Discussion

Sodium L-tenuazonate was found to have a high degree of structural specificity against HAd1 in the egg. All changes in structure led to a marked reduction in activity.

Cytotoxic activities of the C-5 substituted congeners and of the N-methyl derivative of tenuazonic acid against KB cells correlated with antitumor activities in the egg. However, the degree of structural specificity in KB cells was not as great as in the egg tumor system, since N-benzyl compounds were as cytotoxic as L-tenuazonic acid itself.

The least structural specificity was shown in *B. megaterium*. The L-, D-, and D-*allo* isomers of tenuazonic acid had equivalent antimicrobial activities. Miller, *et al.*,[7] found a similar absence of stereospecificity in antiviral studies on sodium tenuazonate and sodium isotenuazonate. Both inhibited Echo-9, *para*influenza, vaccinia, herpes simplex, and measles viruses. However, sodium tenuazonate inhibited polio virus, but sodium isotenuazonate did not.

Some of the C-5 substituted congeners had antimicrobial activities equal to that of sodium L-tenuazonate. Substitution of a benzyl group for the N-hydrogen resulted in a striking enhancement of antimicrobial activity. This might have resulted from an increase in lipid solubility or even from different modes of action for the compounds.

Shigeura and Gordon[8] found that sodium L-tenuazonate inhibits protein synthesis in Ehrlich ascites cells and rat liver. In Ehrlich ascites cells the mode of action was shown to be an inhibition of release of protein from the ribosome. It is not known that this is the mode of action in microorganisms, nor even in other animal cells. Further, since the egg–tumor and *B. megaterium* systems studied here are of such diverse nature, a correlation of activities in the two systems would not necessarily be expected.

Acknowledgment.—The author gratefully acknowledges the technical assistance of Mrs. Barbara L. Bagdi, Mr. George W. Campbell, Mrs. Noreen A. Haag, Miss Joan E. Hitt, and Miss Dolores J. Meltz.

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Polynucleotides. III.[1] Synthesis of (3′→5′)-Linked Diribonucleoside Phosphates Containing 3- and 5-Methyluracil

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The occurrence of 5- and 3-methyluridine in ribonucleic acid has led us to prepare the following dibribonucleoside phosphates in order that their properties may be studied: uridylyl-(3′→5′)-3-methyluridine, 3-methyluridylyl-(3′→5′)-uridine, 3-methyluridylyl-(3′→5′)-3-methyluridine, 5-methyluridylyl-(3′→5′)-5-methyluridine, and 5-methyluridylyl-(5′→5′)-5-methyluridine. These compounds were synthesized by condensing a 3′-nucleotide blocked at the 2′- and 5′-positions by trityl groups with the 2′,3′-iospropylidene derivative of the second nucleoside. The key intermediates, 2′,5′-*O*-ditritylnucleosides were prepared by direct tritylation of the corresponding nucleosides. The location of the trityl groups in the case of 5-methyluridine was demonstrated by conversion of the blocked nucleoside to the xyloside as well as by a methylation procedure which gave 3-*O*-methylribose. The hyperchromic effect of these compounds was compared with that of uridylyl-(3′→5′)-uridine. In comparison with this compound the values for diribonucleoside phosphate methylated in the 3-position are approximately one-third higher than that for the nonmethylated derivative. The introduction of a methyl group at the 5-position causes a more than twofold increase in the hyperchromic effect. The diribonucleoside phosphates containing a 3-methyluridylyl-3′ residue are resistant to the action of pancreatic ribonuclease in contrast to the corresponding 5-methyl derivatives which are substrates for this enzyme.

The four major ribonucleosides comprise about 95–99% of the nucleosides of ribonucleic acid (RNA). The remaining percentage, particularly in the case of soluble RNA, is made up of over 20 minor nucleoside constituents[2] which appear to be distributed throughout the RNA molecule in a specific pattern.[3] The subtleties in physical and biological properties which these minor nucleosides confer upon RNA molecules are not well understood. In order to learn more about the effects of these minor constituents on RNA it is our plan to study model polyribonucleotides containing minor nucleosides. As a step toward preparation of such polynucleotides, diribonucleoside phosphates were synthesized starting from 3- and 5-methyluridine. Soluble RNA contains 0.1–0.5% of 5-methyluridine[4] and about 0.005% of 3-methyluridine.[5]

Preparation of the key intermediates, illustrated by compounds Ia and Ib, was based on an earlier approach to the synthesis of uridylyl-(3′→5′)-uridine[6] which made use of the easily prepared 2′,5′-di-*O*-trityluridine.[7] As in the case of uridine, direct tritylation of 3-methyluridine with an excess of trityl chloride afforded 2′,5′-di-*O*-trityl-3-methyluridine. Proof that the second trityl group is attached at the 2′-position is provided by the observation that methylation of the known 2′,5′-di-*O*-trityluridine affords the same

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(2) R. H. Hall, *Biochemistry*, **4**, 661 (1965).

(3) P. R. Srinivasan and E. Borek, *Science*, **145**, 548 (1964).

