Advanced Synthesis & Catalysis

DOI: 10.1002/adsc.201400966

Synthesis of 2,6-Dihalogenated Purine Nucleosides by Thermostable Nucleoside Phosphorylases

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Received: October 8, 2014; Revised: January 29, 2015; Published online: ■■ ■■, 0000

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201400966.

Abstract: The enzymatic transglycosylation of 2,6-dichloropurine (26DCP) and 6-chloro-2-fluoropurine (6C2FP) with uridine, thymidine and 1-(β-D-arabinofuranosyl)-uracil as the pentofuranose donors and recombinant thermostable nucleoside phosphorylases from *G. thermoglucosidasius* or *T. thermophilus* as biocatalysts was studied. Selection of 26DCP and 6C2FP as substrates is determined by their higher solubility in aqueous buffer solutions compared to most natural and modified purines and, furthermore, synthesized nucleosides are valuable precursors for the preparation of a large number of biologically important nucleosides. The substrate activity of 26DCP and 6C2FP in the synthesis of their *ribo*- and 2′-*deoxyribo*-nucleosides was closely similar to that of related 2-amino- (DAP), 2-chloro- and 2-fluoroadenines; the efficiency of the synthesis of β-D-arabinofuranosides of 26DCP and 6C2FP was lower *vs.* that of DAP under similar reaction conditions. For a convenient and easier recovery of the biocatalysts, the thermostable enzymes were immobilized on MagReSyn® epoxide beads and the biocatalyst showed high catalytic efficiency in a number of reactions. As an example, 6-chloro-2-fluoro-(β-D-ribofuranosyl)-purine (**9**), a precursor of various antiviral and antitumour drugs, was synthesized by the immobilized enzymes at 60 °C under high substrate concentrations (uridine:purine ratio of 2:1, mol). The synthesis was successfully scaled-up [uridine (2.5 mmol), base (1.25 mmol); reaction mixture 50 mL] to afford **9** in 60% yield. The reaction reveals the great practical potential of this enzymatic method for the efficient production of modified purine nucleosides of pharmaceutical interest.

Keywords: biocatalysis; immobilization; nucleoside phosphorylases; purine nucleosides

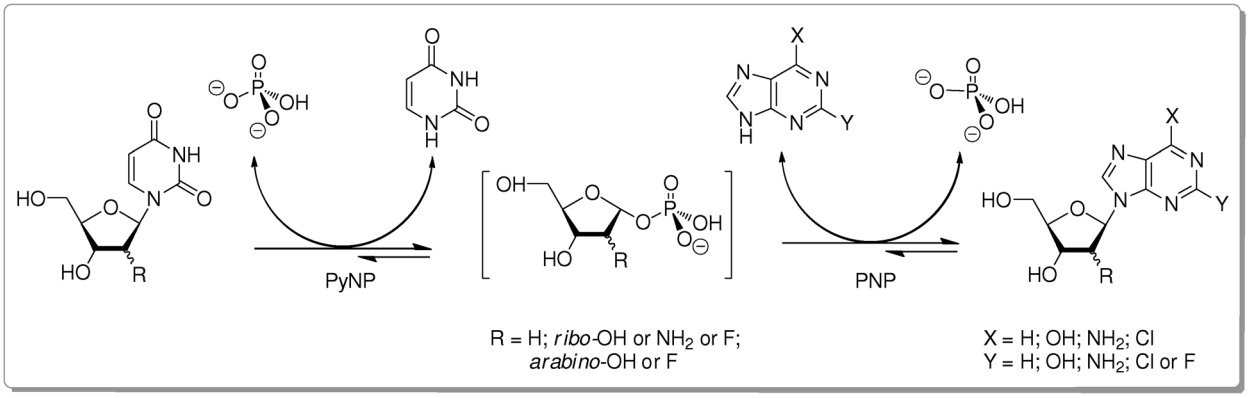
Introduction

Nucleoside analogues are broadly used as antitumour and antiviral agents. In the living cell, they manifest biological effects *via* diverse metabolic transformations, primarily as a result of conversion into derivatives of phosphoric acid, or themselves by inhibiting the metabolic pathways or as blockers of the synthesis of nucleic acids.[1]

