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A novel enzymatic method for the production of purine-2′-deoxyribonucleosides

Kenzo Yokozeki [a,*], Takashi Tsuji [b]

[a] *AminoScience Laboratories, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*
[b] *Pharmaceutical Research Laboratories, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*

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Abstract

The microbial production of purine-2′-deoxyribonucleosides from pyrimidine-2′-deoxyribonucleosides and purine bases was examined by the application of nucleoside phosphorylase using *Enterobacter aerogenes* AJ-11125 as the enzyme source. In this system, 2′-deoxyadenosine (dAR) was efficiently produced from 2′-deoxyuridine (dUR) and adenine. In contrast, 2′-deoxyguanosine (dGR) was scarcely produced from dUR and guanine, because of the low solubility of guanine. Under the conditions using guanosine (GR) with higher solubility than guanine as a guanine source, higher productivity of dGR was obtained, but the maximal molar yield obtained was less than 20%. To improve its productivity, we newly constructed a following enzymatic method via 2,6-diaminopurine-2′-deoxyriboside (dDAPR) as follows: production of dDAPR from dUR and 2,6-diaminopurine (DAP) by *E. aerogenes* AJ-11125, followed by the conversion of dDAPR to dGR by adenosine deaminase. Through the successive reactions, dGR was efficiently produced with high yield.

Keywords: Nucleoside phosphorylase; Purine-2′-deoxyribonucleoside; 2′-Deoxyguanosine; 2′-Deoxyadenosine; 2′-Deoxyuridine; 2,6-Diaminopurine-2′-deoxyriboside; Adenine; Guanine; 2,6-Diaminopurine; *Enterobacter aerogenes*; Adenosine deaminase

1. Introduction

There are pressing worldwide demands for 2′-deoxyribonucleosides as the raw materials of antisense drugs. Natural 2′-deoxyribonucleosides have been supplied from hydrolysate of the raw materials containing DNA. However, this method involves unstable supply of the raw materials and rather complicated isolation processes.

Recently, we have successfully developed the novel processes for the production of various nucleosides by the application of microbial nucleoside phosphorylase at high temperatures such as 60°C [1–14]. The methods developed by us brought about the simple and high-yield processes. The reaction mechanisms of the production system consisted of the following successive two reactions via sugar-1-phosphate as the intermediates; phosphorolysis of a starting nucleoside to form the corresponding sugar-1-phosphate and base (forward reaction of nucleo-

* Corresponding author. Tel.: +81-44-244-6154; fax: +81-44-244-6581.
E-mail address: kenzo_yokozeki@ajinomoto.com (K. Yokozeki).

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side phosphorylase), followed by condensation of sugar-1-phosphate and a new base to form a target nucleosides (reverse reaction of nucleoside phosphorylase).

This transglycosylation system has been applied to produce various physiologically active nucleosides, such as adenine arabinosides [1–4], 2′-amino-2′-deoxynucleosides [5], ribavirin (virazol) [6–11], 5-methyluridine [12,13] and 2′,3′-dideoxynucleosides, i.e. 2′,3′-dideoxyinosine, 2′,3′-dideoxyadenosine [14,15].

This paper describes the construction of a novel production system for purine-2′-deoxyribonucleosides, i.e. 2′-deoxyadenosine (dAR) and 2′-deoxyguanosine (dGR) by the application of the system mentioned above using 2′-deoxyuridine (dUR) and the corresponding bases as the substrates and *Enterobacter aerogenes* AJ-11125 as the enzyme source of nucleoside phosphorylase.

2. Experimental

2.1. Chemicals

dUR, dAR and dGR were purchased from Tokyo Kasei Organic Chemicals, Japan. 2,6-Diaminopurine-2′-deoxyriboside (dDAPR), 2,6-diaminopurine (DAP), adenine and guanine were purchased from Sigma, USA. Adenosine deaminase (EC 3.5.4.4, from calf intestine, 200 U/mg) was the product of Boehringer Mannheim, Germany. Other chemicals used were the best available commercial products.

2.2. Microorganism and medium

E. aerogenes AJ-11125, which was selected as the best strain, was used. The culture medium contained 5 g yeast extract, 10 g meat extract, 10 g peptone and 5 g NaCl in distilled water to make the total volume of 1 l, and was adjusted to pH 7.0 with KOH.

