# Targeted chemoenzymatic synthesis of sugar nucleotide probes reveal an inhibitor of the GDP-D-mannose dehydrogenase from *Pseudomonas aeruginosa*

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Alginate, an anionic polysaccharide composed of acetylated, β-1,4 linked D-mannuronic acid and α-1,4 linked L-guluronic acid residues, constitutes the major exopolysaccharide component of mucoid *Pseudomonas aeruginosa* infections. Such infections are particularly deleterious for cystic fibrosis patients,[1] contributing to the establishment of a biofilm environment and augmenting the antibiotic resistance profile of the bacterium. The molecular genetics and regulation of alginate biosynthesis in *P. aeruginosa* have been well-characterised.[2,3] However, our understanding of the chemical biology underpinning polysaccharide production and modification is still incomplete. This represents a significant opportunity to comprehend and exploit such pathways, especially within the global obligation to develop new anti-bacterial strategies; epitomised by recent work developing small molecule inhibitors for c-di-GMP binding to the receptor protein Alg44, which activates alginate production in *P. aeruginosa*.[4]

Pivotal to the alginate biosynthetic pathway is GDP-D-mannose dehydrogenase (GMD), which is required to form the alginate sugar nucleotide feedstock, guanosine diphosphate mannuronic acid (GDP-D-ManA).[5,6] Since there is no corresponding enzyme in humans, specific inhibition of GMD could be envisaged as a tactic to stop alginate production in chronic, mucoid *P. aeruginosa* infections.[7] GMD belongs to a small group of $NAD^+$-dependent four-electron-transfer dehydrogenases,[8,9] and converts GDP-D-Man **1** to GDP-D-ManA **2** (*Figure 1a*); the $NAD^+$-mediated oxidation is proposed to have four discrete steps, facilitated by an active site $Cys^{268}$ residue.[10] We recently reported the first series of C6-modified sugar nucleotides to investigate this oxidative mechanism, establishing evidence of a C6-ketone intermediate from oxidation of a C6-$CH_3$ modified analogue.[11] Pursuant of a substrate-based inhibitor of GMD and to further expand structure-activity relationship knowledge (to underpin future small molecule inhibition strategies), we report herein the design, synthesis and evaluation of a matrix of C4-, C5- and C6-modified GDP-D-Man analogues (*Figure 1b*). Our strategy focused on analogues of the pyranose component within the sugar nucleotide, specifically: i) C6-OH replacements, removing capability for oxidation, but retaining the potential to bind GMD ii) C6 modifications to mimic the product carboxylic acid in **2** and iii) C4 and C5 functional group changes to probe steric and electronic effects involved in substrate binding.

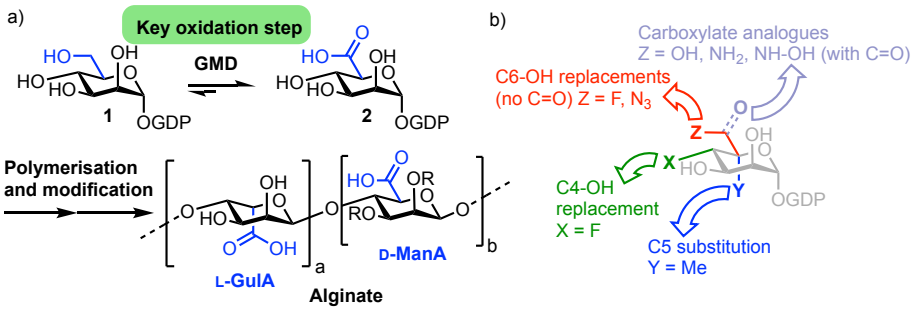



***Figure 1***. ***a)*** Conversion of GDP-D-Man **1** to GDP-D-ManA **2** by GMD ***b)*** Targeted functional group alterations to map GMD structure-function relationships.

