Carbohydrate Research, 97 (1981) 139–146
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

AN ENZYMIC SYNTHESIS OF PURINE D-ARABINONUCLEOSIDES

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(Received May 12th, 1981; accepted for publication, June 8th, 1981)

ABSTRACT

A method is described for the synthesis of purine D-arabinonucleosides that uses purine bases and 2,2′-anhydro-(1-β-D-arabinofuranosylcytosine), AraC-an, as the starting materials. AraC-an was chosen as the precursor to the D-arabinosyl donor, because it is more readily available than any of the products that may be sequentially derived from it, namely, 1-β-D-arabinofuranosylcytosine (AraC), 1-β-D-arabinofuranosyluracil (AraU), and α-D-arabinofuranosyl-1-phosphate (Ara*f* 1-P), a D-arabinofuranosyl donor. Four reactions were involved in the overall process; (*a*) AraC-an was nonenzymically hydrolyzed at alkaline pH to AraC which was then (*b*) deaminated by cytidine deaminase to AraU, a nucleoside, (*c*) phosphorylyzed by uridine phosphorylase to Ara*f* 1-P, and (*d*) this ester caused to react with a purine base to afford a purine D-arabinonucleoside, the reaction being catalyzed by purine nucleoside phosphorylase. All four reactions occurred *in situ*, the first and second being performed sequentially, whereas the third and fourth were combined in a single step. The three enzyme catalysts were purified from *Escherichia coli*. The efficiency of the method is exemplified by the synthesis of the D-arabinonucleosides of 2,6-diaminopurine and adenine; the overall yields, based on AraC-an, were 60 and 80%, respectively.

INTRODUCTION

Current, chemical methods for the synthesis of purine D-arabinonucleosides are generally difficult and inefficient. Indeed, difficulties in their synthesis have impeded acquisition of sufficient quantities of the D-arabinonucleosides of 2,6-diaminopurine[1] and 2-fluoroadenine[2] to permit their chemotherapeutic evaluation. With the antiviral agent adenine D-arabinonucleoside, the difficulties encountered in the chemical synthesis were circumvented by development of a fermentation procedure[3], the biochemical basis for which is the ability of the bacterium *Streptomyces antibioticus* to convert exogenously supplied adenosine into its D-arabinonucleoside without

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0008-6215/81/0000–0000/$ 02.50,

cleavage of the glycosylic bond[4]. This implies the existence of a 2′-epimerase for adenosine.

Recently, the bacterial synthesis of adenine D-arabinonucleoside from exogenously supplied uracil D-arabinonucleoside (AraU) and adenine has been described[5]. The yield, on the basis of the D-arabinosyl donor, was 25%. A similar procedure was used for synthesizing the D-arabinonucleoside of 2-chloro-6-hydroxypurine in a yield[6] of 11% with respect to AraU.

For the synthesis of purine nucleosides, a general method employing, as catalysts, nucleoside phosphorylases purified from *Escherichia coli* has been reported from this laboratory[7]. Herein, procedures for the facile, *in situ* generation of α-D-arabinofuranosyl-1-phosphate (Ara*f* 1-P), and the adaptation of the general, enzymic method to the synthesis of D-arabinonucleosides, are described.

EXPERIMENTAL

Materials. — 2,2′-Anhydro-(1-β-D-arabinofuranosylcytosine) hydrochloride (AraC-an · HCl), ribonuclease B, and deoxyribonuclease I from bovine pancreas were purchased from Sigma Chemical Company, DEAE-cellulose (Microgranular DE52) from Whatman, cellulose thin-layer sheets (No. 6065, with fluorescent indicator) from Eastman, and 2,6-diaminopurine hydrate from Burroughs Wellcome Co.

Enzyme assays. — Nucleoside phosphorylase and cytidine deaminase assays were performed by continuous, spectrophotometric monitoring under the conditions described previously[8], unless noted otherwise. One unit of enzyme activity was that amount of enzyme which catalyzed the formation of 1 μmol of product per min under the standard assay conditions. Concentrations of protein were determined spectrophotometrically[9].

