Polymer-Linked 6-Azauridine 5′-Monophosphate, a Resin of High Bioaffinity to Orotidine-5′-phosphate Decarboxylase

Helmut Rosemeyer and Frank Seela*

Department of Organic Chemistry, Universität Paderborn-Gesamthochschule, Warburger Strasse, 100, D-4790 Paderborn, Federal Republic of Germany. *Received May 7, 1979*

Condensation of 6-azauridine with ethyl levulinate, followed by saponification or phosphorylation, leads to 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]-6-azauridine and its 5′-monophosphate. The latter was coupled to 6-aminohexylagarose via its carboxylic group. Using the same synthetic route, agarose-linked uridine 5′-monophosphate has been prepared. Both polymers show specific binding toward orotidine-5′-monophosphate decarboxylase. The immobilized inhibitor (6-azauridine 5′-monophosphate) binds the enzyme more strongly than the immobilized uridine 5′-monophosphate. Both resins have been used to separate orotidine-5′-monophosphate decarboxylase from orotidine-5′-monophosphate pyrophosphorylase.

In the biosynthesis of pyrimidine nucleosides, orotidine-5′-monophosphate pyrophosphorylase (OMP-PP) and orotidine-5′-monophosphate decarboxylase (OMP-DC) play a key role.[1,2] The former enzyme catalyzes the formation of orotidine 5′-monophosphate from 5′-phospho-β-D-ribofuranosyl pyrophosphate (PRPP) and orotic acid, while the latter effects the decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP). 6-Azauridine and its 5′-monophosphate are strong inhibitors of this biosynthetic pathway; 6-azauridine 5′-monophosphate (6-azaUMP) shows binding to the active site of OMP-DC, which is one order of magnitude higher than the binding of orotidine 5′-monophosphate. This phenomena causes cytostatic activity.[3] The selectivity in the action of 6-azaUMP against cancer cells has been exploited clinically in the treatment of leukemias.[4]

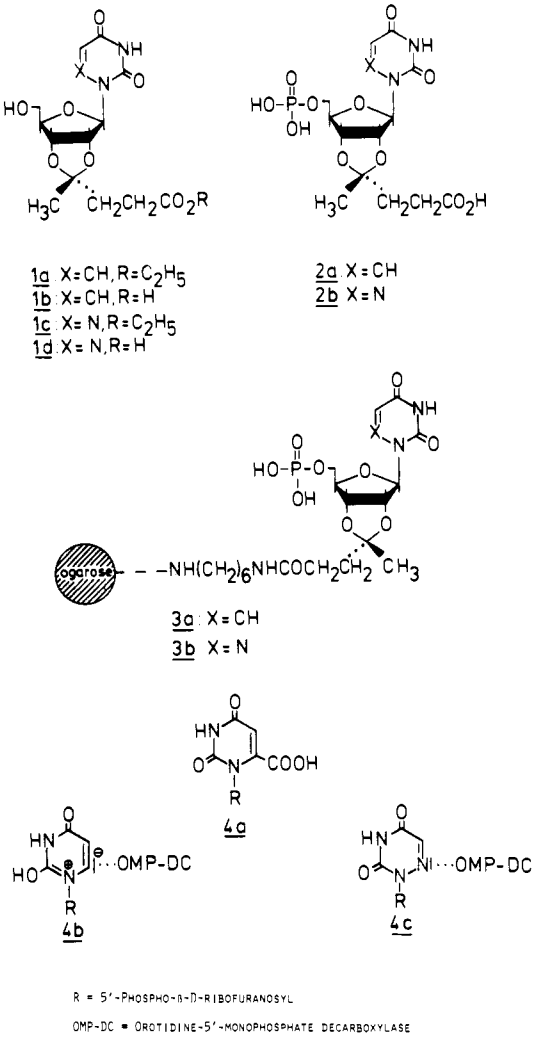
Since 6-azaUMP and UMP 2′,3′-*O*-ethylidene acetals retain most of the activity of the parent compounds, the immobilization via its 2′,3′-hydroxyl groups should lead to polymers with biospecificity to OMP-DC. Insoluble polymers, prepared by this way, may be useful for purification of OMP-DC by one-step affinity chromatography. By immobilization of 6-azaUMP to soluble dextrans by the method we describe in this paper, rapid excretion of 6-azaUMP may be avoided, and prolongated pharmacological activity should be obtained.[5,6]

Synthesis and Properties of the Uridine and 6-Azauridine Derivatives 2a,b. We have already shown that uridine can be condensed with ethyl levulinate to give the acetal **1a**.[7] If **1a** is phosphorylated with phosphoryl chloride in triethyl phosphate, the phosphate **2a** is formed, which can be purified by ion-exchange chromatography on DE-52 cellulose.