Among the great diversity of biologically important nucleosides, adenosine analogues containing a chlorine (**1**–**3**) or fluorine (**4**–**6**) atom at the C-2 carbon atom as well as 2,6-dichloro- and 6-chloro-2-fluoropurine *ribo*- (**7** and **9**) and 2′-*deoxyribo*-nucleosides (**8** and **10**) are of particular interest. Indeed, (i) cladribine (**1**), clofarabine (**3**) and fludarabine (**6**) have attracted much attention during the last two decades as drugs with great therapeutic potentials, in particular for the treatment of leukaemia;[2,4,7] (ii) 2-chloroadenosine (2Cl-Ado, **2**) was shown to be an agonist of the adenosine receptor that induces apoptosis in several cell lines;[3] (iii) 2′-deoxy-2-fluoroadenosine (2F-dAdo, **4**) and 2-fluoroadenosine (2F-Ado, **5**) are the first members of the adenosine analogues and were first synthesized in the middle of the last century; they show high cytostatic activity against tumour cells that, unfortu-

Table 1. Structure (see Scheme 1) and bioactivity of halogenated purine nucleosides.

	Nucleoside	X	Y	R	Bioactivity
1	Cladribine	NH_2	Cl	H	antileukemia; used in clinic[2]
2	2Cl-Ado	NH_2	Cl	OH (*ribo*)	induces apoptosis in several cells[3]
3	Clofarabine	NH_2	Cl	F (*arabino*)	antileukemia; used in clinic[4]
4	2F-dAdo	NH_2	F	H	cytotoxic[5]
5	2F-Ado	NH_2	F	OH (*ribo*)	highly cytotoxic[5,6]
6	Fludarabine	NH_2	F	OH (*arabino*)	antileukemia; used in clinic[7]
7	26DCP-R	Cl	Cl	OH (*ribo*)	smooth muscle relaxant[8]
8	26DCP-dR	Cl	Cl	H	unknown
9	6C2FP-R	Cl	F	OH (*ribo*)	unknown
10	6C2FP-dR	Cl	F	H	unknown



Scheme 1. Enzymatic synthesis of modified nucleosides.

nately, is accompanied by the high toxicity for normal cells.[5,6] Dihalogenated purine nucleosides **7**–**10** (see Table 1) are of great value as precursors for the synthesis of numerous purine nucleosides in the framework of the R&D of efficient routes to the preparation of known substances as well as new nucleosides of potential biological importance.

Until now, the vast majority of nucleoside analogues has been synthesized by chemical methods. Two approaches to the synthesis of purine nucleosides have been widely and extensively studied. The first, convergent approach consists in the condensation of a purine base with a suitable pentofuranose derivative and subsequent deprotection (see, e.g.,[5,6,9] and the reviews[10]). The second comprises the chemical transformation of the purine base or/and the pentofuranose fragment of the natural commercially available *ribo*- or 2′-*deoxyribo*-nucleoside in the desired compound.[11] Despite the very impressive progress achieved in the development of chemical methods, the preparation of purine nucleosides remains a challenging problem. Without going into details, it should be noted that a common drawback of both approaches is the indispensable introduction and subsequent removal of protective groups, which is associated with chromatographic purification in almost every step, a lack of selectivity of chemical reactions, and the problems associated with the poor regio- and stereoselectivity of chemical reactions (see, e.g.[12] and the papers cited therein).

In contrast, the enzymatic synthesis of nucleosides by the transglycosylation reaction proceeds with strict stereo- and regioselectivity (except for a few specific substrates[13]) and requires organic solvents (ethanol, acetonitrile) only for the isolation of the individual desired compound. Obviously, the substrate specificity of the enzyme and the physicochemical properties of the substrates are critically important factors of this reaction. From the viewpoint of the practical synthesis, nucleoside phosphorylases (NPs) of diverse origin are presently very useful biocatalysts for the synthesis of base- and pentofuranose-modified nucleosides,[13,14] which include pyrimidine NPs (PyNP, EC 2.4.2.2; UP, EC 2.4.2.3; TP, EC 2.4.2.4) and purine NPs (PNP, EC 2.4.2.1; MTAP, EC 2.4.2.28). These enzymes catalyse the reversible phosphorolysis of a nucleoside in the presence of inorganic phosphate to afford α-D-ribofuranose-1-phosphate (α-D-Rib-1P) or 2-deoxy-α-D-ribofuranose-1-phosphate (α-D-dRib-1P) and the nucleobase. The basic synthetic strategy is illustrated in Scheme 1.

The rationality of the transglycosylation reaction consists in the transfer of the pentofuranose moiety of a donor, which is a commercially available pyrimidine nucleoside or its pentofuranose-modified derivative, to the purine base; the direction of the reaction is determined by the differences in k_{cat} *vs.* K_m of the purine or pyrimidine phosphorylases, respectively, towards the different substrates. The interplay of two reversible phosphorolysis reactions results usually in the formation of the desired purine nucleoside if the respective PyNP and PNP are combined in a one-pot reaction. Moreover, it is effective to use pyrimidine nucleosides as the pentofuranose donor because the product (a pyrimidine base) from the first step will not become the competitor of the reactant (the purine base) for the second step (Scheme 1).