2.3. Preparation of intact cells

A total of 50 ml of the medium described above in 500-ml flasks was inoculated with one loopful of cells of *E. aerogenes* AJ-11125 subcultured on agar plates of the same medium. After aerobic cultivation at 30°C for 16 h, 2.5 ml of the cultured broth was transferred to 50 ml of the same medium in 500-ml flasks, followed by aerobic cultivation at 30°C for 18 h. The cells were then harvested by centrifugation ($4700 \times g$, 30 min) and rinsed with 50 mM potassium phosphate buffer (pH 7.0). Cells thus prepared were used as intact cells for reaction.

2.4. Reaction with intact cells

Standard reaction mixture in a total volume of 5 ml of 50 mM potassium phosphate buffer (pH 7.0) contained 150 mM dUR and 150 mM base, and 75 or 250 mg of wet cells prepared above, and the reaction was carried out at 60°C with shaking, and was stopped with boiling.

2.5. Analysis

Quantitative determination of dUR, dAR, dGR, dDAPR, uracil, adenine and guanine was carried out by HPLC with a Shodex Asahipak GS-220HQ (7.6×300 mm, Showa Denko) at 50°C with detection at 254 nm. The mobile phase was 200 mM NaH_2PO_4 (pH 3.98 with phosphoric acid) and the flow rate was 1.0 ml/min for dUR, dAR, dDAPR, adenine and DAP, and was 25 mM NaH_2PO_4 (pH 4.30 with phosphoric acid) and the flow rate was 1.0 ml/min for dGR and guanine.

3. Results

3.1. Selection of microorganisms

Various microorganisms with potent nucleoside phospholylase have already been isolated by Utagawa et al. [1–3,5,6] and us [7–12,15].

For the effective production of dAR (or dGR) from dUR and adenine (or guanine) through the successive reactions of nucleoside phosphorylase, it is necessary to use a strain having both the enzymes phosphorolyzing pyrimidine nucleosides [uridine phosphorylase (UPase) or pyrimidine phosphorylase (PyNPase)], and that phosphorolyzing purine nucleosides [purinenucleoside phosphorylase (PNPase)], because the former reaction requires the phosphorolysis of dUR to form deoxyribose-1-phosphate (dR-1-P) and uracil by the forward reaction of UPase or PyNPase, and the latter one requires the condensation of dR-1-P and adenine (or guanine) to form dAR (or dGR) by the reverse reaction of PNPase (Fig. 1).

Judging from these observations, *E. aerogenes* AJ-11125 was selected as the best strain for the production of purine-2′-deoxyribonucleosides from dUR and purine bases, because this bacterium could produce both UPase and PNPase [3], and effectively produce ribavirin, an analogue of purine ribonucleoside, from pyrimidine nucleoside and 1,2,4-triazole-3-carboxamide, a purine base [8].

3.2. Optimal reaction conditions for dAR production

The optimal reaction conditions for dAR production from dUR and adenine were investigated using the intact cells of *E. aerogenes* AJ-11125. Though the optimal pH was found to be around pH 7.0, the activity was observed in a wide pH range from 6.0 to 8.0. The optimal phosphate concentration was 50 mM, but the activity level was almost the same in the phosphate concentration range from 20 to 200 mM.

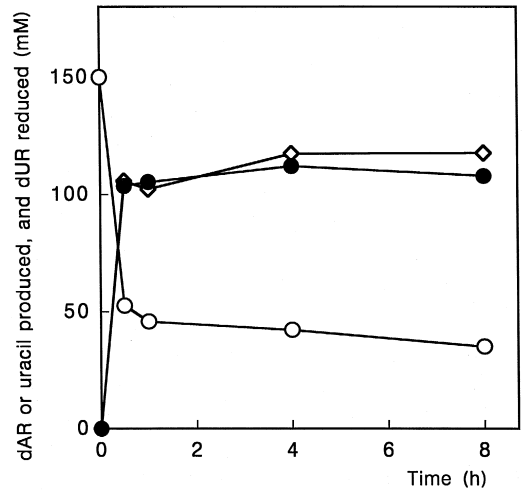


Fig. 2. Time course of dAR production by *E. aerogenes* AJ-11125. Preparation of intact cells was carried out as described in the text. The reaction was carried out at 60°C as described in the text except that 150 mM dUR and 150 mM adenine were used as the substrates and 50 g/l of wet cells were used as the enzyme source. (●) dAR, (◇) uracil, (○) dUR.

