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Immobilized *Drosophila melanogaster* Deoxyribonucleoside Kinase (*Dm*dNK) as a High Performing Biocatalyst for the Synthesis of Purine Arabinonucleotides

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Abstract: Fruit fly (*Drosophila melanogaster*) deoxyribonucleoside kinase (*Dm*dNK; EC: 2.7.1.145) was characterized for its substrate specificity towards natural and non-natural nucleosides, confirming its potential in the enzymatic synthesis of modified nucleotides. *Dm*dNK was adsorbed on a solid ion exchange support (bearing primary amino groups) achieving an expressed activity >98%. Upon cross-linking with aldehyde dextran, expressed activity was 30-40%. Both biocatalysts (adsorbed or cross-linked) were stable at pH 10 and room temperature for 24 h (about 70% of retained activity). The cross-linked *Dm*dNK preparation was used for the preparative synthesis of arabinosyladenine monophosphate (araA-MP) and fludarabine monophosphate (FaraA-MP). Upon optimization of the reaction conditions (50 mM ammonium acetate, substrate/ATP ratio = 1:1.25, 2 mM $MgCl_2$, 37 °C, pH 8) immobilized *Dm*dNK afforded the title nucleotides with high conversion (>90%), whereas with the soluble enzyme lower conversions were achieved (78–87%). Arabinosyladenine monophosphate was isolated in 95% yield and high purity (96.5%).

Keywords: biocatalysis; deoxyribonucleoside kinase; immobilization; nucleotides; phosphorylation

Introduction

Natural nucleosides and nucleotides are implicated in several physiological pathways, being the constituents of nucleic acids, intracellular mediators and coenzymes (i.e., ATP, NADH). Specifically, all organisms require 2′-deoxyribonucleoside triphosphates (dNTPs) for DNA biosynthesis and its regeneration. dNTPs are synthesized by the *de novo* or the salvage pathway. The key step in the *de novo* pathway is through ribonucleotide reductase, where ribonucleoside diphosphates are reduced to the corresponding deoxyribonucleoside diphosphates.[1] The salvage route of dNTPs starts from 2′-deoxyribonucleosides (dNs) that are phosphorylated by intracellular 2′-deoxyribonucleoside kinases (dNKs) to 2′-deoxyribonucleoside monophosphates (dNMPs) which are then converted by deoxyribonucleoside monophosphate and diphosphate kinases into the corresponding diphosphates (dNDPs) and triphosphates (dNTPs), respectively. The number and the specificities of dNKs vary from organism to organism but, in all cases, dNKs phosphorylate 2′-deoxyribonucleosides much more efficiently than they phosphorylate ribonucleoside counterparts.[2]

Natural ribonucleoside monophosphates are not only nucleic acid building blocks but they are also employed as food additives due to their immunostimulant and taste enhancer activity.[3–5]

Nucleoside analogues are widely used as antiviral and antitumour agents.[6,7] They can be incorporated in the viral or cellular DNA by DNA polymerases after phosphorylation by kinases, or they can inhibit the enzymes involved in the biosynthesis of DNA precursors.[8]

Vidarabine (arabinosyladenine, araA), firstly designed as an antineoplastic drug, is now used as a second-line drug for the systemic treatment of

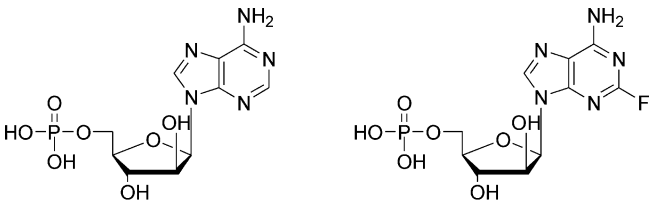
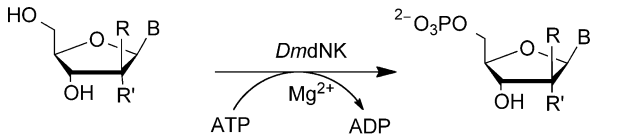


Figure 1. Chemical structures of araA-MP and FaraA-MP.