A number of halogenated nucleosides has been synthesised by NPs from different sources. For instance, 2-Cl (or F) substituted adenine is accepted as substrate of PNPs from *E. coli*,[15] *Geobacillus stearothermophilus*[16] and *Sulfolobus solfataricus*[17] for the synthesis of cladribine **1**,[15a–d,16] Fludarabine **6**[15e,16] and their analogues; 6-Cl substituted purine is accepted by PNPs from *E. coli*[18] and *Aeromonas hydrophila*.[19] The reported conversions resulted in satisfactory yields of the desired nucleosides (mainly HPLC data), and some of them gave yields in the range from 50% to 80% for the pure individual nucleosides. The use of whole bacterial cells as biocatalysts is well documented[14,15] and the synthesis of a number of biologically important nucleosides (AraA, AraG, cladribine, fludarabine, 2-fluorocordycepin etc.) has been described. Whole bacterial cells represent a kind of naturally immobilised nucleoside phosphorylases that are necessary to realise the transglycosylation reactions in combination with further enzymes which provide pro-substrates like arabinose and additional enzymes enabling us to employ pro-substrates like cytosine arabinoside (AraC) which is used as the arabinofuranose donor due to the presence of cytidine deaminase.[14b,15] However, the wider application of whole cell extracts is restricted due to the following limitations.

Firstly, broadly used bacterial whole cells as biocatalysts possess a number of enzymes that can (i) consume substrates; (ii) catalyse undesired transformations of substrates or formed nucleosides; moreover, (iii) secrete cell metabolite(s) in the buffer, which makes the further purification challenging. Purified NPs (e.g., *E. coli* enzymes) typically exhibit lower activity towards the analogues of the natural substrates, and they will become inactive under the harsh conditions including high temperature, high substrate concentration and organic solvents. Even though today molecular evolution techniques are available to improve the *E. coli* enzymes[20] or even for adapting the cell to organic solvents,[21] the genetic reservoir of the variety of microorganisms probably offers better starting points. Enzymes from thermophiles are advantageous over the conventional bacterial enzymes for the synthesis of modified nucleosides. Aside from the advantage that biocatalytic reactions can be performed at higher temperatures, also it is likely that these enzymes provide a different promiscuity to substrate modifications that is, due to their evolutionary distance to *E. coli*.

Table 2. Temperature dependence of the substrate solubility in aqueous solution.[a]

Temp. [°C]	2CA	2FA	Ade	26DCP [mM][b]	6C2FP	6C2FP-R
25	0.1	0.9	5.7[c]	15.8[c]	29.0[c]	79.9
40	0.2	1.4	9.9[d]	17.6	49.3	211.5
55	0.3	2.1	16.5	19.8	53.4	ND[e]
65	0.4	2.2	21.7	34.0	81.8	ND
80	0.5	4.0	36.9	38.4	150.2	ND

[a] Solubility was measured by UV spectroscopy at the maximum wavelength of each compounds (284–288 nm) in 2 mM potassium phosphate buffer (pH 7.0). 2CA = 2Cl-Ade; 2FA = 2F-Ade; Ade = adenine; 26DCP = 2,6-dichloropurine; 6C2FP = 6-chloro-2-fluoropurine; 6C2FP-R = 6-chloro-2-fluoropurineriboside.
[b] The largest relative standard deviation is less than 3% ($n = 4$).
[c] Determined at 22 °C.
[d] Determined at 43 °C.
[e] ND = not determined.

Secondly, the purine bases 2-chloroadenine (2Cl-Ade) and 2-fluoroadenine (2F-Ade) have extremely low solubility in aqueous solutions which are most suitable for the enzymatic reactions. We tested the effect of temperature on the solubility of 2Cl-Ade and 2F-Ade as well as for the other relevant compounds in aqueous solution in the range from 25 °C to 80 °C and found that the use of the halogenated 26DCP and 6C2FP as substrates of the enzymatic synthesis is much more promising than 2Cl-Ade and 2F-Ade from the viewpoint of preparative synthesis because of their greater solubility (Table 2). Nevertheless, the use of 2Cl-Ade and 2F-Ade in the enzymatic synthesis of nucleosides was described in a number of publications (e.g., refs.[15b,16]).

