OLIGONUCLEOTIDIC COMPOUNDS. XXXIX.*
TRIESTER SYNTHESIS OF OLIGONUCLEOTIDES IN THE RIBO SERIES

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The triester synthesis of a ribooligonucleotidic chain starting from 5′-O-formyl-2′-O-tetrahydropyranyluridine 3′-(2-cyanoethyl)phosphate (*VII*) or 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-(2,2,2-trichloroethyl)phosphate (*VIII*) has been studied. Reaction of compounds *VII* or *VIII* with 2′,3′-O-ethoxymethyleneuridine (*IX*) in the presence of 2,3,5-triisopropylbenzenesulfonyl chloride and the subsequent removal of the 5′-O-acyl group affords the 2-cyanoethyl or 2,2,2-trichloroethyl ester of 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneuridine (*XIII* and *XV*, resp.). Condensation of compound *XIII* with *VII* gave 5′-O-formyl-2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneuridine[bis-P^1,P^2-(2-cyanoethyl) ester] (*XVI*). An analogous double extension of compound *XV* afforded after removal of protecting groups the uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine (*XIX*).

The general synthesis of the internucleotide bond consists in activation of the phosphoryl group of one component in the presence of a suitably protected second component, bearing a free hydroxylic function. The activation is performed with N,N′-dicyclohexylcarbodiimide[1] or aromatic sulfonyl chlorides[2,3]. This path affords usually a high yield (55–80%) of the dinucleoside phosphate both in the deoxyribo and the ribo series. In the reported syntheses of oligonucleotides, the 5′-phosphate (deoxyribo series) and the 3′-phosphate (ribo series) are used as the phosphate component. In the former case, the reacting phosphoryl group is in a farly more suitable steric position than in the latter where the 3′-phosphate is in a close neighbourhood of the *cis*-2′-hydroxylic function, bearing a bulky protecting group. This circumstance has a considerable influence on the yields of the trinucleoside diphosphate while in the first step, namely, in the synthesis of the dinucleoside phosphate, this effect is almost negligible. In the deoxyribo series, the yields may be maintained at a satisfactory level by the use of a greater excess of the phosphate component[4]. On the other hand, the yields of trinucleoside diphosphates in the ribo series did not exceed in this Laboratory[5–7] the value of 20%, rarely 25%, in spite of the use of a great

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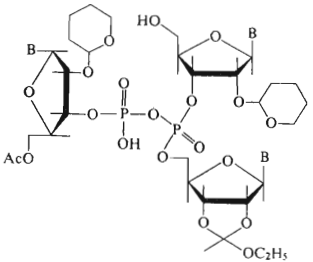
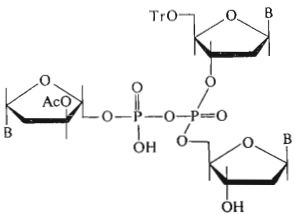
excess of the phosphate component. The main portion of the starting diribonucleoside phosphate is recovered. It is hardly possible to compare our yields with those obtained in other laboratories because of the practice to express the yields on the basis of the recovered unchanged dinucleoside phosphate[8,9] and not to mention the amount of the recovered material. An exception[9] from this practice is the statement of the yield (2 μmol) of uridylyl-uridylyl-uridine from the protected uridylyl-uridine (30 μmol).

As shown by our investigations[5], the diribonucleoside component is in the reaction mixture transformed to an intermediate with a highly decreased reactivity of its 5'-hydroxylic function. In view of the fact that activated phosphomonoesters may react with phosphodiesters under the formation of triesters of pyrophosphoric acid[10], it may be assumed in the present case that the protected nucleoside phosphate reacts in the presence of N,N'-dicyclohexylcarbodiimide with the internucleotidic



I



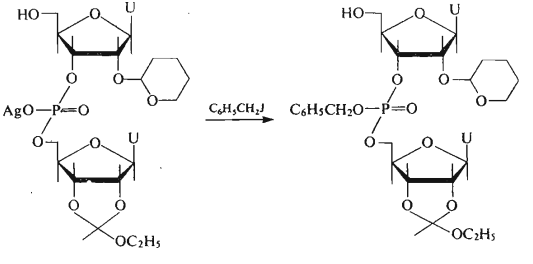
II

B = Heterocyclic base, Ac = $COCH_3$, Tr = substituted triphenylmethyl

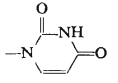
SCHEME 1

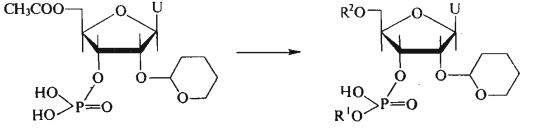
bond of the hydroxylic component under the formation of asymmetric pyrophosphates of the type *I* (ribo series) or *II* (deoxyribo series). There is nothing known on the stereochemistry of the types *I* or *II*. Notwithstanding, it may be assumed from results obtained in phosphorylations of the hydroxylic function (*i.e.*, in the synthesis of a further internucleotidic bond) and from Scheme 1 and 2 that the hydroxylic function is farly more sterically hindered with the ribo compound *I* than with the deoxyribo compound *II*.

The formation of compounds of the type *I* was considerably decreased, at least to our opinion, by the following modification. The protected dinucleoside phosphate[5] was pretreated with N,N'-dicyclohexylcarbodiimide and thereby converted to the symmetric pyrophosphoric acid tetraester with a more favourable stereochemistry of hydroxylic function. In fact, the yield of the trinucleoside diphosphate rose from 13 to 28% with the use of this modification.



