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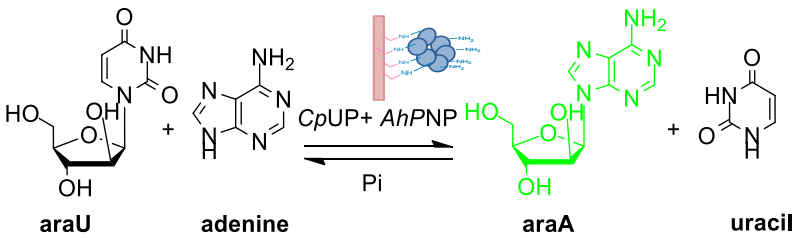
The comparison between the biocatalyzed synthesis of araA here described and the chemical synthesis of this nucleoside as reported in the literature clearly showed that the enzymatic reaction is superior (less number of steps, milder reaction conditions and reagents, easier downstream) also in terms of E factor.



Phosphate buffer pH 7.5; DMF 12.5%-30%; rt
*Cp*UP: *Clostridium perfringens* uridine phosphorylase
*Ah*PNP: *Aeromonas hydrophila* purine nucleoside phosphorylase

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Redesigning the synthesis of Vidarabine *via* a multienzymatic reaction catalyzed by immobilized nucleoside phosphorylases

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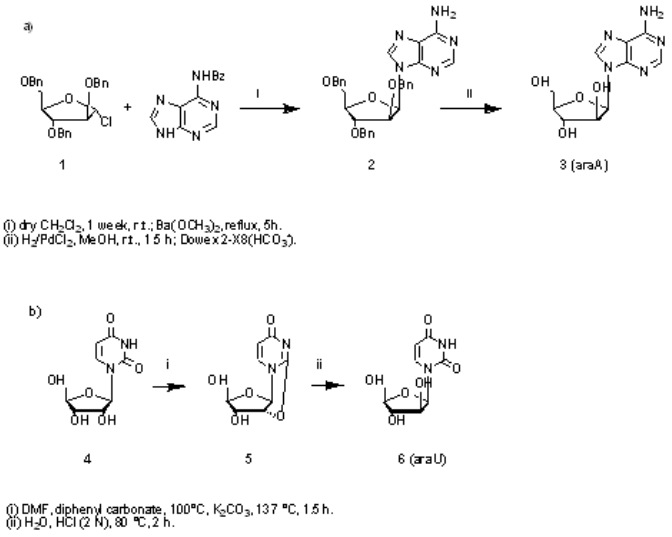
We here report on the enzymatic synthesis of the antiviral drug Vidarabine (arabinosyladenine, araA) starting from arabinosyluracil and adenine. To this aim, uridine phosphorylase from *Clostridium perfringens* (*Cp*UP) and a purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) were used as covalently immobilized biocatalysts. Upon investigation of the optimal conditions for the enzyme activity (phosphate buffer 25 mM, pH 7.5, 25 ° C, DMF 12.5-30%), the synthesis of araA was scaled up (2 L) and the product was isolated in 53% yield (3.5 g/L) and 98.7% purity. A E-factor comparison between the enzymatic synthesis of araA and the classical chemical procedure clearly highlighted the "greenness" of the enzymatic route over the chemical one (E-factor: 423 *vs* 1356, respectively).

Introduction

Nowadays biocatalysis can be considered one of the greenest technologies for the synthesis of high-added value molecules. The synthetic advantages of enzymes mainly lie on their high potential for chemo-, regio- and stereocontrol particularly when working with densely functionalized molecules. In this frame, and specially for multistep processes, the use of highly active and specific biocatalysts allows to circumvent those steps dealing with activation, protection and deprotection of functional groups, so that the biotransformation would produce less waste and would demand a lower energy consumption.[1] The synthesis of a pharmaceutical agent, in particular, is frequently accompanied by the use and generation of large quantities of hazardous substances and is thus considered the worst process in terms of E-factor (mass ratio of waste to the desired product).[2,3] Therefore it is not surprising to recall the high potential that biocatalyzed processes are gaining inside the frame of Pharma Industry.[4] Moreover, avoiding protection/deprotection steps by the introduction of biocatalysis leads to a higher atom economy.[5]

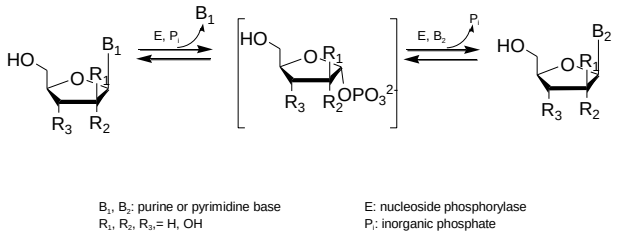
Nevertheless the number of biocatalyzed chemical reactions is still limited due to reaction constraints dictated by the need for enzymes or whole cells to work under physiological conditions. Therefore, enzymes have to be tailored to meet the process requirements that often negatively affect both operational stability and shelf-life of the biocatalyst. Although genetic engineering is increasingly gaining importance for these purposes,[6,7] immobilization is often the key to improve the operational performances of enzymes;[8-11] other potential benefits of enzyme immobilization arise from the prevention of protein contamination of the products and the possibility of recycling the biocatalyst.

