

Efficient synthesis of a nucleoside-diphospho-*exo*-glycal displaying time-dependent inactivation of UDP-galactopyranose mutase†

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A short and efficient synthesis of UDP-*exo*-galactofuranosyl-glycal is presented. This molecule displayed an interesting time-dependent inactivation of UDP-galactopyranose mutase, an essential enzyme of the mycobacterial cell wall biosynthesis.

Galactans of the mycobacterial cell walls are comprised of over 25 galactofuranose (Gal_f) residues and the inhibition of their biosynthesis prevents proliferation of mycobacteria, including important pathogens such as *Mycobacterium tuberculosis*.¹ This biosynthesis involves several enzymes having uridine-diphospho-galactofuranose (UDP-Gal_f) as substrate: a mutase catalysing the interconversion of UDP-galactopyranose (UDP-Galp) and UDP-Gal_f, as well as Gal_f-transferases. On the basis of the respective mechanisms of these enzymes, we are seeking and designing molecules that could be transition state analogues of all enzymes involved in the galactan biosynthesis. The mechanism by which the flavoenzyme UDP-Galp mutase catalyses the interconversion of UDP-Galp into UDP-Gal_f is still unclear. Two main hypotheses have been addressed: i) the involvement of 1,4-anhydrogalactose as intermediate;^{2,3} ii) a single electron transfer from the flavin cofactor to the sugar ring leading to an anomeric galactosyl radical.^{4,5} However, none of these assumptions has been demonstrated yet. Furthermore, Liu *et al.* have shown that UDP-Gal_f analogues fluorinated at the 2- and the 3-positions were substrates of the reduced mutase, whereas they displayed time-dependent inactivation against the native enzyme.⁶ In a previous study, we probed the mutase binding site with conformationally constrained analogues wherein the galactose moiety had been locked in a furanose and a^{1,4B} boat conformation.⁷

The design of glycal-nucleoside-diphosphates (NDPs) as inhibitors is based on the fact that the transition state of enzymatic reactions involving a cleavage of the anomeric C1-O-(NDP) bond might be close to an oxycarbenium species with a flattened conformation. Due to the presence of sp²-hybridized carbons in the glycal skeletons, these species might closely mimic the conformation of oxycarbenium species. The main drawback of the *endo*-glycal-NDP structures is that they lack a hydroxyl group at the 2-position which might be involved in tight interactions with glycosyl processing enzymes.⁸ Although spectacular sialyl trans-

ferase inhibition levels have been observed with nucleotide-*exo*-glycals,⁹ these species have never been prepared in the galactofuranose series.¹⁰ In this context, we designed Gal_f-*exo*-glycal-UDP **1** (Scheme 1) which might be a transition-state analogue inhibitor of the mutase.

The synthesis of *exo*-glycal-NDP has to meet two requirements: i) an efficient methodology for the construction of *exo*-vinylphosphonate; ii) an adequate protection strategy to allow mild deprotection steps without modifying the sensitive enol-ether functionality. Gal_f-*exo*-glycals **3** and **4** were synthesised in two steps from the commercially available 1,4-galactonolactone. Tetra-silylated galactonolactone **2** was condensed with the lithium anion of dialkyl methyl phosphonate,¹¹ yielding an intermediate lactol that was directly subjected to a very efficient elimination procedure recently developed by Lin *et al.*¹² This procedure exclusively gave *exo*-glycals with a (*Z*)-configuration.^{7,12} The complete one-pot deprotection of dimethyl phosphonate **4** with trimethylsilyl iodide mainly led to a decomposition of the sensitive enol-ether functionality. Seeking cleaner conditions, we developed a stepwise procedure from compound **3** based on a preliminary selective debenzylation followed by a tetra-desilylation. The hydrogenolysis had to be performed first since the same reaction on the tetraol **5** led to partial saturation of the double bond and partial hydrolysis. When performed on the tetra-silylated species **3**, standard hydrogenolysis conditions quantitatively yielded the desired intermediate **6**. The *tert*-butyldimethylsilyl (TBS) groups were then removed by