III *IV*

U in formulae *III*—*XIX* represents 



V

VI, $R^1 = CH_2CH_2CN$, $R^2 = COCH_3$
VII, $R^1 = CH_2CH_2CN$, $R^2 = CHO$
VIII, $R^1 = CH_2CCl_3$, $R^2 = COCH_3$

SCHEME 2

A successful synthesis of oligonucleotidic chains, especially in the ribo series, should thus include protection of the acidic function of the internucleotidic bond. This aim might be most advantageously achieved by conversion of the internucleotidic bond into a dinucleoside alkyl phosphate and selective removal of the alkyl group in the final step of the synthesis. The triester of this type was prepared by reaction of the phosphodiester silver salt with an alkyl iodide[11]. Thus, the reaction of benzyl iodide with 2'-O-tetrahydropyranyluridylyl-(3'→5')-2',3'-O-ethoxymethyleneuridine (*III*) silver salt gave the triester *IV*. The latter ester was reacted with two equivalents of the pyridinium salt of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate (*V*) in the presence of N,N'-dicyclohexylcarbodiimide. Removal of the acetyl group with dilute aqueous ammonia, of the benzyl group by hydrogenolysis over palladium, and of the tetrahydropyranyl and ethoxymethylene group with aqueous acetic acid afforded uridylyl-(3'→5')-uridylyl-(3'→5')-uridine in 35% yield while the diester *XI* gives under analogous conditions 13% of the trinucleoside diphosphate It may be seen that the acidic function of the internucleotidic bond clearly interferes in the synthesis of a further internucleotidic bond. Notwithstanding, the overall yield of this modification (preparation of the silver salt, triester, and condensation) is not higher than that obtained by the direct synthesis from the diester *XI*. Consequently, this modification was abandoned.

It was finally the synthesis of phosphoric acid triesters by means of 2,3,5-triisopropylbenzenesulfonyl chloride[12] which opened a passable route to oligonucleotidic compounds containing the phosphotriester bond and made thereby possible investigations on the use of triesters in the synthesis of oligonucleotides. In the deoxyribo series, the internucleotidic bond has been hitherto protected by the 2-cyanoethyl group[12-14] or the 2,2,2-trichloroethyl group[15,16]. In the ribo series this method has been proposed for the 2,2,2-trichloroethyl group[17] and attempted with the 2-cyanoethyl group[18]. The key intermediates are represented by 2',5'-di-O-substituted uridine 3'-alkyl phosphates obtained in turn by reaction of the 2',5'-di-O-substituted uridine with 2-cyanoethyl phosphate or 2,2,2-trichloroethyl phosphate. Because of the difficult preparative accessibility of the starting di-O-substituted nucleosides, the generalisation of this approach is rather problematic.

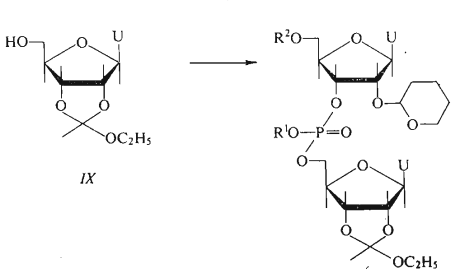
In this Laboratory, we have been for a longer period of time interested in the synthesis of oligonucleotides of the ribo series. The general feature of our approach reported in numerous papers of this Series consists in the use of alkali-stabile protecting groups both for the *cis*-diol system of the nucleoside situated at the 5'-terminus of the chain, and for the 2'-hydroxylic function of the corresponding nucleotidic units. These units are represented by 5'-O-acetyl-2'-O-tetrahydropyranylribonucleoside 3'-phosphates (in the case of the cytidine derivative, the more readily removable 1-ethoxyethyl group is used instead of the tetrahydropyranyl group) which are prepared by the following general procedure (the yields of all steps are almost quantitative): Nucleoside 3'(2')-phosphate → nucleoside 2',3'-cyclic phos-

phate → 5′-O-acetylnucleoside 2′,3′-cyclic phosphate → 5′-O-acetylnucleoside 3′-phosphate → 5′-O-acetyl-2′-O-tetrahydropyranyl (or 1-ethoxyethyl)nucleoside 3′-phosphate.

This approach, designed originally for pyrimidine nucleotides (the 2′,3′-cyclic phosphate is split with the use of pancreatic ribonuclease[19,20]) has been recently extended for purine nucleotides with the use of the extract from Takadiastase[7]. All the specifically protected ribonucleoside 3′-phosphates thus obtained may be readily converted to phosphodiesters by the N,N′-dicyclohexylcarbodiimide induced esterification with 2-cyanoethanol (as shown in an earlier paper of this Series[21]) or 2,2,2-trichloroethanol. The resulting phosphodiesters represent key intermediates for the triester synthesis of the ribooligonucleotidic chain.

In the present paper, we wish to report the stepwise synthesis of the oligonucleotidic chain in the ribo series with the use of the triester method along with some earlier experiments (dinucleoside benzyl phosphate). In the ribo series, the position of the 2′-O-substituted hydroxylic function in the close vicinity of the reacting phosphodiester is expected to exert a deleterious effect on the course of the reaction. Thus, the synthesis of the diribonucleoside phosphate[18] by the triester method gave the yield of about 50% while the yield of an analogous reaction in the deoxyribo series[13] is 64%.