Important classes of antiviral and anticancer drugs are derived from nucleosides.[12] Among them, Vidarabine (arabinosyladenine; araA) is used for systemic treatment of *Herpes simplex* and other viruses.[13,14] Unnatural nucleosides and nucleotides are routinely synthesized by multistep chemical procedures which are often plagued by the formation of undesired by-products and low overall yields. These drawbacks strongly reduce the performance of the processes in terms of purity and costs of the final product as well as of environmental impact. The established chemical synthesis of Vidarabine is depicted in Scheme 1.[15] Following a convergent approach, N^6-benzoyladenine was reacted with 2,3,5-*O*-benzyl-D-arabinofuranosyl chloride (**1**, 90% of 1-α-chloro derivative) achieving compound **2** in 46% yield. The hydrogenation of **2** affords free 9-β-D-arabinofuranosyladenine (**3**) in more than 90% yield (Scheme 1a). On the contrary, pyrimidine arabinonucleosides can be readily prepared from the corresponding pyrimidine nucleoside *via* 2,2'-anhydronucleoside and subsequent hydrolysis (Scheme 2b).[16]



Scheme 1 Chemical synthesis of arabinosyladenine and arabinosyluracil.[15,16]

In this scenario, the use of a biocatalyst appears convenient: in the last decade many reviews or tutorial articles covering the use of enzymes or cells for the synthesis of nucleosides or analogues have been published. [17-19] In this context, nucleoside phosphorylases (NPs; EC: 2.4.2.n) are a class of enzymes reported to catalyze the reversible cleavage of the glycosidic bond of (deoxy)ribonucleosides in the presence of inorganic orthophosphate (P_i) to generate the nucleobase and α-D-(deoxy)ribose-1-phosphate. The presence of an acceptor nucleobase (B_2) in the reaction medium can result in the formation of a new nucleoside (transglycosylation) (Scheme 2). The transfer reaction catalyzed by NPs was first discovered by Krenitsky in 1968[20] and since then the synthetic application of this class of enzymes has been extensively studied, as demonstrated by several examples in literature and patents.[18-19, 21-24] NPs can be, thus, considered a powerful tool for the synthesis of modified nucleosides.

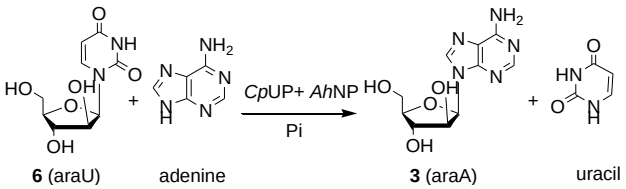


Scheme 2 General scheme of the transglycosylation reaction catalyzed by NPs.

Within this field, our research group has been largely involved in the study of stable and active immobilized enzymes for the synthesis of nucleoside analogues.[21,24-26]

In particular, a collection of immobilized and stabilized NPs has been screened towards different substrates;[27] among them, a purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP; EC: 2.4.2.1) and a uridine phosphorylase from *Clostridium perfringens* (*Cp*UP; EC: 2.4.2.3) resulted interestingly active towards natural as well as modified nucleosides.[22,27] *Ah*PNP coupled with *Cp*UP have been shown to be a good enzymatic twosome for carrying out the synthesis of arabinosyladenine and 2',3'-dideoxyinosine (Scheme 3).[27,28]

In this work, the reaction conditions for the synthesis of araA have been extensively investigated and finally optimized, considering pH and presence of organic cosolvents. The transglycosylation reaction was developed at a preparative scale (2 L), in order to test the suitability of immobilized *Cp*UP and *Ah*PNP to be used as biocatalysts in a large scale process. Isolation and purification of the product has been also studied. The synthesis of araA may be considered a leading reaction not only because of the relevance of the antiviral product obtained, but also because of the drastic conditions required for the development of a preparative process in homogeneous medium; this is ascribed to the very low solubility of the reaction product in fully aqueous medium. Moreover, a comparison in terms of E-factor between the established and the new biocatalyzed reactions was here carried out.



