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Nucleotides. VII.[1] A Procedure for the Preparation of Diribonucleoside Monophosphates having Natural Linkage

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A procedure has been devised whereby out of a pair of 2′-5′- and 3′-5′-positional isomers of 2-thiouridylyl- and uridylyluridine derivatives, the former could be selectively cleaved into halves through the 2,2′-anhydronucleoside formation so that the isolation of the dinucleoside monophosphate having natural linkage may become easier. In this connection, conditions for selective 2,2′ anhydro-bond formation have been examined and 2-(*t*-butoxycarbonyl)phenyl group has been introduced as a protecting group for the phosphate of dinucleoside monophosphates (*e.g.*, **8c** and **9c**).

A simple procedure for the synthesis of 2,2′-(*S*)-anhydro-2-thiouridylyl-(3′-5′)-uridine was also described.

The objective of oligoribonucleotide synthesis is the formation of the internucleotidic phosphodiester bond specifically between the C_3' and C_5' positions of two adjacent nucleosides. A number of procedures for the oligoribonucleoside synthesis have been developed.[3] Among them, there is a procedure, for example "Michelson approach"[4] which may afford a mixture of oligonucleotides of short chain-length having 2′-5′- and 3′-5′-internucleotidic linkage with respect to the joining bond. In this case, the oligonucleotides having natural linkage may be obtained after separation and purification. One of the advantages of this approach is that a mixture of nucleoside 2′- and 3′-phosphate as such can be used as the starting material.

In our laboratory, some attempts have been done to develop another synthetic approach which starts with nucleoside 2′(3′)-phosphates to prepare uridylyl-(3′-5′)-uridine (**9a**) and 2-thiouridylyl-(3′-5′)-uridine (**4a**) by initial preparation of the positional isomers and subsequent cleavage of unnatural isomers by (*O*) or (*S*)-2,2′-anhydro-bond formation (Chart 1).

The present paper reports the preparation of dinucleoside monophosphates (**4a** and **9a**) having the natural linkage by the above approach, along with some results obtained on preliminary experiments done to learn about requirements met by the substrate so as to effect selective anhydronucleoside formation.

We first applied this principle for the preparation of (**4a**) because of the higher nucleophilicity of the 2-thiocarbonyl of the 2-thiouridine moiety of the substrate.

1) Part VI: Y. Mizuno, S. Kitano, and A. Nomura, *Nucleic Acids Research*, **2**, 2193 (1975).
2) Location: *Kita-12 Nishi-6 Kita-ku, Sapporo, 060 Japan.*
3) S.A. Narang and R. H. Wightman, in "The Total Synthesis of Natural Products" vol. 1, ed., by J. ApSimmon, Wiley Intersciences, New York, 1973, pp. 9—12.
4) A.M. Michelson, *J. Chem. Soc.*, **1959**, 1371, 3665.

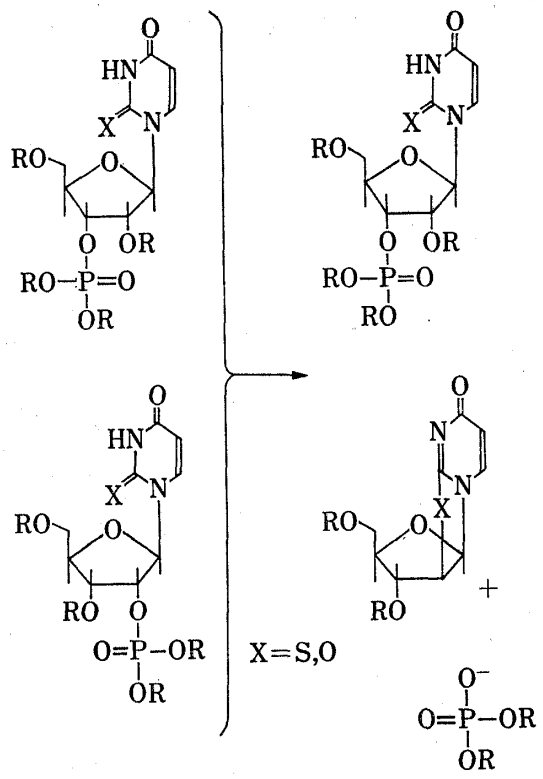

Chart 1

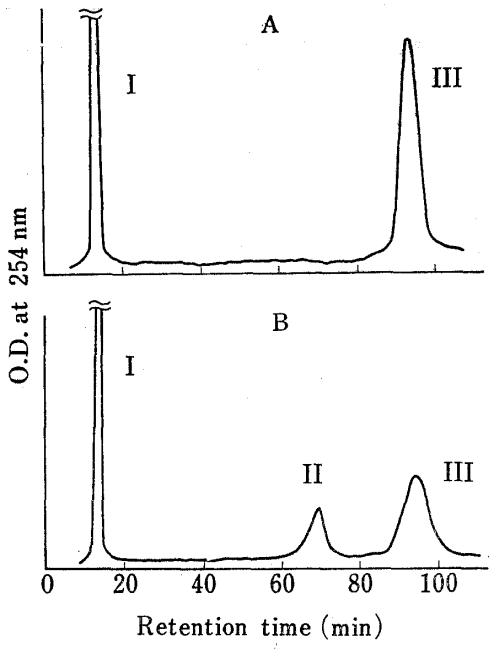

Fig. 1. Chromatographic profiles on a Varian Aerograph LCS 1000 Chromatograph

(A): hydrolysate of 2-thiouridylyl-(3′-5′)-uridine (**4a**) with RNase M; (B): 2-thiouridylyl-(2′-5′)-uridine (**3a**) along with hydrolysate of (**4a**) with the enzyme. (I), uridine; (II), (**3a**); (III), 2-thiouridine 3′-phosphate.

