SYNTHESIS OF OLIGORIBONUCLEOTIDES VIA PHOSPHOTRIESTER INTERMEDIATES

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The synthesis of naturally occurring oligoribonucleotides still presents, despite many efforts made in the past, a tremendous challenge to organic chemists. The reason for the slow progress in the synthesis of RNA, in comparison with DNA, can be ascribed to the complexity and diversity of these compounds. DNA's lack the 2'- OH function and are, in most cases, polymers derived from four common d-nucleosides. These structural elements highly facilitate, from a chemical point of view, the preparation of properly-protected d-nucleosides and the formation of 3'- 5' internucleotide phosphodiester bonds.
On the contrary, the presence of an additional 2'- OH group in ribonucleosides and the fact that RNA's, from natural origin, not only contain the four common but also modified nucleosides make the synthesis of RNA very complex and elaborate.
The aim of this review[1] is to show that most of the different types of naturally occurring RNA can be synthesized exclusively *via* phosphotriester intermediates.
Therefore, in order to make the synthesis of RNA *via* phosphotriester intermediates more accessible, it would be of prime importance to have at our disposal:

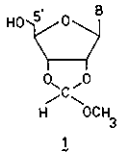
1. general and non *timeconsuming* procedures for the preparation of suitably-protected ribonucleosides(tides);
2. efficient phosphorylating and activating agents which are necessary for the introduction of phosphotriester functions;
3. selective deblocking procedures;
4. rapid and reliable analytical methods to assess homogeneity and structural proof of completely deblocked oligoribonucleotides.

1. Synthesis of protected ribonucleosides and ribonucleotides.

1.1. Properly protected ribonucleosides.

It now seems clear [2] that for a systematic synthesis of oligoribonucleotides *via* the phosphotriester approach several types of building blocks (1, 2, 4 and 5) of each common ribonucleoside are required.

The reason for this is that a proper combination of the above units allows



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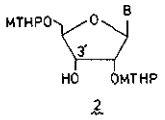

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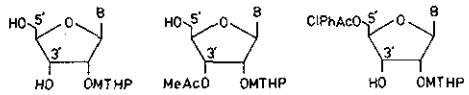
TERMINAL UNITS.



3 4 5

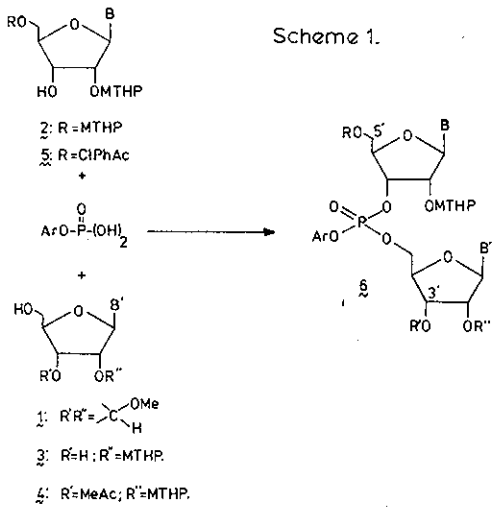
NON-TERMINAL UNITS.

B = U, C^{An}, A^{An}, G^{Bz}. An = $-C(=O)-C_6H_4-OCH_3$. Bz = $-C(=O)-C_6H_5$.

MTHP =  . MeAc = $H_3COCH_2C(=O)-$. ClPhAc = $Cl-C_6H_4-OCH_2C(=O)-$.

the extension of a growing nucleic acid chain in either the 5'- and/or the 3'-direction (Scheme 1). Thus a combination of the non-terminal unit (5) and the terminal unit (1) gives *via* a two-step phosphorylation procedure a fully-protected diribonucleoside monophosphate (6), which by selective removal of the base-labile group (R), can be extended in the 5'- direction.

Scheme 1.



Combination of:	Extension at 5'	3'
2 + 1	-	-
2 + 3	-	+
2 + 4	-	+
5 + 1	+	-
5 + 3	+	+
5 + 4	+	+

Extension of a growing nucleic acid chain, in either the 5'- or 3'-direction, is only successful if the protecting groups, used for the blocking of the 5'- or 3'- OH function, can be removed selectively. The latter means that the protecting group for the phosphodiester linkage should remain intact during this particular deblocking step. It is for this reason that we choose p-chlorophenoxyacetyl as a protecting group for the 5'-hydroxyl function. Indeed, this group could be removed selectively in the presence of an aryl-protected phosphodiester linkage.

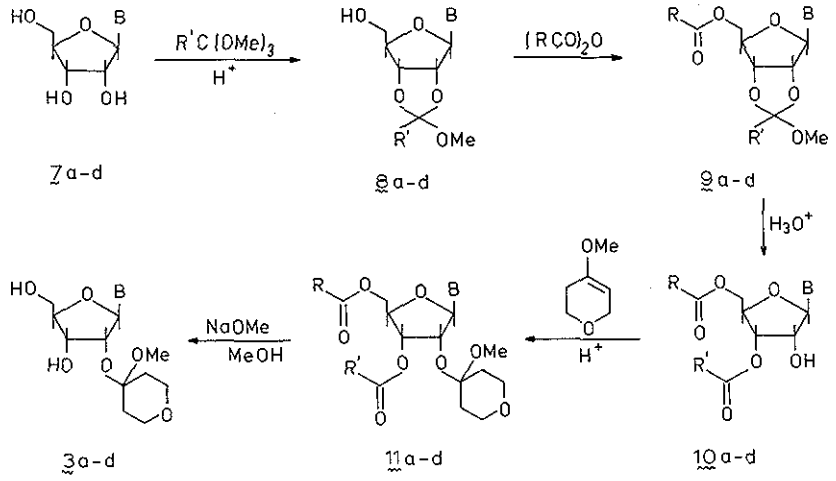
The nature of the acid-labile protecting groups - 4-methoxytetrahydropyranyl and methoxymethylidene - is such that they can be removed, at the final stage of the deblocking process, without isomerization of the 3'- 5' phosphodiester linkages.[3]

Building block (4) which bears a methoxyacetyl group at the 3'-position is, in principle, suitable to extend a growing nucleic acid chain (Scheme 1)

in the 3'- direction. However, this protecting group is too stable to be removed selectively in the presence of suitably aryl-protecting groups for the phosphodiester linkages. Fortunately, however, the monoprotected unit (3) can be used instead of building block (4) for the extension of a growing nucleic acid chain in the 3'- direction.[4] Although the application of unit (3) eliminates an extra deblocking step, it should be pointed out that oligoribonucleotides prepared from this unit may contain small amounts of unnatural (3'-3')-internucleotide linkages.

The importance of units (3) is also underlined by the fact that they are easily converted, by selective acylation [5], into the very valuable non-terminal building blocks (5).

Therefore, we developed [6] a general and convenient method for the synthesis of ribonucleoside-2'-acetals (3a-d) of all four common ribonucleosides (7a-d; Scheme 2).



7a-d 8a-d 9a-d 10a-d 11a-d 3a-d

Scheme 2

a: B = Uracil-1-yl
b: B = 4-N-p-Anisoyl cytosin-1-yl
c: B = 6-N-p-Anisoyladenin-9-yl
d: B = 2-N- Benzoylguanin-9-yl

Treatment of the ribonucleosides (7a-d) with trimethyl orthopropionate [R'C(OMe)$_3$: R' = Et], gave the 2',3'-O-(methoxypropylidene)derivatives (8a-d)

in excellent yields. The latter compounds were, without further purification, treated with methoxyacetic anhydride [$(RCO)_2O$: R = $-CH_2OMe$] in dry pyridine to afford the fully-protected compounds (9a-d) which, after hydrolysis with aqueous acetic acid, were converted into a mixture of the 3',5'-diesters (10a-d) and their 2',5'-isomers. Crystallization afforded the required 3',5'-diesters in 58-70% yield. Only in the case of the compounds (10a) and (10d) was it necessary to purify the products by chromatography before crystallization. In all cases, quantities of up to 15 g of each pure isomer (10) could easily be obtained.