3.3. dAR or dGR production by E. aerogenes AJ-11125

Fig. 2 shows the time course of dAR production by using 50 g/l of intact cells of *E. aerogenes* AJ-11125. dAR was efficiently produced (112 mM, 28.1 g/l) from 150 mM dUR and 150 mM adenine with a molar yield of 75% after 4 h of incubation. In contrast, dGR was scarcely produced from dUR and guanine (4.1 mM dGR with a molar yield of 2.9% after 4 h of incubation). From these results, it was con-

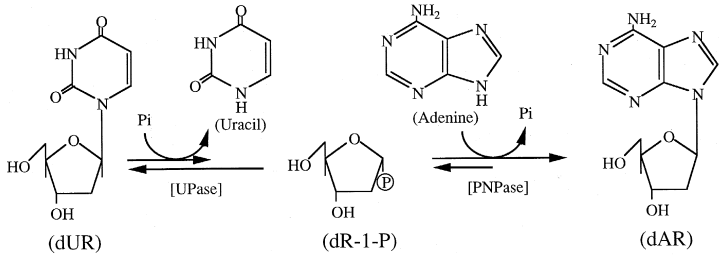


Fig. 1. Proposed mechanism for the production of dAR from dUR and adenine by *E. aerogenes* AJ-11125.

sidered that low productivity of dGR might be due to the poor solubility of guanine.

3.4. dGR production from dUR and GR as a base source

To improve the solubility of guanine, the effect of guanosine (GR) as a base source, which has the higher solubility than that of guanine, on dGR production was investigated by using 15 g/l of intact cells of *E. aerogenes* AJ-11125. As shown in Fig. 3(a), higher productivity of dGR (27.3 mM) from dUR and GR than that from dUR and guanine (Fig. 3(b)) was obtained, but the maximal molar yield obtained was only 18.2% after 2 h of incubation. In this reaction mixture, by-production of uridine (UR) was observed (17.5 mM after 2 h of incubation). This shows that GR used as a guanine source might be converted to UR through the successive reactions of PNPase and UPase (Fig. 4). In addition, the level of the maximal yield of dAR production (20.4%, 31.0 mM) from dUR and AR was similar to that of dGR from dUR and GR, as shown in Fig. 3(a) and (c). This productivity was rather lower than that of dAR from dUR and adenine.

As the other attempts to improve the solubility of guanine, addition of various organic solvents, i.e. methanol, ethanol, dimethylformamide, dimethylsulfoxide, and detergents, i.e. Tween 20, Tween 80, Triton-X in the reaction

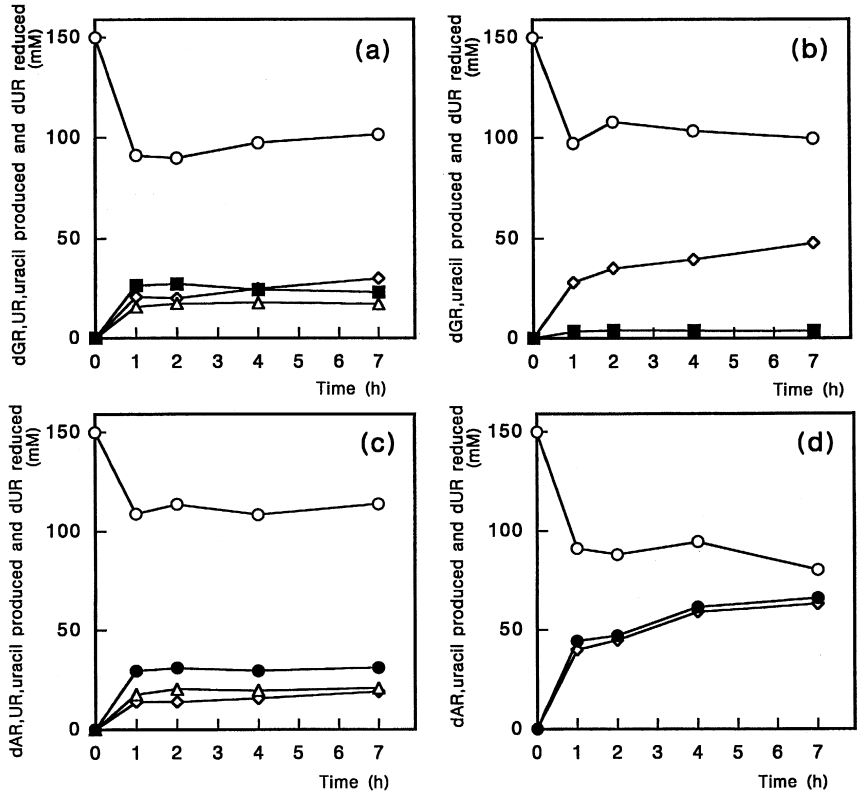
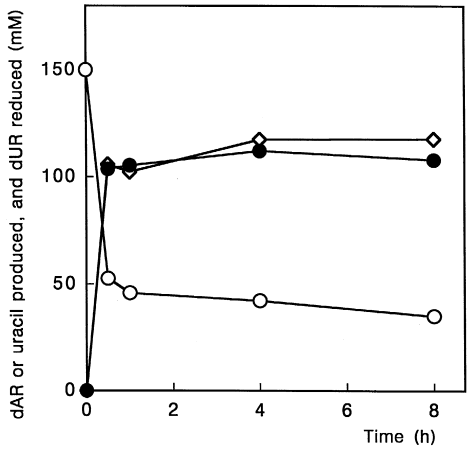


