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Synthetic Nucleosides and Nucleotides. VI.[1] On the Several Routes for the Syntheses of 4-Thiouridylic Acid Homologues[2]

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The following synthetic methods for the preparation of 4-thiouridylic acid homologues from uridine are described.

Route A. Mild acid treatment of 2′,3′-O-isopropylidene-5′-O-benzoyl-4-thiouridine (II) followed by phosphorylation by Tener's procedure gave 4-thiouridine 2′,(3′)-phosphate *via* 5′-O-benzoyl-4-thiouridine (III). Alkali treatment of II followed by phosphorylation by Tener's procedure gave 4-thiouridine 5′-phosphate *via* 2′,3′-O-isopropylidene-4-thiouridine (IV).

Route B. 2′,3′-Di-O-acetyl-5′-O-trityl-4-thiouridine (V) was prepared by thiation of 2,3′-di-O-acetyl-5′-O-trityluridine. Alkali treatment of V followed by Tener's phosphorylation gave 2′,(3′)-phosphate of 4-thiouridine *via* 5′-O-trityl-4-thiouridine (VI). Mild acid treatment of V followed by Tener's phosphorylation gave 5′-phosphate of 4-thiouridine in satisfactory yield *via* 2′,3′-di-O-acetyl-4-thiouridine (VII).

Route C. Direct phosphorylation of 4-thiouridine with excess amounts of cyanoethylphosphate and dicyclohexylcarbodiimide produced 2′,(3′)-mono-, 5′-mono- and 2′,(3′), 5′-diphosphate of 4-thiouridine. Treatment of the diphosphate with dicyclohexylcarbodiimide gave 4-thiouridine 2′,3′-cyclic, 5′-diphosphate (VIII). 4-Thiouridine 2′,3′-cyclic phosphate (IX) was obtained by treatment of 4-thiouridine 2′,(3′)-phosphate with same manner or by digestion of VIII with *E. coli* alkaline phosphomonoesterase. 4-Thiouridine 3′-phosphate was prepared by digestion of IX with bovine pancreatic ribonuclease.

Recently Lipsett reported the isolation and chemical characterization of 4-thiouridylic acid (4-TUMP) as a minor constituent of *E. coli* transfer ribonucleic acid (tRNA),[4] and later this unusual nucleotide was isolated from tyrosine specific tRNA of *E. coli* by several workers.[5] The authors have been greatly interested in the chemical nature and biological function in this unique nucleotide.

Chemical preparation of the titled compounds has been of urgent nessesity in our studies–selective modification of 4-thiouridylic acid homologues and 4-thiouridylate residues in tRNA's,[5b,c] interaction between pyrimidine ribonucleotides and pancreatic ribonuclease (RNase),[6] and photosensitized modification of RNase by 2′(3′)-4-TUMP.[7]

In this paper, the comparative studies on the synthetic routes of several 4-thiouridylic acid homologues using uridine as the starting material are described.

Chemical synthesis of 4-thiouridine 5′-phosphate has already been attempted. Kochetkov, *et al.* prepared this nucleotide, as an intermediate for the synthesis of 4-thiouridine diphos-

1) Part V of this series: M. Araki, *et al.*, *Chem. Pharm. Bull.* (Tokyo), **16**, 1742(1968).
2) A part of this study was reported at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, Apr. 1968.
3) Location: *a*) *No. 1–1, Tsukiji 5–chome, Chuoku, Tokyo*; *b*) *No. 9–1, Anagawa 4–chome, Chiba.*
4) M.N. Lipsett, *Biochem. Biophys. Res. Commun.*, **20**, 224 (1965); *idem*, *J. Biol. Chem.*, **240**, 3975 (1965).
5) *a*) M.N. Lipsett and B.P. Doctor, *J. Biol. Chem.*, **242**, 4072 (1967); *b*) M. Saneyoshi and S. Nishimura, *Biochim. Biophys. Acta*, **145**, 208 (1967); *c*) S. Nishimura, M. Saneyoshi and F. Harada, presented at the 6th International Congress of Biochemistry, Tokyo (1967), Abstr. **I**, p. 51; *d*) F. Harada, F. Kimura and S. Nishimura, *Seikagaku*, **39**, 635 (1967).
6) M. Irie and F. Sawada, *J. Biochem.* (Tokyo), **61**, 282 (1967); F. Sawada and F. Ishii, *ibid.*, **64**, 149 (1968).
7) F. Sawada, Abstracts, 6th International Congress of Biochemistry, General Sess., F-26 (1967); *idem*, *J. Biochem.* (Tokyo), submitted.

phate gulcose, by phosphorylation of 2′,3′-O-isopropylidene-4-thiouridine which was obtained by acetonation of 4-thiouridine.[8] Saneyoshi synthesized this nucleotide by direct thiation of uridine 5′-phosphate.[9] Chemical syntheses of 4-thiouridine 2′(3′)-phosphate and other isomers, however, have not been reported so far.

The most satisfactory methods for the syntheses of 5′- or 2′(3′)-phosphates of ribonucleosides involve, as the key step, the protection of 2′- and 3′-hydroxyl groups and 5′-hydroxyl group in the ribofuranose moiety by the alkali-labile or the acid-labile protecting groups.