Enzyme immobilisation is considered as an advantage when enzymes are applied under the relatively harsh conditions required in industrial processes. Immobilisation also allows for a convenient and easier recovery of the biocatalysts and simplifies the design of a reactor.[22] Considering the multimeric nature of the enzyme,[23] and some examples where NPs were successfully immobilised on a hydrophilic support (e.g., epoxide),[24] we chose MagReSyn microspheres as an immobilisation carrier. These microspheres are made of magnetite particles combined with a hydrophilic polymer with epoxide-functional groups,[25]

which provides high functional group density throughout the flexible fibre network allowing for stabilisation of multimeric enzymes. Besides, the magnetic property makes it easy to remove the biocatalyst from the reaction medium. The detailed immobilisation process and the evaluation are presented elsewhere.[26]

Results and Discussion

Scrutiny of the published methods for the chemo-enzymatic synthesis of base- and pentofuranose-modified purine nucleosides prompted us to formulate a new versatile approach to the synthesis of this class of biologically important compounds. Our approach consists in the enzymatic synthesis of 2,6-dihalogenated purine nucleosides, in particular 2,6-dichloropurine (26DCP) and 6-chloro-2-fluoropurine (6C2FP), as valuable scaffolds for the enzymatic and/or chemical preparation of a broad palette of modified nucleosides. The motivation for this work was connected to a previous screening study which provided us access to a number of PyNPs and PNPs from thermophilic microorganisms (TtPyNP from *Thermus thermophilus*, GtPyNP and GtPNP from *Geobacillus thermoglucosidasius*, DgPNP from *Deinococcus geothermalis*, and ApMTAP from *Aeropyrum pernix*) and the study of their substrate properties,[27] which allowed us to choose the most efficient biocatalysts for the synthesis of halogenated purine nucleosides. It is noteworthy that so far dihalogenated purines were not applied as substrates for the enzymatic synthesis of their nucleosides due to their very poor substrate activity for the so far described PNPs.

The present study was initiated by comparatively testing the activity of DgPNP, GtPNP and ApMTAP under the optimal reaction conditions described earlier.[27] With this aim in view, a two-step analysis was used, that is, phosphorolysis of uridine by TtPyNP or GtPyNP to generate α-D-Rib-1P in step 1. After this a purine base and PNP were added to the reaction mixture (step 2) and the formation of the corresponding purine nucleoside was monitored by HPLC. The activity of 2,6-dihalogenated purines was compared with 2,6-diaminopurine (DAP) that is known to be one of the best purine substrates for PNPs from diverse sources. The results are shown in Table 3. As expected, DAP showed the best substrate properties for all PNPs tested; GtPNP showed superior activities *vs.* those of DgPNP and ApMTAP towards the tested purine bases and thus it was selected for the further study.

In the next series of experiments, the enzymatic syntheses of ribosides (entries 1–5), 2′-deoxyribosides (entries 6–10), and arabinosides (entries 11–13) using GtPNP and TtPyNP or GtPyNP as biocatalysts were investigated, and the results are summarised in Table 4. The choice of combinations of the enzymes as well as the reaction temperatures is based on our previous study of the substrate specificities of the used enzymes.[27] At the indicated temperatures, TtPyNP and GtPyNP are interchangeable for the phosphorolysis of uridine and thymidine, however, AraU showed no little substrate activity for GtPyNP, but is a substrate for TtPyNP (Table 4).

Table 3. Apparent activity of PNPs towards modified purine base.

Purine base[b]	Apparent activity[a] [U mg^{-1}]		
	DgPNP 55 °C	GtPNP 70 °C	ApMTAP 80 °C
DAP	98.96	152.76	10.62
6C2FP	0.33	9.81	0.25
26DCP	0.91	4.30	0.33

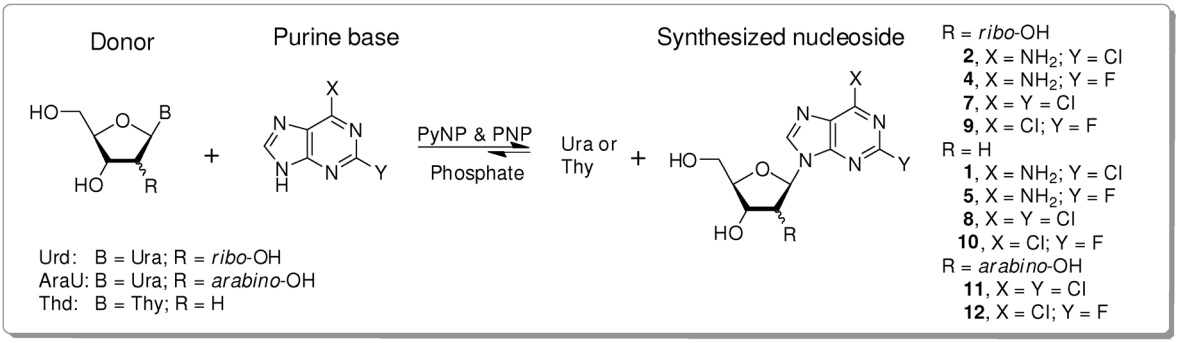
[a] The apparent activity was determined under the conditions such that the ratio of α-D-Rib-1P to purine base was 0.6 mM:1 mM and the base conversion rate was in the linear range. 1 U is the amount of the enzyme converting 1 μmol of purine base per minute under the defined conditions.