The first series of experiments was performed with the use of 2-cyanoethyl as the protecting group. The starting 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-(2-cyanoethyl)phosphate (*VI*) was prepared by modification of an earlier reported method[22] without removing the acetyl group and isolated by precipitation from pyridine–ether. Reaction of phosphate *VI* and two equivalents of 2′,3′-O-ethoxymethyleneuridine (*IX*) in the presence of 2,3,5-triisopropylbenzenesulfonyl chloride afforded 5′-O-acetyl-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2-cya-



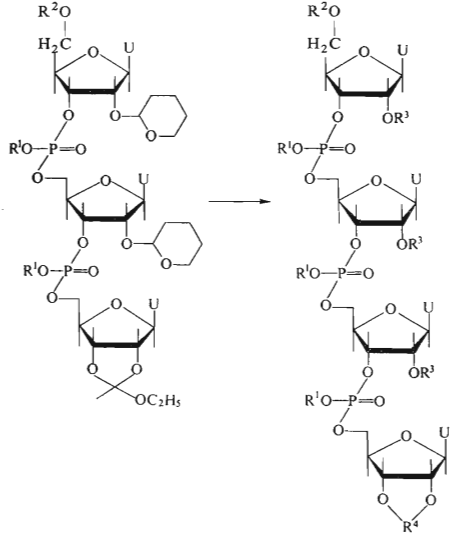
IX

X, $R^1 = CH_2CH_2CN$, $R^2 = COCH_3$
XI, $R^1 = R^2 = H$
XII, $R^1 = CH_2CH_2CN$, $R^2 = CHO$
XIII, $R^1 = CH_2CH_2CN$, $R^2 = H$
XIV, $R^1 = CH_2CCl_3$, $R^2 = COCH_3$
XV, $R^1 = CH_2CCl_3$, $R^2 = H$

SCHEME 3

noethyl) ester] (*X*) in 51% yield. The product was isolated analogously to other triester derivatives reported in the present paper, namely, by preparative chromatography on a thin layer of silica gel in the solvent mixture chloroform–methanol.

Unfortunately, the resulting derivative *VI* cannot be used in further synthetic steps because of the high alkali-lability of the 2-cyanoethyl group. In other words, it is not possible to remove the 5′-O-acetyl group without the simultaneous removal of the 2-cyanoethyl group. For this reason, compound *VI* was replaced by the analogous 5′-O-formyl derivative *VII* which was prepared from compound *VI* by deacetylation and formylation with formic acetic mixed anhydride. The ready removability of the formyl group has been reported in one of the first papers of this Series[23]. The present investigation of the hydrolysis of formyl groups with the use of 2′,3′,5′-tri-O-formyluridine has shown that the formyl group at the 5′-hydroxylic function may be rapidly removed at pH 8·5. At this pH value, the 2-cyanoethyl group at the phosphotriester is relatively stable.



XVI, $R^1 = -CH_2CH_2CN$, $R^2 = -CHO$

XVII, $R^1 = -CH_2CCl_3$, $R^2 = H$

XVIII, $R^1 = -CH_2CCl_3$, $R^2 = -COCH_3$, $R^3 =$  , $R^4 = \rangle CHOC_2H_5$

XIX, $R^1 = R^2 = R^3 = H$, $R^4 = H, H$

Condensation of compounds *VII* and *IX* by the action of 2,3,5-triisopropylbenzenesulfonyl chloride afforded 5'-O-formyl-2'-O-tetrahydropyranyluridylyl-(3'→5')-2',3'-O-ethoxymethyleneuridine [P-(2-cyanoethyl) ester] (*XII*) in 57% yield. Removal of the formyl group at pH 8·5 led to the corresponding derivative *XIII* in 67% yield. The subsequent condensation of compounds *XIII* and *VII* afforded the trinucleotide derivative *XVI* in 35% yield.

The other series of experiments was performed with the use of 2,2,2-trichloroethyl as the protecting group for the phosphodiester bond. The sufficient alkalistability of 2,2,2-trichloroethyl-group-containing phosphotriesters made possible the use of acetyl group for protecting of the 5'-hydroxylic function. The key intermediate, namely, 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-(2,2,2-trichloroethyl) phosphate (*VIII*) was prepared by reaction of the phosphate *V* and 2,2,2-trichloroethanol in the presence of N,N'-dicyclohexylcarbodiimide. Compound *VIII* was isolated both in the form of a pyridinium salt by precipitation from pyridine with ether or as the triethylammonium salt by chromatography on a column of silica gel in chloroform–methanol under the addition of triethylamine.

Reaction of the pyridinium salt of compound *VIII* with 2 equivalents of compound *IX* (two days at room temperature) gave 5'-O-acetyl-2'-O-tetrahydropyranyluridyl-(3'→5')-2',3'-O-ethoxymethyleneuridine [P-(2,2,2-trichloroethyl) ester] (*XIV*) in 93% yield. Under analogous conditions, the triethylammonium salt of compound *VIII* gave the ester *XIV* in 84% yield. Removal of 5'-O-acetyl group with ammonia led to compound *XV* in 85% yield. Condensation of compound *XV* with three equivalents of the diester *VIII* and the subsequent deacetylation afforded 2'-O-tetrahydropyranyluridylyl-(3'→5')-2'-O-tetrahydropyranyluridylyl-(3'→5')-2',3'-O-ethoxymethylene-uridine [bis-P^1,P^2-(2,2,2-trichloroethyl) ester] (*XVII*) in 24% yield (one day at room temperature) or in 32% yield (two days at room temperature).

The further condensation of compound *XVII* with three equivalents of compound *VIII* (two days at room temperature) afforded the fully protected tetranucleotide *XVIII* in 19% yield. Removal of the 2,2,2-trichloroethyl group by the action of zinc in a mixture of pyridine and acetic acid at 20°C, and of the acetyl group and the acidolabile groups gave 49% of uridylyl-(3'→5')-uridylyl-(3'→5')-uridylyl-(3'→5')-uridine (*XIX*).

