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Application of new catalytic phosphate protecting groups for the highly efficient phosphotriester oligonucleotide synthesis

V.A.Efimov*, A.A.Buryakova, I.Y.Dubey, N.N.Polushin, O.G.Chakhmakhcheva and Yu.A.Ovchinnikov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow 117871, USSR

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ABSTRACT

An effective procedure for the synthesis of oligonucleotides by the phosphotriester method has been developed. The procedure is based on the use of phosphate protecting groups enabling O-nucleophilic intramolecular catalysis in the reaction of internucleotide bond formation under the action of arylsulfonyl chlorides and their derivatives. Using this new procedure, the time needed to perform one elongation step on polymer support is 7-8 min. The effectiveness of the methodology has been demonstrated in the syntheses of many oligodeoxyribonucleotides of different length with high yields.

INTRODUCTION

Synthetic oligonucleotides of well-defined sequences have a number of important applications in molecular biology and recombinant DNA research[1]. One of the approaches currently being used for the oligonucleotide synthesis is the phosphotriester methodology[2]. In recent years a variety of methods has been proposed to improve the phosphotriester technique, in particular its solid-phase variant. Earlier we reported the rapid phosphotriester methodology based on the use of arylsulfonyl chlorides in the presence of a nucleophilic catalyst - 1-methylimidazole (MeIm), as effective coupling reagents for the internucleotide bond formation[3,4]. Some recent improvements in the triester approach concerned the use of oxygen-nucleophilic catalysts - 4-substituted derivatives of pyridine N-oxide in conjunction with different condensing and phosphorylating agents[5]. The application of these catalysts allows to reduce the coupling time in homogeneouse solution to 1-2 min and the time needed for the performance of an internucleotide condensation on a polymer support to 4-5 min. It was shown that O-nucleophilic catalysts

are equally effective with the use both aryl and alkyl phosphate blocking groups[6].

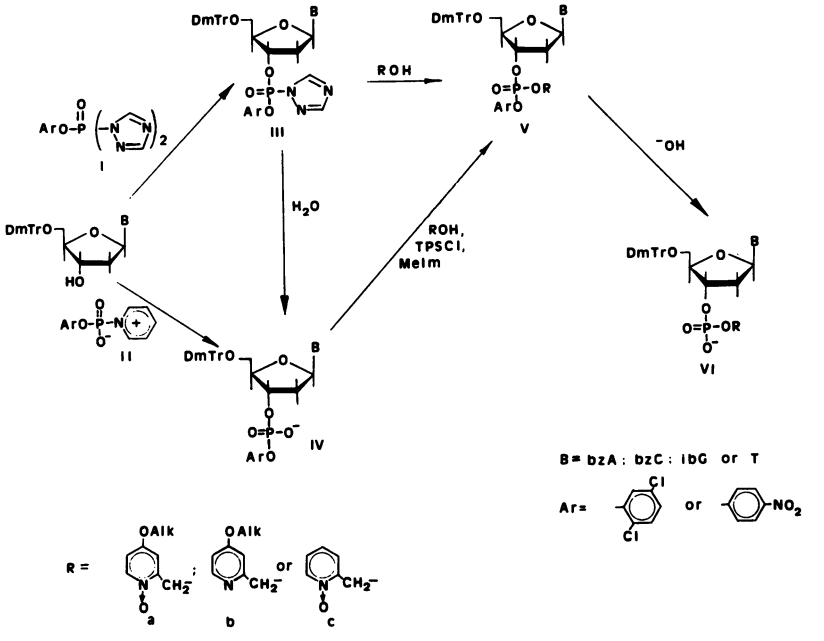
Further improvements in the phosphotriester synthesis suggest the enhancement of the internucleotide bond formation rate owing to neighbouring group participation. The use of 2-(1-methylimidazole-2-yl)phenyl group as catalytic phosphate protecting group has been reported[7,8]. It was shown that such a group bearing a 1-methylimidazole moiety enhance the rate of internucleotide condensation due to an active cyclic intermediate formation giving on solid-phase reaction time of 5-7 min. The similar effect was observed by us with the use of several other N- and O-nucleophilic catalytic blocking groups(such as 1-oxido-4-alkoxy-2-picolyl, 4-alkoxy-2-picolyl and 1-oxido-2-picolyl groups) for the internucleotide phosphate protection[9]. Thus the application of 1-oxido-4-alkoxy-2-picolyl derivatives of nucleotides led to a dramatic increase of the rate of the phosphotriester bond formation. With the use of these monomers, coupling time on solid-phase was 1-2 min depending on the condensing agent.

