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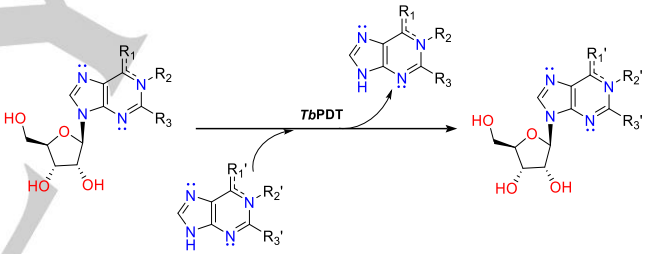
Structure-guided tuning of a selectivity switch towards ribonucleosides in *Trypanosoma brucei* purine nucleoside 2'-deoxyribosyltransferase

Jon Del Arco,[a] Alberto Mills,[b] Federico Gago,[b] Jesús Fernández-Lucas*[a,c]

Abstract: The use of nucleoside 2'-deoxyribosyltransferases (NDTs) as biocatalysts for the industrial synthesis of nucleoside analogues is often hindered by their strict preference for 2'-deoxyribonucleosides. We now show that a highly versatile purine nucleoside 2'-deoxyribosyltransferase from *Trypanosoma brucei* (*Tb*PDT) can also accept ribonucleosides as substrates, most likely because of the distinct role played by Asn53 at a position that is usually occupied by Asp in other NDTs. Moreover, this unusual activity was improved ~3-fold by introducing a single amino acid replacement at position 5 following a structure-guided approach. Biophysical and biochemical characterization revealed that the *Tb*PDT$_{Y5F}$ variant is a homodimer that displays maximum activity at 50 ºC and pH 6.5 and shows a remarkably high melting temperature of 69 ºC. Substrate specificity studies demonstrated that 6-oxopurine ribonucleosides are the best donors (inosine > guanosine >> adenosine) whereas no significant preferences exist between 6-aminopurines and 6-oxopurines as base acceptors. In contrast, no transferase activity could be detected on xanthine and 7-deaza purines. *Tb*PDT$_{Y5F}$ was successfully employed in the synthesis of a wide range of modified ribonucleosides containing different purine analogues.

The use of modified nucleosides as antitumor, antiviral and antimicrobial drugs is well established.[1] Because of this, interest in stereoselective, sustainable and cheap synthetic methods keeps growing in the pharmaceutical industry. Due to their versatility under mild reaction conditions, together with unique chemo-, regio- and enantioselectivities, enzymatic approaches are partially or totally replacing traditional multi-step chemical methods, which often require the presence of organic reagents and tedious purification processes. In this context, glycosyltransferases arise as cost-effective and sustainable alternatives for the enzymatic syntheses of a wide variety of natural nucleosides and nucleoside analogues (NAs) by employing direct one-pot transglycosylation reactions,[2] most frequently those involving a purine and/or pyrimidine nucleoside as the donor and a purine and/or pyrimidine nucleobase as the acceptor. In this regard, nucleoside 2′-deoxyribosyltransferases (NDTs)[3] (EC 2.4.2.6) catalyze a transglycosylation reaction consisting of the exchange of the 2′-deoxyribose moiety between a purine and/or pyrimidine nucleoside and a purine and/or pyrimidine base.[4] NDTs are highly specific for 2′-deoxyribonucleosides and generally display poor activity on modified C2′ and C3′ nucleosides.[4b, 5] The fact that no wild-type NDT or any of their reported variants has shown activity on ribonucleosides so far is seen as an undesirable substrate limitation. The present work describes, for the first time to the best of our knowledge, the ability of a versatile purine NDT (PDT) from *Trypanosoma brucei* (*Tb*PDT)[4b] to use ribonucleosides as substrates (**Scheme 1**) and how this unusual ribosyltransferase activity was enhanced in an engineered Y5F enzyme variant. We also demonstrate that this decreased stringency towards the pentose relies heavily on the presence of Asn53 in a position usually occupied by Asp in other family members.



Scheme 1. Purine nucleoside 2'-ribosyltransferase activity of *Tb*PDT.