Scheme 3 Synthesis of araA by transglycosylation catalyzed by *Cp*UP and *Ah*PNP.

Results and Discussion

Enzyme production and immobilization

Fermentation and purification of both the His-tagged nucleoside phosphorylases was performed up to 200 liter scale (Table 1), affording over 700 kIU of each biocatalyst.

Table 1 Fermentation and purification of the enzymes.

Enzyme	Fermentation volume (L)	Final volume (mL)	Protein amount (mg)	Total units (kIU)	IU/mg
*Cp*UP	15	190	4500	428	95
	200	2000	22000	800	36
*Ah*PNP	15	190	3760	416	110
	200	1000	7200	720	100

The purified enzymes have been immobilized on aldehyde agarose loading different amounts of protein (Table 2).

The load of the protein on the support (mg/g) is, in fact, a crucial factor for the outcome of the immobilization and for the application of the immobilized biocatalysts at a preparative scale. The study of immobilization, carried out on a small scale using minimal amounts of protein (usually 1 mg of enzyme per gram of support), is used to select the experimental conditions for the immobilization, but normally produces a poorly active biocatalyst for a preparative synthetic application. Therefore, it becomes necessary to load on the support increasing amounts of protein so that the interplay of the load and the yield of immobilization can be evaluated.

The results of the immobilization scale up are reported in Table 2. As for *Ah*PNP, the greater the load of protein, the lower was the immobilization yield, differently from *Cp*UP where a trend was not observed. The immobilization was scaled up to prepare 0.2 Kg of each enzyme derivative by loading 10 mg of protein per gram of carrier in both cases. This choice was addressed by the need to obtain a sufficiently active biocatalyst for synthetic applications without losing activity upon immobilization. The results obtained were consistent with the data registered at the lab scale immobilization providing enzyme derivatives with an activity ranging from 70 to 90 IU/g for *Ah*PNP and *Cp*UP, respectively (Table 2).

Table 2 Immobilization scale up.

Enzyme	Load (mg/g)	Carrier (g)	Immobilized protein (%)[b]	Yield % (IU/g)[c]
*Ah*PNP[a]	1	1	100	30 (23)[27]
	5	5	100	17 (85)
	10	5	95	11 (110)
	25	15	95	6 (156)
	10	200	95	7 (70)
*Cp*UP	1	1	100	33 (22)[27]
	5	1	100	19 (34)
	10	5	98	27 (97)
	40	15	98	12 (172)
	10	200	96	25 (90)

Experimental conditions: pH 10, room temperature, 3 h; [a]immobilization performed with 20% glycerol and hypoxanthine 5 mM; [b]determined by Bradford method; [c]on the basis of the expressed activity considering inosine and 2'-deoxyuridine as the reference substrates for *Ah*PNP and *Cp*UP, respectively.

Determination of the activity and stability window

The main problem related to the enzymatic synthesis of araA in fully aqueous medium is the very low water solubility of this nucleoside. The attention was thus focused on the search for the experimental conditions able to enhance the solubility of the product without adversely affecting activity and stability of the enzymes. Since the reaction requires two enzymes in one pot, this study was particularly complex due to the need to identify reaction conditions compatible with the activity and stability of both enzymes.

Activity and stability of the two immobilized derivatives were tested under different conditions, suitable to be used for the development of the process at a high substrate concentration.

As the pKa value for araA is quite high (about 12.5), its solubility is expected to significantly increase through deprotonation as the pH of the reaction medium is increased from acidic to basic conditions. High pH (10) was previously used in the synthesis of 2'-deoxyguanosine to ensure the complete solubility of guanine.[21] However, the mode of binding of a charged substrate in the active site of the enzyme may differ from that one observed for a neutral substrate.[29] Therefore, a balance between substrate solubility, activity and stability of both enzymes must be achieved.

With the idea to perform the synthesis of araA at basic pH, the activity of immobilized *Cp*UP and *Ah*PNP in a wide pH range was evaluated (Figure 1). Except for pH 7 and 7.5, that were compatible with the activity of both enzymes, *Cp*UP and *Ah*PNP showed an opposite activity profile at acid pH (6) and weakly basic pH (8.5). These differences are less evident at pH 10 which, in our intent, could be used for the transglycosylation reaction but that determined a drop of the activity for both catalysts.