In this communication the highly efficient solid-phase phosphotriester synthesis of oligodeoxyribonucleotides with the use of O-nucleophilic intramolecular catalysis and convenient procedures for the introduction and removal of new catalytic phosphate protecting groups are described.

RESULTS AND DISCUSSION

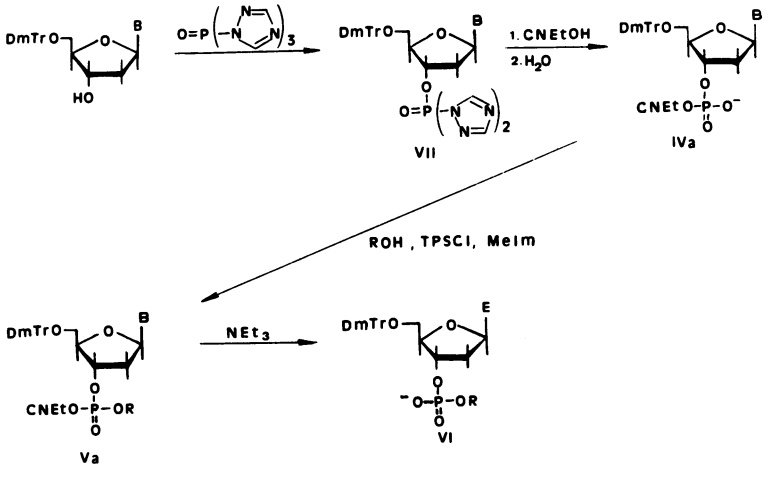
Preparation of Protected Monomers

To prepare monomeric units carrying catalytic phosphate protecting groups we utilized methods shown in Schemes 1 and 2. Similarly to 2-(1-methylimidazole-2-yl)phenyl derivatives[8], the desired nucleoside phosphodiesters (VI) were obtained from the corresponding fully blocked nucleoside phosphotriesters (V) by the selective cleavage of the non-catalytic aryl, or alkyl, phosphate blocking group. The synthesis of the nucleoside phosphotriesters (V) can be performed by several ways. The first way (Scheme 1) suggested the phosphorylation of the 3'-hydroxyl of a deoxynucleoside with arylphosphoroditriazolide (I) (2,5-dichlorophenyl- or p-nitrophenylphosphoroditriazolide) followed by direct conversion of the nucleoside phosphorotriazolide diester



Scheme 1

(III) obtained into the desired triester (V). An alternative procedure included the conversion of a preliminary prepared nucleoside phosphodiester (IV) into the corresponding triester (V) by



Scheme 2

TABLE 1. Yields and selected physical data for the protected mononucleotides

Compound	Phosphate protecting group (R)	Yield, %[a]	Molecular weight[b]	^{31}P NMR δ (ppm)	R_f[c]
[(MeO)$_2$Tr]T-(R)	4-methoxy-2-picolyl	82(56)	846.92	-0.12	0.25
[(MeO)$_2$Tr]T-(R)	1-oxido-2-picolyl	80(51)	832.90	0.14	0.15
[(MeO)$_2$Tr]T-(R)	1-oxido-4-methoxy-2-picolyl	80(52)	862.92	0.28	0.10
[(Meo)$_2$Tr]T-(R)	1-oxido-4-ethoxy-2-picolyl	83(55)	876.95	0.26	0.10
[(MeO)$_2$Tr]T-(R)	1-oxido-4-benzyloxy-2-picolyl	78(54)	939.02	0.25	0.12
[(MeO)$_2$Tr]bzA-(R)	1-oxido-4-methoxy-2-picolyl	76(57)	976.05	0.40	0.17
[(MeO)$_2$Tr]bzC-(R)	1-oxido-4-methoxy-2-picolyl	80(55)	952.02	0.31	0.16
[(MeO)$_2$Tr]ibG-(R)	1-oxido-4-methoxy-2-picolyl	72(50)	958.03	0.34	0.14

[a] The first number indicates the yield of a compound synthesized through the arylphosphotriester (V), the second number - through the β-cyanoethylphosphotriester (Va).

[b] Molecular weights are given for the triethylammonium salts of nucleotides.