[b] In 2 mM phosphate buffer (pH 7.0). DAP = 2,6-diaminopurine; 6C2FP = 6-chloro-2-fluoropurine; 26DCP = 2,6-dichloropurine.

The data of Table 4 confirm the excellent substrate properties of the bases studied in the synthesis of *ribo*- and *2′-deoxyribo* nucleosides. Indeed, the conversion of 26DCP and 6C2FP reached 54–69% without prior optimisation of the experimental conditions in regard to the donor:acceptor ratio and the reaction time (especially in the case of halogenated bases) and thus in our opinion the results demonstrate the great potential of the suggested approach for the synthesis of the corresponding nucleosides on a preparative scale. The excellent substrate activity of DAP towards PNPs of diverse origin is well known,[1b,13,14] however, a high conversion rate of the 2Cl-Ade and 2F-Ade into the corresponding *ribo*- and *2′-deoxyribo* nucleosides was unexpected taking into account the extremely poor solubility of the bases in aqueous media. These data on the whole suggest an unusually high efficiency of the GtPNP-catalyzed practically irreversible coupling of a base with the intermediates α-D-Rib-1P or α-D-dRib-1P, respectively, promoting the solution of 2Cl-Ade and 2F-Ade in the reaction mixture.

In the case of the synthesis of *ara*-nucleosides, the low phosphorolytic efficiency of TtPyNP along with the lower catalytic power of GtPNP toward α-D-ara-1P *vs.* that in regard of the natural α-D-Rib-1P and α-D-dRib-1P are obviously the main reason for the low reaction rate. A careful optimisation of the reaction conditions is the basis to improve the formation rate and yield of the desired nucleosides further.

Table 4. Synthesis of modified purine ribonucleosides *via* enzymatic transglycosylation.[a]



Entry	Pentofuranosyl Donor[b]	R	Purine base[c] X	Y	Synthesized nucleoside	Time/Temp [h/°C]	Conversion [%] Donor[d]	Base[e]
1	Urd	OH (*ribo*)	NH_2	NH_2	DAP-R	1/70	66.9	95.3
2			NH_2	Cl	2Cl-Ado (**2**)	1/70	45.8	93.3
3			NH_2	F	2F-Ado (**5**)	1/70	73.3	91.6
4			Cl	Cl	26DCP-R (**7**)	0.5/65	52.8	53.9
5			Cl	F	6C2FP-R (**9**)	0.5/65	54.1	63.0
6	Thd	H	NH_2	NH_2	DAP-dR	1/70	66.8	80.0
7			NH_2	Cl	cladribine (**1**)	1/70	55.0	80.0
8			NH_2	F	2F-dAdo (**4**)	1/70	83.6	65.9
9			Cl	Cl	26DCP-dR (**8**)	0.5/65	58.6	67.4
10			Cl	F	6C2FP-dR (**10**)	0.5/65	59.7	69.3
11	AraU	OH (*arabino*)	NH_2	NH_2	DAP-araR	3/70	54.6	39.5
12			Cl	Cl	26DCP-araR (**11**)	3/70	40.8	9.9
13			Cl	F	6C2FP-araR (**12**)	3/70	40.0	7.2