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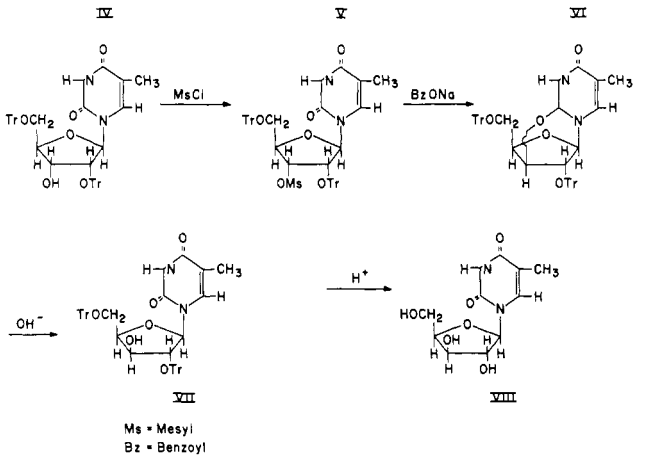
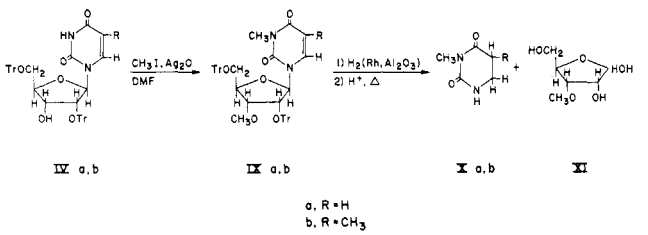
compound. Tritylation of 5-methyluridine under similar conditions produced a 2′,5′-di-*O*-trityl derivative whose structure was established on the basis of two separate pieces of evidence. The free 3′-hydroxyl of this compound (IV) was inverted to yield the known 1-(β-D-xylofuranosyl)-5-methyluridine (VIII) as shown by the series of reactions in Chart I. In addition,

CHART I



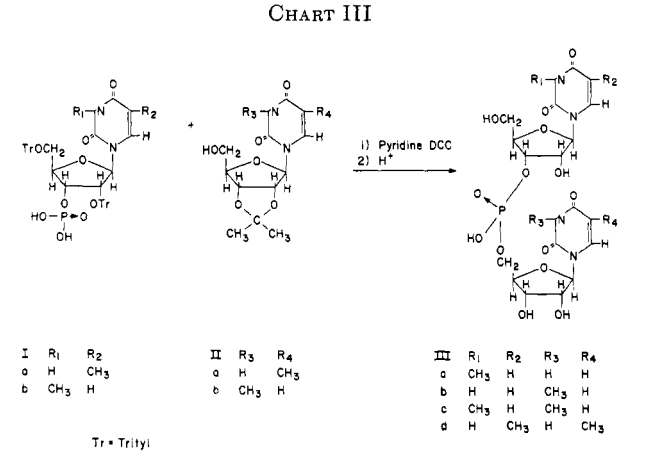
2′,5′-di-*O*-trityl-5-methyluridine was methylated by means of methyl iodide and silver oxide. The methylribose obtained from this nucleoside was identified as 3-*O*-methylribose (XI). Methylation of 2′,5′-di-*O*-trityluridine (IVa) under the same conditions also produced the 3-*O*-methylribose (XI). This series of reactions is shown in Chart II.

CHART II



The 2′,5′-di-*O*-trityl derivatives of 3- and 5-methyluridine were phosphorylated to yield the key intermediates I (Chart III) containing a phosphate residue in the 3′-position. These intermediates were condensed with the appropriate 2′,3′-isopropylidene ribonucleoside (II) to yield intermediates which after removal of blocking groups afforded the (3′→5′)-linked diribonucleoside phosphate. In an analogous procedure 2′,3′-isopropylidene-5-methyluridine 5′-phosphate was condensed with 2′,3′-isopropylidene-5-methyluridine to yield 5-methyluridylyl-(5′→5′)-5-methyluridine.

CHART III



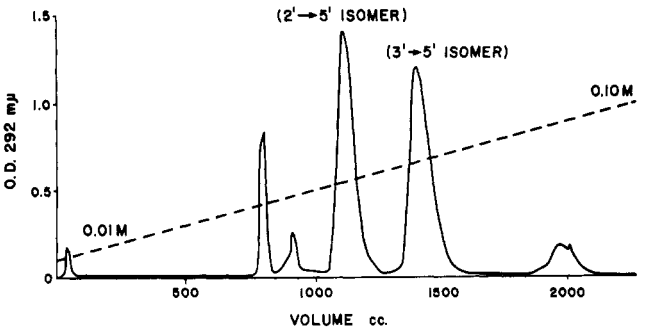


Figure 1.—Resolution of 5-methyluridylyl-(3′(2′)→5′)-5-methyluridine: column, Dowex-1 × 2 (formate) 200–400 mesh, size 1.5 × 40 cm. The mixture of isomers (100 mg.) was placed on the column and the column was developed with a linear gradient of sodium formate buffer (pH 4.5) from 0.01 → 0.1 *M*. Ratio of the amount of the (2′→5′) isomer to that of the (3′→5′) isomer is 45:55.

An important question concerning choice of blocking groups arises in this work. Studies on model phosphate esters of glycols show that under acid conditions the phosphate groups can migrate from one hydroxyl to the neighboring one.[8,9] Thus, removal of acid labile blocking groups from (3′→5′) diribonucleoside phosphates could result in formation of the (2′→5′) isomer. This possibility can be assessed accurately in the case of those diribonucleoside phosphates containing a uridylyl-3′ or 5-methyluridylyl-3′ residue. Uridylyl-(3′→5′)-3-methyluridine (IIIb) prepared in the present study is 98% hydrolyzed by pancreatic ribonuclease. This follows the pattern established previously for other uridylyl-3′ esters.[6] The 5-methyluridylyl-5-methyluridine synthesized in this study, however, actually was a mixture of approximately equal parts of the (3′→5′) (IIId) and (2′→5′) isomers. These isomers were readily separated by ion-exchange chromatography as shown in Figure 1. The (3′→5′) isomer was completely degraded by pancreatic ribonuclease. In the presence of an equal amount of the (2′→5′) isomer, the (3′→5′) isomer was not degraded by this enzyme.