A multiple sequence alignment of *Tb*PDT versus *Leishmania mexicana* PDT (*Lm*PDT) and their closest fully characterized homologous NDTs from *Lactobacillus* species reveals that PDTs from trypanosomatides display the canonical "NDT domain" (Pfam PF05014)[4a] that is also shared by nucleoside and 2´-deoxynucleoside 5'-monophosphate *N*-glycosidases. These latter enzymes catalyse the hydrolytic cleavage of the glycosydic bond to yield a free nucleobase and the corresponding phosphorylated sugar.[6] Interestingly, the strict specificity of *N*-glycosidases towards 2′-ribosyl or 2′-deoxyribosyl groups was attributed, upon resolution of the X-ray crystal structure of *Streptomyces rimofaciens* cytidine-5'-monophosphate (CMP) hydrolase (*Sr*MilB) in complex with CMP, to the presence of either a Phe or Tyr side chain close to the catalytic Glu residue. Further biochemical work indeed revealed that the replacement of Phe with Tyr in *Sr*MilB results in a dramatic reversal of substrate specificity from CMP to dCMP.[6d]

Inspired by these reported findings and knowing the relative laxity of wild-type *Tb*PDT in the recognition of both C2′- and C3′-modified nucleosides,[4b] we reasoned that the reverse replacement of Tyr with Phe at the equivalent position in

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*Tb*PDT could enhance the ability of this enzyme to accept ribonucleosides as substrates. Molecular dynamics (MD) simulations of wild-type and variant enzymes revealed hydrogen-bonding patterns in the active site (**Figure S3**) that made us think that this unusual tolerance to the C2' hydroxyl is likely due to the presence of Asn53 in a position generally occupied by an aspartic acid in other members of the family[3-4] (**Figure 1**). Thus, to assess the role of both Tyr5 and Asn53 in sugar recognition and the distinct interplay among the sugar hydroxyls and the side chains of residues Glu82, Asn53 and Tyr5, the mutant genes ndt_{Y5F}, ndt_{N53D} and $ndt_{Y5F/N53D}$ were cloned and overexpressed in *E. coli* BL21(DE3), as described before, and the recombinant *N*-terminal His_6-tagged variants were purified by using Ni-affinity and size-exclusion chromatography.[4b] SDS-PAGE analysis of the purified enzymes showed only one protein band with an apparent molecular mass of 19.0 kDa, which is very close to the value calculated from their amino acid sequence (**Figure S2**).

