Preparation of 5-Fluoro- and 5-Alkyl-2′-deoxyuridine 5′-Phosphates free of 3′-Phosphates via Phosphorodiamidates

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5-Fluoro- and 5-alkyl-2′-deoxyuridine 5′-phosphates have acquired widespread application for *in vitro* enzyme specificity studies[1,2]. A simple and frequently used method for the preparation of these compounds is the Yoshikawa-procedure: the phosphorylation of unblocked 2′-deoxyribonucleosides with phosphoryl chloride in trialkyl phosphates[3]. This procedure, when combined with hydrolysis, gives predominantly the 5′-

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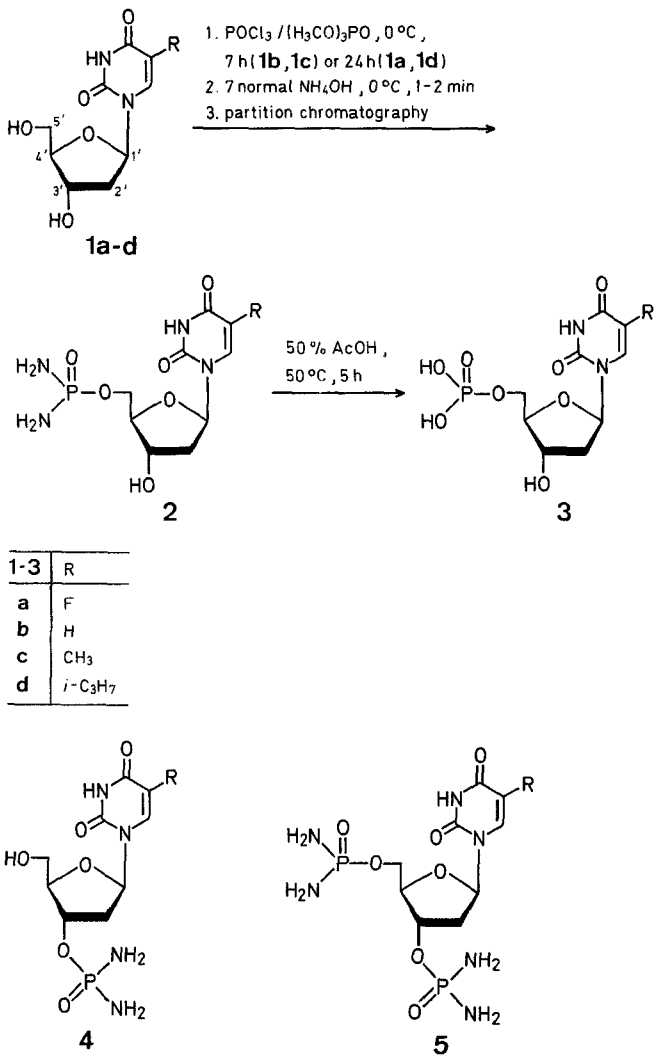
phosphates in high yields with the concomitant formation of the 3′-phosphates and 3′,5′-diphosphates to a much smaller but not negligible extent[4]. The main drawback of this procedure is the problematic separation of the isomeric 5′- and 3′-phosphates.

In general, preparative paper chromatography[5,6,7] or ion-exchange column chromatography[4] are used despite the fact that no clear-cut separation can be attained by either of these two methods. By preparative paper chromatography, only the separation of the mixtures of 3′- and 5′-phosphates from the 3′,5′-diphosphates can be achieved[4]. Therefore, it seems very likely that in earlier works all the 5′-phosphates isolated by paper chromatography and used for enzymic studies had been contaminated with 3′-phosphates. Ion-exchange column chromatography allows a partial resolution of 5′- and 3′-phosphates, the 3′-phosphates appearing as a tail of the peak of 5′-phosphates[4,8]. Sági et al. isolated 5′-phosphates free of 3′-phosphates by repeated fractional precipitation by ethanol of the barium salts from aqueous solution[9].

The present paper offers an alternative method for obtaining pure 5-fluoro- and 5-alkyl-2′-deoxyuridine 5′-phosphates **3** free of 3′-phosphates via phosphorodiamidates, according to the following Scheme.



Phosphorylation of 2′-deoxyribonucleosides **1** according to the Yoshikawa-procedure followed by *in situ* ammonolysis yields mixtures of 5′-phosphorodiamidates **2**, 3′-phosphorodiamidates **4**, and 3′,5′-bis[phosphorodiamidates] **5** as well as hydrolysis products, mainly phosphoramidates (Table).

