OLIGONUCLEOTIDIC COMPOUNDS. XL.*
ASPECTS OF THE TRIESTER SYNTHESIS IN THE RIBO SERIES

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The choice and combination of protecting groups in the triester synthesis of the ribo-internucleotidic linkage is discussed along with the possibility to use a combination of the triester and diester synthesis for the formation of oligonucleotidic chains.

In an earlier paper[1], we have exemplified on uridine-containing oligonucleotides the use of 2,2,2-trichloroethyl and 2-cyanoethyl groups in the triester synthesis of the ribo-internucleotidic linkage. The present paper relates to generalisation of the triester process for nucleotides containing the amino group the presence of which brings some new problems. Novel tactics are also proposed for the synthesis of longer oligonucleotidic chains.

The most atractive approach to the triester synthesis in the ribo series appears to consist in the use of 2,2,2-trichloroethyl esters of protected nucleotides as units for the formation of the chain. The relative stability of the 2,2,2-trichloroethyl group in akaline medium makes possible to protect temporarily the $C_{(5')}$-hydroxylic function by means of the acetyl group. The starting compounds in this approach are represented by specifically protected ribonucleoside 3'-phosphates obtained earlier in this Laboratory, namely, N^4-acetyl-5'-O-acetyl-2'-O-(1-ethoxyethyl)-cytidine 3'-phosphate[2], N^6-acetyl-5'-O-acetyl-2'-O-tetrahydropyranyladenosine 3'-phosphate[3], and N^2-acetyl-5'-O-acetyl-2'-O-tetrahydropyranylguanosine 3'-phosphate[3]. Reaction of pyridinium salts of the latter nucleotide derivatives with 2,2,2-trichloroethanol in the presence of N,N'-dicyclohexylcarbodiimide leads to 2,2,2-trichloroethyl ester *I*–*III*. The application of compounds of this type in the triester synthesis was tested by the synthesis of guanylyl-(3' → 5')-adenosine since the preparation of this dinucleoside phosphate by the diester process gives the lowest yields[3,4]. Consequently, the synthesis of GpA might demonstrate the general applicability of the triester method the steric requirements of which are higher than those of the diester process.

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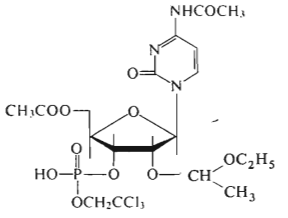
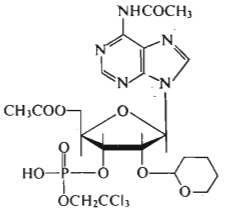
Condensation of the nucleoside component, namely, N^6-dimethylaminomethylene-2′,3′-O-ethoxymethyleneneadenosine (*IV*) with compound *III* was effected by the action of excess triisopropylbenzenesulfonyl chloride since the tertiary amino group of the dimethylaminomethylene grouping consumes one equivalent of the condensing agent[5,6]. The reaction mixture was processed with ammonia (removal of the acetyl and dimethylaminomethylene groups) to afford 2′-O-tetrahydropyranylguanylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneadenosine P-(2,2,2-trichloroethyl) ester (*V*) which was isolated by preparative thin-layer chromatography. The yield of *V* (22%) is similar to those (28%, *cf.*[4], and 37%, *cf.*[3]) obtained in the analogous diester synthesis.



I



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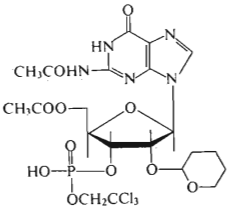
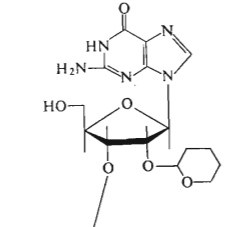
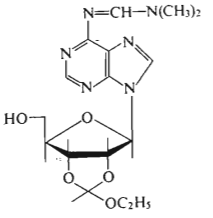
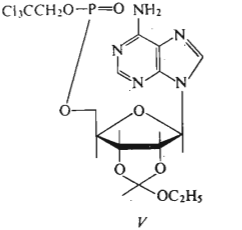


III





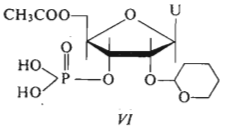
IV



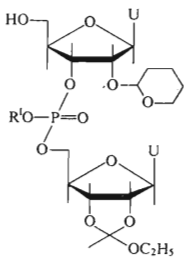
V

The remaining protecting groups of compound *V* were removed by the action of zinc in pyridine–acetic acid and then with 20% aqueous acetic acid to afford the free dinucleoside phosphate GpA in 20% yield. With the use of zinc in 80% aqueous acetic acid, the yield rose to 52%.

