Enzymatic Synthesis of Therapeutic Nucleosides using a Highly Versatile Purine Nucleoside 2'-Deoxyribosyltransferase from *Trypanosoma brucei*

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The use of enzymes for the synthesis of nucleoside analogues offers several advantages over multistep chemical methods, including chemo-, regio- and stereoselectivity as well as milder reaction conditions. Herein, the production, characterization and utilization of a purine nucleoside 2'-deoxyribosyltransferase (PDT) from *Trypanosoma brucei* are reported. *TbPDT* is a dimer which displays not only excellent activity and stability over a broad range of temperatures (50–70 °C), pH (4–7) and ionic strength (0–500 mM NaCl) but also an unusual high stability under alkaline conditions (pH 8–10). *TbPDT* is shown to be proficient in the biosynthesis of numerous therapeutic nucleosides, including didanosine, vidarabine, cladribine, fludarabine

and nelarabine. The structure-guided replacement of Val11 with either Ala or Ser resulted in variants with 2.8-fold greater activity. *TbPDT* was also covalently immobilized on glutaraldehyde-activated magnetic microspheres. *MTbPDT3* was selected as the best derivative (4200 IU/g, activity recovery of 22 %), and could be easily recaptured and recycled for > 25 reactions with negligible loss of activity. Finally, *MTbPDT3* was successfully employed in the expedient synthesis of several nucleoside analogues. Taken together, our results support the notion that *TbPDT* has good potential as an industrial biocatalyst for the synthesis of a wide range of therapeutic nucleosides through an efficient and environmentally friendly methodology.

Introduction

Nucleosides are known to be ubiquitous molecules in crucial biochemical processes including genetic information storage and transfer. There are many examples in the literature of nucleoside analogues that are therapeutically used as antimicrobial, antiviral and anticancer agents or employed as starting materials for the preparation of antisense oligonucleotides.^[1] These molecules have been traditionally synthesized by different chemical methods through multistep processes that require protection and de-protection reactions as well as isolation of intermediates.^[2] In contrast, the enzymatic synthesis of nucleoside analogues is an alternative that shows many advantages, such as (i) high chemo-, regio- and stereoselectivity, (ii) the possibility of being achieved in one-pot, one-step reactions under mild conditions, and (iii) use of an environmentally friendly technology.^[3]

Nucleoside 2'-deoxyribosyltransferases (NDTs) have been generally employed in the synthesis of nucleosides because they catalyze the exchange of 2'-deoxyribose between purine and/or pyrimidine bases.^[3b,4] According to their substrate specificity, NDTs have been classified as type I, which transfer the glycosyl moiety from purine 2'-deoxyribonucleosides to purine bases (Pur↔Pur) such as adenine **1**, guanine **2** or hypoxanthine **3** (Figure 1), and type II, which also accept pyrimidines as substrates (Pur↔Pur, Pur↔Pyr, Pyr↔Pyr).^[3b,4–5] For this reason, type I NDTs are also known as purine-nucleoside 2'-deoxyribosyltransferases (PDTs). Both type I and type II NDTs are (i) specific for 2'-deoxyribonucleosides, (ii) regioselective (N-1 and N-9 glycosylation in pyrimidines and purines, respectively), and (iii) stereoselective (β -anomers are exclusively

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formed). However, some studies have shown that NDTs can tolerate modifications at C2' and C3' of the 2'-deoxyribose sugar.^[3b,4,6]

Despite the advantages of enzyme-mediated bioconversions, their application in industry is limited by several factors, including: i) the high cost of recombinant enzymes, ii) the low stability and short lifespan of the enzyme subjected to the rather harsh conditions often needed in some industrial processes, and iii) difficulties in the isolation of the biocatalyst from the reaction medium for reutilization. Enzyme immobilization may overcome these problems by enhancing protein stability, facilitating separation of products, and allowing the biocatalyst to be reused in various reactor configurations. In this area, our research group has been largely involved in the development of immobilized biocatalysts for the synthesis of therapeutic nucleoside analogues.^[3b,4b,7] Recently, enzyme binding to magnetic supports has emerged as an alternative method that provides immobilized biocatalysts which are easily separated from the reaction medium by applying a magnetic field.^[7–8] Moreover, these magnetic biocatalysts can be employed in fluidized bed reactors, which are stabilized by a magnetic field, or in magnetic rod-driven stirred tank reactors, in which deterioration of the biocatalyst due to mechanical agitation is avoided (Figure 1).

In our search for PDTs suitable as biocatalysts for the synthesis of non-natural therapeutic nucleosides, we found a good candidate in an uncharacterized protein encoded in the genome of *Trypanosoma brucei* (*TbPDT*) that shares 67% sequence identity with a previously reported PDT from *Leishmania mexicana* (*LmPDT*).^[9] The putative enzyme was produced and characterized using both biophysical and biochemical methods. Studying the effects of pH, temperature and ionic strength on enzyme activity and stability allowed us to select a set of optimal operative conditions for the synthesis, in aqueous media, of several nucleoside analogues with therapeutic application (**4–10** in Figure 2). In addition, structure-guided site-directed mutagenesis studies led us to produce two *TbPDT* variants which showed enhanced activity over that of the wild-type enzyme in the synthesis of 2'-deoxyadenosine. Finally, to explore its potential as a biocatalyst, *TbPDT* was immobilized onto magnetic microspheres, which were physically characterized by scanning electron microscopy (SEM) and dynamic light scattering (DLS).

Figure 1. Enzymatic synthesis of nucleosides by 2'-deoxyribosyltransferase immobilized on magnetic microspheres.

Results and Discussion

Production and Purification of *TbPDT*

The *pdt* gene of *Trypanosoma brucei*, annotated as encoding a putative 2'-deoxyribosyltransferase, was cloned and overexpressed in *E. coli* BL21 (DE3). The recombinant *N*-terminal His₆-tagged *TbPDT* was purified by using two chromatographic steps. SDS-PAGE analysis of the purified enzyme showed a single protein band with an apparent molecular mass of 19 kDa (Figure S1, Supporting Information). *N*-terminal His₆-tagged *TbPDT* was predicted to contain 169 amino acid residues and to possess a relative molecular mass of 19.09 kDa (Protparam, <http://web.expasy.org/protparam>). These results agree with those described by Bosch *et al.*^[10] who reported the crystal structure of the same protein but did not carry out any functional characterization that could support its activity as a true NDT.