Fig. 3. Time course of dGR or dAR production from dUR and guanine or adenine, and from dUR and GR or AR as a base sources by *E. aerogenes* AJ-11125. Preparation of intact cells was carried out as described in the text. The reaction was carried out at 60°C as described in the text except that 150 mM dUR and 150 mM guanine, adenine, GR or AR were used as the substrates and 15 g/l of wet cells were used as the enzyme source. Substrates: (a) dUR and GR; (b) dUR and guanine; (c) dUR and AR; (d) dUR and adenine. (■) dGR; (●) dAR; (○) dUR; (◇) uracil; (△) UR.



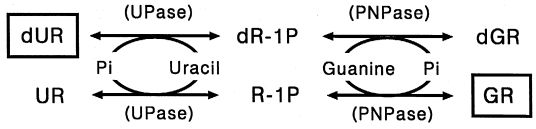


Fig. 4. Proposed scheme of UR by-production from dUR and GR or AR.

mixture were investigated. However, the effective conditions were not found.

3.5. Production of dGR via dDAPR by combining the reactions of transglycosylation and deamination

To improve the productivity of dGR, the combination of transglycosylation reaction for producing 2,6-diaminopurine-2-deoxyriboside (dDAPR) using 2,6-diaminopurine (DAP) with high solubility, as a base source and deamination reaction of dDAPR for producing dGR, was investigated. This system consisted of the following two steps: production of dDAPR from dUR and DAP by the application of nucleoside phospholylase using intact cells of *E. aerogenes* AJ-11125, followed by deamination of dDAPR by adenosine deaminase to produce dGR (Fig. 5).

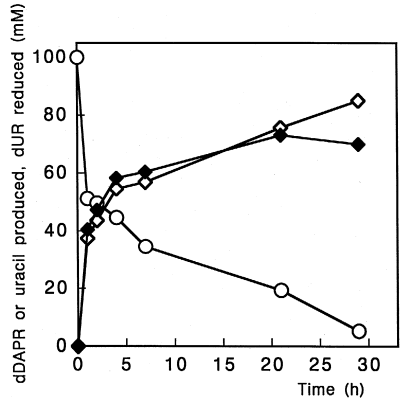


Fig. 6. Time course of dDAPR production from dUR and DAP by *E. aerogenes* AJ-11125. Preparation of intact cells was carried out as described in the text. The reaction was carried out at 60°C as described in the text except that 100 mM dUR and 100 mM DAP were used as the substrates and 50 g/l of intact cells were used as the enzyme source. (♦) dDAPR; (○) dUR; (◇) uracil.

Fig. 6 shows the time course of dDAPR production by intact cells of *E. aerogenes* AJ-11125. dDAPR was effectively produced (73.1 mM, 19.5 g/l) from 100 mM dUR and 100 mM DAP with a molar yield of 73% after 22 h of incubation. The reaction proceeded favorably because of the higher solubility of DAP than that of guanine. The productivity obtained was almost the same as that of dAR from dUR and adenine.