Prerequisite starting materials were an isomeric mixture of 2-thiouridylyl-(2′-5′-, 3′-5′)-uridines (**3a** and **4a**). Initial scheml to prepare this mixture by condensation of 5′-O-benzoyl-2-thiouridine (**1a**) with 2′,3′-di-O-acetyluridine 5′-phosphate[5] (**2a**) was achieved with limited success. Thus, this condensation at ambient temperature followed by deblocking afforded 2,2′-(*S*)-anhydro-2-thiouridylyl-(3′-5′)-uridine (**5**) as a major product, the desired products (**3a** and **4a**) being obtained only in poor yield. It eventually turned out that the relative ratio of (**3a** and **4a**) to (**5**) was temperature-dependent; at lower temperature the yield of (**5**) significantly decreased, with concomitant increase in the desired products (**3a** and **4a**). Howver, even at 5°, the yield of the latter (**3a** and **4a**) amounted to at most 28%. The structures of (**3a** and **4a**) were confirmed by a conventional way (see experimental section) and the structure of (**5**) was determined on the fact that digestion with snake venom phosphodiesterase afforded 2,2′-(*S*)-anhydro-2-thiouridine (**6a**) and uridine 5′-phosphate in a molar ratio of 0.98: 1. Compound (**5**) was formed presumably *via* 5′-O-benzoyl-2-thiouridylyl-(2′,3′-cyclic-5′)-2′,3′-di-O-acetyluridine (**7**). A number of precedents to the formation of the dinucleoside monophosphate *via* the corresponding intermediate of type (**7**), particularly in Michelson synthesis have been noted.

Since the above approach failed to give the required mixture in satisfactory yield, another, but similar approach starting from 2′(3′),5′-di-O-acetyl-2-thiouridines (**1b** and **1c**) was adopted in the hope that the formation of the cyclic triester intermediate could be avoided. Treatment of (**1b** and **1c**), the isomeric ratio being nearly 1: 1, with (**2a**) in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS), followed by deblocking (with methanolic ammonia) and chromatographic purification afforded (**3a** and **4a**) in combined yields of 86%.

Treatment of 2′(3′),5′-di-O-acetyl-2-thiouridylyl-(2′-5′, 3′-5′)-2′,3′-di-O-acetyluridine (**3b** and **4b**) with N,N′-dicyclohexylcarbodiimide (DCC, 5 equivalents) in pyridine at 70° for

5) B.E. Griffin and C.B. Reese, *Tetrahdron Letters*, **1964**, 2925.

4 days afforded, after deblocking and subsequent isolation, 2-thiouridylyl-(3′-5′)-uridine (**4a**) in 89% yield.[6] Although 2,2′-(*S*)-anhydro-2-thiouridine (**6a**) was obtained, no formation of 2,3′-(*S*)-anhydro-2-thiouridine could be observed. The purity as well as the structure of (**4a**) were checked by enzymatic digestion and with a Varian LCS-1000 Chromatograph. Elution profiles of RNase M[7] digest of (**4a**) along with an artificial mixture of uridine, 2-thiouridine 3′-phosphate, and (**3a**) are given in Fig. 1A and 1B. As shown in Fig. 1A, no peak is observed at the retention time (68 min) where the peak due to (**3a**) appears in Fig. 1B.

It is worthy of note that both (**3b**) and (**4b**) were stable in pyridine and that (*S*)-anhydronucleoside formation could be observed on heating at 60° in the presence of TPS or DCC. This means that 2,2′-anhydronucleoside was formed presumably by 2,2′-cyclization of either a pyrophosphate arising from (**3b**) under the action of DCC or TPS or a phosphodiester-TPS mixed anhydride.

In view of comparatively low nucleophilicity of 2-carbonyl group of uridine and high temperature (60°) required to effect the 2,2′-(*S*)-anhydronucleoside formation, we felt it appropriate to find out the requirements met by the substrate to effect the 2,2′-cyclization under reasonable conditions before we moved on to the synthesis of uridylyl-(3′-5′)-uridine [UpU (**9a**)].

To this end, we prepared 3′,5′-di-O-acetyluridine 2′-diphenylphosphate (**2c**), 3′,5′-di-O-acetyluridine 2′-diethylphosphate (**2d**), and 3′,5′-di-O-acetyluridylyl-(2′,5′)-2′,3′-di-O-acetyluridine *p*-phenylester (**8b**) from 3′,5′-di-O-acetyluridine[8] (**2b**) in 54%, 46%, and 71%, respectively.[9] The spectral (UV and NMR) data along with combustion values readily confirmed the assigned structures of (**2c**) and (**2d**). The structure of (**8b**) was confirmed by spectral (UV and NMR) data. The former two (**2c**) and (**2d**) were stable at 70° for *ca.* 20 hr in DMF or in a solution of tri-*n*-butylamine in DMF and no 2,2′-cyclization was observed. However, treatment of (**2c**) with 1,5-diazabicyclo[5,4,0]undec-7-ene (DBU),[10] in DMF at 25° for 40 hr afforded after hydrolysis of 1-β-D-arabinofuranosyluracil (**10**) in 80% isolated yields. In the case of (**2d**), however, rather streneous conditions of 60° and 72 hr were required, resulting in reduced yield of the cyclized product. The yield of (**10**) was only 59%. Such a remarkable difference in ease with which the cyclization occurs was not unexpected because a leaving group that corresponds to the stronger acid may be usually better leaving group. Since with (**8b**) the 2,2′-cyclization proceeded sluggishly at 25° even in the presence of DBU, a solution of (**8b**) in DMF (containing three equivalents of DBU) was heated at 40° for 25 hr. After work-up and subsequent hydrolysis the product (**10**) and uridine 5′-phenylphosphate were isolated by column chromatography in 69% and 70%, respectively.

Thus it is clearly shown that in order for the 2,2′-cyclization to be effected under mild conditions, the phenyl group is suitable as a protecting group for the phosphodiester backbone.

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6) The yield was based on (**4b**) which had been contained in the starting isomeric mixture (**3b** and **4b**).

7) Ribonuclease M has a base specificity similar to that RNase T_2[M. Irie, *J. Biochem.* (Tokyo) **62**, 509 (1967)].

8) H.P.M. Fromageot, B.E. Griffin, C.B. Reese, and J.E. Sulston, *Tetrahedron*, **23**, 2315 (1967).

9) Since 2′-O- or 3′-O-acetyl group of the ribonucleoside tends to migrate in pyridine, [C.B. Reese and D.R. Trentham, *Tetrahedron Letters*, **1965**, 2465] care has been taken to avoid the migration of the acetyl group, by adding the phosphorylating agent as rapidly as possible immediately after the dissolution of the substrate in pyridine is completed.

