Studies on the substrate specificity of a GDP-mannose pyrophosphorylase from *Salmonella enterica*

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**Abstract**

A series of methoxy and deoxy derivatives of mannopyranose-1-phosphate (Manp-1P) were chemically synthesized, and their ability to be converted into the corresponding guanosine diphosphate mannopyranose (GDP-Manp) analogues by a pyrophosphorylase (GDP-ManPP) from *Salmonella enterica* was studied. Evaluation of methoxy analogues demonstrated that GDP-ManPP is intolerant of bulky substituents at the C-2, C-3, and C-4 positions, in turn suggesting that these positions are buried inside the enzyme active site. Additionally, both the 6-methoxy and 6-deoxy Manp-1P derivatives are good or moderate substrates for GDP-ManPP, thus indicating that the C-6 hydroxy group of the Manp-1P substrate is not required for binding to the enzyme. When taken into consideration with other previously published work, it appears that this enzyme has potential utility for the chemoenzymatic synthesis of GDP-Manp analogues, which are useful probes for studying enzymes that employ this sugar nucleotide as a substrate.

**Introduction**

Modified sugar nucleotide analogues are valuable probes to study glycosyltransferases and other enzymes that use these activated glycosylating agents as substrates [1-5]. The synthesis of natural and non-natural sugar nucleotides is therefore a topic of continuing interest [6]. The classical method for chemically synthesizing sugar nucleotides involves the preparation of a sugar 1-phosphate derivative followed by its coupling to an activated nucleoside monophosphate to form the key pyrophosphate moiety (Figure 1A) [7]. In general, the yield of this process is low, and the purification of the product can be tedious; hence, the development of new methods to prepare sugar nucleotides remains an area of active research [6].
Figure 1: (A) Conventional approach for the chemical synthesis of sugar nucleotides from sugar 1-phosphates; (B) enzymatic conversion of sugar 1-phosphates into sugar nucleotides.

Although improved chemical methods have been developed [8-13], another attractive strategy is to employ a chemoenzymatic approach, in which a synthetic sugar 1-phosphate derivative is converted to the sugar nucleotide by a pyrophosphorylase (Figure 1B) [14,15]. This approach is increasingly used for the synthesis of sugar nucleotides, but a limitation is that the specificity of the pyrophosphorylase must be sufficiently broad to recognize the synthetic sugar 1-phosphate derivative. However, some of these enzymes have been demonstrated to have broad specificity, or can be engineered to have broad specificity, with regard to both the sugar 1-phosphate and nucleotide substrates [16-19].

As part of a larger study on the specificity of mannosyltransferases involved in mycobacterial glycan biosynthesis [20-22], we had the need for a panel of singly deoxygenated and methylated guanosine diphosphosphate mannopyranose (GDP-Man) derivatives. In developing a strategy for the synthesis of these compounds, we chose to take advantage of a GDP-mannose pyrophosphorylase (GDP-ManPP) from Salmonella enterica [23], which had previously been shown to have a relaxed specificity for the sugar 1-phosphate moiety [24,25]. In particular, it has been shown that the enzyme will accept mannoypyranosyl 1-phosphate (Manp-1P) derivatives deoxygenated at C-2, C-3 and C-4 (1–3, Figure 2), as well as a substrate lacking the hydroxymethyl group at C-5 (4) [24]. A series monoazido derivatives (5–8) were also shown to be substrates [25]. To further probe the potential of this enzyme for the chemoenzymatic synthesis of modified GDP-Man derivatives, we describe here the preparation of all four singly methylated Manp-1P analogues 9–12, as well as the 6-deoxy-Manp-1P derivative 13, and an initial evaluation of their ability to serve as a substrate for S. enterica GDP-ManPP.

Results and Discussion

Synthesis of 2-methoxy derivative 9

The synthesis of sugar 1-phosphate 9 containing a methyl group at O-2 commenced from 3-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside 14 [26] as illustrated in Scheme 1. Methyl-
Synthesis of 3-methoxy derivative 10

The preparation of the 3-methoxy Manp-1P analogue 10 followed a route similar to that used for the synthesis of 9 (Scheme 2). Methyl 2-0-benzyl-4,6-O-benzylidene-α-D-mannopyranoside 19 [26] was first methylated giving 20 and then converted into glycosyl acetate 21 in 49% yield over the two steps. Subsequent thioglycosylation provided a 52% yield of 22. The protected dibenzyl phosphate 23 was next formed by the NIS–AgOTf promoted glycosylation of dibenzyl phosphate with 22, which afforded the desired compound, 23, in 75% yield. Hydrogenolysis of the benzyl groups and deacylation led to the formation, in 67% yield, of Manp-1P derivative 10.

