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Microbial synthesis of 2,6-diaminopurine nucleosides

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Abstract

2,6-Diaminopurine nucleosides are used as pharmaceutical drugs or prodrugs against cancer and viral diseases.

The synthesis of 2,6-diaminopurine riboside, -2′-deoxyriboside, -2′,3′-dideoxyriboside and -arabinofuranoside was efficiently carried out by transglycosylation using bacterial whole cells as biocatalysts. The preparation of 2,6-diaminopurine-2′,3′-dideoxyriboside catalysed by whole cells is here reported for the first time.

Keywords: 2,6-Diaminopurine nucleoside; Whole cells; Microbial transglycosylation; Nucleoside analogues

1. Introduction

Nucleoside analogues are used in the treatment of cancer and viral infections. As antiviral agents, these compounds inhibit replication of the viral genome, whereas anticancer compounds inhibit cellular DNA replication and repair [1].

Anticancer nucleoside analogues used in the clinic include 2-chloro-2′-deoxyadenosine (cladribine), 2-fluoro-9-β-D-arabinofuranosyladenine (fludarabine), 1-β-D-arabinofuranosylcytosine (cytarabine) and 2′,2′-difluorodeoxycytidine (gemcitabine). Nucleosides such as 2′,3′-dideoxyinosine (didanosine), 2′,3′-dideoxycytidine (zalcitabine), 2′-deoxy-3′-thiacytidine (lamivudine), 3′-azido-3′-deoxythymidine (zidovudine), 2′,3′-didehydro-3′-deoxythymidine (stavudine) and 6-cyclopropylamino-2′,3′-didehydro-2′,3′-dideoxyguanosine (abacavir) are used in antiviral treatment [2].

Major problems of nucleoside therapies are acquirement of resistance and side effects such as delayed cytotoxicity. Therefore, the need for developing new nucleoside analogues still exists.

Purine arabinosides such as 9-β-D-arabinofuranosyladenine (AraA), which is being used in the treatment of different viral infections [3], and fludarabine, which is active in chronic lymphocytic leukaemia therapies [4], have generated considerable interest as chemotherapeutic drugs.

2,6-Diaminopurine arabinoside (DAPA) and 2-amino-6-methoxypurine arabinoside [5] are potential prodrugs since they are rapidly hydrolysed in vivo by adenosine deaminase (ADA) to 9-β-D-arabinofuranosylguanine.

2,6-Diaminopurine-2′,3′-dideoxyriboside (ddDAPR) is, like 2′,3′-dideoxyadenosine (ddA), a potent and selective inhibitor of HIV [6] and HBV [7]. Moreover, ddDAPR is a strong inhibitor of human ADA and therefore, it is used in combination with other nucleosidic drugs to potentiate their activity, such as AraA in the treatment of herpes simplex and vaccinia virus infections [8].

2,6-Diaminopurine nucleosides are also used in the synthesis of modified oligonucleotides [9] and as intermediates in the synthesis of guanosine derivatives [10]. For example, 2′-deoxynucleosides are prepared by hydrolysis of DNA, although this methodology is rather complicated. Chemical, enzymatic and microbial syntheses have been successfully developed but 2′-deoxyguanosine was scarcely obtained by these methodologies because of the low solubility of guanine. More soluble 6-substituted purine nucleosides, like 2,6-diaminopurine-2′-deoxyriboside (dDAPR), are employed since they can be further converted to 2′-deoxyguanosine [10,11] making use of ADA activity. The same strategy has been reported employing dDAPR as a prodrug of deoxyguanosine in L1210 cells [12] and in ducks [7].

This paper describes the identification of efficient biocatalysts for the preparation of 2,6-diaminopurine nucleosides using the microbial transglycosylation procedure that we have previously applied to the synthesis of other purine nucleosides

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[13–15]. This methodology includes the selection of the appropriate biocatalysts by systematic screening of several bacterial genera, followed by optimisation of the reaction conditions.