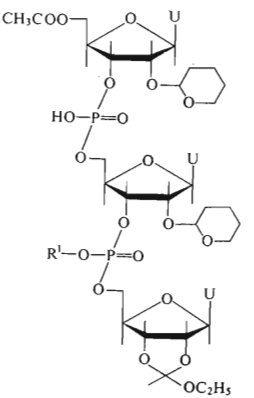
Consequently, the triester method may be used as a general procedure in the ribo series. Owing to the steric influence of the substituent at the $C_{(2')}$-hydroxylic function in *cis*-position to the reacting phosphodiester, the yield of the diribonucleoside phosphate is somewhat lower, but this drawback is compensated by at least twofold yields in the synthesis of the trinucleoside phosphate and tetranucleoside triphosphate[1]. The yields fall with the growing chain. The drop of yields in the synthesis of higher oligonucleotidic systems could be prevented, *inter alia*, by a combination of the triester and diester method. As shown by kinetics of the formation of the internucleotidic linkage by the action of triisopropylbenzenesulfonyl chloride, the reaction of the hydroxylic function with a phosphomonoester[7,8] is at least ten times as fast as that of the phosphodiester[9]. Consequently, a process starting from an oligonucleotide the internucleotidic bonds of which would be protected in the form of a triester, and using an activated phosphomonoester for the chain extension, should give better



VI



VII

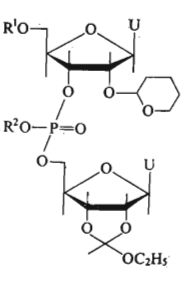


VIII

VIIa, *VIIIa*, $R^1 = -CH_2CCl_3$
VIIb, $R^1 = -CH_2CH_2CN$
VIIc, *VIIIc*, $R^1 = -CH_2C_6H_4NO_2\text{-}p$

yields than the triester method. This approach is exemplified on the reaction of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate (*VI*) with 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine P-(2,2,2-trichloroethyl) ester (*VIIa*). With two equivalents of the phosphate *VI* and in the presence of triisopropylbenzenesulfonyl chloride, there was obtained 63% of the trinucleoside diphosphate *VIIIa* (33% in the presence of N,N'-dicyclohexylcarbodiimide). The analogous phosphates *VIIIb* and *VIIIc* were obtained by reaction of compound *VI* with 2-cyanoethyl ester *VIIb* and *p*-nitrobenzyl ester *VIIc*, resp. The result indicates some advantageous features of the combined triester and diester synthesis.

The above approach is also promissing for connection of oligonucleotidic systems the internucleotidic linkages of which would occur in the form of triesters; the connection would be performed by activation of the phosphomonoester. Realisation of this process is accompanied, however, by a new problem consisting in conversion of the diester internucleotidic linkage into the triester one. This problem was approached by reaction of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine (*IX*) with 2-cyanoethanol, 2,2,2-trichloroethanol, and benzyl alcohol in the presence of triisopropylbenzenesulfonyl chloride. Conversions of the diester internucleotidic linkage of compound *IX* into the triester linkage occurred in satisfactory yields. Thus, the triesters *Xa* (70%), *Xb* (52%), and *Xc* (38%) were obtained in the yields given from 2-cyanoethanol, 2,2,2-trichloroethanol, and benzyl alcohol, resp. 2-Cyanoethanol was also used in the conversion of the dinucleoside phosphate *XI* bearing a free $C_{(5')}$-hydroxylic function into the triester *XIIa* (yield, 58%). Another possibility for the formation of the triester consists in reaction of the phosphodiester *XI* silver salt with a suitable halo derivative. Thus, the reaction of the silver salt with 3-iodopropionitrile gave only 22% of the triester *XIIa*, the principal process being the β-elimination. On the other hand, *p*-nitrobenzyl bromide afforded at 100°C 70% of the *p*-nitrobenzyl ester *XIIb*. The conversion of a diester internucleotidic linkage into a triester bond is therefore possible. It may be also assumed that the diester internucleotidic linkage will not interfere in the subse-



IX, $R^1 = -C(C_6H_5)(p\text{-}C_6H_4OCH_3)_2$, $R^2 = H$

Xa, $R^1 = -C(C_6H_5)(p\text{-}C_6H_4OCH_3)_2$, $R^2 = -CH_2CH_2CN$

Xb, $R^1 = -C(C_6H_5)(p\text{-}C_6H_4OCH_3)_2$, $R^2 = -CH_2CCl_3$

Xc, $R^1 = -C(C_6H_5)(p\text{-}C_6H_4OCH_3)_2$, $R^2 = -CH_2C_6H_5$

XI, $R^1 = R^2 = H$

XIIa, $R^1 = H$, $R^2 = -CH_2CH_2CN$

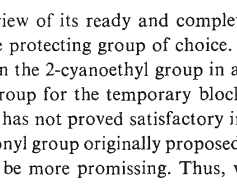
XIIb, $R^1 = H$, $R^2 = -CH_2C_6H_4NO_2$

quent diester synthesis when located in a greater distance from the $C_{(5')}$-hydroxylic function; in this case, the transformation of the diester into the triester could be omitted.