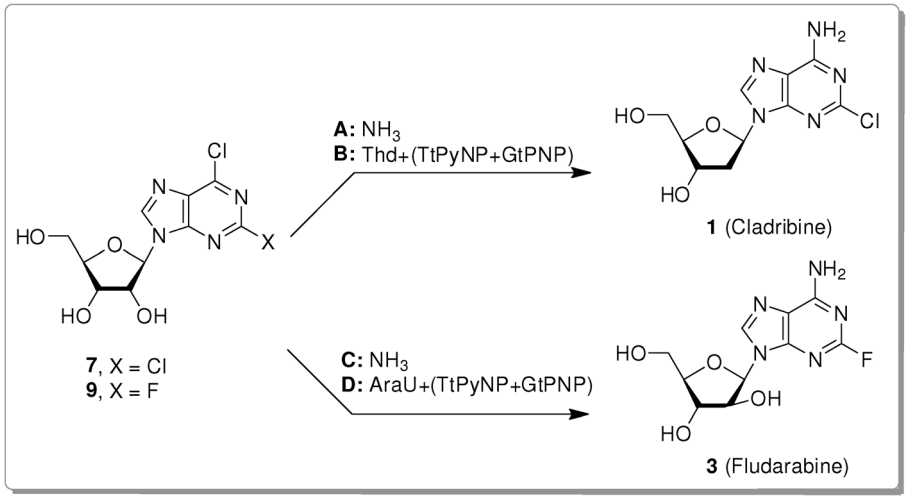
[a] Enzymes: TtPyNP 0.1 mg mL^{-1} (5.3 U mL^{-1} towards Urd at 70 °C) and GtPNP 0.2 mg mL^{-1} (2.0 U mL^{-1} towards 6C2FP at 70 °C) (entries 1–3, 6–8, 11–13); GtPyNP (5.1 U mL^{-1} towards Urd at 65 °C) and GtPNP (0.9 U mL^{-1} towards 6C2FP at 65 °C), 0.1 mg mL^{-1} each (entries 4–5, 9–10); medium: 2 mM K-phosphate buffer (pH 7.0).
[b] Pentofuranosyl donor: 2 mM. Urd = uridine, Thd = thymidine, AraU = 1-(β-D-arabinofuranosyl)uracil.
[c] Purine base: 1 mM. 2Cl-Ade (entries 2 and 7) can only reach 0.6 mM.
[d] Yield (according to HPLC) based on pyrimidine nucleosides (donor) conversion.
[e] Yield (according to HPLC) based on purine base conversion.

With this aim in view, TtPyNP and GtPNP were immobilised on MagReSyn® epoxide beads [polyethyleneimine-based iron oxide magnetic microspheres with epoxide functional groups (from ReSyn Biosciences, Pretoria, South Africa] for a convenient and easier recovery of the biocatalysts. The activities of the immobilized enzymes were measured by the activity assay as previously described[27] and the following data were obtained: TtPyNP – 4.8 mg enzyme were loaded per mL beads, 41% residual activity; GtPNP – 16.5 mg enzyme were loaded per mL beads, 83% residual activity. It should be stressed that a high residual activity of immobilised enzymes points to the presence of all subunits in the biocatalyst. Noteworthy, the enzymatic activity of *E. coli* PNP that is a trimer of dimers was recently investigated in detail and it was convincingly proven that only the hexameric structure displays activity.[23] It was found that Urd and 6C2FP in concentrations up to 80 mM and 55 mM, respectively, do not inhibit the immobilised enzymes.

In small-scale reactions (0.4 mL), under optimal conditions, the conversion of 26DCP and 6C2FP into the corresponding *ribo*-nucleosides **7** and **9** was verified by HPLC to be 78% and 85%, respectively (see the Experimental Section for details).

To validate the efficiency of the immobilised enzymes for the preparation of pure nucleosides on the multimilligram scale, the synthesis of 6-chloro-2-fluoro-9-(β-D-ribofuranosyl)-purine (**9**) was studied. The reaction mixture (in 2 mM K-phosphate buffer, pH 7.0; total volume 50 mL) containing 50 mM Urd, 25 mM 6C2FP was kept at 60 °C for 20 h and the reaction progress was monitored by HPLC. During this period, the formation of nucleoside **9** reached 75% and the reaction mixture was worked-up (removing the biocatalyst by a magnet, silica gel column chromatography) and resulted in a 60% yield of the product which had a purity of 98% (HPLC).

The results of the synthesis of nucleoside **9** prompted us to extend this work to the preparation of nucleosides **7**, **8** and **10** aiming to exploit the new effi-



Scheme 2. Chemo-enzymatic routes for the synthesis of cladribine and fludarabine.

cient chemo-enzymatic routes to known and new modified purine nucleosides. This work is still in progress. Emphasis is made on the role of the successive treatments for the efficient synthesis of purine nucleosides, e.g., cladribine (**1**) and clofarabine (**3**), using either ribonucleosides **7** and **9** (first B then A, and first D then C) or their corresponding 2-chloro- and 2-fluoroadenine derivatives (first A then B, and first C then D) as donors for the relevant purine bases in the transglcosylation reactions (Scheme 2) (*cf.* ref.[28]).