As indicated by the above experiments, the triester synthesis affords even in the ribo series higher yields in all steps. Also in this case, the yields decrease with the growing chain-length because of the statistically lower accessibility of the free hydroxylic function. This effect may be mitigated by the use of a greater excess of the active component and higher concentrations of the reaction mixture. In comparison to the 2-cyanoethyl group, the 2,2,2-trichloroethyl group proved more suitable in the synthesis of protected oligonucleotidic compounds since the removal of the 5'-O-acetyl group may be performed in a high yield. The final result, namely, the preparation of the unprotected oligonucleotide is somewhat less satisfactory since

the removal of the 2,2,2-trichloroethyl group and the isolation of the product is not by far quantitative[16]. From methods serving to remove the 2,2,2-trichloroethyl group[16]), the action of zinc in pyridine–acetic acid at room temperature proved as the most advantageous. In contrast to the other method[16], this does not affect the acidolabile protecting groups.

The possibility to separate the intermediates from the starting material by thin-layer chromatography decreases with the growing chain-length and it is practically impossible to isolate the tetranucleotide in a pure state. For the synthesis of longer chains it will be necessary to apply condensation of oligonucleotidic blocks instead of the one-unit extension.

EXPERIMENTAL

Methods

Paper chromatography was performed by the descending technique on paper Whatman No 1 in the solvent systems S_1, 2-propanol–concentrated aqueous ammonia–water (7 : 1 : 2), and S_2, 2-propanol–concentrated aqueous ammonia–water (6 : 1 : 3). For the R_F values see Table I.

Paper electrophoresis was performed by the technique of Markham and Smith[24] on paper Whatman No 1 in the buffer solution E_1, 0·05M triethylammonium hydrogen carbonate (pH 7·5). For the electrophoretical mobilities see Table I.

Thin-layer chromatography was performed on Silufol UV_{254} sheets (produced by Kavalier Glass Works, Votice, Czechoslovakia). Preparative thin-layer chromatography was performed on a loose layer (20 × 20 × 0·3 cm) of silica gel according to Pitra (60—100 micron; produced by Service Laboratories of our Institute, Prague-Suchdol) in the solvent systems T_1, chloroform–methanol (9 : 1); T_2, chloroform–methanol (8 : 2); T_3, chloroform–methanol (95 : 5); T_4, chloroform–methanol–triethylamine (90 : 9 : 1); T_5, chloroform–methanol–triethylamine (85 : 14 : 1); and T_6, chloroform–methanol–triethylamine (93 : 6 : 1). Elution of compounds from the thin layer was performed with the solvent mixture T_E, chloroform–methanol (1 : 1).

Pancreatic ribonuclease degradations were performed with the use of about 2 μmol of the test substance in 50 μl of 0·1M-Tris-HCl buffer solution (pH 8·5), containing 100 μg of the enzyme (pancreatic ribonuclease Sigma, St. Louis, U.S.A.).

Spectroscopic measurements were performed on a Beckman DU type apparatus in methanol (with protected nucleotides) and 0·01M-HCl (with unprotected substances). Quantitative measurements were performed at 260 nm with the use of the extinction coefficient equal to 10000 for the uridine unit. One optical density unit is defined as that amount of the test substance which dissolved in 1 ml of a solvent (methanol or 0·01M-HCl) shows in 1 cm cell at 260 nm the absorbancy equal to one.

Starting Compounds and Reagents

Pyridine was dried for several weaks over calcium hydride, filtered, and stored over molecular sieves Potassit 3A *in globulis* (Slovnaft, Bratislava, Czechoslovakia). Dimethylformamide was distilled with 5% by weight of phosphorus pentoxide and the distillate stored over molecular sieves. 2,3,5-Triisopropylbenzenesulfonyl chloride was purchased from Aldrich Chemical Company, Milwaukee, U.S.A.

Prior to the synthesis of the internucleotidic bond, the reaction mixtures were dehydrated by evaporation with five portions of pyridine at 20°C/1 Torr and stored in well-stoppered vessels in desiccators.

Pyridinium Salt of 5′-O-Acetyl-2′-O-tetrahydropyranyluridine 3′-Phosphate (*V*)

The calcium salt[20] of compound *V* (25 mmol) is dissolved by shaking with 50 ml of pyridinium Dowex 50 X 8 ion exchange resin (100—200 mesh) and 50 ml of 30% aqueous pyridine and the suspension is applied to a column (80 ml) packed with the same ion exchange resin. The column is eluted with 30% aqueous pyridine and the eluate (400 ml) is evaporated at 20°C/1 Torr under occasional additions of pyridine to the consistence of a sirup. The sirup is evaporated with six 100 ml portions of pyridine and the final residue is dissolved in 100 ml of pyridine. This stock solution is stored at +4°C. As shown by chromatography of an aliquot in the solvent system S_1 and absorbancy of eluates of the corresponding bands, the stock solution contains 20 mmol (80%) of compound *V* and 0·8 mmol of 5′-O-acetyluridine 3′-phosphate.

2′-O-Tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine P-Benzyl Ester (*IV*)

Silver trifluoroacetate (100 mg) in ether (20 ml) was added to a solution of 2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine triethylammonium salt[5] (*XI*; 0·2 mmol) in ethanol (2 ml). The precipitate was collected by centrifugation and washed with two 20 ml portions of 1 : 20 ethanol–ether and finally with ether. After air-drying for 2 hours, the precipitate was dried for several hours at 40°C/0·1 Torr. The resulting silver salt *III* (160 mg) was heated for 2 hours at 60°C with 50 mg of benzyl iodide in acetonitrile (2 ml). The mixture was allowed to cool, diluted with acetonitrile (10 ml), centrifuged, and the supernatant evaporated at 35°C/15 Torr. The residue was dissolved in dioxane (2 ml), the weak turbidity removed by centrifugation, the supernatant diluted with n-heptane to the volume of 20 ml, the precipitate collected with suction, and washed with n-heptane to afford 82 mg (53 %) of compound *IV*. The analytical sample was reprecipitated from dioxane with n-heptane. For $C_{33}H_{41}N_4O_{16}P$ (780·7) calculated: 50·70% C, 5·28% H, 7·18% N, 3·98% P; found: 51·26% C, 5·43% H, 6·67% N, 3·52% P.