Herpex simplex viruses. Upon administration, this compound is phosphorylated to the triphosphate (araA-TP) and competes with 2′-deoxyadenosine triphosphate (dAdo-TP) as substrate for the viral DNA polymerase leading to the formation of a defective DNA and resulting in the prevention of viral infection.[9] However, the potential usefulness of araA is limited by its poor solubility and the treatment with araA requires a continuous intravenous infusion. To overcome this drawback, araA-5′-monophosphate (araA-MP, Figure 1) has been proposed as a soluble prodrug which can be administered intermittently intramuscularly or intravenously.[10] The fluorinated counterpart of araA-MP, fludarabine monophosphate (FaraA-MP, Figure 1), is used against hematological malignancies such as leukemias and lymphomas.[11]

In contrast to mammals, the fruit fly *Drosophila melanogaster* was found to have only one multisubstrate deoxyribonucleoside kinase (*Dm*dNK; EC: 2.7.1.145). This dNK is able to phosphorylate all four natural 2′-deoxyribonucleosides with high efficiency. The broad specificity and high turnover towards the naturally occurring 2′-deoxyribonucleosides by *Dm*dNK is reflected in a similar broad specificity towards nucleoside analogues used in chemotherapy.[12]

*Dm*dNK is active as a homodimer with a global molecular weight of 58 kDa. *Dm*dNK is an Mg^{2+}-dependent 5′-phosphotransferase and catalyzes the formation of a 5′-phosphate nucleoside and ADP from a nucleoside and a phosphate donor like ATP (Scheme 1).[2]



nucleoside — nucleoside 5'-MP

2'-deoxyribonucleosides	R = R' = H
ribonucleosides	R = H, R' = OH
arabinonucleosides	R = OH, R' = H

Scheme 1. Phosphorylation reaction of nucleosides catalyzed by *Dm*dNK.

This enzyme is particularly interesting from an industrial viewpoint for the environmentally sustainable large-scale production of dNTPs and their analogues.[13] Chemical synthesis of mononucleotides is routinely performed by treating the precursor nucleoside with a large excess of $POCl_3$ in acetic acid in the presence of pyridine or using short-chain trialkyl phosphates (i.e., trimethyl phosphate) as solvents under anhydrous conditions at 0 °C.[14–16] In both cases, these synthetic routes usually provide poor or moderate yields, low product purity and are also associated with harsh reaction conditions and waste disposal issues.

In this frame, the application of white biotechnology in the synthesis of nucleotides is an attractive field since biotransformations do not usually require tedious protection/deprotection steps and frequently occur under milder and greener conditions due to the high regio- and stereospecificity of the enzymes. The chemical production of active pharmaceutical ingredients is reported as the worst process for the mass ratio of waste *versus* product (E-factor).[17–19] The use of biocatalysts can thus provide novel and straightforward synthetic schemes for developing sustainable industrial processes that turn in high yields and purity of the products.[20–22]

To date, an efficient alternative to dNKs is represented by class A bacterial non-specific acid phosphatases (NSAPs, EC: 3.1.3.2) which have been reported to catalyze the phosphorylation of nucleosides[23,24] and other compounds (i.e., carbohydrates)[25] using pyrophosphate as a phosphate donor. However, final yields are plagued by the hydrolysis of the products, thus requiring a laborious reaction optimization or the use of specific inhibitors.[25] For some substrates, in fact, dephosphorylation is much faster than phosphorylation.[26] NSAPs display higher affinities for ribonucleosides than 2′-deoxyribonucleosides.[27] This feature complements the specificity of dNKs, mostly directed *versus* 2′-deoxyribonucleosides, thus implementing the toolset of phosphorylating enzymes.

The bottleneck in the use of enzymes as biocatalysts is often their instability and poor performance under reaction conditions, their high cost and solubility in the reaction medium. These issues, which may affect the economy of a biocatalytic process, can be frequently overcome by immobilizing the enzyme on a solid support, especially in the case of multimeric enzymes. In fact, these enzymes may be easily inactivated by dissociation of the protein subunits.[28–31] A convenient strategy to achieve immobilization and stabilization of multimeric enzymes is the immobilization *via* ionic exchange of the enzyme on a carrier (epoxy Sepabeads) coated with polyethylenimine (PEI) followed by cross-linking with a polyaldehyde macromolecule (oxidized dextran). This process permits one to obtain a multisubunit immobilization.[32,33]