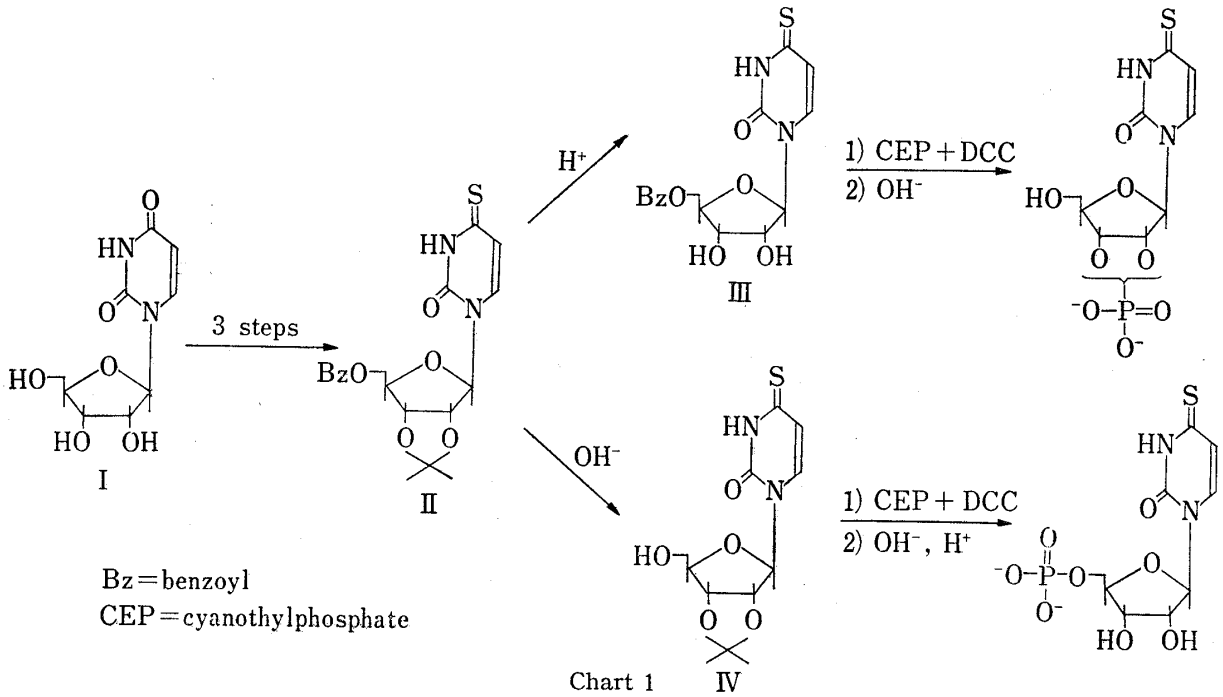

Chart 1

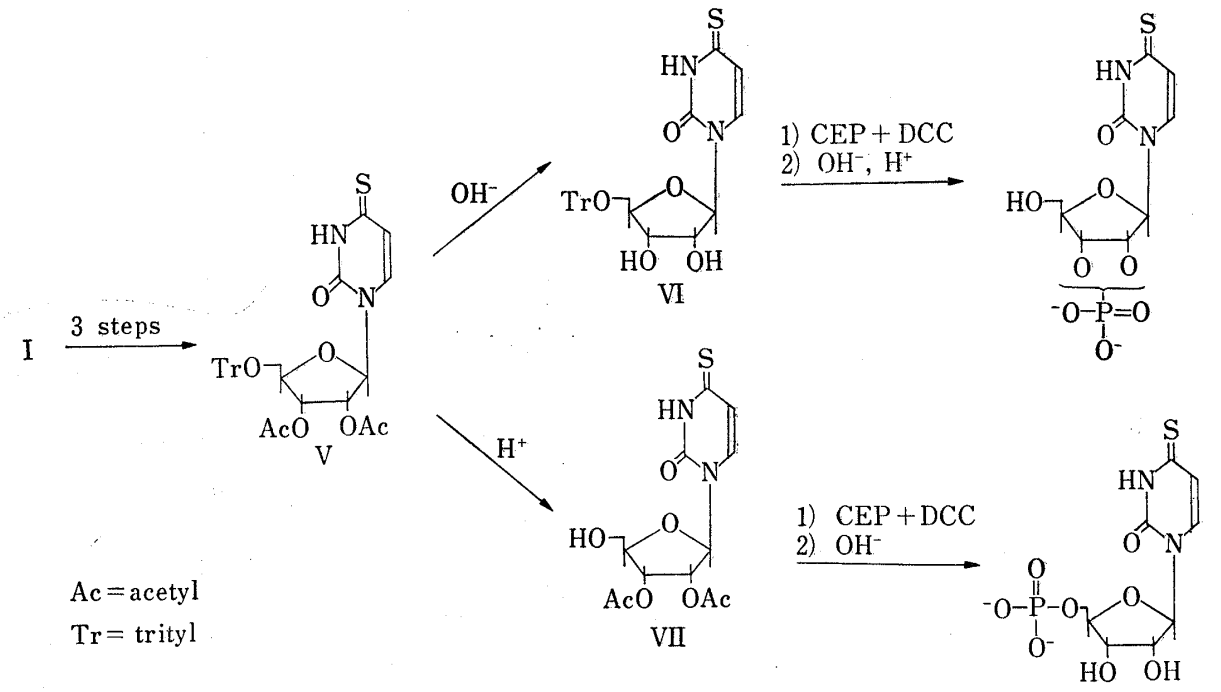

Chart 2

8) N.K. Kocketkov, E.I. Budowski, V.N. Shibaev, G.I. Yeliseeva, M.A. Grachev and V.P. Demushkin, *Tetrahedron*, **19**, 1207 (1963).
9) M. Saneyoshi, *Chem. Pharm. Bull.* (Tokyo), **16**, 1400 (1968).

Preparation of protected ribonucleosides bearing free 5′-hydroxyl group involves the alkylidenation or alcoxyalkylidenation of the ribonucleosides and deacylation of 2′,3′-O-alkylidene-5′-O-acylribonucleosides under alkaline condition or detritylation of 2′,3′-di-O-acyl-5′-O-tritylribonucleosides under acidic condition. Similarly, preparation of protected ribonucleosides bearing free 2′,3′-hydroxyl groups involve the removal of alkylidene group of 2′,3′-O-alkylidene-5′-O-acylribonucleosides under acidic condition or deacylation of the 2′,3′-di-O-acyl-5′-tritylribonucleosides under alkaline condition.

As the first step to synthesize 4-thiouridine 2′(3′)-phosphate (2′(3′)-4-TUMP), 2′,3′-O-isopropylidene-5′-benzoyl-4-thiouridine (compound II in Chart 1)[10] was heated with 70% acetic acid at 100° for 30 min or treated with 98% formic acid at room temperature for 3 hr to give 5′-O-benzoyl-4-thiouridine (III). After recrystallization from ethyl acetate/*n*-hexane mixture, the product gave yellow rods in 65—75% yield. mp 198—199°. Compound III was then phosphorylated with cyanoethylphosphate (CEP) and dicyclohexylcarbodiimide (DCC) after Tener[11] to give 2′- and 3′-phosphate mixture of 4-thiouridine, and 5′-O-benzoyl and cyanoethyl groups were removed by alkali treatment.

The phosphorylated products were chromatographed on a DEAE-cellulose or DEAE-Sephadex column in order to separate the nucleosides and the phosphate derivatives. Small amounts of contaminating uridine and its phosphates were thus separable easily from thiouridine derivatives.

