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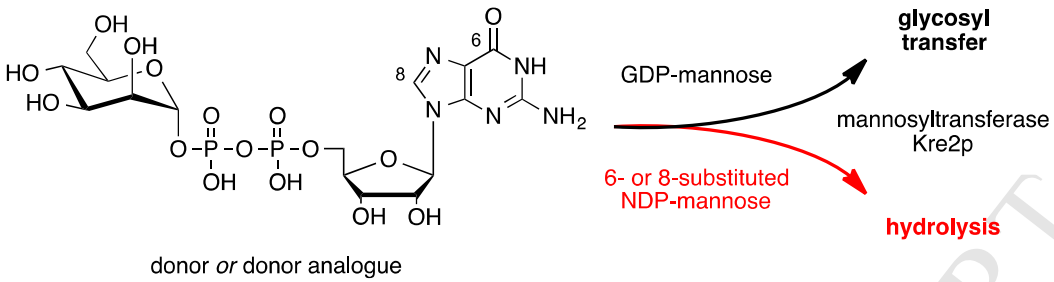
Graphical Abstract



Base-modified GDP-mannose derivatives and their substrate activity towards a yeast mannosyltransferase

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19 September 2017

Keywords: mannosyltransferase, NDP-mannose, donor analogue, substrate

Abstract

We have previously developed a new class of inhibitors and chemical probes for glycosyltransferases through base-modification of the sugar-nucleotide donor. The key feature of these donor analogues is the presence of an additional substituent at the nucleobase. To date, the application of this general concept has been limited to UDP-sugars and UDP-sugar-dependent glycosyltransferases. Herein, we report for the first time the application of our approach to a GDP-mannose-dependent mannosyltransferase. We have prepared four GDP-mannose derivatives with an additional substituent at either position 6 or 8 of the nucleobase. These donor analogues were recognised as donor substrates by the mannosyltransferase Kre2p from yeast, albeit with significantly lower turnover rates than the natural donor GDP-mannose. The presence of the additional substituent also redirected enzyme activity from glycosyl transfer to donor hydrolysis. Taken together, our results suggest that modification of the donor nucleobase is, in principle, a viable strategy for probe and inhibitor development against GDP-mannose-dependent GTs.

Introduction

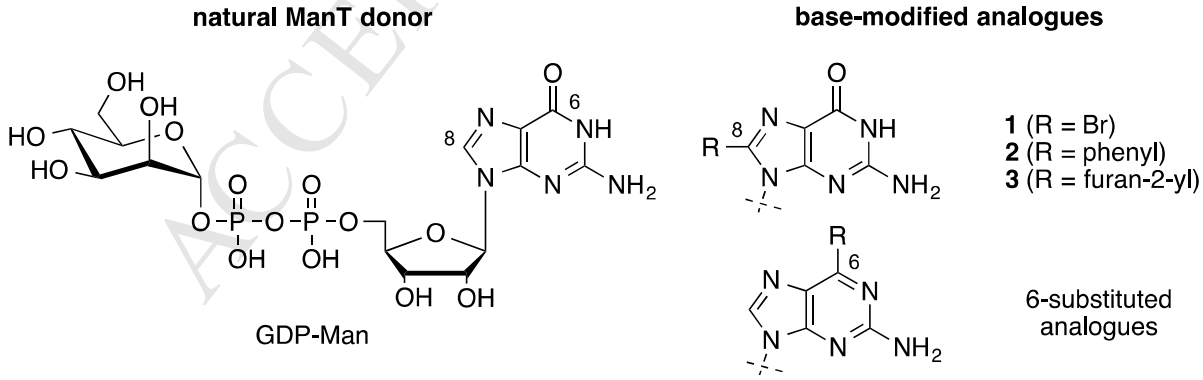
Glycosyltransferases (GTs) are a large family of enzymes that catalyse the transfer of a sugar from an activated glycosyl donor, frequently a sugar-nucleotide, to a specific acceptor [1]. Mannosyltransferases (ManTs) are a subfamily of GTs, which use either dolicholphoshate-linked mannose or the sugar-nucleotide GDP-α-D-mannose (GDP-Man) as their donor. ManTs play important roles in both eukaryotic and prokaryotic organisms, including many human pathogens such as *Candida albicans* [2], *Mycobacterium tuberculosis* [3], and *Trypanosoma brucei* [4]. Small molecular inhibitors and probes for ManTs are therefore of considerable interest for mechanistic, structural and functional studies with these enzymes, and also as potential candidates for anti-infective drug discovery [5].

We have recently developed a new class of inhibitors and fluorescent probes for GTs by installing an additional substituent at the nucleobase of the sugar-nucleotide donor [6,7]. Examples for base-modified sugar-nucleotides, containing either non-natural sugar/nucleotide combinations [8] or additional substituents at the nucleobase [9], are still relatively rare. We have shown that 5-substituted derivatives of UDP-α-D-galactose are poor substrates, but potent inhibitors for galactosyltransferases [6]. The additional substituent at the nucleobase of these donor analogues interferes with crucial conformational changes during the GT catalytic cycle, resulting in the observed substrate/inhibitor switch [6]. To date, we have applied this design concept only to UDP-sugar-dependent GTs [6,7]. In this study, we describe, for the first time, the application of this general strategy to a GDP-sugar-dependent enzyme, the ManT Kre2p.