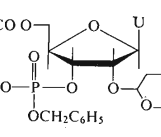
In the synthesis of longer chains, it is necessary to consider the yields of all steps including the final removal of the protecting group at the internucleotidic linkage. The stability of the 2,2,2-trichloroethyl group in alkaline media facilitates the preparation and isolation of starting compounds and fully protected intermediates; with longer chains, however, the removal of the 2,2,2-trichloroethyl group in the last step does not give satisfactory yields[9].

In the search for other more readily removable protecting groups, we have preliminarily tested the properties of benzyl and *p*-nitrobenzyl esters derived from uridylyl-(3′ → 5′)-uridine. The benzyl ester *XV* was prepared by the triester synthesis starting from 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-benzyl phosphate (*XIII*) and 2′,3′-O-ethoxymethyleneuridine (*XIV*). Both the benzyl[1] and *p*-nitrobenzyl groups may be quantitatively removed by hydrogenolysis over palladium. It has been also found that the *p*-nitrobenzyl group may be selectively removed at 80°C by the action of a tertiary base, namely, N-methylmorpholine. As shown be these preliminary tests, both the benzyl and *p*-nitrobenzyl group are promissing for the use in the triester synthesis. On the other hand, the methyl group (preliminarily tested on the basis of its ready removal from phosphoric acid triesters) did not prove suitable as a protecting group for the internucleotidic linkage. The alkaline cleavage of dinucleoside methyl phosphates was accompanied to a small extent by a partial cleavage of the internucleotide linkage[10].

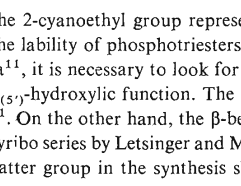
In view of its ready and complete removal, the 2-cyanoethyl group represents so far the protecting group of choice. Because of the lability of phosphotriesters which contain the 2-cyanoethyl group in alkaline media[11], it is necessary to look for a suitable group for the temporary blocking of the $C_{(5')}$-hydroxylic function. The formyl group has not proved satisfactory in this respect[1]. On the other hand, the β-benzoylpropionyl group originally proposed for the deoxyribo series by Letsinger and Miller[11] might be more promissing. Thus, we used the latter group in the synthesis starting



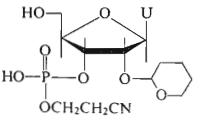
XIII



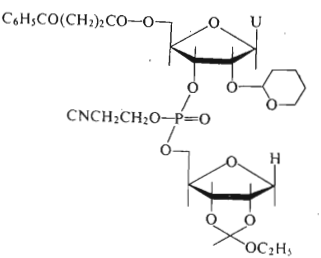
XIV



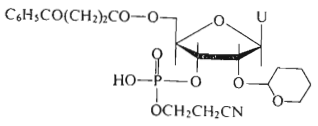
XV



XVI



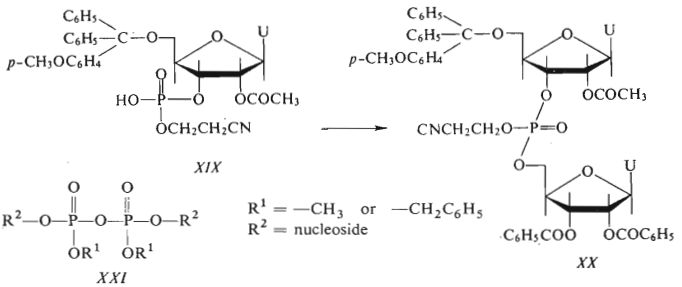
XVIII



XVII

from 2′-O-tetrahydropyranyluridine 3′-(2-cyanoethyl) phosphate (*XVI*). Treatment of compound *XVI* with β-benzoylpropionic acid and N,N′-dicyclohexylcarbodiimide yielded the 5′-O-(β-benzoylpropionyl) derivative *XVII* the reaction of which with 2′,3′-O-ethoxymethyleneuridine (*XIV*) and triisopropylbenzenesulfonyl chloride led to the triester *XVIII* in 58% yield. The β-benzoylpropionyl group was removed with hydrazine acetate in a mixture of pyridine and acetic acid under the formation of the triester *XIIa* in 80% yield. This approach makes somewhat difficult the choice of other protecting groups for blocking of the nucleotidic amino groups, since hydrazine acetate splits both the benzoyl[11] and the N-dimethylaminomethylene derivatives. The re-introduction of the dimethylaminomethylene group by the action of dimethylformamide dialkylacetals is not possible in the case of the 2-cyanoethyl triesters because of the high basicity of these acetals.

Taking into consideration the lability of 2-cyanoethyl triesters in alkaline media and the requirement to keep the other protecting groups intact, the $C_{(5')}$-hydroxylic function could be blocked by an acidolabile group, the removal of which would not endanger the 2′-O-tetrahydropyranyl group. Thus, *e.g.*, we have found that the dimethoxytrityl group of compound *IX* may be selectively removed in 80% aqueous acetic acid at 0°C in the course of 80 minutes. At 20°C, a partial removal of the tetrahydropyranyl and ethoxymethylene groups was observed. It is noteworthy in this connection that the stability of the tetrahydropyranyl group in the neighbourhood to a neutral phosphotriester will be higher than in the vicinity of a phosphodiester. This selective removal of the dimethoxytrityl group provides access to the utilisation of ribonucleoside 3′-phosphate 2′-O-tetrahydropyranyl derivatives in combination with the 2-cyanoethyl group and is being object of further investigations.