It is noteworthy (see Section 1.2) that compounds (10a-d) are also key intermediates in the synthesis of 2'-O-diphenylphosphoryl-3'-O-propionyl nucleosides (15).

Acid (MSA) catalyzed acetalization of (10a-d) with 5,6-dihydro-4-methoxy-2H-pyran [7] afforded the fully-protected derivatives (11a-d) which, without further purification by column chromatography, were treated with sodium methoxide to yield the required crystalline 2'-O-acetal derivatives (3a-d) in good yields. Only compound (3d: B = N^2-benzoylguanin-9-yl) had to be purified, prior to crystallization, by short column chromatography [8].

The usefulness of compounds (3a-d) is also demonstrated (see Section 1.2) in the synthesis of 2'-O-methoxytetrahydropyranyl-3'-O-diphenylphosphoryl nucleosides (16).

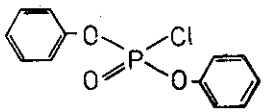
Convenient synthetic procedures for the preparation of building blocks (1, 2, and 3), by similar methods as depicted in Scheme 2, are described in the literature [9,10,11].

1.2. Properly-protected ribonucleosides bearing a masked phosphate at the 3'- or 2'- position.

In the preceding section convenient synthetic methods for the preparation of properly-protected ribonucleosides were described. These building blocks make it possible to synthesize RNA's with a defined sequence but without phosphate

groups at the head (5'-end) or tail (3'-end).

Recently, we introduced the monofunctional phosphorylating agent (12) and showed [12,13] that this reagent, in the presence of the tertiary base (13), was very convenient for the introduction of a phosphate group at the 3'-, 2'- or 2',3'-position of ribonucleosides or oligoribonucleotides.



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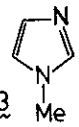
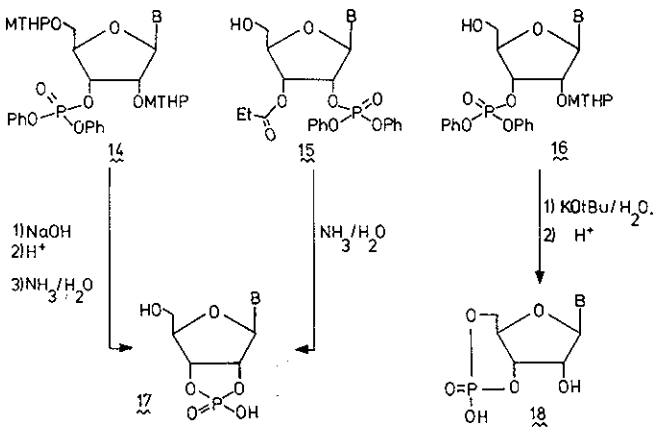


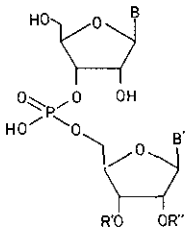
13

Thus when 2',5'-bis-O-4-methoxytetrahydropyran-4-yluridine (2;B=U) was treated with a slight excess of diphenyl phosphorochloridate (12) in acetonitrile solution, in the presence of 1-methylimidazole (13), phosphorylation occurred readily at 20°C and 2',5'-bis-O-4-methoxytetrahydropyran-4-yluridine-3'-diphenyl phosphate (14) was obtained in 60% yield. Base hydrolysis of (14) - 0.1 M-sodium hydroxide in aqueous dioxan at 20°C - gave 2',5'-bis-O-4-methoxytetrahydropyran-4-yluridine 3'-monophenyl phosphate in quantitative yield. Acid hydrolysis (0.01 N-HCl,pH 2) of the latter intermediate afforded uridine-3'-monophenyl phosphate, which was quantitatively converted - aqueous 0.155 M-ammonia - into uridine-2',3'-cyclic phosphate (17;B=U).

B	Yield, % 15
U	62
C^{an}	70
A^{an}	71
G^{bz}	57



The above described phosphorylation procedure could also be used in the synthesis of the diribonucleoside phosphate 2',3'-cyclic phosphate (19c;B=B'=U). Thus reaction between the partially-protected diribonucleoside monophosphate (6;B=B'=U;R'=H; R=R''=MTHP;Ar=2-ClC_6H_4) and diphenyl phosphorochloridate (12) in the presence of (13) gave the corresponding 3'-diphenyl phosphate in 60% yield. Complete deblocking of the latter compound, under the conditions as used for the preparation of (17;B=U), gave the required 2',3'-cyclic phosphate (19c;B=B'=U) in virtually quantitative yield.



	19	20	21	22
B	U	U	U	A
B'	U	A	C	G

a) R'= $-P(O)(OH)_2$, R''=H.

b) R'=H, R''= $-P(O)(OH)_2$.

c) R'R''= $>P(O)OH$.

Despite the positive results obtained in the synthesis of the 2',3'-cyclic phosphate (17;B=U), we developed [14] a more convenient procedure for the synthesis of 2',3'-cyclic phosphates of all four common ribonucleosides. Thus phosphorylation of the easily accessible 3',5'-diesters (10a-d;Scheme 2) with diphenyl phosphorochloridate (12) in the presence of (13), followed by selective 5'-deacylation (K_2CO_3/MeOH), afforded the phosphotriester derivatives (15) of all four common ribonucleosides (B=U,C^{an},A^{an} or G^{bz}) in good yields. Alkaline treatment (aqueous ammonia) of the nucleoside 2'-diphenyl phosphates (15) results in the formation of the nucleoside 2',3'-cyclic phosphates (17;B=U,C,A or G), which could be isolated in overall good yields.

The usefulness of the phosphotriester derivatives (15), which can be regarded as terminal nucleotide building blocks, is also demonstrated in the synthesis of the nucleotidyl-(3'- 5')-nucleoside-3'-phosphates UpUp (19a), UpAp (20a), UpCp (21a) and ApGp (22a). For instance, removal of the propionyl, phenyl and aryl groups from the fully-protected dinucleoside diphosphate (6;B=B'=U;R=THP;R''=diphenyl phosphate;R'=propionyl;Ar=2-ClC_6H_4), which was prepared by using the nucleotide unit (15;B=U) as the second component in a two-step phosphorylation procedure (Scheme 1), with aqueous ammonia afforded the 2',3'-cyclic intermediate (6;B=B'=U; R'R''=2',3'-cyclic phosphate;R=THP;Ar=H). Incubation of the latter intermediate

with pancreatic RNase gave crude 3'-phosphate (6;B=B'=U;R=THP;R'=phosphate; R''=H;Ar=H) which, after purification, was treated with aqueous hydrochloric acid to give pure UpUp (19a) in 80% yield (based on the 2',3'-cyclic intermediate). In the same way, UpCp (21a) was prepared in 86% yield. The other two diribonucleoside diphosphates UpAp (20a) and ApGp (22a) were obtained in good yields (81 and 62%, respectively) by treatment of the corresponding partially-protected 2',3'-cyclic phosphate intermediates with T_2 takadiastase followed by acid hydrolysis. However, complete deprotection of the afore mentioned fully-protected dinucleoside diphosphate (6;B=B'=U;R=THP;R''=diphenyl phosphate;R'=propionyl;Ar=2-ClC_6H_4) with aqueous ammonia followed by acid resulted in the formation of a mixture of 2'- and 3'-phosphates of the dinucleoside diphosphates (19a and 19b;B=B'=U).