On the other hand, when II was treated with 2 N sodium hydroxide at room temperature for 15—20 min or it was refluxed with 0.3 N sodium methoxide in methanol for 3 hr and was neutralized with Amberlite IR-120 or Dowex 50 resin it gave 2′,3′-O-isopropylidene-4-thiouridine (IV) in good yield. After having been recrystallized from ethanol containing cyclohexane, it was obtained in a crystalline state. mp 171—173°. This product was identified by mixed melting point test, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopic measurements with an authentic specimen.[8,12] IV could be easily methylated with methyl iodide in alkaline solution to 2′,3′-O-isopropylidene-4-methylthiouridine.[10] When IV was heated with 40% methylamine in a seald tube at 100° for 6 hr 2′,3′-O-isopropylidene-N_4-methylcytidine[10] was given. Compound IV was phosphorylated by the method described by Tener to give 5′-4-TUMP in 46% yield.

An attempt to find out the other routes for the convenient synthesis of 4-thiouridine 2′(3′)- or 5′-phosphate was made. 2′,3′-Di-O-acetyl-5′-O-trityluridine, which was obtained by acetylation of 5′-O-trityluridine[13] with acetic anhydride in pyridine, was refluxed with phosphorus pentasulfide. The resulting thiated product was purified by silica gel column chromatography and recrystallized from benzene–cyclohexane to give light yellow rods. mp 123—126°. The elemental analysis, ultraviolet absorption (UV), IR and NMR spectroscopic data strongly suggest the structure to be 2′,3′-di-O-acetyl-5′-O-trityl-4-thiouridine (V).

Compound V was treated with 85% formic acid at room temperature for 1.5—2 hr to release trityl alcohol, and 2′,3′-di-O-acetyl-4-thiouridine (VII) was obtained. After chromatographed on silica gel it was obtained in pure state in 58% yield. This product was difficult to be crystallized from several solvents, but no further purification was needed to perform the next steps. VII was phosphorylated by the Tener's procedure to give 5′-4-TUMP in 48% yield.

When compound V was deacetylated with 0.15N sodium methoxide or with 2N NaOH–dioxane (1:1 v/v), 5′-O-trityl-4-thiouridine (VI) was obtained. Compound VI was phosphorylated by the Tener's method to give 2′,(3′)-4TUMP in 35—50% yield.

10) M. Ikehara, T. Ueda and K. Ikeda, *Chem. Pharm. Bull.* (Tokyo), **10**, 767 (1962).
11) G.M. Tener, *Chem. Ind.* (London), **72**, 1002 (1959); *idem*, *J. Am. Chem. Soc.*, **83**, 159 (1961).
12) K.H. Scheit, *Tetrahedron Letters*, **2**, 103 (1967).
13) D.H. Rammler and H.G. Khorana, *J. Am. Chem. Soc.*, **84**, 767 (1962).

For purposes of identification, compound VI was heated with methanol saturated with anmonia in a sealed tube at 100° for 10 hr and the content was evaporated. The resulting dark gum was directly benzoylated with an equivalent amount of benzoic anhydride in pyridine[14] to give a crystalline derivative which melted at 196—199°. Further recrystallization of the derivative from ethyl acetate/*n*-hexane raised the melting point to 201—203°. This sample was identified by mixed melting point test, UV and IR spectroscopic measurements with authentic N_4-benzoyl-5′-O-tritylcytidine.[13]

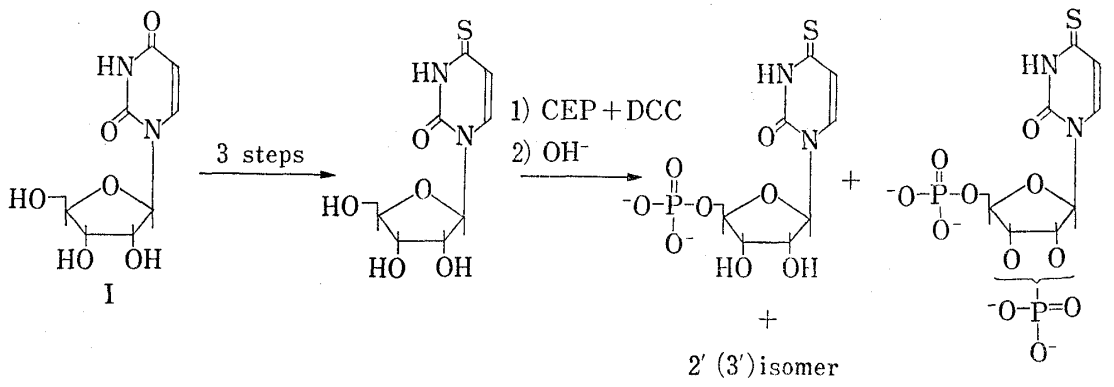

Chart 3

For the synthesis of 4-thiouridine 2′(3′),5′-diphosphate, the purified 4-thiouridine was treated with excess amounts of CEP and DCC at 37° for 48 hr, decyanoethylated by alkali treatment and finally chromatographed on a DEAE-cellulose column. Mixture of 4-thiouridine 2′,5′- and 3′,5′-diphosphates was isolated in 40% yield as their triethylammonium salts (Yield of 2′,(3′) or 5′-monophosphate was *ca.* 20%). The resulting isomers, 2′(3′)-mono-, 5′-mono- and 2′(3′),5′-diphosphates of 4-thiouridine were hydrolyzed by *E. coli* alkaline phosphomonoesterase (PMase) to 4-thiouridine in all cases. These compounds were converted