10) DBU has been shown to be quite effective for the proton abstraction [H. Oedinger and G. Moller, *Angew. Chem. Intern. Ed. Engl.*, **6**, 76 (1967)]. It was conceivable that in the system (**2c**) this reagent may abstract the proton on nitrogen-3 and promote the O^2-2′ cyclization.

11) Some attempts to try popular phosphate protecting groups such as benzyl and 2,2,2-trichloroethyl for the present purpose were found unrewarding. Diethyl 2-carboxyphenyl phosphate[12] which had been prepared as a model compound to find out the deblocking conditions was converted smoothly into salicylic acid and diethyl phosphate on treatment with aqueous 50% pyridine at 37° for 2 days or 60° for 5 hr.

12) C.M. Blackburn and M.J. Brown, *J. Am. Chem. Soc.*, **91**, 523 (1969), where this compound has been reported without detailed preparative procedures.

Unfortunately, however, deblocking of protected oligo-ribonucleotides such as (**8b**) and its positional isomer (**9b**) bearing both O-acyl and phenyl group on the phosphate may result in hydrolytic cleavage of the phosphodiester linkage owing to prior deblocking of the O-acyl group. In order to avert this serious shortcoming with phenyl group we adopted salicyl

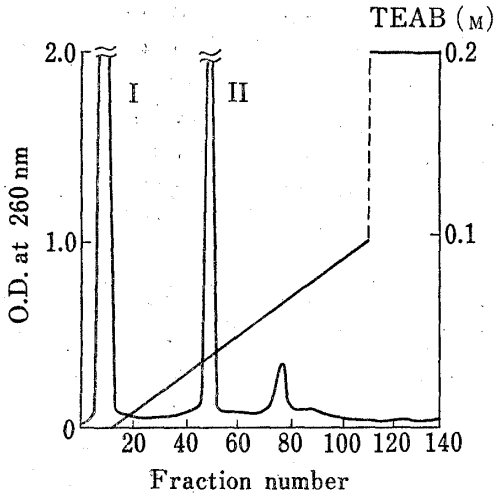
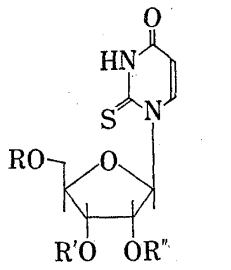
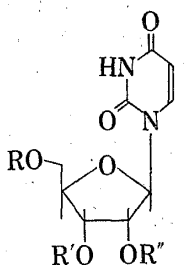


Fig. 2. Reaction products of 3′,5′-Di-O-acetyluridine 2′-Diphenylphosphate (**2c**) with DBU in DMF at 25° for 40 hr and Subsequent Treatment with 0.1 N NaOH

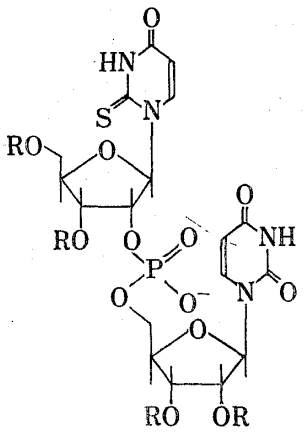
Seperation of the products on a DEAE-cellulose column (2.0×46 cm). Elution was carried out with a linear gradient of H_2O (1 l iter) and 0.1M TEAB solution (1 l iter), and fractions of 20 ml were collected. (I), 1-β-D-arabinofuranosyluracil; (II), diphenyl phosphate



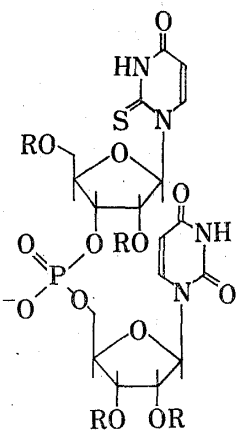
1a : R=Bz, R′=R″=H
1b : R=R″=Ac, R′=H
1c : R=R′=Ac, R″=H



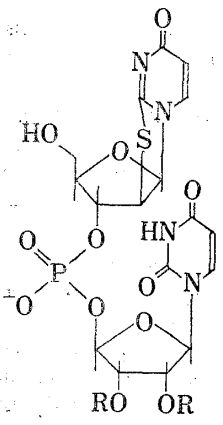
2a : R=PO_3H_2, R′=R″=Ac
2b : R=R′=Ac, R″=H
2c : R=R′=Ac, R″=$(PhO)_2P=O$
2d : R=R′=Ac, R″=$(EtO)_2P=O$
2e : R=R′=Ac, R″=PO_3H_2
2f : R=R″=Ac, R′=PO_3H_2
2g : R=H, R′=R″=Ac



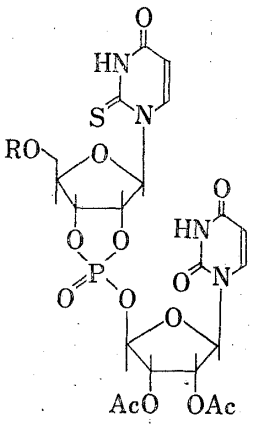
3a : R=H
3b : R=Ac



4a : R=H
4b : R=Ac



5 : R=H



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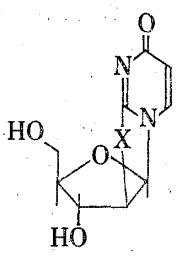
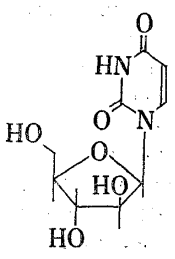


6a : X=S
6b : X=O



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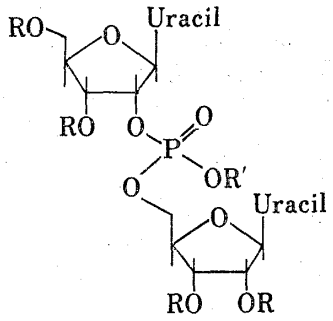
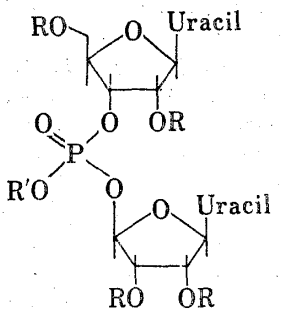