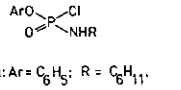
Another interesting application of the above described phosphorylation procedure is the synthesis and smooth conversion [15] of nucleoside-2'-acetal-3'-diphenyl phosphates (16) into the nucleoside-3',5'-cyclic phosphates (18) in good overall yields. For instance, the uridine derivative (16;B=U) was prepared (79% yield) by treatment of the 2'-acetal-5'-ester of uridine (5;B=U) with a slight excess of diphenyl phosphorochloridate (12), in the presence of 1-methylimidazole (13), followed by removal of the 5'-O-p-chlorophenoxyacetyl group with potassium carbonate in methanol. Treatment of (16;B=U) with an excess of potassium t-butoxide in dimethyl sulphoxide solution followed by mild acidic hydrolysis (pH 2, 0.01 N-HCl) afforded the unprotected uridine 3',5'-cyclic phosphate (18;B=U) in 90% yield, based on (16;B=U).

The general usefulness of the present approach to the synthesis of nucleoside 3',5'-cyclic phosphates was demonstrated by the preparation of the biologically-important adenosine compounds (18;B=A;m_1^6A or m_2^6A, respectively). Thus the crystalline 2'-O-methoxytetrahydropyranyl-3'-O-diphenyl phosphate esters of N^6-p-anisoyl-, N^6-methyl- and N^6,N^6-dimethyladenosine (16;B=A^{an}, m_1^6A or m_2^6A) were obtained in 65, 70 and 71% yield, respectively, from the

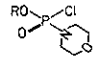
appropriate 2'-acetal-5'-esters (5;B=A^{an},m_1^6A or m_2^6A). The phosphotriesters (16;B=A^{an},m_1^6A or m_2^6A) were converted into the corresponding 3',5'-cyclic nucleotides (18;B=A^{an},m_1^6A or m_2^6A) which were then isolated in 82, 90 and 80% yields, respectively. Adenosine 3',5'-cyclic phosphate (18;B=A) itself was obtained by treating its N^6-p-anisoyl derivative (18;B=A^{an}) with aqueous ammonia.

1.3. Properly-protected ribonucleosides bearing a masked phosphate at the 5'-position.

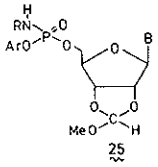
For the introduction of phosphate groups at the 5'-position of partially-protected ribonucleosides or nucleic acids *via* phosphotriester intermediates we developed [14,16] the monofunctional phosphorylating agents (23a-e and 24a-b). The latter aryl or alkyl phosphoroamidochloridates are readily available and stable crystalline compounds and are, furthermore, effective phosphorylating agents.



23 a: Ar= C_6H_5; R = C_6H_{11}.
b: Ar= 2-ClC_6H_4; R = C_6H_{11}.
c: Ar=2,4-$Cl_2C_6H_3$; R=C_6H_{11}.
d: Ar=2,6-$Cl_2C_6H_2$-4-t-pentyl; R= C_6H_{11}.
e: Ar=C_6H_5; R= CH_2CH_2OMe.

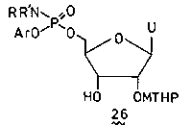


24 a: R =2,4-$Cl_2C_6H_3$.
b: R= CH_2CBr_3



25

B	Yield, % 25
U	88
C^{an}	90
A^{an}	88



26

a: Ar= C_6H_5; R= C_6H_{11}; R'=H.
b: Ar= 2,4-$Cl_2C_6H_3$; RR'= $(CH_2)_2O(CH_2)_2$

Aryl or alkyl phosphoroamidochloridates may readily be prepared by treating aryl or alkyl phosphorochloridates [17] with two molecular equivalents of the appropriate amines in ether solution. In this way we prepared: four crystalline

aryl phosphorocyclohexylamidochloridates (23a-d) in yields of 80% or more; one aryl methoxyalkylamidochloridate (23e) which was obtained as an oil in 80% yield; one aryl phosphoromorpholidochloridate (24a) and one alkyl phosphoromorpholidochloridate (24b), both were obtained as crystalline compounds in yields of 80%.

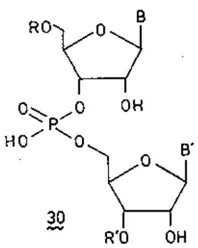
All of the above obtained aryl or alkyl phosphoramidochloridates were found to be effective phosphorylating agents. Thus treatment of 2',3'-O-methoxymethyleneuridine (1,B=U) with a twofold excess of phenyl, o-chlorophenyl or 2,4-dichlorophenyl phosphorocyclohexylamidochloridate (23a, 23b, 23c or 23e), in the presence of 1-methylimidazole (13), gave the corresponding 2',3'-O-methoxymethyleneuridine-5'-aryl phosphoramidates (25;B=U) in overall yields of 88%. In the same way: high yields of products (respectively, 25;B=A^{an};Ar=2,4-$Cl_2C_6H_3$;R=-C_6H_{11} and 25;B=C^{an};Ar=2,4-$Cl_2C_6H_3$;R=-C_6H_{11}) were obtained, when N^4-anisoyl-2',3'-O-methoxymethylenecytidine (1;B=C^{an}) and N^6-anisoyl-2',3'-O-methoxymethyleneadenosine (1;B=A^{an}) were phosphorylated with 2,4-dichlorophenyl phosphorocyclohexylamidochloridate (19b). Furthermore, the aryl and alkyl phosphoramidates displayed a high degree of regioselectivity. Thus phosphorylation of 2'-O-methoxytetrahydropyranyluridine (3;B=U) with both (19b) and (24a) gave crystalline (26a) and (26b) in 60 and 50% yield, respectively. The tribromoethyl phosphoromorpholidates of the three modified nucleosides of adenosine (27a;B=iPA; 27b;B=m_2^6A and 27c;B=m_1^6A) were prepared by allowing the corresponding modified nucleosides and 2,2,2-tribromoethyl phosphoromorpholidochloridate (24b) to react together in dry pyridine at 20°C. The required phosphotriesters (27a-c) could be isolated as colourless solids in 75-80% yield.

Complete deprotection of nucleoside aryl phosphoramidates (25) and (26) was performed by treating these nucleoside phosphotriester derivatives with 0.1 M-sodium hydroxide in aqueous dioxan (4:1,v/v) at 20°C followed by acid hydrolysis (0.01 N-HCl, pH 2) at 20°C.

In all cases, alkaline hydrolysis of the nucleoside 5'-aryl phosphoramidates (25 and 26) led, although with different rates, to the formation of the corres-

ponding nucleoside 5'-phosphoramidates which could be isolated in virtually quantitative yield. Acid hydrolysis of the thus obtained 5'-phosphorocyclohexylamidates and 5'-phosphoromorpholidates afforded the nucleoside 5'-phosphates as the sole nucleotide product. The half-times of acid hydrolysis of these two phosphoramidates were found to be 516 and 10 min, respectively. However, the relatively slow acidic hydrolysis rate of nucleoside phosphoroclyclohexylamidates was not especially disadvantageous. Thus uridine, cytidine and adenosine 5'-phosphates were readily obtained in virtually quantitative yields from their respective precursors (25;B=U, C^{an} or A^{an}, respectively) and uridine 5'-phosphate was also obtained in high yield by acidic treatment of the alkaline hydrolysis products of the above described 2'-O-methoxytetrahydropyranyluridine 5'-aryl phosphoramidates (26a-b).