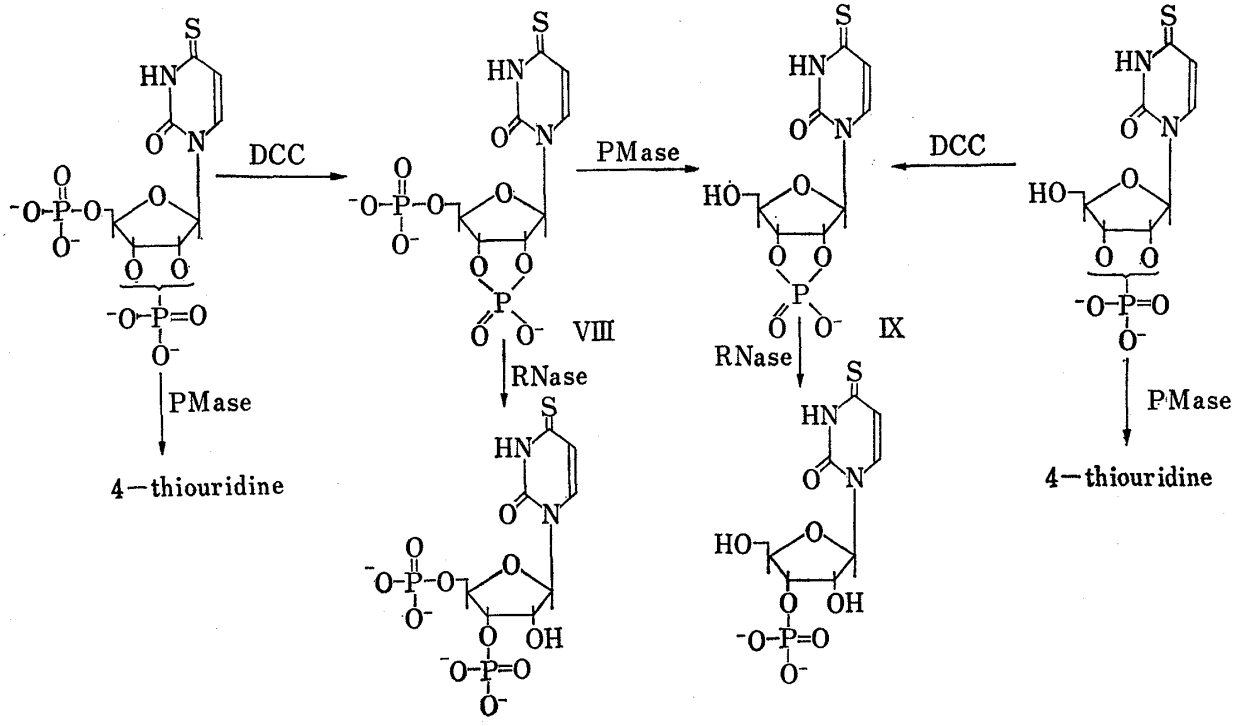

Chart 4

14) T. Sasaki and Y. Mizuno, *Chem. Pharm. Bull.* (Tokyo), **15**, 894 (1967).

to the corresponding uridine phosphates by heating in 0.1N hydrochloric acid at 100° for 1 hr. They were also converted to the corresponding cytidine phosphates by heating in methanol saturated with ammonia at 70—80° for 5 hr.

Both 2′(3′)-mono- and 2′(3′),5′-diphosphates of 4-thiouridine reacted easily with DCC in *tert*-butanol and dimethylformamide in the presence of tri-*n*-butylamine and gave their 2′,3′-cyclic phosphates. 4-Thiouridine 2′,3′-cyclic, 5′-diphosphate (VIII) was hydrolyzed by *E. coli* alkaline PMase to give 4-thiouridine 2′,3′-cyclic phosphate (IX). This cyclic phosphate was hydrolyzed easily by bovine pancreatic RNase give 4-thiouridine 3′-phosphate under a conventional condition. Treatment of VIII with the same RNase gave 4-thiouridine 3′,5′-diphosphate.

It should be mentioned that the synthetic methods by three different routes described above are most useful for the large scale preparation of 4-thiouridylic acid homologues. Polymerization of these nucleotides by chemical or enzymatic procedures will be reported in a forthcoming paper and photosensitized modification by a 4-thiouridine nucleotide of bovine pancreatic RNase is another report.[7]

Experimental

General Methods——Reagent grade pyridine was refluxed with potassium permanganate for oxidation of contaminating alkylpyridines and was distilled. Pyridine used for phosphorylation reactions was further purified in order to remove water and small amounts of primary and secondary amines as follows. Pyridine was distilled over potassium hydroxide, and *p*-toluenesulfonyl chloride (10 g for 1000 ml of pyridine) was added cautiously, and the solution was distilled using a short fractionating column. The distilled product was kept over molecular seive (Type 4A) beads in the dark.

Paper chromatography was performed by descending technique using Toyo Roshi No.51A or No.53 papers. The solvent systems used for paper chromatography were: solvent A, 2-propanol–1N NH_4OH (7:3, v/v); solvent B, 95% ethanol–1N ammonium acetate (5:2, v/v, pH 7.5); solvent C, saturated aqueous ammonium sulfate–1N sodium acetate–2-propanol (80:18:2, v/v/v). The protected nucleosides were checked for purity by thin-layer chromatography on silica gel using chloroform or chloroform containing methanol as solvent.

5′-O-Benzoyl-4-thiouridine (III) Method A——2′,3′-O-Isopropylidene-5′-O-benzoyl-4-thiouridine (II) (180 mg) was suspended in 70% acetic acid (18 ml), heated at 100° for 30 min, and acetic acid was removed by repeated flash evaporation with water. The residue was recrystallized from cold ethyl acetate–*n*-hexane to give yellow rods. Yield 113 mg (67%), mp 198—199°. UV λ_{max}^{EtOH} mμ: 230, 331 and a shoulder at 248. UV λ_{min}^{EtOH} mμ: 214, 278. *Anal.* Calcd. for $C_{16}H_{16}O_6N_2S$: C, 52.75; H, 4.43; N, 7.69. Found: C, 52.77; H, 4.53; N, 7.78.

Method B——Compound II(1.8 g) was added to 280 ml of 98% formic acid and the suspension was stirred at room temperature. After 1 hr the mixture became clear. After 3 hr stirring, formic acid was removed at room temperature under reduced pressure as rapidly as possible. Evaporation with water was repeated three time. Resulting yellow gum was recrystallized from hot ethanol to give yellow prisms. Yield 1.35 g (80%). This sample was identified with a specimen prepared by method A.

2′,3′-O-Isopropylidene-4-thiouridine (IV) Method A——Compound II (1.8 g) was treated with 20 ml of 0.5 N aqueous sodium hydroxide solution and the mixture was stirred until it became clear (about 30 min at room temperature). The resulting solution was neutralized with Dowex 50 (H^+-form) resin to pH 8. After the resin was filtered off, the solvent was removed under reduced presure and the yellow glassy residure was crystallized from ethanol–cyclohexane. Light yellow fine needles (1.1 g) was obtained. mp 171—173°. UV λ_{max}^{EtOH} mμ: 245, 329. This product was confirmed to be 2′,3′-O-isopropylidene-4-thiouridine[8] by comparison with the authentic specimen by mixed melting point test, UV, IR and NMR spectrophotometries.

