}$ 3.1, PCH_2CH_2), 26.7 (NCH_2CH_2), 35.6 (d, $J_{C,P}$ 3.1, PCH_2), 53.7 (NCH_2), 67.1 (d, $J_{P,C}$ 6.0, C-5′), 72.4 (C-2′), 75.0 (C-3′), 85.4 (d, $J_{P,C}$ 6.0, C-4′), 89.4 (C-1′), 118.0 (C-5), 136.5 (C-8), 153.3 (C-4), 155.3 (C-2) and 158.4 (C-6); δ_P(101 MHz; CD_3OD) −9.1 (d, $J_{P,P}$ 35, PO_4) and +99.6 (d, $J_{P,P}$ 35, Bu_2P=S); *m/z* (FAB) 725 $(M + H)^+$, 540 $[M + H - N(Bu)_3]^+$ and 186 $[HN(Bu)_3]^+$ [Found: $(M + H)^+$, 725.3450. $C_{30}H_{59}N_6O_8P_2S$ requires *m/z*, 725.3590].

GDP-Mannose **1**.—The mono-triethylammonium salt of Man-1-P **5** (9 mg, 25 mmol) and the tributylammonium salt of GMP-anhydride **11** (36 mg, 50 μmol) were combined and repeatedly concentrated to dryness with dry distilled pyridine (4 × 1 cm^3) to remove moisture. The residue was taken up in dry pyridine (1 cm^3) to give a yellow solution, and AgOAc (17 mg, 0.10 mmol) was added. The flask was covered in foil and the mixture was stirred under nitrogen for 4 h. Water (3 cm^3) was added to the stirred mixture to give a whitish emulsion, then hydrogen sulfide was bubbled through the mixture, with the formation of a heavy black precipitate of silver sulfide, which was removed by vacuum filtration through Celite. The solvent

was taken off by rotary evaporation and the remaining aqueous solution was freeze dried. Water (5 cm^3) was added to the residue and the mixture was stirred with alkaline phosphatase (E.C. 3.1.3.1) (10 U, 10 cm^3) at pH 7.50 (20–25 °C) for 2 h, then the enzyme was precipitated by heating of the mixture at 100 °C for 45 s, and was removed by centrifugation. The entire product was purified by ion-exchange chromatography using AG 1X8-200 (HCO_3^- form) (1.5 cm^3). After application of the crude sugar nucleotide **1**, the resin was washed with deionised water (10 cm^3), and the column was eluted with 50 mmol dm^{-3} (30 cm^3), 150 mmol dm^{-3} (40 cm^3), and 250 mmol dm^{-3} (150 cm^3) aq. TEAB. Fractions eluted with 250 mmol dm^{-3} aq. TEAB contained GDP-Man **1**, and these were combined, and concentrated to dryness by rotary evaporation under reduced pressure with *very* gentle warming. The residue was repeatedly redissolved and evaporated with water (2 × 50 cm^3) then with MeOH (3 × 10 cm^3) to remove excess of TEAB. The resulting solid was taken up in water (5 cm^3) and the mixture stirred with Dowex-50 (Na^+ form), then was filtered, and the filtrate and washings were freeze dried to yield the disodium salt of GDP-mannose **1** (13 mg, 40%). A small sample of product **1** (2.5 mg) was purified to homogeneity by reversed phase HPLC; [Gilson HPLC system with two pumps: λ_{abs} 254 nm; absorbance range 1.0; flow rate 2.0 cm^3 min^{-1}. Philips 8251 chart recorder: range 10 mV; chart speed 10 mm min^{-1}. Column: Spherisorb 5μ ODS-2, 25 × 0.80 cm semi-preparative column. Mobile phase: 50 mmol dm^{-3} TEAB (adjusted to pH 6.0 with glacial acetic acid *after* filtration)–acetonitrile (93:7)]. The sugar nucleotide **1** was taken up in HPLC-grade water (2 cm^3) and the mixture was filtered through a 0.2 m filter. The solution was applied to the column in 150–200 mm^3 aliquots, t_R 6.25 min (GDP-man **1**). Fractions containing GDP-man **1** were pooled, and concentrated under reduced pressure. The residue was treated with Dowex-50 (Na^+ form) to give the disodium salt of phosphate **1** as a whitish, hygroscopic, semi-crystalline solid, R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (6:4)] 0.58; δ_H(250 MHz; D_2O) 3.78–4.08 (4 H, m, 4″- and 5″-H and 6″-H_2), 4.07 (1 H, m, $J_{H2'',H3''}$ 3.3, $J_{H3'',H4''}$ 9.7, 3″-H), 4.19 (1 H, $J_{H1'',H2''}$ 1.6, $J_{H2'',H3''}$ 3.3, 2″-H), 4.35 (2 H, m, 5′-H_2), 4.49 (1 H, br m, 4′-H), 4.66 (1 H, t, 3′-H), 4.90 (hidden, 2′-H), 5.65 (1 H, dd, $J_{H1'',H2''}$ 1.6, $J_{H1'',P}$ 7.4, 1″-H), 6.08 (1 H, 2, $J_{H1',H2'}$ 6.1, 1′-H) and 8.25 (1 H, s, 8-H).