The monophosphorylating agents (23a) and (23d) showed also to be very suitable for the introduction of terminal 5'-phosphate groups in the phosphotriester approach to oligonucleotide synthesis. For instance, the partially-protected uridylyl-(3'-5')-uridine derivative (6;B=B'=U;R=H; Ar=2,4-$Cl_2C_6H_3$;R'R''=methoxymethylene) was phosphorylated with an excess of phosphorylating agent (23a) in acetonitrile solution, in the presence of 1-methylimidazole (13), to give a fully-protected dinucleoside phosphate phosphoramide in 85% yield. Treatment of the latter compound at 20°C with 0.1 M sodium hydroxide solution for 15 hr followed by dilute hydrochloric acid (pH 2) for three days gave uridylyl-(3'-5')-uridine 5'-phosphate (30a, pUpU) in virtually quantitative yield.



30

a; B = B' = U; R = P(O)(OH)$_2$; R' = H.

b; B = B' = U; R = P(O)(OH)-O-P(O)(OH)$_2$; R' = H.

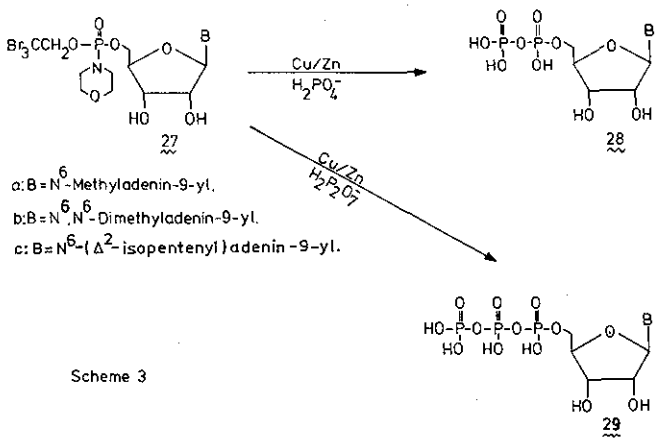
c; B = B' = U; R = P(O)(OH)-O-P(O)(OH)-O-P(O)(OH)$_2$; R' = H

d; B = A; B' = U; R = R' = P(O)(OH)$_2$.

Phosphorylation of the partially-protected adenylyl-(3'-5')-uridine 3'-phos-

phate (6;B=A^{an};B'=U;R=H;Ar=2-ClC_6H_4;R'=diphenylphosphate;R''=MTHP) with phosphorylating agent (23d) gave a fully-protected dinucleoside triphosphate. Deblocking of the latter compound with base and acid followed by a short ammonia treatment afforded pApU>p in very good yield. This 2',3'-cyclic derivative was quantitatively converted, by enzymic digestion with pancreatic RNase, into (30d, pApU(3')p.

The availability of the modified adenosine tribromoethyl phosphoromorpholidates (27a-c) enabled us to synthesize [18] the respective di- and triphosphates of these modified adenosine nucleosides. (See Scheme 3.)



27

a: B = N^6-Methyladenin-9-yl.
b: B = N^6,N^6-Dimethyladenin-9-yl.
c: B = N^6-(Δ^2-isopentenyl)adenin-9-yl.

28

29

Scheme 3

So far, complete deblocking of nucleoside aryl phosphoramidates (25 and 26) was performed by alkaline followed by acid hydrolysis.

However, recently, we showed that the 2,2,2-tribromoethyl group can be removed selectively, by Cu/Zn couple [19], from the nucleoside tribromoethyl phosphoromorpholidates (27a-c) to give the respective nucleoside 5'-phosphoromorpholidates which, in turn, could be easily converted, in the presence of phosphoric or pyrophosphoric acid, into the corresponding di-(28a-c) and triphosphates (29a-c), respectively.

For example, removal of the 2,2,2-tribromoethyl group from N^6-(Δ^2-isopentenyl)adenosine 5'-tribromoethyl phosphoromorpholidate (27a) with Cu/Zn couple during 10 min at 20°C gave the corresponding N^6-(Δ^2-isopentenyl)adenosine

5'-phosphoromorpholidate which was treated with bis(tri-n-butylammonium) pyrophosphate in DMF solution at 20°C for 3 hr. Work-up and purification of the crude reaction mixture gave pure N^6-(Δ^2-isopentenyl)adenosine 5'-triphosphate (29a;B=iPATP). N^6-methyl- (29c;B=m_1^6A) and N^6,N^6-dimethyladenosine (29b;B=m_2^6A) were obtained similarly in 84 and 81% yields, respectively.

In the above experiment, when mono(tri-n-butylammonium)phosphate salt in dry pyridine was used instead of the pyrophosphate salt in dry DMF the corresponding modified nucleoside diphosphates (28a-c) could be isolated in 71, 72 and 75% yields, respectively.

Finally, 2,2,2-tribromoethyl phosphormorpholidochloridate (24b) proved [20] also to be a convenient reagent for the introduction of 5'-terminal di- and triphosphates in the phosphotriester approach to oligoribonucleotides.

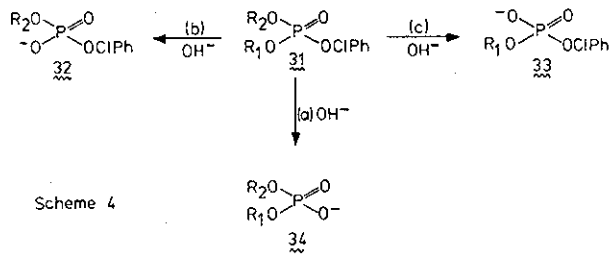
Thus the partially-protected uridylyl-(3'-5')-uridine derivative (6;B=B'=U; R'R''=methoxymethylene;R=H;Ar=2-ClC_6H_4) was phosphorylated with an excess of (24b) in the presence of 1-methylimidazole (13) to give a fully-protected dinucleoside phosphate phosphoramide in 70% yield. Removal of the tribromoethyl group from the latter derivative gave the corresponding 5'-phosphormorpholidate derivative which was converted, in the presence of phosphoric and pyrophosphoric acid,respectively, into the respective di- and triphosphates. Complete deblocking of the latter derivatives with base followed by acid afforded ppUpU (30b) and pppUpU (30c), respectively.

2. The formation and deprotection of internucleotide phosphotriester bonds.

Nowadays, the development of successful procedures, with the ultimate goal in mind, to synthesize oligoribonucleotides exclusively *via* phosphotriester intermediates is one of the main and difficult tasks in nucleic acid chemistry. The reason for this is, that it is very difficult to develop a proper protecting group for the masking of the internucleotide phosphodiester linkages. To be successful the nature of this group must be such that the following conditions are met: (a) it should remain stable during the whole course of

the synthesis; (b) it should allow a selective deblocking of one of the protecting groups at either the 5'- or 3'- position of a growing nucleic acid chain; (c) its final deblocking should not give rise to internucleotide bond fission.

After a careful analysis [21] of different aryl-protecting groups for the masking of phosphodiester linkages, we have come to the conclusion that the 2-chlorophenyl group meets, in a satisfactory way, the above formulated conditions. This group is stable under the conditions as used for the formation of phosphotriester bonds and survives usual work-up procedures. Its stability towards base allows a selective removal of a p-chlorophenoxyacetyl group at the 5'- position of a growing nucleic acid chain. Furthermore, removal of the 2-chlorophenyl from the phosphotriester intermediate (31; R_1 and R_2 are properly-protected nucleosides) with aqueous sodium hydroxide in dioxan indicates that, on the average, alkaline hydrolysis proceeds (Scheme 4) 98% by pathway (a) and 2% by pathways (b) and (c) together (wrong breakdown).