Method B——Compound II (3.6 g) was dissolved in 100 ml of anhydrous methanol and solution of 1 N sodium methoxide in methanol (10 ml) was added to this solution drop by drop. The resulting clear solution was refluxed for 4 hr (the pH of the solution was maintained at pH 8), treated with a few drops of acetic acid (to pH 5) and then evaporated to dryness. The residue was taken up in water and extracted several times with chloroform to remove methyl benzoate and any starting materials. The aqueous layer was treated with charcoal and concentrated to dryness. The yellow residue was dried further by azeotroping with benzene and crystallized from ethanol–cyclohexane. Yellow fine needles, mp 172—173°, 1.9 g. This product was identified with a sample which was prepared by method A.

Methylation of IV with Methyl Iodide——One gram of IV was dissolved in 50 ml of 0.5 N aqueous sodium hydroxide at room temperature. Methyl iodide (800 mg) and 30 ml of water were added into this solution

and the solution stirred for 1.5 hr. After adding 400 mg of methyl iodide, stirring was continued for 1.5 hr. The white precipitate formed was collected by filtration, washed thoroughly with water and dried. Recrystallization from ethanol resulted in white needles (1 g). mp 209—211° (lit. 214°).[10] UV λ_{max}^{EtOH} mμ: 271, 301. UV λ_{min}^{EtOH} mμ: 240. This sample was identified with authentic 2′,3′-isopropylidene-4-methylthiouridine[10] by mixed melting point test, UV, IR and NMR spectroscopic measurements.

Direct Conversion of IV to 2′,3′-O-Isopropylidene-N_4-methylcytidine——Compound IV (200 mg) was treated with 40% solution of methylamine (30 ml) in a sealed tube at 100° for 18 hr. The tube was opened and the reaction mixture was concentrated to dryness. Water (3 ml) was added to the residue and extracted three times with 10 ml of chloroform. The chloroform layer was dried with anhydrous sodium sulfate and evaporated *in vacuo*. The residue was crystallized from ethanol–cyclohexane mixture to give white needles (154 mg). mp 163—164°. It was identified with authentic 2′,3′-O-isopropylidene-N_4-methylcytidine[10] by mixed melting point test, UV and IR spectroscopic measurements.

2′,3′-Di-O-acetyl-5′-O-trityl-4-thiouridine (V)—Crude 5′-O-trityluridine[13] (18.5 g) was dissolved in dry pyridine (300 ml), and freshly distilled acetic anhydride (10 ml) was added to this solution. The resulting solution was kept for 48 hr in the dark at room temperature. The reaction mixture was concentrated to small volume and was poured into ice–water mixture while stirring, and white precipitate was obtained. The yield of crude tritylacetyluridine in this stage was 20.5 g. A freshly ground phosphorus pentasulfide (30 g) was added to a pyridine solution containing 20.5 g of crude tritylacetyluridine. The resulting solution was refluxed for 3 hr. After the reaction mixture was cooled, the pyridine solution containing thiated product was decanted from dark orange hygroscopic precipitate. The solid was extracted twice with pyridine and combined pyridine solution was evaporated to dryness under reduced pressure. The remaining dark orange syrup was treated with 50% ethanol and the mixture was evaporated. Evaporation with 50% ethanol was repeated. The orange residue was dissolved in chloroform and a small amount of insoluble material was removed by filtration. The chloroform solution was dried over magnesium sulfate and concentrated to small volume. The solution was applied on a silica gel column (3.5 × 30 cm) and was eluted with chloroform. The fractions eluted at 1—1.5 liters were collected and the solvent was removed. The residue was dissolved in benzene and the solution was concentrated to small volume. The solution was kept at 4° for 10 hr and yellow precipitate separated. The precipitate was collected by filtration and washed with anhydrous petroleum ether. This product was recrystallized from benzene–cyclohexane. Light yellow rods (12.3 g) was obtained. mp 126—128°. UV λ_{max}^{EtOH} mμ (ε): 242 (2400), 329 (19200). IR ν_{max}^{KBr} cm^{-1}: 3000—3060 (CH_3 of acetyl group), 1730 (C=O of acetyl), 1070 (ribofuranose C-O-C), 750 and 700 (trityl). NMR (in $CDCl_3$)τ: 7.98 (CH_3 of acetyl group). *Anal.* Calcd. for $C_{32}H_{30}O_7N_2S$: C, 65.85; H, 5.14; N, 4.80. Found: C, 65.77; H, 5.21; N, 4.92.

2′,3′-Di-O-acetyl-4-thiouridine (VII)——Ninty-eight percent formic acid (300 ml) was added to 5 g of the dried compound V, and the resulting suspension was stirred at room temperature. After 30 min the suspension became clear, and light yellow precipitate appeared. After stirring for 1.5 hr, precipitated trityl alcohol was removed by filtration. The colored solid was washed with cold 98% formic acid to give white product. The combined filtrate and washings were evaporated under reduced pressure at room temperature as rapidly as possible. The yellow residue was treated with 50% ethanol and was concentrated. This process was repeated three times. The formic acid–free residue was applied onto a silica gel column (2 × 15 cm) and the column was first with 300 ml of dry benzene and then with chloroform–methanol (9:1, v/v). The desired compound was eluted at 700—760 ml. The solvent was removed and the residue was triturated with dry ether to obtain yellow precipitate, 1.8 g. This product failed to be crystallized from several solvent systems but was homogeneous on thin-layer chromatography (see Table I). UV λ_{max}^{EtOH} mμ: 245 (shoulder), 330. IR ν_{max}^{KBr} cm^{-1}: 1730 (C=O of acetyl), no absorption at 700 and 750 (no trityl group). No further purification of this product was needed for the next step of phosphorylation.

Deacetylation of VII with Sodium Methoxide in Methanol——Compound VII (300 mg) was added to 0.5 N sodium methoxide in absolute methanol (25 ml) and the resulting clear solution was refluxed for 3 hr. The solvent was removed and the residue was dissolved in 2 ml of water. The solution was neutralized with Dowex 50 (H^+-form) resin and the resin was filtered off. The yellow solution was concentrated carefully to small volume. The product was purified chromatographically on Whatman 3MM paper with isobutylic acid–concentrated ammonia–water (66:1:33, v/v/v, pH 3.7) or *n*-butyl alcohol–water (86:14) by descending technique. The band corresponding to 4-thiouridine was cut out and was eluted with acetone. The pure compound thus obtained was recovered by evaporation under reduced pressure and the resulting yellow glass was recrystallized from ethanol–cyclohexane. Yellow micro-needles which melted at 138°—140° was obtained. This product was identified with authentic 4-thiouridine,[8,15] by IR spectrophotometry and mixed melting point test (lit. mp 135—138°[8]).