The isomerization of the 5-methyluridylyl derivative appears to be due to a labile acid-catalyzed transesterification of the phosphodiester bond during removal of the trityl groups. This lability contrasts sharply with the stability of the uridylyl derivatives under similar conditions The question as to whether the 3-methyl group has a similar labilizing effect as the 5-methyl group was harder to assess since these derivatives are resistant to the action of pancreatic ribonuclease.[10] The preparation of 3-methyluridylyl-(3′→5′)-3-methyluridine (IIIc) was subjected to ion-exchange chromatography under conditions in which the (2′→5′) and (3′→5′) isomers of compound IIId were resolved and a single symmetrical peak was obtained. This suggests that only a single isomer was present.

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(9) D. M. Brown, D. I. Magrath, A. H. Neilson, and A. R. Todd, *Nature*, **177**, 1124 (1956).
(10) W. Szer and D. Shugar, *Acta Biochim. Polon.*, **7**, 491 (1960).

Polynucleotides exhibit a substantial hyperchromic effect when they are hydrolyzed to their constituent nucleotides. This effect affords a measure of the interaction of the constituent nucleotides with each other. The forces responsible for this interaction have been ascribed to coulombic interactions between base residues.[11,12] Studies of these forces are generally made on long-chain polynucleotides; however, Michelson[13,14] has also observed hyperchromic effects at the level of a diribonucleoside phosphate.

Comparison of the hyperchromicity of the methylated diribonucleoside phosphates with that of the nonmethylated diribonucleoside phosphate, uridylyl-(3′→5′)-uridine, shown in Table I, provides some insight into

TABLE I

HYPERCHROMICITY AT pH 6.0

Compd.	Optical density increase at λ_{max} %
Uridylyl-(3′→5′)-uridine	4.8
Uridylyl-(3′→5′)-3-methyluridine	6.4
3-Methyluridylyl-(3′→5′)-uridine	6.4
3-Methyluridylyl-(3′→5′)-3-methyluridine	5.8
5-Methyluridylyl-(2′→5′)-5-methyluridine	17.9
	18.1[a]
5-Methyluridylyl-(3′→5′)-5-methyluridine	10.2
	10.3[a]
5-Methyluridylyl-(5′→5′)-5-methyluridine	7.6

[a] These values were obtained by treating the respective compounds with 0.5 *N* NaOH for 24 hr. at 37°.

the effect of a methyl group on the interaction of two adjacent uracil residues of an oligoribonucleotide. The methyl group at position 3, regardless of configuration of the internucleotide bond, *i.e.*, (3′→5′) or (5′→3′), causes a slight increase in hyperchromicity. This effect is not augmented when two 3-methyluracil residues are joined together in this fashion. The methyl group at position 5 on the other hand causes a substantial increase in base–base interaction as indicated by the pronounced hyperchromicity. The strong base–base interaction exhibited by this compound presumably has some bearing on the stable secondary structure of polyribothymidylic acid which exhibits a hyperchromic effect of 35%[15] and a T_m of 36°.[16] The methyl groups at position 3 of polyuridylic acid, conversely, eliminate all vestiges of a secondary structure.[17] The marked changes in physical structure brought about by the introduction of a methyl group into the uracil residue of oligonucleotides indicates one of the ways in which minor nucleosides may influence the biological properties of RNA.

Experimental[18]

Paper Chromatography.—Whatman filter paper No. 1 was used. The solvent systems used were as follows: A, isopropyl alcohol–1% aqueous ammonium sulfate (2:1); B, isopropyl alcohol–concentrated NH_4OH–water (7:1:2); C, ethyl acetate–*n*-propyl alcohol–water (4:1:2); D, *n*-butyl alcohol–water (86:14); E, *n*-butyl alcohol–water–concentrated NH_4OH (86:14:5); F, isopropyl alcohol–concentrated HCl–water (680:176:144). See Table II.

Electrophoresis was conducted on Whatman 3 MM paper in a Gilson Electrophorator for 1 hr. at 50 v./cm. in 0.05 *M* ammonium formate buffer, pH 3.5. See Table II.

3-Methyluridine.—Uridine (4.0 g., 16.4 mmoles) was methylated according to the procedure of Miles.[19] Purification of the product was simplified by employing a column partition chromatography technique.[20] The column contained 150 g. of Celite-545[21] size 2.54 × 80 cm., and the solvent system used was ethyl acetate–*n*-propyl alcohol–water (4:1:2). The solvent eluted a small fraction (about 10% of total) which consisted of a dimethyl derivative of uridine and this was followed by a fraction containing the product. The effluent containing the product was concentrated on a flash evaporator and to the resulting sirup methanol (1 ml.) and anhydrous ether (5 ml.) were added. The solution was maintained at 0° for several hours during which time the product slowly crystallized. The yield was 3.2 g. (74%). The compound melted at 118–119° after recrystallization from ethanol and cyclohexane (lit.[19] m.p. 119–120°).

Anal. Calcd. for $C_{10}H_{14}N_2O_6$: C, 46.51; H, 5.45; N, 10.85. Found: C, 46.06; H, 5.70; N, 10.60.

2′,5′-Di-*O*-trityl-3-methyluridine. A.—3-Methyluridine (1.0 g., 3.87 mmoles) was treated with triphenylmethyl chloride by the method which Yung and Fox[7] used for uridine. The crude product was dissolved in a mixture of hot benzene and methanol. Upon cooling, a small amount of a minor product was obtained (75 mg., m.p. above 250°). As the solution was concentrated on a steam bath, the major product precipitated (1.1 g., 38%). It was crystallized from hot benzene and anhydrous ether; m.p. 238–239°.