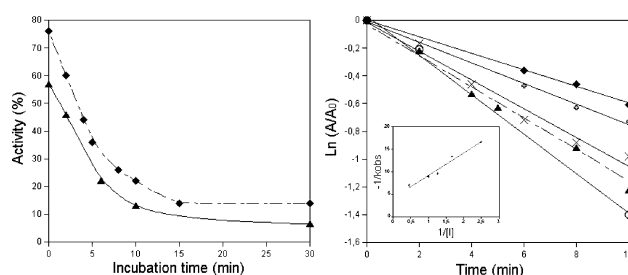
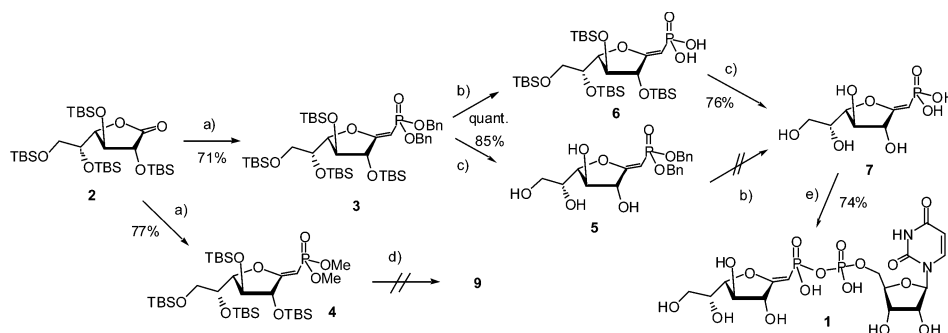
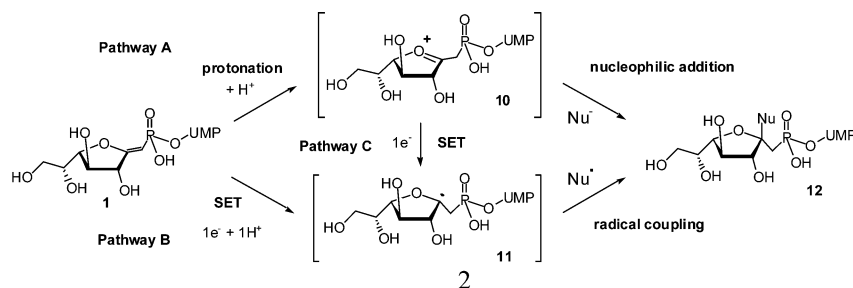


Fig. 1 (left) Residual enzymatic activity at different incubation times of inactivator **1** (residual concentration of **1** \blacklozenge 1 mM, \blacktriangle 2.2 mM); (right) k_{obs} determination at different inactivator concentrations (\circ 2.2 mM, \blacktriangle 1 mM, \times 0.8 mM, \diamond 0.6 mM, \blacklozenge 0.4 mM); (inset) double reciprocal plot of k_{obs} versus concentration of **1**.



Scheme 1 Reagents and conditions: a) (RO)₂POCH₂Li, THF, concentrated then THF/Py, (CF₃CO)₂O; b) H₂, Pd/C, Et₃N, CH₂Cl₂; c) TBAF, THF; d) TMSI; e) UMP activated by the Bogachev procedure (see text). TBS (*tert*-butyldimethylsilyl), Bn (benzyl).

† Electronic supplementary information (ESI) available: experimental section. See <http://www.rsc.org/suppdata/cc/b4/b402469a/>



Scheme 2 Hypothetical inactivation pathways.

tetrabutylammonium fluoride, giving phosphonate **7** in 76% yield after ion-exchange chromatography. As outlined in Scheme 1, the choice of TBS as a protective group happened to be critical: the silyl ethers were not only compatible with the chemistry of the *exo*-glycal construction but also prevented side-reactions occurring during the key hydrogenolysis step. Phosphonate **7** was then coupled to UMP following a procedure recently developed by Bogachev.¹³ Thus, NDP-*exo*-glycal **1** was efficiently obtained in 5 steps and 40% overall yield from commercial 1,4-galactono-lactone.¹⁴