Kre2p is a retaining α-1,2-ManT from *S. cerevisiae*, which catalyses the transfer of α-D-mannose from a GDP-Man donor to an α-mannoside acceptor [10]. Kre2p is involved in the biosynthesis of cell wall O- and N-glycoproteins in yeast, and a homologue of the *Candida albicans* enzyme Mnt1p [11], which is essential for the viability and virulence of this pathogenic fungus.

We have prepared four base-modified derivatives of the Kre2p donor GDP-Man, and evaluated their activity towards this enzyme. The design of our target molecules was inspired by our previous discovery that base-modified UDP-sugars with an additional, bulky substituent in position 5 are potent inhibitors, but poor substrates for UDP-sugar-dependent GTs [6,7], due to interference of the additional 5-substituent with conformational changes during the enzyme catalytic cycle. In direct analogy to the UDP-sugar series, we therefore wanted to specifically explore the effect of bulky, aromatic or heteroaromatic substituents in position 6 or 8 of the GDP-Man nucleobase on Kre2p activity (Fig. 1). We have found that in the case of Kre2p, these modifications do not abolish donor substrate activity, in contrast to our observations in the UDP-sugar series [6]. The presence of an additional substituent at position 6 or 8 of the donor does, however, redirect Kre2p activity from mannosyl transfer to hydrolysis. These results suggest that modification of the nucleobase is tolerated by this ManT, but that the functional consequences of this modification are different from those observed with base-modified UDP-sugars and UDP-sugar-dependent GTs.

Figure 1 GDP-α-D-mannose (GDP-Man) and base-modified target molecules of the present study



Results & discussion

Chemical synthesis

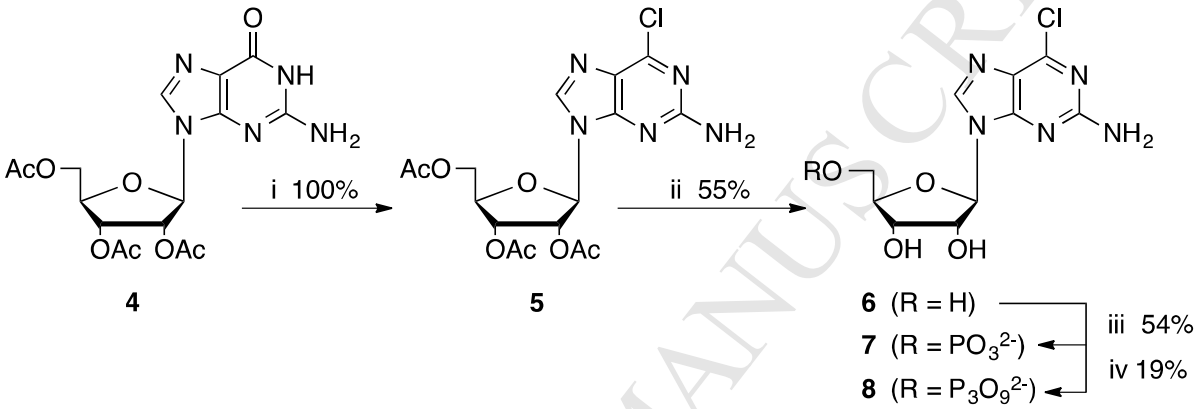
Installation of an aromatic or heteroaromatic substituent at position 8 of GDP-Man was achieved via palladium-catalysed Suzuki-Miyaura cross-coupling chemistry. Thus, the 8-substituted GDP-Man derivatives **2** and **3** (Fig. 1) were prepared from 8-bromo GDP-Man **1** and the requisite phenyl- or furan-2-yl boronic acid as previously reported [12]. For the synthesis of 6-substituted NDP-mannose derivatives, we intended to use a similar cross-coupling strategy. Ideally, we wanted to carry out the cross-coupling step at the stage of the sugar-nucleotide, to facilitate structural diversification. We therefore required a suitable 6-halo nucleoside precursor, which could be readily elaborated into the corresponding NDP-mannose derivative as the cross-coupling substrate. We chose 2-amino-6-chloro-purine riboside **6** as a suitable building block, as purine nucleosides with a chloro substituent at position 6 have been shown to readily undergo Suzuki-Miyaura cross-coupling reactions [13].

2-Amino-6-chloro-purine riboside **6** was prepared as depicted in Scheme 1. Peracetylated guanosine **4** was chlorinated using $POCl_3$ in acetonitrile to give 2-amino-6-chloro-2',3',5'-tri-*O*-acetyl purine riboside **5** in quantitative yield. Unfortunately, the chemical deprotection of **5** under either acidic or basic conditions was unsuccessful. Thus, the use of acetyl chloride in methanol resulted in cleavage of the ribose bond, while the use of K_2CO_3 in methanol led to the substitution of the 6-chloro substituent with a methoxy group. Successful deprotection of **5** was finally achieved enzymatically [14], using a commercially available lipase. With this method, the protecting groups were readily removed under mild, neutral conditions to give 6-chloro-2-amino-purine riboside **6** in 55 % yield.