8a : R=R′=H
8b : R=Ac, R′=Ph
8c : R=Ac,
R′=*o*-(*t*-butoxycarbonyl)phenyl



9a : R=R′=H
9b : R=Ac, R′=Ph
9c : R=Ac,
R′=*o*-(*t*-butoxycarbonyl)phenyl

protecting group[11] whose carboxyl group has been shown by Kirby and his coworkers to assist the hydrolysis of phosphotriester to the enormous extent.[13]

Thus we prepared a nearly 1: 1 mixture of 3′,5′-di-O-acetyluridylyl-(2′-5′)-2′,3′-di-O-acetyluridine *p*-(2-*t*-butoxycarbonylphenyl)ester (**8c**) and 2′,5′-O-acetyl-(3′-5′)-2′,3′-di-O-acetyluridine *p*-(2-*t*-butoxycarbonylphenyl)ester (**9c**) in the following way. 3′(2′),5′-Di-O-acetyluridine 2′(3′)-phosphates (**2e** and **2f**) were treated with *t*-butyl salicylate[14] in the presence of TPS in pyridine at 25° for 24 hr. After this period, the resulting mixture was treated with 2′,3′-di-O-acetyluridine (**2g**)[15] and TPS to afford the required products which were isolated as an isomeric mixture by preparative (TLC) in 18% yield. The structural confirmation of (**8c** and **9c**) rests upon spectral and analytical data. NMR spectra of this mixture were also compatible with the assigned structure. Treatment of this isomeric mixture in DMF in the presence of DBU at 37° for 40 hr afforded a mixture of products which still contained intact blocked dinucleoside monophosphate (**9c**) which was isolated by TLC in 75% yield.[16] For the deblocking of (**9c**), this compound was initially treated with methylene chloride-5% trifluoroacetic acid. The resulting de-butylated product was isolated by paper electrophoresis and then heated at 60° for 16 hr in 50% aqueous pyridine (removal of salicylate). Deacetylation was finally carried out by treatment of O-acetylated nucleotide with 7M ammonium hydroxide. The product was purified by a preparative paper chromatography. On paper electrophoresis, this sample had the same mobility as an authentic specimen of UpU (**9a**) and was completely hydrolyzable with pancreatic RNase A. UV spectra were also compatible with the structure assigned. Yield of UpU (**9a**) was 64%, based on (**9c**).

Experimental

General. Melting points were determined by a capillary method and uncorrected. The UV spectra were determined with a Hitachi recording spectrophotometer Model 3T. The NMR spectra were determined with a Hitachi high resolution NMR spectrometer R24. The chemical shifts were reported in parts per million downfield from tetramethylsilane as internal standard. Shake venom phosphodiesterase (*Clotalus adamanteus*) and pancreatic RNase A were obtained from Worthington Biochemical Co. RNase M was prepared according to a reported procedure[17] and a gift from Dr. Masachika Irie of Hoshi College of Pharmacy. Digestion with these enzymes was carried out as reported.[18] Paper electrophoresis (PEP) was performed on Toyo-Roshi paper No 51A at *ca.* 20 V/cm. The buffers used were as follows: (A) 0.05 M triethylammonium bicarbonate (TEAB,

13) H. Bramilow, S.A. Khan, and A.J. Kirby, *J. Chem. Soc.*, (*B*) **1971**, 1091.
14) G.E. Cwalina and A. Gringanz, *J. Org. Chem.*, **26**, 3344 (1961).
15) G.W. Kenner, A.R. Todd, R.F. Webb, and F.J. Weymouth, *J. Chem. Soc.*, **1954**, 2288.
16) The yield was based on the amount of the nucleotide (**9c**) which had been contained in the mixture prior to the DBU treatment.
17) M. Irie, *J. Biochem.* (Tokyo), **62**, 509 (1967).
18) Y. Kawamura and Y. Mizuno, *Biochim. Biophys. Acta*, **277**, 323 (1972).

pH 8.0); (B) 0.02 M sodium borate (pH 9.2); (C) 0.05 M ammonium formate (pH 3.2). Paper chromatography (PPC) was carried out by the ascending technique on Toyo-Roshi paper No 51A. The solvent systems used were as follows: (A) EtOH–1 M NH_4OAc (5: 2); (B) iso-PrOH–conc. NH_4OH–H_2O (7: 1: 2); (C) *n*-BuOH–AcOH–H_2O (5: 2: 3). DEAE-cellulose refers to the product of Jujo Seishi Co. and a gift therefrom which was used in the bicarbonate form. Silica gel for the column chromatography refers to Kiesel gel 60 (Merck). Unless otherwise stated, solvent system used for TLC (silica gel) refers to the solvent system: $CHCl_3$–10% MeOH. Silica gel for TLC refers to Kiesel gel HF 254 (Merck). Detection of phosphate and *cis*-diol system was performed by acid molybdate method[19] and HIO_4–benzidine method,[20] respectively.

Sugars were detected by charcoaling with hot sulfuric acid. Conditions used for a Varian Aerograph LCS 1000 column chromatography were as follows. A column used was packed with PA 38 pellicular exchange resin (column size, 1 mm × 300 cm); temperature: 70°; flow rate: 10 ml/hr; elution was performed by a linear gradient from 0.02 M KH_2PO_4 (pH 3.25) to 1.0 M KH_2PO_4 (pH 3.85); initial gradient chamber volume: 40 ml; gradient delay: 10 min. The following extinction coefficients (at 260 nm in H_2O) were used: uridine, 9000; 2-thiouridine, 7400; 2,2′-anhydro-2-thiouridine, 7000.