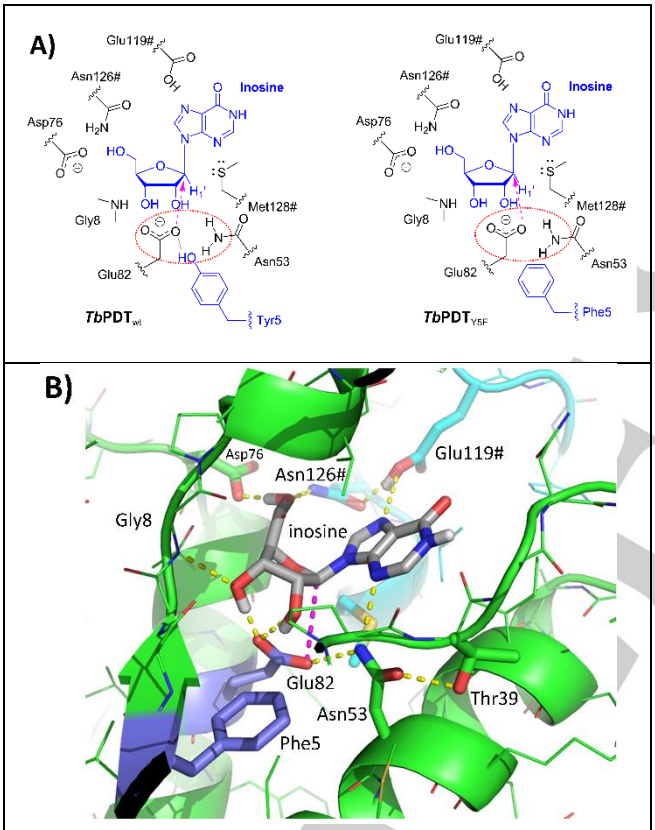

Figure 1. A) Schematic drawing of the binding of Ino within the active site of wild-type *Tb*PDT (left) and *Tb*PDT_{Y5F} (right). The encircled areas focus on the hydrogen-bonding side chains involved in orienting the carboxylate for nucleophilic attack on C1' (magenta arrow). **B)** 3D view of the active site of *Tb*PDT_{Y5F} (C atoms in green for one monomer and cyan for the other) in complex with Ino (C atoms in grey). Enzyme residues relevant for the discussion are shown as sticks and have been labeled. Yellow dashed lines represent hydrogen bonds whereas the magenta broken line stands for the direction of nucleophilic attack.

Wild-type *Tb*PDT (*Tb*PDT_{wt}) and its *Tb*PDT_{Y5F}, *Tb*PDT_{N53D}, and *Tb*$PDT_{Y5F/N53D}$ variants were assayed for the enzymatic synthesis of Ado and dAdo using Ino or dIno as the nucleoside donors, respectively, and Ade as the base acceptor. The single amino acid replacement Y5F resulted in a ~4-fold decrease in 2'-deoxyribosyltransferase activity and a ≥3-fold increase in ribosyltransferase activity depending on the pH (**Table 1**). Thus, the discrimination between 2'-deoxyribo- and ribonucleosides was reduced by a factor of 10. Interestingly, our MD simulation results showed that the single replacement of Tyr by Phe at position 5 leads to an improved hydrogen-bonding interaction between the 2′-OH from the ribose moiety and one of the Glu82 carboxylate oxygens (OE1), so that the other carboxylate oxygen (OE2) more easily adopts a proper orientation for attack on the anomeric C1′ of the ribose (**Figure 1**). We note, however, that while this indicates feasibility of the Michaelis complex, it does not inform about possible changes in the energy barriers separating substrates and products along the coordinates of the two half-reactions.

Table 1. 2'-deoxyribosyl- (dR) and ribosyltransferase (R) activities of wild-type and variant *Tb*PDTs using either deoxyinosine (dIno) or inosine (Ino) as the nucleoside donor and adenine (Ade) as the base acceptor.

	Specific activity (IU/mg protein)		
	dIno + Ade[a]	Ino + Ade[b]	dR/R selectivity ratio
pH 6.0[c]			
Wild type	38.5 ± 3.3	0.9 ± 0.1	42.8
Y5F	11.3 ± 2.2	2.7 ± 0.3	4.2
N53D	11.0 ± 2.3	0.2 ± 0.0	55.0
Y5F/N53D	7.3 ± 1.2	1.6 ± 0.0	4.6
pH 4.0[d]			
Wild type	47.5 ± 1.3	<0.1 ± 0.0	475
Y5F	12.3 ± 2.4	0.5 ± 0.3	24.6
N53D	47.5 ± 1.0	n.d.	47.5
Y5F/N53D	11.1 ± 1.5	<0.1 ± 0.1	11.1

[a] 0.33 µg of enzyme in 40 µL at 50 °C, 5 min. [Substrates] = 10 mM.
[b] 1 µg of enzyme in 40 µL at 50 °C, 5 min. [Substrates] = 2 mM.
[c] 50 mM sodium phosphate buffer.
[d] 50 mM sodium citrate buffer.