Sedimentation velocity experiments reveal *TbPDT* as a single species with an experimental sedimentation coefficient of 3.17 S ($s_{20,w} = 3.19$) compatible with an obligate homodimer of 39.07 kDa molecular weight. Thus, the single monomer affords a value of 19.53 kDa, which is very close to the molecular mass calculated from the amino acid sequence (19.09 kDa). In this respect, different oligomeric states have been described for various bacterial and protozoan NDTs. On the one hand, NDTs from *Lactobacillus leichmanii*, *L. helveticus* (*LhNDT*) and *L. reuteri* (*LrNDT*) appear as hexamers (displaying a molecular weight ranging from 105 to 120 kDa),^[4,11] whereas a type II NDT from *Lactococcus lactis* was characterized as a tetramer of 69 kDa.^[12] On the other hand, we recently reported *LmPDT* as a dimer^[9] whose activity is similar to that of its bacterial counterparts (Table 1). These findings support the view that a higher oligomerization state for NDTs is not essential for catalysis and/or stabilization of the active conformation.

Figure 2. Chemical structures of the most energetically stable tautomeric forms of (i) purine bases adenine (**1**), guanine (**2**) and hypoxanthine (**3**), (ii) 2'-deoxyribonucleosides cladribine (**4**), 2F-dAdo (**5**) and 6-MPdRib (**6**), (iii) arabinosyl nucleosides vidarabine (**7**), fludarabine (**8**) and nelarabine (**9**), and (iv) 2',3'-dideoxyinosine (**10**).

Table 1. Synthesis of natural nucleosides catalyzed by *TbPDT* and other PDTs.

Acceptor ^[b] /Donor	Ade	<i>TbPDT</i>		Specific activity (IU/mg protein)						<i>LmPDT</i> ^[a]	
		Gua	Hyp	<i>LhNDT</i> ^[a]		<i>BbPDT</i> ^[a]				Gua	Hyp
dAdo	–	10.5 ± 2.3	9.5 ± 2.4	–	n.d.	0.06	–	0.09	0.02	–	75.4
dGuo	21.4 ± 3.1	–	39.0 ± 2.6	0.05	–	0.05	0.08	–	0.02	52.7	68.6
dIno	38.5 ± 3.3	11.0 ± 2.1	–	0.08	n.d.	–	0.04	0.04	–	73.1	–

[a] Activities for *LhNDT*, *BbPDT* and *LmPDT* were taken from Refs. [4a], [5] and [9], respectively; [b] reaction conditions were 0.3 µg of enzyme in 40 µL at 50 °C for 5 min. Ade and Hyp concentrations were 10 mM in 50 mM sodium phosphate buffer, pH 6.0, whereas that of Gua was 1 mM in 50 mM borate buffer, pH 8.5; n.d.: not detected.

Biochemical Characterization of *TbPDT*

TbPDT is highly active (> 63%) across a broad temperature range (from 50 to 70 °C), with an optimum at 60 °C (Figure 3A). The pH profile reveals that *TbPDT* is more than 60% active over a broad pH range (from 4 to 7) and displays maximum activity at a pH between 5 and 6 (Figure 3B). In addition, negligible loss of activity is observed in the presence of 500 mM NaCl, and only a slight decrease is observed when this concentration is increased (i.e. 70% activity remains at 1 M NaCl) (Figure 3C).

The effect of pH on *TbPDT* stability was evaluated by incubating the enzyme for 30 h at 50 °C at different pH values (ranging from 6.5 to 10). The results suggest that alkaline environments (pH 8–10) do not inactivate the enzyme during

this time period to a significant extent (Figure 3D). This unusual tolerance to alkaline conditions was unexpected for a mesophilic enzyme and is a very interesting advantage for enzyme immobilization, as will be described below.

The stability of *TbPDT* as a biocatalyst was assessed by showing that the enzyme stored at 4 °C still displays 85% relative activity after 180 days (data not shown). Nonetheless, this percentage drops to ~23% when *TbPDT* is stored at –20 °C or –80 °C.

Figure 3. Dependence of *TbPDT* activity and stability on pH and temperature. A) Effect of temperature on *TbPDT* activity. B) Effect of pH on *TbPDT* activity, (Δ) sodium citrate 50 mM (pH 4–6), (●) sodium phosphate 50 mM (pH 6–8), (○) sodium borate 50 mM (pH 8–10). C) Effect of ionic strength on *TbPDT* activity, and D) Time course of the thermal inactivation of *TbPDT* at 50 °C in 10 mM sodium phosphate pH 6.5 (●), and 10 mM sodium borate pH 8 (○) and pH 10 (▲).

Substrate Specificity and Structural Considerations

Our experimental data show that *TbPDT* should be classified as a type I NDT since it exclusively catalyzes the transfer of 2'-deoxyribose between purine bases. In common with other previously described PDTs,^[4a,5,9] *TbPDT* recognizes 2'-deoxyinosine (dIno) and hypoxanthine (Hyp) as the best nucleoside donor and base acceptor, respectively (Table 1).

Because type II NDTs offer several advantages over PDTs, such as higher activity values under the same experimental conditions or the possibility of using a broad range of substrates, type II NDTs usually represent the best option for the enzymatic synthesis of nucleoside analogues. Nevertheless, the specific activities of *TbPDT* are higher than those reported for other PDTs from *Lactobacillus* and *Borrelia* species under similar enzymatic assay conditions (Table 1) and comparable to those previously described for several type II NDTs from *Lactobacillus reuteri*^[4b] and *Lactobacillus delbrueckii*.^[13] *TbPDT* can also use as substrates the manifold of purine derivatives and nucleoside analogues displayed in Tables 2 and 3, and it is to be preferred, in principle, over *LmPDT*^[9] because it can transglycosylate on a larger number of purine analogues when dIno is used as the donor nucleoside (data not shown). These results encouraged us to consider *TbPDT* a good candidate for the enzymatic synthesis of nucleoside analogues.