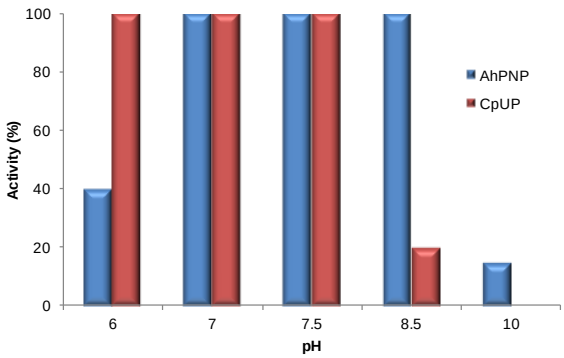


Fig. 1 Activity of the immobilized *Ah*PNP and *Cp*UP toward the standard substrates (2'-deoxyuridine and inosine for *Cp*UP and *Ah*PNP, respectively) at different pHs. Activities are normalized toward the values obtained with the standard assay performed in phosphate buffer at pH 7.5.

The use of non physiological pH values appeared thus to be not applicable for developing preparative processes using *Cp*UP and *Ah*PNP.

Alternatively, the use of different cosolvents can be considered. It is already well known that switching from hazardous solvents to more environmentally-friendly alternatives is currently a matter of intense research, aligned with the Green Chemistry philosophy,[30-31] and really crucial because solvents constitute the major source of waste in chemical processes;[32] furthermore, if we focus this fact for Pharma Industry, the situation is even more dramatic, because it is well known that solvents usually account for 80-90% of the non-aqueous mass of material usage for APIs synthesis, and consume about 60% of the overall energy used to the produce them.

For this reason, the activity of *Ah*PNP in mixtures of phosphate buffer with green solvents (such as glycerol, glycerol formal, and dimethylcarbonate) and *tert*-butanol (regarded as preferred)[33] at different proportions was measured and compared to the standard medium. As it can be seen in Figure

2, results were not very encouraging as the measured activity of the enzyme in those media was always lower than that one registered using only buffer.

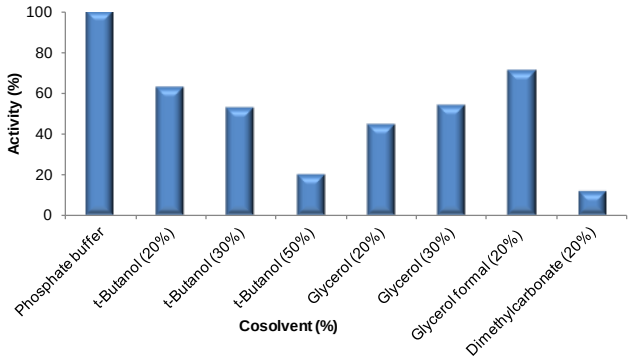


Fig 2 Activity of the immobilized *Ah*PNP toward inosine in mixtures of green solvents. Activities are normalized toward the values obtained with the standard assay performed in phosphate buffer at pH 7.5.

If one considers the enzymatic activity, some mixtures would have been acceptable (such as *tert*-butanol or glycerol formal); however, the solubility of araA in those media was quite poor. As an alternative, the use of less green cosolvents, frequently used in nucleoside chemistry and able to enhance product solubility, was indeed considered.

As depicted in Figure 3, dimethylformamide (DMF) and dimethylacetamide (DMA) allow to maintain completely the activity of *Ah*PNP, and more than 50% of the activity of *Cp*UP. A higher inhibition was observed in presence of acetonitrile (MeCN): at 30% of concentration of this cosolvent, about 50% of the activity was maintained by *Ah*PNP, and only 30% with *Cp*UP.

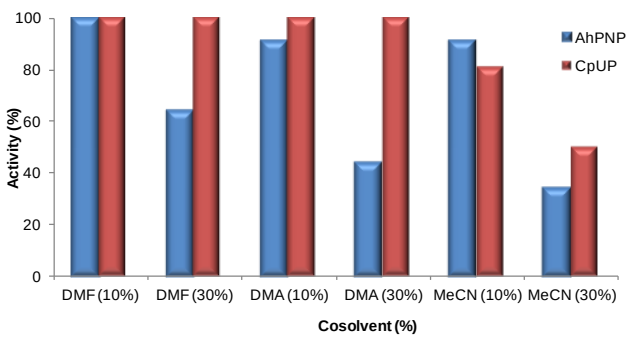


Fig 3 Activity of the immobilized *Ah*PNP and *Cp*UP toward the standard substrates (2'-deoxyuridine and inosine for *Cp*UP and *Ah*PNP, respectively) at different concentrations of cosolvents at pH 7.5. Activities are normalized toward the values obtained with the standard assay performed in phosphate buffer at pH 7.5.

Also for that concerning the stability, MeCN highly affected the two enzymes and in both cases about 50% of activity was retained in presence of 30% of this cosolvent, while with DMF and DMA a complete stability was observed even when concentration was increased to 50% (Table 3).