[c] The solvent is chloroform-methanol-triethylamine (89:10:1, v/v).

the action of a 2-pyridinemethanol derivative in the presence of triisopropylbenzenesulfonyl chloride (TPSCl) and MeIm. The starting nucleoside arylphosphodiesters can be obtained by phosphorylation of a protected nucleoside with bis(triazolyl)arylphosphate followed by the treatment with aqueous triethylammonium bicarbonate (TEAB), or by the action on arylphosphorylpyridinium derivative (II) to the protected nucleoside[3]. The other way included the phosphorylation of a nucleoside 3'-OH group with phosphorotritriazolide and subsequent replacement of a triazole moiety of a resulting nucleoside 3'-phosphoroditriazolide (VII) by β-cyanoethanol (Scheme 2). Then, the β-cyanoethyl ester of a nucleoside phosphorotriazolide can be smoothly converted into the phosphodiester (IVa). The treatment of (IVa) with corresponding derivative of 2-pyridinemethanol in the presence of TPSCl and MeIm gives the triester (Va). The arylphosphotriesters (V) can be converted quantitatively into the desired phosphodiesters (VI) without any isolation by the action of some bases (such as an oximate reagent[8] or LiOH), whereas the cyanoethyl blocking group can be removed from the crude phosphotriester (Va) by the action of triethylamine.

The resulting monomeric nucleotide components (VI) were isolated by a silica gel column chromatography and characterised by ^{1}H and ^{31}P NMR spectroscopy. According to the above described procedures, substituted 2-pyridinemethyl esters of all the four 5'-dimethoxytritylnucleoside 3'-phosphates were obtained on a 5 mmole scale (Table 1).

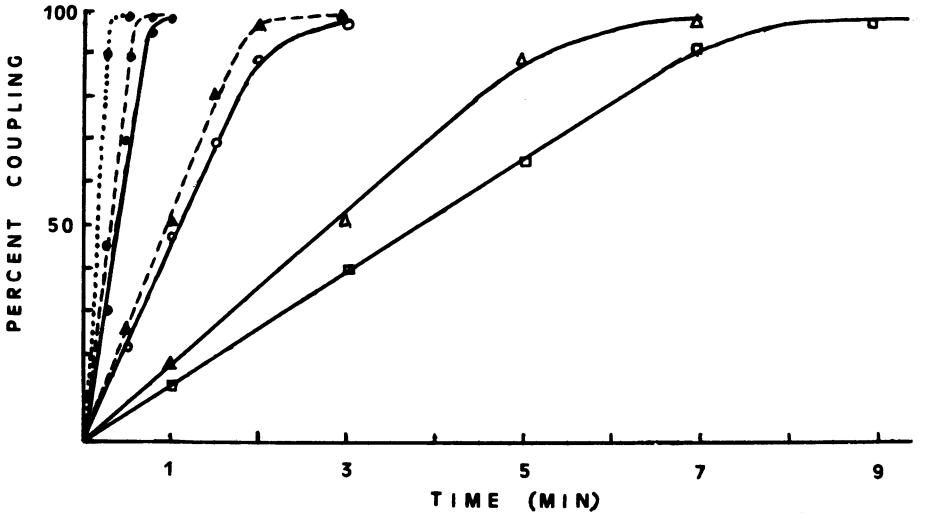


Figure 1. Coupling rates in the synthesis of $[(MeO)_2Tr]Tp(R)T(Ac)$. The coupling reactions were carried out in pyridine (1 ml) using 0.05 mmol of the OH-component, T(Ac), and 0.05 mmol of a P-component: (VIa) - (●), (VIb) - (Δ), (VIc) - (□), 2-(1-methylimidazole-2-yl)phenyl phosphodiester - (▲), or o-chlorophenyl phosphodiester (o), in the presence of 0.1 mmol of a condensing agent: MSCl (dotted line), MSNT (dashed line) or TPSCl (solid line). In the case of the o-chlorophenyl derivative, 0.2 mmol of 4-methoxypyridine N-oxide was added.