TbPDT is a homodimer which displays the typical 2'-deoxyribose binding site motif made up of two acidic residues from one subunit (Asp76 and Glu82) and one polar residue from the characteristic NLM motif in the neighboring monomer (Asn126#). The 5'-hydroxyl group of the incoming 2'-deoxynucleoside hydrogen bonds to the carboxylate of Asp76 and is hydrogen bonded by the carboxamide of Asn126#. The nucleophilic attack on C1' of the sugar is effected by the side-chain carboxylate of Glu82, which is held in position by hydrogen bonds donated by the phenol group of Tyr5 and the 3'-hydroxyl of the sugar (Figure 4). In the near-attack conformation,^[14] one of the lone pairs of the M128# sulfur is poised to interact with the developing positive charge on the oxocarbenium ion of the transition state. The purine ring is sandwiched

between the hydrophobic side chains of Val11 and Phe12, on one side, and those of Ile49 and Leu127# on the other side, whereas the nucleobase's N-3 atom is recognized by the carboxamide nitrogen of Asn53. This is a rather unique feature of PDTs from trypanosomatids (family Trypanosomatidae), as recently reported for *LmPDT*,^[9] because NDTs from *Lactobacillus* have an aspartic acid at the equivalent position, which means that this side chain needs to be protonated as a carboxylic acid.^[15] The occurrence of Asn53 in *TbPDT* (and Asn56 in *LmPDT*) in a position usually occupied by Asp in other NDTs may be advantageous for resilience at high pH values.

The results of our molecular dynamics (MD) simulations reveal that, in the Michaelis complex (Figure 4B), the flexible ³⁹TDNEATEA⁴⁶ and ¹¹⁶LRVEGF¹²¹ loops connecting β 2- α 3 and β 6- α 6, respectively, become more ordered because of the involvement of the side chain of Glu42 in base recognition (Figure 4A) and the stacking of the phenyl ring of Phe121# on Ile49 and Leu127#, which results in the approximation of Glu119# towards the N-7 position of the base. In this "closed" conformation, the substrate is placed within a preorganized active site in an orientation conducive to reaction. Thereafter, we postulate that glycosidic bond cleavage by nucleophilic attack of Glu82 on C1' is accompanied by proton transfer from Glu119# to N-7 so that this tautomer is initially generated, in agreement with early data,^[16] prior to departure of the energetically more stable N-9 tautomer.^[17]

To understand the structural determinants of *TbPDT* stabilization, the dimer interface was analyzed with the aid of programs MM-ISMSA^[18] and HBplot,^[19] which provided valuable information about those residues that contribute the most to stabilizing the dimer. The former dissected, on a per-residue basis, the monomer-monomer binding energy (Figure 5), including desolvation, and the latter analyzed the types of hydrogen bonds (backbone-backbone, side chain-backbone, and side chain-side chain) between the two monomers (Figure S2). Our analyses reveal the importance of Phe12, Arg50, Arg71, Phe81, Leu116, Arg117, and Met128 for dimer stabilization. In this respect, it is noteworthy that Arg71 is part of an insertion particular to trypanosomatids that is located in a

Figure 4. A. Schematic drawing of the binding of 2'-deoxyinosine (dIno) within the active site of *TbPDT*. B. Detail of a 3D model of the *TbPDT* active site (C atoms colored in green in one monomer and in cyan in the other) with a bound dIno molecule (C atoms in orange) in the near-attack conformation.^[14] Enzyme residues making up the active site and relevant for catalysis are labeled and shown as sticks.

Table 3. Synthesis of nucleoside analogues from 2',3'-modified nucleosides catalyzed by *TbPDT*.^[a]

Donor	Acceptor	Product	Conversion [%]
ddA	Gua	ddG	24 ± 3
	Hyp	ddl	27 ± 1
	2-FAde	dd2-FAde	16 ± 2
	2-ClAde	dd2-ClAde	n.d.
	2,6-DAP	dd2,6-DAPRib	16 ± 2
	6-ClPur	dd6-ClPurRib	10 ± 3
	6-MP	dd6-MPRib	2
	6-MetPur	dd6-MetPurRib	6 ± 1
	6-MeOGua	dd6-MeOGuo	18 ± 1
	Ade	ddl	17 ± 1
ddl	Gua	ddG	20
	2-FAde	dd2-F-dAdo	–
	2-ClAde	dd2-Cl-dAdo	n.d.
	2,6-DAP	dd2,6-DAPRib	10
	6-ClPur	dd6-ClPurRib	6
	6-MP	dd6-MPRib	2
	6-MetPur	dd6-MetPurRib	4
	6-MeOGua	dd6-MeOGuo	7
ara A	Gua	ara G	43 ± 1
	Hyp	ara H	55 ± 1
	2-FAde	ara 2-FAde	23
	2-ClAde	ara 2-ClAde	13
	2,6-DAP	ara 2,6-DAP	29
	6-ClPur	ara 6-ClPur	7
	6-MP	ara 6-MP	n.d.
	6-MetPur	ara 6-MetPur	15
	6-MeOGua	ara 6-MeOGuo	38 ± 1
ara G	Ade	ara A	37 ± 1
	Hyp	ara H	50 ± 1
	2-FAde	ara 2-FAde	37
	2-ClAde	ara 2-ClAde	17 ± 1
	2,6-DAP	ara 2,6-DAP	28
	6-ClPur	ara 6-ClPur	8
	6-MP	ara 6-MP	21
	6-MetPur	ara 6-MetPur	14
	6-MeOGua	ara 6-MeOGuo	52
2'-F-dIno	Ade	2'-F-dAdo	29
	Gua	2'-F-dGuo	36
	2-FAde	2'-F-2-F-dAdo	21
	2-ClAde	2'-F-2-Cl-dAdo	15
	2,6-DAP	2'-F-2,6-DAPdRib	15
	6-ClPur	2'-F-6-ClPurdRib	4
	6-MP	2'-F-6-MP dRib	10 ± 1
	6-MetPur	2'-F-6-MetPurdRib	4
	6-MeOGua	2'-F-6-MeOdGuo	15

[a] Reaction conditions were 8 µg of enzyme in 40 µL at 50 °C for 4 h. Substrate concentration was 1 mM in 50 mM sodium phosphate buffer, pH 6.0, except for Gua, in which case it was 10 mM in 50 mM sodium borate buffer, pH 8.5. n.d. not detected.