Table 3 Residual activity of the immobilized preparations after 24 h of incubation in presence of cosolvents at pH 7.5 and room temperature.

Enzyme	Solvent	Residual activity (%)
*Ah*PNP	DMF 20%	115
	DMF 30%	109
	DMF 50%	105
	DMA 30%	105
	MeCN 30%	51
*Cp*UP	DMF 20%	103
	DMF 30%	103
	DMF 50%	98
	DMA 30%	108
	MeCN 30%	48

Enzymatic synthesis of araA

The enzymatic reaction that from arabinosyluracil and adenine leads to the formation of arabinosyladenine (Scheme 3) has been optimized in order to maximize the yield, balancing catalytic activity of enzymes and solubility of the product.

For this purpose, the influence exerted by the cosolvent (DMF, DMA, MeCN) and phosphate concentration on the highest conversion and on the reaction rate was evaluated.

In agreement with the activity data, the addition of a small percentage of organic co-solvent (MeCN or DMF at 10% v/v) did not influence the outcome of the bioconversion, both in terms of reaction rate and the maximum conversion, which was in fact similar to that obtained in the absence of cosolvent (Table 4).

The effect of phosphate concentration was evaluated using a stoichiometric ratio of araU: adenine equal to 20:10: as reported in Table 4; the decrease of the phosphate concentration from 50 mM to 10 mM (entries 5-7) did not have a significant effect on the extent of the final conversion (about 72% in 48 h), although a decrease in the initial rate (v_s) was observed. For an easier purification, a phosphate concentration of 25 mM, which represents a good compromise between reaction rate and final conversion, was then used for the scale up of the bioconversion. In the same conditions, the use of DMF or DMA (entries 8 and 9) allowed to reach the highest conversion compared to MeCN. Moreover, increasing the concentration of these solvents from 10% to 20% no relevant reduction of yield was observed with DMF, while the yield decreased from 80% to 75% using DMA.

In conclusion, the results obtained indicate that the use of DMF could be preferred to other cosolvents in order to ensure the complete solubilization of araA during the synthesis. With this cosolvent (up to 30% of concentration) the enzymes retain their activity and the yields are similar or even higher compared with other cosolvents. Furthermore, the stability of both NPs was complete using up to 50% of DMF in buffer.

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Table 4 Study of the bi-enzymatic synthesis of araA.

Entry	AraU (mM)	Ade (mM)	Phosphate (mM)	Solvent (%)	*Cp*UP (IU)	*Ah*PNP (IU)	v_s[a]	Conversion % (h)
1	4	2	50	-	6	12	0.0251	63 (24)
2	4	2	50	DMF 10%	6	12	0.0211	63 (48)
3	4	2	50	MeCN 10%	6	12	0.0251	68 (48)
4	10	5	50	MeCN 10%	12	15	0.0372	74 (48)
5	20	10	50	MeCN 10%	12	15	0.0687	74 (48)
6	20	10	25	MeCN 10%	12	15	0.0664	72 (48)
7	20	10	10	MeCN 10%	12	15	0.0572	72 (48)
8	20	10	25	DMF 10%	12	15	0.0802	80 (48)
9	20	10	25	DMA 10%	12	15	0.0824	80 (48)
10	20	10	25	DMF 20%	12	15	0.0698	78 (48)
11	20	10	25	DMA 20%	12	15	0.0604	75 (48)

Experimental conditions: pH 7.5, 10 mL and room temperature. [a](μmol/min)

Based on the results obtained in the optimization study, and taking into account the conditions that could conjugate activity and stability of both enzymes, the reaction was scaled up using a concentration of 50 mM of araU and 25 mM of adenine in a final volume ranging from 20 mL to 2 L, in phosphate buffer 25 mM, pH 7.5 and at 25 ° C. Given the poor solubility of the reaction product, it was necessary the use of an automatic dispenser to control the progressive addition of DMF in order to enhance the solubilization of araA. The choice for a gradual increase of the cosolvent was dictated by the need to combine the activity of the enzymes with the solubility of araA. In fact, as shown in Figure 3, the activity of the two enzymes decreases significantly upon increasing concentration of DMF which is, however, essential to prevent the precipitation of the reaction product and thus to facilitate the separation from the immobilized biocatalysts.

The transglycosylation reaction was then performed in the presence of 12.5 % of DMF v/v for the initial 6 h, followed by the dropwise addition of DMF until 30% concentration over the following 18 h. The product was isolated in about 50% yield and high purity. In particular, 7.1 g of araA (purity 97.7 %, Figure S1, Supporting information) were isolated from the 2 L scale reaction. Identity of the isolated compound was confirmed by NMR and MS analyses (Figure S2 and Figure S3, Supporting information).