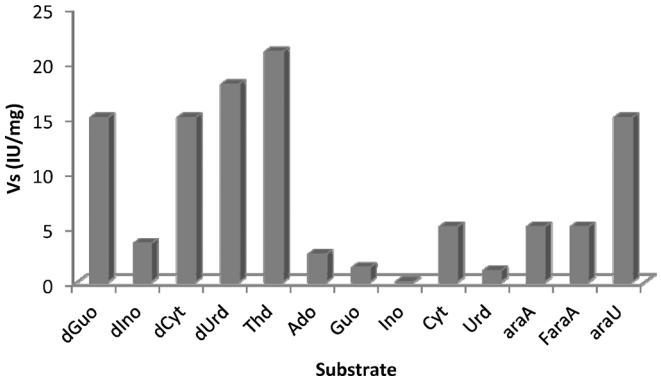


Figure 2. Substrate specificity of the soluble *Dm*dNK. IU: μmol min^{-1}.

Furthermore, in some cases, enzyme properties have been enhanced upon immobilization.[34]

The present work describes the study of substrate specificity of *Dm*dNK from the standpoint of its application as a biocatalyst, its immobilization on a solid support and the use of the resulting biocatalyst for the preparative synthesis of araA-MP and FaraA-MP.

Results and Discussion

Substrate Specificity

The soluble *Dm*dNK was tested for its substrate specificity towards 2′-deoxyribo-, ribo- and arabinonucleosides (Figure 2, Table 1).

Table 1. Phosphorylation catalyzed by *Dm*dNK as depicted in Scheme 1. In parentheses, the maximum percentages of conversion after 24 h are given.[a]

Nucleoside	R	R′	B	Nucleotide (%)
dAdo (**1**)	H	H	adenine	**1a** (>98)
dGuo (**2**)	H	H	guanine	**2a** (>98)
dIno (**3**)	H	H	hypoxanthine	**3a** (30)
dCyt (**4**)	H	H	cytosine	**4a** (>98)
dUrd (**5**)	H	H	uracil	**5a** (>98)
Thd (**6**)	H	H	thymine	**6a** (>98)
Ado (**7**)	H	OH	adenine	**7a** (5)
Guo (**8**)	H	OH	guanine	**8a** (<5)
Ino (**9**)	H	OH	hypoxanthine	**9a** (<5)
Cyt (**10**)	H	OH	cytosine	**10a** (81)
Urd (**11**)	H	OH	uracil	**11a** (64)
araA (**12**)	OH	H	adenine	**12a** (>98)
FaraA (**13**)	OH	H	2-F-adenine	**13a** (>98)
araU (**14**)	OH	H	uracil	**14a** (>98)

[a] *Experimental conditions:* substrate, 1 mM; ATP, 2 mM; buffer, 50 mM Tris HCl pH 8; $MgCl_2$, 2 mM; temperature, 37 °C; volume, 1 mL.

This enzyme efficiently catalyzed the phosphorylation of natural 2′-deoxyribonucleosides allowing >98% transformation of these substrates; with dIno (2′-deoxyinosine, **3**) the enzyme displayed a reaction rate five-fold lower than with dAdo (2′-deoxyadenosine, **1**) achieving only 30% conversion after 24 h (Table 1). *Dm*dNK showed, after 24 h, less than 5% conversion of Ado (**7**), Guo (**8**) and Ino (**9**). Cytidine (**10**) was converted in up to 81% after 24 h with a reaction rate of 6 IU/mg. A good conversion (64%) was achieved also with uridine (**11**) even if the specific activity was less than 1 IU/mg (Table 1). In agreement with previous results,[35] in general *Dm*dNK preferentially phosphorylated pyrimidine derivatives and was also able to synthesize the nucleotide analogues araA-MP (**12a**) and FaraA-MP (**13a**). In the latter case final conversions after 24 h were comparable with those of the natural substrates (2′-deoxyribonucleosides) (Table 1).

*Dm*dNK Immobilization

With the aim to prepare an active, stable and recyclable biocatalyst, *Dm*dNK was immobilized on a solid support.

Immobilization was performed through a two-step procedure, previously reported for immobilization and stabilization of multimeric enzymes.[32,33] Initially, the experiments were performed by loading a low amount of enzyme on the carrier (0.1–0.5 mg g^{-1}) in order to control diffusion problems. The enzymatic activity was completely retained after adsorption on the ionic carrier and after cross-linking in almost all cases while, as expected, the crucial step was the chemical reduction of imines to stable C–N bonds which resulted in a striking loss of activity of the biocatalyst (about 2/3 of initial activity was recovered) (Table 2).