Table 4. Enzymatic activities of wild-type and variant *TbPDT*s in two transglycosylation reactions.^[a]

	Specific activity [IU/mg protein]	
	dIno + Ade	dGuo + Ade
Wild type	38.5 ± 4.2	21.4 ± 3
V11A	84.7 ± 2	50.3 ± 2
V11S	90.7 ± 3	59.9 ± 2

[a] Reaction conditions: 0.3 µg of enzyme in 40 µL at 50 °C, 5 min. [Substrates] = 10 mM, 50 mM sodium phosphate buffer, pH 6.0.

dual role; on the one hand, by stacking on the heterobase, it contributes to the binding energy; on the other hand, its apolar character prevents the approach of incoming water molecules that might be activated for attack on the deoxyribosyl-enzyme intermediate to release the bonded sugar.

To assess this dual role, two variants (*TbPDT*_{V11A} and *TbPDT*_{V11S}) were generated by single amino acid substitutions at position 11 and their transferase activities were determined using Ade as the acceptor base and either dIno or dGuo as the nucleoside donor. The specific activity of each of these two variants was > 2-fold that of the wild-type enzyme (Table 4). To gain further insight into the factor (s) responsible for these differences, steady-state kinetic studies were performed. Our results show that the rates of catalysis for the two half-reactions, i.e. nucleobase release and transglycosylation, are enhanced in both variants, despite the higher K_M values, which probably reflect the expected loss in van der Waals and hydrophobic interactions between enzyme and substrates (Table 5). Since enhanced transition state stabilization associated to increased flexibility of the active site is the single factor thought to be a requirement for reducing the free energy barrier and accelerate the enzymatic reaction,^[27] this must be the overall effect of replacing Val11 with the less bulky Ala or Ser residues. Nonetheless, further experimental and theoretical work will be necessary to ascertain the origin of the improved catalytic efficiencies of these variants.

Immobilization of recombinant *TbPDT* onto glutaraldehyde-activated MagReSyn®Amine microspheres Since immobilization is an essential requirement for the use of enzymes as industrial biocatalysts, we assayed the stability of *TbPDT* under immobilization conditions. Covalent immobilization of proteins is usually achieved via multipoint covalent attachment through

Table 5. Kinetic parameters of wild-type and variant *TbPDT*s for the Glu82-assisted cleavage of the C1'-N9 glycosidic bond of Ino (first half-reaction, leading to enzyme glycosylation and release of free Hyp) and for the overall conversion of dIno + Ade to dAdo + Hyp (transglycosylation reaction).^[a]

Parameter	Wild type	V11A Variable dIno	V11S	Wild type	V11A Variable Ade	V11S
Nucleobase release						
K_M [mM]	0.7 ± 0.2	5.1 ± 1.1	6.8 ± 1.6	0.6 ± 0.2	2.7 ± 0.9	1.4 ± 0.4
k_{cat} [s ⁻¹]	7.9 ± 1.3	39.6 ± 4.0	40.7 ± 3.9	8.7 ± 0.95	21.5 ± 1.6	19.6 ± 1.9
k_{cat}/K_M [s ⁻¹ M ⁻¹]	11314	7765	5985	14500	7962	14000
V_{max}	0.21 ± 0.01	1.05 ± 0.06	1.08 ± 0.09	0.23 ± 0.04	0.57 ± 0.10	0.52 ± 0.09
Transglycosylation						
K_M [mM]	0.2 ± 0.1	1.2 ± 0.4	2.0 ± 0.9	1.8 ± 0.2	2.9 ± 0.7	2.9 ± 0.6
k_{cat} [s ⁻¹]	6.4 ± 1.3	14.3 ± 3.4	17.0 ± 2.6	10.2 ± 1.1	23.4 ± 2.3	23.8 ± 2.9
k_{cat}/K_M [s ⁻¹ M ⁻¹]	30476	11440	8415	5600	8068	8206
V_{max}	0.17 ± 0.01	0.38 ± 0.03	0.45 ± 0.06	0.27 ± 0.04	0.62 ± 0.04	0.63 ± 0.05

the region with the highest density of primary amino groups, especially the ϵ -NH₂ of lysine residues.^[28] These covalent immobilization techniques usually employ long reaction times (4–10 h) and alkaline conditions (pH 8–10). For this reason, we thought that the exceptional stability displayed by *TbPDT* under alkaline conditions could offer a substantial advantage for efficient immobilization on magnetic particles. *TbPDT* immobilization was carried out by covalent attachment of the enzyme to glutaraldehyde-activated MagReSyn®Amine microspheres (Scheme 1).

Different immobilized biocatalysts (*MTbPDT*1–*MTbPDT*5) were prepared by increasing the enzyme load and *MTbPDT*3 was selected as the optimum construct (Table 6) because it showed a remarkably high activity of 4200 IU/g of wet biocatalyst (in the synthesis of dAdo from dlno and adenine at 50 °C and 50 mM sodium phosphate pH 6) and an activity recovery^[29] of ~22%. Noteworthy, *MTbPDT*3 displays the highest activity ever described for an immobilized NDT. The calculated specific activities for *MTbPDT*3 are much higher (between 64.2 and 1615 times) than those reported for other NDTs, such as that from *Lr*NDT immobilized on epoxy-activated Sepabeds® for the enzymatic synthesis of dAdo (65.4 IU/g of wet biocatalyst)^[30] or that from *Bacillus psychrosaccharolyticus* (*Bp*NDT) immobilized on PEI 600 Da agarose followed by crosslinking with 70% oxidized dextran (20 kDa) (11.3 IU/g of wet biocatalyst).^[31] Other similar examples, such as the enzymatic synthesis of thymidine by *Lr*NDT immobilized on magnetic chitosan beads (2.10 IU/g of wet biocatalyst)^[7] or the enzymatic synthesis of halogenated nucleosides by an NDT from *Lactobacillus animalis* (*La*NDT) immobilized on DEAE-Sepharose (2.6–5.4 IU/g of wet biocatalyst)^[32] displayed very low activity values compared to *MTbPDT*3.