Table 5 Scale- up of the synthesis of araA.

Volume (mL)	*Cp*UP (IU)	*Ah*PNP (IU)	v_s[a]	Conv. % (h)	Isolated yield (%)	Purity (%)
20	48	60	0.6	73 (48)	nd	nd
100	250	300	3.8	77 (26)	45	96
500	1250	1500	20.1	77 (26)	56	96
2000	5000	6000	nd	78 (26)	53	98.7

Reaction conditions: 50 mM araU, 25 mM Ade, 25 mM phosphate buffer, pH 7.5, 25 °C, DMF 12.5%→30%. [a]μmol/min; nd: not determined

Biocatalyst recycling

Immobilized *Cp*UP and *Ah*PNP have been also tested in the *in continuum* synthesis of araA. The reactions were carried out in a final volume of 20 mL in the conditions described for the preparative synthesis (50 mM araU, 25 mM Ade, 25 mM phosphate buffer, pH 7.5, DMF 12.5→30%). When the highest conversion was achieved, DMF was added up to 50% to allow for the complete solubilization of the product. After filtration, the enzymes were further washed with 50% of DMF for 30 minutes and then re-used for a new bioconversion. As depicted in Figure 4, the biocatalysts completely retained their activity after 5 reactions (that corresponds to 1 working week) showing an optimal recyclability and indicating that they could be re-used for additional reactions.

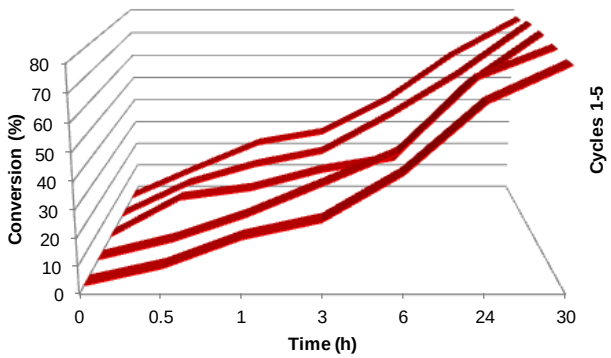

Fig 4 Cycles of transglycosylation reaction catalyzed by immobilized *Cp*UP and *Ah*PNP.

Comparison between chemical and enzymatic synthesis.

Drawing a straightforward comparison between different synthetic methods is not always easy, because many different parameters must be taken into account. Of course, to reach the higher reaction yield is the desired target but, adopting Green Chemistry postulates, many other chemometrics must be considered; between them, E-factor (mass ratio of waste to the desired product) is one of the more frequently applied.[2-3]

Thus, a E-factor comparison has been established between the enzymatic synthesis proposed in this paper and the classical chemical methodology described by Glaudemans and Fletcher (see Supporting information for details on the calculations).[15] The enzymatic protocol (at 2 L scale) would lead to different E-values depending on the reagents which are taken into account, as shown in Table 6.

As it can be seen, the enzymatic methodology presents clear advantages over the chemical procedure. For sure, the inclusion of solvents is the main factor to contribute to E-factor, as we have stated before. Furthermore, as the enzymatic derivative can be efficiently re-used at least five times without a noticeable decline of the activity, the waste contribution of the biocatalyst is clearly reduced, and also the economical balance of the overall protocol is undoubtedly improved.

Table 6 Comparison of E-factor values for the enzymatic synthesis (2 L scale).

Enzymatic synthesis				
E Values	***No enzyme waste No solvent***	***Enzyme waste No solvent***	***No enzyme waste Solvent***	***Enzyme waste Solvent***
Based on analytical yield	2.45	26.25	285	309
Based on final yield	3.65	39.13	423	460
Chemical synthesis (Glaudemans and Fletcher)[15]				
	No solvent		***Considering solvents***	
First step	2		201	
Second step	3.05		1155	
Overall	5.05		1356	

Very recently, a new methodology for the synthesis of Vidarabine has been described by Xia et al., through the dehydrazination of 1.5 kg of 8-hydrazine-vidarabine (obtained from a non-described Vidarabine producing company) catalyzed by $CuSO_4$ in water, on a 100 L reaction vessel at 80 ºC for 6 h, to produce 0.975 kg of crude Vidarabine (yield 75%), and 0.923 kg (yield 71%) after recrystallization.[34] These Authors claimed the greenness of this process as compared to a previous one, which required an excess of HgO as the dehydrazination reagent, with Hg produced as a side product and causing serious environmental pollution. As only this step is described in the paper, no direct comparison in terms of E-factor can be established. In any case, the mildness of our enzymatic protocol, carried out at room temperature, is a clear advantage compared to the dehydrazination methodology, as clearly pointed in the 6th Principle of Green Chemistry.[35]