To minimize the loss of activity upon chemical reduction, two strategies were pursued: reduction was performed by using a lower amount of $NaBH_4$ (entry 3 Table 2) or by replacing $NaBH_4$ with the same amount (1 mg mL^{-1}) of $NaBH_3CN$ (entry 4 Table 2) which is a milder reducing agent. However, in both cases, no significant improvement of the final activity was observed. Moreover, not even the addition of dAdo to the immobilization medium was helpful in preserving the enzyme activity during reduction (entry 5 Table 2). dAdo, in fact, could shield the enzyme active site by binding to it, thus avoiding possible distortions occurring during the immobilization process.[36]

Using the standard immobilization conditions, a higher amount of protein was also loaded per gram of carrier (2 mg g^{-1}) and 40% of the total activity was

Table 2. Immobilization of *Dm*dNK on Sepabeads PEI 600 cross-linked with aldehyde dextran.

Entry	IU/g ($mg\,g^{-1}$)	Adsorbed activity (%)	Activity after cross-linking IU/g (%)	Reducing agent	Final activity (IU/g)	Recovered activity (%)[a]
1	2 (0.1)	>98%	2.0 (100)	$NaBH_4$	0.6	27
2	10 (0.5)	>98%	8.5 (85)	$NaBH_4$	2.7	27
3[b]	10 (0.5)	>98%	10.0 (100)	$NaBH_4$	2.9	29
4	13 (0.6)	>98%	11.5 (89)	$NaBH_3CN$	1.5	12
5[c]	10 (0.5)[c]	>98%	nd	$NaBH_4$	2.7	28
6	40 (2.0)	>98%	nd	$NaBH_4$	16.0	40

[a] Calculated on the basis of the expressed activity considering dAdo as the reference substrate.
[b] [$NaBH_4$] was reduced by half.
[c] 2 mM dAdo were added to the immobilization suspension; nd: not determined.

recovered obtaining a solid biocatalyst with 16 IU/g of activity (entry 6 in Table 2).

The stability at pH 10 of immobilized *Dm*dNK before and after cross-linking was investigated. The results obtained could not highlight any difference between the two enzyme preparations under these conditions; in fact, after 24 h of incubation, both preparations retained more than 70% of the initial activity (Figure 1 in the Supporting Information).

Moreover, the possible leakage of the protein from the support under the reaction conditions was evaluated, because of the reversibility associated to this immobilization.[37,38] To this aim, the cross-linked and non-cross-linked preparations were incubated at either 50 mM Tris HCl pH 8 or 50 mM ammonium acetate pH 8 and no activity could be detected in solution. These results indicate that both the enzyme preparations can be used indistinctly. However, for the preparation of active pharmaceutical ingredients, a covalently bound enzyme (thus cross-linked) is highly desirable to avoid the possibility of product contamination. For this reason, the cross-linked immobilized *Dm*dNK was used in this report.

Preparative Enzymatic Synthesis of araA-MP (12a) and FaraA-MP (13a)

The reaction conditions for the enzymatic synthesis of nucleotides araA-MP and FaraA-MP have been extensively explored in order to develop a preparative process. The reaction parameters that have been considered are: reactant concentration (stoichiometric ratio), type of buffer and $MgCl_2$ concentration. The effect of these parameters was evaluated both with the soluble and the immobilized enzyme.

Role of ATP

The molar ratio between nucleoside and ATP was investigated (Table 3) aiming at maximizing the conversion with a small excess of ATP.

Considering the soluble enzyme, in the phosphorylation of dAdo (**1**) using 1.25 or 1 equivalent of ATP, the yield slightly decreased, compared with the reaction performed with 2 equivalents of ATP (from 99 to 95% and 90%, respectively) and, to a higher extent, the same trend was observed in the phosphorylation of araA (**12**) where 52% and 89% of conversion was achieved using a 1:1 and 1:2 molar ratio of ATP, respectively.