In addition, *MTbPDT*3 could be recycled after at least 25 consecutive batch reactions with negligible loss of catalytic activity in the synthesis of dAdo from dlno and Ade.

A physical characterization of magnetic particles can be of help to establish the optimal conditions in the bioreactor. SEM micrographs of *MTbPDT*3 (Figure S3) showed individual particles in a micro-size scale and with a rough surface. This irregular surface might enhance enzyme attachment and expand the number of internal spaces available for substrate binding. Thus, this carrier material might be close to ideal due to its high accessible surface area and the nanoscaled intrapore system that allows both stable enzyme binding and high flow permeability.^[33] Furthermore, DLS analysis of these systems revealed a Z-average size between 24.6–113.42 μ m and 4.96–19.50 μ m for the free MagReSyn®Amine beads and *MTbPDT*3, respectively. As Manoel *et al.* demonstrated with poly(styrene-co-divinylbenzene)-based supports,^[34] this micrometric size is normally desirable to enable the industrial implementation of biocatalysts because it avoids the use of complex methods for separation of the biocatalyst from the reaction media and the diffusional problems associated to very small or very large particles, respectively. These results reveal that the enzyme displayed on the surface of magnetic devices can reduce the presence of aggregates in water dispersion and, therefore, promote the stability of individual particles. This explanation is in accordance with zeta potential measurements.

Thus, the zeta potentials of free MagReSyn®Amine beads were between –0.5 and –0.8 mV and decreased to –64.1 and –67.6 mV upon enzyme immobilization due to surface charge modification. The measurement conditions and the immobilized enzyme seem to reduce the flocculating behavior of free MagReSyn®Amine beads. The zeta potential is used mainly as an indicator of electrostatic stabilization of colloidal particles, in agreement with the DLVO theory,^[35] according to which large

Scheme 1. Schematic representation of immobilization of recombinant *TbPDT* on glutaraldehyde-activated MagReSyn®Amine microspheres.

Table 6. Effect of enzyme/support mass ratio on the activity of *TbPDT* immobilized on glutaraldehyde-activated MagReSyn®Amine microspheres.

Derivative	Biocatalyst load [mg _{enz} /g _{support}]	Immobilization yield [%] ^[c]	Activity [IU/g _{support}]	Specific activity [IU/mg protein]	Recovery [%]
<i>TbPDT</i> ^[a]	–	–	–	38.5 ± 3.3	–
<i>MTbPDT</i> 1 ^[b]	152	100 ± 2	2027 ± 70	13.1 ± 0.8	34 ± 2
<i>MTbPDT</i> 2 ^[b]	310	93 ± 4	2482 ± 55	9.6 ± 0.8	25 ± 1
<i>MTbPDT</i> 3 ^[b]	620	91 ± 2	4200 ± 27	8.5 ± 0.5	22 ± 2
<i>MTbPDT</i> 4 ^[b]	928	80 ± 0	4400 ± 45	5.4 ± 0.6	14 ± 4
<i>MTbPDT</i> 5 ^[b]	992	62 ± 2	3100 ± 30	5.0 ± 0.5	13 ± 1

[a] Reaction conditions: 0.3 μ g of enzyme in 40 μ L at 50 °C for 5 min. [dlno] = [Ade] = 10 mM in 50 mM sodium phosphate buffer, pH 6.0, at 50 °C, 5 min, 300 r.p.m. Vr = 40 μ L (0.4 μ mol dlno = A); [b] reaction conditions: 2.64 μ g enzyme (2.6–26.4 μ g beads), [dlno] = [A] = 10 mM in 50 mM sodium phosphate buffer, pH 6.0 at 50 °C, 10 min, 300 r.p.m. Vr = 320 μ L (3.2 μ mol dlno = A); [c] percentage of immobilized enzyme.

magnitude zeta potential values, such as those measured for the MTbPDT3 beads, indicate that an electrostatically stabilized suspension will remain stable, unlike free MagReSyn®Amine beads, which have a zeta potential close to zero.

Finally, as a proof of concept, MTbPDT3 was successfully employed in the one-pot, one-step synthesis of several nucleoside analogues of pharmaceutical interest using dGuo as the nucleoside donor with conversion rates approaching 50 % (Table 7).

Table 7. MTbPDT3-catalyzed synthesis of nucleoside analogues. ^[a]				
Donor	Acceptor	Product	Activity [IU/g _{support}]	Nucleoside conversion [%]
dGuo	2-FAde	2-FdAdo	3424 ± 30	40 ± 2
	2-ClAde	2-ClAdo	3521 ± 35	41 ± 2
	2,6-DAP	2,6-DAPdRib	3057 ± 50	33 ± 5
	6-MeOGua	6-MeOdGuo	5101 ± 75	46 ± 3
[a] Reaction conditions: 2.64 µg prot (4.25 µg beads), [dGuo] = [Base] = 1 mM in 50 mM sodium phosphate buffer, pH 6.0 at 50 °C, 20 min, 300 r.p.m. Vr = 320 µL (3.2 µmol dlno = Base).				