The biochemical characterization confirmed that *Tb*PDT_{Y5F} is, like *Tb*PDT_{wt}, a homodimer and revealed that it is active in a 40-60 °C temperature interval (>60 % retained activity), with a peak value at 50 °C (**Figure 2A**). Moreover, the pH profile showed >60 % retained activity in the pH range 5-7 and optimal activity at pH 6-7 (**Figure 2B**). In view of these findings, 50 mM sodium phosphate pH 6.5 and 50 °C were chosen as suitable conditions for further studies. The thermal denaturation curves[7] allowed us to estimate for *Tb*PDT_{Y5F} a T_m of 69 °C (**Figure 2C**), which compares favorably with those of NDTs from the mesophilic *Lactobacillus reuteri* (T_m = 64 °C)[5a] and *Bacillus psychrosaccharolyticus* (T_m = 49 °C).[8]

This unusually high T_m value is most likely due to the presence of a disulfide bridge between the spatially very close Cys77 and Cys77# residues from each monomer, as suggested earlier for *Tb*PDT_{wt}.[4b] This hypothesis is supported by the fact that an engineered C77S variant (*Tb*PDT_{C77S}) displayed, unlike *Tb*PDT_{wt}, a drastic loss of activity over the course of 2 h upon incubation at 50 °C for 8 h (**Figure S3**).

The hydrogen-bonding interaction network depicted in **Figure 1** is facilitated by the presence of Asn53 in a position that is occupied by an aspartic acid in many other members of the NDT family. The finding that this asparagine is strictly conserved in trypanosomatidae[4] led us to assess the interplay between the catalytic Glu82 and the spatially close Tyr5 and Asn53 residues. The N53D substitution resulted in

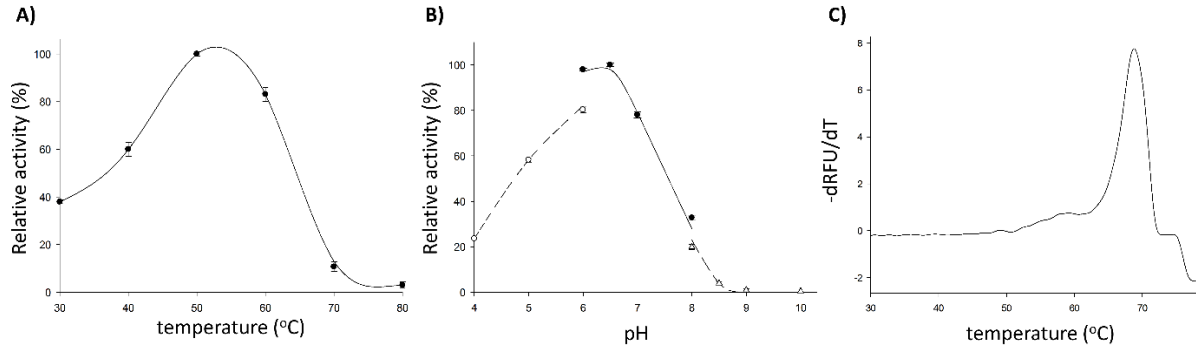

Figure 2. Ribosyltransferase activity and stability profiles of *Tb*PDT$_{Y5F}$. **A)** Temperature dependence of *Tb*PDT$_{Y5F}$ activity. **B)** *Tb*PDT$_{Y5F}$ activity in (○) 50 mM sodium citrate (pH 4-6), (●) 50 mM sodium phosphate (pH 6-8), and (Δ) 50 mM sodium borate (pH 8-10). **C)** Melting of *Tb*PDT$_{Y5F}$ as monitored by differential scanning fluorimetry.[7]

a loss of activity at pH 6 comparable to that previously found for *Tb*PDT$_{Y5F}$ whereas the activity at pH 4 was the same as in *Tb*PDT$_{wt}$ (**Table 1**). Remarkably, catalysis by the *Tb*PDT$_{Y5F/N53D}$ variant containing both amino acid substitutions was as pH-dependent as that of *Tb*PDT$_{wt}$.