TABLE I

Paper Chromatography (solvent system S_1), Thin-Layer Chromatography (solvent system T_1) and Electrophoresis (buffer solution E_1), R_F Values and Mobilities

Compound	S_1	T_1	E_1	Compound	S_1	T_1	E_1
Up	0·12	—	1	*IX*	0·71	0·30	—
UpU	0·21	—	0·42	*X*	—	0·50	—
UpUpU	0·06	—	0·65	*XI*	0·54	0·10	0·40
UpUpUpU (*XIX*)	0·02	—	0·78	*XII*	—	0·46	—
IV	—	0·49	—	*XIII*	—	0·36	—
V	0·25	—	—	*XIV*	—	0·48	—
VI	0·68	—	0·55	*XV*	—	0·36	—
VII	0·65	—	0·59	*XVI*	—	0·35	—
VIII	0·78	—	0·47	*XVII*	—	0·24	—

Hydrogenolysis. A mixture of compound *IV* (3 mg), ethanol (0·5 ml), dioxane (0·5 ml), palladium oxide (20 mg), and 10% palladium on active charcoal (2 mg) was shaken under hydrogen for one hour, the catalyst removed by centrifugation, and the supernatant chromatographed in the solvent system S_1 to afford a single ultraviolet-absorbing spot, identical with that of the authentic diester *XI*.

Uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine

A solution of compound *IV* (0·1 mmol) and the pyridinium salt of 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate (*V*) in pyridine was dried by repeated coevaporations with pyridine. A mixture of the final residue, pyridine (5 ml), and N,N′-dicyclohexylcarbodiimide was allowed to stand at room temperature for 3 days and then treated with water (0·1 ml). After additional one hour, the solution was evaporated under diminished pressure and the residue kept in a mixture of methanol (10 ml), concentrated aqueous ammonia (10 ml), and n-heptane (10 ml) for 20 hours. The mixture was filtered, the aqueous layer evaporated under diminished pressure, the residue repeatedly coevaporated with ethanol to remove pyridine, and finally dissolved in ethanol. Palladium oxide (200 mg) and 10% palladium on active charcoal (20 mg) were added to the ethanolic solution, the whole shaken under hydrogen for four hours, and the catalyst filtered off. The filtrate was evaporated under diminished pressure to dryness and the residue heated in 20% aqueous acetic acid one hour at 50°C. The mixture was evaporated to dryness at 20°C/1 Torr, the residue dissolved in a little water, and the aqueous solution chromatographed for 2 days on one sheet of paper Whatman No 3 MM. The ultraviolet-absorbing band (R_{Up} value, 0·72) was eluted as usual to afford $1050 A_{260}$ (35%) of uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine, the pancreatic ribonuclease degradation of which led to uridine and uridine 3′-phosphate in the ratio 1 : 2·03 (by 99%).

2′,3′-O-Ethoxymethyleneuridine (*IX*)

The title compound was prepared according to the ref.[25] with small modifications. A mixture of uridine (10 mmol), dimethylformamide (5 ml), ethyl orthoformate (2·5 ml), and 6M-HCl in dimethylformamide (0·2 ml) was shaken until a homogeneous solution was obtained (about one hour) and then allowed to stand at room temperature for 20 hours. Pyridine (10 ml) was then added, the mixture evaporated at 20°C/1 Torr, and the residue coevaporated five times with pyridine to remove dimethylformamide. The final residue was made up with pyridine to the volume of 25 ml. The stock solution was stored at +4°C.

5′-O-Acetyl-2′-O-tetrahydropyranyluridine 3′-(2-Cyanoethyl) Phosphate (*VI*)

A mixture of the pyridinium salt of compound *V* (5 mmol), pyridine (25 ml), 2-cyanoethanol (5 ml), and N,N′-dicyclohexylcarbodiimide (5 g) was shaken for one hour and then allowed to stand at room temperature for 2 days. Water (10 ml) was added, the mixture kept one hour, washed with cyclohexane (20 ml), and filtered. The aqueous-pyridine layer was evaporated at 20°C/1 Torr, the residue coevaporated twice with pyridine, and finally dissolved in pyridine (25 ml). The solution was treated dropwise under stirring with ether (30 ml), the precipitate collected with suction, and washed with a mixture of pyridine and ether (1 : 1; 10 ml). The filtrate and washing was then added dropwise over 15 minutes into magnetically stirred ether (500 ml). The precipitate was collected with suction, washed with ether, and made up with pyridine*

* We do not recommend to store the pyridinium salts in a dry state since the salts release pyridine and the free acids are decomposed by the action of atmospheric moisture.

to the volume of 50 ml. An aliquot was chromatographed in the solvent system S_1 and the ultraviolet-absorbing band eluted. As shown by absorbancy of the eluate at 260 nm, the stock solution contains 3·2 mmol of compound *VI*.

5′-O-Acetyl-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2-Cyanoethyl) Ester] (*X*)

The stock solution of 2′,3′-O-ethoxymethyleneuridine (*IX*; 2 mmol) is added to a solution of the pyridinium salt of compound *VI* (1 mmol) in pyridine, the whole is coevaporated with pyridine, and the residue is dissolved in pyridine (5 ml). 2,3,5-Triisopropylbenzenesulfonyl chloride (600 mg) is then added, the whole shaken for 20 minutes and then allowed to stand for 20 hours. Water (3 ml) is added, the mixture kept at room temperature for one hour, and extracted with chloroform (20 ml). The extract is evaporated at 35°C/15 Torr, the residue coevaporated with three 10 ml portions of 1-propanol at 20°C/1 Torr, dissolved in chloroform, and chromatographed on two plates of loose silica gel in the solvent system T_1. The ultraviolet-absorbing bands (R_F value, 0·5) are eluted with the solvent mixture T_E to afford $11\,200 A_{260}^{CH_3OH}$ of compound *X* containing 8% of 2′,3′-O-ethoxymethyleneuridine. The analytical sample was rechromatographed in the solvent mixture T_3. For $C_{31}H_{40}N_5O_{17}P$ (785·3) calculated: 47·35% C, 5·14% H, 8·91% N; found: 46·77% C, 5·01% H, 8·55% N.