Conclusions

The presence of NDTs in *Trypanosoma* species has not been ascertained yet, despite a report^[10] on the crystal structure of a protein annotated in bioinformatic databases as a putative NDT from *Trypanosoma brucei*, the function of which has not been described, to the best of our knowledge. We now report the cloning of the *pdt* gene, its expression in *E. coli*, and the purification and biochemical characterization of the encoded protein, TbPDT. Our experimental results reveal TbPDT as a homodimeric enzyme endowed with significant activity in a broad range of temperature (50–70 °C), pH (4–7) and ionic strength (0–500 mM) values. Additionally, TbPDT was found to exhibit great stability in a pH interval from 6.5 to 10.0 and display negligible loss of activity at 50 °C for 30 h. The potential of TbPDT for biocatalysis was demonstrated by achieving the enzymatic synthesis of numerous therapeutic nucleosides, including 2-ClAdo (4), vidarabine (7), fludarabine (8), nelarabine (9), and ddl (10), among others (Tables 2 and 3).

The covalent immobilization of TbPDT on glutaraldehyde-activated MagReSyn®Amine microspheres led to derivative MTbPDT3, which displayed a remarkable activity of 4200 IU/g of wet biocatalyst and retained 22% activity in the enzymatic synthesis of dAdo from dlno and adenine. Furthermore, MTbPDT3 was reutilized for at least 25 times without any apparent loss of catalytic activity, an essential prerequisite for its application in industrial bioreactors, and allowed the expedient enzymatic synthesis of several nucleoside analogues at moderate conversion rates.

Experimental Section

Materials

Cell culture medium reagents were from Difco (St. Louis, United States). Trimethyl ammonium acetate buffer was purchased from Sigma-Aldrich (Madrid, Spain). All other reagents and organic solvents were purchased from Scharlab (Barcelona, Spain) and Symta (Madrid, Spain). Nucleosides and nucleobases used in this work were provided by Carbosynth Ltd. (Compton, United Kingdom).

Gene Expression and Protein Purification

The *pdt* gene, which encodes a protein annotated as nucleoside 2'-deoxyribosyltransferase from *Trypanosoma brucei gambiense* (European Nucleotide Archive code: AAX70453.1; UniProtKB Q57VC7, was purchased from Genscript (USA). The coding sequence appeared as a *NdeI-EcoRI* fragment subcloned into the expression vector pET28b (+). The resultant, recombinant vector pET28bTbPDT provided an N-terminal His₆-tagged fusion with a thrombin cleavage site between the tag and the enzyme. TbPDT was expressed in *E. coli* BL21 (DE3) grown in LB medium at 37 °C with kanamycin 50 µg/mL. Protein overexpression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside and the cells were further grown for 3 h. These were harvested via centrifugation at 3500 × g. The resulting pellet was resuspended in 10 mM sodium phosphate buffer pH 7. Crude extracts were prepared by French press lysis of cell suspensions. The lysate was centrifuged at 17500 × g for 30 min and the supernatant was filtered through a 0.22 µm filter (Millipore). The cleared lysate was loaded onto a 5-mL HisTrap FF column (GE Healthcare) pre-equilibrated in a binding buffer (20 mM Tris-HCl buffer, pH 8.0, with 100 mM NaCl and 10 mM imidazole) and the column was washed. Bound proteins were eluted using a linear gradient of imidazole (from 10 to 500 mM). Fractions containing TbPDT were identified by SDS-PAGE, pooled, concentrated and loaded onto a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) pre-equilibrated in 50 mM sodium phosphate, pH 7.0. Fractions with the protein of interest identified by SDS-PAGE were pooled and the protein was dialyzed against 10 mM sodium phosphate, pH 7.0, and concentrated and stored at 4 °C until its use. Electrophoresis was carried out on a 15% polyacrylamide slab gel with 25 mM Tris-HCl buffer, pH 8.6, 0.1% SDS.^[36] Protein concentration was determined spectrophotometrically by UV absorption at 280 nm using $\epsilon_{280} = 8,940 \text{ M}^{-1} \text{ cm}^{-1}$.^[37]

Analytical Ultracentrifugation Analysis

Sedimentation velocity experiments for TbNDT were carried out in 10 mM sodium phosphate (pH 8.0, 20 °C, 50,000 × g) using an Optima XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-VIS absorbance and Raleigh interference detection systems, using an An-60Ti rotor and standard (12 mm optical path) double-sector centre pieces of Epon-charcoal. Sedimentation profiles were recorded at 292 nm. Sedimentation coefficient distributions were calculated by least-squares boundary modeling of sedimentation velocity using the continuous distribution *c(s)* Lamm equation model as implemented in SEDFIT 14.7 g.^[38] (<https://sedfitsedphat.nibib.nih.gov/software/default.aspx>). Baseline off-sets were measured afterwards at 200,000 × g. The experimental sedimentation coefficients were corrected to standard conditions (water, 20 °C, and infinite dilution) using SEDNTERP software to obtain the corresponding standard values ($s_{20,w}$).^[39]

Enzyme Activity Assay

The standard activity assay was performed by incubating 0.3 μg of pure enzyme with 10 mM 2'-deoxyinosine (dIno) and 10 mM adenine in 50 mM sodium phosphate buffer pH 6.5 in a final volume of 40 μL . The reaction mixture was incubated at 40 °C for 10 min (300 rpm). The enzyme was inactivated by adding 40 μL of cold methanol in an ice bath and heating for 5 min at 100 °C. After centrifugation at 9000 $\times g$ for 2 min, the samples were half-diluted with water and frozen at -20 °C. Nucleoside production was analyzed using HPLC to measure the reaction products quantitatively, as described below. All determinations were carried out in triplicate and the maximum error was less than 5%. Under such conditions, one international activity unit (IU) was defined as the amount of enzyme producing 1 $\mu\text{mol}/\text{min}$ of 2'-deoxyadenosine under the assay conditions.

Influence of pH and Temperature on Enzyme Activity

The pH profile of purified recombinant enzyme was initially determined using the standard assay, as described above, with sodium citrate (pH 4–6), sodium phosphate (pH 6–8) and sodium borate (pH 8–10) as reaction buffers (50 mM). The optimum temperature was determined using the standard assay across a 20–90 °C range. A similar approach was followed to characterize the effect of ionic strength on enzyme activity. *TbPDT* activity was measured at different concentrations of NaCl, ranging from 0 to 1 M.