2. Experimental

2.1. Chemicals and microorganisms

Nucleosides and bases were purchased from Sigma or ICN. The culture media components were obtained from Merck and Difco. HPLC grade methanol and acetonitrile were from Sintorgan. Most of the microorganisms were supplied by the *Sociedad Española Microbiología*.

2.2. Growth conditions

The strains were cultured in liquid media at the below detailed optimum temperature (T) and time (t), according to the American Type Culture Collection (ATCC): *Aeromonas* (T: 30 °C, t: 1 day), *Pseudomonas* (T: 26 °C, t: 1 day), Bacillus (T: 30 °C, t: 1 day), *Achromobacter* (T: 30 °C, t: 2 days), *Citrobacter* (T: 37 °C, t: 1 day), *Enterobacter* (T: 37 °C, t: 1 day), *Klebsiella* (T: 37 °C, t: 2 days), *Escherichia* (T: 37 °C, t: 1 day), *Proteus* (T: 37 °C, t: 1 day), *Xanthomonas* (T: 26 °C, t: 1 day), *Cellulomona* (T: 30 °C, t: 1 day), *Staphylococcus* (T: 37 °C, t: 1 day), *Micrococcus* (T: 30 °C, t: 1 day), *Agrobacterium* (T: 26 °C, t: 2 days) and *Serratia* (T: 26 °C, t: 5 days) were grown in Luria Broth medium; *Erwinia* (T: 30 °C, t: 1 day) and *Arthrobacter* (T: 26 °C, t: 2 days) in Agar II; *Corynebacterium* (T: 30 °C, t: 2 days) and *Brevibacterium* (T: 30 °C, t: 2 days) in *Corynebacterium* medium; *Lactobacillus* (T: 37 °C, t: 1 day) in MRS broth (oxoid CM359); *Streptomyces* (T: 28 °C, t: 5 days) in Streptomyces medium, while *Nocardia* (T: 30 °C, t: 1 day) in YEME (Bennett's agar) medium.

The saturated cultures broths were centrifuged at 12,000 × g for 10 min and the pellets used as the biocatalysts.

2.3. Standard conditions

The standard reaction mixture comprising: wet cell paste containing 1.1×10^{10} cells, 7 mM 2,6-diaminopurine, 21 mM uridine, thymidine, 2′,3′-dideoxyuridine or 1-β-arabinofuranosyluracil and 30 mM pH7 potassium phosphate buffer (final volume 1ml), was stirred at 200 rpm and 60 or 45 °C. Samples were centrifuged at 10,000 × g for 30 s and the supernatants were analysed by HPLC. The products were further characterized by LC/MS.

2.4. Screening procedure

The standard reaction medium containing 4.5×10^8 cells in a final volume of 40 μl was treated as above described. Aliquots were taken at 4, 8, 12, 24 and 48 h and centrifuged as above. Supernatants were analysed by HPLC.

2.5. Analytical methods

HPLC analysis was performed using a C-18 column (150 mm × 4 mm) at a flow rate of 0.9 ml min^{-1}. The UV detector was set at 254 nm and the column was operated at room temperature.

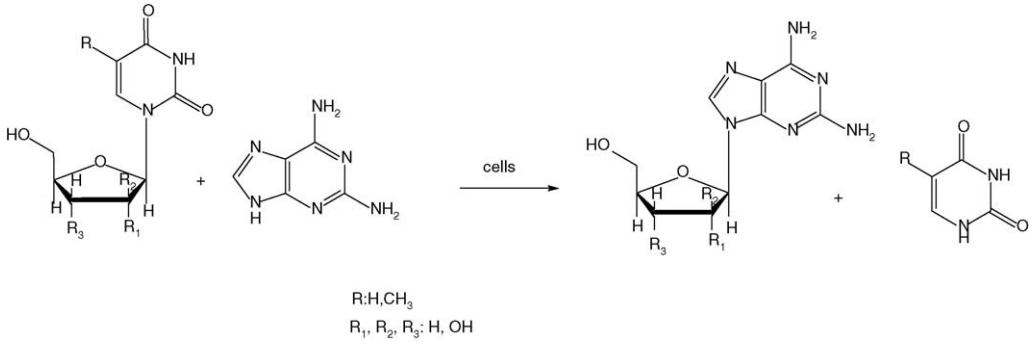
The other operating conditions were as follows:

DAPR from uridine and 2,6-dDAPR from thymidine: 7 min water/acetonitrile (98/2, v/v), 3 min gradient to water/acetonitrile (90/10, v/v), and 1 min water/acetonitrile (90/10, v/v).
dDAPR from 2′-deoxyuridine: 4 min water/methanol (95/5, v/v), 5 min gradient to water/methanol (80/20, v/v), 1 min water/methanol (80/20, v/v).
ddDAPR from 2′,3′-dideoxyuridine: 8 min water/acetonitrile (91/9, v/v).
DAPA from 1-β-arabinofuranosyluracil: 8.5 min water/methanol (90/10, v/v), flow rate: 1 mil min^{-1}.

LC/MS analysis of 2,6-diaminopurine nucleosides was carried out using a Finnigan LCQ Duo spectrometer (positive mode, solvent: methanol/water), affording the expected molecular mass as $M^+ + H$: DAPR and DAPA 283.1 (expected M^+: 282.2560); dDAPR 267.1 (expected M^+: 266.2566); ddDAPR 251.1 (expected M^+: 250.2572).

3. Results and discussion

During the last years, we have been applying a screening methodology to select useful whole cell biocatalysts for the synthesis of modified nucleosides by transglycosylation (Scheme 1).



Scheme 1. Synthesis of 2,6-diaminopurine nucleosides by microbial transglycosylation.

This method was used in the present work to obtain DAPR, dDAPR, ddDAPR and DAPA from uridine, thymidine, 2′,3′-dideoxyuridine and 1-β-D-arabinofuranosyluracil, respectively, as sugar donors and 2,6-diaminopurine (DAP) as the purine base (Table 1).

Screenings were carried out at 60 °C to avoid the deleterious effects of ADA, but in cases that thymidine phosphorylase (TP) was involved in the reaction, lower temperatures were used and therefore, only microorganisms containing low ADA activity afforded good yields of the target nucleosides.

Table 1
Screening of transglycosylases for the synthesis of 2,6-diaminopurine nucleosides