Enzyme preparations. — Cytidine deaminase (EC 3.5.4.5) was purified from *Escherichia coli* B-96/1 (ATCC 13,863). Frozen cell-extracts were prepared as previously described[7]. Thawed cell-extract (1 L) was centrifuged at 27,000*g* for 30 min. The supernatant liquor contained 1.4 units of cytidine deaminase/mL. Ribonuclease B (70 mg) and deoxyribonuclease I (80 mg) (7,100 and 140,000 Kunitz units, respectively) were added, and the solution was dialyzed at 25° in tubing (diam. 2.5 cm) against 5mM potassium phosphate, pH 7.0, according to the following schedule: 16 L for 7 h, 22 L for 18 h, and 16 L for 1.5 h. The dialyzed material was centrifuged at 27,000*g* for 30 min, and then filtered through a Millipore Type AA filter. The filtrate contained 1.3 units of enzyme activity per mL. This nuclease treatment lowered the nucleic acid content to <1.5%, as determined spectrophotometrically[10]. DEAE-Cellulose (105 g) was added to the filtrate, and the suspension was stirred slowly for 30 min, and then poured onto a column (diam. 5 cm) in which a bed of DEAE-cellulose (95 g) had been pre-equilibrated with 5mM potassium phosphate buffer, pH 7.4. When all of the DEAE-cellulose had been packed and washed with this buffer, the final height of the resin bed was 31 cm. The enzyme was eluted with a linear gradient (1.9 L)

of 5mM to M potassium phosphate, pH 7.4. Fractions containing the bulk of the cytidine deaminase activity were combined, and the solution had a phosphate concentration of 0.6M. This chromatographic procedure increased the specific activity of cytidine deaminase from 0.1 to 2.3 units/mg of protein. A total of 750 units was obtained, and the recovery was 61%. The enzyme was stored (in aliquots) at −73°, and was stable for at least 1 year under these conditions.

Uridine phosphorylase (EC 2.4.2.3) and purine nucleoside phosphorylase (EC 2.4.2.1) were isolated[11] from *Escherichia coli B* (ATCC 11303). Thymidine phosphorylase (EC 2.4.2.4) was purified from *E. coli* B-96 as previously described[7].