If 6-azauridine is treated with ethyl levulinate under the conditions already described for uridine, the ester **1c** is obtained. Its ^{13}C NMR spectrum shows that the acetalation occurs at the 2′,3′-hydroxyls, since only the C-2′/C-3′ signals are shifted to higher field compared to the nucleosides. In addition ^{13}C and ^{1}H NMR spectra show that only one diastereoisomer with the *R* configuration at the acetal carbon is formed, as has been found for other nucleosides.[7-9]

Alkaline hydrolysis of **1c** leads to the acid **1d**, whose carboxylic group can be observed by its higher mobility in the pherogram (silica gel, 0.1 M sodium citrate buffer, pH 6.5). Because the acid does not crystallize, it was chromatographed on a Dowex 1X2 anion-exchange resin for purification.

The synthesis of **2b** was the same as that for **2a**; treatment of the ester **1c** with phosphoryl chloride and hydrolysis of the ester group yields the monophosphate. The



removal of the byproducts was realized by DE-52 cellulose chromatography. By quantitative phosphate determination using the ammonium molybdate method it could be shown that the nucleotides **2a,b** contained 1 mol of phosphate/mol of nucleotide. In addition, the phosphate residues can be cleaved with alkaline phosphatase to form the derivatives **1b,d** as the only reaction products. To show that the derivative **2b** is bound to the active site of OMP-DC as well as its parent nucleotide,[3] **2b** was tested as an inhibitor in the enzymatic decarboxylation reaction. The enzymatic assay depends upon the alteration of the UV spectrum of orotidine 5′-monophosphate compared to uridine 5′-monophosphate. The decrease of absorbance was followed at 285 nm.[10] It could be shown that the relative initial velocities of the enzymatic decarboxylation decline

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to 85% at a molar ratio of substrate to inhibitor of 1:1 and to 35% at a ratio of 1:7.3.

This finding verifies that mainly the nucleobase is involved in the enzymatic mechanism. Since the decarboxylation of orotidine 5′-monophosphate (**4a**) takes place without any coenzyme, a dipolar-ionic tautomer of the substrate may be formed, which is stabilized by OMP-DC and can be decarboxylated via the carbanion **4b**.[11]

6-Azauridine 5′-monophosphate (**4c**) is a transition-state analogue of this carbanion and shows stronger binding to OMP-DC than orotidine 5′-monophosphate itself.[3]

The acetals **2a,b** were coupled to 6-aminohexylagarose in *p*-dioxan/water in the presence of water-soluble carbodiimide. The concentration of bound ligand was determined after incineration of the gels, by quantitative phosphate determination using the molybdenum blue method. The concentration of ligand was found to be 124 μmol/g of **3a** and 26 μmol/g of **3b**. The polymer **3b** was then tested as an inhibitor in the OMP/OMP-DC system. At a molar ratio (1:1.5) of substrate to immobilized 6-azaUMP, 80% inhibition was found. In order to show the biospecificity of the new polymers, 1-mg samples of enzyme mixtures of OMP-DC and OMP-PP in 200 μL of Sörensen phosphate buffer, pH 7.6, were injected onto thermostated chromatography columns (10 °C, 0.9 × 10 cm) containing either **3a** or **3b**. By eluting with $^1/_{15}$ M Sörensen phosphate buffer, pH 7.6, crude OMP-PP, in contrast to OMP-DC, was not retarded. OMP-DC was bound to both gels. By increasing the salt concentration to 0.5 M NaCl, OMP-DC could be eluted from the product gel **3a**, while **3b** still bound the enzyme. At a salt concentration of 1 M NaCl, OMP-DC was eluted from the inhibitor gel **3b**.

During elution, conductivity was measured at the column base to show that the enzyme was eluted after 1 void volume. After chromatography, the content of both peaks was tested, and it could be demonstrated by thin-layer chromatography that only the retarded protein had OMP-DC activity, while the prezone showed no decarboxylase activity. These experiments show the high specificity of both resins, especially for separation of OMP-DC from mixtures containing OMP-PP.

