Chemoenzymatic synthesis of GDP-azidodeoxymannoses: non-radioactive probes for mannosyltransferase activity†

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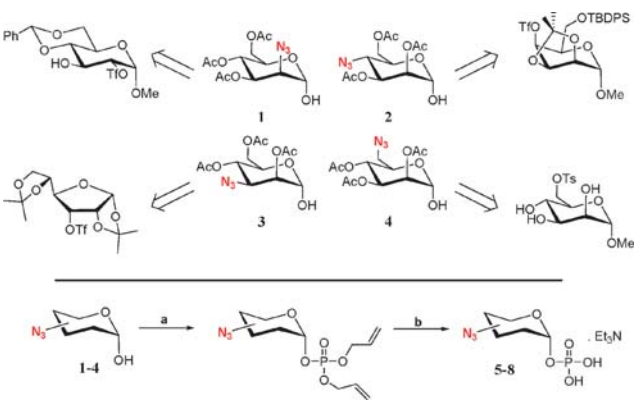
GDP-2-, 3-, 4- or 6-azidomannoses can be successfully prepared from the corresponding azidomannose-1-phosphates and GTP using the enzyme GDP-Mannosepyrophosphorylase (GDP-ManPP) from *Salmonella enterica* and may serve as useful probes for mannosyltransferase activity.

The discovery of promiscuous carbohydrate processing enzymes has provided chemists with powerful tools to readily access unnatural glycoconjugates with complete regio- and stereoselectivity, in water, and with minimal protecting group manipulation.[1] It has also facilitated metabolic engineering approaches employing tagged sugars displaying functional groups, such as ketones, which may alter the properties of various targeted cellular components.[2] This methodology was rapidly extended to sugars containing azides or acetylenes combined with the Staudinger ligation, or "click" chemistry for further manipulation.[3] Such methods have also been used to explore glycosyltransferase activity and substrate specificity *in vitro* and *in vivo*. In most cases, it is the *N*-acetyl group of glucosamine, mannosamine or galactosamine which carries the azide functionality.[4] Herein we report the first chemoenzymatic synthesis of four GDP-azidodeoxymannoses and investigate their use as substrates for a mannosyltransferase (ManT). Being a common constituent of *N*-linked glycoproteins, and a direct modification of mammalian glycoproteins in the extracellular matrix, it is clear that the availability of tools to profile mannose transfer may have valuable applications in disease diagnosis, discovery of novel mannosyltransferase activities and in glycoprotein remodelling.[5]

A key requirement is access to activated sugar nucleotides. We investigated the potential for GDP-mannose pyrophosphorylase (GDPManPP, EC. 2.7.7.13)[6] to produce our desired azidodeoxy products. Ultimately this provides the potential for conducting GDP-azidomannose synthesis and glycosyl transfer in one pot, which is not likely to be possible if they were prepared synthetically.[7] First, the azide was installed at four positions on the pyranose ring (**1–4**), by using displacement reactions of sodium azide on suitably protected tosylate and triflate precursors (Scheme 1).[8] Phosphoramidite chemistry at the anomeric centre allowed for the insertion of the α-phosphate group, required for GDP-ManPP activity, to yield 2-, 3-, 4- and 6-azidodeoxy mannose compounds **5–8** respectively.

To test **5–8** as unnatural substrates for GDP-ManPP, we overexpressed the protein in *E. coli* (strain ER2566(DE3)) transformed with the plasmid pET16b-GDP-ManPP. Induction at OD_{600} = 0.6 with 1 mM IPTG at 30 °C for 5 h yielded the poly-histidine tagged protein, which was purified from the cell free extract by Nickel affinity chromatography. SDS-PAGE and Western blot analysis (using an antibody raised against the poly-histidine tag) confirmed the identity and purity of GDP-ManPP. Typical yields of 1.1 mg L^{-1} GDP-ManPP were obtained using this method. The enzyme was concentrated and the solution exchanged against 50 mM Tris; pH 7.6, 8 mM $MgCl_2$, 1 mM DTT to test for activity, initially with 6-azidomannose-1-phosphate (**8**). The reaction was monitored by HPLC and the formation of the corresponding GDP-6-azidodeoxy derivative **9** was observed (Fig. 1(a)). After 60 h at 37 °C, the protein content was removed by filtration of the reaction mixture through a 10 kDa molecular weight cut-off membrane, and the resulting solution was purified by anion-exchange HPLC in a gradient of ammonium formate. The desired GDP-6-azidodeoxymannose **9** (33 mg, 63%), was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and high-resolution mass spectrometry. Having demonstrated enzymatic activity with 6-azidomannose-1-phosphate, we next optimised a colourimetric activity assay in order to compare the remaining isomers with the natural substrate in a high



Scheme 1 *Reagents and conditions*: (a) i-$Pr_2NPO(OAll)_2$, 1*H*-tetrazole, DCM, −40 °C to room temp., 46–72%. (b) *p*-$TolSO_2Na$, $Pd(PPh_3)_4$, THF–MeOH, room temp.; then MeOH–H_2O–Et_3N (5 : 2 : 1), 86–99%.