Synthesis of 4-methoxy derivative 11

As illustrated in Scheme 3, the synthesis of the 4-methoxy Manp-1P analogue 11 started by treatment of methyl α-D-mannopyranoside 24 with trityl chloride in pyridine. The product, 25, was then converted to the isopropylidene acetal 26 in 65% overall yield from 24. The hydroxy group in 26 was
methylated under standard conditions (CH$_3$I, NaH) to give the 4-methoxy analogue 27 in 91% yield. Acetolysis of 27 to the corresponding glycosyl acetate 28, followed by reaction with ethanol and BF$_3$OEt$_2$, yielded thioglycoside 29, in a modest 39% yield from 27 over two steps. This compound was then converted to 11, in 56% yield, as outlined above, by successive phosphorylation and deprotection.

**Synthesis of 6-methoxy derivative 12**

Two routes, differing in the choice of protecting groups, were explored to produce the 6-methoxy Man$_p$-1P derivative 12 (Scheme 4 and Scheme 5). In one route, the C-2, C-3, and C-4 hydroxy groups of the mannose residues were protected with benzyl ethers and in the other they were protected with benzoyl esters. The overall yields of these two methods were 30% and 17%, respectively. In the first method (Scheme 4), the initial step was the conversion, in 78% yield, of the fully acetylated thioglycoside 31 into silyl ether 32 by treatment with sodium methoxide and then tert-butyldiphenylchlorosilane in DMF. Benzylation of 32 using benzyl bromide and sodium hydride gave 33 in 84% yield. The TBDPS group was then cleaved and replaced with a methyl group to give the 6-methoxy compound 35 in 72% yield over two steps. The protected dibenzyl phosphate 36 was formed in 70% yield by phosphorylation as described for the synthesis of 9–11. Catalytic hydrogenolysis in the presence of NaHCO$_3$ was used to cleave all the benzyl groups, which gave the 6-methoxy Man$_p$-1P derivative 12 in 91% yield.

The second route to 12 began with methyl 2,3,4-tri-O-benzoyl-α-D-mannopyranoside (37) [29] and is illustrated in Scheme 5. Methylation of the free OH, even under mildly basic conditions (e.g., Ag$_2$O–CaSO$_4$), led to significant amounts of acyl group migration, and the desired product was obtained in only 52% yield. Nevertheless, enough material was produced to move forward. Acetolysis conditions were used to replace the methyl group at the anomeric center in 38 with an acetyl group, resulting in a 96% yield of 39. Thioglycosylation, followed by coupling of the resulting thioglycoside donor 40 (obtained in 75% yield) with dibenzyl phosphate, gave phosphonate 41 in a yield of 67% over the two steps. The 6-methoxy Man$_p$-1P analogue 12 was obtained by catalytic hydrogenolysis of the benzyl ethers followed by treatment with CH$_3$OH–H$_2$O–Et$_3$N 5:2:1 providing 12 in 85% yield over two steps.

**Synthesis of 6-deoxy derivative 13**

The synthesis of the 6-deoxy Man$_p$-1P analogue 13 used an intermediate (37) prepared in the course of the synthesis of the 6-methoxy analogue (Scheme 6). First, the hydroxy group of 37 was converted to the corresponding iodide in 65% yield, by using triphenylphosphine and iodine. The product, 42, was then subjected to acetolysis and catalytic hydrogenation, which gave 6-deoxy glycosyl acetate derivative 43 in 72% yield. The subsequent thioglycosylation, phosphorylation and deprotection steps proceeded, as outlined above, to give the 6-deoxy Man$_p$-1P 13 in 43% yield over four steps.
Evaluation of 9–13 as substrates for GDP-Man pyrophosphorylase

With 9–13 in hand, each was evaluated as a substrate for the S. enterica GDP-ManPP. Before doing that, the recombinant protein was produced and the natural substrate for the enzyme, Manp-1P (46, Figure 3), was evaluated by incubation with the enzyme and GTP. The reaction was monitored by HPLC (Figure S1 in Supporting Information File 1) and stopped when the complete consumption of GTP was observed. Simultaneous with the loss of the GTP was the appearance of the signal for a new product, which was found to elute at a retention time similar to that for an authentic sample of GDP-Manp. The product was isolated, and analysis by high-resolution electrospray ionization mass spectrometry revealed an ion with m/z = 604.0691, which corresponds to the [M − H]− ion (calcd m/z = 604.0699) of GDP-Manp.

Having established that the enzyme GDP-ManPP was active, we carried out the same incubations for 9–13, and in all cases the corresponding GDP-Manp analogue peaks could be observed (Figure S2 in Supporting Information File 1). However, in the case of 11 and 9, a peak corresponding to GDP, resulting from hydrolysis of the GDP-sugar, was also observed, and, in the case of 9, a much smaller amount of the GDP-Manp analogue was produced. To confirm the identity of each GDP-Manp analogue, the product peaks were isolated and analysed by electrospray ionization mass spectrometry. For the reactions involving 9–12 a signal at m/z ≈ 618 was observed, as would be expected for the [M − H]− ion of the methylated GDP-Man derivatives (48–51, Figure 4). Similarly, for the reaction with 13, a signal at m/z ≈ 588 was observed in the mass spectrum consistent with the 6-deoxy GDP-Man derivative 52.