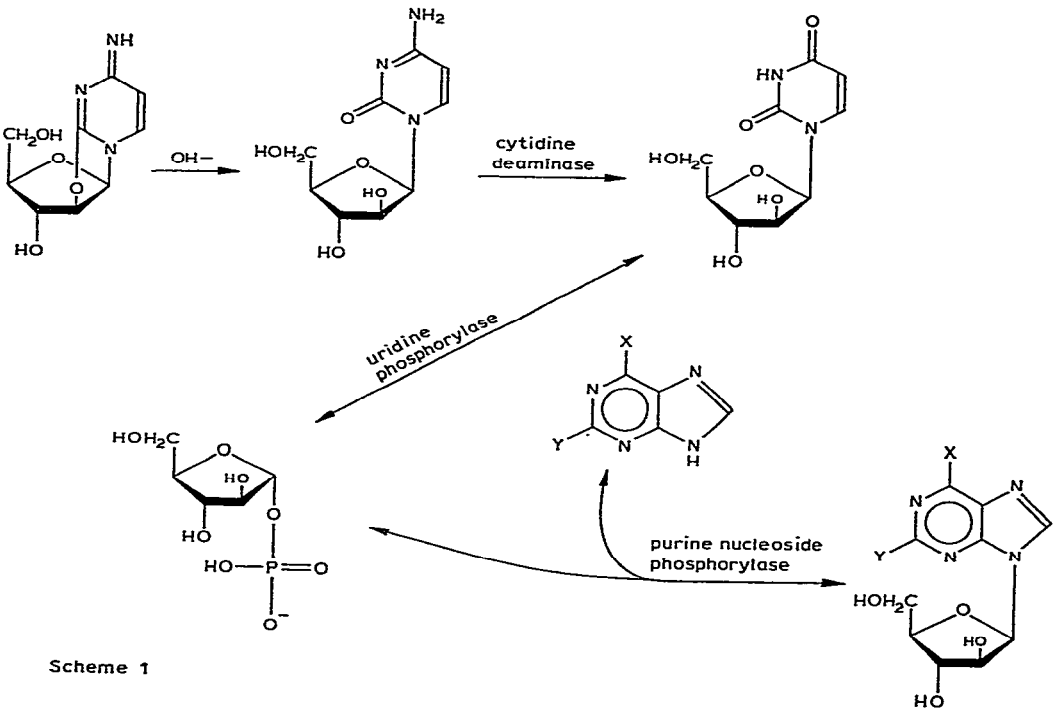
RESULTS AND DISCUSSION

The method for the synthesis of D-arabinonucleosides presented here was based on the general, enzymic method involving the reaction of a purine base with an aldopentosyl-1-phosphate[7]. At the beginning of these studies, some obstacles to the adaptation of this method to D-arabinonucleosides were apparent. It had been reported that purine nucleoside phosphorylase from human erythrocytes does not phosphorylyze hypoxanthine D-arabinonucleoside[12]. Furthermore, it had been averred[13] that adenine D-arabinonucleoside is not phosphorylyzed by purine nucleoside phosphorylase from *E. coli*. However, on reinvestigation, a different result was obtained with the enzyme from *E. coli*. At 0.1mM nucleoside and 100mM phosphate (K), pH 7.3, the rate of phosphorolysis of adenine D-arabinonucleoside determined spectrophotometrically ($\Delta\varepsilon_{256} = 2.13\ \text{mM}^{-1}.\text{cm}^{-1}$) was one-tenth that with adenosine. Under these conditions, the K'_m value for adenine D-arabinonucleoside was 0.4mM. These results suggested that purine nucleoside phosphorylase from *E. coli* might be useful as a catalyst in the synthesis of purine D-arabinonucleosides.

Another obstacle was a source of Ara*f* 1-P, a D-arabinofuranosyl donor[14]. The chemical synthesis of this ester is at present exceedingly difficult[15]. However, it had been shown that Ara*f* 1-P can be generated from AraU by an enzyme-catalyzed phosphorolysis[16]. This nucleoside is less rare than Ara*f* 1-P, and can be synthesized in good yield by the method of Nishimura and Shimizu[17]. However, it was more suitable to utilize a sequence of reactions designed to generate Ara*f* 1-P *in situ*, starting from 2,2′-anhydro-(1-β-D-arabinofuranosylcytosine) (AraC-an), a precursor that is relatively inexpensive when obtained from commercial sources, or that can be synthesized by published procedures[18,19]. The sequence of reactions used in the total synthesis of a purine D-arabinonucleoside from AraC-an and a purine base is shown in Scheme 1.

AraC-an was converted into cytosine arabinonucleoside (AraC) by alkaline hydrolysis[19], and the AraC was then deaminated to AraU by cytidine deaminase purified from *E. coli* (see Experimental section). The details of this hydrolysis and deamination are described in Example I.

Ara*f* 1-P was next generated by phosphorolysis of AraU. Two distinct, pyrimidine nucleoside phosphorylases, a thymidine phosphorylase and a uridine phosphorylase,



Scheme 1

are readily isolated[7] from *E. coli*. In theory, either of these enzymes might catalyze the desired phosphorolysis. However, under the standard conditions of assay, the rate of phosphorolysis of AraU by thymidine phosphorylase was so low that it was difficult to detect; one unit of thymidine phosphorylase cleaved AraU at <2.5 nmol per min, in agreement with results of Tono and Cohen[16]. In contrast, phosphorolysis of AraU by uridine phosphorylase was readily demonstrated. The rates of the uridine phosphorylase-catalyzed phosphorolysis of uridine and AraU as a function of pH are shown in Fig. 1. The shape of the pH-dependence curve with AraU was similar to that with uridine, but the pH optimum was ~1 pH unit higher. The kinetic constants of uridine and AraU as substrates at pH 6 and 8 are given in Table I. For AraU at the lower pH, the V'_{max} value was lower and the K'_m value was higher. Both trends contributed to a decreased substrate efficiency at the lower pH value.