Protection of the $C_{(2')}$-hydroxylic function of the *cis*-diol system by acidolabile groups has proved useful in this Laboratory for the synthesis of shorter ribooligonucleotides. The acidolabile protecting groups are conveniently removed by the action of 20% aqueous acetic acid at 50°C to yield compounds containing almost 100% of natural internucleotidic linkages. The rate of removal of these acidolabile protecting groups may be, however, considerably decreased with longer oligonucleotidic systems which contain basic nucleotides: the longer action of acidic media may lead to an appreciable cleavage and isomerisation of some ribo-internucleotidic linkages. It appears therefore advisable to attempt the synthesis of longer chains with the use of alkalilabile protecting groups for the $C_{(2')}$-hydroxylic function and the *cis*-diol system[12]. In this respect, we have performed a preliminary experiment, namely,

TABLE I

Paper Chromatography (S_1), Thin-Layer Chromatography (T_{system}), and Electrophoresis (E_1; Based on Uridine 3'-Phosphate)

Compound	S_1	E_1	Compound	T	Compound	T
Up	0·10	1	*IV*	$0{\cdot}40_2$	*Xc*	$0{\cdot}50_2$
UpU	0·20	0·42	*V*	$0{\cdot}47_5$	*XIIa*	$0{\cdot}32_2$
UpUpU	0·06	0·65	*VIIc*	$0{\cdot}50_2$	*XIIb*	$0{\cdot}35_2$
GpA	0·14	0·35	*VIIIa*	$0{\cdot}29_3$	*XV*	$0{\cdot}46_2$
I	0·62	0·53	*VIIIc*	$0{\cdot}32_3$	*XVII*	$0{\cdot}51_4$
II	0·67	0·45	*IX*	$0{\cdot}10_2$	*XVIII*	$0{\cdot}53_2$
III	0·55	0·42	*Xa*	$0{\cdot}45_2$	*XIX*	$0{\cdot}40_3$
XIII	0·60	0·45	*Xb*	$0{\cdot}48_2$	*XX*	$0{\cdot}43_2$
XIX	0·89	—				

a triester synthesis of 5'-monomethoxytrityl-2'-O-acetyluridylyl-(3' → 5')-2',3'-di-O-benzoyluridine P-(cyanoethyl) ester (*XX*) from the diester *XIX* and 2',3'-di-O-benzoyluridine. The separation of the product *XX* from the unreacted 2',3'-di-O-benzoyluridine was effected by processing the crude reaction mixture with dimethoxytrityl chloride, *i.e.*, by conversion of 2',3'-di-O-benzoyluridine into the more mobile 5'-O-dimethoxytrityl derivative.

In connection with the preparation of nucleotidic esters (used in the triester synthesis) by reaction of nucleotides with alcohols in the presence of N,N'-dicyclohexylcarbodiimide, we should like to mention the formation of N-alkylpyridinium compounds when methanol[13] or benzyl alcohol is used. With 2-cyanoethanol and 2,2,2-trichloroethanol, these by-products have not been observed. It may be assumed that the smooth N-alkylation of pyridine cannot be achieved by the action of phosphoric acid diesters; more probably, the alkylating agent is represented by symmetrical diphosphates of the type *XXI*, formed from diesters by the action of N,N'-dicyclohexylcarbodiimide. This quaternisation of pyridine is analogous to the recently reported quaternisation with methylphosphodichloridate[14].

EXPERIMENTAL

Methods

Descending paper chromatography was performed on paper Whatman No 1 in the solvent system S_1. 2-propanol–concentrated aqueous ammonia–water (7 : 1 : 2). Paper electrophoresis was effected on paper Whatman No 1 in the buffer solution E_1, 0·05M triethylammonium hydrogen carbonate, pH 7·5. Thin-layer chromatography was carried out on ready-for-use Silufol UV_{254} (Glassworks Kavalier, Votice) silica gel foils (Table I). Preparative chromatography was performed on a loose 20 × 20 × 0·3 cm layer of 10—60 micron silica gel containing incorporated fluorescent indicator according to Pitra (produced by our Service Laboratories in Prague - Suchdol) in the solvent system T_1, chloroform–methanol (95 : 5); T_2, chloroform–methanol (9 : 1); T_3, chloroform–methanol–triethylamine (85 : 14 : 1); T_4, chloroform–methanol–triethylamine (75 : 24 : 1); T_5, chloroform–methanol–triethylamine (40 : 59 : 1); T_6, methanol–ethanol–triethylamine (60 : 39 : : 1). The substances were eluted with the solvent systems T_{e1}, chloroform–methanol (1 : 1); T_{e2}, chloroform–methanol–triethylamine (50 : 49 : 1); T_{e3} chloroform–methanol–triethylamine (9 : 90 : : 1); T_{e4} chloroform–methanol–pyridine (50 : 45 : 5). Detection of dimethoxytrityl and monomethoxytrityl derivatives on preparative thin layers was performed with the use of 1 cm wide strips of paper Whatman No 1, previously pressed to the moist developed layer; the strips were sprayed with 10% aqueous perchloric acid. Enzymatic degradations were performed with about 2 μmol of the test substance in 50 μl of a 0·1M Tris-HCl buffer solution (pH 8·5) containing 100 μg of pancreatic ribonuclease (Sigma, St. Louis, U.S.A.) or 100 units of T 1 ribonuciease (Calbiochem, Los Angeles, U.S.A.). Spectroscopic measurements were performed on a Beckman DU apparatus in methanol (with protected compounds) and 0·01M-HCl (with unprotected compounds). Quantitative determinations were performed at 260 nm with the use of the following extinction coefficients: 10000 for the uridine unit, 11800 for the guanosine unit, and 14200 for the adenosine unit. The hyperchromicity of oligonucleotide derivatives was not taken into account. One absorption optical unit $A_{260}^{solvent}$ is defined as such an amount of the substance which dissolved in 1 cm of a solvent (methanol, 0·01M-HCl) shows at 260 nm in an 1 cm cell the absorbancy equal to one.