*GDP-α-*D*-Galactose* (*GDP-Gal*) **12**.—GDP-Gal **12** was prepared, according to the method described for GDP-Man **1** from the mono-triethylammonium salt of galactose 1-phosphate (9.8 mg, 25.8 μmol) prepared from its $2K^+$, 5.5 H_2O salt (10.9 mg, 25 μmol) to give the disodium salt of compound **12** (crude yield 14 mg); δ_H(250 MHz; D_2O) 3.82–3.89 (2 H, m, 6″-H_2), 3.89–3.99 (1 H, m, 2″-H), 3.99–4.08 (1 H, dd, 3″-H), 4.10–5.15 (1 H, br, d, 4″-H), 4.24–4.33 (1 H, 2 overlapping d, 5″-H), 4.33–4.41 (2 H, m, 5′-H_2), 4.45–4.55 (1 H, m, 4′-H), 4.61–4.69 (1 H, 2 overlapping d, 3′-H) (2′-H hidden under HOD peak), 5.77 (1 H, dd, $J_{H1'',H2''}$ 3.4, $J_{H1'',P}$ 7.3, 1″-H), 6.10 (1 H, d, $J_{H1',H2'}$ 5.5, 1′-H) and 8.55 (1 H, s, 8-H); δ_P(101 MHz; D_2O) −11.0 (2 d, $J_{P,H}$ 17, PO_4).

A portion of the part-purified material (6.8 mg) was subjected to HPLC {t_R [50 mmol dm^{-3} aq. TEAB, pH 6.0–acetonitrile (97:3)] 6.2 min} to give the bis-triethylammonium salt of GDP-gal **12** as a powdery solid (7.4 mg, 70%), R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (6:4) 0.64].

*GDP-α-*D*-Xylose* (*GDP-Xyl*) **13**.—GDP-Xyl **13** was prepared from the mono-triethylammonium salt of xylose 1-phosphate (9.0 mg, 25.8 μmol) prepared from its bis-cyclohexylammonium salt (10.5 mg, ~25 μmol) to give the disodium salt of compound **13** (crude yield 32 mg); δ_H(250 Mz; D_2O) 3.60–3.95 (m), 4.30–4.45 (m), 4.45–4.55 (m), and 4.70–4.80 (m) (9 H, 3′-, 4′-, 2″-, 3″- and 4″-H and 5′- and 5″-H_2) (2′-H hidden under HOD peak), 5.68 (1 H, dd, $J_{H1'',H2''}$ 3.5, $J_{H1'',P}$ 7.0, 1″-H), 6.09 (1 H, d, $J_{H1',H2'}$ 7.4, 1′-H) and 8.40 (1 H, s, 8-H).

A portion of this material (9.2 mg) was subjected to HPLC {t_R [50 mmol dm^{-3} TEAB, pH 6.0–acetonitrile (6:4)] 6.4 min} and purified to give the bis-triethylammonium salt of GDP-Xyl **13** as a powdery solid (5.0 mg, 87% overall); R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (6:4)] 0.60.