The data in Fig. 1 and Table I, together with the relatively high, pH optimum for nucleoside synthesis with purine nucleoside phosphorylase[20], suggest that the pH of reaction mixtures should be adjusted to values lying between 7 and 8. Another reason for avoiding pH values higher than 8 is that the stabilities of uridine phosphorylase and purine nucleoside phosphorylase have been shown[7] to decrease at higher pH values.

The last step in the synthesis, the reaction of Ara*f* 1-P with a purine base to afford the nucleoside product, was catalyzed by purine nucleoside phosphorylase purified from *E. coli*. A guide to the variety of purine bases that are substrates for this enzyme was provided by the specificity study of Doskocil and Holý[13], which showed

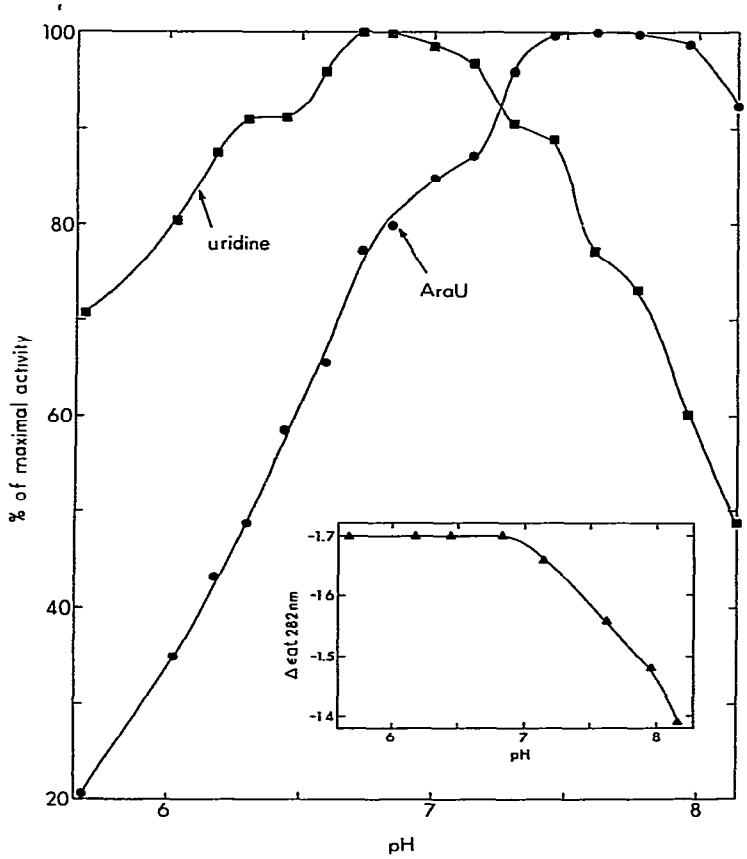


Fig. 1. Dependence on pH of the phosphorolysis of uridine (■) and AraU (●) catalyzed by uridine phosphorylase. [$\Delta\varepsilon$ Values used to calculated rates from spectral changes at 282 nm are given in the insert (▲).] Concentrations of nucleoside and phosphate in the reaction mixture were 0.5mM and 200mM, respectively. The maximal velocity with uridine was 9.4 units/mg of protein, whereas that with AraU was 0.18 unit/mg of protein.]

TABLE I

KINETIC CONSTANTS FOR URIDINE PHOSPHORYLASE

Substrate	*pH*	K'$_m$ (±*S.E.*)	*Rel.* V'$_{max}$[a] (±*S.E.*)
Uridine	6	0.079 (±0.004)	100 (±2)
AraU	6	1.5 (±0.2)	2.6 (±0.2)
Uridine	8	0.050 (±0.003)	64 (±2)
AraU	8	0.72 (±0.22)	4.2 (±1)

[a]Rel. V'_{max} was expressed as the percentage of that for uridine at pH 6.0 (6 μmol/min/mg of protein).

that certain heterocycles other than purines can act as substrates for the enzyme. However, in the experience of this laboratory, the only bases that were found to be practical substrates for the synthesis of D-arabinonucleosides were those containing the purine ring-system.