Anal. Calcd. for $C_{48}H_{42}N_2O_6$: C, 77.6; H, 5.69; N, 3.77. Found: C, 77.87; H, 5.75; N, 3.56.

The minor product was not completely characterized; however, the analysis corresponded to a tritrityl-3-methyluridine.

Anal. Calcd. for $C_{67}H_{56}N_2O_9$: C, 81.68; H, 5.73; N, 2.84. Found: C, 80.93; H, 5.79; N, 2.89.

B.—2′,5′-Di-*O*-trityluridine (0.82 mmole)[7] was added to boiling methanol (75 ml.), and benzene was added until all the material had dissolved. After cooling the solution to 4°, it was treated with an ethereal solution of diazomethane. The reaction mixture was allowed to stand at room temperature for 3.5 hr. Evaporation of the solvent yielded a white solid which was recrystallized from benzene and anhydrous ether. The yield was 450 mg. (73%), m.p. 238–239°.

Anal. Calcd. for $C_{48}H_{42}N_2O_6$: C, 77.6; H, 5.69; N, 3.77. Found: C, 77.56; H, 5.79; N, 3.86.

The mixture melting point of the two preparations obtained by methods A and B was 239°. Their infrared spectra were also the same and each gave 3-methyluridine when hydrolyzed by 80% acetic acid for 30 min. at 100°.

2′,5′-Di-*O*-trityl-3-methyluridine 3′-Phosphate (Ib).—2′,5′-Di-*O*-trityl-3-methyluridine (4 mmoles) was phosphorylated with β-cyanoethyl phosphate by a method used for the preparation of 2′,5′-di-*O*-trityluridine 3′-phosphate.[6] The product was obtained as the pyridinium salt (2.8 g., 70.8%).

Anal. Calcd. for $C_{53}H_{48}N_3O_9P \cdot H_2O$: C, 69.12; H, 5.47; N, 4.56. Found: C, 69.00; H, 5.50; N, 4.43.

3-Methyluridine 3′-Phosphate.—A mixture of 2′,5′-di-*O*-trityl-3-methyluridine 3′-phosphate (0.2 mmole of pyridinium salt) and 100 ml. of 80% acetic acid was heated on a steam bath for 1.5 hr. The mixture was cooled and filtered, and the filtrate was concentrated to dryness. The residue was dissolved in a mixture of water and ethanol (1:1) and the solution was evaporated to dryness. This process was repeated several times to remove excess acetic acid. Paper chromatography of the crude reaction

(11) H. DeVoe and I. Tinoco, *J. Mol. Biol.*, **4**, 500 (1962).
(12) D. M. Crothers and B. H. Zimm, *ibid.*, **9**, 1 (1964).
(13) A. M. Michelson, *Biochim. Biophys. Acta*, **55**, 841 (1962).
(14) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press Inc., New York, N. Y., 1963.
(15) B. F. Griffin, A. Todd, and A. Rich, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1123 (1958).
(16) D. Shugar and W. Szer, *J. Mol. Biol.*, **5**, 580 (1962).
(17) W. Szer and D. Shugar, *Acta Biochim. Polon.*, **8**, 235 (1961).
(18) Melting points were determined by the capillary method and are uncorrected. Microanalyses were performed by George I. Robertson, Jr., Fordham, N. J. The nucleotides were dried over phosphorus pentoxide *in vacuo*. Since no attempt was made to render them completely anhydrous, due to their lability to heat, some of these compounds were analyzed as hydrates.
(19) H. T. Miles, *Biochim. Biophys. Acta*, **22**, 247 (1956).
(20) R. H. Hall, *J. Biol. Chem.*, **237**, 2283 (1962).
(21) Johns-Manville Co. brand of diatomaceous earth.

TABLE II

Compd.	Spectrophotometric data: pH	λ_{max}, mμ	$\epsilon_{max} \times 10^3$	Paper chromatography: R_f values, Solvent A	Solvent B	Electrophoresis: Distance moved toward anode, cm.
	4.9	262	9.02	0.77	0.75	2.9
3-Methyluridine	11.6	263	9.00			
5-Methyluridine	...	...	...	0.73	0.56	0.7
2′,3′-Isopropylidene-3-methyluridine	5.4	261	9.49	0.88	0.85	3.4
	11.5	259	9.45			
2′,3′-Isopropylidene-5-methyluridine	...	...	...	0.88	0.79	1.2
3-Methyluridine 3′-phosphate	5.6	261	9.52	0.32	0.46	31.6
	11.6	260	9.34			
5-Methyluridine 3′-phosphate	...	...	...	0.61	0.12	27.3
5-Methyluridine 5′-phosphate	...	...	...	0.50	0.03	25.4
2′,5′-Di-*O*-trityl-3-methyluridine 3′-phosphate	2.4	257	9.91	0.83	0.88	...
	11.4	256	9.69			
2′,5′-Di-*O*-trityl-5-methyluridine 3′-phosphate	...	...	...	0.35	0.14	...
Uridylyl-(3′→5′)-3-methyluridine	2.4	261	18.03	0.37	0.52	20.2
	10.2	260	15.6			
3-Methyluridylyl-(3′→5′)-uridine	2.4	260	18.1	0.38	0.52	20.6
	11.6	259	15.14			
3-Methyluridylyl-(3′→5′)-3-methyluridine	3.2	261	17.44	0.63	0.62	21.4
	11.5	260	16.74			
5-Methyluridylyl-(2′→5′)-5-methyluridine	2.0	267	...	0.51	0.28	23.7
	10.0	267	...			
5-Methyluridylyl-(3′→5′)-5-methyluridine	...	...	...	0.51	0.28	22.8
5-Methyluridylyl-(5′→5′)-5-methyluridine	...	...	...	0.48	0.14	20.0