Nucleoside **6** was converted into the corresponding mono- (**7**) and triphosphate (**8**) by selective phosphorylation at 5'-OH with $POCl_3$ using Ikemoto's protocol for the Yoshikawa reaction (Scheme 1) [15,16]. Previously, we found that the Yoshikawa phosphorylation of 8-substituted guanosine derivatives can be complicated by acid-catalysed cleavage of the glycosidic bond, and therefore requires the presence of a base, e.g. proton sponge [17]. In

contrast, 2-amino-6-chloropurine riboside **6** proved to be less sensitive to acidic conditions and the phosphorylation could be performed under the original Yoshikawa-Ikemoto conditions, providing nucleoside monophosphate **7** in 54% yield. The corresponding triphosphate **8** was obtained in analogous fashion in 19% yield from **6**, using Ludwig's procedure [18], once again without the requirement for the use of proton sponge.

Scheme 1 Synthesis of key intermediates **6**-**8**[a]

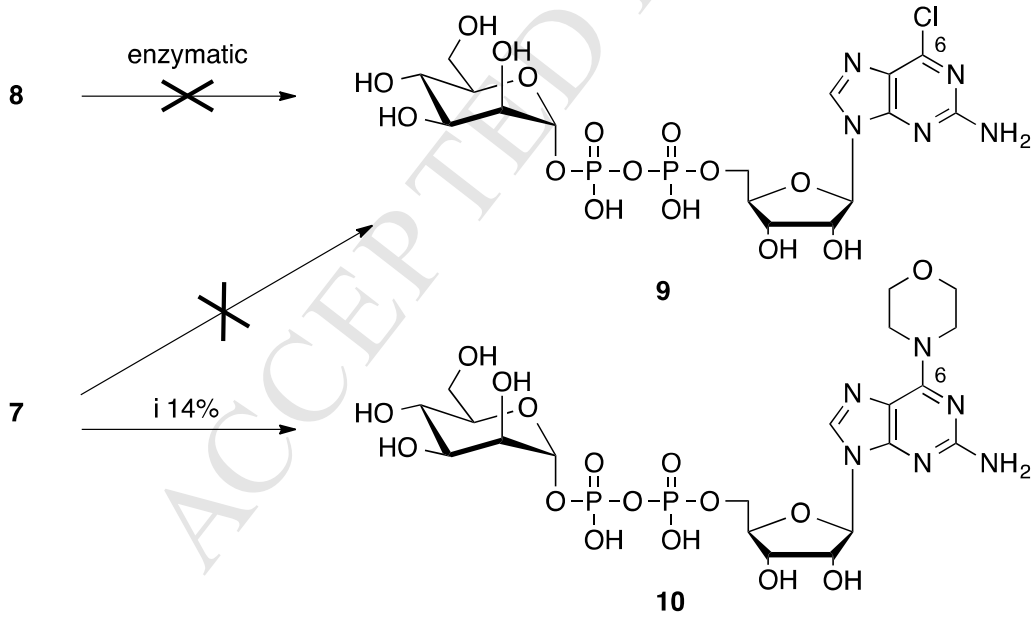


[a]*Reagents and conditions*: (i) $POCl_3$, benzyltriethylammonium chloride, dimethylaniline, MeCN, 90°C, 10 min; (ii) Novozyme, phosphate buffer (30 mM, pH 8.0), 60°C, 8 days; (iii) $POCl_3$, triethylphosphate, H_2O, 0°C; (iv) $O(PO_3)_2(Bu_3NH)_4$, DMF, 0°C; then TEAB (0.2M).

With the mono- (**7**) and triphosphate (**8**) of 2-amino-6-chloro-purine riboside **6** in hand, we explored both an enzymatic and a chemical route towards the corresponding NDP-mannose derivative **9** (Scheme 2). The enzymatic synthesis of **9** from triphosphate **8** and mannose-1-phosphate using GDP-mannose pyrophosphorylase was unsuccessful. The chemical synthesis of sugar-nucleotide **9** required the activation of nucleoside monophosphate **7** prior to pyrophosphate bond formation. Thus, **7** was converted into the corresponding phosphoromorpholidate via a dipyridyl disulfide/triphenylphosphine-mediated redox condensation with morpholine [19, 20]. The resulting phosphoromorpholidate was reacted successfully with mannose-1-phosphate under $MnCl_2$ catalysis to form the pyrophosphate bond, as evidenced by the ^{31}P NMR spectrum of the reaction product.