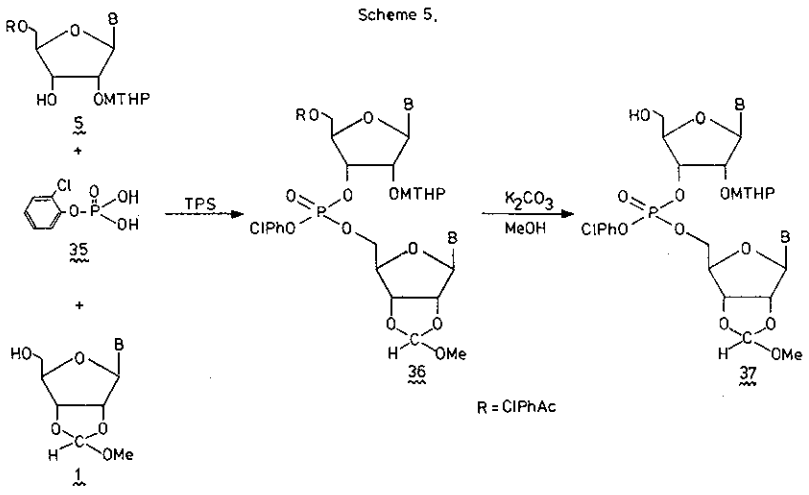


Scheme 4

The introduction of the internucleotide phosphotriester bond can be accomplished as follows (Scheme 5.). A protected nucleoside (5 ; B=U), having a free 3'- hydroxyl function, is added to a solution of a slight excess of 2-chlorophenyl dihydrogen phosphate [17] (35) in pyridine in the presence of the activating agent 2,4,6-tri-isopropylbenzenesulphonyl chloride [22] (TPS). After 8 hr, the protected nucleoside (1 ; B=U), having a free 5'- hydroxyl function, and more TPS were added and the second step of the phosphorylation was

allowed to proceed to completion. The products were then worked-up and fractionated by short column chromatography [8] on silica gel to give the fully-protec-

Scheme 5.

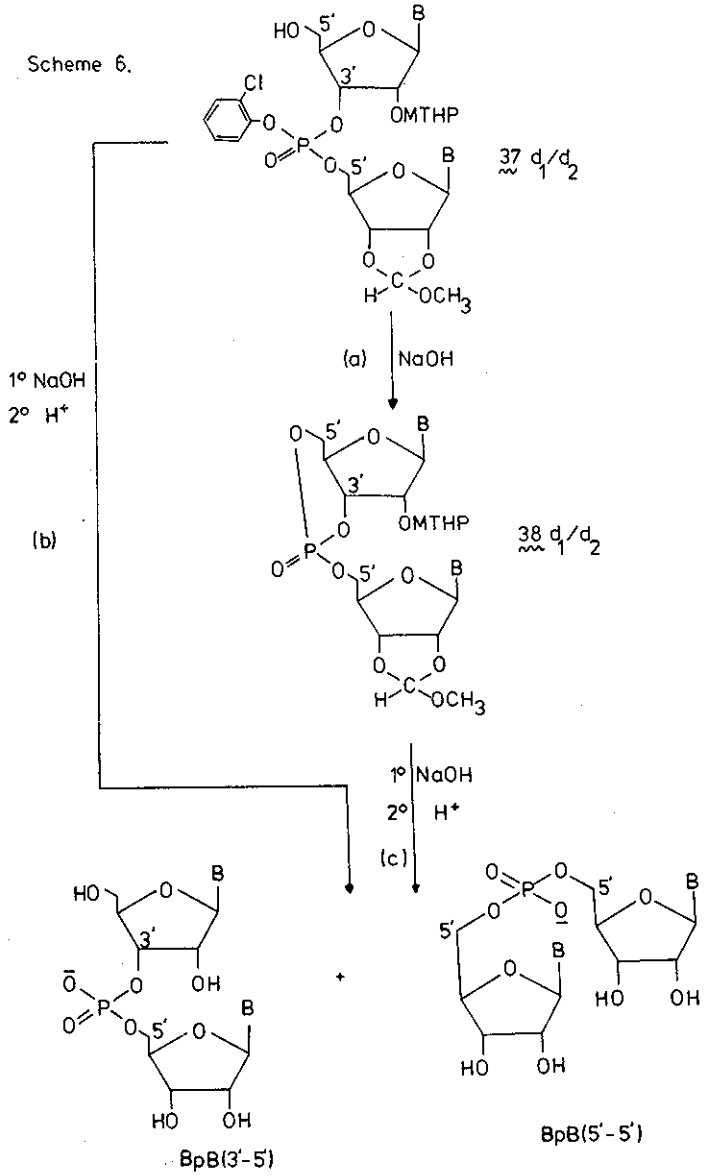


ted dinucleoside monophosphate (36 ; B=U) as a glass in 75% yield. Selective deblocking of the p-chlorophenoxyacetyl group, at the 5'- position of (36;B=U), with K_2CO_3/MeOH affords the dinucleoside monophosphate (37;B=U) which can be isolated as a colourless solid in 85% yield. The latter derivative (37) can now be extended in the 5'- dirction (tail-to-head synthesis) with another suitably protected ribonucleoside.

The synthesis of diribonucleoside monophosphates with the possibility for extension in the 3'- direction (head-to-tail synthesis) can be accomplished by adding, in the second step of the previously described phosphotriester method, the 2'- acetal nucleosides (3;B=U,C^{an},A^{an} or G^{bz}). Coupling of these units , which have the 3'- and 5'- OH unblocked, may give rise to the formation of small amounts of unwanted symmetrical 3'-3' triesters.

Complete deblocking of the dinucleoside monophosphate (36;B=U) or (37;B=U) with aqueous sodium hydroxide in dioxan followed by acid hydrolysis (0.01 N-HCl, pH 2) doesn't afford the required uridylyl-(3'-5')-uridine (UpU). Instead,

Scheme 6.



Experiment no.	Substrate	Diastereoisomers	Distribution of Products %(3'-5')	%(5'-5')
1	37	d_1	61	39
2	37	d_2	80	20
3	37	d_1 + d_2	72	28
4	38	d_1	58	42
5	38	d_2	79	21

the deblocked product consists of a mixture of dinucleoside monophosphates with natural (3'-5') and unnatural (5'-5') internucleotide phosphodiester linkages. Examination [23] of this phenomenon revealed that, during the base hydrolysis of (36;B=U) or (37;B=U), neighbouring group participation of the 5'- OH group with the phosphotriester had occurred. The result of this participation is the rapid formation of the cyclic phosphotriester (38;B=U) which, after prolonged treatment with base followed by acid, hydrolyses to give a mixture of two isomeric dinucleoside monophosphates (Scheme 6). The quantities of isomeric diribonucleoside monophosphates which are formed during alkaline hydrolysis of (37;B=U) depend on the diastereoisomeric composition of the starting product. Thus both pure diastereoisomers d_1 and d_2 of starting product (37;B=U) give 39 and 20%, respectively, uridylyl-(5'-5')-uridine (experiments no. 1 and 2 in Scheme 6). Furthermore, both pure diastereoisomers d_1 and d_2 of the cyclic intermediate (38;B=U) afford (experiments no. 4 and 5) virtually identical amounts of uridylyl-(5'-5')-uridine as obtained in the previous two experiments. These results indicate that alkaline hydrolysis of (37;B=U) proceeds almost exclusively *via* the cyclic intermediate (38;B=U).