Internucleotide Coupling Reactions

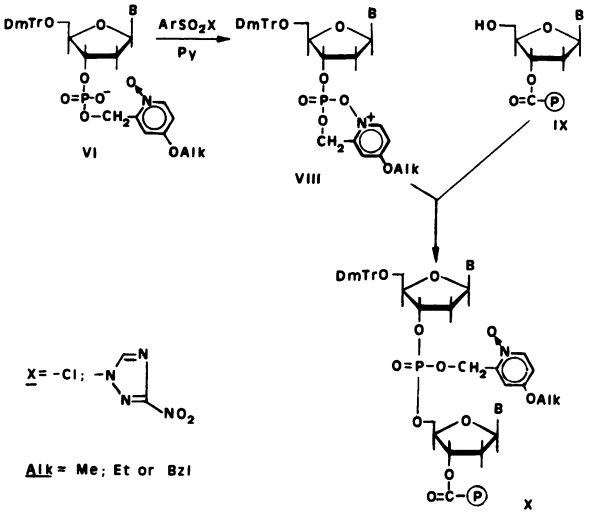
The results on the comparison of the reaction rates in the synthesis in solution of the dinucleotide $[(MeO)_2Tr]Tp(R)T(Ac)$ using different catalytic groups (R) are shown in Fig. 1. The reactions between monomeric P-component (1 mol equiv.) and OH-component (1 mol. equiv.) were carried out in the presence of 2-fold excess of a condensing agent in pyridine. As it follows from Fig. 1, the highest rate of the phosphotriester bond formation is provided by 1-oxido-4-alkoxy-2-picolyl derivatives (VIa). The condensation with the use of this O-nucleophilic intramolecular catalysts and TPSCl is complete in <1 min. The application of other condensing agents (mesitylenesulfonyl chloride, MSCl, and 1-mesitylenesulfonyl 3-nitro-1,2,4-triazole, MSNT) gives the reaction time of 0.5-0.75 min. At the same time, on polymer support the use of MSCl and MSNT in conjunction with the same protecting groups provides 97-98% yields in coupling reac-

tions within 1-1.5 min, whereas in the presence of TPSCl the coupling reaction is complete in 2 min. The reaction rate is not practically dependent on the structure of the substituent in the fourth position of the pyridine ring of the 1-oxido-4-alkoxy-2-picolyl group.

It should be noted that the use of catalytic protecting groups provides a minimum of 5'-O-sulfonated by-products. Apparently, it is due to the differential catalysis of the phosphorylation and sulfonation reactions. The first reaction is intramolecular process, which is speeded up by powerful O-nucleophilic catalyst, whereas the second reaction is intermolecular process, which is accelerated only by pyridine. Thus, the comparison of the 5'-sulfonation rates under the action of TPSCl with the use of the 1-oxido-4-alkoxy-2-picolyl group and with the use of o-chlorophenyl protecting group plus 4-methoxypyridine N-oxide as a catalyst has shown that in normal coupling reaction conditions the amount of sulfonated by-product was much lower in the first case (<0.2%) than in the case of O-nucleophilic intermolecular catalysis (2-3%)[10].

The acceleration of the condensation rate with the use of catalytic phosphate blocking groups may be accounted for the formation of an active cyclic intermediate[7,11]. Earlier, we investigated a mechanism of the phosphotriester bond formation in the presence of O-nucleophilic catalysts with the help of ^{31}P NMR spectroscopy. It was shown that in the presence of 4-substituted pyridine N-oxide reactive intermediate formed under the action of condensing agent on phosphodiesters represents the N-phosphoryloxypyridinium salt[6]. As it can be supposed on the analogy with the action of these catalysts, the highly active intermediate formed in the case of 1-oxido-4-alkoxy-2-picolyl derivatives represents a cyclic N-phosphoryloxipyridinium salt (VIII) (Scheme 3).

The examination of the stability of new O-catalytic protecting groups in various conditions have revealed that these groups are fully stable during internucleotide condensation. They are also stable to the action of acidic reagents, which are used for the removal of 5'-trityl protecting group, and triethylamine. At the same time, they can be easily removed by the action of nuc-



Scheme 3

leophilic reagents, such as triethylammonium thiophenate or piperidine.

Solid-phase oligonucleotide synthesis

The monomers carrying O-nucleophilic catalytic blocking groups (VIa) were successfully employed for the syntheses of oligodeoxyribonucleotides ranging from 8 to 50-mers. The most part of oligomers was obtained with the use of non-swellable polymer supports on the base of porous glass beads or paper disks. The growing oligonucleotide chain was elongated from 3'-end to 5'-end by a successive addition of the appropriately protected mononucleotides containing 1-oxido-4-methoxy-2-picolyl protected 3'-phosphates. At each step, the 10-fold excess of a P-component over the resin capacity was used. The coupling reactions were performed in the presence of 3-fold excess of a condensing agent with respect to a P-component using pyridine as a solvent. It was found that P-components carrying O-nucleophilic catalytic phosphate protecting groups are fully stable in pyridine solution during several days at room temperature.