Relatively small amounts of cytidine deaminase were needed in order to catalyze the deamination of AraC. On the other hand, the practicality of this method could be limited by the relatively large amounts of uridine phosphorylase and purine nucleoside phosphorylase needed for catalyzing the last two reactions in the synthetic scheme. This potential disadvantage was somewhat ameliorated by recycling the enzyme catalysts. In instances where the D-arabinonucleoside product was sparingly soluble, the nucleoside phosphorylases were reused by simply adding more reactants to the soluble portion of a reaction mixture after the insoluble product had been removed by filtration or centrifugation. This enzyme recycling is demonstrated in Example I.

The n.m.r. spectra of the D-arabinonucleoside products of this synthetic procedure indicated the absence of ribonucleoside contaminants. If any appreciable ribonucleoside were present in either product, resonances for H-1′ and H-2′ would be detectable in the δ 5.9 or 4.7 regions of the spectrum[21]. No signals were detected in these regions for adenine D-arabinonucleoside (Example II). In the spectrum of 2,6-diaminopurine D-arabinonucleoside, resonances of exchangeable protons obfuscated these regions (Example I). However, after D_2O exchange, these regions were free from signals.

This method for the synthesis of purine arabinonucleosides has the obvious disadvantage of requiring the purification of the enzymes employed. To counterbalance this, it has definite advantages over the chemical and bacterial procedures currently available. The use of purified enzymes as catalysts provides greater assurance of the purity of the products. Furthermore, the yields of product are superior. This enzymic method provides a new and attractive alternative to existing procedures for the synthesis of purine D-arabinonucleosides.

EXAMPLES OF THE METHOD

I. *2,6-Diamino-9-β-D-arabinofuranosylpurine*[1]. — A solution of AraC-an hydrochloride (57 mmol) in water (965 mL) was stirred at 4°, and sufficient, freshly prepared 2M KOH was added dropwise to raise the pH from 3.4 to 10 and maintain it near there. After a notable decrease in the rate of addition of KOH necessary to maintain the pH at 10, the solution was allowed to warm to room temperature. The addition of KOH was continued until the pH remained steady at 10.3 for at least 20 min and only arabinonucleoside was detectable by thin-layer chromatography (t.l.c.) on cellulose with 2:79:19 (v/v)[18] 2-propanol–saturated ammonium sulfate–M sodium acetate. The R_F value of AraC-an was 0.74, and that of AraC was 0.59.

After the hydrolysis was complete, the pH was adjusted to 6.8 with 17M acetic acid, and then 30 units of cytidine deaminase purified from *E. coli* (see Experimental

section) were added. Deamination was monitored by t.l.c. on cellulose with water-saturated 1-butanol. The R_F value of AraC was 0.37, and that of AraU was 0.67. After 2 days at 37°, deamination was complete. The pH of the reaction was then adjusted to 7.1 with 17M acetic acid.

2,6-Diaminopurine (118 mmol), K_2HPO_4 (3 mmol), and sodium azide (3 mmol) were added. The pH of the mixture was adjusted to 7.1, and then 15,000 units of purine nucleoside phosphorylase and 8,000 units of uridine phosphorylase were added. After 7 days at 37°, the insoluble material in the reaction mixture was collected by centrifugation, and extracted with water (700 mL) at 50°. After mechanical stirring for 30 min, the suspension was allowed to settle for 17 h at 3°. The solid was collected by filtration, suspended in anhydrous methanol (1.8 L), and the suspension gently boiled and immediately filtered; the cake was washed, resuspended in methanol (350 mL), the suspension boiled, filtered, and the cake washed as before, and dried *in vacuo*, yielding 8.07 g of product, designated "first crop".