Reaction with methanolic ammonia. Compound *X* ($5200 A_{260}$) is dissolved in 20% methanolic ammonia (5 ml), the solution kept at room temperature for 3 hours, and chromatographed on two sheets of paper Whatman No 3 MM in the solvent system S_1. Elution of bands (R_F value, 0·55) affords $4700 A_{260}$ of the dinucleoside phosphate *XI*.

5′-O-Formyl-2′-O-tetrahydropyranyluridine 3′-(2-Cyanoethyl) Phosphate (*VII*)

The pyridine solution of the pyridinium salt of compound *VI* (5 mmol) is evaporated at 20°C at 1 Torr and the residue is dissolved in a mixture of methanol (50 ml) and concentrated aqueous ammonia (50 ml). The mixture is allowed to stand at room temperature for 20 hours, diluted with pyridine (100 ml), and evaporated at 35°C/15 Torr. The residual sirup is coevaporated three times with pyridine at 20°C/1 Torr and dissolved in pyridine. The solution is cooled down to —30°C, treated with the mixed formic acetic anhydride (1·1 ml), and the whole kept at -10°C overnight. Ethanol (10 ml) is added, the solution kept at room temperature for one hour, evaporated at 20°C/1 Torr, and the residue evaporated three times with pyridine. The final residue is dissolved in pyridine (50 ml) and the solution added dropwise under stirring into 700 ml of ether. The precipitate is collected with suction, washed with ether, and dissolved with pyridine to the volume of 50 ml. An aliquot is chromatographed in the solvent system S_1; as shown by absorbancy of the eluate, the stock solution contains 4·2 mmol of compound *VII*. The product was characterised by electrophoresis (phosphodiester) and inability to afford the dimethoxytrityl derivative (blocking of 5′-hydroxylic function).

5′-O-Formyl-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2-Cyanoethyl) Ester] (*XII*)

The pyridine solutions of compound *VII* (1 mmol) and *IX* (2 mmol) were evaporated, the residue dissolved in pyridine (5 ml) and shaken with 900 mg of 2,3,5-triisopropylbenzenesulfonyl chloride for 20 minutes. The mixture is allowed to stand overnight and evaporated at 20°C/1 Torr. The residue is repeatedly coevaporated with dioxane (to remove pyridine) and finally dissolved in chloroform. The chromatography is performed on two plates of loose silica gel in the solvent system

T_1. The ultraviolet-absorbing bands (R_F value, 0·4—0·5) are eluted with the solvent mixture T_E, the eluates evaporated, and the residue rechromatographed under the same conditions. Elution of bands of the R_F value 0·45 afforded 11 400 $A_{260}^{CH_3OH}$ (57%) of compound *XII*. The product was characterised by a quantitative conversion into the compound *XI* by the action of methanolic ammonia.

Removal of Formyl Groups from 2′,3′,5′-Tri-O-formyluridine[23]

One mg of the test substance was treated with the following reagents (0·05 ml each): *a*) methanol, 45°C, 5 hours; *b*) 0·1M morpholine acetate, pH 7·5, in 80% aqueous methanol, 30 min, 20°C; *c*) 0·1M morpholine acetate, pH 8·5, in 80% aqueous methanol, 30 min, 20°C; *d*) 0·1M triethylmamonium hydrogen carbonate, pH 8·5, 90% aqueous methanol, 30 min, 20°C; *e*) 0·1M-NH_3 in methanol, 30 min, 20°C. The reaction mixtures were chromatographed on a thin layer of silica gel in the solvent system T_1. The spots of tri-O-formyluridine (R_F, 0·81), di-O-formyluridines (R_F, 0·44), 5′-O-formyluridine (R_F, 0·26), and uridine were evaluated spectrophotometrically. Table II shows the ratio of components.

TABLE II
Removal of Formyl Groups: Ratio of Products

Test	Tri-O-formyl	Di-O-formyl	Mono-O-formyl	Uridine
a	10	42	48	0
b	0	48	52	0
c	0	0	65	35
d	0	0	0	100
e	0	0	0	100

Stability of the Cyanoethyl Group in the Triester *XII*

Compound *XII* (about 1 mg) was dissolved in *a*) 0·05 ml of 0·1M-NH_3 in methanol, *b*) 0·05 ml of 0·1M triethylammonium hydrogen carbonate (pH 8·5) in 90% aqueous methanol. After 30 min at 20°C, the solutions were chromatographed on a thin layer of silica gel in the solvent system T_1. The spots of the phosphotriester *XIII* (R_F, 0·36) and the phosphodiester *XI* (R_F, start line) were eluted and evaluated spectrophotometrically. Test *a*) afforded 85% of the diester, test *b*) 28% of the diester.

2′-O-Tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2-Cyanoethyl) Ester] (*XIII*)

The formyl derivative *XII* (6 760 $A_{260}^{CH_3OH}$) was dissolved in 0·1M triethylammonium hydrogen carbonate (pH 8·5) in 90% aqueous methanol (10 ml). After 20 minutes, the solution was chromatographed on two plates of silica gel in the solvent system T_1. Elution of the ultraviolet-ab-

sorbing band (R_F value, 0·68) with the solvent system T_E gave 4540$A_{260}^{CH_3OH}$ (67%) of compound *XIII*.

