COMPARATIVE CHARACTERISTICS OF METHODS FOR OBTAINING OLIGODEOXYRIBONUCLEOTIDE 3'-PHOSPHATES

E. M. Volkov, S. M. Gryaznov,
N. F. Krynetskaya, T. S. Oretskaya,
and V. K. Potapov

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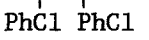
Methods of obtaining oligodeoxyribonucleotide 3'-phosphates based on the triester synthesis of oligodeoxyribonucleotides are discussed. These methods can be divided into two classes: I) phosphorylation of the 3'-OH group in a protected oligo-deoxyribonucleotide; and II) modification of a terminal nucleotide 3'-phosphate. It has been shown that a universal method is the preparation of an oligonucleotide with a 3'-terminal uridine residue followed by its oxidative elimination. The methods discussed in the paper have been used for obtaining a set of oligodeoxyribonucleotide 3'-phosphates required for the chemical "cross-linking" of oligonucleotides in DNA duplexes.

Investigations connected with obtaining fragments of DNAs (including those containing modified internucleotide bonds [1]) with chemical ligation [2] require the development of a universal chemical method for synthesizing oligodeoxyribonucleotides with 3'-terminal phosphate groups.

In the present paper we give the comparative characteristics of methods of obtaining oligodeoxyribonucleotide 3'-phosphates that have been developed in our laboratory. The syntheses were performed within the framework of the triester method. The methods of introducing a 3'-terminal phosphate group into oligodeoxyribonucleotides presented in this paper can be divided into two classes: 1) phosphorylation of the 3'-OH in a 5'MeOTr-N-bz-oligodeoxyribonucleotide; and II) modification of a 3'-terminal nucleotide unit, leading to the production of an oligodeoxyribonucleotide 3'-phosphate. In the first class of methods, the following are used as phosphorylating agents: Ia) a mixture of $POCl_3$ and 3 equivalents of triazole [3], and Ib) β-cyanoethyl phosphate in the presence of TPS. The second class of methods includes the preparation of oligonucleotides of the type of d[> Np Np(CE)] followed by transesterification to d[> Np $Np(CE)_2$] in method (IIa) [3] and the preparation of oligonucleotides with 3'-terminal uridine residues followed by the cleavage of the ribose units in method (IIb).



The use of methods of Ia and Ib is possible if the 3'-OH protection used is a levulinyl grouping [4], which can be eliminated selectively under the action of hydrazine. After the elimination of the levulinyl group it is possible to phosphorylate the 3'-hydroxy group in the oligonucleotide with β-cyanoethyl phosphate or a mixture of $POCL_3$ and TriH (1:3) in the concluding stage of the synthesis of the oligodeoxyribonucleotide. This method is particularly valuable where the desired product is required in the subsequent work both in the form of a component bearing a 3'-phosphate group and also without it.

The oligonucleotide synthesized (III) is stable under the conditions of deblocking the 5'-monomethoxytrityl group and of the chromatographic isolation of the substance on silica gel [3] and can be used as the 3'-terminal block in subsequent condensation.

When a mixture of $POCl_3$ and 3 equivalents of TriH is used, the phosphorylating agent is phosphorotritriazolide (by analogy with the phosphorylimidazole formed in a mixture of $POCl_3$ and 3 equivalents of ImH [5]).

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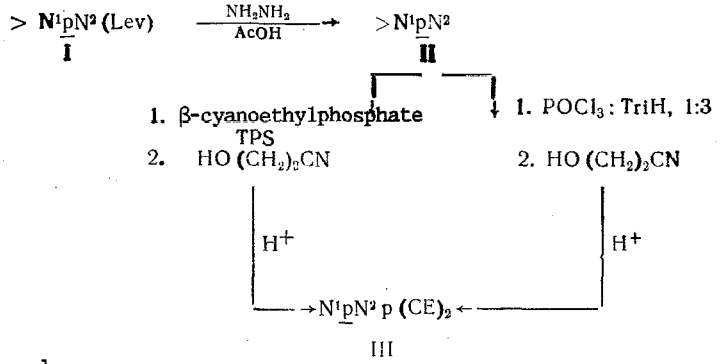
TABLE 1. Conditions of Preparation and Yields of Oligonucleotide 3'-Phosphates

Method	Oligonucleotide	Excesses of the reagent	Time, h	Temp., °C	Yield, %
I. 3'-Phosphorylation of protected oligonucleotides					
Phosphorylating agent					
Ia—PO(Tri)$_3$	$d(>\underline{T}pT)$	2 eq. $POCl_3$ 6 eq. TriH	0,25 1	0 20	80 60
	$d\left(\overset{\vee}{A}\underline{p}\overset{\vee}{G}\right)$				
Ib, β-cyanoethyl phosphate	$d\left(\overset{\vee}{A}\underline{p}\overset{\vee}{G}\right)$	3 eq. β-CEtP 6 eq. TPS Pyridine	1 1	20 20	85 80
	$d\left(\overset{\vee}{A}\underline{p}\overset{\vee}{A}\underline{p}\overset{\vee}{G}\right)$				
II. Modification of a terminal nucleotide unit:					
IIa, transesterification	$d\left[>N^1_{\underline{p}}N^2_{\underline{p}}(CE)\right]$ (see text)	Saturated sol. in CsF in abs. cyanoethanol	10—14	20	35—8
IIb, selective cleavage of 3'-uridine residue	$d\left(N^1_pN^2_p\right)rU$ (see text)	20 eq. $NaIO_4$ (0,025 M) 20 eq. CHA (0,3 M)	0,5 1,5	20 20	75—95