5′-O-Trityl-4-thiouridine (VI) Method A——Five grams of compound V was dissolved in 25 ml of 0.5 N sodium methoxide in anhydrous methanol and the resulting clear solution was heated under reflux for

15) J.J. Fox, D. Van Praag, I. Wempen, I.L. Doerr, L. Cheong, J.E. Knoll, M.L. Eidinoff, A. Bendich and G.B. Brown, *J. Am. Chem. Soc.*, 81, 178 (1959).

3 hr. The residue, which was obtained after removal of the solvent, was treated with 50 ml of water and an equal volume of chloroform and it was shaken in a separatory funnel. The water layer was discarded and the chloroform layer was treated three times with 50 ml of water. The chloroform layer was dried on anhydrous sodium sulfate and filtered. The filtered was concentrated to approximately 3 ml. The solution was applied on a silica gel column (2.5 × 30 cm) and the column was washed first with benzene (300 ml) and then with 15% methanol in chloroform. The desired compound was eluted at 500—600 ml. After removal of the solvent, yellow gum, which was homogenous on thin-layer chromatography, remained. This material precipitated as yellow powder from chloroform–ether, but was not crystallizable from several solvent systems. Data on thin-layer chromatography are shown in Table I. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1070 (ribofuranose C–O–C), 700 and 750 (trityl), no absorption at 1730 (no C=O of acetyl). No methyl signal in NMR spectrum. This sample was used for the next step of synthesis without further purification.

Table I. *Rf* Values of Protected Derivatives of 4-Thiouridine on Thin-Layer Chromatography

Substance	*Rf* Solvent A[a]	*Rf* Solvent B[b]
2′,3′-Isopropylidene-5′-benzoyl-4-thiouridine (II)	0.43	0.67
5′-Benzoyl-4-thiouridine (III)	0.21	0.39
2′,3′-Isopropylidene-4-thiouridine (IV)	0.09	0.26
2′,3′-Diacetyl-5′-trityl-4-thiouridine (V)	0.76	0.92
5′-Trityl-4-thiouridine (VI)	0.52	0.76
2′,3′-Diacetyl-4-thiouridine (VII)	0.46	0.68

a) Solvent A, $CHCl_3$; b) Solvent B, $CHCl_3$-MeOH (9:1, v/v)

Method B——Compound V was treated with a mixture of dioxane and 2 N NaOH (1:1, v/v) and the resulting solution was stirred at room temperature for 20 min. The reaction mixture was poured into large excess of ice–water mixture and was extracted with chloroform. The chloroform layer was washed with water, dried and evaporated. The purification process described in method A was satisfactorily applied.

Conversion of VI to N_4-Benzoyl-5′-O-tritylcytidine——Compound VI (1.3 g) was placed in a tube containing 40 ml of anhydrous methanol previously saturated with ammonia at 0° and the tube was sealed and heated in a boiling water–bath for 10 hr. The tube was cooled, opened and the content was concentrated to dryness. The UV absorption spectrum of the residue showed a maximum at 272 mμ in ethanol. The gum (1.2 g) was added to a solution of benzoic anhydride (1.2 g) in 70 ml of dry pyridine. The resulting clear solution was stirred for 18 hr at room temperature in the dark, poured into ice–water and stirred for 3 hr. Chloroform(300 ml)was added to the aqueous suspension. The chloroform layer was washed with water(100 ml) and with 5% aqueous sodium bicarbonate (five times with 75 ml) and was dried over magnesium sulfate. The dried chloroform solution was evaporated to dryness and the residue was dissolved in hot ethyl acetate. The solution made opalescent by addition of *n*-hexane and light prism separated (1.3 g) which melted at 196—199°. The analytical sample was prepared by further recrystallization from ethylacetae and *n*-hexane. mp 201—203° (lit. 202°).[13] UV λ_{max}^{EtOH} mμ: 219, 260, 305. This sample was identified with authentic N_4-benzoyl-5′-O-tritylcytidine prepared after Khorana[13] by mixed melting point test and IR spectroscopy.

4-Thiouridine 2′,(3′)-phosphate *via* Phosphorylation of 5′-O-Benzoyl-4-thiouridine (III)——To a mixture of an anhydrous solution of pyridinium β-cyanoethylphosphate (1 mmole) and 5′-O-benzoyl-4-thiouridine (III) (0.5 mmole) in dry pyridine (1 ml), 1.5 mmoles of DCC was added. The mixture was sealed in a tube and was kept at room temperature in the dark with the exclusion of atmospheric moisture. After 48 hr, 1 ml of water was added and was kept at room temperature for 12 hr. The resulting suspension was added to the solution of 2 N lithium hydroxide. The mixture was heated at 90° for 3 hr to remove benzoyl and cyanoethyl groups and was kept at room temperature for additional 18 hr. The precipitated dicyclohexylurea was removed by filtration and the filtrate was neutralized with Dowex 50 (H^+-form) resin and was evaporated to dryness. The residue was dissolved in samll amount of water and was chromatographed on a DEAE–cellulose (bicarbonate form) column (1.5 × 30 cm) as follows. Trace of uridine followed by unreacted 4-thiouridine was eluted with water. Linear gradient elution, consisting of 0.01 M aqueous triethylammonium bicarbonate (pH 7.9, 1.5 liters) in a mixer and 0.2 M solution of the same buffer (1.5 liters) in a reserver, was made. Contaminating uridine phosphate was eluted in 0.9—1.0 liter and 4-thiouridine 2′,(3′)-phosphate in 1.3—1.56 liters. Fractions containing 4-thiouridine 2′,(3′)-phosphate was collected and evaporated under reduced pressure at room temperature. The residue was treated with 50% ethanol and evaporated repeatedly to remove triethylamine and its bicarbonate salt. 4-Thiouridine 2′,(3′)-phosphate (0.2—0.25 mmole) as triethylammonium salt, which was estimated spectrophotometrically, was obtained. UV $\lambda_{max}^{0.1N\ HCl}$ and $\lambda_{max}^{pH\ 5.6}$ mμ (ε): 245 (3980), 331 (20600); $\lambda_{max}^{0.1N\ NaOH}$ mμ (ε): 315 (18300); $\lambda_{min}^{0.1NHCl}$ and $\lambda_{min}^{pH\ 5.6}$ mμ: 225 and 276; $\lambda_{min}^{0.1N\ NaCH}$ mμ; 257. *Rf* values on paper chromatography in three solvent systems are shown

in Table II. This nucleotide was eluted from a Dowex 1 (formate form) column with 0.2 N sodium formate–0.02N formic acid system (pH 4.6) at four-fold elution volumes of that with uridine 2′, (3′)-phosphate. *Anal.* Calcd. for $C_9H_{15}O_9N_2SP$ (as free acid monohydrate)[16]: P, 8.66. Found: P, 8.4.

