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Production of a Novel *N*-Monomethylated Dideoxysugar

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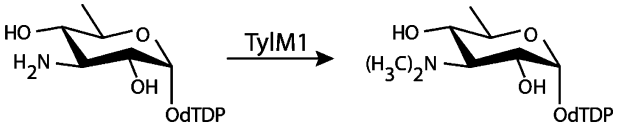
Supporting Information

ABSTRACT: The importance of unusual deoxysugars in biology has become increasingly apparent over the past decade. Some, for example, play key roles in the physiological activities of the natural products to which they are attached. Here we describe a study of TylM1, a dimethyltransferase from *Streptomyces fradiae* involved in the production of dTDP-mycaminose. From this investigation, the manner in which the enzyme binds its dimethylated product has been revealed. More significantly, by providing the enzyme with an alternative substrate, it was possible to produce a monomethylated product not observed in nature. This has important ramifications for the production of unique carbohydrates that may prove useful in drug design.

Dimethylated aminosugars such as D-desosamine and D-mycaminose are found on a variety of natural products, including erythromycin, azithromycin, spiramycin, and tylosin. Studies suggest that these unusual carbohydrates play key roles in the biological activities of the compounds to which they are attached.[1] In bacteria, the first step for the production of these methylated sugars is the attachment of a nucleoside monophosphate to glucose 1-phosphate.[2] This is followed by a series of enzymatic transformations, including dehydrations, isomerizations, and aminations. In all the pathways, the final step involves the dimethylation of the dTDP-linked sugar by an *N*,*N*-dimethyltransferase using *S*-adenosylmethionine (SAM) as the methyl donor. The first model of an *N*,*N*-dimethyltransferase to be reported was that of DesVI from *Streptomyces venezuelae*.[3] It is involved in the production of D-desosamine. Whereas the DesVI structure provided an initial glimpse into the three-dimensional architecture of a sugar *N*,*N*-dimethyltransferase, details of its active site geometry were limited because of the lack of a bound dTDP-sugar substrate. Several years later the structure of TylM1 from *Streptomyces fradiae* was determined in the presence of *S*-adenosylhomocysteine (SAH) and its natural substrate, dTDP-3-amino-3,6-dideoxyglucose (Scheme 1).[4]

Scheme 1. Reaction Catalyzed by TylM1



dTDP-3-amino-3,6-dideoxyglucose dTDP-mycaminose

A model of TylM1 with bound SAM and dTDP-phenol was also reported. Taken together, these two structures provided a snapshot of the Michaelis complex as shown in Figure 1. As can

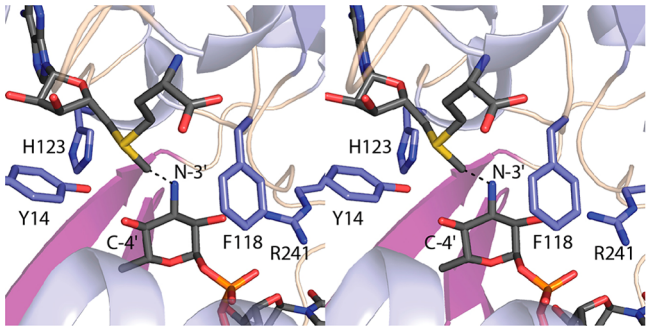


Figure 1. Model of the Michaelis complex in stereo. The C-3′ amino group is positioned to attack the methyl group of SAM as indicated by the dashed line.

be seen, the SAM and dTDP-3-amino-3,6-dideoxyglucose ligands are aligned for a direct in-line displacement reaction. Interestingly, the active site is devoid of catalytic bases near the sugar C-3′ amino group, suggesting that the proton on the nitrogen is transferred directly to one of the water molecules lining the active site cleft.

We were curious about the manner in which a dimethylated dTDP-sugar binds in the active site of TylM1 and, more importantly, whether we could produce a new dimethylated sugar using dTDP-3-amino-3,6-dideoxygalactose as a substrate.

The first structure determined for this investigation was that of TylM1 in complex with SAH and dTDP-mycaminose [the dimethylated sugar (Scheme 1)]. The model was refined to a nominal resolution of 1.6 Å with an $R_{overall}$ of 18.2% and an R_{free} of 21.6% (Tables S1 and S2 of the Supporting Information).

The observed electron density corresponding to dTDP-mycaminose is shown in Figure 2. Within experimental error, the structures of TylM1 with either bound dTDP-substrate or dTDP-product are virtually identical (225 α-carbons superimpose with a root-mean-square deviation of 0.32 Å). Only a water molecule is expelled from the active site to accommodate one of the methyl substituents on the C-3′ amino group.