With the soluble enzyme it is evident that the concentration of ATP exerts a remarkable effect on the non-natural substrate, i.e., araA, poorly affecting the outcome of the phosphorylation of dAdo. On the contrary, immobilization resulted in high conversion at all three molar ratios of nucleoside and ATP (Table 3). Consequently, besides the well known advantages of

Table 3. Phosphorylation of nucleosides with different amounts of ATP.[a]

Substrate	*Dm*dNK	ATP (mM)	Product	Conversion % (hours)
dAdo (**1**)	soluble	2	**1a**	99 (3)
dAdo (**1**)	immobilized	2	**1a**	99 (3)
dAdo (**1**)	soluble	1.25	**1a**	95 (3)
dAdo (**1**)	immobilized	1.25	**1a**	99 (3)
dAdo (**1**)	soluble	1	**1a**	90 (3)
dAdo (**1**)	immobilized	1	**1a**	96 (3)
araA (**12**)	soluble	2	**12a**	89 (6)
araA (**12**)	immobilized	2	**12a**	99 (8)
araA (**12**)	soluble	1.25	**12a**	59 (12)
araA (**12**)	immobilized	1.25	**12a**	97 (8)
araA (**12**)	soluble	1	**12a**	52 (12)
araA (**12**)	immobilized	1	**12a**	95 (12)
AraA (**12**)[b]	immobilized	1	**12a**	60 (12)
FaraA (**13**)[c]	soluble	1:1.25	**13a**	59 (4)
FaraA (**13**)[c]	immobilized	1:1.25	**13a**	99 (4)

[a] *Experimental conditions:* substrate, 1 mM; buffer, 50 mM Tris·HCl pH 8; $MgCl_2$, 2 mM; temperature, 37 °C; volume, 5 mL. Soluble enzyme: 0.2 IU; immobilized enzyme: 0.16 U.
[b] Addition of K_2CO_3 50 mM, pH 10.5.
[c] Volume 1 mL; enzyme 0.02 IU.

Table 4. Influence of reaction conditions on the enzymatic phosphorylation.[a]

Substrate	*Dm*dNK (IU)	Buffer	$MgCl_2$ (mM)	Vs (IU/mg)	Product	Conversion % (hours)
araA (**12**)	soluble (0.4)	Tris	2	4.4	**12a**	90 (6)
araA (**12**)	soluble (0.4)	acetate	2	4.9	**12a**	86 (6)
araA (**12**)	immobilized (0.2)	Tris	2	0.9	**12a**	96 (6)
araA (**12**)	immobilized (0.2)	acetate	2	0.8	**12a**	98 (6)
FaraA (**13**)	soluble (0.02)	Tris	2	5.0	**13a**	>98 (4)
FaraA (**13**)	soluble (0.02)	acetate	2	3.9	**13a**	>98 (4)
FaraA (**13**)	soluble (0.02)	Tris	0.2	3.7	**13a**	>98 (4)
FaraA (**13**)	soluble (0.02)	acetate	0.2	3.9	**13a**	>98 (4)
FaraA (**13**)	soluble (0.02)	Tris	0.04	3.4	**13a**	96 (6)
FaraA (**13**)	soluble (0.02)	Tris	0.02	3.1	**13a**	93 (6)
FaraA (**13**)	immobilized (0.02)	acetate	2	0.2	**13a**	91 (6)

[a] *Experimental conditions:* substrate, 1 mM; ATP, 2 mM; buffer, 50 mM pH 8; temperature, 37 °C; volume, 5 mL for the synthesis of araA-MP and 1 mL for the synthesis of FaraA-MP. IU: μmol min^{-1}.

an immobilized enzyme (stability, recyclability, recovery), in this case the immobilization appeared to exert a dramatic influence on the catalytic properties of the enzyme allowing the reaction to achieve almost a quantitative conversion even when using a equimolar ratio of nucleoside/ATP. The improved performances obtained with the immobilized *Dm*dNK, compared to the soluble enzyme, might be ascribed to the microenvironment generated by the carrier surface. In fact, the strongly cationic surface, resulting after coating the carrier with PEI, could create a driving force to direct the negatively charged ATP to the immobilized enzyme. The absorption of ATP on the carrier was experimentally confirmed by detecting (HPLC monitoring) the decrease of ATP concentration (but not of the monophosphate) in the supernatant upon incubation with the support.