5′-O-Benzoyl-2-thiouridine (1a)——To a solution of 2′,3′-O-isopropylidene-2-thiouridine[14] (1.20 g, 4 mmol) in pyridine (20 ml) was added benzoyl chloride (1 ml, 8.5 mmol) at room temperature. After 14 hr, the solvent was evaporated off and the residue was dissolved in chloroform (50 ml). The solution was washed successively with water, aqueous 5% sodium hydrogen carbonate, and water. The organic layer was concentrated to dryness and the residue was treated with 70% acetic acid (50 ml) at 95° for 1 hr. The solvent was evaporated off and the residue was applied to a column of silica gel (20 g). Elution with chloroform–3% methanol gave, after evaporation of the solvent a glass, which was triturated with ether to afford 5′-O-benzoyl-2-thiouridine (**1a**) as a pale yellow powder (772 mg, 53%), mp 84—85°. UV $\lambda_{max}^{95\%\ EtOH}$ nm: 224 and 274. *Anal.* Calcd for $C_{16}H_{16}O_6N_2S$: C, 52.74; H, 4.39; N, 7.69; S, 8.78. Found: C, 52.96; H, 4.45; N, 7.59; S, 8.67.

Condensation of 5′-O-Benzoyl-2-thiouridine (1a) with 2′,3′-Di-O-acetyluridine 5′-phosphate (2a)——(a) With TPS: To a solution of (**1a**) (0.3 mmol) and (**2a**) (pyridinium salt, 0.3 mmol) in pyridine (5 ml) was added TPS (0.6 mmol). The solution was allowed to stand at room temperature for 14 hr. Water (2 ml) and tri-*n*-butylamine (1 ml) were added to the ice-cooled solution which was then kept at the same temperature for 30 min. The solvent was evaporated off and the residue was dried by co-distillation with pyridine and then treated with methanolic ammonia (30 ml) at room temperature overnight. After evaporation of the solvent, water (50 ml) was added to the residue and filtered. The filtrate was washed with ether (10 ml). The aqueous layer was concentrated to dryness and the residue was applied to a column (DEAE-cellulose, 2.3 × 50 cm). The column was washed with a linear gradient of water (1 liter) and 0.1 M TEAB solution (1 liter.). Fraction containing 2,2′ (*S*)-anhydro-2-thiouridylyl-(3′-5′)-uridine (**5**), eluted at the ionic strength of 0.04 M, was concentrated to dryness *in vacuo*. Yield, $A_{260\ nm}(H_2O)$ 2120 units (41%), UV $\lambda_{max}^{H_2O}$ nm: 229 and 261 nm. The structural confirmation comes from the fact that digestion with venom phosphodiesterase afforded uridine 5′-phosphate and 2,2′-(*S*)-anhydro-2-thiouridine (**6a**) in a molar ratio of 1: 0.98. Compound (**6a**) was found to be identical with an authentic specimen[21] in the criteria of PPC (system B and C) and PEP (buffer A) behavior. 2-Thiouridylyl-(2′-5′, 3′-5′)-uridines (**3a** and **4a**) were eluted at the ionic strength of 0.07 M. UV $\lambda_{max}^{H_2O}$ nm: 268. Yield, A_{260} (H_2O) 850 units (16%). The structural confirmation comes from the fact that digestion with venom phosphodiesterase afforded uridine 5′-phosphate and 2-thiouridine in a molar ratio 1: 0.96. The latter was found to be identical with an authentic sample[22] on the criteria of PPC (system B and C) and PEP (buffer A) behavior.

(b) With DCC: To a solution of (**1a**) (0.1 mmol) and (**2a**) (pyridinium salt, 0.15 mmol) in pyridine (2 ml) was added DCC (0.75 mmol). The mixture was allowed to stand at 37° for 44 hr. Water (1 ml) was added to the mixture, and kept at room temperature for 2 hr. After evaporation of the solvent, water (20 ml) was then added and filtered. The filtrate was washed with ether (5 ml) and the aqueous layer was concentrated. The residue was then treated with methanolic ammonia (10 ml). The solvent was evaporated off and the residue was dissolved in water and chromatographed as in (a). (**5**) was obtained in 64% yield.

Table I. Temperature Effect on Yields of **3a**, **4a**, and **5**

Temperature °C	Yield (%) (**3a** and **4a**)	(**5**)
5	28	31
12	16	38
37	—	44

19) C.S. Hanes and F.S. Isherwood, *Nature*, **164**, 1107 (1949).
20) M. Viscontini, O. Hoch, and P. Karrer, *Helv. Chim. Acta*, **38**, 642, (1955).
21) T. Ueda and S. Shibuya, *Chem. Pharm. Bull.* (Tokyo), **18**, 1078 (1970).
22) D.M. Brown, D.B. Parihar, A.R. Todd, and S. Varadarajan, *J. Chem. Soc.*, **1958**, 3028.

(c) Temperature Effect: To a solution of (**1a**) (0.1 mmol) (**2a**) (0.1 mmol) in DMF (2 ml) was added TPS (0.2 mmol) at *ca.* 0°. The mixture was allowed to stand at 5°, 12°, or 37° for 34 hr. After work-up as in (a), the products were separated by chromatography on DEAE-cellulose (1.1 × 42 cm) and estimated. Results obtained are summarized in Table I.

2′(3′),5′-Di-O-acetyl-2-thiouridine (1b and 1c)——To a solution of 2′,3′-O-iso-propylidine-2-thiouridine (1.5 g, 5 mmol) in pyridine (15 ml) was added acetic anhydride (5 ml) at *ca.* 5° and the solution was kept at room temperature for 15 hr and excess acetic anhydride was then decomposed with methanol (7 ml). The mixture was concentrated to dryness. The residue was treated with 60% formic acid (30 ml) for 4.5 hr at room temperature to remove isopropylidene group. The mixture was again concentrated to dryness. The residue was codistilled with ethanol and dried (P_4O_{10}) *in vacuo*. The dried residue was dissolved in DMF (3 ml) and there was then added ethyl orthoacetate (10 ml) and toluene *p*-sulfonic acid (monohydrate, 122 mg). The mixture was stirred at room temperature for 7 hr. After evaporation of the solvent, the residue was dissolved in dioxane (10 ml) and 5% acetic acid (40 ml) and the stirring was continued for 30 min. The solvent was evaporated and the residue was chromatographed on silica gel (70 g). Elution was performed with chloroform–2.5% methanol. Fraction containing (**1b** and **1c**) was pooled and concentrated to dryness to give a glass, which was triturated with ether to give the products as a pale yellow powder (1.42 g, 83%), mp *ca.* 60°. UV $\lambda_{max}^{95\%\ EtOH}$ nm: 221 and 277; NMR[$(CD_3)_2SO$] δ: 2.06 (6H, s, CH_3CO), 6.05 (1H, d, J=8 Hz, H_5), 6.66 (d, J=1 Hz, $H_{1'}$), 6.73 (s, $H_{1'}$) and 7.77 (1H, d, J=8 Hz, H_6). Anal. Calcd. for $C_{13}H_{16}O_7N_2S$: C, 45.35; H, 4.65; N, 8.14; S, 9.32. Found: C, 45.49; H, 4.64; N, 7.87; S, 9.10.