A close-up view of the dTDP-mycaminose binding pocket is displayed in Figure 3. The sugar C-2′ hydroxyl lies within hydrogen bonding distance of the guanidinium group of Arg 241, whereas the C-4′ hydroxyl participates in hydrogen

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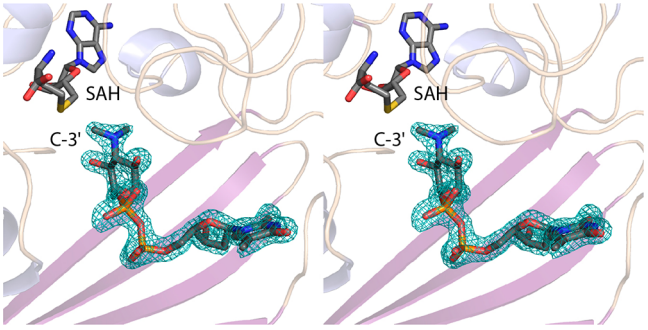

Figure 2. Electron density for dTDP-mycaminose. The map, contoured at 4σ, was calculated with coefficients of the form $F_o - F_c$, where F_o was the native structure factor amplitude and F_c was the calculated structure factor amplitude. Coordinates for the dTDP-mycaminose ligand were never included in the map calculation. All figures were prepared with PyMOL.[5]

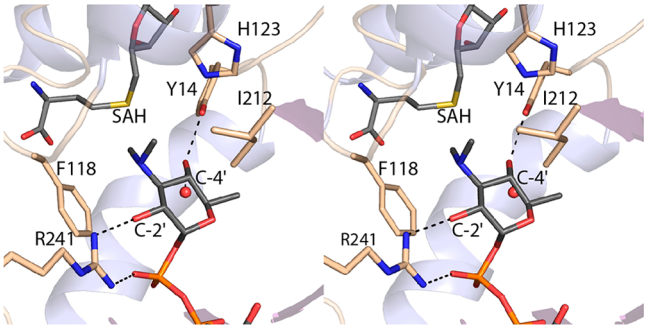

Figure 3. Binding pocket for dTDP-mycaminose. Potential hydrogen bonding interactions are indicated by the dashed lines.

bonding interactions with the side chain of Tyr 14 and a water molecule. One of the methyl substituents on the sugar C-3′ nitrogen lies within 3.6 Å of the sulfur of SAH and the side chain of Phe 118. The other methyl group projects toward a rather open pocket bounded by His 123 and Ile 212.

Given that the protein region surrounding the C-4′ hydroxyl group of dTDP-3-amino-3,6-dideoxyglucose is quite open (Figure 1), we reasoned that TylM1 could also function as an *N*,*N*-dimethyltransferase on dTDP-3-amino-3,6-dideoxygalactose. These two substrates differ only in the stereochemistry about C-4′. Accordingly, we first investigated the kinetic properties of the two substrates (details of the assay can be found in the Supporting Information). For dTDP-3-amino-3,6-dideoxyglucose, the K_m and k_{cat} values were determined to be 0.079 ± 0.015 mM and 0.75 ± 0.09 s^{-1}, respectively. Analysis by mass spectroscopy in negative ion mode gave the expected mass of 574 for an *N*,*N*-dimethylated product.

Using dTDP-3-amino-3,6-dideoxygalactose as the substrate, the K_m and k_{cat} values were determined to be 1.54 ± 0.08 mM and 0.61 ± 0.07 s^{-1}, respectively. Importantly, mass spectroscopic data yielded only a peak at 560, which is consistent for a monomethylated rather than a dimethylated dTDP-sugar product (namely dTDP-3-*N*-methylamino-3,6-dideoxygalactose). Indeed, under all experimental conditions employed, there was never any evidence for the production of the dimethylated dTDP-sugar product. To the best of our knowledge, this represents the first report of the enzymatic production of dTDP-3-*N*-methylamino-3,6-dideoxygalactose, which is not observed in nature.

To further explore the manner in which this unusual monomethylated sugar binds to TylM1, the second structure determined in this investigation was that of the enzyme in complex with SAH and dTDP-3-*N*-methylamino-3,6-dideoxygalactose. The structure was determined to a nominal resolution of 2.2 Å ($R_{overall}$ = 21.9%; R_{free} = 27.7%). Electron density corresponding to the bound dTDP-sugar is displayed in Figure 4.