The manipulations of a complete elongation cycle are summarized in Table 2. The time needed to perform one cycle is 7-8

TABLE 2. Reaction cycle for the synthesis of oligonucleotide on a polymer support

Step	Reagents and solvents	Time (min)
1.	1,2-dichloroethane wash	0.5
2.	3% dichloroacetic acid in 1,2-dichloroethane	1.5
3.	1,2-dichloroethane wash	0.5
4.	Pyridine wash	1.0
5.	Coupling mixture in pyridine[a] (stop flow)	1-2
6.	Pyridine wash	0.5
7.	Pyridine-MeIm-acetic anhydride (8:1:1, v/v)	1.5
8.	Pyridine wash	0.5

[a]The nucleotide component (0.1 M, 100 µl) is added to the solution of condensing agent (TPSCl, MSCl or MSNT) (0.3 M, 100 µl), and the mixture obtained is immediately injected into a reaction vessel containing 30 mg of a glass support, or 5 paper disks.

min depending on the condensing agent used. The average yield per step was 97-98%.

The structures of several oligonucleotides obtained by this method and their yields are shown in Table 3. The undecamer (XI) pentadecamer (XII) and hexadecamer (XIII) are fragments of a gene for *E.coli* β-galactosidase. The 25-mer (XIV) and 28-mer (XV) were designed as primers to obtain directed mutations in the synthetic gene for human proinsulin. The 31-mer (XVI) is a molecular probe for the isolation of a gene for tobacco-plant herbicide-binding protein.

The removal of the protecting groups from the final oligonucleotides after completion of the synthesis was carried out in several steps. The substance was treated with piperidine (or

TABLE 3. Overall yields of oligonucleotides prepared with the use of O-nucleophilic intramolecular catalysis

Compound	Sequence (length)	Support[a]	Overall yield,%[b]	
			coupling	isolated[c]
(XI)	d(TCCTGTGTGAC) (11)	CPG	67	25
(XII)	d(CGGTCGCTACCATTA) (15)	CPG	52	18
(XIII)	d(AATTTCAGCTGAGCGC) (16)	PD	45	16
(XIV)	d(TCTTCGAATTCATGGATCCGCGTCG)(25)	CPG	50	21
(XV)	d(GCAACCGTAGAGATCTGTAAGCTT-TAAT) (28)	CPG	32	12
(XVI)	d(CTATGCATGGTTCCTTGGTAACTT-CTAGTTT) (31)	CPG	29	11

[a]CPG - long chain alkylamine controlled pore glass; PD - Whatmann 3 MM paper disks.
[b]Yields are based on the first nucleoside attached to a resin.
[c]After the removal of blocking groups and reversed-phase HPLC.

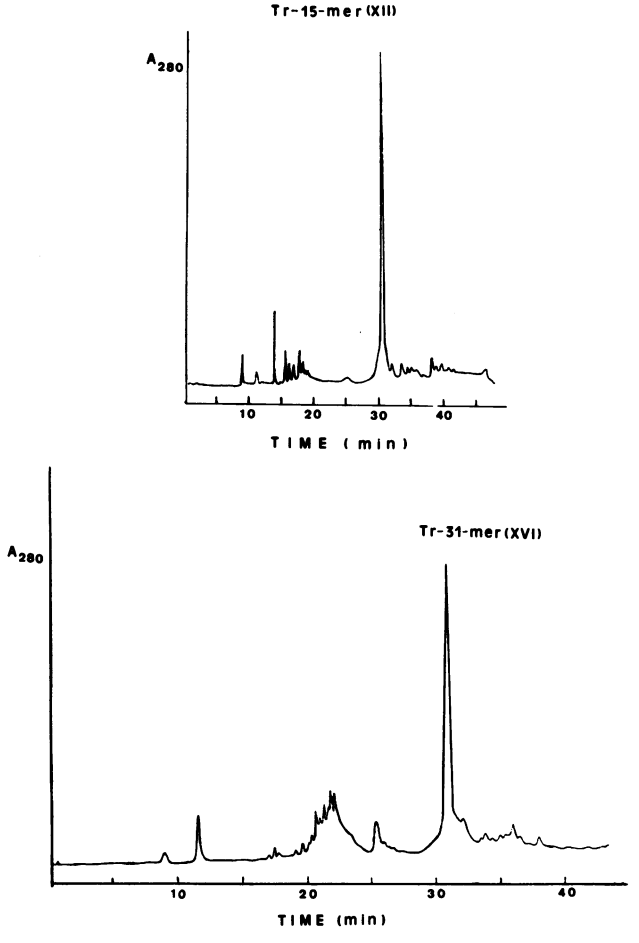


Figure 2. The HPLC analysis on Lichrosorb RP-18 column of the dimethoxytrityl-containing synthetic oligonucleotides after the removal of amino and phosphate protecting groups.