Reaction of 2′,(3′),5′-Di-O-acetyl-2-thiouridine (1b and 1c) and 2′,3′-Di-O-acetyluridine 5′-phosphate (2a)——To a solution of (**1b** and **1c**) (0.3 mmol) and (**2a**) (pyridinium salt, 0.45 mmol) in pyridine (5 ml) was added TPS (0.9 mmol). The solution was allowed to stand at room temperature for 15 hr. Water (2 ml) and tri-*n*-butylamine (0.5 ml) were added to the ice-cooled solution and the resulting solution was kept at room temperature for 30 min. The residue obtained after evaporation of the solvent was dried by co-distillation with pyridine and then treated with methanolic ammonia (30 ml) at room temperature overnight. After evaporation of the solvent, water (30 ml) was added and filtered. The filtrate was washed with ether (5 ml). The aqueous layer was concentrated *in vacuo* to a small volume and applied to a column of DEAE-cellulose (2.3 × 50 cm). The column was washed with linear gradient of water (1.5 liter) and 0.1 M TEAB solution (1.5 liter). Fraction containing (**3a** and **4a**), eluted at the ionic concentration of 0.06 M was collected. Yield, A_{260nm} (H_2O) 4480 units (86%). On digestion of the mixture of (**3a**) and (**4a**) with RNase M, (**4a**) alone was hydrolyzed to 2-thiouridine 3′-phosphate and uridine, whose molar ratio was 0.96: 1. On the basis of these data, isomeric ratio of (**3a**) to (**4a**) was estimated to be 1.36: 1.

2′(3′),5′-Di-O-acetyl-2-thiouridylyl-(2′-5′, 3′-5′)-2′,3′-di-O-acetyluridine (3b) and (4b)——2-Thiouridylyl-(2′-5′, 3′-5′)-uridines (**3a** and **4a**) (0.1 mmol) were treated with tetraethylammonium acetate (1 mmol) and acetic anhydride (1 mmol) essentially according to Rammler, *et al.*[23] to afford (**3b** and **4b**). They were both resistant to digestion with RNase M. Treatment of (**3b**) and (**4b**) with methanolic ammonia gave (**3a**) and (**4a**).

Treatment of (3b and 4b) with DCC——A solution of (**3b** and **4b**) (0.1 mmol, isomeric ratio: 1.4: 1) and DCC (0.5 mmol) in pyridine (2 ml) was heated at 70° for 4 days. There was then added water (2 ml) to the cooled mixture. The solution was allowed to stand at room temperature for 2 hr and then concentrated to dryness. The residue was dissolved in water (20 ml) and filtered. The filtrate was washed with ether (5 ml). The aqueous layer was evaporated off and the residue was dissolved in methanolic ammonia (10 ml) and the solution was kept at room temperature overnight. The solvent was evaporated off and the residue was chromatographed on DEAE-cellulose (1.1 × 40 cm). Elution was performed with a linear gradient of water (500 ml) and 0.1 M TEAB solution (500 ml). Compound (**4a**) was eluted at the ionic concentration of 0.06 M, yield, A_{260nm} (H_2O) 640 units (89%), based on (**4b**) which had been contained in the starting material. This sample was completely hydrolyzed with RNase M to give 2-thiouridine 3′-phosphate and uridine in a molar ratio 0.97: 1. In addition, 2,2′-(*S*)-anhydrouridine[21] was isolated. UV $\lambda_{max}^{H_2O}$ nm: 229. 2,3′-(*S*)-Anhydrouridine[21] was not detected by PPC (system B and C).

3′,5′-Di-O-acetyluridine 2′-diphenylphosphate (2c)——The title compound was prepared essentially according to a procedure which had been used by Gulland and Smith[24] for the preparation of 2′,3′-O-benzylidene 5′-diphenylphosphate. To a cooled (to −15——17°), stirred solution of 3′,5′-di-O-acetyluridine[8] (**2b**) (1.64 g, 5 mmol) in dry pyridine (10 ml) was added a solution of diphenyl phosphorochloridate[25] (2.68 g, 10 mmol) in pyridine (5 ml) over a period of 20 min. The solution was kept at −10° for 15 hr and then allowed to return to room temperature. The solution was again cooled to *ca.* −15°. There was then added water (5 ml). The solution was kept at the same temperature for 10 min and allowed to return to room temperature. After 1 hr, the solution was concentrated to dryness at 30°. The residue was dissolved in $CHCl_3$ (100 ml) and the solution was successively washed with water, 5% aqueous $NaHCO_3$ and water. The solution was dried

23) D.H. Rammler, Y. Lapidot, and H.G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963).
24) J.M. Gulland and H. Smith, *J. Chem. Soc.*, **1947**, 338.
25) P. Brigle and H. Muller, *Chem. Ber.*, **72**, 2121 (1937).

(Na_2SO_4), and filtered. The filtrate was concentrated to dryness. The residue was chromatographed on silica gel (50 g). Elution was performed with ethyl acetate–benzene (3: 2). Fraction containing (**2c**) was collected. Evaporation of the solvent left a colorless glass which was triturated with dry ether, mp 52—55°, yield, 1.52 g (54%). UV $\lambda_{max}^{95\% EtOH}$ nm: 261; NMR ($CDCl_3$): 1.95 (3H, s, CH_3CO), 2.10 (3H, s, CH_3CO), 4.33 (3H, m, $H_{4'}$ and $H_{5'}$), 5.35 (2H, m, $H_{2'}$ and $H_{3'}$), 5.70 (1H, d, J=8 Hz, H_5), 5.96 (1H, d, J=4 Hz, $H_{1'}$), 7.29 (11H, m, phenyl and $H_{1'}$) and 9.60 (1H, bs, NH). *Anal.* Calcd. for $C_{25}H_{25}O_{11}N_2P$: C, 53.52; H, 4.50; N, 5.00. Found: C, 53.72; H, 4.50; N, 5.03.