mixture in solvent systems A and B indicated the presence of a single ultraviolet-absorbing compound. The crude product was dissolved in 5 ml. of a 0.01 *M* triethylammonium carbonate solution (pH 8.6), and the solution was placed on a column (1.9 × 30 cm). of DEAE cellulose (carbonate form). The column was eluted with 600 ml. of 0.01 *M* triethylammonium carbonate solution (pH 8.6) at the rate of 30 ml./hr., and the fraction containing the product was evaporated to dryness. The brownish residue was dissolved in ethanol which was evaporated; this was repeated several times, and the residue was dissolved in 1 ml. of alcohol. Upon addition of anhydrous ether a white solid precipitated. The triethylammonium salt of 3-methyluridine 3′-phosphate (47 mg., 80%) was collected by centrifugation, washed with anhydrous ether, and dried over P_2O_5 for 24 hr.

Anal. Calcd. for $C_{16}H_{30}N_3O_9P \cdot 3H_2O$: C, 38.94; H, 7.35; N, 8.52; P, 6.28. Found: C, 39.00; H, 6.56; N, 8.82; P, 6.07.

2′,5′-Di-*O*-trityl-5-methyluridine was prepared from 5-methyluridine[22] by a procedure identical with that for 2′,5′-di-*O*-trityl-3-methyluridine (method A). The product melted at 273–274°, yield 37%.

Anal. Calcd. for $C_{48}H_{42}N_2O_6$: C, 77.63; H, 5.66; N, 3.77. Found: C, 77.53; H, 5.93; N, 3.67.

2′,5′-Di-*O*-trityl-5-methyluridine 3′-phosphate (IIIa) was prepared by a procedure similar to that for 2′,5′-di-*O*-trityluridine 3′-phosphate.[6] Lithium hydroxide was used in place of NaOH for removal of the cyanoethyl group; yield 69%. This product sublimes.

Anal. Calcd. for $C_{48}H_{41}Li_2N_2O_9 \cdot 4H_2O$: C, 63.57; H, 5.41; N, 3.10. Found: C, 63.55; H, 5.57; N, 3.33.

5-Methyluridine 3′-Phosphate.—The lithium salt of 2′,5′-di-*O*-trityl-5-methyluridine 3′-phosphate (90.6 mg., 0.1 mmole) was suspended in 6 ml. of 80% acetic acid. The mixture was heated for 2 hr. at 100° and filtered, and the filtrate was evaporated to dryness. Traces of acetic acid were removed from the residue by repeated dissolution in water and ethanol and evaporation. The product was dissolved in water and passed through a column of Dowex-50 (H^+) ion-exchange resin to remove Li^+. The free acid was neutralized with NaOH. This product was homogeneous on the basis of paper chromatography and electrophoresis. The yield was 80% based on spectrophotometric analysis (ϵ_{267} 9.6 × 10^3).

2′,3′-*O*-Isopropylidene-5-methyluridine (IIa).—This method is based on that of Hampton.[23] To a suspension of 1.032 g. (4 mmoles) of 5-methyluridine[22] in 40 ml. of acetone was added 0.136 g. (0.4 mmole) of di-*p*-nitrophenyl phosphate and 4 ml. (33 mmoles) of 2,2-dimethoxypropane. The mixture was stirred 2 hr. at room temperature to give a clear solution. Dowex-1 × 8 (OH^-) ion-exchange resin (washed with acetone and air dried, 2.4 g.) was added, and the mixture was stirred 3 hr. at room temperature. Paper chromatography of the reaction mixture in solvents A and B showed a single ultraviolet-absorbing spot. The resin was filtered and washed with acetone, and the combined filtrates were evaporated to dryness. The product was subjected to partition chromatography on a column (2.54 × 76 cm.) containing 150 g. of Celite-545 using solvent C. The fraction containing the product was evaporated to dryness and the residue was recrystallized from ethyl acetate and petroleum ether (b.p. 66–75°); yield 0.944 g. (79%), m.p. 124°. The product prepared by another method melted at 112°.[15]

Anal. Calcd. for $C_{13}H_{18}N_2O_6$: C, 52.3; H, 6.04; N, 9.38. Found: C, 51.94; H, 6.45; N, 8.41.

2′,3′-*O*-Isopropylidene-5-methyluridine 5′-Phosphate.—2′,3′-*O*-Isopropylidene-5-methyluridine (0.61 g., 2.05 mmoles) was phosphorylated by cyanoethyl phosphate.[24] The crude pyridinium salt of the product was purified by converting it to the barium salt. To a solution of 1.46 g. of crude product in 25 ml. of water was added 1.42 g. of barium acetate (5.2 moles) and, after standing at room temperature for 2 hr., the barium phosphate was filtered off. Three volumes of ethanol was added to the solution, and the product was collected by centrifugation. It was redissolved in water and passed through a column of Dowex-50 × 8 (pyridinium form). The filtrate was lyophilized and the pyridinium salt thus obtained was dried over P_2O_5 in a desiccator at room temperature for 2 days (yield 98%). It moved as a single ultraviolet-absorbing spot on electrophoresis.

Anal. Calcd. for $C_{18}H_{24}N_3O_9P \cdot H_2O$: C, 45.3; H, 5.48. Found: C, 44.58; H, 5.59.