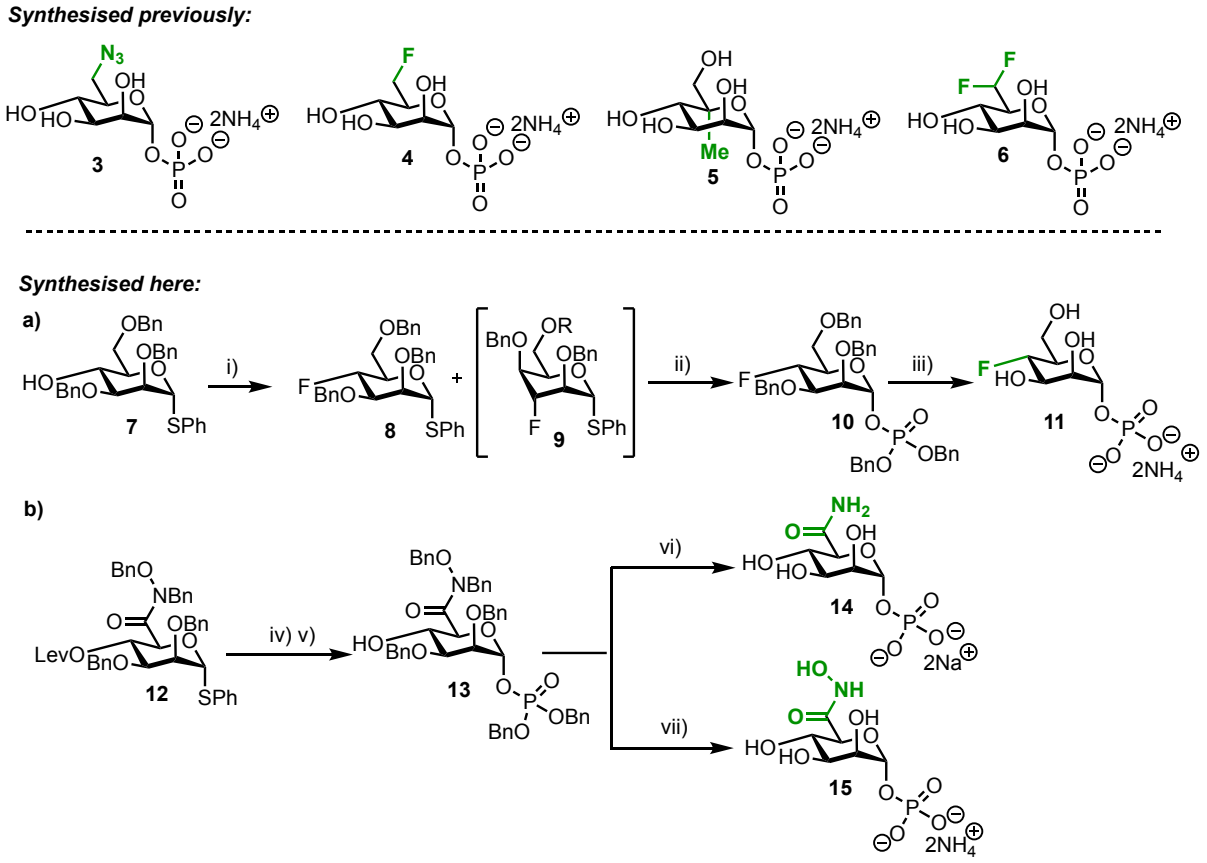
In order to access these materials a chemoenzymatic strategy was proposed, first using chemical synthesis to functionalise the pyranose ring and deliver the required glycosyl 1-phosphates, followed by nucleotide diphosphate formation with a pyrophosphorylase from *Salmoella enterica*.

### *Chemical synthesis of glycosyl 1-phosphates*

A recently developed synthesis of glycosyl 1-phosphates **3-6**, using a modified MacDonald anomeric phosphorylation procedure, meant these compounds were already available for enzymatic pyrophosphorylation.[12] Accordingly, the chemical synthesis of 4- and 6-position analogues **11**, **14** and **15** was completed (*Scheme 1*).