3′,5′-Di-O-acetyluridine 2′-Diethylphosphate (2d)——To a cooled (with ice), stirred solution of 3′,5′-di-O-acetyluridine (**2b**) (1.64 g, 5 mmol) in dry pyridine (10 ml) was added a pyridine solution (5 ml) of diethyl phosphorochloridate[26] (1.73 g, 10 mmol) over a period of 40 min. The solution was kept at 5° for 18 hr and then at room temperature for 1 hr. The solution was again cooled to 0° and there was then added water (5 ml). The solution was kept at the same temperature for 15 min and allowed to return to room temperature and kept at the same temperature for 1 hr. The solution was concentrated to dryness at 30° and the residue was dissolved in $CHCl_3$ (100 ml). The solution was successively washed with 0.1 N HCl, water, 5% aqueous $NaHCO_3$, and water and dried (Na_2SO_4). The salt was filtered off. The filtrate was concentrated to dryness and the residue was applied to a silica gel column (60 g). The column was washed with ethyl acetate–benzene (7: 4). Evaporation of fraction containing (**2d**) left yellow glass, yield, 1.00 g (46%), mp 45—50°. UV $\lambda_{max}^{95\% EtOH}$ nm: 259; NMR ($CDCl_3$) δ: 1.30 (6H, t, J=7 Hz, $CH_3\underline{CH_2}$), 2.10, 2.13 (each, 3H, s, CH_3CO), 4.12 (4H, quintet, J=7 Hz, $CH_3\underline{CH_2}$), 4.37 (3H, b, $H_{4'}$ and $H_{5'}$), 5.1—5.3 (2H, m, $H_{2'}$ and $H_{3'}$), 5.77 (1H, d, J=8 Hz, H_5), 6.02 (1H, d, J=4 Hz, $H_{1'}$) and 7.48 (1H, d, J=8 Hz, H_6). *Anal.* Calcd. for $C_{17}H_{26}O_{11}N_2P$: C, 43.93; H, 5.42; N, 6.04. Found: C, 44.22; H, 5.95; N, 5.96.

3′,5′-Di-O-acetyluridylyl-(2′-5′)-2′,3′-di-O-acetyluridine-*p*-phenylester (8b)——To a solution of phenyl phosphorodichloridate[25] (313 mg, 1.5 mmol) in pyridine (10 ml) was added with stirring crystalline 3′,5′-di-O-acetyluridine (**2b**) (492 mg, 1.5 mmol) over a period of 3 hr. The resulting solution was allowed to stand at room temperature for 15 hr. There was then added in drops a solution of 2′,3′-di-O-acetyluridine (**2g**) (1.00 g, 3 mmol) in pyridine (10 ml). The mixture was stirred at room temperature for 10 hr. There was then added TPS (445 mg, 1.5 mmol). Stirring was continued for 21 hr. Acetate buffer (1M, pH 7, 100 ml) was added and the solution was allowed to stand at room temperature for 30 min. The solution was extracted with $CHCl_3$ (3×70 ml). Combined extracts were washed with acetate buffer (1M, 40 ml) and dried (Na_2SO_4), and filtered. The filtrate was concentrated to dryness. The residue was applied to a silica gel (40 g) column and the column was washed with ethyl acetate. Fraction containing (**8b**) was collected and rechromatographed on a silica gel (40 g). The column was washed with increasing proportions of MeOH (from 2 to 4%) in $CHCl_3$. Evaporation left a colorless glass after trituration with ether, yield, 840 mg (71%). NMR ($CDCl_3$) δ :2.10 (12H, bs, CH_3CO), and 7.30 (5H, m, C_6H_5); UV $\lambda_{max}^{95\% EtOH}$ nm: 259.

Cleavage Reaction of (2c)——To a solution of 3′,5′-di-O-acetyluridine 2′-diphenyl phosphate (**2c**) (116 mg, 0.2 mmol) in DMF (2 ml) was added DBU (0.1 ml, 0.66 mmol). The solution was allowed to stand at 25° for 40 hr. The progress of the reaction was monitored by TLC (silica gel) and PEP (buffer A). After ascertaining by TLC that (**2c**) was completely consumed, there was then added 0.1 N NaOH solution (10 ml). The solution was allowed to stand at room temperature overnight to effect hydrolysis. PEP examination revealed that the mixture contained exclusively D-arabinofuranosyluracil (araU)[27] (**10**) and diphenyl phosphate. This reaction mixture was passed through a Dowex (50 W×8, H^+ form, 10 ml) to remove the cations including DBU. The column was washed with water. The eluate was rechromatographed on a DEAE cellulose column (2.0×46 cm). The column was washed with a linear gradient of water (1 liter) and 0.1 M TEAB solution (1 liter). The elution profile is given in Fig. 2. The structure of (**10**) was confirmed by the UV spectral and electrophoretic (buffer A and B) comparison with an authentic specimen of araU.[27] The product (**10**) also showed the negative metaperiodate-benzidine test. Yield, A_{260nm} (H_2O): 1570 units (80% yield).

Cleavage Reaction of (2d)——To a solution of 3′,5′-di-O-acetyluridine 2′-diethyl phosphate (**2d**) [A_{260} (95% EtOH) 2000 units (0.2 mmol)] in DMF (2 ml) was added DBU (0.1 ml, 0.66 mmol). No reaction took place at room temperature or even at 40°. The mixture was heated at 60° for 72 hr. After ascertaining by TLC and PEP that (**2d**) completely disappeared, there was then added 0.1N NaOH (10 ml). The mixture was allowed to stand at room temperature for 24 hr. The solution was passed through a Dowex (50 W×8, H^+ form, 10 ml) column to remove cations. The eluate [A_{260} (H_2O): 2080 units] was applied to a column on DEAE-cellulose (2.0×54 cm). Elution was carried out with a linear gradient of water (1 liter). and 0.1M TEAB solution (1 liter). Arabinofuranosyluracil (**10**) was eluted first, yield, being A_{260} (H_2O) 1170 units (59% yield) and then two phosphorous-containing, UV-absorbing materials were eluted in two separate peaks at ionic concentrations of 0.025 M and 0.033 M TEAB. The structures of these compounds remained unclarified.