Conclusions

In summary, we have studied the possibility of application of different enzyme pairs (GtPNP and TtPyNP, GtPNP and GtPyNP) as biocatalysts for the synthesis of various *ribo*-, *2′-deoxyribo*- and *arabino*-purine nucleosides. We found that DAP, 2Cl-Ade, 2F-Ade, 26DC-P and 6C2FP are effectively transformed into the corresponding *ribo*- and *2′-deoxyribo*-nucleosides; while the formation of the *arabino*-nucleosides of DAP, 26DC-P and 6C2FP had a lower efficiency. Immobilisation of GtPNP and TtPyNP on MagReSyn® epoxide beads resulted in efficient transformation of 26DC-P and 6C2FP into the corresponding *ribo*-nucleosides as it was exemplary shown by the synthesis of 6-chloro-2-fluoro-9-(β-D-ribofuranosyl)-purine (**9**) on a hundred milligram scale. The results of this study *in toto* represent an interesting example of the opportunities that are offered by the exploitation of new enzymes from thermophilic organisms for the efficient production of base and pentofuranose modified purine nucleosides.

Experimental Section

General

All chemicals and solvents were of analytical grade or higher and purchased, if not stated otherwise, from Sigma–Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), TCI Deutschland (Eschborn, Germany), and VWR (Darmstadt, Germany). Water was purified by a purification system from Merck Millipore (Schwalbach, Germany). MagReSyn® Epoxide beads were a kind gift from ReSyn Biosciences (Pretoria, South Africa). The UV spectra and absorption of purine bases were recorded with a Biochrom Ultrospec 3300 pro UV/Visible Spectrophotometer (Cambridge, England). HPLC analyses were carried out with an Agilent 1200 series system equipped with an Agilent DAD detector using a Phenomenex (Torrance, United States) reversed phase C18 column (150×4.60 mm). Column chromatography was performed on silica gel 60 (0.043–0.06 mm, 230–400 mesh, ASTM) from Grace GmbH. NMR data were recorded on Bruker AV 500 instruments. High resolution mass spectrometry (HR-MS) analyses were performed by the Analytical Facility at the Institut für Chemie of the Technische Universität Berlin.

Solubility Test

Calibration curves were made by UV spectroscopy for each compound to be tested. The wavelengths of maximum absorption are: 285 nm (2Cl-Ade, $\varepsilon = 624.6\ M^{-1}cm^{-1}$), 284 nm (2F-Ade, $\varepsilon = 284.9\ M^{-1}cm^{-1}$), 284 nm (Ade, $\varepsilon = 261.5\ M^{-1}cm^{-1}$), 288 nm (26DCP, $\varepsilon = 3215\ M^{-1}cm^{-1}$), 285 nm (6C2FP, $\varepsilon = 2335\ M^{-1}cm^{-1}$; 6C2FP-R, $\varepsilon = 1627\ M^{-1}cm^{-1}$). The saturated solutions suspended in 2 mM phosphate buffer (pH 7.0) were incubated at different temperatures in a water bath or on a thermomixer for 30 min. The supernatant was withdrawn and cooled down shortly, and then diluted to the suitable concentration for the absorption measurement. Solubility was calculated according to the saturation concentration at the defined temperature.

Expression and Preparation of Enzymes

TtPyNP, GtPyNP, GtPNP, DgPNP and ApMTAP were expressed in *E. coli* BL21 and purified by heat treatment and standard Ni-NTA affinity chromatography as previously described.[27] Purified TtPyNP has a specific activity of 53 U mg^{-1} at 70 °C and GtPyNP 51 U mg^{-1} at 65 °C towards Urd in 50 mM phosphate buffer (pH 7).

Determination of PNP Apparent Activity towards Purine Base

The procedure contains two steps: (i) 0.5 mL 4 mMUrd was incubated with 0.1 mg GtPyNP or TtPyNP in 2 mM phosphate buffer (pH 7) for 5 min at the temperature of the second step. At the end of the reaction, the equilibrium was reached (30% Urd was converted to Ura and α-D-Rib-1P). (ii) 0.5 mL 2 mM purine base in 2 mM phosphate buffer (pH 7) with PNP (the concentration was selected in such a way that the reaction rate was in the linear range) was added into the first step reaction for 2 min at 55 °C (DgPNP), 65 °C (GtPNP) or 80 °C (ApMTAP). The reaction mixtures were analysed by HPLC (see the Supporting Information). One unit PNP apparent activity was defined as the amount of PNP which catalyses the conversion of 1 μmol purine base per minute under the aforementioned conditions.