Experimental

General

Adenine, inosine and solvents were purchased from Sigma Aldrich and/or VWR International (Milano, Italy). 2'-Deoxyuridine was supplied by Pro.Bio.Sint. (Varese, Italy). 6% Crosslinked agarose beads were from ABT (Burgos, Spain). Arabinosyluracil (**6**) was purchased from Xinxiang Tuoxin Biochemical Technology & Science Co., Ltd (China). Arabinosyladenine was purchased from Jena Bioscience (Jena, Germany). Enzymatic reactions were monitored by using a HPLC Merck Hitachi L-7100 equipped with a UV detector L-7400 and column oven L-7300 (Darmstadt, Germany). All solvents were HPLC grade.

AccuPrime Pfx DNA Polymerase, pET151/D-TOPO vector and *Echerichia coli* BL21 Star (DE3) chemically competent cells were purchased from Invitrogen (San Giuliano Milanese, Italy). Protein concentration assay was performed on a Shimadzu spectrophotometer UV 1601 by Bradford method using bovine serum albumin as standard. All experiments were performed at least as duplicate.

^{1}H NMR spectrum of Vidarabine was recorded DMSO-d_6 at 400.13 Hz on a Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running a Windows operating system equipped with a XWIN-NMR or TOPSPIN software package, at 300 K (Figure S2 Supporting information)..

Electrospray ionization mass spectrum (ESI-Q-Tof-MS) was recorded on a Micromass Q-Tof micro mass spectrometer (Waters, Milford, Massachusetts, U.S.A.) (Figure S3 Supporting information).

Enzyme production

Cloning and expression of *Clostridium perfringens* udp gene in *E. coli*. Uridine phosphorylase from *Clostridium perfringens* (*Cp*UP) is coded by the udp gene that has been amplified from the *Cp*UP pGEX-2T plasmid [27] by PCR, using gene specific primers (OL128: 5'-CACCATTTATACACAAGGTTCAGACAA-3'; OL129: 5'-

CTATGCATTATTTTTCATTTTATCTTCTTT-3').

The amplified gene has been ligated into the pET151/D-TOPO vector (for the expression of the recombinant protein with an N-terminal 6xHis-tag) and the correct construction of the expression plasmid, named PL50, has been verified by direct sequencing of the cloned region.

Preparation of the enzymes. The expression of the recombinant *Cp*UP and *Ah*PNP was obtained from cultures of *E. coli* BL21 Star (DE3) transformed with the corresponding plasmids.

Expression of both enzymes carried out at 15 L and 200 L fermentation volume consisted of a fermentation process composed of 3 phases: preseed, seed, fermentation.

The preseed cells were prepared in LB medium, incubated at 37 °C for 6 h and then inoculated in the pre-fermentative medium (yeast extract 1 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 15 g/L, $MgSO_4$ 5 g/L, dextrose 10 g/L) to a final concentration of 0.5% (v/v). The seed culture was incubated at 30 °C for 16 h and was subsequently diluted at 5% into a stirred bioreactor containing the fermentative medium ($(NH_4)_2SO_4$ 2 g/L, K_2HPO_4 15 g/L, KH_2PO_4 7.5 g/L, $MgSO_4$ 2 g/L, dextrose 50 g/L). The pH was controlled by automatic addition of NH_4OH 25%; the stirring rate was adjusted to 500 rpm and the pO_2 was maintained automatically at 50% with aeration and agitation. The strain was grown to mid-exponential phase at 30 °C and then the protein expression was induced by adding 0.5 mM isopropylthio-β-D-galactoside (IPTG). After a 24 h expression phase, the cells were separated from the broth by centrifugation and disrupted by two cycles of homogenization using a GEA Niro Soavi Homogenizer at a pressure of 1000 bar. The suspension was then clarified by the addition of 0.5% quaternary ammonium salt and centrifugation (Avant J25 Beckman Coulter).

The enzymes were purified by column chromatography with Ni Sepharose™ 6 Fast Flow (GE Healthcare). The adsorbed enzymes were then eluted with a linear gradient of 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8. Fractions containing the active enzymes were collected, dialyzed by 5 kDa ultrafiltration and stored at -20°C.

The purity of both the proteins (>90%) was assessed by SDS-PAGE electrophoresis (4-12%).

Standard activity assay. The standard enzyme activity assay was performed as previously reported.[27] One international unit (IU) corresponds to the amount of enzyme that converts 1 μmol of substrate per minute at room temperature. The specific activity is defined as units of enzyme activity per milligram of protein.