In the synthesis of the internucleotidic linkage, the reaction mixtures were freed from water by evaporating with five portions of pyridine at 20°C/1 Torr; the residues were stored in closed

vessels in a desiccator. Pyridine was dried over calcium hydride, filtered, and stored over molecular sieves Potassit 3 A *in globulis* (Slovnaft, Bratislava). Dimethylformamide was distilled over 5% of phosphorus pentoxide and stored over molecular sieves.

2,2,2-Trichloroethyl Esters of N^4-Acetyl-5′-O-acetyl-2′-O-(1-ethoxyethyl)cytidine 3′-Phosphate (*I*), N^6-Acetyl-5′-O-acetyl-2′-O-tetrahydropyranyladenosine 3′-Phosphate (*II*), and N^2-Acetyl-5′-O-acetyl-2′-O-tetrahydropyranylguanosine 3′-Phosphate (*III*)

2,2,2-Trichloroethanol (1 ml) and N′,N′-dicyclohexylcarbodiimide (1 g) is added to a solution of the pyridinium salt of the nucleotide (1 mmol) in pyridine (10 ml), the mixture is shaken for several hours and kept at room temperature overnight. Water (2 ml) is then added, the mixture left to stand for one hour, shaken with cyclohexane (10 ml), and filtered. The aqueous pyridine layer is evaporated, the residue coevaporated with three portions of pyridine to remove water, and the final residue dissolved in 5 ml of pyridine. The solution is added dropwise into ether (200 ml), the precipitate is collected with suction, washed with ether, dried under diminished pressure, and dissolved in pyridine. An aliquot is chromatographed in the solvent system S_1, the spots are eluted, and the ultraviolet absorption of the eluates measured to indicate about 90% yields of compounds *I*–*III*.

2′-O-Tetrahydropyranylguanylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneadenosine (*V*)

A solution containing 2′,3′-O-ethoxymethyleneadenosine (800 mg), dimethylformamide (2 ml), and dimethylformamide dimethylacetal (2 ml) is kept at room temperature for 20 hours, evaporated, and the residual N^6-dimethylaminomethylene derivative *IV* repeatedly coevaporated with pyridine to remove the dimethylformamide. A solution of the pyridinium salt of the protected guanosine 3′-(2,2,2-trichloroethyl) phosphate *III* (1·25 mmol) in pyridine (10 ml) and triisopropylbenzenesulfonyl chloride (1·8 g) is then added, the whole shaken for several minutes, concentrated to the consistence of a thick sirup, and kept at room temperature for 2 days. Concentrated aqueous ammonia (10 ml) and pyridine (5 ml) is added, the mixture left to stand at room temperature for 3 hours, evaporated, and the residue coevaporated with two portions of 1-propanol to remove pyridine. The final residue was chromatographed on four loose layers of silica gel in the solvent system T_5. The dark bands (R_F value 0·2–0·5) were eluted with the solvent mixture T_{e3} and rechromatographed in the solvent system T_6. The ultraviolet-absorbing bands (R_F value 0·6–0·8) were eluted with the solvent mixture T_{e3} to afford 7200 $A_{260}^{methanol}$ (22%) of compound *V*. Ultraviolet spectrum (methanol): λ_{max} 258 nm, λ_{min} 230 nm; $A_{250/260}$ 0·88, $A_{280/260}$ 0·58.

Removal of the dimethylaminomethylene group. A solution containing 25 μmol of N^6-dimethylaminomethylene-2′,3′-O-ethoxymethyleneadenosine (*IV*), 0·1 ml of 4 : 1 pyridine–acetic acid, and 1 μl of 90% hydrazine hydrate was shaken overnight and chromatographed in the solvent system T_1 to exhibit a single ultraviolet-absorbing spot of 2′,3′-O-ethoxymethyleneadenosine.