Enzymatic Synthesis of Halogenated Purine Nucleosides

The reaction mixture of 0.4 mL contained 2 mM pentofuranosyl donor (Urd, Thd or AraU), 1 mM purine base, 2 mM phosphate buffer (pH 7.0), 0.1 mg mL^{-1}TtPyNP (or GtPyNP) and 0.1–0.2 mg mL^{-1}GtPNP. Reactions were performed on at thermomixer at defined temperatures for 0.5 to 3 h (Table 4) and stopped by adding ice-cold water at a 1:1 dilution. Controls (Table 4, entries 2–3, 7–8, 11–13) without enzyme were performed in parallel. The conversion rates were calculated from the integration of the corresponding HPLC peaks and are presented as the percentage of converted donor or base, respectively.

Immobilisation of TtPyNP and GtPNP

TtPyNP and GtPNP were immobilised on MagReSyn® epoxide beads (ReSyn Biosciences, Pretoria, South Africa) according to ReSyn's manual with a slight modification. Briefly, 0.04 mL suspension beads (1 mg dry beads) were loaded with TtPyNP (0.19 mg; 4.1 U towards Urd at 70 °C) or GtPNP (0.66 mg; 2.4 U and 5.4 U towards 26DCP and 6C2FP at 70 °C, respectively) in a total volume of 0.40 mL binding buffer (50 mM potassium phosphate buffer, pH 6.0) and incubated for 4 h on the thermomixer at 950 rpm, 50 °C. The quenching step was skipped. Beads were washed and stored at 4 °C in 50 mM potassium phosphate buffer (pH 7.0) until further use.

The optimal conditions for the synthesis of the nucleoside **7** were: 0.4 mL reaction containing 20 mM Urd, 7 mM 26DCP, 1.4 mM K-phosphate (pH 7.0), 4.1 U TtPyNP (towards Urd at 70 °C) and 2.4 U GtPNP (towards 26DCP at 70 °C). The reaction mixture was shaken at 1000 rpm for 30 min at 70 °C and HPLC analysis showed a 78% base conversion for the nucleoside **7**.

The optimal conditions for the synthesis of nucleoside **9** were: 0.4 mL reaction containing 50 mM Urd, 20 mM 6C2FP, 2 mM K-phosphate (pH 7.0), 4.1 U TtPyNP (towards Urd at 70 °C) and 5.4 U GtPNP (towards 6C2FP at 70 °C). The reaction mixture was shaken at 1000 rpm for 180 min at 70 °C and HPLC analysis showed an 85% base conversion for the nucleoside **9**.

Synthesis and Purification of 6-Chloro-2-fluoro-9-(β-D-ribofuranosyl)-purine (9)

The reaction mixture (50 mL) containing uridine (0.61 g, 2.5 mmol), 6-chloro-2-fluoropurine (0.216 g, 1.25 mmol), potassium phosphate (0.1 mmol, pH 7.0) and immobilized biocatalysts (TtPyNP: 8 mg, *ca.* 67 U, towards Urd at 60 °C; GtPNP: 15 mg, *ca.* 87 U, towards 6C2FP at 60 °C) was prepared in a 50 mL-falcon tube. This tube was closed and rotated in an end-over-end rotator at 55–60 °C in a water bath for 20 h. A 75% conversion for the base was determined by HPLC. The biocatalyst was removed by a permanent magnet and the product was isolated by silica gel column chromatography (dichloromethane/ethanol 15:1 and then ethyl acetate/acetone 7:3) to afford the desired product **9** as a white powder; yield: 0.230 g (0.75 mmol, 60%; HPLC purity 98%) (see the Supporting Information for more details).

Acknowledgements

We are grateful to Dr. Justin Jordaan (ReSyn Biosciences, Pretoria, South Africa) for the kind donation of the MagReSyn® Epoxide beads as well as useful suggestions for the immobilization experiments. This work is part of the Cluster of Excellence "Unifying Concepts in Catalysis" coordinated by the Technische Universität Berlin. Financial support by the Deutsche Forschungsgemeinschaft (DFG) within the framework of the German Initiative for Excellence is gratefully acknowledged (EXC 314). X.Z. gratefully acknowledges financial support by an ESF-grant of the TU Berlin. I.A.M. is deeply thankful to the A. von Humboldt-Stiftung (Bonn-Bad-Godesberg, Germany) and the Byelorussian Republican Foundation for Fundamental Research (www.fond.bas-net.by; project #X13MC-027) for partial financial support. L.-Y.J. thanks the China Scholarship Council (CSC) for a predoctoral fellowship (2011–2015).

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