with triethylammonium thiophenate) to remove phosphate protecting groups, then with concentrated ammonia to remove acyl protecting groups. To cleave an oligomer from a polymer support on the base of paper disks, the additional treatment with oximate solution was introduced. The terminal 5'-dimethoxytrityl group was removed by the action of 80% acetic acid.

The deprotected oligonucleotides were isolated by preparative electrophoresis on denaturing polyacrylamide gels and/or

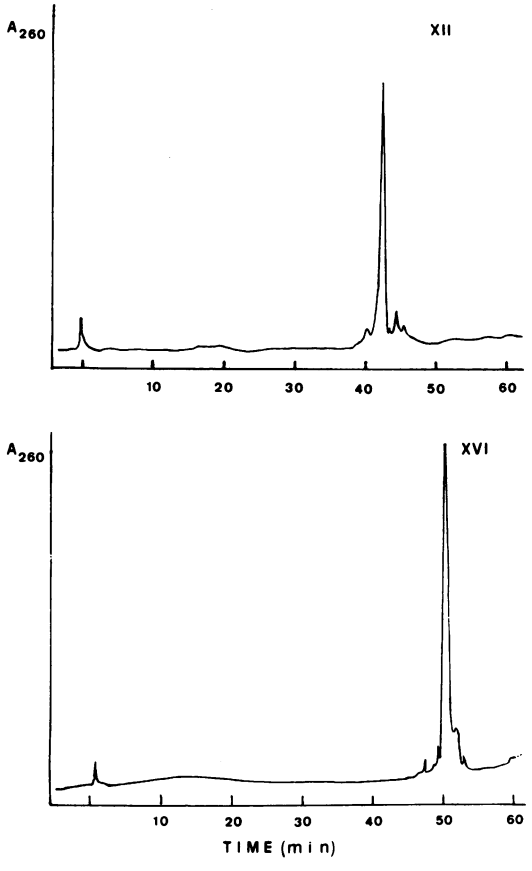


Figure 3. The HPLC analysis of fully deprotected oligonucleotides on Zorbax C-8 column.

reversed-phase chromatography. The purity of the synthetic oligomers was checked by the reversed-phase HPLC before and after the removal of a trityl blocking group (Figs. 2 and 3). After the ^{32}P-labelling, the homogeneity of the oligonucleotides synthesized was estimated by a polyacrylamide gel electrophoresis (Fig. 4), and their primary structure was confirmed by the sequence analysis (Fig. 5).

Conclusion

The results obtained have demonstrated the effectiveness of improved phosphotriester synthesis with the use of 1-oxido-4-al-

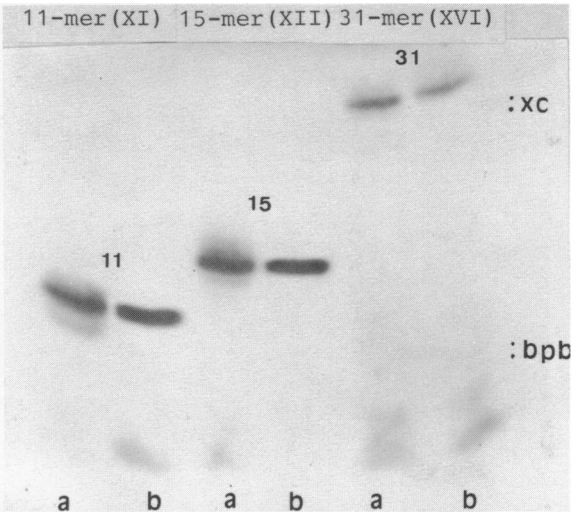


Figure 4. Analysis of the synthetic oligonucleotides after labelling their 5'-termini by electrophoresis on 20% polyacrylamide gel containing 7 M urea. Lanes "a" show the crude oligonucleotide mixtures, lanes "b" - the same oligonucleotides after purification.

koxy-2-picolyl phosphate protecting groups. The application of these blocking groups, which are at the same time intramolecular catalysts, provides dramatic increasing the rate of phosphotriester internucleotide condensation. It allows to decrease the extent of side-reactions, such as sulfonation of a nucleoside 5'-hydroxyl group, that is especially important for the synthesis on polymer supports. Now the rate of the solid-phase phosphotriester synthesis and the efficiency of this method are the same as those in the phosphoramidite method[12]. Moreover, the phosphodiesters (VI) are more stable than phosphoramidites, and the yields of oligonucleotides synthesized with the use of the modified triester methodology are not inferior to those obtained with the aid of the modern phosphite methodology.