Experimental Section

^{13}C NMR spectra were measured in Me_2SO-d_6 (internal Me_4Si) with a Bruker HX 60 spectrometer, and chemical shifts are reported in δ values (ppm). UV spectra were measured with a Zeiss PMQ 3 or Shimadzu UV-200 spectrometer. Microanalyses were performed by Mikroanalytisches Labor Beller (Göttingen, FRG). Where analyses are indicated by symbols of the elements, analytical results obtained for those elements were within ±0.3% of the calculated values.

Chromatography and Electrophoresis. Column chromatography was performed on DE-52 cellulose (Whatman, Springfield, UK). Thin-layer chromatography (TLC) was performed on silica gel F-254 or polyethylenimine cellulose plates (Woelm, Eschwege, FRG); solvent systems used were the following: A, water/methanol (4:1); B, 0.25 M LiCl. Thin-layer electrophoresis (TLE) was performed on silica gel F-254 thin-layer plates in a TLE double chamber (Desaga, Heidelberg, FRG) with 0.1 M sodium citrate buffer, pH 6.5. Ion-exchange chromatography was performed on Dowex 1X2 ion-exchange resin, OAc^-, 200–400 mesh (Serva, Heidelberg, FRG). The $(NEt_3)HCO_3$ buffer was prepared by bubbling CO_2 through a 14.4% (v/v) aqueous NEt_3 solution until pH 7.0 was obtained. 6-Aminohexylagarose (AH-Sepharose 4B) was purchased from Pharmacia (Uppsala, Sweden), and nucleosides were obtained from Pharma Waldhof (Düsseldorf, FRG) and from Sigma Chemical Co. (St. Louis). Orotidine-5′-monophosphate decarboxylase (OMP-DC, EC 4.1.1.23), orotidine-5′-monophosphate pyrophosphorylase (OMP-PP, EC 2.4.2.10), mixed enzymes, were purchased from Sigma (St. Louis).

Phosphate Determination. An aliquot (0.7 mL) of a freshly prepared 1:6 mixture of 10% aqueous L-ascorbic acid and 0.42% ammonium molybdate tetrahydrate in 0.5 M H_2SO_4 was added to a solution (0.3 mL) of the unknown phosphate. The mixture was kept at 37 °C for 1 h and the absorbance was read at 820 nm.

Cleavage of 5′-Phosphates with Alkaline Phosphatase. A suspension (10 μL) of *Escherichia coli* alkaline phosphatase (EC 3.1.3.1, Boehringer, Mannheim, FRG, 1 mg/mL) was diluted with 0.25 M Tris-HCl buffer (90 μL, pH 8.0). A portion (10 μL) of this solution was added to the substrate (10 μL) and incubated at 37 °C for 2 h.

2′,3′-*O*-[1-[2-(Ethoxycarbonyl)ethyl]ethylidene]-6-azauridine (1c). To a suspension of 6-azauridine (5 g, 20.4 mmol) in dry *N*,*N*-dimethylformamide (70 mL) were added ethyl levulinate (6.25 mL, 40 mmol) and triethyl orthoformate (4.65 mL, 30 mmol) and insoluble material was dissolved by adding a solution (8.5 mL) of 7 M hydrogen chloride in dry *p*-dioxane. The mixture was kept for 24 h at room temperature and then poured into ether (750 mL). The upper layer was decanted, and the oily residue was washed twice with ether and then dissolved in chloroform by the addition of 2% aqueous sodium hydrogen carbonate. The organic layer was washed with water, dried, and concentrated, giving a heavy oil of **1c**: yield 3.42 g (46%); TLC (solvent B) R_f 0.58; UV (MeOH) λ_{max} 263 nm (ε 4800); ^{13}C NMR δ 172.58 (C=O), 156.59 (C-4), 147.97 (C-2), 136.32 (C-5), 113.13 (acetal C), 90.92 (C-1′), 87.94 (C-4′), 83.15 (C-3′), 81.73 (C-2′), 61.97 (C-5′), 59.84 (O-CH_2), 33.29 (CH_2CO), 28.04 (acetal CH_2), 23.44 (acetal Me), 13.99 (ester Me). Anal. ($C_{15}H_{21}N_3O_8$) C, H, N.