For the synthesis of 4-deoxy 4-fluoro mannose 1-phosphate **11** the synthesis began with available thioglycoside **7**[13] (*Scheme 1a*). Treatment of **7** with DAST to effect 4-position fluorination was low yielding (45%) and afforded a mixture of D-mannose and D-idose configured products **8** and **9**, which were inseparable by silica gel flash chromatography. Instead, the next transformation was completed, glycosylating dibenzyl phosphate with the C4-fluorinated thioglycoside mixture which enabled separation of the required protected 1-phosphate **10,** in an acceptable yield of 41%. A final global hydrogenolysis afforded the target 4-deoxy 4-fluoro mannose 1-phosphate **11** in 85% yield.

For C6-amide and C6-hydroxamic acid 1-phosphate targets **14** and **15** the synthesis started from recently reported thioglycoside hydroxamate building block **12** (*Scheme 1b*).[14] Glycosylation of dibenzyl phosphate with **12** proceeded in moderate yield (54%) and, following removal of the 4-position *O*-Lev group, delivered **13** which was then subjected to hydrogenolysis. Mindful that N-O bond cleavage under these deprotection conditions was a possibility, standard conditions were utilised,[11] but only for 5 h. Pleasingly, after this time only complete debenzylation to give **15** was observed, confirmed by $^{13}$C NMR (δ 167.5 ppm for C(O)NH-OH) and HRMS analysis. Extending the time of this reaction to 32 h also cleaved the N-O bond, delivering C6-amide **14** in 80% yield ($^{13}$C NMR δ 174.7 ppm for C(O)NH$_2$). With access to the desired series of mannose 1-phosphate derivatives **3-6**, **11** and **14-15** conversion to their GDP-D-Man form was evaluated.



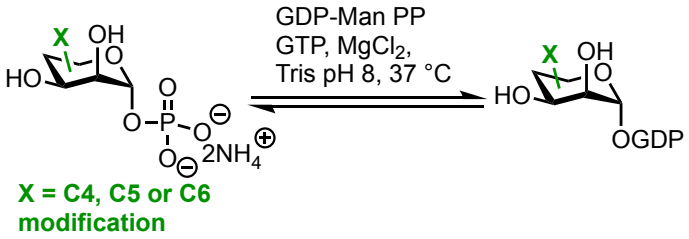

***Scheme 1***. i) DAST, DCM, 45% ii) $(BnO)_2P(O)OH$, NIS, AgOTf, DCM, 41% iii) $H_2$, Pd/C, $Pd(OH)_2$, $NaHCO_3$, THF, EtOH, 85% iv) $(BnO)_2P(O)OH$, NIS, AgOTf, DCM, 54% v) $H_2NNH_2{\cdot}AcOH$, pyr, AcOH, 62% vi) $H_2$, Pd/C, $Pd(OH)_2$, $NaHCO_3$, THF, EtOH, 32 h, 80% vii) $H_2$, Pd/C, $Pd(OH)_2$, $NaHCO_3$, THF, EtOH, 5 h then SAX with $NH_4HCO_3$ 67%.

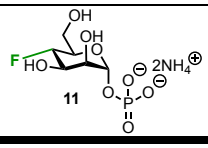
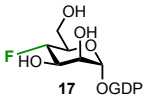
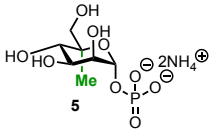
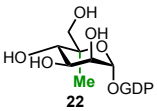
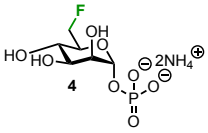
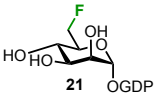
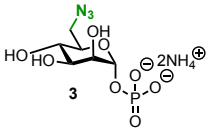
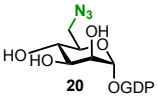
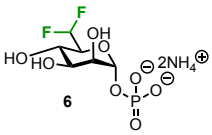
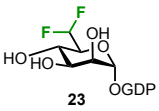
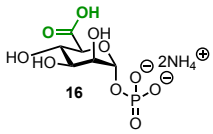
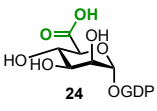
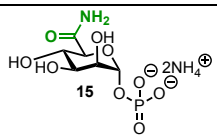
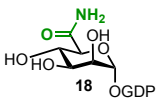
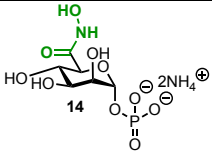
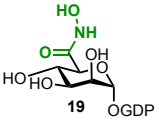
## *Enzymatic sugar nucleotide synthesis*

Utilising a GDP-mannose pyrophosphorylase (GDP-Man PP) from *S. enterica*,[15–17] nucleoside diphosphate formation was evaluated using modified glycosyl 1-phosphates **3**-**6**, **11** and **14**-**15**. The results of these experiments are shown in *Table 1* and discussed below.