Cleavage Reaction of (8b)——To a solution of 3′,5′-di-O-acetyl-(2′-5′)-2′,3′-di-O-acetyluridine *p*-phenylester (**8b**) (159 mg, 0.2 mmol) in DMF (2 ml) was added DBU (0.1 ml, 0.66 mmol). The solution was kept at 25° for 72 hr and then at 40° for 25 hr. After ascertaining by both TLC and PEP that the cleavage reaction

26) H. McCobie, B.C. Sanders, and G.J. Stacy, *J. Chem. Soc.*, **1945**, 380.
27) D.M. Brown, A.R. Todd, and S. Varadarajan, *J. Chem. Soc.*, **1956**, 2388.

was almost complete, there was then added 0.1N NaOH (10 ml) to the mixture. The solution was allowed to stand at room temperature for 24 hr, and then passed through a Dowex (50×8, H^+ form, 10 ml) column to remove DBU and cations. The eluate was applied to a DEAE-cellulose (2.0×48 cm). The column was washed with linear gradient of water (1 liter) and 0.1M TEAB solution (1 liter). Fraction containing araU (10) was pooled, yield, A_{260} (H_2O) 1380 units, (70% yield). Fraction containing uridine 5′-phenyl phosphate was then pooled, yield, being 69%. This sample showed the positive metaperiodate-benzidine-htest and was completely digested with venom phosphodiesterase to give Up, NMR (D_2O) δ: 7.2 (5H, br, C_6H_5).

3′,5′-Di-O-acetyl-(2′-5′)-2′,3′-di-O-acetyluridine-*p*-(2-*t*-butoxycarbonylphenyl) ester (8c) and 3′-5′-Positional Isomer (9c)——To a solution of 2′(3′), 5′-di-O-acetyluridine 3′(2′)-phosphates (2e), (2f) which had been prepared from uridine 2′(3′)-phosphate (1 mmol) according to a general method[23] in pyridine (5 ml) was added *t*-butyl salicylate[14] (234 mg, 1.2 mmol) and TPS (600 mg, 2 mmol). After the period of 24 hr at 25°, 2′,3′-di-O-acetyluridine (2g) (657 mg, 2 mmol) and TPS (606 mg, 2 mmol) were added. The solution was allowed to stand at room temperature for 24 hr. There was then added ice-water (*ca.* 2 ml) to the cooled solution. After 30 min $CHCl_3$ (50 ml) was added and the whole was washed with acetate buffer (1M, pH 7) and then with water. The organic layer was separated, dried (Na_2SO_4), and evaporated. The residue was co-distilled with ethanol and the final residue was chromatographed on TLC (silica gel). The products (**8c** and **9c**) were obtained as pale yellow powder after trituration with ether, yield, 169 mg (18% yield), UV $\lambda_{max}^{95\%\ EtOH}$ nm (ε): 260 (20600); NMR ($CDCl_3$) δ: 1.58 (9H, s, *t*-butyl), 2.09 (12H, bs, CH_3CO), 7.1—8.0 (6H, m, aromatic protons and H_6), 10.3 (2H, br, NH). *Anal.* Calcd. for $C_{37}H_{43}O_2N_4$; C, 49.67; H, 4.84: Found: C, 49.35; H, 4.83.

Deblocking of (8c and 9c)——3′,5′-Di-O-Acetyluridylyl-(2′-5′)-2′,3′-di-O-acetyluridine-*p*-(2-*t*-butoxycarbonylphenyl)ester (**8c**) and its 3′-5′-positional isomer (c) [total A_{260nm} (95% EtOH) 119 units] were dissolved in methylene chloride–5% trifluoroacetic acid (1 ml) and the solution was allowed to stand at room temperature for 24 hr. TLC examination revealed the absence of the remaining starting meterials and PEP examination showed the presence of a single product (R_{UMP}[28] 0.66 in buffer A; R_{UMP} 0.41 in buffer C). The solvent was evaporated off and the residue was subjected to preparative PEP (buffer C), and a band (R_{UMP} 0.41) was eluted with aqueous 50% pyridine (20 ml) and the solution was heated at 60° for 16 hr. The resulting solution was concentrated to dryness and the residue was dissolved in 7 M ammonium hydroxide (5 ml). The solution was allowed to stand at room temperature overnight. After evaporation of the solvent, the residue was subjected to preparative PPC (solvent system A). Uridylyl-(2′-5′, 3′-5′)-uridines (**8a** and **9a**) were obtained in 66% yield, A_{260} (H_2O) 76 units, UV $\lambda_{max}^{H_2O}$ nm: 263. This sample was found to be identical with an authentic sample on the criteria of chromatographic behavior and digested completely with venom phosphodiesterase to give uridine and uridine 5′-phosphate. Based on the data obtained by digestion with RNase A, the isomeric ratio in the starting material was estimated to be 1.11: 1.

Treatment of (8c and 9c) with DBU in DMF——To a solution of (**8c** and **9c**) [total A_{260nm} (5% EtOH) 1640 units] in DMF (1 ml) was added DBU (50 μl) (0.33 mmol). The solution was allowed to stand at 37° for 40 hr. After this period, the products were isolated by preparative TLC, yield of (**9c**), 75% [A_{260} (95% EtOH) 647 units] based on the amount of (**9c**) which had been contained in the starting materials. An aliquot [A_{260} (95% EtOH) 134 units] was withdrawn, and removal of salicylate protecting group was carried out as above. For complete deblocking the final residue was dissolved in 7M NH_4OH (5 ml). After 18 hr, the solution was concentrated to dryness and the residue was purified by PPC (solvent system A), yield, 64% [A_{260} (H_2O) 82 units], UV $\lambda_{max}^{H_2O}$ nm: 263. This sample was found to be identical with an authentic specimen of UpU (**9a**) on the criteria of the chromatographic behavior and completely (checked by Varian LCS 1000) digested with pancreatic RNase A to give uridine and Up in a molar ratio 0.98: 1.

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28) R_{UMP} refers to relative mobility with respect to Up on paper electrophoresis.