The phosphorodiamidates can be clearly separated by partition chromatography on a cellulose column with a mixture of *n*-butanol/ethanol/0.1 molar triethylammonium hydrogen carbonate, pH 7.5, in the order of **4** > **2** > **5** (see R_f values in Table). A column of 2.2 × 40.0 cm gives a clear-cut resolution of reaction mixtures produced from 1.0 mmol of **1a** or **1c**, 0.5 mmol of **1b**, and 0.1 mmol of **1d**. Unreacted starting materials **1** are eluted ahead of the phosphorodiamidates[10]. The structure of the phosphorodiamidates was confirmed by hydrolytical degradations and ^{31}P-N.M.R. spectrometry.

The desired 5′-phosphates **3** were obtained by hydrolyzing the 5′-phosphorodiamidates **2** in 50% aqueous acetic acid at 50 °C for 5 h. The absence of 3′-phosphates in the 5′-phosphates was checked by hydrolysis with a 5′-nucleotidase specific for P—O—C$^{5'}$ phosphomonoester bonds.

^{31}P-N.M.R. spectra were recorded under proton decoupling on a JEOL FX60 spectrometer in FT mode at 24.2 MHz.

5-Fluoro-2′-deoxyuridine 5′-Phosphorodiamidate (2a):
Freshly distilled phosphoryl chloride (0.2 ml, 2.2 mmol) is added to a well-stirred solution of compound **1a** (246 mg, 1.0 mmol) in trimethyl phosphate (2.5 ml), at 0 °C. Stirring is continued at 0 °C for 24 h, 7.0 normal aqueous ammonium hydroxide (10.0 ml) is then added under vigorous stirring at 0 °C, and the solution is evaporated to dryness under reduced pressure. The residue is dissolved in deionized water (50.0 ml). The solution is percholated through a DEAE-cellulose [$HCO_3^{\ominus}$] column (1.6 × 53.0 cm), and the column is washed with water (elution rate: 20.0 ml/20 min/fraction). The first nine fractions containing ammonium hydrogen carbonate, phosphoric triamide and practically no U.V.-absorbing material are discarded. The mixture of compounds **2a**, **4a**, **5a**, and **1a** (about 88% of the total absorbancy applied) emerge in fractions 10-30. These are pooled and evaporated to dryness under reduced pressure. The residue is applied onto a cellulose (Whatman CC 31) column (2.2 × 40.0 cm) equilibrated with *n*-butanol/ethanol/0.1 molar triethylammonium hydrogen carbonate, pH 7.5, 16:2:5, v/v, and the column is developed with the same solvent mixture (elution rate: 4.5 ml/20 min/fraction). Four large peaks appear between fractions 12-31 (**1a**), 42-50 (**4a**), 52-80 (**2a**), and 124-160 (**5a**). T.L.C. pure, solid phosphorodiamidates can be obtained by evaporating the appropriate fractions under reduced pressure; yield of **2a**: 0.155 g (45%).

$C_9H_{14}FN_4O_6P \cdot H_2O$	calc.	C 31.55	H 4.67	N 16.36
(342.2)	found	31.95	4.82	15.90

U.V. (pH = 2.0): λ_{max} = 268 nm, λ_{min} = 233.5 nm; (pH = 10.0): λ_{max} = 268 nm, λ_{min} = 247 nm.

^{31}P-N.M.R. (D_2O, pD = 7.0/H_3PO_4): δ = 20.61 (**2a**), 19.79 (**4a**), 19.79 and 20.24 ppm (two signals of equal intensity, **5a**).

After standing 12 h, at pD = 1.0 and 25 °C[11]:
^{31}P-N.M.R. (D_2O, pD = 1.0/H_3PO_4): δ = −0.16 (**2a**), −0.89 (**4a**), −0.26 and −0.93 ppm (two signals of equal intensity, **5a**)[13].

Compounds **2a**, **4a**, and **5a** are quantitatively converted to **1a** upon standing in 0.1 normal aqueous sodium hydroxide at 25 °C for 5 min[12]. The alkaline hydrolysis of **5a** proceeds via **2a** and **5a** (as detected by T.L.C.).

2′-Deoxynucleoside 5′-Phosphorodiamidates 2b, 2c, and 2d:
The syntheses are performed with 1.0 mmol of **1c**, 0.5 mmol of **1b**, and 0.1 mmol of **1d** as described for **2a** except that the reaction time is only 7 h for **1b** and **1c**. Except for the partition column, everything is proportionally reduced.

Yield of **2b**: 0.091 g (56%).

$C_9H_{15}N_4O_6P \cdot H_2O$	calc.	C 33.31	H 5.24	N 17.27
(324.2)	found	33.52	5.38	16.99

Yield of **2c**: 0.177 g (52%).