However, ^{1}H NMR and mass spectroscopic analysis of the resulting sugar-nucleotide showed that during this reaction sequence, the chloro substituent in position 6 had unexpectedly been replaced with a morpholino group, resulting in the formation of NDP-mannose derivative **10** instead of **9**. This side reaction can be attributed to the pronounced susceptibility of the 6-position in **7** to nucleophilic attack. Such S_NAr displacement reactions of 6-halopurines with amines have been employed e.g. for the preparation of N^6-substituted adenosine derivatives [21]. Unfortunately, all efforts to suppress this chloro/morpholino exchange reaction during the pyrophosphate bond formation step remained unsuccessful. As our primary interest was to explore the tolerance of Kre2p towards additional, bulky substituents in position 6 of the sugar-nucleotide donor, we therefore decided at this stage to evaluate sugar-nucleotide **10** as a potential donor substrate of Kre2p.

Scheme 2 Synthesis of 6-substituted NDP-mannose derivative **10**[a]



[a]*Reagents and conditions*: (i) (a) morpholine, PPh_3, dipyridyl disulfide, DMSO, rt; (b) mannose-1-phosphate, $MnCl_2$, $MgSO_4$, formamide, rt.

Biochemical evaluation

Sugar-nucleotides **1**-**3** and **10** were tested as donor substrates for Kre2p, with methylmannoside as the acceptor substrate. To follow the enzymatic reactions, the consumption of the respective starting NDP-mannose was monitored by HPLC. Aliquots were removed from each reaction and analysed by RP-HPLC with UV detection. To account for non-specific as well as enzyme-catalysed hydrolysis, two control experiments were carried out in each assay: one in the absence of enzyme, and another in the absence of acceptor.

First, the enzymatic conditions were optimized to achieve complete conversion of the natural donor substrate GDP-Man within 15 mins. In the absence of enzyme, no change in the GDP-mannose peak was observed over 60 min, demonstrating the chemical stability of GDP-Man under the assay conditions. In the absence of acceptor, a decrease of GDP-Man was observed but at a much slower rate (<10% after 60 min) than in the presence of acceptor. The primary product of this slow, enzymatic hydrolysis of GDP-Man was GDP, indicating that hydrolysis occurred at the anomeric position of the donor.

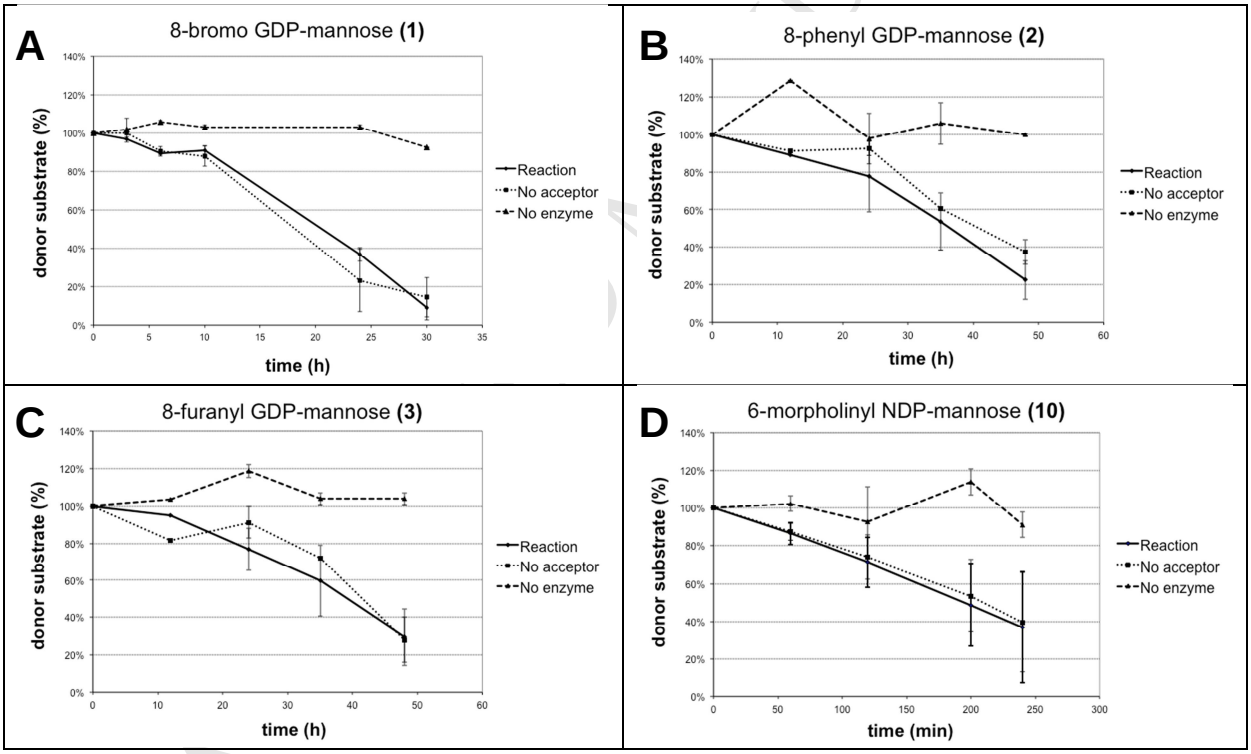
The time course of the enzymatic reactions with all four base-modified NDP-mannose derivatives is shown in Fig. 2. Table 1 shows the % conversion based on peak area for each of the analogues after maximum incubation time (4-48h). For all four compounds, no, or almost no, conversion was observed in the control experiment without enzyme (Fig. 2, Table 1). This demonstrates that **1**-**3** and **10** are all chemically stable under the conditions of the enzyme assay. In the presence of both Kre2p and acceptor, all four donor analogues were turned over, albeit at different rates, and much more slowly than the natural donor GDP-Man. This suggests that all four NDP-mannose derivatives are recognised as donor substrates by Kre2p, but less well than the parent sugar-nucleotide GDP-Man. Moreover, for each of the donor analogues a similar conversion profile was observed in the presence and absence of