The same phenomenon was observed during the deblocking of a dinucleoside o-chlorophenyl phosphotriester having a 3'- OH group free but all other hydroxyl groups protected with base-stable groups. In this case, deblocking affords a mixture of dinucleoside monophosphates containing (3'-5') and (3'-3') internucleotide phosphodiester linkages .

However, neighbouring group participation can be prevented by tetrahydropyranylation [24,25] prior to unblocking of partially-protected oligonucleotides. Thus complete deblocking of dinucleoside monophosphate (36;B=U;R=THP) affords solely uridylyl-(3'-5')-uridine.

In table 1 are summarized some of the oligoribonucleotides which were prepared according to the above described two-step phosphorylation procedure.

Another way to prevent neighbouring group participation in the deblocking

process of oligoribonucleotides, with a 5'- end free or protected with a base labile group which is hydrolysed faster than the 2-chlorophenyl group for the protection of phosphodiester linkages, is to introduce one 2,2,2-tribromoethyl group at the head (5'- terminus) of the oligonucleotide to be synthesised. Complete deblocking is now performed in the following order: firstly the 2,2,2-tribromoethyl group is removed with Cu/Zn-couple (5 min in DMF at 20°C); secondly the remaining 2-chlorophenyl groups and other base-labile groups are removed with aqueous ammonia at 50°C and, finally, the acid-labile groups with aqueous hydrochloric acid (pH 2). In applying this modification, we were able to synthesize [26] the two hexamers m_2^6ApCpCpUpCpC and ApCpCpUpCpm_2^4C without recurrence to tetrahydropyranylation.

Table 1.

Oligoribonucleotides prepared *via* the two-step phosphorylation procedure.

Compounds	Lit.	Compounds	Lit.
UpUpU	25	ApG(3')p	14
pUpU	16	i^6Ap4sU	27
ppUpU	20	pAmpU	20
pppUpU	20	CGAA	20
UpU>p	12	UCCC	20
pApU>p	13	UUCA	20
pApU(3')p	13	AGGA	20
UpC(3')p	14	m_2^6ACCUCC	26
UpU(3')p	14	ACCUCm_2^4C	26
UpA(3')p	14	UCCA	20

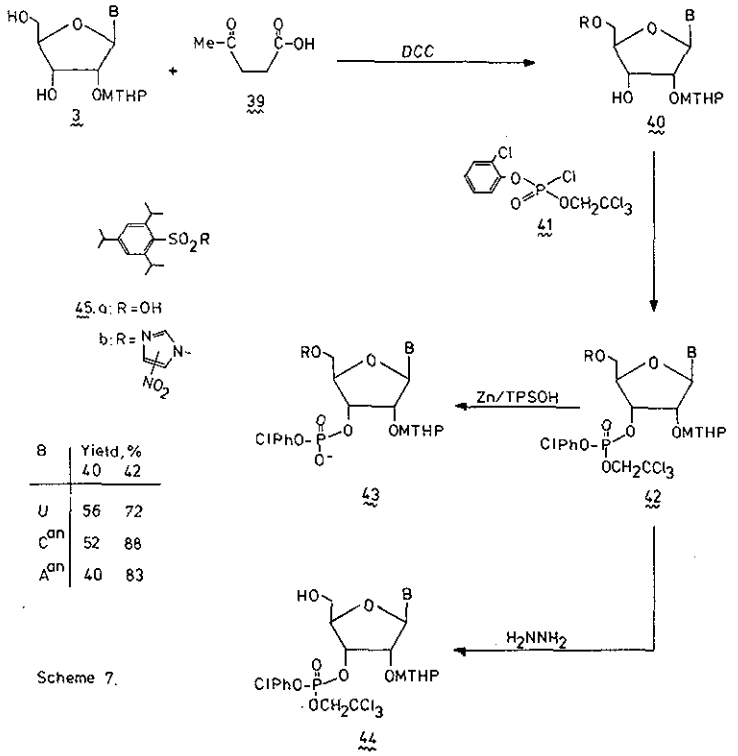
Am = 2'-O-methyladenosine; i^6A = N^6-(Δ^2-isopentenyl)adenosine; 4sU = 4-thiouridine; m_2^6A = N^6,N^6-dimethyladenosine; m_2^4C = N^4,N^4-dimethylcytidine.

The results obtained (Table 1) in the synthesis of oligoribonucleotides *via* the two-step phosphorylation procedure looked very promising. Nevertheless, the following disadvantages still adhere to this approach. Firstly, the p-chlorophenoxyacetyl group which was used to block the 5'- position is too labile and sometimes loss (of up to 5%) occurred during silica-gel chromatography. Besides,this group is too stable to be removed with complete selectivity from oligoribonucleotides (three ore more units long) having 2-chlorophenyl as protective groups for the internucleotide linkages. Secondly, phosphorylation with bifunctional phosphorylating agents, e.g. 2-chlorophenyl dihydrogen phosphate (35)[17], leads to the formation of unwanted (3'-3') symmetrical products in the first step of the phosphotylation and, because unreacted phosphorylating agent remains in the reaction mixture, (5'-5') symmetrical products in the second step of the reaction. Thirdly, deblocking of phosphodiester linkages protected with aryl groups requires that, in order to suppres neighbouring group participation, all terminal hydroxyl groups must be protected with base-stable groups. Finally, TPS is a rather sluggish activating agent, gives rise to the formation of sulphonylated [28] products and loss of required products occurs, especially, in a coupling process in which guanosine participates.

To overcome the above mentioned disadvantages we successively replaced:the p-chlorophenoxyacetyl by the levulinyl group; the bifunctional phosphorylating agent (35) by the monofunctional o-chlorophenyl 2,2,2-trichloroethyl phosphorochloridate (41). Furthermore, deblocking of phosphodiester protecting groups was performed with fluoride ion instead of sodium hydroxide and activation during the formation of phophotriester bonds was performed with 2,4,6-triisopropylbenzenesulphonyl-4(5)-nitroimidazolide (45b).

By using the levulinyl group we found several properties which will make this group very attractive for further use: (i) it can easily be introduced, although with not high selectivity, by dropwise addition of a solution of DCC in dioxan to a solution of a ribonucleoside-2'-acetal (3; B=U, C^{an} or

A^{an}) (Scheme 7) and levulinic acid in 2,6-lutidine/dioxan; (ii) it can be remo-



B	Yield,% 40	42
U	56	72
C^{an}	52	88
A^{an}	40	83

Scheme 7.

ved selectively by hydrazine hydrate in pyridine-acetic acid (conversion of 42 into 44, Scheme 7); the reaction time for its complete removal (3-4 min) is independent of the nature of the substrate; this feature is a great improvement over the use of base-labile protecting groups, in the latter case the reaction time depends on the sequence [29] as well as the length [30] of the oligonucleotides; (iii) despite its non-lipophilic character a reasonable drop in R_f value is observed upon its removal, which facilitates the monitoring of this deblocking step. Another very important property, which enables deblocking of 2-chlorophenyl groups in phosphotriester intermediates bearing ester functions at the 5'- position, is that the levulinyl group is stable under the conditions necessary for the removal of phosphate protecting 2-chlo-

rophenyl groups by fluoride ion.