MATERIALS AND METHODS

Reagents, solvents and methods not mentioned below were as described previously[3-6,13]. HPLC was performed with a Du Pont preparative 830 System and LKB HPLC System. 2-Pyridinemethanol

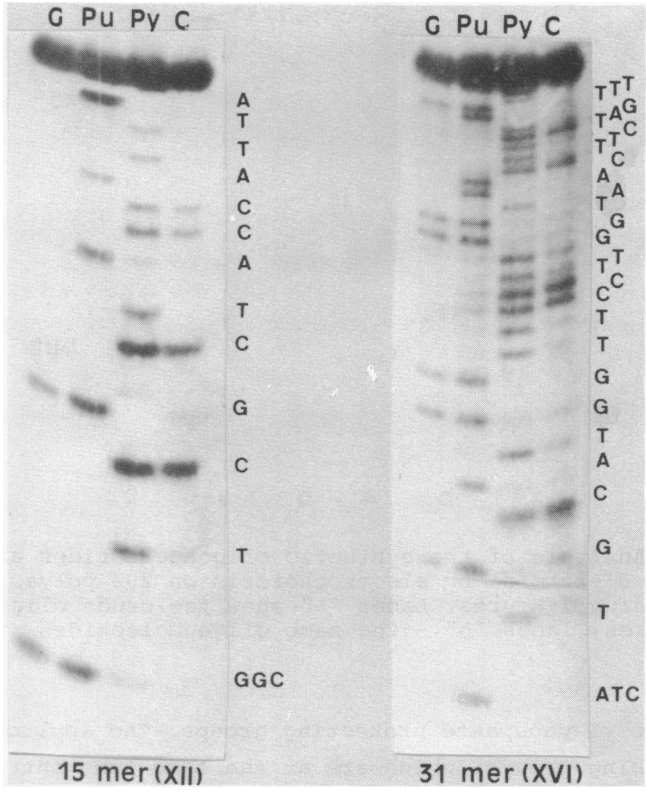


Figure 5. Sequence analysis of the 5'-labelled oligonucleotides (XII) and (XVI).

was purchased from Aldrich. The derivatives of 2-pyridinemethanol 1-oxide and 2-pyridinemethanol were obtained essentially as described[11,14], and their structures were confirmed by ^{1}H NMR analysis.

^{31}P NMR spectra were obtained at 500 MHz with a Bruker WM500 spectrophotometer operating in the Fourier transform mode. The chemical shifts are given in ppm relative to 85% H_3PO_4 as an external standard. Broad-band proton decoupling was used.

The determination of the phosphotriester bond formation rate and the rate of 5'-OH sulfonation were performed with the help of TLC and HPLC as described earlier[5,15].

Preparation of 5'-O-dimethoxytrityl deoxynucleoside 3'-(1-oxido-4-alkoxy-2-picolylphosphates (VIa).

Syntheses of nucleoside 1-oxido-4-alkoxy-2-picolylarylphosphates (V) was performed by the following procedures.

a) Solution of arylphosphoroditriazolide (I) (7.5 mmol) in 50 ml of dry dioxan was prepared as described[8] and mixed with a N-protected 5'-O-dimethoxytritylnucleoside (5 mmol) dried by coevaporation with pyridine. After 1 h (control by TLC), dry 1-oxido-4-alkoxy-2-pyridinemethanol (12.5 mmol) was added to clear solution of phosphorotriazolide (III). After 1.5 h, the solution was evaporated in vacuo, the residues was dissolved in chloroform (200 ml). The solution obtained was washed with 0.5 M TEAB (pH 8.0) and by 0.05 M TEAB (2 x 100 ml). The solvent was evaporated in vacuo, and crude phosphotriester (V) was obtained as a foam.