Microorganism	DAPR		dDAPR		dDAPR		ddDAPR		DAPA	
	60 °C		60 °C		45 °C		45 °C		60 °C	
	Yield (%)	Time (h)	Yield (%)	Time (h)	Yield (%)	Time (h)	Yield (%)	Time (h)	Yield (%)	Time (h)
Pseudomonas putida (*CECT324*)	0	48	–	48	61	4	–	48	15	48
Erwinia chrysanthemi (*CECT509*)	ND		ND		ND		–	48	11	48
Lactobacillus acetotolerans (*CECT4019*)	ND		–	48	77	4	–	48	ND	
Klebsiella sp (*CECT367*)	84	4	82	3	65	8	49	7	7	8
Chromobacterium violaceum (*CECT4226*)	72	4	79	1.3	89	2	6	24	4	24
Streptomyces badius (*CECT3275*)	8	8	–	48	1	4	–	48	–	24
Enterobacter aerogenes (*CECT684*)	88	4	61	4	71	8	10	48	22	24
Citrobacter amalonaticus (*CECT863*)	ND		ND		ND		32	48	14	24
Proteus rettgeri (*CECT171*)	80	4	69	4.7	67	4	16	48	5	24
Pseudomonas stuzeri (*CECT930*)	ND		74	4.7	36	4	–	48	–	48
Proteus rettgeri (*CECT865*)	ND		ND		ND		12	48	3	24
Aeromonas salmonicida (*CECT896*)	90	4	18	4.7	81	4	ND		26	48
Proteus vulgaris (*CECT174*)	92	4	52	4	88	0.5	7	24	7	48
Proteus vulgaris (*CECT4077*)	78	4	ND		ND		8	48	–	24
Proteus rettgeri (*CECT4557*)	ND		ND		ND		2	48	2	24
Citrobacter freundii (*CECT401*)	ND		69	4.7	68	4	ND		14	24
Proteus vulgaris (*CECT165*)	ND		ND		ND		ND		6	8
Streptomyces sp (*CECT3145*)	4	4	–	48	2	8	16	48	–	24
Serratia rubidaea (*CECT868*)	57	4	27	4	72	8	7	24	–	24
Klebsiella planticola (*CECT843*)	ND		ND		ND		10	24	11	4
Escherichia coli (*CECT877*)	ND		ND		ND		56	48	22	24
Achromobacter cycloclastes (*CECT333*)	91	4	–	48	76	8	3	24	4	24
Proteus mirabilis (*CECT4101*)	ND		ND		ND		ND		4	48
Xanthomona translucens (*CECT4643*)	1	4	–	48	27	4	–	48	5	24
Escherichia coli (*CECT731*)	ND		ND		ND		42	48	13	8
Escherichia coli (*CECT105*)	68	4	56	4	77	4	53	48	6	24
Xanthomona campestris (*CECT95*)	7	8	ND		ND		–	48	ND	
Serratia marescens (*CECT977*)	ND		ND		ND		ND		–	48
Arthrobacter oxydans (*CECT4368*)	–	48	2	0.5	5	4	–	48	–	48
Erwinia amylovora (*CECT222*)	83	4	10	4.7	52	8	–	48	5	24
Serratia marescens (*CECT159*)	82	4	46	4.7	91	3.5	1	48	3	24
Bacillus cereus (*CECT193*)	ND		50	24	3	8	–	48	–	48
Escherichia coli (*CECT433*)	ND		ND		ND		ND		12	24
Erwinia carotovora (*CECT314*)	84	4	–	48	69	4	–	48	–	48
Erwinia carotovora (*CECT225*)	ND		ND		ND		9	24	2	8
Enterobacter gergoviae (*CECT857*)	ND		ND		ND		ND		72	48
Escherichia coli (*CECT100*)	ND		ND		ND		ND		14	4
Enterobacter cloacae (*CECT4214*)	ND		ND		ND		ND		7	24
Enterobacter cloacae (*CECT960*)	88	4	73	4.7	77	8	38	48	9	48
Enterobacter cloacae (*CECT194*)	83	4	32	1.5	2	8	–	48	11	24
Bacillus stearothermophilus (*CECT43*)	91	12	ND		ND		ND		ND	
Brevibacterium linens (*CECT76*)	0	48	87	1.5	1	8	–	48	–	48
Brevibacterium helvolum (*CECT73*)	0	48	70	4.5	1	8	–	48	–	48
Escherichia coli BL 21 (*ATCC47092*)	75	4	85	1.5	75	1.5	66	48	11	4
Celullomonas cellulans (*CECT3050*)	30	4	10	3	ND		–	48	ND	
Staphylococcus capitis (*CECT233*)	29	12	–	48	28	4	4	24	7	24
Micrococcus luteus (*CECT241*)	9	12	–	48	76	4	–	48	ND	
Agrobacterium tumefaciens (*CECT4067*)	0	48	–	48	–	48	4	24	–	48
Corynebacterium ammoniagenes (*CECT72*)	4	8	–	48	11	8	ND		ND	

DAPR: 2,6-diaminopurine riboside; dDAPR: 2,6-diaminopurine-2′-deoxyriboside; DAPA: 2,6-diaminopurine arabinoside; ddDAPR: 2,6-diaminopurine-2′-3′-dideoxyriboside; ND: not determined.

Table 2
Influence of experimental conditions on dDAPR synthesis

Entry	Uridine (mM)	2,6-Diaminopurine (mM)	Cells/ml 1 $X = 1.108$	Temperature (°C)	Time (h)	Yield (%)
1	30	10	10*X*	30	6	19*
2	30	10	10*X*	45	1.5	47*
3	30	10	10*X*	60	1	95*
4	30	10	0.5*X*	60	1	21
5	30	10	1*X*	60	1	38
6	30	10	5*X*	60	1	92
7	30	10	20*X*	60	1	96
8	10	10	10*X*	60	1	70*
9	30	10	75*X*	60	0.6	98*
10	30	10	150*X*	60	0.6	90*
11	30	10	300*X*	60	0.3	37*

* Maximum yield.