5′-O-Formyl-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [Bis-P^1,P^2-(2-cyanoethyl) Ester] (*XVI*)

2,3,5-Triisopropylbenzenesulfonyl chloride (350 mg) was added to a solution of the pyridinium salt of compound *VII* (0·3 mmol) in pyridine. The mixture was shaken for two hours, treated with a solution of the dinucleoside phosphate *XIII* (0·1 mmol) in pyridine (2 ml), and the whole kept at room temperature for two days. Water (2 ml) was added and the mixture extracted with two 20 ml portions of chloroform. The extracts were evaporated under diminished pressure, coevaporared three times with 1-propanol to remove pyridine, the residue dissolved in chloroform and chromatographed on one preparative thin layer of silica gel in the solvent system T_2. The ultraviolet-absorbing band (R_F value, 0·74) was eluted and rechromatographed on one plate in the solvent system T_3 to afford 2 bands, R_F 0·12 (1470A_{260}, 25%, compound *XVI*) and R_F 0·29 (1270A_{260}, the starting compound *XIII*). The product *XVI* was characterised by conversion to uridylyl-uridylyl-uridine by the action of the mixture concentrated aqueous ammonia–ethanol followed by heating in 20% aqueous acetic acid at 50°C.

5′-O-Acetyl-2′-O-tetrahydropyranyluridine 3′-(2,2,2-Trichloroethyl) Phosphate (*VIII*)

a) *Pyridinium salt.* 2,2,2-Trichloroethanol (5 ml) and N,N′-dicyclohexylcarbodiimide (5 g) was added to a solution of the pyridinium salt of compound *V* (5 mmol) in pyridine (30 ml). The mixture was shaken for one hour and then kept at room temperature for 3 days. Water was added (20 ml), the mixture kept for additional 20 hours, shaken with cyclohexane (50 ml), and filtered. The aqueous pyridine layer was evaporated at 20°C/1 Torr, the residue coevaporated three times with pyridine, and finally dissolved in pyridine (25 ml). Ether (30 ml) was added dropwise under stirring to this solution, the precipitate filtered off and washed with a mixture (10 ml) of ether and pyridine (1 : 1). The filtrate and washings were added dropwise under stirring into 500 ml of ether, the precipitate collected with suction, washed with ether, and dissolved in pyridine (20 ml). The solution was evaporated and the residue dissolved in pyridine to the volume of 25 ml. An aliquot of the stock solution was chromatographed in the solvent system S_1 and the single ultraviolet-absorbing band evaluated spectrophotometrically. The stock solution contained 3·2 mmol (64%) of compound *VIII*.

b) *Triethylammonium salt.* The same amounts of reactants were used. After the extraction with cyclohexane, the aqueous pyridine solution was treated with triethylamine (5 ml), the mixture evaporated at 20°C/1 Torr, the residue coevaporated twice with pyridine, and finally dissolved in pyridine. The solution was added dropwise under stirring into 900 ml of ether, the precipitate collected with suction, washed with ether, and dissolved in a mixture of 1-propanol (30 ml) and triethylamine (5 ml). The solution was evaporated under diminished pressure, the residue dissolved in a mixture (20 ml) of chloroform and methanol (2 : 1) and applied to a column (500 ml) of silica gel equilibrated with chloroform. The column was successively eluted with the solvent mixtures chloroform–methanol–triethylamine (80 : 19 : 1; 500 ml) and chloroform–methanol–triethylamine (60 : 39 : 1; 1 500 ml). The ultraviolet-absorbing peak (appearing after elution of 900 ml) was collected in ten 100 ml fractions. Compound *VIII* was identified in fractions 1—8 by paper chromatography. The combined fractions (22400A_{260}) were evaporated to afford 1·76 g of the triethylammonium salt of compound *VIII*. The analytical sample was dried at 40°C and 0·1 Torr for 40 hours. For $C_{18}H_{24}Cl_3N_2O_{11}P.C_6H_{15}N$ (682·9) calculated: 42·18% C, 5·71% H, 15·38% Cl, 6·15% N; found: 42·40% C, 6·14% H, 15·68% Cl, 5·92% N.

5′-O-Acetyl-2′-O-tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2,2,2-Trichloroethyl) Ester] (*XIV*)

2,3,5-Triisopropylbenzenesulfonyl chloride (700 mg) was added to a dry solution of the pyridinium salt of compound *VIII* (1·05 mmol) and compound *IX* (2 mmol), the mixture shaken for 20 minutes, and then allowed to stand at room temperature for 2 days. Sodium acetate (5 ml of 1M solution) was then added and the mixture extracted twice with chloroform. The extract was evaporated under diminished pressure, the residue coevaporated three times with 1-propanol to remove pyridine, the final residue dissolved in chloroform and chromatographed on two preparative layers of loose silica gel in the solvent mixture T_4. Elution of ultraviolet-absorbing bands (R_F value, 0·55) with the solvent mixture T_E afforded 19 700$A_{260}^{CH_3OH}$ units (93%) of compound *XIV* which was characterised by conversion to uridylyl-(3′→5′)-uridine by the successive action of ammonia, powdered zinc in pyridine–acetic acid, and finally aqueous acetic acid. The analogous preparation starting from the triethylammonium salt of compound *VIII* afforded the ester *XIV* in 84% yield.