A dependency on pH for NDTs in general and *Tb*PDT in particular can be expected from the fact that attack of the catalytic glutamate on C1' is accompanied by proton transfer to N7 of the purine ring from a carboxylic acid, which in the case of *Tb*PDT (**Figure 1A**) belongs to the side chain of Glu$_{119}$ in the other subunit making up the dimer (**Figure 1B**).[4b] Besides, the significant loss of activity at pH 6 observed for *Tb*PDT$_{N53D}$ (**Figure 3**) highlights a significant evolutionary advantage of the wild-type enzyme brought about by use of a protonation-independent asparagine versus a protonation-dependent aspartic acid as the hydrogen bond donor for purine N3 recognition. Poisson Boltzmann electrostatic calculations[9] on *Tb*PDT$_{N53D}$ provide an estimated pK_a value for Asp53 of ~5.2 in both monomers in the absence of substrate. Taken together, our results attest to the importance of subtle hydrogen-bonding rearrangements in active site residues to tip the balance towards catalysis or the stabilization of a non-productive complex (**Figure S1**). These results are in accordance with previous reports for NDTs from *Bacillus psychrosaccharolyticus*[8] and *Lactobacillus delbrueckii*[10] describing the interaction between the side chains of the catalytic Glu and a positionally equivalent Tyr residue as essential for catalysis.

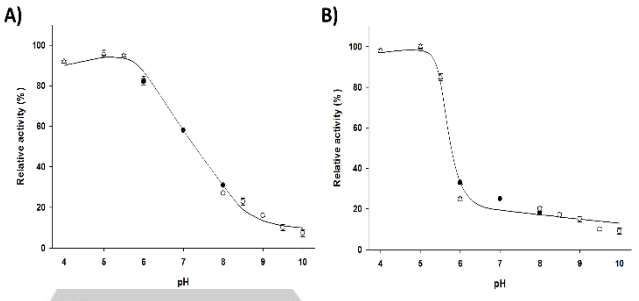

Figure 3. Effect of pH on 2'-deoxyribosyltransferase activity in **(A)** *Tb*PDT$_{wt}$ and **(B)** *Tb*PDT$_{N53D}$. (Δ) 50 mM sodium citrate (pH 4-6), (●) 50 mM sodium phosphate (pH 6-8), and (○) 50 mM sodium borate (pH 8-10).

The biochemical characterization of *Tb*PDT$_{Y5F}$ was completed by assessing its potential to use different natural purine nucleosides and purine bases as donors and acceptors, respectively (**Table 2**).

Table 2. Substrate specificity of *Tb*PDT$_{Y5F}$.

	*Tb*PDT$_{Y5F}$			
Donor \ **Acceptor**	**Ade**	**Gua**	**Hyp**	**Xan**
Ado[a]	-	0.1 ± 0.0	0.1 ± 0.0	n.d.
Guo[b]	2.3 ± 0.1	-	1.9 ± 0.1	n.d.
Ino[b]	2.7 ± 0.1	1.8 ± 0.1	-	n.d.

[a] Reaction conditions: 1 µg of enzyme in 40 µL at 50 °C, 10 min. [Substrates] = 1 mM, 50 mM sodium phosphate buffer, pH 6.5.
[b] Reaction conditions: 1 µg of enzyme in 40 µL at 50 °C, 5 min. [Substrates] = 2 mM, 50 mM sodium phosphate buffer, pH 6.5. n.d. = not detected.

It is noteworthy that *Tb*PDT$_{Y5F}$ shows a remarkable preference for ribonucleosides containing 6-oxopurine over those possessing a 6-amino group; thus, the specific activities were 18-19 times higher when Ino or Guo were used as donors relative to Ado. In contrast, the nature of the base acceptor (Ade, Hyp and Gua) has a negligible effect on the catalytic efficiency save for Xan, which is inactive. In view of our MD simulation results, this finding is most likely due to electrostatic repulsion between the 2-oxo group of the base and carbonyl groups from the flexible recognition loop encompassing ^{39}TDNEATEA^{46} that connects β2 and α3 secondary structure elements (see Pro38 and Thr39 in **Figure 1B**).