Starting with C4-fluoro 1-phosphate **11**, smooth conversion (96% after 27 h) was observed and GDP-Man derivative **17** was isolated in 52% yield (Table 1, Entry 1). For C5-Me derivative **5**, conversion after 48 h was significantly slower and after this time the reaction was stopped and **22** isolated in a low 16% yield (Table 1, Entry 2). For 6-deoxy 6-fluoro 1-phosphate **4** a reaction time of 24 h enabled isolation of **21** in 41% yield (Table 1, Entry 3). 6-Deoxy 6-azido mannose 1-phosphate **3** was converted successfully after 16 h with an isolated yield of 16% for **20** (Table 1 Entry 4), affirming activity data reported previously for this substrate.[17] Lastly, for C6-modified substrates, gem-difluoro 1-phosphate **6** was evaluated (Table 1, Entry 5), but showed no conversion to **23** after multiple attempts. For C6-carboxylate level substrates **14**-**16** (Table 1, Entries 6-8) only C6-amide 1-phosphate **14** was a substrate for the enzyme (79% conversion after 27 h), enabling isolation of C6-amido GDP-Man **18** in 41% yield.

***Table 1***. Enzymatic synthesis of C4, C5 and C6-modified GDP-D-Man derivatives.




| Entry | Substrate | Product | Isolated yield (%) | Scale (mg)[a] |
|---|---|---|---|---|
| 1 |  |  | 52 | 1.7 |
| 2 |  |  | 16 | 1.0 |
| 3 |  |  | 41 | 1.9 |
| 4 |  |  | 16 | 1.5 |
| 5 |  |  | 0 | -- |
| 6 |  |  | 0 | -- |
| 7 |  |  | 41 | 1.6 |
| 8 |  |  | 0 | -- |

[a]of isolated product

Taken together, these findings present an important development in structure-activity information for this pyrophosphorylase in accessing non-native sugar nucleotides (*Figure 2*),

building on previous reports.[11] Replacement of mannose C4-OH with fluorine is well tolerated, similarly at C6-OH for fluorine and azide, but not for gem difluorination. A C5 modification with axial Me is also possible, but turnover is sluggish and inefficient. Finally, for C6 carboxylate oxidation-level substrates, charge does not appear to be tolerated at this position, but a neutral substrate is turned over. With access to milli-gram quantities of **17**-**18** and **20**-**22** their capability to compete with oxidation of **1** under GMD catalysis was examined.

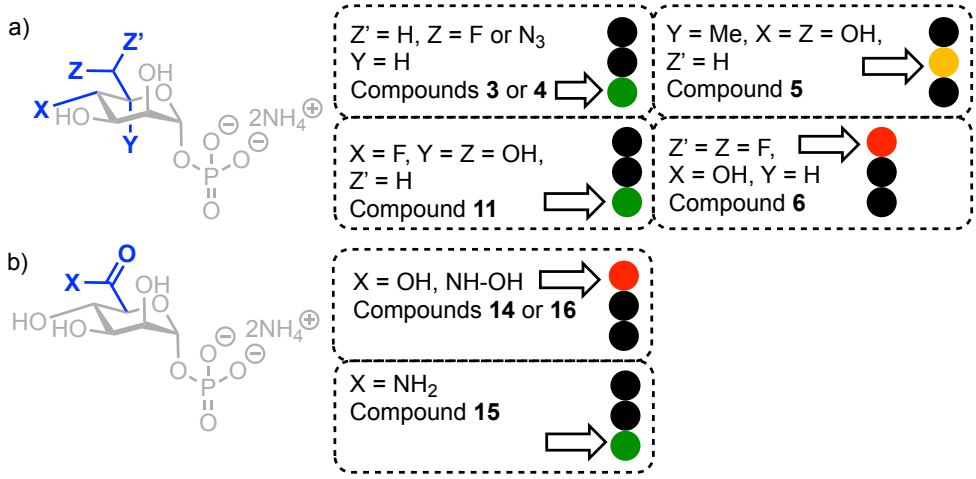



***Figure 2***. Expanded SAR profile for GDP-D-Man PP from *S. enterica* a) D-Man 1-phosphate analogues b) D-ManA 1-phosphate analogues. Activity indicated by good (green), moderate (amber) or none (red).