Guanylyl-(3′ → 5′)-adenosine

A solution of compound *V* (500 $A_{260}^{methanol}$) in 80% aqueous acetic acid (0·1 ml) was shaken with powdered zinc (50 mg) for one hour, the mixture diluted with water (0·1 ml), and heated at 50°C for one hour. The solution was diluted with 30% aqueous pyridine (1 ml), passed through a column of pyridinium Dowex 50 ion exchange resin (1 ml), and the column eluted with 30% aqueous pyridine (10 ml). The eluates were evaporated and the residue chromatographed on one sheet of paper Whatman No 1 in the solvent system S_1. The ultraviolet-absorbing band (R_F value 0·15) was eluted with 1% aqueous ammonia to afford 290 $A_{260}^{pH\ 1}$ of guanylyl-(3′ → 5′)-adenosine (58%).

The ribonuclease T 1 degradation was quantitative and led to guanosine 3'-phosphate and adenosine in the ratio of 1 : 0·88. An analogous deblocking with the same amount of zinc but in a mixture of pyridine acetic acid (4 : 1) gave only a 20% yield of guanylyl-(3' → 5')-adenosine.

5'-O-Acetyl-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2'-O-tetrahydropyranyluridyiyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine P^2-(2,2,2-Trichloroethyl) Ester Triethylammonium Salt (*VIIIa*)

A solution of the pyridinium salt of the phosphate *VI* (0·2 mmol) and of the ester *VIIa* (0·1 mmol) in pyridine (5 ml) was shaken for several minutes with triisopropylbenzenesulfonyl chloride (120 mg) and then concentrated to the volume of about 2 ml. The concentrate was kept at room temperature for 20 hours, diluted with water (2 ml), kept for additional 2 hours, evaporated, and the residue coevaporated (to remove pyridine) with 1-propanol–triethylamine (5 : 1; 5 ml). The final residue was chromatographed on one plate of silica gel in the solvent system T_4. The ultraviolet-absorbing band (R_F value 0·50–0·60) was eluted with the solvent mixture T_{e2} to afford 1 880 $A_{260}^{methanol}$ (63%) of compound *VIIIa*. The substance was characterised by conversion to uridylyl-uridylyl-uridine (yield 48%) by a successive treatment with dilute aqueous ammonia, with zinc in a mixture of pyridine and acetic acid, and with 20% aqueous acetic acid. With the use of N,N'-dicyclohexylcarbodiimide (0·3 g), the analogous reaction (reaction period, 3 days) afforded 33% of compound *VIIIa*.

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

A solution of the pyridinium salt of the phosphate *VI* (0·4 mmol) and compound[1] *VIIb* (0·2 mmol) in pyridine (5 ml) was shaken for several minutes with triisopropylbenzenesulfonyl chloride, concentrated to the volume of about 2 ml, the concentrate kept at room temperature for 20 hours, treated with water, kept for additional one hour, treated with concentrated aqueous ammonia (2 ml), kept for 2 hours more, evaporated, the residue coevaporated with 1-propanol to remove pyridine, and the final residue dissolved in 20% aqueous acetic acid (5 ml). The solution was heated for one hour at 50°C and then chromatographed on one sheet of paper Whatman 3 MM in the solvent mixture S_1 for 48 hours. The slowest ultraviolet-absorbing band was eluted with 1% aqueous ammonia to afford 3800 A_{260} (63%) of uridylyl-(3' → 5')-uridylyl-(3' → 5')-uridine which was characterised on comparison with an authentic sample and pancreatic ribonuclease degradation.

5'-O-Acetyl-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine P^2-(*p*-Nitrobenzyl) Ester Triethylammonium Salt (*VIIIc*)

Triisopropylbenzenesulfonyl chloride (600 mg) was dissolved in an anhydrous solution of the triester *VIIc* (0·55 mmol) and the phosphate *VI* (1·1 mmol) in pyridine (5 ml), the resulting solution concentrated to the volume of about 3 ml, the concentrate kept at room temperature for 20 hours, and evaporated. The residue was dissolved in a mixture of 1-propanol (10 ml) and triethylamine (1 ml), the solution evaporated, and the residue chromatographed on two plates in the solvent system T_3 to afford two ultraviolet-absorbing bands. Elution of one band (R_F value 0·75) with the solvent mixture T_{e2} afforded 228 mg of the starting compound *VIIc*. The other band (R_F value 0·5) was eluted with the solvent mixture T_{e2} and rechromatographed on two plates in the solvent system T_4 to afford 532 mg (55%) of compound *VIIIc*. On successive treatment with dilute aqueous ammonia, hydrogenation over palladium oxide in 50% aqueous ethanol and finally, on heating in 20% aqueous acetic acid for one hour, compound *VIIIc* was converted to uridylyl-(3' → 5')-uridylyl-(3' → 5')-uridine in 78% yield.