The target molecule was assayed against UDP-Galp mutase following a described procedure.^{6,7} Figure 1 shows the time dependency of the native enzyme activity when incubated with **1** at different times. As already observed with 3F-Galf-UDP,⁶ the inactivation was only observed with the native enzyme, and not when a strong reducing agent such as dithionite was used. As in the case of Liu's study, sodium dithionite could restore enzymatic activity of the inactivated mutase. After an incubation time of 30 minutes at a high inactivator concentration (2.2 mM), the mutase was almost totally inactivated (> 95%). The inactivated enzyme did not recover its activity after extensive dialysis against the assay buffer (8 hours, with 3 buffer changes). These results suggest that a covalent intermediate between the inactivator and the enzyme is involved. Competition experiments such as the ones depicted in Fig. 1 (left) showed that the residual mutase activity depended on the ratio UDP-Galf/**1**, suggesting that the inactivation is active-site-directed.

The kinetic characteristics K_I and k_{inact} were determined from the double reciprocal plot of k_{obs} versus concentration of **1** (Fig. 1, inset), the k_{obs} values being obtained from the natural logarithm of residual activity versus time at different inactivator concentrations. The K_I value thus determined was 0.9 mM which is significantly higher than the K_m value of UDP-Galf (0.2 mM). This probably signifies that the relative position of the Galf and the UDP moieties of **1** does not provide an optimal fit within the enzyme binding site. Interestingly the k_{inact} value found (0.23 min⁻¹) is very similar to that of UDP-3F-Galf (0.19 min⁻¹), the only inactivator of this enzyme reported to date, despite the major structural difference between the two molecules. Inactivation of glycosyl-processing enzymes by fluorinated molecules is well documented, especially in the glycosidase series,⁸ wherein the electron-withdrawing character of the fluorine atom dramatically slows down the deglycosylation of the covalent glycosyl-enzyme intermediate. On the contrary, glycals are generally designed as transition state analogues. Thus, rather than a time-dependent inactivation, a competitive inhibition with molecule **1** was expected.

The surprising behaviour of *exo*-glycal **1** has to be directly related to the unique but still unresolved isomerization mechanism of UDP-galactopyranose mutase. Although the reaction catalysed by this flavoenzyme does not imply any redox change, the role of the flavin cofactor has been addressed.¹⁵ Potentiometric titration experiments have shown the stabilisation of FADH[•], the semiquinone form of FAD, in presence of the substrate, suggesting the possibility of a crypto redox process in which a single electron transfer (SET) from FADH[•] to the nucleotide sugar would generate an anomeric radical.⁵ A recent study of the catalytic properties of the mutase reconstituted with 1- and 5-deaza-FAD supports this hypothesis.¹⁶ Thus, several putative pathways might

explain the inactivation of the mutase by *exo*-glycal **1** (Scheme 2). Pathway A describes the usual bis-electronic reactivity of enol-ethers upon acid activation of the double bond. Taking into account the possibility of a SET, pathways B and C can also be envisioned: either via an oxycarbenium intermediate **10** (pathway C) or via a SET preceding a protonation (pathway B). In the two former cases a coupling with a radical species leading to a covalent adduct **12** might be invoked. We extensively tried to characterise the formation of such an adduct by mass spectrometry but all attempts have failed so far. This may be due to the instability of **12**, if formed, in the conditions required for mass spectrometry.¹⁷ Three conserved tyrosine residues, located in the binding site, have been shown to play an important role in the mechanism.⁴ Since a tyrosine has recently been demonstrated to be the catalytic nucleophile of a trans-sialidase,¹⁸ a tyrosine of the mutase could possibly display a nucleophilic role in this ring contraction. On the other hand, FADH[•] might also be a good candidate for a radical coupling.¹⁶

In conclusion, this work presents the first synthesis of an NDP-*exo*-furanoglycal that displays an interesting time-dependent inactivation of UDP-galactopyranose mutase. This study opens the way to the design and the synthesis of a new generation of UDP-galactopyranose mutase inactivators with improved binding and kinetic properties that would ultimately facilitate the characterisation of the still hypothetical catalytic nucleophile involved in this intriguing enzymatic transformation.

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