acceptor. This indicates that these base-modified analogues are used by Kre2p as donors not for glycosyl transfer, but for hydrolysis.

Table 1 Conversion (%) of NDP-mannose derivatives **1**-**3** and **10** by the yeast mannosyltransferase Kre2p after maximum incubation time (4-48h). Data extracted from Figure 2.

Cmpd	Time (h)	Conversion (%)		
		+ acceptor + enzyme	- acceptor + enzyme	+ acceptor - enzyme
1	30	91%	86%	8%
2	48	78%	63%	0%
3	48	71%	72%	0%
10	4	63%	60%	9%

Fig. 2 Donor substrate activity of NDP-mannose derivatives **1**-**3** and **10** towards the yeast mannosyltransferase Kre2p.[a]



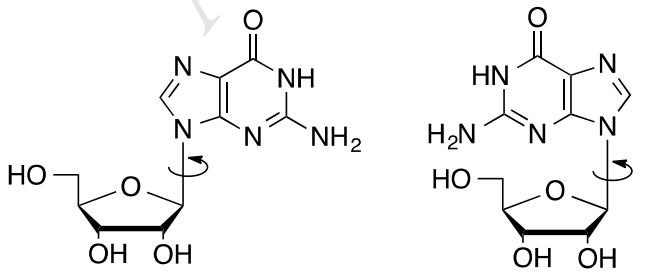
[a]*Conditions*: donor analogue (0.1 mM), α-methyl-mannoside acceptor (4 mM), Kre2p (0.05 mU/µL), Tris-HCl buffer (50 mM, pH 7.6), $MnCl_2$ (10 mM), 37 °C, 4-48h. Each experiment was carried out in duplicate.

A possible explanation for this behaviour is, that these base-modified NDP-Man derivatives are bound by Kre2p in a non-productive orientation, which allows the hydrolytic cleavage of

the mannose, but not its transfer to acceptor. This is in keeping with a recent theoretical study, which indicates that the Kre2p acceptor itself plays a crucial role in protecting the anomeric carbon of the donor against unwanted hydrolysis [22]. Very likely, the acceptor cannot perform this protective function if either the donor, or the acceptor, or both are not oriented correctly – as a result, for example, of the donor base modification. In this case, enzymatic donor hydrolysis will occur both in the presence and absence of acceptor, as observed for all four base-modified NDP-Man derivatives.

Interestingly, the 8-substituted derivatives showed a much slower turnover rate (30-48h) than the 6-substituted derivative (4h), suggesting that additional substitutents in the 6-position are better tolerated by the enzyme than substituents in the 8-position. This could be due to the difference in conformation between the 6- and 8-substituted analogues. The crystal structure of Kre2p in complex with GDP shows this nucleotide bound at the nucleotide binding pocket in the *anti* conformation (Fig. 3), stabilised by hydrogen bonds between Kre2p and N-1 and N-2 of the guanine base [10]. It is well known that in solution, guanosine analogues with bulky substituents in the 8-position adopt preferentially the *syn* conformation. Binding of the 8-substituted GDP-Man derivatives **1**-**3** therefore requires a conformational change from the *syn* to the *anti* conformation, which may contribute to the prolonged time course we observed for these donor analogues. In contrast, the 6-substituted NDP-mannose **10** adopts the *anti* conformation already in solution. This facilitates its binding at Kre2p and may explain the considerably shorter time course compared to **1**-**3**.

Fig. 3 *Syn* and *anti* conformation of guanosine



anti conformation *syn* conformation

Conclusion

Four base-modified derivatives of GDP-Man have been prepared and tested as donor substrates for the yeast α-1,2-mannosyltransferase Kre2p. Our results suggest that an additional substituent at either the 6- or 8-position of the donor nucleobase is, in principle, tolerated by this enzyme. The additional substituent does, however, alter both the outcome and the time course of the enzymatic reaction: the presence of the additional substituent redirects the outcome of the Kre2p reaction from glycosyl transfer to hydrolysis. Moreover, all four donor analogues are turned over much more slowly than the natural donor GDP-Man. Contributing factors to the observed rank order of turnover rates (8-phenyl, 8-furanyl < 8-bromo < 6-morpholinyl << GDP-Man) may be the steric bulk of the additional substituent, but also the different conformational preferences between the 8- and 6-substituted derivatives. Taken together, our results suggest that modification of the donor nucleobase is, in principle, a viable strategy for probe and inhibitor development against GDP-Man-dependent GTs.