Yield of **2d**: 0.016 g (43.1%).

$C_{12}H_{21}N_4O_6P \cdot H_2O$	calc.	C 39.34	H 6.27	N 15.28
(366.3)	found	39.50	6.32	15.04

Table. Distribution [%][a] of Intermediary Phosphorodiamidates (cf. Scheme) and their R_f values[b]

R=	F (a)		H (b)		CH_3 (c)		i-C_3H_7 (d)	
Compound	[%]	R_f	[%]	R_f	[%]	R_f	[%]	R_f
1	18.6	0.63	6.7	0.50	19.1	0.66	26.6	0.67
2	63.4	0.26	81.3	0.20	69.5	0.28	51.1	0.38
4	6.5	0.31	2.4	0.21	4.4	0.35	8.6	0.45
5	7.6	0.13	6.3	0.09	5.2	0.12	11.4	0.18

[a] Determined by U.V. spectrophotometry at λ_{max}^{pH7} assuming the absorptivities to be identical to that of the respective nucleoside. Hydrolysis products (5–20%) were neglected for calculation, i.e. 100% is equal to the sum of the absorptivities of the compounds **1**, **2**, **4**, and **5**. Differences from 100% were caused by slight degradation during the separation of reaction mixtures.

[b] On cellulose (MN-300)/silica gel (HF_{254}), 8:2, w/w, home-made thin-layer plates in *n*-butanol/ethanol/0.1 molar triethylammonium hydrogen carbonate, pH 7.5, 16:2:5, v/v.

5-Fluoro-2′-deoxyuridine 5′-phosphate (3a):
Compound **2a** produced (~0.45 mmol) is dissolved in 50% aqueous acetic acid (45 ml), the solution is left at 50 °C for 5 h, and then evaporated to dryness under reduced pressure. Last traces of acetic acid are removed by repeated evaporation with water. The residue is dissolved in deionized water (10.0 ml). The solution is applied onto a DEAE-cellulose [HCO_3^-] column (1.6 × 53.0 cm). Elution is carried out with a linear gradient of deionized water/0.2 molar aqueous triethylammonium hydrogen carbonate, pH 7.5 (2000 ml, elution rate: 20 ml/20 min/fraction). The main peak is pooled and evaporated to dryness under reduced pressure. Triethylammonium hydrogen carbonate is removed by repeated evaporation with methanol. Finally the residue is dissolved in a small amount of water. The solution is freeze-dried to give the bis-triethylammonium salt of **3a**; yield: 0.231 g (97%); homogeneous by T.L.C. on silica gel (Kieselgel 60 F_{254}) in *n*-propanol/conc. aqueous ammonium hydroxide/water, 11:7:2, v/v.

^{31}P-N.M.R. (D_2O, pD = 1.0/H_3PO_4): $\delta = -0.26$ ppm[13].

On treatment with 5′-nucleotidase from *Crotalus atrox* venom (Sigma), **3a** is quantitatively hydrolysed to **1a** (1.0 A_{max} unit of **3a**, 1.0 unit of the enzyme in 10 µl of 0.1 molar aqueous glycine/sodium hydroxide buffer, pH 9.0, 37 °C, 6 h, detected by T.L.C.).

2′-Deoxynucleoside 5′-Phosphates 3b, 3c, and 3d:
These compounds are prepared from the respective phosphorodiamidates **2b**, **2c**, and **2d** in exactly the same manner as described for **3a**; yield of **3b**: 0.133 g (93%); of **3c**: 0.260 g (95%); of **3d**: 0.023 g (95%).

^{31}P-N.M.R. (D_2O, pD = 1.0/H_3PO_4): $\delta = -0.22$ **(3b)**, -0.28 **(3c)**, -0.3 ppm **(3d)**.

Enzymic hydrolysis with 5′-nucleotidase yielded the respective nucleosides **1b**, **1c**, and **1d**.

^{31}P-N.M.R. measurements performed by Dr. W. S. Zieliński (Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Łódź, Poland) are gratefully acknowledged.

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Phelps et al. have recently reported on the synthesis of **2a** from 5-fluoro-2′-deoxy-3′-*O*-acetyluridine and found the compound to be stable in normal sodium hydroxide solution at 65 °C for 2 h: M. E. Phelps, P. W. Woodman, P. V. Danenberg, *J. Med. Chem.* **23**, 1229 (1980).
On the basis of our present and previous results we think, that the compound in the hands of Phelps et al. was not **2a**, but the alkali stable 5′-phosphoramidate, which might be formed e.g. during deacetylation, by the participation of the 5′-diamidophosphoryl group in the hydrolysis of the 3′-*O*-acetyl group.
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