Thermal and pH Stability of *TbNDT*

TbPDT was stored at 4 °C in 10 mM sodium phosphate, pH 7.0 for 135 days. Samples were taken periodically for enzymatic activity evaluation. Storage stability was defined as the relative activity between the first and successive reactions. Moreover, the thermal stability of *TbNDT* was assessed by incubating 0.3 μg of pure enzyme in a pH range from 6.5 to 10, at 50 °C for a period of 30 h. Thereafter, the activity was measured using the standard assay.

Substrate Specificity

To explore the substrate specificity of *TbPDT*, 0.3 μg of recombinant enzyme were incubated with 10 mM of purine and pyrimidine nucleosides and bases, in 50 mM sodium phosphate buffer pH 6.0 or 50 mM sodium borate pH 8.5 at 50 °C and 300 rpm orbital shaking at different reaction times. Samples were extracted at regular intervals and enzymatic activity was evaluated as described in the Analytical methods section.

Enzymatic Synthesis of Nucleoside Analogues by Soluble *TbPDT*.

The synthesis of non-natural nucleosides was achieved at variable amounts of enzyme, using different nucleoside and base analogues. Reactions were carried out incubating 0.3–8 μg of *TbPDT* with 1 mM nucleoside and base, in 50 mM sodium phosphate buffer pH 6.0 or sodium borate pH 8.5 at 50 °C and 300 rpm orbital shaking at different reaction times. Samples were taken at regular intervals and enzymatic activity was evaluated as described below.

Kinetic Parameters and Initial Velocity Pattern

The steady-state kinetic parameters, K_M , k_{cat} and V_{max} were determined under standard assay conditions at varying concen-

trations of one substrate (dIno or Ade, 0.25–15 mM), while the concentration of the other substrate was fixed at constant saturating level (dIno or Ade, 5 mM). Apparent K_M , k_{cat} and k_{cat}/K_M values were determined by non-linear regression assuming Michaelis-Menten kinetics.

Enzyme Immobilization

TbPDT immobilization was carried out by covalent attachment of the enzyme to glutaraldehyde-activated MagReSyn®Amine microspheres (from ReSyn Biosciences, Pretoria, South Africa) according to the recommendations of the manufacturer. 25 μL of the bead suspension (20 mg mL^{-1}) were washed and equilibrated in binding buffer (50 mM potassium phosphate buffer, pH 8.5) during 4 h at 25 °C. After the equilibration procedure, the beads were collected by a magnetic separator and the supernatant was discarded. Activation of the support was accomplished by contacting MagReSyn®Amine microspheres with 200.0 μL of 50 mM sodium phosphate buffer pH 8.5 containing 4% (w/v) glutaraldehyde during 3 h at 25 °C, and then washed extensively with distilled water to remove the excess of the activating agent. Finally, activated microspheres were washed and equilibrated in binding buffer (50 mM potassium phosphate buffer, pH 8.5) shortly prior to use.

Subsequently, different amounts of enzyme (25–125 μg) were mixed with glutaraldehyde-activated MagReSyn®Amine microspheres. The enzyme solution with the binding buffer was adjusted to 10 $\times$ the volume of the initial beads suspension. The enzyme and bead suspensions were mixed thoroughly and incubated at room temperature under orbital shaking using a Sunflower 3D Mini-Shaker (BioSan, Latvia) for 10 h. After the binding procedure, the beads were collected by a magnetic separator and the supernatant was discarded. The beads were washed with washing buffer (50 mM potassium phosphate buffer, pH 8.5; 1 M NaCl) for three times to remove non-covalently bound enzyme. After this, the beads were also treated with 1 M Tris-HCl, pH 9.0 for 3 h at room temperature to quench any remaining amine reactive residues on the microparticles. The beads were washed with 50 mM potassium phosphate buffer (pH 8.5) for three times and then stored at 4 °C.

Enzyme Activity Assay for Immobilized *TbPDT*

As performed with the soluble enzyme, the enzymatic synthesis of dAdo from dIno and Ade was established as the standard trans-glycosidase reaction. The enzymatic activity of the immobilized enzyme was measured using 2.6–26.4 μg of different *MTbPDT* derivatives (each containing 2.64 μg of immobilized *TbPDT*) which were added to a 320 μL solution containing 10 mM dIno and 10 mM Ade in 50 mM phosphate buffer, pH 6.0. The reaction mixture was incubated at 50 °C for 10 min under magnetic stirring. The reaction was stopped by collecting the beads with a magnetic separator and the supernatant was treated following the general procedure described above. Enzymatic activity (IU/mg of enzyme) was evaluated as described above to measure specific activity of the biocatalyst (IU/g derivative).

Enzymatic Synthesis of Nucleoside Analogues by Immobilized *TbPDT*

Non-natural nucleosides were synthesized by adding 4.25 μg of *MTbPDT3* (containing 2.64 μg of immobilized *TbPDT*) to a 320 μL solution containing 1 mM dGuo and 1 mM purine base analogues in 50 mM sodium phosphate buffer pH 6.0 at 50 °C for 20 min, under magnetic shaking. Enzymatic activity (IU/mg of enzyme) was

evaluated as described above to measure specific activity of the biocatalyst (IU/g derivative).

Analytical Methods

The production of nucleosides was quantitatively measured with an ACE EXCEL 5 μ m CN-ES 250 $\times$ 4.6 mm equilibrated with 100% trimethyl ammonium acetate at a flow rate of 0.8 mL/min. Retention times for the reference natural and non-natural bases (hereafter abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature) were as follows: adenine (Ade), 10.2 min; guanine (Gua), 7.3 min; hypoxanthine (Hyp), 7.5 min; 2-fluoro-adenine (2-FAde), 14.4 min; 2-chloro-adenine (2-ClAde), 17.3 min; 2,6-diaminopurine (2,6-DAP), 11.1 min; 6-chloropurine (6-ClPur), 16.7 min; 6-mercaptopurine (6-MP), 10.5 min; 6-methylpurine (6-MetPur), 13.8 min; 6-methoxyguanine (6-MeOGua), 16.4 min; benzimidazole (Bzm), 29.6 min, and theophylline (Theo), 18.42 min.