The filtrates from the afore-described extractions were lyophilized and the residues returned to the supernatant liquor of the original reaction-mixture. Additional uracil D-arabinonucleoside (44 mmol), prepared as just described, was added to this mixture. After 5 days at 37°, the insoluble material present in the mixture was treated as described, yielding 9.08 g of product, designated "second crop", m.p. >250°, $[\alpha]_D^{25}$ +30.6° (*c* 0.52, 1:3 H_2O–$HCONMe_2$); λ_{max}^{pH1} 252 (ε_{mM} 11.0) and 291 nm (9.9); λ_{max}^{pH13} 256 (ε_{mM} 9.3) and 280 nm (10.5); ^{1}H-n.m.r. (Varian CFT-20, Me_2SO-d_6): δ 7.77 (s, 1 H, H-8), 6.06 (d, 1 H, *J* 4.1 Hz, H-1), and 6.66 and 5.77 (2 s, 2 H each, 2- and 6-NH_2).

The overall yield was 60.7 mmol, constituting a 51% yield of D-arabinonucleoside with respect to the amount of free base used, and 60% with respect to the amount of D-arabinosyl donor used.

Anal. Calc. for $C_{10}H_{14}N_6O_4$: C, 42.55; H, 5.00; N, 29.78. Found: (1st crop) C, 42.67; H, 5.10; N, 29.82; (2nd crop) C, 42.67; H, 5.03; N, 29.65.

II. *6-Amino-9-β-*D*-arabinofuranosylpurine*[22]. — The reaction mixture consisted of an aqueous suspension (0.37 L) that contained AraU (60 mmol) prepared from AraC-an as described in Example I. Other components were: adenine (118 mmol), KH_2PO_4 (9 mmol), purine nucleoside phosphorylase (4,000 units), and uridine phosphorylase (2,900 units). The pH of the suspension was adjusted to 7.7 before addition of the enzymes. After 6 days at 42°, the mixture was filtered. The solid was washed with water (50 mL), dried, suspended in anhydrous dimethyl sulfoxide (40 mL), and the suspension warmed to 55°, filtered, and the cake reserved. Addition of water (250 mL) to the filtrate precipitated the product. After being kept for 16 h at 3°, the precipitate was collected by filtration, and washed with anhydrous methanol (20 mL). The reserved filter-cake from the original extraction was re-extracted a second and a third time with Me_2SO (33 mL), and the product was precipitated with water as before. These three precipitates weighed 12.42 g after being dried. The filtrates and washings were combined, and evaporated *in vacuo*. After extraction with Me_2SO, and precipitation of the product with water as before, another 0.51 g of

product was obtained. The total weight of product was 12.93 g (48 mmol), a yield of 80%, based on the D-arabinosyl donor; m.p. 258–261° (lit.[22,23] m.p. 257–257.5; 258–260), $[\alpha]_D^{20}$ −1.4° (*c* 1, $HCONMe_2$) {lit.[22,23] $[\alpha]_D^{27}$ −5° (0.25% in H_2O), $[\alpha]_D^{20}$ −1.7° (*c* 0.54, pyridine)}; λ_{max}^{pH1} 257 nm (ε_{mM} 15.2); λ_{max}^{pH13} 260 nm (ε_{mM} 15.4) [lit.[22,23] λ_{max}^{pH1} 257.5 nm (ε_{mM} 12.7); λ_{max}^{pH13} 259 nm (ε_{mM} 14.0); λ_{max}^{EtOH} 258 nm]; ^{1}H-n.m.r. (Varian XL-100, Me_2SO-d_6); δ 8.18 and 8.14 (2 s, 1 H each, H-2, 8), 6.26 (d, 1 H, *J* 4.4 Hz, H-1′), and 7.22 (s, 2 H, NH_2).

Anal. Calc. for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.20. Found: C, 45.04; H, 4.95; N, 26.16.

ACKNOWLEDGMENT

The authors are indebted to Janie W. Peck for technical assistance.

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