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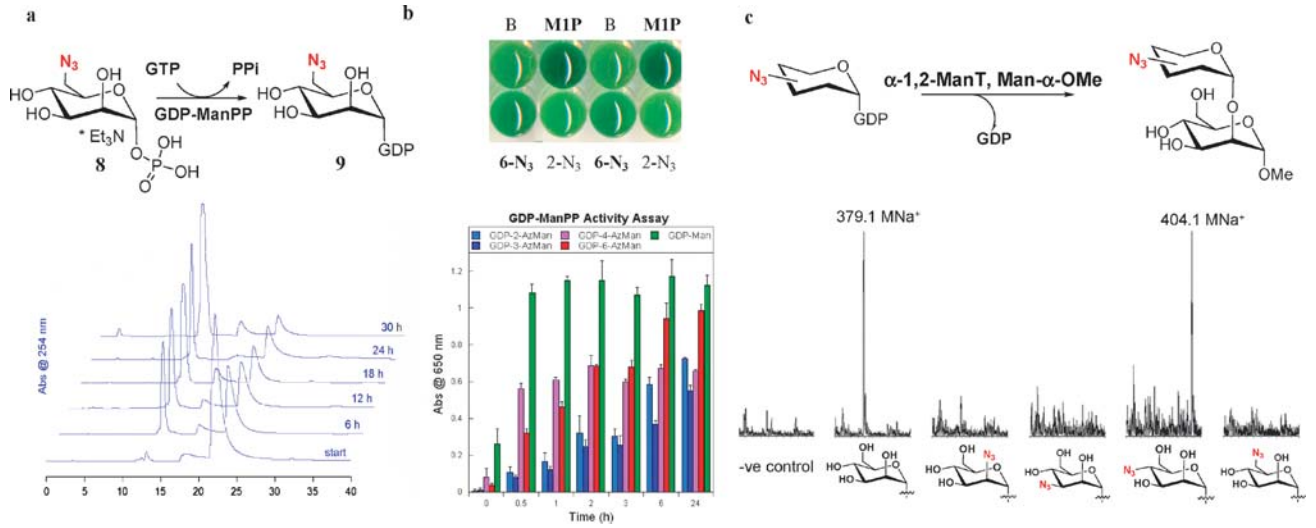

Fig. 1 (a) Anion-exchange HPLC monitoring of the GDP-ManPP catalyzed synthesis of GDP-6-azido-6-deoxymannose **9** (peak at 13 min = **9**, peak at 22 min = GTP). (b) This methodology is applicable to the synthesis of all the azidodeoxymannose isomers as observed by a colourimetric activity assay: B = blank, M1P = mannose-1-phosphate as substrate, 6/2-N_3 = 6/2-azidodeoxy mannoses as substrates. (c) ESI-MS analysis of the enzymatic reaction mixture shows that GDP-4-azidodeoxymannose is incorporated most readily by the α-1,2-ManT into the disaccharide 4-AzMan(α-1,2)Man-α-OMe.

throughput fashion (Fig. 1(b)). The screen was based on the inorganic pyrophosphatase-mediated hydrolysis of this reaction byproduct, and subsequent formation of a complex between phosphate, ammonium molybdate, and malachite green dye, which shifted the dye absorbance maximum to 650 nm.[9] Typically, the reagent was calibrated against a series of phosphate standards and a standard curve was generated against which 25 μL reaction samples were analysed at timed intervals.[8] Subsequently the 2-, 3- and 4-azido derivatives were also isolated in 41, 52 and 55% yield, respectively, and characterised by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and high-resolution mass spectrometry.

If the GDP azidomannose products were to find utility, for example, as probes for mannosyltransferase activity or glycoprotein remodelling, it was essential to investigate whether they can subsequently be transferred by ManTs to potential substrates. In a model system we over-expressed the soluble fragment of the *S. cerevisiae* α-1,2-ManT from *E. coli*.[10] After partial purification, the protein was concentrated (to 1 ml L^{-1} culture), exchanged against an appropriate buffer for subsequent MS analysis (100 mM ammonium formate, pH 7.5), and characterised by SDS-PAGE and Western blot. The reaction mixture containing the protein (107.2 μL), the GDP-mannose donor (1.25 mM) and the α-methyl mannoside acceptor (7.5 mM), in a total volume of 160 μl of 50 mM ammonium formate buffer pH 7.5, 10 mM $MnCl_2$, was incubated at 30 °C for 24 h. The protein content was removed and the filtrate was analysed by ESI-MS. Unsurprisingly, not all the isomers appeared to be converted to the desired product, however GDP-4-azidodeoxymannose was successfully incorporated into the disaccharide 4-AzMan(α-1,2)Man-α-OMe (Fig. 1(c)). This preliminary observation opens the door to *in vitro* profiling of mannosyltransferase activity and to the remodelling of mannose-containing glycoconjugates such as glycoproteins by using the azide as a synthetic handle.

In conclusion, we have developed an effective chemoenzymatic route to four GDP-azidodeoxymannoses. The colourimetric GDP-ManPP activity assay proved to be a useful analytical tool to monitor product formation and will facilitate optimisation of the enzymatic syntheses of azide-containing compounds. We also demonstrated that a recombinant α-1,2-ManT from *S. cerevisiae* can successfully process GDP-4-azidomannose. Consequently GDP-azidomannoses should function as useful non-radioactive probes to investigate ManT activity and, when successfully transferred to substrates, these compounds provide useful synthetic handles for further engineering *via* azide chemistry.[11]

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