2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]-6-azauridine (1d). A solution of **1c** (1 g, 2.7 mmol) in a 1:1 mixture (60 mL) of ethanol and 1 M sodium hydroxide was kept for 30 min at room temperature and then neutralized (glass electrode) with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated. After evaporation to dryness, 650 mg (70%) of a colorless glass was obtained. The residue was dissolved in water and, after adding a few drops of concentrated ammonia, chromatographed on an ion-exchange resin (Dowex, 1X2, acetate form) eluting with a linear gradient of 0.3 M acetic acid/water (1 L). The acid was found in the main peak. Fractions were pooled and evaporated to dryness, giving 600 mg of the acid (65%): R_e relative to 6-azaU +1.7; TLC (solvent B) R_f 0.70; UV (MeOH) λ_{max} 264 nm (ε 4500); ^{13}C NMR δ 174.07 (C=O), 156.52 (C-4), 147.91 (C-2), 136.25 (C-5), 113.20 (acetal C), 90.86 (C-1′), 87.94 (C-4′), 83.02 (C-3′), 81.66 (C-2′), 61.91 (C-5′), 33.48 (CH_2CO), 28.11 (acetal CH_2), 23.38 (acetal Me). Anal. ($C_{13}H_{17}N_3O_8$) C, H, N.

2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]-6-azauridine 5′-Monophosphate (2b). To a solution of **1c** (1 mmol, 370 mg) in triethyl phosphate (5 mL) was added $POCl_3$ (180 μL, 2 mmol) at 4 °C. After leaving for 24 h at 4 °C, the solution was neutralized with 1 M sodium hydroxide and then concentrated in vacuo. A suspension of the residue in 0.5 M sodium hydroxide (20 mL) was stirred for 30 min and then neutralized with 1 M hydrochloric acid. The solution was diluted with water (300 mL), adsorbed on a DE-52 cellulose column (25 × 3.5 cm), and eluted with a linear gradient of water/0.5 M TBK buffer (1000 mL). Unphosphorylated **1d** was eluted first, followed by the phosphate **2b**, at about 0.3 M TBK. The phosphate-containing fractions were combined and concentrated in vacuo, and excess buffer was removed by repeated evaporation of aqueous ethanol from the residue to yield **2b** (6700 A_{264} units, 32%, using ε of 6-azauridine) as a colorless amorphous solid: TLC (solvent B) R_f (silica gel) 0.60, R_f (PEIC) 0.54. Cleavage of the phosphate with alkaline phosphatase gave **1d** as the only reaction product: 0.335 A_{264} unit of **2b** (0.056 μmol) gave 0.152 A_{820} unit of molybdate complex, corresponding to 0.06 μmol of phosphate.

2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]uridine 5′-Monophosphate (2a). **1a**, 370 mg, was phosphorylated as described for **1c**: yield 120 mg (32%) of amorphous **2a**; TLC (solvent B) R_f (silica gel) 0.60, R_f (PEIC) 0.57. Cleavage of the phosphate with alkaline phosphatase gave 2′,3′-*O*-[1-(2-carboxyethyl)-ethylidene]uridine (**1b**) as the only reaction product: 0.04 μmol (0.348 A_{261} unit, ε 8700) gives 1.08 A_{820} units of molybdate complex, corresponding to 0.045 μmol of phosphate.

Condensation of 2a,b with 6-Aminohexylagarose. To a solution of **2a** (42, 2 mg, 0.1 mmol) in water (10 mL) was added 6-aminohexylagarose (5 mL) at pH 6. The suspension was shaken for 10 min, and the ligand was coupled by the addition of *N*-[3′-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride

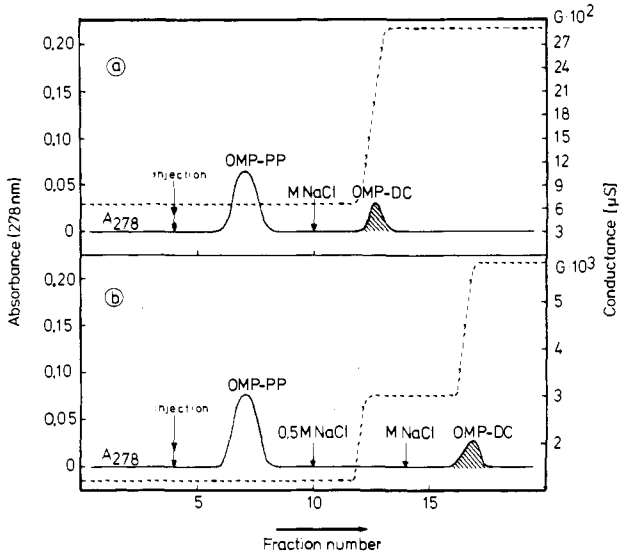

Figure 1. Biospecific chromatography of orotidine-5′-monophosphate decarboxylase/orotidine-5′-monophosphate pyrophosphorylase on the polymers **3a** (a) and **3b** (b); 1 mg of the mixture of the enzymes in 200 μL of $^1/_{15}$ M phosphate buffer (pH 7.6) was applied to columns (20 × 0.9 cm) equilibrated with the same buffer and eluted with sodium chloride.