Finally, to study the potential of *Tb*PDT$_{Y5F}$ as a biocatalyst, the enzymatic synthesis of several ribonucleosides was carried out using Guo as the donor and different substituted purines (2-ClAde, 2-FAde, 2,6-DAP, 6-ClPur, 6-MP, 6-MeOGua, 6-MetPur, 7-deazaHyp, and 7-deazaXan) or benzimidazole (Bzm) as the acceptors (**Table 3**). As was shown to be the case with *Tb*PDT$_{wt}$,[4b] *Tb*PDT$_{Y5F}$ is highly tolerant to a broad range of purine modifications but 7-deazapurine derivatives are not accepted as substrates. This finding is in consonance with the proposed

catalytic mechanism, which involves glycosydic bond cleavage by nucleophilic attack of Glu82 on C1′ accompanied by proton transfer from Glu119# to N7 of the purine ring (**Figure 1B**).[4b]

To avoid the reverse reaction that could be detrimental to overall yields, the effect on enzyme activity of molar ratio (1 *vs* 3 mM Guo) was also studied. In all cases, an excess of Guo led to improved conversion rates (**Table 3**). Thus, it seems that an excess of ribonucleoside partially shifts the reaction equilibrium towards product formation. Consequently, these experimental results support 3:1 as the optimal nucleoside:base molar ratio for biotransformation using *Tb*PDT$_{Y5F}$.

Table 3. Synthesis of ribonucleoside analogues by *Tb*PDT$_{Y5F}$ using guanosine as the ribosyl donor and several purine base derivatives or analogues as the acceptors.

Donor	Acceptor	Product	Molar ratio	Conversion (%) [a]
Guo	Ade	Ado	1:1	35 ± 4
			3:1	50 ± 2
Guo	Hyp	Ino	1:1	35 ± 4
			3:1	55 ± 1
Guo	Xan	Xao	1:1	n.d.
			3:1	n.d.
Guo	2-ClAde	2-ClAdo	1:1	37 ± 1
			3:1	69 ± 2
Guo	2-FAde	2-FAdo	1:1	48 ± 2
			3:1	68 ± 2
Guo	2,6-DAP	2,6-DAPRib	1:1	60 ± 2
			3:1	67 ± 3
Guo	6-ClPur	6-ClPurRib	1:1	18 ± 1
			3:1	24 ± 4
Guo	6-MP	6-MPRib	1:1	14 ± 3
			3:1	50 ± 2
Guo	6-MeOGua	6-MeOGuo	1:1	65 ± 4
			3:1	83 ± 2
Guo	6-MetPur	6-MetPurRib	1:1	29 ± 4
			3:1	41 ± 2
Guo	7-deazaHyp	7-deazaIno	1:1	n.d.
			3:1	n.d.
Guo	7-deazaXan	7-deazaXao	1:1	n.d.
			3:1	n.d
Guo	Bzm	BzmdRib	1:1	30 ± 3
			3:1	35 ± 4

[a] Reaction conditions: 1 µg of enzyme in 40 µL at 50 ºC, 30 min. [Guo] = 1 or 3 mM, [Base] = 1 mM, 50 mM sodium phosphate buffer, pH 6.5. n.d. = not detected.

In conclusion, *Trypanosoma brucei* purine nucleoside 2'-deoxy-ribosyltransferase (*Tb*PDT) is shown to be the first NDT that accepts ribonucleosides as substrates and the deoxyribose/ribose selectivity ratio improves ~10 fold when Tyr5 is replaced with a phenylalanine using site-directed mutagenesis. Biophysical and biochemical characterization revealed that the *Tb*PDT$_{Y5F}$ variant is a homodimer that displays maximum activity at 50 ºC and pH 6.5 and shows a remarkably high melting temperature of 69 ºC. Substrate specificity studies demonstrated that 6-oxopurine ribonucleosides are the best donors (inosine > guanosine >> adenosine) whereas no significant preferences exist between 6-aminopurines and 6-oxopurines as base acceptors. In contrast, no transferase activity could be detected on xanthine and 7-deaza purines. *Tb*PDT$_{Y5F}$ was successfully employed in the synthesis of a wide range of modified ribonucleosides containing different purine analogues.