### *Evaluation of sugar nucleotide probes with GMD*

Sugar nucleotides **17**-**18** and **20**-**22** were incubated with GMD from *P. aeruginosa*, and the release of NAD(H) monitored colorimetrically at 460 nm, as described previously.[11] For 4-deoxy 4-fluoro derivative **17** no production of NAD(H) was observed over 1 h (see SI, Figure S5). The experiment was repeated, but after 40 minutes the well containing **17** was spiked with **1** and production of NAD(H) began. This suggests that **17** was either oxidised by GMD very slowly or is not a substrate as it is unable to bind in the active site. Consulting the crystal structure of GMD (PDB: 1MV8) with reaction product **2** bound,[5] the C4-OH makes three hydrogen bonds with the carboxylate groups of proximal $Leu^{159}$ and $Glu^{157}$ residues (see SI, Figure S6). The same capability may be required in **1** to bind with GMD and enable $Cys^{268}$-mediated oxidation at C6. This capability would be diminished upon substituting C4-OH with fluorine. Whilst analogue **17** was not evaluated further as an inhibitor of GMD, it does provide important information regarding sugar nucleotide binding requirements in this enzyme active site.

For derivatives **20** and **21**, which replace the required C6-OH with azide or fluoride respectively, establishing whether these analogues could compete with **1** and prevent its conversion to **2** was evaluated. Analogue **20** (50 µM) and **1** (10 µM) were incubated with GMD and after 45 minutes conversion to the product had reached 89% (see SI, Figure S7), suggesting **20** had little influence in preventing the oxidation. Repeating the experiment with a 2 h pre-incubation of **20** with GMD also had little effect, with conversion to **2** still reaching 83% after 50 minutes. Similar observations were made for C6-fluoro derivative **21**, indicating that these analogues are not substrates of GMD and do not inhibit the enzyme.

For C5-Me derivative **22**, which could be oxidised at C6, no NAD(H) production after 36 minutes was observed. When the plate was subsequently spiked with **1**, NAD(H) production was observed, suggesting that the inclusion of an axial methyl group at C5 in the substrate may

be tolerated for binding but that this was not a substrate for GMD, showing no inhibitory activity.

Finally, for C6-amide derivative **18**, the assay conditions for **20** were repeated using **18** (50 μM) and **1** (10 μM) and a substantial reduction in the conversion of **1** was observed over 45 minutes. The experiment was repeated in triplicate using seven different concentrations of **18** (from 1 to 200 μM), noting a clear relationship between increased conversion of **1** and decreased concentration of **18** (*Figure 3*). These data reveal that at high concentrations of **18** (200 μM) inhibition of GMD was >70%, but at lower concentrations (10 μM) this dropped to 20%. This constitutes the first example of a sugar nucleotide analogue inhibitor for GMD.