3′-Mesyl-2′,5′-di-*O*-trityl-5-methyluridine (V).—Methane sulfonyl chloride (0.08 ml., 1 mmole) was added to an ice-cold solution of 2′,5′-di-*O*-trityl-5-methyluridine (0.35 g., 0.47 mmole) in 5 ml. of anhydrous pyridine. The mixture was shaken and kept at 5° for 16 hr., after which time it was filtered and washed with 10 ml. of pyridine. Ethanol (0.1 ml.) was added to the pyridine solution and after 2 hr. the solvent was evaporated. The residue was crystallized from aqueous ethanol mixture; yield 0.285 g. (74%), m.p. 222–223°.

Anal. Calcd. for $C_{49}H_{44}N_2O_8S$: C, 71.70; H, 5.37; N, 3.41; S, 3.90. Found: C, 71.58; H, 5.53; N, 3.61; S, 3.98.

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(23) A. Hampton, *ibid.*, **83**, 3640 (1961).

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TABLE III

ANALYSIS OF DIRIBONUCLEOSIDE PHOSPHATES

Dinucleoside phosphate (triethylammonium salt)	Formula	Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N	Yield, %
Uridylyl-(3′→5′)-3-methyluridine·3H₂O (IIIb)	$C_{25}H_{40}N_5O_{14}P\cdot 3H_2O$	41.72	6.44	9.73	41.95	6.08	9.68	20
Uridylyl-(5′→3′)-3-methyluridine·2H₂O (IIIa)	$C_{25}H_{40}N_5O_{14}P\cdot 2H_2O$	42.79	6.32	9.98	42.84	6.21	10.24	17
3-Methyluridylyl-(3′→5′)-3-methyluridine (IIIc)	$C_{26}H_{42}N_5O_{14}P$	45.94	6.23	10.34	45.73	6.37	10.02	14
5-Methyluridylyl-(3′(2′)→5′)-5-methyluridine[a] (IIId)	$C_{33}H_{58}N_6O_{17}P$	47.08	6.90	9.98	47.00	7.15	9.23	15

[a] One molecule of triethylammonium carbonate crystallized with the product.

2,3′-Anhydro-1-(2,5-di-*O*-trityl-β-D-xylofuranosyl)thymine (VI).—3′-Mesyl-2′,5′-di-*O*-trityl-5-methyluridine (0.155 g., 0.188 mole) and sodium benzoate (0.5 g.) were dissolved in 10 ml. of dimethylformamide and the solution was maintained at 130° for 18 hr., then cooled and poured into 200 ml. of water. This solution was stirred 2 hr. and the product was obtained by filtration. The product was not purified further but was treated directly with alkali as described below.

1-(2,5-Di-*O*-trityl-β-D-xylofuranosyl)-5-methyluridine (VII).—The anhydro derivative obtained from the above reaction was dissolved in 25 ml. of ethanol containing 1 ml. of 1 *N* NaOH solution. The solution was refluxed 45 min. and cooled, and the solution was neutralized with 80% acetic acid. The precipitated product was obtained by filtration and recrystallized from ethanol; m.p. 261–262°.

Anal. Calcd. for $C_{48}H_{42}N_2O_6\cdot H_2O$: C, 75.8; H, 5.79; N, 3.68. Found: C, 76.45; H, 6.10; N, 3.85.

1-(β-D-Xylofuranosyl)-5-methyluridine (VIII).—The preceding product was suspended in 20 ml. of ethanol and 0.1 ml. of concentrated HCl was added; the solution was refluxed for 1 hr., cooled, and filtered, and the clear filtrate was evaporated to a sirup. The sirup was dissolved in 10 ml. of water and extracted twice with $CHCl_3$ to remove triphenylcarbinol. The product, when examined by paper chromatography in solvents A through F, exhibited R_f values which agreed with those of an authentic sample of 1-(β-D-xylofuranosyl)-5-methyluridine[22] and did not agree with those of the ribo- and arabinofuranosyl derivatives.

This nucleoside was further characterized by removing and identifying the sugar. A sample of the nucleoside (3 mg.) and rhodium-on-alumina catalyst (0.1 g.)[25] were added to 3 ml. of water and, after adjusting to pH 4.0 with HCl, the mixture was hydrogenated at atmospheric pressure for 4 hr. The catalyst was removed, the pH was adjusted to 2 with HCl, and the solution was heated at 100° for 90 min. This solution contained only one sugar whose R_f value corresponded to that of xylose on paper chromatography in solvent systems A–E.

N^3,$O^{3'}$,5-Trimethyl-2′,5′-di-*O*-trityluridine (IVb).—This reaction was carried out according to the method of Kuhn and coworkers.[26] 2′,5′-Di-*O*-trityl-5-methyluridine (0.83 g., 1.12 mmoles), silver oxide (2.6 g. 11.2 mmoles), and iodomethane (26.1 g., 184 mmoles) were mixed together, and 3 ml. of N,N-dimethylformamide was added. The mixture was stirred and refluxed for 9 hr. Chloroform (35 ml.) was added, and the silver residue was filtered off. The precipitate was washed on the filter with six 10-ml. portions of $CHCl_3$, and the total chloroform filtrate was extracted with 250 ml. of water containing 2 g. of sodium cyanide. The chloroform was washed twice with water, dried (Na_2SO_4), and evaporated to a gum *in vacuo*. The residue was dissolved in hot benzene and ether was added to incipient cloudiness after which the product crystallized; yield 0.73 g. (84%), m.p. 263°.

Anal. Calcd. for $C_{50}H_{46}N_2O_6$: C, 77.92; H, 5.97; N, 3.64. Found: C, 77.71; H, 6.16; N, 3.56.

N^3,$O^{3'}$-Dimethyl-2′,5′-di-*O*-trityluridine (IXa) was prepared in 80% yield from 2′,5′-di-*O*-trityluridine as described above for the 5-methyluridine derivative. It melted at 228°.