(100 mg, 0.52 mmol). Shaking was continued overnight, and the agarose derivative was collected, washed with 0.1 M sodium hydrogen carbonate (pH 8, 250 mL), 0.5 M sodium chloride (250 mL), and water to give the gel **3a** (5 g).

A portion of the packed gel was dried for 72 h at 95 °C in vacuo to constant weight; there was a 96% loss in weight. The dry powder (14.3 mg) was mixed with water (0.5 mL) and 10% $Mg(NO_3)_2$ in ethanol–water (1.5 mL, 1:1), swollen for 15 min, and then incinerated. The residue was hydrolized with 1 M hydrochloric acid (10 mL) for 30 min at 100 °C. An aliquot (300 μL) of the solution was mixed with the phosphate determination reagent (700 μL) and stored at 37 °C for 1 h. The absorbance at 820 nm (1-cm path length) was then 1.27 and, since 1 μmol of phosphate is 24 A_{820} units, this corresponds to 124 μmol of ligand/g of dry gel **3a**, which is estimated to be 5 μmol/g of moist **3a**. The coupling of **2b** to 6-aminohexylagarose was carried out as described for **2a**. The concentration of ligand, measured as described above, was 1.04 μmol/g of moist **3b** which is estimated to be 26 μmol/g of dry **3b**.

A. Affinity Chromatography of OMP-DC on 3a,b. The gels **3a,b** were thoroughly washed with water and packed into jacketed columns (10 × 0.9 cm), which were thermostated at 10 °C. The flow rate was adjusted with a peristaltic pump and was 20 mL/h. The columns were first equilibrated with 2–3 void volumes of phosphate buffer ($^1/_{15}$ M, pH 7.6). For each run, 1 mg of OMP-DC/OMP-PP (mixed enzymes), dissolved in 200 μL of Sörensen phosphate buffer ($^1/_{15}$ M, pH 7.6), was applied to the resins **3a** or **3b**. The columns were attached to a Uvicord 3 photometer (LKB Instruments, Bromma, Sweden), on which absorbance was read at 280 nm. Conductance was constantly measured by using a conductivity cell with a cell constant K = 12.6 cm^{-1}. Two-milliliter fractions were collected with a fraction collector.

In every case, the OMP-PP was eluted with the void volume of buffer and did not contain any decarboxylase activity; this was shown by qualitative TLC after incubating OMP with the contents of the breakthrough peak for 1 h at 37 °C in Sörensen buffer ($^1/_{15}$ M, pH 7.6). OMP-DC (Figure 1) can be eluted from the product gel **3a** with the void volume using a 0.5 M NaCl solution only, while for elution from the inhibitor gel **3b** 1 M NaCl has to be used.

B. Enzymatic Assay. Relative initial velocities of the decarboxylation reaction of OMP by OMP-DC in the presence of **2b** were determined by addition of 10^{-3} units of OMP-DC to 1 mL of $^1/_{15}$ M Sörensen phosphate buffer, pH 7.6, containing 0.1 μmol of OMP and 0.1–0.73 μmol of **2b**. The decrease of OMP at room temperature was followed at 285 nm within 10 min. The slope between the first and second minute was defined as the initial velocity and arbitrarily taken as 100 for OMP. At a molar ratio of substrate to inhibitor the relative initial velocity was found to be 85% and at a ratio 1:7.3 it was 35%. The inhibition of the decarboxylation reaction by immobilized 6-azaUMP (**3b**) was determined by the addition of 3×10^{-3} units of OMP-DC to 1 mL of $^1/_{15}$ M Sörensen phosphate buffer containing 0.3 μmol of OMP and 0.46 μmol of immobilized 6-azaUMP (400 μL; **3b**). At this molar ratio (substrate to immobilized inhibitor 1:1.5), 80% inhibition was measured.

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