To further corroborate this hypothesis, the percentage of conversion of araA (**12**) using 1 equivalent of phosphate donor was evaluated in conditions that avoid the adsorption of ATP (i.e., pH 10.5). At this pH, ATP was not adsorbed on the carrier and the immobilized enzyme was stable and active. After 12 h, the conversion obtained was similar to that of the soluble enzyme (60%), thus indicating that the adsorption of ATP on the carrier is a key point to achieve high conversions with the immobilized enzyme.

We concluded that a molar ratio of 1:1.25 was the optimal condition in terms of percentage of conversion, consumption of reagents and impurity profile for the synthesis of araA-MP. These conditions were used for the synthesis of the fluorinated counterpart FaraA-MP (**13a**) as well confirming the trend observed for araA and resulting in 99% conversion.

Optimization of Experimental Conditions

A further optimization concerned the use of different buffers and concentrations of $MgCl_2$. The results reported in Table 4 reveal that both Tris HCl and acetate buffer can be used: the possibility of using acetate buffer is of outstanding importance, in fact, due to its volatility, it can be easily removed by lyophilization. The reduction of the concentration of Mg^{2+}, which is essential for catalysis,[2] at a concentration lower than 0.2 mM delivered a slight reduction of the reaction rate and yields. The threshold concentration of $MgCl_2$ was thus set to 2 mM.

Scale-Up and Purification

By using the immobilized enzyme prepared by loading 2 mg of *Dm*dNK per gram of carrier (final activity 16 IU/g) the phosphorylation of **12** and **13** in acetate buffer was then developed at increased substrate concentration and using a 1:1.25 ratio of nucleoside/ATP.

Quantitative conversions were obtained at all concentrations of substrates (Table 5) using a small amount of immobilized enzyme in 10 mL of reaction volume (about 1% weight/volume). When the soluble kinase was used the reactions were slower; for example, starting from 12 mM of araA, the reaction reached only 78% of conversion in 24 h, while the immobilized derivative achieved the almost complete phosphorylation of the substrate after only 12 h.

Arabinosyladenine monophosphate (25 mM) was purified by preparative HPLC. The collected fractions were pooled together affording, upon lyophilization, 95% of final yield. The identity of the isolated product was confirmed by NMR and MS analysis; chromatographic analysis revealed the presence of 3.5% of ADP (see the Supporting Information for details).

Conclusions

Fruit fly deoxyribonucleoside kinase (*Dm*dNK) can be used as biocatalyst for the synthesis of a wide

Table 5. Scale-up of araA-MP and FaraA-MP.[a]

Substrate	ATP (mM)	Enzyme	Product	Conversion % (hours)
12 (6.5 mM)	8	immobilized	**12a**	>98 (12)
12 (12 mM)	15	soluble	**12a**	78 (24)
12 (12 mM)	15	immobilized	**12a**	>98 (12)
12 (25 mM)	30	soluble	**12a**	87 (24)
12 (25 mM)	30	immobilized	**12a**	97 (12)
13 (6.5 mM)	8	immobilized	**13a**	93 (4)
13 (12 mM)	15	immobilized	**13a**	91 (4)

[a] *Experimental conditions*: buffer, 50 mM ammonium acetate pH 8; $MgCl_2$, 2 mM; temperature, 37 °C; volume, 10 mL; soluble enzyme, 2.8 IU; immobilized enzyme, 1 IU. In the case of 25 mM araA, soluble enzyme, 5.6 IU; immobilized enzyme, 2.5 IU.

range of nucleoside monophosphates and it can be thus a convenient alternative to $POCl_3$-based phosphorylation. Upon immobilization, productivity and rate of synthesis of *Dm*dNK in the bioconversion of araA (**12**) were dramatically enhanced with respect to the non-immobilized enzyme, affording the title compound in 95% yield and 96.5% of purity.