Table II. *Rf* Values of 4-Thiouridylic Acid Homologues on Paper Chromatography

Substance	*Rf* Solvent A[b]	Solvent B[c]	Solvent C[d]
2′(3′)-4TUMP	0.24	0.26	0.54
2′,3′-Cyclic 4TUMP	0.46	0.51	0.32
3′-4TUMP	0.24	0.26	0.55
5′-4TUMP	0.25	0.25	0.56
2′(3′),5′-4TUDP	0.08	0.11	0.78
2′,3′-Cyclic,5′-4TUDP	0.14	0.20	0.66
3′,5′-4TUDP	0.08	0.11	0.76
2′(3′)-UMP	0.23	0.21	0.79

a) 4TUMP, 4-thiouridine monophosphate; 4TUDP, 4-thiouridine diphosphate; UMP, uridine-monophosphate
b) solvent A, 2-propanol–1N NH_4OH (7:3, v/v)
c) solvent B, 95% ethanol–1N ammonium acetate (5:2, v/v, pH 7.5)
d) solvent C, saturated ammonium sulfate–1 N sodium acetate–2–propanol (80:18:2, v/v/v)

4-Thiouridine 2′, (3′)-phosphate *via* Phosphorylation of 5′-O-trityl-4-thiouridine (VI)——A mixture of pyridinium β-cyanoethylphosphate (2.5 mmoles) and 5′-O-trityl-4-thiouridine (1 mmole) (VI) was dissolved in anhydrous pyridine (5 ml) and evaporated *in vacvo* two times to remove a trace of water. The syrupy mixture was dissolved in 10 ml of purified anhydrous pyridine, and DCC (6 mmoles) was added to this solution. The solution was kept at room temperature for 4 days with exclusion of moisture. To the reaction mixture 10 ml of water was added. The mixture was extracted with cyclohexane and the aqueous pyridine phase was kept at room temperature overnight. The resulting suspension was filtered from dicyclohexylurea and evaporated *in vacuo*. To the residue 10 ml of 0.5 N lithium hydroxide was added. And the solution was kept at 95—100° for 1.5 hr to eliminate cyanoethyl group. After cooling, the solution was neutralized with Dowex 50 (H^+–form) resin and filtered. The resin was washed with methanol, then filtrate and washings was evaporated to dryness. The residue was suspended in 85 or 98% formic acid and was stirred vigorously at room temperature for 2 hr.

Trityl alchol precipitated was filtered off and formic acid was removed as rapidly as possible by flash evaporation at room temperature. The resulting residue was treated with 50% ethanol and was evaporated. This process was repeated three times. The formic acid-free residue was treated in 10 ml of water and applied on a DEAE-cellulose column (2.5 × 30 cm, bicarbonate form). The product was eluted with a linear salt gradient system consisting of water (2 liters) in a mixer and 0.2 M triethylammonium bicarnonate (pH 7.9; 2 liters) in a reserver. Contaminating uridine was eluted first in 120—160 ml, free 4-thiouridine in 200—260 ml, small amounts of uridine 2′, (3′)-phosphate in 1.2—1.32 liters and finally the desired product in 1.4—1.84 liters. Fractions containing 4-thiouridylic acid were collected and evaporated. The residue was treated with 50% ethanol and evaporated repeatedly to remove triethylamine and its bicarbonate salt. Triethylammonium 4-thiouridine 2′, (3′)-phosphate (0.35—0.5 mmoles, estimated spectrophotometrically) was obtained. This sample was confirmed for its purity by paper chromatography in three different solvent systems.

Phosphorylation of 2′,3′-O-Isopropylidene-4-thiouridine (IV)——Compound IV was phosphorylated with CEP and DCC and deisopropylidenated with acid in the same manner as Kochetkov, *et al.*,[8] and 4-thiouridine 5′-phosphate was obtained. Separation of the phosphorylated product was carried out by chromatography on DEAE-cellulose as described above.

Phosphorylation of 2′,3′-Di-O-acetyl-4-thiouridine (VII)——Compound VII was phosphorylated with CEP and DCC and was treated with alkali in the same manner as was described in the phosphorylation of III, and 4-thiouridine 5′-phosphate in a satisfactory yield. The product was identified with an authentic sample by paper chromatography in three solvent systems and by spectrophotometric measurements.

Direct Phosphorylation of 4-Thiouridine by Exeess Amounts of CEP and DCC——Purified 4-thiouridine (1 mmole) was treated with pyridinium β-canoethylphosphate (4 mmoles) and DCC (10 mmoles) in anhydrous pyridine at room temperature for 48 hr. Conventional alkali treatment eliminated cyanoethyl group, and unreacted DCC and dicyclohexylurea was removed. The resulting phosphorylated product was dis-

16) The free acid monohydrate was obtained by treatment of the salt with Dowex 50 (H^+-form) resin and lyophilization.

solved in 10 ml of water and applied on a column of DEAE-cellulose (bicarbonate form) (2.5×30 cm). The product was eluted with a linear gradient system consisting of water (2.5 liters) in a mixer and 0.5 M triethylammonium bicarbonate (pH 7.9, 2.5 liters) in a reserver. Contaminating nucleosides were eluted in 200—240 ml, uridine monophosphate mixture in 1.38—1.46 liters, 4-thiouridine monophosphate mixture in 1.66—1.98 liters, and 4-thiouridine 2′, (3′), 5′-diphosphate(s) in 4.0—4.8 liters. Yield; 4-thiouridine monophosphate 20%, 4-thiouridine diphosphate 40%. *Rf* values of this diphosphate in three solvent systems are summerized in Table II. *Anal.*, 4-thiouridine: P=1: 1.89.