Anal. Calcd. for $C_{49}H_{44}N_2O_6$: C, 77.78; H, 5.82; N, 3.70. Found: C, 77.90; H, 6.03; N, 3.45.

3-*O*-Methylribose Obtained from N^3,$O^{3'}$-Dimethyl-2′,5′-di-*O*-trityluridine (IXa) or N^3,$O^{3'}$,5-Trimethyl-2′,5′-di-*O*-trityluridine (IXb).—The nucleoside derivative (20 mg.) was suspended in 10 ml. of 80% acetic acid, and the mixture was heated at 100° for 90 min. The clear solution was evaporated *in vacuo* to dryness and 4 ml. of water was added to the residue. Triphenylcarbinol was filtered off. The methylated nucleoside was reduced and hydrolyzed as described above for 1-(β-D-xylofuranosyl)-5-methyluridine. Examination of the products of hydrolysis showed in each case the presence of only one sugar which corresponded to an authentic sample of 3-*O*-methylribose, prepared by the method of Barker[27] on paper chromatography in solvent systems B–E and on electrophoresis in the presence of borate ion.

Diribonucleoside Phosphates (General Procedure.) The pyridinium salt of the 2′,5′-di-*O*-tritylribonucleoside 3′-phosphate (I, 0.5 mmole) and the 2′,3′-isopropylideneribonucleoside (II, 1 mmole) were dissolved in 10 ml. of anhydrous pyridine and the solution was concentrated to dryness. Evaporation with pyridine was repeated several times and finally the residue was dissolved in 25 ml. of anhydrous pyridine and dicyclohexylcarbodiimide (DDC, 4.1 g.) was added. The reaction flask was flushed with dry nitrogen, stoppered tightly, and allowed to stand at room temperature for 6 days. On day 3, more DCC (2 g.) was added. On day 6, water (5 ml.) was added and the mixture was left at room temperature overnight. After filtering, the mixture was evaporated to dryness *in vacuo* and traces of pyridine were removed by evaporating with water, then ethanol. The residue was treated with 80% acetic acid for 30 min. at 100° in order to remove blocking groups and the product was purified on a column (1.9 × 36 cm.) of DEAE cellulose (carbonate). The column was developed with a linear gradient of triethyl ammonium carbonate (0.01 *M* → 0.1 *M*, pH 8.6, total volume 2 l.) according to the general procedure described previously.[6] The fraction containing the product was concentrated to dryness *in vacuo*, and excess triethylammonium bicarbonate was removed by repeated suspension of the residue in absolute ethanol and followed by evaporation of the solvent. The residue was dissolved in a minimum amount of absolute ethanol (2 ml.) and, on addition of anhydrous ether, the product precipitated out of solution. The product (triethylammonium salt) was collected by centrifugation, washed several times with anhydrous ether, and dried over P_2O_5 for 24 hr. The yield and analytical data of the four diribonucleoside phosphates synthesized are presented in Table III. The mixed isomers of 5-methyluridylyl-(3′(2′)→5′)-5-methyluridine were separated on a column of Dowex-1 × 2 residue as shown in Figure 1. Other intermediates utilized in this work, 2′,3′-isopropylidene-3-methyluridine[28] and 2′,5′-di-*O*-trityluridine 3′-phosphate,[6] were prepared as described previously.

5-Methyluridylyl-(5′→5′)-5-methyluridine.—Pyridinium 2′-3′-*O*-isopropylidene-5-methyluridine 5′-phosphate (2.2 mmoles) was condensed with 2′,3′-*O*-isopropylidene-5-methyluridine (4.4 mmoles) by the method described above. The crude product was dissolved in 50 ml. of 80% acetic acid, and the mixture was heated at 100° for 1 hr. The acetic acid was evaporated *in vacuo*, and the residue was dissolved in water which was evaporated. This was repeated until all traces of acetic acid had been removed. The product was dissolved in 20 ml. of water and the pH was adjusted to 8.5 with NH_4OH. The solution was applied to a column (42 × 1.2 cm.) of Dowex-1 × 2 (formate) 200–400 mesh. The column was developed with 3750 ml. of a linear gradient of ammonium formate buffer, pH 4.5, 0.02 *M* → 0.13 *M*. The product was eluted between the 1950 and the 2925 ml. marks. The solution corresponding to this fraction was passed through a column of Dowex-50 × 8 (H^+) in order to remove ammonium ions. The filtrate was lyophilized to yield 188 mg. (15%) of 5-methyluridylyl-(5′→5′)-5-methyluridine. This material moved as a single spot when subjected to electrophoresis and on paper chromatography in solvents A and B. The product was completely degraded by snake venom phosphodiesterase to yield 1 mole each of 5-methyluridylic acid and 5-methyluridine.

(25) W. E. Cohn and D. G. Doherty, *J. Am. Chem. Soc.*, **78**, 2863 (1956).

(26) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).

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(28) N. K. Kochetkov, E. I. Budowsky, and V. N. Shibaev, *Biochim. Biophys. Acta*, **53**, 415 (1961).