Fig. 7 shows the time course of dGR production from dDAPR by adenosine deaminase using 48-h incubation mixture containing 68 mM

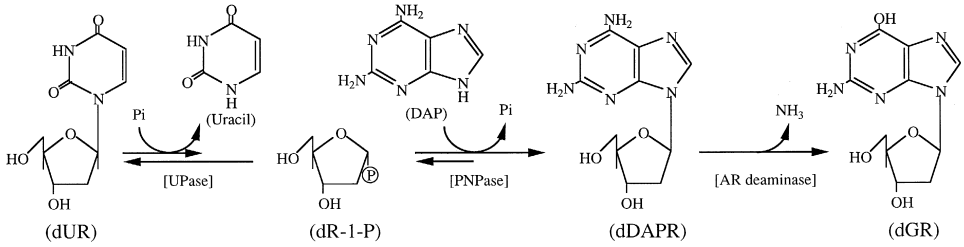


Fig. 5. Biosynthesis of dGR via dDAPR by combining the reactions by nucleoside phosphorylase and adenosine deaminase.

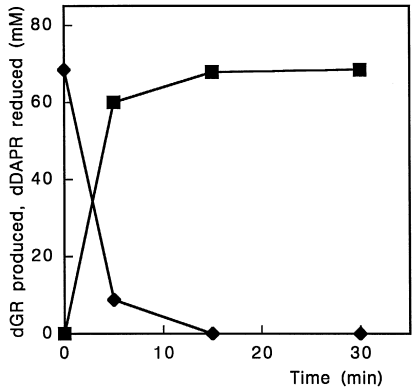


Fig. 7. Time course of dGR production from dDAPR by adenosine deaminase. A total of 5 ml of the 48 h of incubation mixture described in Fig. 8 (containing dDAPR 68 mM) was used as the reaction mixture. The reaction was carried out at 25°C using 20 units of adenosine deaminase by adjusting the pH at 7.0 with 6 N HCl. (■) dGR; (♦) dDAPR.

dDAPR. The productivity of dGR reached molar yield of 100% after 1 h of incubation.

4. Discussion

We developed a novel enzymatic method for the production of purine-2′-deoxyribonucleosides by the application of nucleoside phosphorylase from UR and the corresponding bases in the presence of inorganic phosphate.

E. aerogenes AJ-11125 selected as the best strain, which could produce both UPase and PNPase [3], effectively produced dAR from dUR and adenine. The mechanism underlying this reaction might consist of the following successive reactions similarly as the reaction mechanism for the production of the other various nucleosides [3,6–12,15]; dR-1-P formation from dUR and inorganic phosphate through the forward reaction of UPase, followed by dAR formation from dR-1-P and adenine through the reverse reaction of PNPase (Fig. 1). In contrast, dGR was scarcely produced in this system. This result shows that poor solubility of guanine might be a rate-limiting step in this reaction. In fact, dGR was well produced under the condition using GR, with higher solubility than that of guanine, as a guanine source. However, the productivity of dGR was lower than that of dAR from dUR and adenine. In case of dAR production from dUR and AR, the productivity of dAR was also lower than that of dAR from dUR and adenine. In this system, using GR or AR as a base source, by-produced UR was detected in each reaction mixture. This shows that GR (or AR) used as guanine (or adenine) source might be phosphorolyzed to guanine (or adenine) and R-1-P through the forward reaction of PNPase of *E. aerogenes* AJ-11125, and UR might be by-produced from R-1-P and uracil by the reverse reaction of UPase (Fig. 4). Judging from these results, the reason of the lower productivity of dGR (or dAR) under the condition using GR (or AR) as a guanine (or adenine) source, might be due to the competitive reactions between the reaction of dUR phosphorolysis and UR phosphorolysis by UPase, and between the reaction of dGR phosphorolysis and GR phosphorolysis by PNPase (Fig. 4).

A Novel system for dGR production via dDAPR developed in this study brought about the efficient productivity. In this system, dGR was produced with high yield through the successive reactions of dDAPR production from dUR and DAP by the application of nucleoside phosphorylase using *E. aerogenes* AJ-11125 and dGR production from dDAPR by adenosine deaminase. The productivity of dDAPR in the former reaction was almost at the same level compared with that of dAR from dUR and adenine because of the higher solubility of DAP than that of guanine. In the second step, adenosine deaminase could catalyze the deamination of dDAPR to form dGR quantitatively.

In the transglycosylation reaction by the application of nucleoside phosphorylase shown in this paper, thymidine was also a good substrate like dUR, and dAR or dDAPR was efficiently produced from thymidine and adenine, or thymidine and DAP, respectively, similarly as [7-^{15}N]-labeled deoxynucleoside production [16] (data not shown).

This system, which we developed by combining the reactions of transglycosylation and

deamination, has a wide range of application, and various nucleosides with guanine as a base moiety can be produced from the corresponding nucleosides with DAP as a base moiety as far as lies in the substrate specificity of adenosine deaminase.

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