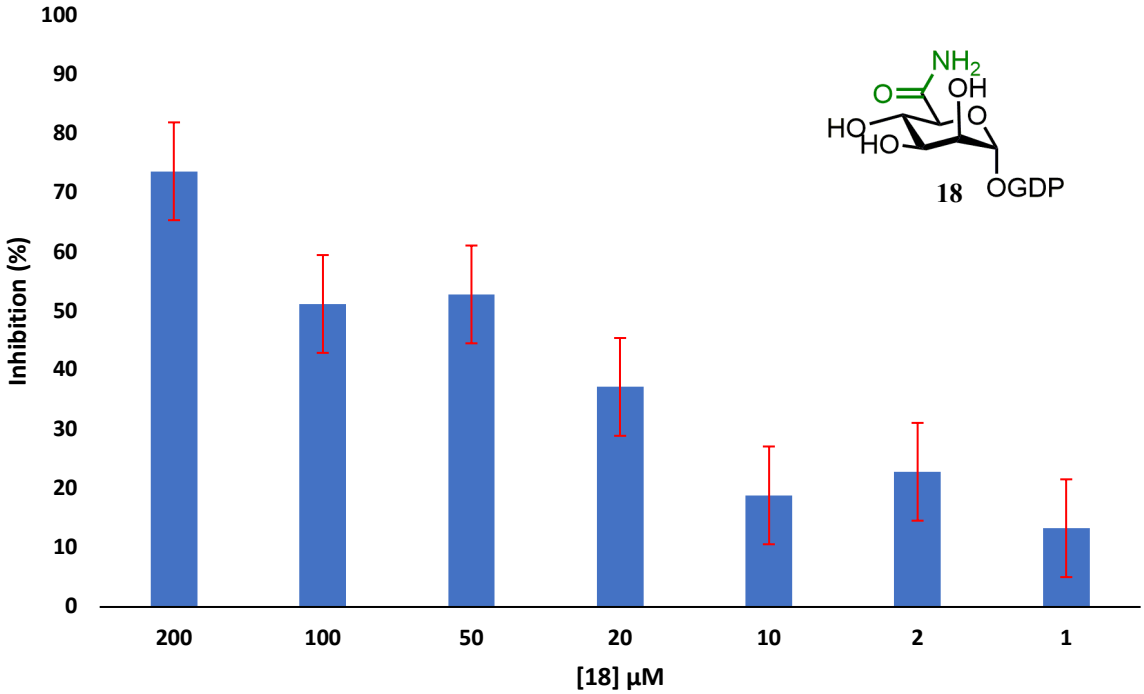



***Figure 3***. Evaluation of **18** at concentrations from 1 to 200 μM in the presence of **1** (50 μM) against GMD (50 μg/mL) and $NAD^+$ (200 μM). The % inhibition is derived from conversion of **1** to **2**, detecting formation of NADH at 460 nm and is a mean ± standard error of three repeats. A negative control experiment was run with no GMD (data not shown).

To probe this result further, consideration as to whether **18** was being hydrolysed to **2** was made. The native carboxylate product of GMD oxidation is a feedback inhibitor of GMD; 44% inhibition of NAD(H) production at 50 μM for **2** was observed (see SI, Figure S8). To rule this out a 3 h incubation of **18** with GMD was followed by HRMS analysis and revealed no evidence of amide hydrolysis (only molecular ions for $[\mathbf{18} + H]^+$ were observed). This further affirms **18** as an inhibitor of GMD.

To support these experimental data and inform molecular detail as to how a C6-amide of GDP-D-ManA may inhibit GMD, molecular modelling of **18** and **1** with GMD was completed (*Figure 4*). For **18** (*Figure 4a*) the amide component establishes hydrogen bond networks with a proximal $Glu^{157}$ and the key $Cys^{268}$ which may provide additional bonding capability and preference of this analogue to remain in the active site and not be as easily displaced by **1**. *Figure 4b* depicts **18** and **1** overlaid in the enzyme active, illustrating their similarity in binding and proximity to the key $Cys^{268}$ residue, thus offering potential for **18** to act as a substrate mimic and compete for the binding site.