Acknowledgement

We thank the University of East Anglia for a studentship (to A.C.), and the National Mass Spectrometry Faciltiy (Swansea) for the recording of mass spectra. This study was supported by a Leverhulme Trust Research Fellowship (to G.K.W.).

Experimental section

General

All chemicals were obtained commercially and used as received unless stated otherwise. MeCN was dried (sodium) and stored over activated 4 Å molecular sieves. TLC was performed on precoated aluminium plates (Silica Gel 60 F_{254}, Merck). Compounds were visualized by exposure to UV light. NMR spectra were recorded at 298 K on a Varian VXR 400 S spectrometer at 400 MHz (^{1}H) or on a Bruker Avance DPX-300 spectrometer at 300 MHz (^{1}H). Chemical shifts (δ) are reported in ppm and either referenced to residual solvent or to acetone (^{1}H δ 2.05, ^{13}C δ 30.83 for solutions in D_2O). Coupling constants (*J*) are reported in Hz. Resonance allocations were made with the aid of COSY experiments. Accurate electrospray ionisation mass spectra (HR ESI-MS) were obtained on a Finnigan MAT 900 XLT mass spectrometer at the EPSRC National Mass Spectrometry Facilty, Swansea. Analytical chromatography (HPLC) was carried out on an Agilent 1200 machine or a Perkin Elmer Series 200 machine equipped with a Supelcosil LC-18T column (25 cm × 4.6 mm, particle size 5 μm) and a UV detector. Preparative column chromatography was performed on silica gel 60 (particle size 0.063-0.2 mm). Ion-pair and reversed phase chromatography were performed on a Bio-Rad BioLogic LP system. TEAB (triethylammonium bicarbonate) buffer was prepared by bubbling CO_2 gas through a mixture of Et_3N in water until saturation was achieved.

Purification method 1. Ion-pair chromatography was performed using Lichroprep RP-18 resin, gradient 0-15% MeCN against TEAB (0.05M) over 400 mL, at a flow rate of 3 mL/min. Product-containing fractions were combined and reduced to dryness. The residue was co-evaporated with methanol to remove residual TEAB.

Purification method 2. Anion exchange chromatography was performed using MacroPrep 25Q resin, gradient 0-100% TEAB (1M, pH 7.3) against H_2O over 400 mL, then 100% TEAB (1M, pH 7.3) over 80 mL, at a flow rate of 3 mL/min. Product-containing fractions were combined and reduced to dryness. The residue was co-evaporated with methanol to remove residual TEAB.

8-Bromo GDP-mannose **1**, 8-phenyl GDP-mannose **2,** and 8-(2-furanyl) GDP mannose **3** were prepared as previously described [12].

2-Amino-6-chloro-2',3',5'-tri-O-acetyl purine riboside (5).

In an oven-dried flask, peracetylated guanosine **4** [23] (1.56 g, 3.81 mmol), benzyltriethylammonium chloride (1.74 g, 7.64 mmol) and N,N-dimethylaniline (530 μL, 4.18 mmol) were dissolved in dry MeCN (15 mL). $POCl_3$ (1.8 mL, 19.3 mmol) was added and the reaction immersed in a pre-heated oil bath at 90^{o}C for 10 min. All volatiles were flash-evaporated and the blue residue dissolved in chloroform and stirred with crushed ice for 15 min. The organic phase was washed with water (3 x 5 mL), and 5% aq. $NaHCO_3$ solution (3 x 10 mL), and dried over $MgSO_4$. All solvents were removed *in vacuo* to give **5** as an oil in quantitative yield, which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.87 (s, 1H, H-8), 6.01 (d, *J* = 4.93 Hz, 1H, H-1'), 5.95 (t, *J* = 5.07, 5.07 Hz, 1H, H-2'), 5.74 (t, *J* = 4.97, 4.97 Hz, 1H, H-3'), 5.20 (s, 2H, NH_2), 4.42 (m, 3H, H-4', H_2-5'), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.09 (s, 3H, OAc); ^{13}C NMR (100 MHz, DMSO-d_6)

ppm 170.5, 169.6, 169.3, 159.1, 153.0, 151.9, 140.7, 125.8, 86.6, 80.0, 72.7, 70.4, 62.9, 20.7, 20.5, 20.4.

2-Amino-6-chloropurine riboside (6).