Hyperchromicity (Table II).—Alkaline digestion could not be used for those compounds with a 3-methyluracil residue since this moiety undergoes degradation in alkali.[10] Hyperchromicity was determined by measuring the optical density at the λ_{max} of a solution of the diribonucleoside phosphate at pH 6.0 before and after digestion with purified snake venom diesterase (Worthington Biochemicals). The digestion was carried out as follows. To 2 ml. of an aqueous solution of the diribonucleoside phosphate was added 0.2 ml. of 0.1 *M* Tris buffer (pH 8.2), 0.025 ml. of 0.1 *M* $MgSO_4$ solution, and 0.1 ml. of a freshly prepared enzyme solution (5 mg./ml.). Incubation was carried out at 37° in a jacketed compartment of a Cary Model 14 spectrophotometer and was judged complete when the optical density at λ_{max} remained steady for several hours. In one experiment 5-methyluridylyl-(3′→5′)-5-methyluridine was hydrolyzed with 0.5 *N* NaOH and the hyperchromicity measured at pH 6.0 was similar to that obtained by use of the enzyme. In separate experiments the effectiveness of the enzyme treatment was tested on more concentrated samples of each of the diribonucleoside phosphates under similar conditions. The products of the digestion were separated by electrophoresis and in no case could the unchanged diribonucleoside phosphate be detected.

Ribonuclease Treatment.—To the diribonucleoside phosphate (0.2 mg.) in 100 λ of water was added 25 λ of 0.1 *M* Tris buffer solution pH 7.0 and 25 λ of pancreatic ribonuclease solution (1 mg./ml. of Worthington crystalline, Code R), and the solution was incubated at 37° for 15 hr. The reaction mixture was examined by means of electrophoresis.

The following compounds were not hydrolyzed: 3-methyluridylyl-(3′→5′)-3-methyluridine, 3-methyluridylyl-(3′→5′)-uridine, and 5-methyluridylyl-(2′→5′)-5-methyluridine. The following compounds were completely hydroyzed to the 3′ nucleotide and nucleoside: uridylyl-(3′→5′)-3-methyluridine and 5-methyluridylyl-(3′→5′)-5-methyluridine.

When a mixture of equal parts of 5-methyluridylyl-(2′→5′)-5-methyluridine and 5-methyluridylyl-(3′→5′)-5-methyluridine was subjected to these hydrolytic conditions, no breakdown products were detected.

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Derivatives of Fluorene. XXI. New Halogenofluorenes. II.[1a] Further Potential Antitumor Agents

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Further polyhalofluorene derivatives have been found effective against certain tumors in mice, and data are presented for some of the compounds reported earlier.[1a] On the basis of present data, the most active compounds in this group are: N-2-(1,3-dichloro-7-nitrofluorenyl)acetamide (Adenocarcinoma 755), N-2-(7-bromo-1,3,4-trichlorofluorenyl)acetamide (Sarcoma 180), N-2-(1,3,7-trichlorofluorenyl)acetamide (Cloudman melanoma), 1,3-dibromo-7-nitro-2-fluorenamine (Adencarcinoma and Sarcoma), 1,3,7-tribromo-2-fluorenamine (Adenocarcinoma), and N-2-(1,3,4,6,7-pentachlorofluorenyl)acetamide (Sarcoma). The position of the chlorine atom, designated earlier[1a] as 4(?) on the basis of infrared absorption, is confirmed by synthesis. Some compounds we described as 2,3,7-trisubstituted fluorenes are shown to be 2,4,7-derivatives, in agreement with recent reports,[2,3] but still in marked disagreement with an earlier paper.[4] 2,7-Dibromo-, -dichloro-, and -difluorofluorenes and the three corresponding 9-oxofluorenes are all nitrated at the 4-position. Chlorination of N-2-(4-chlorofluorenyl)acetamide, N-2-fluorenylformamide, and ethyl N-2-fluorenylcarbamate, as found earlier[1a] with N-2-fluorenylacetamide, leads to the 1,3,4,7-tetrachloro compounds. 2,3,7-Trichlorofluorene is prepared by three routes. Acetylation of some relatively weak amines by merely shaking at room temperature with glacial acetic acid is described. Bromination of N-2-(7-bromofluorenyl)acetamide with N-bromosuccinimide in dimethylformamide gives the 3-bromo derivative.

Our previous report in this series[1a] included a number of polyhalogenated fluorene derivatives which show antitumor activity in animals. In expanding this area, in a search for further compounds with biological activity, we have first confirmed the supposed 4-position for a number of these substances. In all cases in the previous paper[1a] the position designation 4(?) is, in fact, 4. In addition to this minor point, we wish, in the present paper, to clarify a discrepancy in the literature and, in so doing, correct a position designation in the names of a number of the reported compounds in the previous paper. In brief, eight di- and trichlorofluorene derivatives (shown in Table I), named 2,3,7-substituted fluorenes, are 2,4,7-substituted fluorenes.

TABLE I

No.[a]	Correct name
XIX	9-Bromo-2,4,7-trichlorofluorene
XX	2,4,7-Trichlorofluoren-9-ol
XXI	9-Oxo-2,4,7-trichlorofluorene
LII	4-Amino-2,7-dichlorofluoren-9-ol
LIII	2,7-Dichloro-9-oxo-4-fluorenamine
LXXIII	2,4,7-Trichlorofluorene
In text only	2,7-Dichloro-4-nitrofluorene
In text only	2,7-Dichloro-4-fluorenamine

[a] See ref. 1a, Table II, or text.

As noted,[1a] there are discrepancies apparent in comparing some of our compounds with those of the same name described by Kretov, *et al.*[4] The latter claimed to have obtained the 3-nitro derivative, by nitrating 2,7-dichlorofluorene, and from this 2,3,7-trichloro-

(1) (a) For Halogenofluorenes. I, see H.-L. Pan and T. L. Fletcher, *J. Med. Chem.*, **7**, 31 (1964). (b) Supported in part by a grant (Ca-01744) from the National Cancer Institute, National Institutes of Health, and in part by Research Career Development Award 5-K3-GM-14,991 (T. L. F.).

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(3) A. Sieglitz, *ibid.*, **97**, 3392 (1964).

(4) In particular see paragraph 2 and footnotes 6–8 in ref. 1a.