Conversion of 4-Thiourdirine Phosphates to Uridine Phosphates——A 4-thiouridine phosphate (1 mg) was dissolved in 0.1N hydrochloric acid (0.2 ml) and heated at 90—100° for 1 hr. The hydrolysate was proved to be corresponding uridine phosphate by paper chromatography with three solvent systems and by UV specrtometries in acidic and alkaline media.

Conversion of 4-Thiouridine 2′,(3′)-Phosphate to Cytidine 2′,(3′)-Phosphate——Cyclohexylammonium salt of 4-thiouridine 2′, (3′)-phosphate[17] (4 μmoles) was dissloved in anhydrous methanol saturated with ammonia at 0° (1 ml) and heated at 75° for 8 hr in a sealed tube. The reaction mixture was evaporated to dryness and chromatographed on a Dowex 1 (formate form) column by eluting with 0.02 M formic acid[18]. The chromatographic pattern revealed that 4-thiouridine 2′, (3′)-phosphate was converted to a mixture of cytidine 2′- and 3′-phosphate in a good yield (80%). It was supported by UV spectrophotometry in both acidic and neutral media too.

4-Thiouridine 2′,3′-Cyclic Phosphate (IX)——4-Thiouridine 2′,(3′)-phosphate (free acid, 12 μmoles) and DCC (120 μmoles) were reacted in pyridine (0.8 ml) in presence of tri-*n*-butylamine (80 μmoles)at 25° for 48 hr. The reaction mixture was diluted with water (4 ml) and extracted with ether (8 ml), and the aqueous phase was evaporated to dryness. The residue was dissolved in water (4 ml), treated with Dowex 50 (ammonium form) resin and evaporated to dryness. The product gave only one spot in two solvent systems (see Table II).

4-Thiouridine 2′,3′-Cyclic, 5′-diphosphate (VIII)——To 40 mg of 4-thiouridine 2′,(3′),5′-diphosphate-(cyclohexylammonium salt) and 160 mg of DCC in a 25 ml flask was added 3 ml of dimethylformamide. All the materials dissolved rapidly on gentle stirring. *tert*-Butanol (2 ml) was added to this solution and refluxed for 15 min. The solution cloudy and precipitates appeared. Paper chromatography with solvent system B showed absence of the starting materials and presence of 4-thiouridine 2′,3′-cyclic, 5′-diphosphate (VIII) as the main product along with a trace of thiouridylylurea, the *Rf* values being 0.20 and 0.48, respectively. About 5 ml of water was added to the reaction mixture and dicyclohexylurea precipitated was filtered off. The filtrate was extracted three times with 3 ml of ether and concentrated to small volume at room temperature. Paper chromatography at this stage indicated the presence of only the cyclic phosphate. This product was purified by preparative paper chromatography on Whatman 3 MM paper with solvent B. Elution of the band with water and lyophilization produced yellow hygroscopic powder (28 mg).

Enzymatic Hydrolysis of 4-Thiouridylic Acid Homologues with *E. coli* Alkaline Phosphomonoesterase —— 4- Thiouridine 2′,(3′)-mono, 5′-mono- or 2′(3′), 5′-diphosphate (*ca.* 1 mg) was dissolved in 0.2 ml of 0.05 N ammonium bicarbonate (pH 8.5) and 0.02 ml of *E. coli* alkaline PMase solution (6.68×10^{-5} units)[19] was added to this solution. The mixture was incubated at 37° for 18 hr.

The reaction mixture was developed on Toyo Roshi No. 51 paper with *n*-butanol–water (86:14, v/v) as solvent. No spot of the phosphate and only one spot (*Rf*=0.36) corresponding to 4-thiouridine was observed in all the cases.

Preparation of 4-Thiouridine 2′,3′-Cyclic Phosphate (IX) *via* Dephosphorylation of VIII with *E. coli* PMase——Compound VIII (10 mg) was dissolved in 0.8 ml of 0.05N ammonium bicarbonate (pH 8.5) and 0.05 ml of PMase solution (see above)was added the mixture was incubated at 37° for 18 hr and the solution was carefully concentrated to small volume under reduced pressure at room temperature. Paper chromatography at this stage showed only one spot corresponding to 4-thiouridine 2′,3′-cyclic phosphate (IX) in both solvent A and B, *Rf*=0.46 and 0.51, respectively. The product was purified by preparative paper chromatography on Whatman 3MM paper with solvent A by descending technique. The band corresponding to IX was cut out and eluted with water. The solution was lyophilized to give yellow powder, which was identified with an authentic specimen by paper chromatography in three solvent systems.

Digestion of IX with Bovine Pancreatic RNase ——Compound IX (1.2 μmoles) was dissolved in 0.1M ammonium acetate (0.3 ml) containing bovine pancreatic RNase (0.012 μmoles) and incubated at 25° for 4 hr in the dark. The reaction mixture was evaporated repeatedly with water (1 ml) and was applied on

17) The cyclohexylammonium salt was prepared by treatment of the free acid with Dowex 50 (cyclohexylammonium form resin and by flash evaporation. The product was purified by dissolving the residue in anhydrous methanol and precipitating with ether.

18) W.E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).

19) The enzyme was kindly supplied from Dr. K. Tanaka, Shionogi Research Laboratory, Osaka.

paper chromatography. No spot was detected at the position of IX, but one spot was observed at the position of 4-thiouridine 2′(3′)-phosphate in either system A and B.

Chromatographic behavior on Dowex 1 and UV absorption properties were the same as those of 4-thiouridine 2′,(3′)-phosphate. These data indicated that the cyclic phosphate was digested by RNase to give 4-thiouridine 3′-phosphate as deduced from the specificity of the enzyme.

Cleavage of VIII by Pancreatic RNase——Compound VIII (3 μmoles) and bovine pancreatic RNase dissolved in 0.2 ml of 0.05 M Tris–HCl buffer (pH 7.8) was incubated at 37° for 18 hr. A control containing no enzyme was incubated in the same manner. The reaction mixture was spotted on paper and developed with solvent A, B and C. The substrate was cleaved by the enzyme and a spot corresponding to 4-thiouridine 2′,(3′),5′-phosphate was detected in all cases. In the control solution the cyclic phosphate completely remained.

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