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Note

Synthesis of 5-fluorouridine 5′-diphosphate galactose from 5-fluorouridine by chemical phosphorylation and microbial uridylyl transfer

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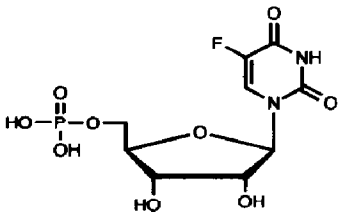
The sugar nucleotide analogue 5-fluorouridine 5′-diphosphate galactose (**2**) has been detected in several lines of tumor cells treated with 5-fluorouridine or 5-fluorouracil [1], though the physiological meaning of accumulated **2** has not been assessed so far. The intracellular pool of fluorinated sugar nucleotides may account for delayed drug effects by gradually liberating 5-fluorouridine monophosphate (**1**) or diphosphate, which is subsequently converted into 5-fluoro-2′-deoxyuridine monophosphate, an inhibitor of thymidylate synthase, or perhaps incorporated into RNA. The chemotherapeutic effect of **2** is presumably exerted via enzymatic degradation to 5-fluorouridine monophosphate by nucleotide pyrophosphatase (phosphodiesterase) [2].



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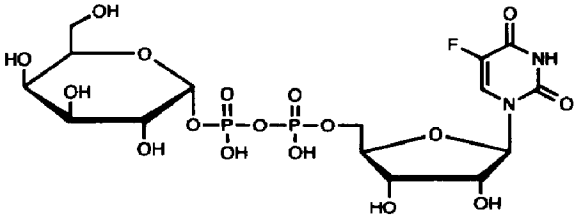

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Compound **2** has previously been prepared by chemical coupling of the imidazolidate of **1** with the tributylammonium salt of D-galactose 1-phosphate in hexamethylphosphoric triamide [3]. On the other hand, the preparation of uridine 5′-diphosphate galactose has been achieved by enzymatic transfer of a uridylyl group to D-galactose 1-phosphate [4], or directly from D-galactose and uridine 5′-monophosphate by microbial fermentation [5]. We have applied the method for the microbial production of uridine 5′-diphosphate galactose to the synthesis of **2**, and describe the results here. Our procedure involves the fermentative formation of **2** from **1** and added D-galactose, in the presence of inorganic phosphate and magnesium ions, by dried cells of *Candida saitoana* IFO 0768 (formerly *Torulopsis candida* IFO 0768) grown on a lactose medium. The necessary starting material **1** was obtained by the reaction of 5-fluorouridine with phosphoryl chloride in anhydrous trimethyl phosphate at 0°C [6].

1. Experimental

General methods.—HPLC analyses were performed on an AM-312 ODS reversed phase column (6×150 mm, YMC, Japan). The conditions were as follows: isocratic elution at room temp with 50 mM $NH_4H_2PO_4$; flow rate, 1 mL min^{-1}; detector sensitivity at 260 nm, 0.02 AUFS. The ^1H and ^{13}C NMR spectra were recorded in D_2O at 40°C with H_2O (δ 4.70) and pyridine-d_5 (δ 149.5), respectively, as internal references, on a Jeol JNM-400GX spectrometer operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR.

5-Fluorouridine 5′-monophosphate (**1**), *barium salt.*—Phosphoryl chloride (400 μmol, 330 μL) was mixed with 5 mL of anhyd trimethyl phosphate in an ice bath, and 1.9 mmol of 5-fluorouridine (Sigma Chemical Co., St. Louis, MO, USA) was added with stirring to the above solution. After standing at 0°C for 16 h the mixture was poured into 100 mL of cold water. Compound **1**, obtained in 73% yield based on HPLC analysis, was then purified by ion-exchange chromatography on Dowex 1-X8 (Cl^-) resin. The mixture was applied to the column (3×15 cm), and the resin was washed with 2 L of aq 50% MeOH. The product was eluted with 4 L of 0.01 N HCl containing a linear gradient of 0–0.2 M LiCl. The fractions containing **1** were combined, adjusted to pH 7.5 with 1 N LiOH, and concentrated to 50 mL at 45°C in vacuo. Active carbon (4 g) was added to the solution which had been adjusted to pH 3.8 with 1 N HCl, and then filtered off and washed thoroughly with distilled water. Compound **1** was eluted with 200 mL of 45:50:5 EtOH–H_2O–NH_4OH, and the eluate was concentrated to 10 mL by evaporation at 45°C. Barium acetate (400 μmol) was added to the concentrated solution to form a precipitate of the barium salt of **1**, and the excess was removed by two extractions of the precipitate with aq 70% EtOH and one with abs EtOH. The purified **1** (506 mg, 53%) was a white powder; NMR data are given in Table 1. Anal. Calcd for $C_9H_{10}FN_2O_9P \cdot Ba \cdot 3H_2O$: C, 20.34; H, 3.03; N, 5.27; P, 5.83. Found: C, 19.93; H, 2.92; N, 4.97; P, 5.75.