For the synthesis of high-added value products, such as the monophophates of araA **12a** and fludarabine **13a**, the use of stoichiometric ATP could be justified. Otherwise, for the synthesis of other monophosphates, the recycle of ADP produced during the reaction should be considered using, for instance, pyruvate, acetate or polyphosphate kinases.[39]

Experimental Section

General

Arabinosyladenine (**12**) and thymidine 5′-monophosphate (**6a**) were purchased from Jena Biosciences (Jena, Germany). Arabinosyladenine 5′-monophosphate (**12a**) was from Acros Organics (Geel, Belgium). 2′-Deoxyuridine (dUrd, **5**) was a gift from Pro.Bio.Sint. (Varese, Italy). Fludarabine (**13**) and fludarabine 5′-monophosphate (**13a**) were from Adorkem Technologies (Costa Volpino, Bergamo, Italy). All the other nucleosides and nucleotides were from Sigma Aldrich and/or Alfa Aesar (Milano, Italy). Solvents, polyethylenimine (PEI) MW 600, DL-dithiothreitol and dextran MW 6000 were purchased from Sigma Aldrich or VWR International (Milano, Italy). Sepabeads EC-EP was kindly supplied by Resindion (Mitsubishi, Binasco, Italy). Regenerated cellulose tubular membrane for dialysis was from Membrane Filtration Products, Inc. (Seguin, TX, USA). 10 kDa Nanosep® centrifugal devices were from Pall (Buccinasco, Milano, Italy). All solvents were HPLC grade. Enzymatic reactions were monitored by using HPLC Merck Hitachi L-7100 equipped with a UV detector L-7400 and column oven L-7300 (Darmstadt, Germany).

Protein concentration assay was performed on a Shimadzu spectrophotometer UV 1601 by the Bradford method using bovine serum albumin as standard.[40] ^{1}H NMR spectra were recorded at 400.13 MHz, on a Bruker AVANCE 400 spectrometer equipped with the XWIN-NMR software package (Bruker, Karlsruhe, Germany) at 300 K. ^{1}H chemical shifts (δ) were referenced to the solvent signal ($\delta_H = 3.31$ for CD_3OD). Electrospray mass spectroscopy (ESI-MS) was performed in MeOH/H_2O on an LTQ linear ion trap mass spectrometer (Thermo Finnigan) in negative ion mode.

All experiments were performed at least as duplicate.

Enzyme Production and Purification

Recombinant *Dm*dNK was expressed and purified as previously described.[41] The purity of the enzyme in the collected fractions was assessed by SDS-PAGE (>90%) and the final enzyme concentration was about 3 mg mL^{-1}.

Preparation of Sepabeads-PEI

Activation of epoxy Sepabeads with PEI was performed as previously reported.[33]

Preparation of Aldehyde Dextran

Dextran (1.67 g) was suspended in deionized water (50 mL) and $NaIO_4$ (0.43 g) was added to obtain 10% of oxidation degree. The reaction was carried out for 2 h at room temperature and then the solution was dialyzed (3500 Da MWCO) against deionized water. The oxidized dextran was stored at −20 °C.[32]

Immobilization on Sepabeads-PEI and Cross-Linking with Aldehyde Dextran

Immobilization on Sepabeads-PEI and stabilization by cross-linking with 10% oxidized dextran was performed by slightly modifying the procedure previously described.[32] Briefly, the activated carrier (1.0 g) was suspended in 5 mM phosphate buffer pH 7.5 containing the soluble *Dm*dNK (0.1–2.0 mg) in a total volume of 14 mL. The suspension was kept under mechanical stirring at room temperature and after 1 hour aldehyde dextran (1.4 mL) was added and allowed to stir for an additional hour. The temperature was then lowered to 4 °C, the pH was adjusted to 10.05 and 15.4 mg of $NaBH_4$ were added (1 mg mL^{-1} of suspension); the reaction was carried out for 30 min under mechanical stirring. The immobilized preparation was then filtered and washed with 10 mM potassium phosphate buffer pH 5 and deionized water.

Standard Enzymatic Assay

The standard enzymatic assay was carried out by adapting the previously reported radiometric assay.[12]

In a final volume of 1 mL a solution of Tris-HCl 50 mM pH 8.0 containing dAdo (1 mM), ATP (2 mM), $MgCl_2$ (2 mM) was prepared. The reaction was started by the addition of the enzyme (soluble or immobilized) and the mixture was stirred at 37 °C. At different times (5, 10, and 15 min), samples were withdrawn, filtered (either by centrifugation with 10 kDa MWCO Nanosep at 4 °C and 13,200 rpm for

2 min for the soluble enzymes, or by using pipette filter devices for the immobilized enzymes) and analyzed by HPLC (see below for chromatographic conditions and t_R). The activity of *Dm*dNK was expressed in international units (IU). One IU corresponds to the amount of enzyme that produces 1 μmol of dAdo-MP (**1a**) per min.