Experimental Section

Thermal denaturation

The melting temperature (T_m) was measured using differential scanning fluorimetry in a CFX96 Real-Time PCR Detection System (Bio-Rad, Spain) essentially as described.[7] Briefly, *Tb*PDTY5F was diluted to a final concentration of 20 µM and 18 µL were mixed with 2 µL 100x diluted SYPRO Orange (Sigma-Aldrich, St. Louis, MO). The samples were heated from 35 to 95 °C at 1 °C/min. The fluorescent signal was measured with excitation and emission filters of 500 and 580 nm, respectively. The T_m was approximated to the melt peak obtained from a graph plotting the negative first derivative of the melting curve with respect to the temperature (dRFU/dT *vs* T), where RFU stands for relative fluorescence units.

Computational Methods

The Protein Data Bank entry 2A0K provided the Cartesian coordinates for a 3D model of the apo form of *Tb*PDT solved by X-ray crystallography.[11] Replacement of Tyr5 by a Phe residue in both subunits was accomplished by means of the interactive molecular graphics program PyMOL,[12] which was also employed for structure visualization, molecular editing and figure preparation. Ado and Ino were docked into the active site of *Tb*PDT$_{Y5F}$ as reported earlier for dAdo in *Lm*PDT[4a] and dIno in *Tb*PDT.[4b] Each complex of *Tb*PDT$_{Y5F}$ with either Ino or dIno bound in the active site of both monomers was immersed in a box of ~ 13,400 TIP3P water molecules that extended 25 Å away from any solute atom and 4 Na^+ ions were added to ensure electrical neutrality. Energy minimization followed by unrestrained molecular dynamics simulations for 150 ns were carried out as described before[4b] using the *pmemd_cuda.SPFP* module and the standard ff14SB force field parameter set in AMBER14.[13] The *cpptraj* module[14] in AMBER14 was employed for data processing and geometry analysis of the calculated trajectories.

ABBREVIATIONS

Ade, Adenine; Gua, Guanine; Hyp, Hypoxanthine; Xan, xanthine, 2-ClAde, 2-chloroadenine; 2-FAde, 2-fluoroadenine; 2,6-DAP, 2,6-diaminopurine; 6-ClPur, 6-chloropurine; 6-MP, 6-mercaptopurine; 6-MeGua, 6-methoxyguanine; 6-MetPur, 6-methylpurine; 7-deazaHyp, 7-deazahypoxanthine; 7-deazaXan, 7-deazaxanthine; Bzm, benzimidazole.

dAdo, 2'-deoxyadenosine; dIno, 2'-deoxyinosine; Ado, adenosine; Ino, inosine Guo, guanosine; 2-ClAdo, 2-chloroadenosine; 2F-Ado, 2-fluoroadenosine; 2,6-DAPdRib, 2,6-DAP riboside; 6-ClPurRib, 6-ClPur riboside; 6-MPRib, 6-MP riboside; 6-MeOGuo, 6-methoxyguanosine, 6-MetPurRib, 6-MetPur riboside; 7-deazaIno, 7-deazainosine, 7-deazaXao, 7-deazaxanthosine and BzmRib, Bzm riboside.

Acknowledgements

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Keywords: biocatalysis • nucleoside analogues • 2'-deoxy-ribosyltransferase • enzyme immobilization • molecular dynamics

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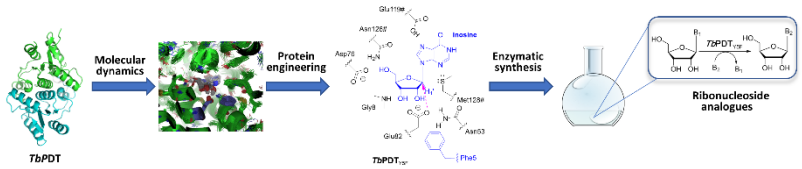
Structure-guided tuning of a selectivity switch towards ribonucleosides in *Trypanosoma brucei* purine nucleoside 2'-deoxyribosyltransferase



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A ~3 fold improvement in enzyme activity towards ribonucleosides of a purine 2'-deoxyribosyltransferase from *Trypanosoma brucei* (*Tb*PDT) was achieved upon replacement of Tyr with Phe at position 5 following a structure-guided approach. The *Tb*PDT$_{Y5F}$ variant was biophysically and biochemically characterized and successfully employed in the synthesis of a wide range of modified ribonucleosides containing different purine analogues.