5'-O-Dimethoxytrityl-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine Triethylammonium Salt (*IX*)

A solution of the triethylammonium salt of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine (1 mmol) in pyridine (10 ml) was treated with dimethoxytrityl chloride (500 mg) and the reaction mixture kept at 20°C for 20 hours. Triethylamine (0·5 ml) was then added, the mixture evaporated and chromatographed on two plates in the solvent system T_3. Bands positive to perchloric acid (R_F 0·8) were eluted with the solvent mixture T_{e2} and rechromatographed on two plates in the solvent system T_1. The ultraviolet-absorbing bands (R_F 0·4—0·5) were eluted with the solvent mixture T_{e2} to afford 850 mg of the triethylammonium salt of compound *IX*. Ultraviolet spectrum (methanol): λ_{max} 233 nm, 261 nm; λ_{min} 225 nm, 248 nm. The analytical sample was rechromatographed on a thin layer in the solvent system T_1. For $C_{47}H_{53}N_4O_{18}P.C_6H_{15}N$ (1094) calculated: 58·15% C, 6·23% H, 6·40% N, 2·83% P; found: 57·55% C, 6·53% H, 6·55% N, 2·43% P.

Removal of the dimethoxytrityl group. Solutions of compound *IX* (5 mg) in 80% aqueous acetic acid (50 µl) were kept at *a*) 0°C, *b*) 20°C, the samples withdrawn in 15 min intervals and chromatographed on a thin layer in the solvent system T_2. The dimethoxytrityl group was completely removed in the course of 90 minutes at 0°C and of 30 minutes at 20°C. As shown by chromatography on paper in the solvent system S_1, the sample detritylated at 0°C contains exclusively 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine, while the sample deblocked at 20°C contains additional 12% of the detetrahydropyranylated substance and 6% of uridylyl-(3' → 5')-uridine.

5'-Dimethoxytrityl-2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine P-(2-Cyanoethyl) Ester (*Xa*)

A solution of compound *IX* (0·05 mmol) in pyridine (1 ml) was treated with triisopropylbenzenesulfonyl chloride (0·15 mmol) and then, after one hour, with 2-cyanoethanol (0·15 mmol). The reaction mixture was kept at room temperature for 20 hours and then chromatographed on a thin layer in the solvent system T_2. The band positive to perchloric acid (R_F 0·6) was eluted with the solvent mixture T_{e1} and rechromatographed under the same conditions to afford 715 $A_{260}^{methanol}$ (73%) of compound *Xa* which was characterised by a quantitative conversion to compound *IX* by the action of ammonia in methanol. The 2,2,2-trichloroethyl ester and the benzyl ester were obtained analogously in 52 and 38% yields, resp.

2'-O-Tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-ethoxymethyleneuridine P-(2-Cyanoethyl) Ester (*XIIa*)

A solution of the diester *XI* (0·5 mmol) in pyridine (5 ml) was treated with 2-cyanoethanol (0·38 ml) and triisopropylbenzenesulfonyl chloride (450 mg) and the whole kept at room temperature for 20 hours. Water (1 ml) was then added, the mixture extracted with chloroform (15 ml), the extract evaporated, the residue coevaporated twice with 1-propanol to remove pyridine, and the final residue chromatographed on two plates in the solvent system T_2. The coloured bands (R_F value 0·5) were eluted with the solvent mixture T_{e1}, and rechromatographed on two plates in the solvent system T_1. Elution (solvent mixture T_{e1}) of the ultraviolet-absorbing bands (R_F value 0·2) afforded 5 500 $A_{260}^{methanol}$ of compound *XIIa* which was identified on comparison with an authentic sample.

Removal of the 2-cyanoethyl group. A solution of compound *XIIa* (3 mg) in dimethylformamide (0·1 ml) and dimethylformamide dineopentylacetal (10 µl) was kept at 20°C for 20 hours and chromatographed on a thin layer in the solvent system T_2 to exhibit a single spot of the diester *XI*.

2′-O-Tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneuridine P-(*p*-Nitrobenzyl) Ester (*XIV*)

A suspension of the silver salt of compound[1] *XI* (0·8 mmol; previously dried at 30°C/0·1 Torr) in acetonitrile (10 ml) and *p*-nitrobenzyl bromide (1·2 g) was heated at 100°C for 4 hours under stirring and chromatographed on two plates in the solvent system T_2. The ultraviolet-absorbing bands (R_F value 0·5) were eluted with the solvent mixture T_{e1} to afford 459 mg of compound *XIIb*. The analytical sample was rechromatographed under the same conditions. For $C_{33}H_{40}N_5 \cdot O_{18}P$ (825·6) calculated: 47·95% C, 4·86% H, 8·49% N; found: 47·47% C, 5·12% H, 8·18% N.

Reaction of the Silver Salt *XI* with 3-Iodopropionitrile

A suspension containing the silver salt *XI* (0·1 mmol), acetonitrile (1 ml), and 3-iodopropionitrile (0·1 ml) was shaken with glass beads for 4 hours at room temperature, the mixture diluted with acetonitrile (2 ml), the silver iodide removed by centrifugation, and the supernatant chromatographed on a loose layer (10 × 20 × 0·3 cm) of silica gel in the solvent system T_2. Elution of the ultraviolet-absorbing band (R_F value 0·40) afforded 450 $A_{260}^{methanol}$ (22%) of the triester *XIIa*.