Retention times for the nucleosides (hereafter abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature) were as follows: 2'-deoxyadenosine (dAdo), 15.50 min; 2'-deoxyguanosine (dGuo), 11.6 min; 2'-deoxyinosine (dIno), 12.0 min; 2-fluoro-2'-deoxyadenosine (2-F-dAdo), 14.4 min; 2-chloro-2'-deoxyadenosine (2-Cl-dAdo), 23.2 min; 2,6-diaminopurine-2'-deoxyribose (2,6-DAPdRib), 16.04 min; 6-chloropurine-2'-deoxyribose (6-ClPurdRib), 22.9 min; 6-mercaptopurine-2'-deoxyribose (6-MPdRib), 13.5 min; 6-methylpurine-2'-deoxyribose (6-MetPurdRib), 17.7 min; 6-methoxy-2'-deoxyguanosine (6-MeOdGuo), 21.8 min; benzimidazole-2'-deoxyribose (BzmdRib), 33.5 min; arabinosyl adenine (ara A), 15.00 min; arabinosyl guanine (ara G), 11.4 min; arabinosyl hypoxanthine (ara H), 11.6 min; arabinosyl 2-fluoro-adenine (ara 2-FAde), 13.8 min; arabinosyl chloro-adenine (ara 2-ClAde), 22.6 min; arabinosyl 2,6-diaminopurine (ara 2,6-DAP), 15.8 min; arabinosyl 6-chloropurine (ara 6-ClPur), 22.1 min; arabinosyl 6-mercaptopurine (ara 6-MP), 13.2 min; arabinosyl 6-methoxyguanine (ara 6-MeOGua), 21.4 min; 2',3'-dideoxyadenosine (ddAdo), 19.7 min; 2',3'-dideoxyguanosine (ddGuo), 16.4 min; 2',3'-dideoxyinosine (ddIno), 15.9 min; 2-fluoro-2',3'-dideoxyadenosine (dd2-Fado), 18.3 min; 2-chloro-2',3'-dideoxyadenosine (dd2-Clado), 27 min; 2,6-diaminopurine-2',3'-dideoxyribose (dd2,6-DAPRib), 20.9 min; 6-chloropurine-2',3'-dideoxyribose (dd6-ClPurRib), 27 min; 6-mercaptopurine-2',3'-dideoxyribose (dd6-MP Rib), 19.5 min; 2',3'-dideoxy-6-methoxyguanosine (dd6-MeOGuo), 25.4 min. 2'-fluoro-2'-deoxyadenosine (2'-F-dAdo), 17.8 min; 2'-fluoro-2'-deoxyguanosine (2'-F-dGuo), 16.3 min; 2'-fluoro-2'-deoxyinosine (2'-F-dIno), 13.9 min; 2'-fluoro-2-fluoro-adenosine (2'-F-2FdAdo), 17.4 min; 2'-fluoro-2-chloro-adenosine (2'-F-2Cl-dAdo), 33.9 min; 2'-fluoro-2,6-diaminopurine-2'-deoxyribose (2'-F-2,6-DAPdRib), 18 min; 2'-fluoro-6-chloropurine-2'-deoxyribose (2'-F-6-ClPurdRib), 26 min; 2'-fluoro-6-mercaptopurine-2'-deoxyribose (2'-F-6-MPdRib), 19 min; 2'-fluoro-6-methoxyguanosine (2'-F-MeOdGuo), 24.6 min.

Scanning Electron Microscopy

The morphology and particle structure of MagReSyn®Amine and TbpPDT-MagReSyn®Amine beads (MTbpPDT beads) were evaluated by SEM. SEM images were collected using a JEOL JSM-6400F electron microscopy equipped with a field emission gun at 10.0 kV (Centro de Microscopía Electrónica, UCM, Madrid). The magnetic devices were dispersed in distilled water at room temperature (25 °C), deposited onto a metal support and coated with an ultrathin layer of gold.

Particle size distribution and zeta potential were also analyzed by DLS (Nanopartica, Horiba SZ-100, Braga, Portugal) at 25 °C. For this

purpose, homogeneous dispersions of MagReSyn®Amine beads and MTbpPDT beads in distilled water were prepared inside two different types of disposable cells. Scattered light was collected at a 90° angle for size determinations and data were processed by Nanotracs software. All quantifications were performed in quintuplicate.

Computational Methods

The AMBER force field^[40] was used for energy refinement and molecular dynamics simulations^[41] of an all-atom representation of PDB entry 2 A0 K^[10] (upon removal of the N-terminal His tag) in explicit solvent, following a previously described protocol.^[42] An ensemble of 10 representative low-energy snapshots of TbpPDT in complex with 2'-deoxyinosine (docked as reported earlier for 2'-deoxyadenosine in LmPDT)^[9] was used to calculate the solvent-corrected intersubunit energy, as well as its decomposition into van der Waals, coulombic, apolar, and desolvation contributions by means of our in-house tool MM-ISMSA.^[18] The non-electrostatic calculations involve a van der Waals term, as in molecular mechanics (MM), and the change in solvent-accessible surface area (SASA) of both subunits brought about by dimer formation. The former, which accounts for shape complementarity, is calculated with the standard AMBER 12–6 Lennard-Jones potential whereas the latter ("apolar" in the Table), representing the nonpolar part of the desolvation process, is assumed to be linearly related to a combination of the cavitation term and the van der Waals solute-solvent interactions. The solvent-screened coulombic interactions were estimated by means of an implicit solvent model (ISM) that makes use of a sigmoidal, distance-dependent dielectric function.^[43] The pattern of interchain hydrogen bonds present in the TbpPDT dimer was calculated and visualized using the PHP-based web application HBplot (www.virtuadrug.com/hbplot)^[19], which employs the HBplus algorithm.^[44]

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Conflict of Interest Statement

Dr. Justin Jordaan has an interest in ReSyn Biosciences. For the purpose of this study, he advised on immobilization strategy but did not directly participate in the experimentation and did not generate any data.

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

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