Novozyme (1.6 g) in phosphate buffer (58 mL, pH 8) was added to a solution of **5** (1.56 g, 3.64 mmol) in acetone (10 mL). The mixture was stirred at 60°C for 8 days. The enzyme was removed by filtration and washed with ethanol (50 mL) and acetone (50 mL). The filtrate was evaporated *in vacuo* and the crude residue purified by column chromatography (SiO_2, CH_2Cl_2:MeOH, 85:15) to yield 0.61 g (55%) of **6** as a pale blue powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.38 (s, 1H, H-8), 6.99 (s, 1H, NH_2), 5.81 (d, J = 5.80 Hz, 1H, H-1'), 5.49 (br s, 1H, OH-2'), 5.19 (br s, 1H, OH-3'), 5.05 (t, J = 5.24, 5.24 Hz, 1H, OH-5'), 4.47 (t, J = 4.85, 4.85 Hz, 1H, H-2'), 4.20-4.02 (m, 1H, H-3'), 3.90 (q, J = 3.98, 3.95, 3.95 Hz, 1H, H-4'), 3.69-3.60 (m, 1H, H_2-5'), 3.59-3.52 (m, 1H, H_2-5'); ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.5, 154.7, 150.2, 141.9, 124.2, 87.4, 86.0, 74.2, 70.9, 61.9; High-resolution ES-MS *m/z* 302.0649 ($[M + H]^+$, $C_{10}H_{13}{}^{35}ClN_5O_4$ calcd 302.0651).

2-Amino-6-chloropurine riboside monophosphate (7).

2-Amino-6-chloropurine riboside **6** (220 mg, 0.745 mmol) was dissolved in triethylphosphate (2 mL) by gentle heating. H_2O (6.7 µL, 0.37 mmol) was added and the solution cooled to 0°C in an ice-salt bath. $POCl_3$ (280 µL, 3.00 mmol) was added dropwise and the solution stirred at 2-4°C for 1 hr. The reaction was quenched by stirring with crushed ice for 15 min. The pH was adjusted to 7 with 2M NaOH, the aqueous phase washed with EtOAc (3 x 30 mL) and all solvents removed *in vacuo.* The residue was purified by method 1 to remove excess salts, then by method 2 (fractions 44-60) to yield 220 mg of **7** (54%, 1.6 equiv. triethylammonium as determined by NMR). ^{1}H NMR (400 MHz, D_2O): δ 8.23 (s, 1H, H-8), 5.78 (d, 1H, J = 5.1 Hz, H-1'), 4.59 (t, J = 5.1, 5.1 Hz, 1H, H-2'), 4.35-4.33 (m, 1H, H-3'), 4.18 (m, 1H, H-4'), 3.98 – 3.90 (m, 2H, H_2-5'), 3.00 (q, J = 7.1, 7.3, 7.3 Hz, 9.5H, CH_2), 1.08 (t, J = 7.1, 7.3 Hz, 14.5H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ 159.8, 153.6, 150.7, 142.6, 124.0, 87.9, 84.6 (d, $J_{c,p}$ = 9.1 Hz), 74.6, 71.0, 64.6, 47.1, 8.8. ^{31}P NMR (162 MHz, D_2O): δ 7.31; High-resolution ES-MS *m/z* 380.0163 (monoanion, $C_{10}H_{12}{}^{35}ClN_5O_7P$ calcd 380.0168).

2-Amino-6-chloropurine riboside triphosphate (8).

2-Amino-6-chloropurine riboside **6** (100.5 mg, 0.33 mmol) was dissolved in triethylphosphate (2.5 mL) by gentle heating, H_2O (3 µL, 0.167 mmol) was added and the solution cooled to 0°C in an ice-salt bath. $POCl_3$ (124 µL, 1.32 mmol) was added dropwise and the solution stirred at 2-4°C for 3 hr. A cold mixture of Bu_3N (330 µL, 1.39 mmol) and tributylammonium pyrophosphate (2.33 mmol) in DMF (2.5 mL) was added in one portion and the reaction stirred at 4°C for 30 min. The reaction was quenched by stirring with ice cold TEAB (0.2M) for 15 min. The reaction was washed with EtOAc (3 x 30 mL) and all solvents removed *in vacuo.* The residue was purified by method 1 to remove excess salts, then by method 2 (fractions 18-24) to yield 54.1 mg of **8** (19%, 3 equiv. triethylammonium as determined by NMR). ^{1}H NMR (400 MHz, D_2O) δ 8.29 (s, 1H, H-8), 5.85 (d, J = 5.7 Hz, 1H, H-1'), 4.44 (br s, 1H, H-3'), 4.21 (br s, 1H, H-4'), 4.08 (m, 2H, and H_2-5'), 3.01 (q, J = 7.3 Hz, 25H, CH_2), 1.09 (t, J = 7.3 Hz, 38H, CH_3); ^{13}C NMR (100 MHz, D_2O): δ 159.4, 153.2, 150.3, 142.0, 123.7, 86.7, 83.7 (d, $J_{c,p}$ = 8.0 Hz), 73.3, 70.0, 64.9, 46.3, 7.9; ^{31}P NMR (121 MHz, D_2O): δ -4.04 (d,

J = 20.6 Hz), -9.01 (d, *J* = 19.4 Hz), -20.16 (t, *J* = 20.6, 19.4 Hz); High-resolution ES-MS *m/z* 539.9491 (monoanion, $C_{10}H_{14}N_5O_{13}{}^{35}Cl\,P_3$ calcd 539.9495).