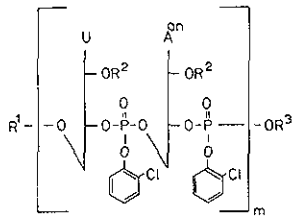
To eliminate the formation of unwanted symmetrical products in the two-step phosphorylation method, we introduced the easily accessible [31] monofunctional reagent (41, Scheme 7). Thus treatment of the partially-protected nucleoside (40) with reagent (41) in acetonitrile solution, using 1-methylimidazole (13) as tertiary base, afforded the fully-protected ribonucleotide (42) in good yield. The fully-protected nucleotide (42) is a crucial intermediate in the modified phosphotriester approach; to be effective in the synthesis of oligoribonucleotides it is essential that the removal of the 2,2,2-trichloroethyl group, to give the phosphotriester intermediate (43), is selective. Fortunately, we found that this group could be removed fastly (≤5 min) and selectively from the phosphotriester (42) with the reagent Zn/TPSOH (45a)/pyridine. After removal of excess zinc by filtration, the filtrate was diluted with chloroform and the zinc ions extracted from the organic layer with aqueous triethylammonim bicarbonate buffer (TEAB) to give the triethylammonium salt of phosphodiester (43) as the sole nucleotide product in quantitative yield.

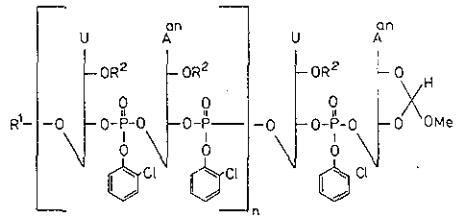
The usefulness of the intermediates (42) and (43), together with the activating agent TPSNI (45b), will be demonstrated [32] in the synthesis of the fully-protected tetradecamer [53b, $(UpA)_7$].

First the fully-protected dinucleoside monophosphate (48a)was prepared by condensation of (43;B=U) with 2',3'-O-methoxymethylene-N^6-anisoyladenosine (1;B=A^{an}), using TPSNI (45b) as activating agent. Work-up of the reaction mixture afforded (48a, n = 0) in 82% yield.

The fully-protected dinucleoside diphosphate (46a; m = 1), which was used in most steps of the chain-lengthening block-condensations, was prepared *via* the general procedure outlined above. Thus mononucleotide (43;B=U) was condensed with the partially-protected mononucleotide (44;B=A^{an}) and TPSNI (45b) to give, after work-up and purification by short column chromatography, (46a)

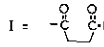
in 87% yield.





46 : m = 1
47 : m = 2
a : R^1 = I, R^2 = II, R^3 = Cl_3CCH_2-
b : R^1 = I, R^2 = II, R^3 = H
c : R^1 = H, R^2 = II, R^3 = Cl_3CCH_2-

48 : n = 0
49 : n = 1
50 : n = 2
51 : n = 3
52 : n = 4
53 : n = 6
a : R^1 = I , R^2 = II
b : R^1 = H , R^2 = II

I =  II= 

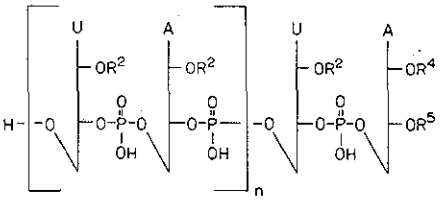
The dinucleoside diphosphate (48a), bearing the chain-terminating 2',3'-O-methoxymethylene group, constitutes the 3'- terminus of the oligomer to be synthesized. Starting from this end the molecule was extended towards the 5'- direction by: (i) removal of the levulinyl group from (48a); (ii) removal of the trichloroethyl group from (46a) and finally coupling of resulting (48b) and (46b), in the presence of TPSNI (45b), to give fully-protected tetramer (49a) in 72% yield. In the same way hexanucleotide (50a, n = 2), octanucleotide (51a, n = 3) and decanucleotide (52a, n = 4) were prepared in reasonable yields (69, 74 and 61%, respectively) by zinc treatment of (46a, m = 1) and subsequent condensation of (46b, m = 1) with (49b, n = 1), (50b, n = 2) and (51b, n = 3), respectively.

Finally, the fully-protected tetradecamer (53a, n = 6) was obtained by block-condensation of a tetramer with a decamer. Tetranucleoside tetraphosphate (47a, m = 2) was prepared in 75% yield by condensation of dinucleoside diphosphate (46a, m = 1) with dinucleoside diphosphate (46c, m = 1), obtained by hydrazine treatment of (46a), and TPSNI (45b). The trichloroethyl group was removed from (47a, m = 2) with zinc in pyridine/TPSOH (45a), and the resulting phosphodiester (47b) was condensed with the partially-pro-

tected decanucleotide (52b, n = 4) in the presence of TPSNI to give the fully protected tetradecamer in 58% yield.

The usefulness of TPSNI (45b) should be pointed out in this study. In comparison with TPS it gives far less sulphonylation of the 5'- hydroxy function of the incoming nucleoside and, besides, a higher yield of the desired product is obtained. Furthermore, block-condensations between small and large oligonucleotides are much faster (two times) with TPSNI than with TPS. Recently, arylsulphonyltriazolides have been used by Neilson [33] and Hata [34] in the synthesis of oligoribonucleotides with a defined sequence.

Deblocking of the fully-protected tetradecamer (53a, n = 6) can now be performed. The whole process comprises the successive removal of: o-chlorophenyl groups with fluoride ion ; N-acyl and levulinyl groups with aqueous ammonia at 50°C and, finally, all acid-labile groups with aqueous hydrochloric acid (pH 2). Thus tetradecamer (53a) was treated with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran-pyridine-water for 16 hr at 20°C. The remaining base-labile groups were removed by treatment with 25% aqueous ammonia for 24 hr at 50°C. The resulting partially-protected tetradecamer (54a) was analyzed by high-performance liquid chromatography and purified by DEAE-Sephadex chromatography. A consistent average of *ca* 2% internucleotide cleavage per



54 n = 6

a: R^2=MTHP, R^4R^5= 

b: $R^2=R^4=R^5$=H

phosphotriester linkage was found by using fluoride ion as the deblocking agent.

The fact that phosphotriesters are susceptible to nucleophilic attack by fluoride ion has been known for many years [35] and, recently, two groups have employed [36] this nucleophile for the unblocking of aryl-protected oligodeoxynucleotides.

Finally, removal of the acid-labile protective groups (0.01 N-HCl, pH 2) afforded the unprotected tetradecamer [54b, $(UpA)_7$] in a yield of 37%.

By applying the same procedures, as outlined above, we were able to synthesize the oligoribonucleotides $r\text{-}(UpA)_5$ [37], r-ApCpCpUpCpC [26], r-UpCpCpUpUpA [38] and r-ApCpCpUpCpCpUpUpA [38].

3. Analysis of synthetically-prepared oligoribonucleotides.

Existing analytical methods which are frequently used by organic chemists to assess homogeneity and structural proof of synthetically-prepared oligoribonucleotides are rather cumbersome and not very reliable.

However, many groups have reported on the use of high-performance liquid chromatography (HPLC) for the analysis of nucleic acid components [39], and showed that this technique could be used successfully for the identification and quantitation of nucleosides and nucleotides.

Indeed, inspection of the analytical data presented in Table 2 clearly shows that: (i) the identity of different nucleotides can be rapidly and unambiguously achieved by a comparison of their respective retention times; (ii) the ε values allow, in principle, a direct quantitation of ribonucleotides(sides).

Analysis of synthetically-prepared oligoribonucleotides, which consists of assessing base composition, ascertaining the nature of the internucleotide phosphodiester linkages and sequence analysis, can easily be performed by using HPLC.