Immobilization. Immobilization on aldehyde agarose was performed following the procedure previously reported.[26]

Stability. The immobilized enzyme preparation (200 mg) was added to a solution (1.5 mL) containing 50 mM phosphate buffer and the solvent at the desired concentration (v/v) at pH 7.5. The mixture was stirred at room temperature for 24 h. At fixed times, 40 μL of suspension were withdrawn and activity was tested by standard activity assay.

General procedure for transglycosylation. A) Analytical scale. A solution of phosphate buffer (10 mL) with the appropriate amount of solvent (v/v) at pH 7.5 containing araU (**6**) and adenine at the desired concentration was prepared. The enzyme preparations were added to the reaction and the mixture was kept at room temperature under mechanical stirring till the highest conversion was achieved. The reaction was monitored by HPLC (λ= 260 nm) identifying the products by comparing their retention times with those of authentic samples. Mobile phase: 0.01 M KH_2PO4 buffer pH 4.6/methanol 90% (90:10). Uracil, R_t=3.85 min, adenine, R_t=7.42 min, arabinosyluracil (**6**), R_t=5.62 min, arabinosyladenine (**3**), R_t=12.33 min. The percentage of conversion was calculated on the basis of the depletion of sugar acceptor compound (heterocyclic base) and monitoring the formation of the nucleoside products: Conversion (%)= [product area / (product area + base area)]× 100. The reaction was stopped by *s.v.* filtration of the immobilized biocatalyst. B) Preparative scale. A solution of 25 mM adenine in phosphate buffer (25 mM) containing 10% of DMF (v/v) was prepared by adding 6 N HCl until the complete dissolution of adenine was achieved. The solution was set to pH 7.5 with NaOH and 50 mM araU was then added. The reaction was started by adding the enzyme preparations (see Table 5) and the mixture was kept at 25 °C under mechanical stirring. After 6 h, DMF was added dropwise at 0.3 mL/min to reach 30% of DMF (v/v). At the end of the reaction, in order to completely dissolve araA, an additional 20% of DMF was added to the reaction and the mixture was allowed to stir for 1 h. The enzyme was then filtered off and washed twice with phosphate buffer containing 50% DMF (v/v) at pH 7.5 for 30 minutes in order to recover the residual araA. Conversion was determined quantitatively by means of external standard procedure.

Enzyme recycling. Recycling of immobilized *Ah*PNP and *Cp*UP was performed by evaluating the outcomes of synthesis of araA by transglycosylation, in the same conditions described in Section General procedure for transglycosylation (B, preparative scale) in a final volume of 20 mL. At the end of each cycle the final mixture was filtered under reduced pressure and the immobilized biocatalyst was re-used for the following reaction.

Product purification. After removing DMF under reduced pressure, the mixture was cooled at 4 °C for 24-72 h till a white precipitate (araA) was formed. The suspension was then kept at -10 °C for at least 3 h to allow for the complete precipitation of araA. The white solid was recovered by filtration *s.v.* and dried in the oven at 50 °C and 5-10 mbar till the weight remained constant. Arabinosyladenine (4 g) was obtained in 97.77% purity. The mother liquor was concentrated to dryness under vacuum affording 39 g of a wet solid that was immediately dissolved in demineralized water (250 mL) and refluxed. Once a clear solution was obtained (around 70 °C), the mixture was cooled to 35 °C. A precipitate was recovered by filtration *s.v.* and dried (50 °C, 5-10 mbar) affording a second crop of raw araA (4 g, purity 77.34%). The solid was suspended in demineralized water (1/6 w/V) and refluxed for 1 h. The

suspension was cooled to 50 °C and then filtered *s.v.* The recovered white solid (araA) was dried at 50 °C and 5-10 mbar (3.1 g, purity 98.73%). The overall yield was 7.1 g (53%).

Conclusions

We have developed a new synthesis of the antiviral drug Vidarabine *via* a transglycosylation reaction catalyzed by a twosome of immobilized nucleoside phosphorylases (*Cp*UP and *Ah*PNP). The reaction was scaled up till 2 L (yield= 3.5 g/L) in phosphate buffer, containing DMF as the cosolvent, at room temperature and neutral pH in a "one-pot, one-step" mode. The downstream processing was performed by a simple precipitation and crystallization from water. Moreover, the biocatalysts were recycled and successfully reused for five reactions (corresponding to one working week) without loss of activity and thus proving that they could be further used to catalyze this bioconversion. Overall, the biocatalyzed synthesis of araA here described is superior to the chemical synthesis described in the literature[19] as it is characterized by less steps, milder reaction conditions and reagents, easier downstream, lower E factor.

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