*GDP-α-*N*-Acetylglucosamine* (*GDP-GlcNAc*) **14**.—GDP-GlcNAc **14** was prepared from the mono-triethylammonium salt of *N*-acetylglucosamine 1-phosphate (10.7 mg, 24.4 μmol), which was prepared from its $2Na^+$, $0.5H_2O$ salt (8.9 mg, 25 μmol), to give the disodium salt of compound **14** (crude yield 22 mg); δ_H(250 MHz; D_2O) 3.60–3.80 and 3.85–4.06 (5 H, 2 m, 3″-, 4″- and 5″-H and 6″-H_2), 4.16–4.15 (1 H, m, $J_{H1'',H2''}$ 3.2, $J_{H2'',H3''}$ 10.7, 2″-H), 4.31–4.39 (2 H, m, 5′-H_2), 4.45–4.52 (1 H, m, 4′-H), 4.61–4.67 (1 H, 2 overlapping d, 3′-H), ~4.85 (1 H, hidden under HOD signal, 2′-H), 5.64 (1 H, dd, $J_{H1'',H2''}$ 3.2, $J_{H1'',P}$ 7.3, 1″-H), 6.08 (1 H, $J_{H1',H2'}$ 5.5 1′-H) and 8.40 (1 H, s, 8-H). Purification by HPLC {t_R [50 mmol dm^{-3} aq. TEAB, pH 6.0–acetonitrile (97:3)] 7.34 min} gave the bis-triethylammonium salt of GDP-GlcNAc **14** as a powdery solid (7.2 mg, 36%) R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (6:4)] 0.53.

Generation of GTP from GMP.—GMP **9** (disodium salt) (25 mg, 55 μmol), phospoenol pyruvate monopotassium salt (PEP^- K^+) (22 mg, 108 μmol), catalytic ATP (0.6 mg, 1 μmol), and $MgCl_2 \cdot 6H_2O$ (25.5 mg, 125 μmol) were dissolved in 0.1% aq. NaN_3 (12.5 cm^3) and the pH was adjusted to 7.50. Guanylate kinase (0.1 U, 15 cm^3) and pyruvate kinase (1.0 U, 10 cm^3 of a solution of 10 cm^3 pyruvate kinase diluted to 333 cm^3 with 0.1% aq. NaN_3) were added and the solution was adjusted to pH 7.50 and stirred under nitrogen at room temperature for 24 h, after which the reaction mixture was freeze dried. Yield by ^{31}P NMR spectrometry was 88%; δ_P(101 MHz; D_2O) −18.1 (2 overlapping d, $J_{P,P}$ 15, P–O–*P*–O–P), −9.9 (d, *J* 17, POC), −9.1 (d, *J* 21, POC of trace GDP), −5.0 (d, *J* 21, *P*–O–POC of trace GDP), −4.5 (d, *J* 16, *P*–OPOP) and +0.2 (trace PEP^- K^+).

Enzymic Synthesis of D*-Mannose 6-Phosphate* (*Man-6-P*) **8**.—D-Mannose **4** (1.44 g, 8.0 mmol), PEP^- K^+ (1.66 g, 8.0 mmol), $MgCl_2 \cdot 6H_2O$ (71 mg, 350 μmol) and 2-mercaptoethanol (8 mg, 100 μmol) were dissolved in deionised water (12 cm^3) and the solution was purged with nitrogen. The solution was adjusted to pH 7.5–7.6 with conc. aq. KOH (10 mol dm^{-3}). ATP (8 mg, 12 μmol) was added followed by pyruvate kinase (20 U) and hexokinase (41.5 U) [commercial suspensions in 3.2 mol dm^{-3} aq. ammonium sulfate, prediluted with water (4 cm^3)]. The pH was adjusted to 7.5–7.6 and the reaction mixture was stirred under nitrogen for 24 h at room temperature, after which time more pyruvate kinase (10 U) and hexokinase (41.5 U) were added. The pH was adjusted as before and the mixture was stirred for a further 6 days, with further adjustment of pH as required. After 7 days, TLC indicated considerable conversion of mannose **4** into its 6-phosphate **8**, {R_f [acetonitrile–0.1 mol dm^{-3} aq. NH_4Cl (7:3)] 0.12 (Man-6-P) and 0.40 (mannose)}. The degree of conversion was calculated to be 83% by ^1H NMR spectrometry. $BaCl_2 \cdot 2H_2O$ (2.04 g, 8.0 mmol, based on 83% conversion of mannose) was added to the stirred mixture, followed by absolute ethanol and the mixture was kept overnight at 0–4 °C. The copious precipitate was collected by vacuum filtration, washed with cold absolute EtOH (5 cm^3), and dried under high vacuum, to give Man-6-P **8** as the crude barium salt (2.29 g, 55%) (assuming the heptahydrate).