2′-O-Tetrahydropyranyluridylyl-(3′→5′)-2′,3′-O-ethoxymethyleneuridine [P-(2,2,2-Trichloroethyl) Ester] (*XV*)

A solution containing 3 900A_{260} units of compound *XIV*, concentrated aqueous ammonia (3 ml), and methanol (3 ml) was allowed to stand at 20°C for 2 hours, diluted with 1-propanol, and the whole evaporated at 35°C/15 Torr. The residue was chromatographed on a preparative layer of silica gel in the solvent mixture T_5. Elution of the ultraviolet-absorbing band (R_F value, 0·6) with the solvent mixture T_E afforded 3 320$A_{260}^{CH_3OH}$ units (85%) of compound *XV*. The analytical sample was rechromatographed on a thin layer of silica gel and dried at 40°C/1 Torr for 40 hour. For $C_{28}H_{36}Cl_3N_4O_{16}P$ (822·0) calculated: 40·85% C, 4·38% H, 12·79% Cl, 6·82% N; found: 41·35% C, 4·86% H, 12·09% Cl, 7·12% N.

2′-O-Tetrahydropyranyluridylyl-(3′→5′)-2′-O-tetrahydropyranyluridylyl-(3′→5′)2′,3′-O-ethoxymethyleneuridine [Bis-P^1,P^2-(2,2,2-trichloroethyl) Ester] (*XVII*)

2,3,5-Triisopropylbenzenesulfonyl chloride (540 mg) was added to a solution of the pyridinium salt of compound *VIII* (0·9 mmol) in pyridine (2 ml), the mixture shaken for two hours, treated with a solution of compound *XV* (0·3 mmol) in pyridine (3 ml), the whole concentrated at 20°C at 1 Torr to a half of the original volume and then allowed to stand at room temperature for two days. The concentrate was treated with 1M sodium acetate (5 ml) and pyridine (5 ml), and extracted with two portions of chloroform. The extract was evaporated under diminished pressure, the residue coevaporated three times with 1-propanol to remove pyridine, and finally chromatographed on two preparative layers of silica gel in the solvent mixture T_5. The ultraviolet-absorbing bands (R_F value, 0·6—0·7) were eluted with the solvent mixture T_E, the eluate evaporated, and the residue rechromatographed on two plates in the solvent mixture T_6 to afford two ultraviolet-absorbing bands R_F 0·32 (3 650A_{260} units) and R_F 0·41 (1 040A_{260} units), representing the fully protected uridylyl-uridylyl-uridine (R_F 0·32) and the starting compound *XV* (R_F 0·41). The trinucleotidic portion was dissolved in a mixture of methanol (5 ml) and concentrated aqueous ammonia (5 ml), the solution allowed to stand at room temperature for 90 minutes, diluted with 1-propanol (10 ml), and evaporated under diminished pressure. The residue was chromatographed on one plate of silica gel in the solvent mixture T_5 and the ultraviolet-absorbing band (R_F value, 0·56) eluted with the solvent mixture T_E to afford 2 840A_{260} units (overall yield, 32%) of compound *XVII*. When the analogous reaction with 2,3,5-triisopropylbenzenesulfonyl chloride was performed for 20 hours, the yield of compound *XVII* was 24%.

Uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine (*XIX*)

2,3,5-Triisopropylbenzenesulfonyl chloride (200 mg) was added to a solution of the pyridinium salt of compound *VIII* (0·3 mmol) in pyridine (2 ml), the mixture shaken for 2 hours, treated with a pyridine solution of the ester *XVII* (2800A_{260} units), the whole concentrated to the volume of 2 ml, and the concentrate kept at room temperature for two days. After the addition of pyridine (5 ml) and 1M sodium acetate (5 ml), the mixture was extracted twice with chloroform. The extract was evaporated under diminished pressure, the residue coevaporated three times with 1-propanol to remove pyridine, and finally dissolved in a mixture of concentrated aqueous ammonia (5 ml) and methanol (6 ml). The solution was heated at 37°C for 30 minutes, diluted with 1-propanol (10 ml), the whole evaporated under diminished pressure, and the residue chromatographed on a loose layer of silica gel in the solvent system T_5. Elution of the ultraviolet-absorbing band (R_F value, 0·63) with the solvent mixture T_E afforded 2 130A_{260} units of the nucleotidic material. The complete deblocking of a sample afforded uridylyl-uridylyl-uridine and uridylyl-uridylyl-uridylyl-uridine in the ratio of 2 : 1, *i.e.*, the condensation gave the protected tetranucleotide *XVIII* in 19% yield.

A part of the mixture (1 100A_{260} units) was dissolved in pyridine (3·5 ml), the solution treated with acetic acid (0·2 ml) and powdered zinc (1 g), the mixture stirred for 90 minutes. The excess zinc filtered off, washed with 80% aqueous acetic acid (10 ml) and 80% aqueous pyridine (10 ml), the filtrate and washings evaporated at 20°C/1 Torr, and the residue coevaporated twice with pyridine. The final residue was dissolved in 30% aqueous pyridine (2 ml) and the solution passed through a column (10 ml) of pyridinium Dowex 50 ion exchange resin. The column was washed with 100 ml of 30% aqueous pyridine, the effluent evaporated under diminished pressure, the residue coevaporated twice with 1-propanol, and finally dissolved in 20% aqueous acetic acid. The solution was heated at 50°C for one hour under occasional shaking and evaporated under diminished pressure. The residue was dissolved in 20% aqueous ethanol and applied to one sheet of paper Whatman No 3 MM. Chromatography (2 days) in the solvent system S_2 afforded two ultraviolet-absorbing bands. Elution of the band possessing the R_{U_p} value 0·48 with 1% aqueous ammonia gave 175 A_{260} units of the tetranucleotide *XIX* the pancreatic ribonuclease degradation of which led by 99% to uridine and uridine 3′-phosphate in the ratio 1 : 3·04. The other band (R_{U_p} 0·73) afforded 265A_{260} units of uridylyl-uridylyl-uridine.

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