5′-O-Acetyl-2′-O-tetrahydropyranyluridine 3′-Benzyl Phosphate (*XIII*)

A solution of the pyridinium salt of compound *VI* (10 mmol) in pyridine (30 ml) was treated with benzyl alcohol (12 ml) and N,N′-dicyclohexylcarbodiimide (10 g) and the whole mixture left to stand at room temperature for two days. Water (5 ml) was then added, the mixture kept for one hour, washed with cyclohexane (30 ml), and filtered. As shown by electrophoresis, the aqueous pyridine layer contained the product and the N-benzylpyridinium ion (identified on comparison with an authentic sample). The solution was passed through a column of pyridinium Dowex 50 ion exchange resin (50 ml) and the column washed with 30% aqueous pyridine. The effluents were evaporated, the residue dried by repeated coevaporations with pyridine, and the final residue was dissolved in pyridine (20 ml). The solution was added dropwise under stirring into ether (700 ml), the precipitate collected with suction, washed with ether, and dried under diminished pressure. Yield, 5·5 g of the pyridinium salt of compound *XIII* which was dissolved in pyridine and the solution stored at 0°C.

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

A solution containing compound *XIII* (0·5 mmol), 2′,3′-O-ethoxymethyleneuridine (*XIV*) pyridine (5 ml), and triisopropylbenzenesulfonyl chloride (300 mg) was concentrated to the volume of about 3 ml and the concentrate allowed to stand at room temperature for 20 hours. Concentrated aqueous ammonia (5 ml) was then added, the mixture kept for additional 3 hours, and extracted with chloroform. The extracts were evaporated, the residue coevaporated with 1-propanol, and the final residue chromatographed on two plates in the solvent system T_2. Elution of the ultraviolet-absorbing band (R_F value 0·47) afforded 180 mg of compound *XV*, identical with an authentic specimen[1].

5′-O-(β-Benzoylpropionyl)-2′-O-tetrahydropyranyluridine 3′-(2-Cyanoethyl) Phosphate Triethylammonium Salt (*XVII*)

The pyridinium salt of 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-(2-cyanoethyl) phosphate[1] (0·5 mmol) is dissolved in a mixture of concentrated aqueous ammonia (10 ml) and methanol (10 ml), the solution is kept at room temperature for 8 hours, evaporated, the residue dissolved

in 30% aqueous pyridine (5 ml), the solution passed through a column of pyridinium Dowex 50 ion exchange resin (5 ml), the effluent evaporated, and the residue dried by coevaporation with pyridine. The dry residue is dissolved in pyridine (3 ml), the solution is treated with β-benzoylpropionic acid (240 mg) and N,N′-dicyclohexylcarbodiimide (0·5 g), the whole is allowed to stand at room temperature for 20 hours, shaken with 70% aqueous pyridine (10 ml) for one hour, and filtered. The filtrate is evaporated and the residue coevaporated (to remove pyridine) with a mixture of 1-propanol (10 ml) and triethylamine (1 ml). The final residue is chromatographed on two plates in the solvent system T_3. The ultraviolet-absorbing bands (R_F value 0·37) are rechromatographed (after the elution with the solvent mixture T_{e2}) under analogous conditions. Yield, 250 mg (68%) of the triethylammonium salt of compound *XVII*.

5′-O-(β-Benzoylpropionyl)-2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneuridine P-(2-Cyanoethyl) Ester (*XVIII*)

A solution of the triethylammonium salt of compound *XVII* (0·3 mmol) and 2′,3′-O-ethoxymethyleneuridine (1 mmol) in pyridine (5 ml) was treated with triisopropylbenzenesulfonyl chloride (200 mg) and the whole was concentrated to the volume of about 2 ml. After 20 hours at room temperature, pyridine (10 ml) and 1M sodium acetate (5 ml) were added and the mixture extracted with chloroform (25 ml). The extract was evaporated, the residue coevaporated with 1-propanol, and the final residue chromatographed on two plates in the solvent mixture T_2. The ultraviolet-absorbing bands (R_F value 0·53) were eluted with the solvent mixture T_{e1} to afford 174 mg (58%) of compound *XVIII* which was characterised by conversion to uridylyl-(3′ → 5′)-uridine by the successive treatment with a mixture of concentrated aqueous ammonia and methanol and then 20% aqueous acetic acid.

Removal of the β-benzoylpropionyl group. A solution of the triester *XVIII* (23 mg) in a mixture (0·1 ml) of pyridine and acetic acid (4 : 1) was treated with 90% hydrazine hydrate (1 μl) and the whole shaken overnight. The mixture was evaporated and the residue chromatographed on a 10 × × 20 × 0·3 cm plate in the solvent system T_2. Elution of the ultraviolet-absorbing band (R_F value 0·45) afforded 400 $A_{260}^{methanol}$ (80%) of compound *XIIa*.

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