Enzyme Stability Assay

The residual activity was determined by modifying the general procedure previously reported.[33]

Synthesis of AraA-MP and FaraA-MP

The suitable amount of nucleoside and ATP was dissolved in 50 mM ammonium acetate containing 2 mM $MgCl_2$. The final pH was set to 8.0 with NaOH. *Dm*dNK (soluble or immobilized) was then added and the mixture was kept under mechanical stirring at 37 °C. The reaction was stopped by filtering the enzyme. The filtered solution was analyzed by HPLC (see below for chromatographic conditions and t_R).

Chromatographic Conditions

The reaction was monitored by HPLC ($\lambda = 260$ nm), identifying the products by comparing their retention times with those of authentic samples. The column was a Kromasil RP18 (5 μm, 250×4.6 mm) kept at 35 °C; flow rate was 1 mL min^{-1}.

Mobile phase: eluent A = 2 g $(NH_4)H_2PO_4$ + 0.5 g $(NH_4)_2HPO_4$/L + 30 mL MeOH; eluent B = 90% CH_3CN. Gradient: 0 (100% A)→8 min. (100% A)→22 min. (70% A–30% B)→23 min. (100% A).

Retention times (nd = not detected): ATP (adenosine triphosphate) 3.2 min; ADP (adenosine diphosphate) 3.6 min; dAdo (2′-deoxyadenosine, **1**) 16.5 min, dAdo-MP (2′-deoxyadenosine monophosphate, **1a**) 10 min; dGuo (2′-deoxyguanosine, **2**) 15 min, dGuo-MP (2′-deoxyguanosine monophosphate, **2a**) 5.5 min; dIno (2′-deoxyinosine, **3**) 14.1 min, dIno-MP (2′-deoxyinosine monophosphate, **3a**) 5.1 min; dCyt (2′-deoxycytidine, **4**) 6.9 min, dCyt-MP (2′-deoxycytidine monophosphate, **4a**) 2.9 min; dUrd (2′-deoxyuridine, **5**) 9.4 min, dUrd-MP (2′-deoxyuridine monophosphate, **5a**) 5.1 min; Thd (thymidine, **6**) 15.4 min, Thd-MP (thymidine monophosphate, **6a**) 5.6 min; Ado (adenosine, **7**) 17.73 min, Ado-MP (adenosine monophosphate, **7a**) 5.3 min; Guo (guanosine, **8**) 12.2 min, Guo-MP (guanosine monophosphate, **8a**) nd; Ino (inosine, **9**) 12.7 min, Ino-MP (inosine monophosphate, **9a**) nd; Cyt (cytidine, **10**) 4.9 min, Cyt-MP (cytidine monophosphate, **10a**) 2.5 min; Urd (uridine, **11**) 6.7 min, Urd-MP (uridine monophosphate, **11a**) 2.7 min; araA (arabinosyladenine, **12**) 15.5 min, araA-MP (arabinosyladenine monophosphate, **12a**) 5.6 min; FaraA (2-fluoro-arabinosyladenine, **13**) 21.31 min, FaraA-MP (2-fluoroarabinosyladenine monophosphate, **13a**) 9.09 min; araU (arabinosyluracil, **14**) 8.6 min, araU-MP (arabinosyluracil monophosphate, **14a**) 2.8 min.

The percentage of conversion was calculated on the basis of the depletion of nucleoside compound and monitoring the formation of the nucleotide product: conversion (%) = [product area/(product area + nucleoside area)] × 100.

In the case of araA-MP (**12a**) and FaraA-MP (**13a**) synthesis scale-up, due to the low solubility of the starting nucleosides, the conversion was determined using the monophosphates as external standards.

Purification of AraA-MP (12a)

Purification of **12a** was accomplished by preparative HPLC using an AKTA Basic100 instrument (Pharmacia, Uppsala, Sweden); chromatographic conditions were as follows: column, Phenomenex Jupiter RP-18 (10 μm, 250×10 mm, Merck); flow rate, 5 mL min^{-1}; detector, $\lambda = 260$ nm and 280 nm; mobile phase MeOH/$(NH_4)HCO_3$, isocratic elution at 3% MeOH for 2 column volumes, then gradient elution from 3% to 40% MeOH in 2 column volumes. After lyophilization the product appeared as a white solid.

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