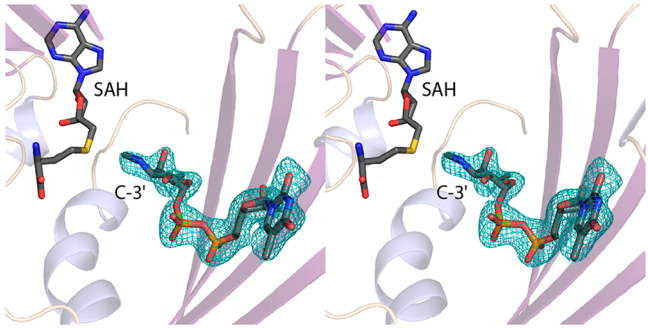

Figure 4. Electron density for dTDP-3-*N*-methylamino-3,6-dideoxygalactose. The map was contoured at 4σ and calculated as described in the legend of Figure 2.

The α-carbons for the two models, with bound dTDP-mycaminose or dTDP-3-*N*-methylamino-3,6-dideoxygalactose, superimpose with a root-mean-square deviation of 0.32 Å. Within experimental error, there is no difference in the active site geometry upon the binding of either dTDP-linked sugar.

On the basis of the kinetic parameters determined *in vitro*, TylM1 functions more efficiently on dTDP-3-amino-3,6-dideoxyglucose versus dTDP-3-amino-3,6-dideoxygalactose. The parameter affected is the K_m. Assuming that the dTDP-sugar products bind similarly to TylM1 as the dTDP-sugar substrates, a superposition of the two structures determined in this investigation suggests a possible explanation. As shown in Figure 5, and with the knowledge that O^{η} of Tyr 14 is sp^2-hybridized, the geometry for the hydrogen bond in the TylM1/dTDP-mycaminose complex is more optimal in terms of distance and angle. Specifically, in the TylM1/dTDP-mycaminose complex, the distance is 2.6 Å and the angle is 117°, whereas in the TylM1/dTDP-3-*N*-methylamino-3,6-

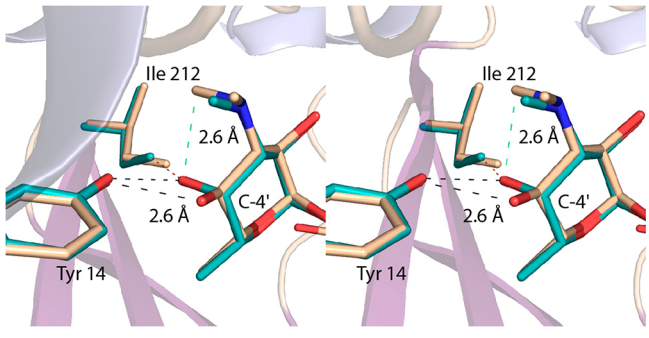

Figure 5. Comparison of the binding of dTDP-mycaminose and dTDP-3-*N*-methylamino-3,6-dideoxygalactose. Only the region immediately surrounding the pyranosyl moiety of the dTDP-sugars is shown. The model of dTDP-mycaminose and the associated protein side chains are shown in wheat. The model of dTDP-3-*N*-methylamino-3,6-dideoxygalactose and the associated protein side chains are displayed in teal.

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dideoxygalactose model, the distance is 3.0 Å and the angle is 154°. Furthermore, because of the change in configuration about the C-4′ hydroxyl group in dTDP-3-*N*-methylamino-3,6-dideoxygalactose, steric hindrance occurs between it and $C^{\delta 1}$ of Ile 212 (3.2 Å). This does not occur when dTDP-mycaminose is bound in the active site region.

One question that arises from this investigation is why dTDP-3-amino-3,6-dideoxygalactose can be only monomethylated under all the experimental conditions employed. The answer lies not in the protein region surrounding the dTDP-sugar but rather in the sugar itself. Placing a second methyl group on the C-3′ amino moiety would create an unacceptable steric clash between it and the C-4′ hydroxyl of dTDP-3-*N*-methylamino-3,6-dideoxygalactose as indicated by the green dashed line in Figure 5.

In summary, we have shown the manner in which a sugar *N*,*N*-methyltransferase can accommodate a dimethylated product within its active site pocket. More importantly, however, this investigation demonstrates that TylM1 can be utilized for the production of a novel monomethylated sugar. Indeed, the biosynthesis of rare sugars via enzymatic routes has attracted significant research attention over the past several years, in part because of the key biological roles they often play in anticancer, antibacterial, antifungal, and antiviral agents.[2] Others have been found, for example, to function as insecticides or as food sweeteners.[6,7] By the judicious use of various dTDP-linked sugars, it should be possible to produce additional novel carbohydrates. This work is in progress.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and Tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates have been deposited in the Protein Data Bank as entries 4OQD and 4OQE.

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Notes

The authors declare no competing financial interest.

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