Several out of the 100 bacteria screened, catalysed the synthesis of DAPR in high yields, but considering the short reaction time, *Aeromonas salmonicida* was selected for the further assessment of the influence of different experimental conditions.

Reactions were carried out using the standard conditions described in Section 2.

As reported in Table 2, yields of DAPR improved when the temperature is increased up to 60 °C (entry 1–3), while reaction times decreased. This kinetic behaviour was previously observed by other bacteria [16]. As expected, using a low amount of biocatalyst (entry 4–5), comparable yields were achieved (95%) but considerably longer reaction times were required. When the amount of cells was between 10 and 20×10^8, the time needed to reach the maximum yield was similar (entry 3, 7). This reaction is also dependent on the relative concentration of uridine: DAP, since a 1:1 ratio afforded a lower yield (entry 8).

Regarding dDAPR, this compound has been previously synthesised using isolated enzymes [11] (*N*-deoxyribosyltransferase from *Lactobacillus helveticus* and phosphorylases from *Bacillus stearothermophyllus*), and whole cells of *Enterobacter aerogenes* [10]. The sugar donor was thymidine in the first report and 2′-deoxyuridine in the second one, which is in agreement with the specificity of the enzymes involved in the reactions.

Since thymidine is readily available, screenings at both, 45 and 60 °C were carried out using this starting material. As expected, bacteria that contain *N*-deoxyribosyltransferase, like *Lactobacillus,* or TP, like *Escherichia, Aeromonas, Citrobacter, Serratia and Proteus* [17], afforded yields higher than 70%. The shortest time was shown by *Proteus vulgaris* that produced 88% of dDAPR in 30 min.

When conducting the reaction at 60 °C, only bacteria that contain pyrimidine nucleoside phosphorylases (PyNPs), like *Bacillus*, or uridine phosphorylases (UPs) that accept also thymidine, like *Escherichia, Aeromonas and Enterobacter,* produced dDAPR in high yields.

Preliminary experiments were performed in order to assess the ability of 2′-deoxyuridine as substrate in dDAPR synthesis. Using bacteria carrying active UP [18], *Enterobacter cloacae* appeared to be the best biocatalyst affording dDAPR in 80% yield at 60 °C in 1 h.

For the preparation of dideoxynucleosides, previous reports used isolated enzymes of *Lactobacillus helveticus* [19] and *Escherichia coli* whole cells [20]. In our attempts to obtain ddDAPR, low yields were obtained when the reaction was carried out at 60 °C, as expected. In contrast, several bacteria catalysed the transglycosylation at 45 °C, especially those belonging to *Escherichia* and *Klebsiella* genera. Despite of the long reaction time required, no ADA activity was observed which proves that ddDAPR is a poor substrate of this enzyme.

Arabinonucleosides have been previously synthesised using isolated UP and purine nucleoside phosphorylases (PNP) from *E. coli* since it had been demonstrated that 1-β-D-arabinofuranosyluracil is not a substrate for TPs [21]. Consequently, Utagawa [20] reported the preparation of adenine- and other purine substituted arabinosides using whole cells of *Enterobacter aerogenes* at 60 °C. The results obtained in the present screening showed that only *Enterobacter gergoviae* afforded the best yield of DAPA.

4. Conclusions

DAP mimics adenine in many reactions and is accepted by adenine-metabolizing enzymes. Moreover, diaminopurine nucleosides can act as prodrugs or drugs depending on their susceptibility to ADA activity in vivo.

In this paper, screening of a cell collection allowed the identification of alternative biocatalysts useful for the synthesis of DAPR, dDAPR, ddDAPR and DAPA, through microbial transglycosylation.

It has been previously reported that DAP is an acceptor in the 2′,3′-dideoxyribosyl transfer using the isolated trans-*N*-deoxyribosylase from *Lactobacillus helveticus* [19], but as far as we know, this is the first report of the whole cell catalysed preparation of ddDAPR.

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