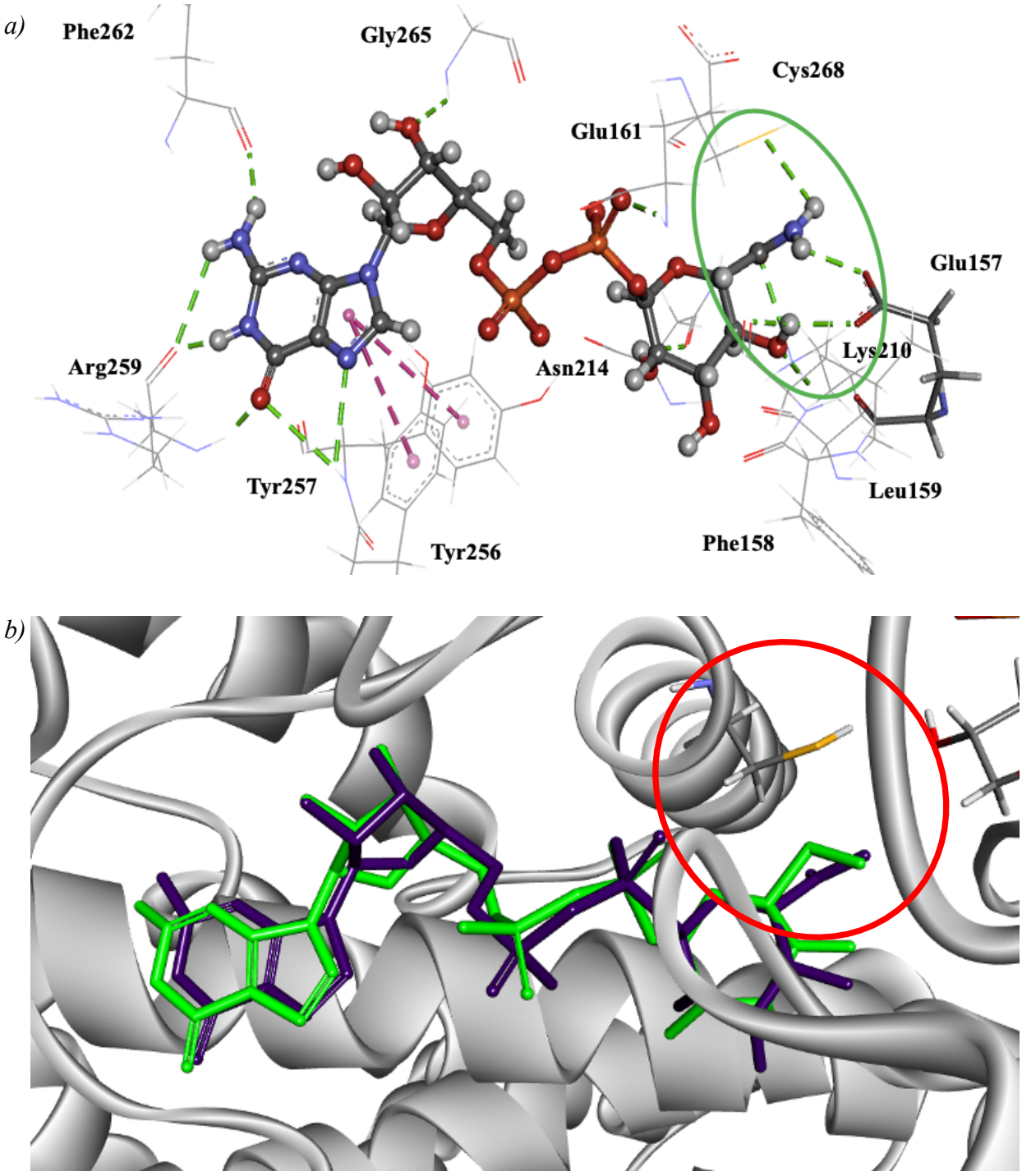



***Figure 4. a)*** Molecular modelling of **18** in the active site of GMD showing hydrogen bonds between $C(O)NH_2$ with $Glu^{157}$ and $Cys^{268}$ (green oval) ***b)*** Overlays of **1** (green) and **18** (purple) in the GMD binding site with similar proximity of C6 substituents to $Cys^{268}$ shown within red oval; hydrogen atoms are removed for clarity.

***Conclusions***

Using a chemoenzymatic strategy a series of GDP-D-Man probes containing C4-, C5- and C6-position modifications can be accessed. These materials have been evaluated as structure-function tools for the mannose dehydrogenase from *P. aeruginosa*. Fluorine replacement of the C4-OH in mannose removes substrate binding capability, suggesting this position is key for recognition. Introduction of a C6-position amide identified the first example of a sugar nucleotide inhibitor for this enzyme. These findings will inform the design of a next

generation of GDP-D-Man analogues to target GMD inhibition and underpin the development of small molecule inhibition strategies against an enzyme critical to bacterial exopolysaccharide biosynthesis.

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