b) The nucleoside phosphorotriazolide (III) obtained as described above was treated with 50 ml of water-dioxan-triethylamine (10:17:3) during 10 min at room temperature. Chloroform (150 ml) was added, and the solution was extracted with 0.2 M TEAB (150 ml). The organic layer was washed with 0.2 M TEAB (2 x 50 ml), dried (anhydrous Na_2SO_4) and evaporated. 1-Oxido-4-alkoxy-2-pyridinemethanol (7 mmol) and 1-methylimidazole (20 mmol) were added to the residue, and the mixtire obtained was dried by coevaporation with dichloromethane. Then, TPSCl (10 mmol) in 50 ml of dry dichloromethane was added. After 3-5 min (control by TLC) the reaction was stopped by the addition of chloroform (100 ml) and the mixture was extracted with 0.5 M TEAB (2 x150 ml). Chloroform phase containing phosphotriester (V) was evaporated to a foam.

The crude phosphotriester (V) was dissolved in 150 ml of 0.2 M LiOH in 60% aqueous dioxan. Then, 0.5 M TEAB (300 ml) was added in 3-5 min, and the phosphodiester obtained was extracted with chloroform (2 x 200 ml). The chloroform solution was washed with 0.5 M TEAB (100 ml) and with water and evaporated to a gum. The residue was dissolved in chloroform (100 ml). Crude phosphodiester (VIa) was purified by a silica gel column chromatography using chloroform-methanol-triethylamine (88:10:2 v/v) as an eluent. After removal of solvent, the phosphodiester (VIa)

was dissolved in chloroform and precipitated by the addition of this solution into a large volume of stirred pentane-ether (1:1). The precipitated material was collected by centrifugation and dried in vacuo. Yields were in the range of 75-80% based on the starting 5'-O-dimethoxytrityl deoxynucleoside. The removal of an aryl phosphate blocking group can be also performed by the action of the oximate reagent as described[8].

Syntheses of the phosphodiesters (VIa) through the β-cyanoethyl phosphotriesters (Va) was performed similar to the above described procedures. The nucleoside β-cyanoethyl phosphates (IVa) were prepared with the use of phosphorotritriazolide as described earlier[6]. The crude phosphotriester (Va) was treated with triethylamine-acetonitrile (1:1) (15 ml per mmol) during 10 min at 50°C. The mixture was evaporated and the crude phosphodiester (VIa) was purified by a silica gel column chromatography. Yields were 50-60%.

The corresponding nucleoside 1-oxido-2-picolyl phosphates (VIb) and 4-alkoxy-2-picolyl phosphates (VIc) were obtained similarly to the above described procedures. The chemical shifts of 5'-dimethoxytrityl nucleoside phosphodiesters (VI) are shown in Table 1.

Assembly of oligonucleotide chains

The functionalized support on the base of long chain alkylamine pore glass (about 30 μmol of a nucleoside per g) or Whatman 3MM disks (diameter 6 mm, 0.3-0.5 μmol of nucleoside per disk) was packed into a glass column (6 x 50 mm). The flow rates of solvents was adjusted to 2 ml/min. The various chemical operations performed for the addition of one coupling unit to the polymer supports, amount of reagents and the reaction times are listed in Table 2. The monomeric nucleotide components are dried by coevaporation with dichloromethane and dissolved in anhydrous pyridine to obtain 0.1 M solutions. The condensing reagent (MSCl, TPSCl or MSNT) is prepared as 0.3 M solution in pyridine. The coupling mixture consists of the equal volumes of the appropriate monomer solution and the condensing reagent solution.

Deprotection and purification of oligonucleotides

After the appropriate number of reaction cycles, the support was treated with piperidine for 6-12 h. After removal of

piperidine by filtration, the glass support is treated with concentrated ammonia for 6 h at 50°C, whereas the paper disks are preliminary treated with oximate solution as described[16]. The remaining work up procedure with 80% acetic acid was as described[3,13].

The isolation of deprotected oligonucleotides was accomplished by preparative electrophoresis on denaturing polyacrylamide slab gels[4,13]. After desalting the oligonucleotides were subjected to reversed phase HPLC on a Zorbax C-8 column (4 x 250 mm) using methanol concentration gradient in 0.1 M ammonium acetate solution or on Lichrosorb RP-18 column (4.6 x 120 mm) using acetonitrile concentration gradient in 0.1 M triethylammonium acetate solution. 5'-^{32}P-labelling, chemical cleavage and gel electrophoresis in oligonucleotide sequence analysis were performed as described[17].

*To whom reprint requests should be sent.

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