2-Amino-6-morpholinylpurine riboside diphosphate mannose (10).

Nucleoside monophosphate **7** (125 mg, 0.231 mmol) was dissolved in dry DMSO (0.5 mL) and co-evaporated with dry DMF (3 x 2 mL). Morpholine was added (121 µL, 1.39 mmol) and the reaction stirred at rt for 5 min. Dipyridyl disulfide (154 mg, 0.70 mmol) was added and stirring continued for 5 min. Finely ground triphenylphosphine (177 mg, 0.67 mmol) was added and stirring continued for 3 hr. 0.1 M NaI solution in acetone was added dropwise until precipitation was complete. The white precipitate formed was collected by filtration and washed with acetone. The crude morpholidate was used in the next step without further purification. The morpholidate and mannose-1-phosphate (70 mg, 0.162 mmol) were dissolved in dry pyridine (5 mL) and evaporated to dryness several times. $MgSO_4$ (36 mg, 0.30 mmol) and dry 0.2 M $MnCl_2$ in formamide (1.1 mL) were added and the solution stirred at rt under N_2 for 6 days. MeCN was added dropwise until precipitation was complete and the supernatant was removed. The red oily residue was purified by method 1 followed by method 2. The thin film obtained was dissolved in H_2O (5 mL) and treated with Chelex 100 resin Na^+ form. The resin was filtered off and washed with water and the filtrate reduced to dryness to yield 24.5 mg (14%) of **10** as a glassy solid. ^{1}H NMR (400 MHz, D_2O): δ 7.93 (s, 1H, H-8), 5.76 (d, *J* = 6.4 Hz, 1H, H-1'), 5.29 (d, *J* = 8.0 Hz, 1H, H-1''), 4.52 (m, 2H, H-2', H-3'), 4.28 (m, 1H, H-4'), 4.14 (m, 1H, H-5'a), 3.98 (m, 1H, H-5'b), 3.89 (br s, 4H, 2 x CH_2), 3.83 (m, 1H, H-2''), 3.75 (m, 1H, H-3''), 3.69 (d, *J* = 9.6, 3.2 Hz, 1H, H-4''), 3.53 (m, 7H, H-6'', H-5'', 2 x CH_2); ^{31}P NMR (121 MHz, D_2O): δ -7.89 (d, *J* = 20.3 Hz), -10.17 (d, *J* = 20.2 Hz); High-resolution ES-MS *m/z* 673.1272 (monoanion, $C_{20}H_{31}N_6O_{16}P_2$ calcd 673.1277).

Biochemical assays.

Analytical chromatography

Analytical chromatography (HPLC) was carried out on an Agilent 1200 machine or a Perkin Elmer Series 200 machine equipped with a Supelcosil LC-18T column (25cm ×4.6mm, particle size 5 µm) and a UV detector. Methanol used was HPLC grade. 0.05 M TEA-phosphate buffer (1L) was prepared by mixing TEA (12.4 mL) and H_3PO_4 (6.2 mL) in MilliQ water, adjusting the pH to 7.0 with TEA and passing through a 0.45 □m filter. Injection volume 20 □L. HPLC conditions used for each substrate were as follows: 8-bromo-GDP-mannose (**1**): isocratic 10% MeOH against 0.05 M TEA-Phosphate buffer (pH 7.0), run time 25 min, detection at 262 nm. 8-Phenyl and 8-furanyl GDP-mannose (**2** & **3**): gradient of 15-30% MeOH against TEA-Phosphate buffer (0.05M, pH 7.0), run time 15 min, detection at 280 nm. 2-Amino-6-morpholinylpurine riboside diphosphate mannose (**10**): isocratic 15% MeOH against TEA-Phosphate buffer (0.05M, pH 7.0), run time 30 min, detection at 254 nm.

Enzyme assay

Recombinant Kre2p was purchased from Sigma. Donor (GDP-mannose) or donor analogue (1-3, 10, 0.1 mM), α-methyl-mannoside acceptor (4 mM) and Kre2p (0.05 mU/µL) in a total volume of 200 µL of Tris-HCl buffer (50 mM, pH 7.6) containing $MnCl_2$ (10 mM) were incubated at 37 ^{o}C for the required time (4-48h). Aliquots of 20 µL were taken at regular intervals, diluted to 40 µL and kept at 0 ^{o}C to quench the reaction, prior to analysis by RP-HPLC. The residual amount of donor or donor analogue at each time point was determined

by HPLC and calculated (in %) relative to the corresponding amount at t = 0. Control experiments contained buffer instead of acceptor or enzyme. Each assay was carried out in duplicate.

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Highlights

(1) GDP-mannose derivatives with an additional substituent in position 6 or 8 of the nucleobase are recognised as donor substrates by the *S. cerevisiae* α-1,2-mannosyltransferase Kre2p

(2) The additional substituent redirects donor substrate activity from glycosyl transfer to donor hydrolysis

(3) Position and steric bulk of the additional substituent, as well as conformational preferences, are determining factors for the observed activities