Relative activity of Manp-1P analogues with GDP-ManPP

After it was established that all five Manp-1P analogues could serve as substrates for GDP-ManPP, the relative activity with each was assessed. This was done by using an established colorimetric activity assay, which relies on the detection of the pyrophosphate (PPi, Figure 3) formed as a byproduct of the
enzymatic reaction [30]. As illustrated in Figure 5, all five synthetic derivatives 9–13 were active as substrates, although at lower levels than the parent compound 46. The 6-methoxy (12) and 6-deoxy (13) analogues, demonstrated moderate to good relative activities, while the 2-methoxy (9), 3-methoxy (10), and 4-methoxy (11) compounds showed much lower activities. For example, the 2-methoxy, 3-methoxy, and 4-methoxy analogues displayed a 6-, 14-, and 17-fold decrease relative to 46, respectively. Because both the 6-deoxy and 6-methoxy analogues (12 and 13) showed relatively good activity it is likely that this hydroxy group does not interact significantly with the enzyme. On the other hand, because the 2-methoxy, 3-methoxy, and 4-methoxy compounds all showed a large decrease in activity, it is likely that these positions are bound tightly in the active site of the enzyme. A graphical summary of the substrate specificity for GDP-ManPP is shown in Figure 6.

Kinetic analysis of Manp-1P analogues with GDP-ManPP

To better understand how these 9–13 interact with GDP-ManPP, kinetic analyses were performed by using the colorimetric activity assay mentioned above (Table 1). Both the 6-methoxy Manp-1P (12) and 6-deoxy Manp-1P (13) derivatives bind relatively well to the enzyme, showing only a two- or three-fold increase in $K_M$ respectively, compared to the native Manp-1P donor 46. The turnover rate of 6-methoxy analogue 12 is, however, much lower than the 6-deoxy counterpart (13) and the natural substrate 46, as substantiated by a greater than 10-fold decrease in $k_{cat}$. Taken together, these results suggest that the C-6 hydroxy group does not engage in any critical hydrogen-bonding interactions and that a bulky substituent interferes with the rate of substrate turnover. The binding of the 2-methoxy (9) and 4-methoxy (11) analogues is very weak compared to the native substrate, as seen by the greater than 100-fold increase in $K_M$; consequently, the turnover rates are also low. The binding between 3-methoxy analogue 10 is moderate, with only a five-fold increase in the observed $K_M$, but it shows an extremely low turnover rate. These results all suggest that GDP-ManPP is not tolerant of bulky substituents at the C-2, C-3, and C-4 positions, which is consistent with the results obtained from their relative activity. It should be noted that these trends are consistent with earlier studies of the enzyme using deoxygenated or azido analogues [24,25].

Table 1: $K_M$, $k_{cat}$, and $k_{cat}/K_M$ of GDP-ManPP kinetic studies.

| compound | $K_M$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_M$ (min$^{-1}$·μM$^{-1}$) |
|----------|-----------|------------------------|--------------------------------------|
| 9 (2-methoxy analogue) | 4000 ± 1100 | 70 ± 11 | (2 ± 1) × 10$^{-2}$ |
| 10 (3-methoxy analogue) | 200 ± 72 | 5.2 ± 0.7 | (2.6 ± 0.1) × 10$^{-2}$ |
| 11 (4-methoxy analogue) | 3400 ± 870 | 31 ± 4.7 | (9 ± 5) × 10$^{-3}$ |
| 12 (6-methoxy analogue) | 120 ± 18 | 27 ± 1 | 0.23 ± 0.06 |
| 13 (6-deoxy analogue) | 70 ± 13 | 300 ± 13 | 4 ± 1 |
| 46 (Man-1P) | 40 ± 6 | 360 ± 16 | 9 ± 3 |

Conclusion

In this paper, we report the synthesis of a panel of methoxy and deoxy analogues of Manp-1P. Five analogues, 9–13, in which one of the hydroxy groups was methylated or deoxygenated were generated by chemical synthesis, and the ability of these compounds to be converted to the corresponding GDP-Manp analogues by GDP-ManPP from S. enterica was evaluated. All the derivatives acted as substrates for GDP-ManPP, but with uniformly lower activity than the natural substrate Man-1P. The
results suggest that the C-2, C-3, and C-4 hydroxy groups of Man-1P are bound within the active site of GDP-ManPP and the addition of a methyl group at these positions is tolerated very poorly. Conversely, the addition of a methyl group to, or deoxygenation of, O-6 had a much smaller effect, suggesting that this position protrudes from the active site, or is accommodated in a pocket that can tolerate either of these modifications. These results are consistent with earlier studies of this enzyme, which were focused on deoxygenated and azido derivatives [24,25]. Considered together, our studies and those published previously suggest that this enzyme can be used to access deoxy and azido derivatives of GDP-Man on a preparative scale, but that the synthesis of analogues containing more sterically demanding groups is likely to be only possible when the modifications are present on O-6.

Experimental
Detailed experimental procedures can be found in Supporting Information File 1.

Supporting Information
Supporting Information File 1
Detailed experimental procedures.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-8-136-S1.pdf]

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