Compound **8** (250 mg) was converted into the disodium

dihydrate salt by ion-exchange with Dowex 50W-X8 (Na^+ form) in deionised water (4 cm^3). The resulting solution was filtered and freeze dried to give compound **8** (194 mg); δ_H(250 MHz; D_2O) 3.55 and 3.98 (6 H, non-anomeric CH and CH_2 of **5**), 5.02 (0.34 H, d, $J_{H1,H2}$ 1.3, 1-H of α-Man-6-P) and 5.29 (0.66 H, d, $J_{H1,H2}$ 2.0, 1-H of β-Man-6-P).

GDP-Mannose Pyrophosphorylase: Purification from S. cerevisiae.[20]—Boot's Genuine Brewers' Yeast (2 g) was autolysed in aq. K_2HPO_4 (70 mmol dm^{-3}; 8 cm^3) by sonication followed by centrifugation at 15 000 rpm for 10 min. The pellet that resulted was discarded and the supernatant was reserved. Saturated aq. $(NH_4)_2SO_4$ (Analar®, saturated at 0–4 °C) (5.6 cm^3) was slowly added to the stirred supernatant, then the mixture was stirred gently for 30–45 min and centrifuged as above. The supernatant was discarded, and the residue was taken up in deionised water (3 cm^3) and adjusted to pH 7.50 with 2 mol dm^{-3} NaOH. Saturated aq. $(NH_4)_2SO_4$ (6 cm^3) was added slowly to the stirred solution, which was stirred gently for 30–45 min, and centrifuged as before. The supernatant was discarded, and the precipitate was dissolved in aq. K_2HPO_4 (20 mmol dm^{-3}; pH 7.50) (1 cm^3) to give a volume of 2 cm^3. The resulting solution was stirred, cooled to −1 °C (ice–salt-bath), and 50% aq. EtOH (1.6 cm^3) was added dropwise. The temperature of the mixture was gradually lowered to −10 °C during the addition process. The mixture was centrifuged at −10 °C to 12 °C for 20 min at 15 000 rpm, and the supernatant was retained, being used directly for the next step.

Enzymic Synthesis of GDP-Mannose.—Man-1-P·$2Na^+$·$2H_2O$ (6.8 mg, 20 μmol), $MgCl_2$·$2H_2O$ (184 mg, 0.90 mmol), GTP·$3Na^+$·H_2O (14.8 mg, 25 μmol), neutral aq. EDTA (0.90 cm^3; 0.1 mol dm^{-3} solution of sodium salt adjusted to pH 7–7.5 with 1 mol dm^{-3} HCl), and aq. NaF (0.10 cm^3; 1.0 mol dm^{-3}) were dissolved in TRIS–DTT buffer* (8 cm^3; 50 mmol dm^{-3} TRIS–4 mmol dm^{-3} DTT) and the pH was adjusted to 7.5. To this solution was added the partially purified yeast extract containing GDP-mannose pyrophosphorylase (1 cm^3) and the pH was readjusted to 7.5. After overnight incubation at room temperature, the reaction mixture was heated at 100 °C for 45 s and then centrifuged to remove precipitated protein. The supernatant was freeze dried. The resulting solid was dissolved in water (0.50 cm^3) and loaded onto a column of Dowex 1X8-200 (HCO_3^- form) (2 cm^3). The column was eluted stepwise with increasing concentrations of aq. TEAB: 50 mmol dm^{-3} (40 cm^3), 100 mmol dm^{-3} (40 cm^3), 150 mmol dm^{-3} (20 cm^3), 200 mmol dm^{-3} (30 cm^3), and 250 mmol dm^{-3} (70 cm^3). Fractions containing GDP-mannose **1** were pooled and worked up as described above [6.0 mg, 37% as the bis-triethylammonium salt, from M-1-P (6.8 mg)].

* 2-Amino-2-(hydroxymethyl)propane-1,3-diol–dithiothreitol.

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