Thus, for example, the identity of the (3'-5')-internucleotide linkages in the tetradecamer $r\text{-}(UpA)_7$ was established [32] by its complete digestion with

phosphodiesterase, spleen phosphodiesterase (Figure 1) and pancreatic ribonuclease (Figure 2) to the expected products. Quantitation of the enzymic products

Table 2.

Retention times[a] and ε_{254} (pH 4.5) values of the common nucleotides(sides)

Ribonucleosides	Retention times (min)					ε_{254} values[b]	
	M	pM	M>p	M(2')p	M(3')p	M(3')p or pM	M
uridine	0	9.1	2.3	7.3	8.8	9.1	8.9
adenosine	0	14.0	3.6	9.1	13.5	14.0	13.0
cytidine	0	5.3	1.8	5.6	7.1	5.3	6.6
guanosine	0	13.6	6.8	20.7	27.0	13.6	13.5

a) Relative to injection peak and under isocratic elution conditions (0.005 M-KH_2PO_4, pH 4.5).

b) Expressed as l/mmole.cm.

thus obtained affords the base composition of the tetradecamer. The sequence analysis of synthetically-prepared oligoribonucleotides, using HPLC, is based on the stepwise degradation of these oligoribonucleotides by the exonucleolitic action (the digestion of oligonucleotides starts at the righthand-end and proceeds towards the left producing the mononucleoside 5'-phosphates) of the enzyme venom phosphodiesterase and the simultaneous recording of the amounts of ribonucleotides and ribonucleosides evolved. Plotting of molar fractions of released monomers against time results in curves that relate the concentration of each ribonucleotide/ribonucleoside evolved to the time of venom phosphodiesterase action. For instance, the sequence r-ApCpCpUpCpC is easily derived [40] from the curves in Figure 3.

Furthermore, HPLC proved also [26,38] to be a very useful technique for monitoring the efficacy of the removal of protecting groups from masked phosphodiester linkages.

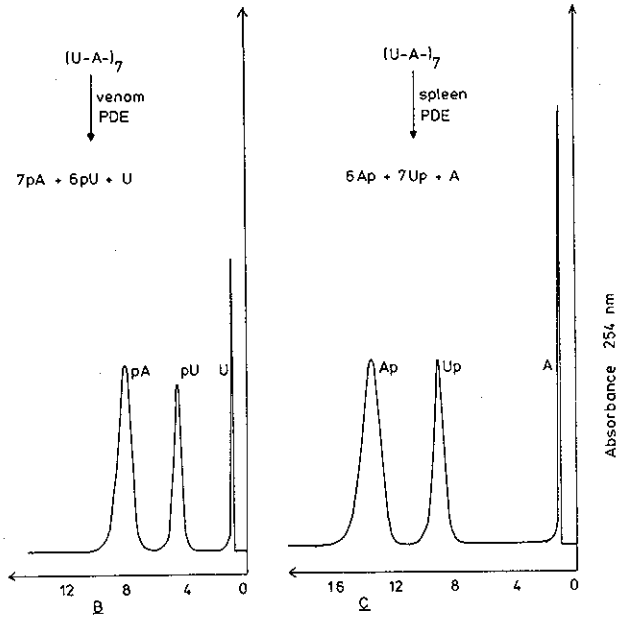


Figure 1. HPLC analysis of venom and spleen digestions of tetradecamer r-$(UpA)_7$; B,C = isocratic elution.

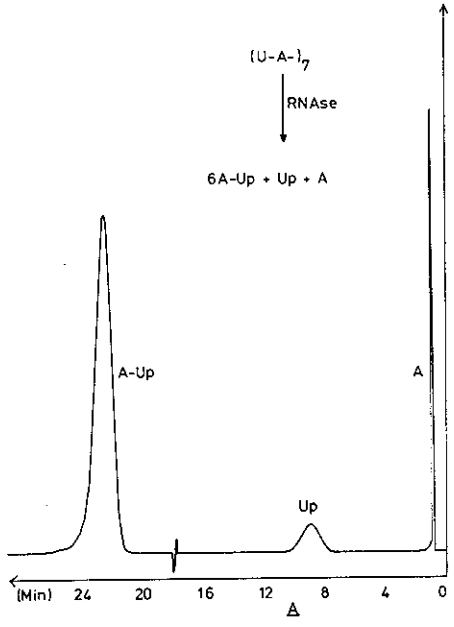


Figure 2. HPLC analysis of RNase digestion of tetradecamer r-$(UpA)_7$; A = gradient elution.

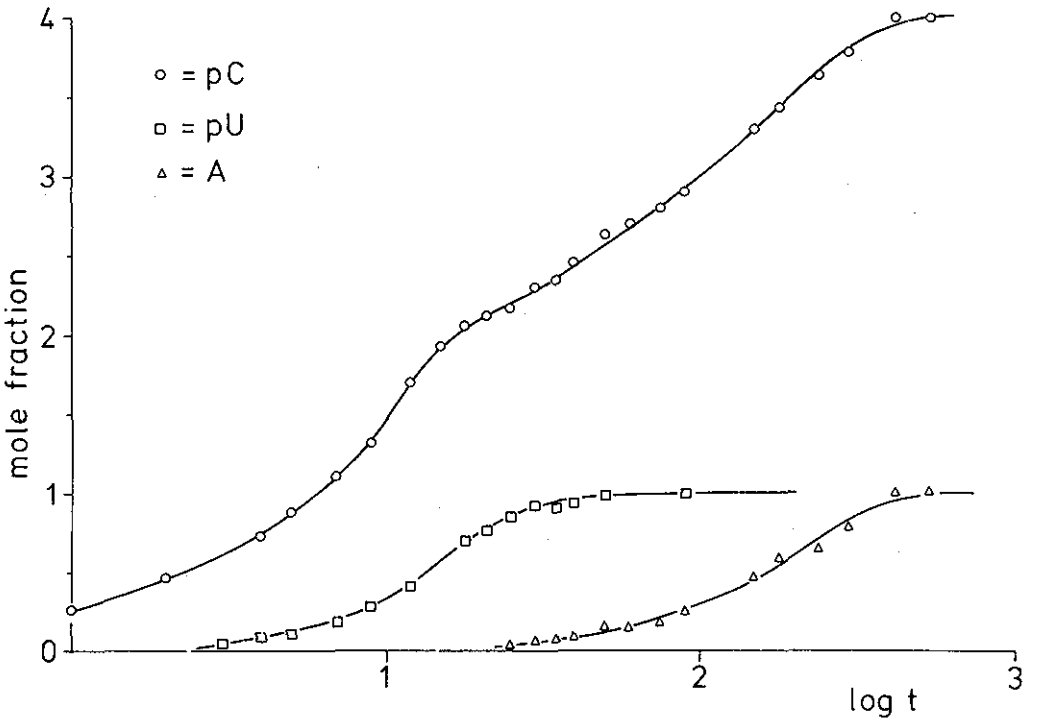


Figure 3. Curves of the time of appearance of nucleotides (pC and pU) and nucleoside (A) from venom phosphodiesterase action. Synthetic r-ApCpCpUpCpC.

Conclusion.

The data presented in this review demonstrate that the phosphotriester approach is, at the present time, the method of choise for the synthesis of oligoribonucleotides with a defined sequence and length. However, it must also be stated that the synthesis of these molecules is by no means a routine method. To achieve this more research dealing with the development of reliable deblocking procedures and more selective protecting groups has to be done. In this respect the recent results of Reese [41], Adamiak [42] and van Tamelen [43] on the use of new procedures for the demasking of protective groups from phosphotriester internucleotide linkages are noteworthy.

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