5-Fluorouridine 5′-(α-D-galactopyranosyl diphosphate) (**2**, *5-fluorouridine 5′-diphosphate galactose*), *barium salt.—Candida saitoana* IFO 0678 was grown in 5% lactose, 0.5% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$, and 0.1% $MgSO_4 \cdot 7H_2O$ (pH 6.2) at 30°C for 24 h with vigorous shaking. The cells were harvested by centrifugation and washed 3 times with cold distilled water. The washed cells were then

Table 1
NMR spectra of the fluorinated derivatives

Position	5-Fluorouridine [a]		5-Fluorouridine 5′-monophosphate		5-Fluorouridine 5′-diphosphate galactose	
	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift
6	7.83 (d, 6.4)		8.11 (d, 6.4)		7.98 (d, 6.4)	
1′	5.87 (dd, 4.3, 1.5)	91.33	5.90 (dd, 4.9, 1.5)	90.79	6.03 (dd, 4.9, 1.83)	90.13
2′	4.19 (t, 5.5)	75.48	4.29 (t, 5.2)	75.86		74.99
3′	4.26 (t, 5.5)	71.14	4.31 (t, 5.2)	71.98		70.53 [b]
4′	4.10 (m)	85.67	4.20 (m)	85.91 (d, 8.8)		84.20 (d, 10)
5′	3.89 (dd, 12.8, 3.0)	62.53	4.02 (ddd, 12.0, 5.5, 3.1)	65.97	3.89 (dt, 10.4, 3.2)	66.35 (d, 6)
	3.80 (dd, 12.8, 4.3)		3.97 (ddd, 12.0, 5.5, 3.4)		3.98 (dd, 10.4, 3.3)	
1″					5.72 (dd, 7.0, 3.4)	97.04 (d, 6)
2″						69.76 (d, 7)
3″						70.96 [b]
4″						70.72 [b]
5″						73.15
6″					3.79 (dd, 11.9, 5.5)	62.35
					3.82 (dd, 11.9, 7.3)	

[a] Values measured on an authentic sample. [b] These assignments may be interchanged.

dried thoroughly under reduced pressure over P_2O_5. The mixture for the production of **2** contained 400 μmol of **1** barium salt, 4 mmol of D-galactose, 240 μmol of $MgSO_4 \cdot 7H_2O$, and 2 g of the dried cells in 20 mL of 200 mM potassium phosphate buffer (pH 7.0). This mixture was incubated at 30°C for 16 h with continuous shaking, the microbial reaction was stopped by heating at 100°C for 10 min, and the mixture was cooled immediately in an ice bath, then centrifuged at 10 000*g* for 10 min. Compound **2** was obtained in 96% yield based on HPLC analysis. The supernatant from the centrifugation was adjusted to pH 3.8 with 1 N HCl, and 4 g of active carbon was added. The active carbon was washed thoroughly with distilled water, and the adsorbed nucleotides were eluted with 200 mL of 45:50:5 EtOH–H_2O–NH_4OH. The eluate was concentrated to 5 mL by evaporation under vacuum at 45°C, and the purification of **2** was accomplished by ion-exchange chromatography on Dowex 1-X8 (Cl^-) resin. The concentrated mixture was applied to the column (3 × 30 cm), and the resin was washed with 2 L of distilled water. Compound **2** was eluted with 4 L of 0.01 N HCl containing a linear gradient of 0–0.2 M LiCl. Fractions from the column were combined, adjusted to pH 7.5 with 1 N LiOH, and concentrated to 50 mL. Active carbon (4 g) was added to the solution which had been adjusted to pH 3.8 with 1 N HCl, then filtered off and washed thoroughly with distilled water, and compound **2** was eluted with of 200 mL 45:50:5 EtOH–H_2O–NH_4OH. The eluate was concentrated to 10 mL by evaporation at 45°C. Barium acetate (600 μmol) was then added to the concentrated solution

to form a precipitate of the barium salt of **2**, and the excess reagent was removed by extraction of the precipitate with aq 70% EtOH (2×), then abs EtOH. Purified **2** (252 mg, 74%) was a slightly yellow powder; NMR data are shown in Table 1. Anal. Calcd for $C_{15}H_{21}FN_2O_{17}P_2 \cdot 2/3Ba \cdot 4H_2O$: C, 20.93; H, 3.40; N, 3.25; P, 7.20. Found: C, 20.54; H, 3.34; N, 3.27; P, 7.10.

Structural elucidation.—The structures of **1** and **2** were elucidated by comparison of their ^1H and ^{13}C NMR data with those of authentic 5-fluorouridine, shown in Table 1. The positions of attachment of the phosphate groups were confirmed by observation of the ^{31}P–^1H and ^{31}P–^{13}C hetero spin–spin couplings. Thus, in the spectra of **1** the signals assigned to C-4′ and C-5′ were observed as doublets due to ^{31}P–^{13}C coupling, and the two H-5′ signals at δ 4.02 and 3.97 appeared as ddd due to ^{31}P–^1H coupling. Also, in the spectra of **2** couplings similar to those shown by **1** were observed in the signals assigned to C-4′, C-5′, C-1″, C-2″, H-5′, and H-1″. Finally, elemental analyses supported the formulas assigned to **1** and **2** by their good agreement with the calculated values.

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