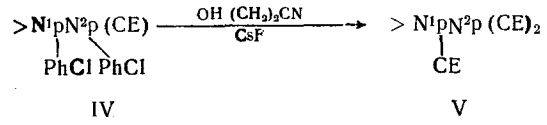
Note. The symbols recommended be the IUPAC-IUB Nomenclautre Commission have been used, and: $>\overset{\vee}{N}p$) a 5'-monomethoxy trityl-N-benzoylnucleoside 3'-(p-chlorophenyl phosphate); TriH) 1,2,4-triazole; CE) 2-cyanoethyl; β-CEtP) β-cyanoethyl phosphate.



N^1 and N^2-nucleosides with protected amino groups.

The conditions of phosphorylation by methods Ia and Ib are given in Table 1. The yield at the stage of introducing the 3'-phosphate group is 60-85% and falls with a lengthening of the chain and with an increase in the number of purine bases in the desired oligonucleotide. Using the procedures developed, 174 OU_{260} of d(CCTGGAATTp) has been obtained by method Ia, and 21 OU_{260} of d(TCGAAATCGAAGp) by method Ib. The protective groups were eliminated by standard procedures [6], and the oligonucleotides were isolated by ion-exchange chromatography on DEAE-cellulose at pH 7.5 and 3.5. The homogeneity of the oligonucleotides synthesized was confirmed by high-performance chromatography and their structures by the Maxam-Gilbert method [7].

In method IIa, to obtain an oligonucleotide 3'-phosphate the transesterification of the 3'-terminal chlorophenyl ester of an oligonucleotide to form the cyanoethyl ether in the presence of cesium chloride is used [3]:



IV V

Thus, compound (V), which is an analog of the final product in methods Ia and Ib, can be obtained in the synthesis of an oligonucleotide from the standard components of the triester synthesis.

The yields of oligodeoxynucleotide 3'-phosphates at the transesterification stage [3] (transesterification of chlorophenyl esters of mono- and oligonucleotides under the action of cyanoethanol in the presence of CsF) are given below:

Initial oligonucleotide	Yield,% (bis-CE-derivative)
d[>$\overset{\vee}{G}$p(GlPh, CE)]	80
d[>$\overset{\vee}{C}$pTp(ClPh, CE)]	73
d[>$\overset{\vee}{G}$p$\overset{\vee}{A}$pTp$\overset{\vee}{A}$p(ClPh, CE)]	68
d[>$\overset{\vee}{G}$p$\overset{\vee}{C}$p$\overset{\vee}{A}$pTp(ClPh, CE)]	60
d[>$\overset{\vee}{G}$p$\overset{\vee}{A}$pTp$\overset{\vee}{C}$p$\overset{\vee}{C}$p$\overset{\vee}{A}$p(ClPh, CE)]	44

As we see, with an increase in the length of the oligonucleotide the degree of its degradation in the presence of cesium fluoride falls and the yield of the 3'-dicyanomethyl derivative, then leading to the production of an oligonucleotide with a free 3'-phosphate group, falls sharply. The conditions for performing the transesterification reaction are given in Table 1.

In method IIb in order to obain an oligonucleotide with a free 3'-phosphate group an oligonucleotide with a 3'-terminal uridine residue is synthesized (within the framework of the triester synthesis), i.e., $dN\underset{\vee}{p}rU(Bz)_2$ is used as the 3'-terminal oligonucleotide block. The use of rU, rather than other ribonucleosides [8], eliminates the introduction of protective groups into the heterocyclic bases. After the end of the synthesis, the oligonucleotide is deblocked by standard methods [6], and then the cis-glycol group of the uridine residue is selectively oxidized under the action of $NaIO_4$ to a dialdehyde fragment [9], and the phosphomonoester grouping is liberated by a β-elimination reaction in the presence of cyclohexylamine [10]. The conditions for the reaction are given in Table 1. An oligonucleotide 3'-phosphate obtained by method IIb was isolated by ion-exchange chromatography on Lichrosorb-NH_2, and the presence of a 3'-phosphate group was confirmed by an analysis of the products of hydrolysis of the oligonucleotide by alkaline phosphatase.

Below we give the yields of oligonucleotide 3'-phosphates at the stage of the selective cleavage of the 3'-terminal ribose unit and subsequent purification on LiChrosorb-NH_2. The yields of oligodeoxyribonucleotide 3'-phosphates amounted to 75-95% and were practically independent of the length and composition of the initial oligonucleotide. The use of method IIb for obtaining oligonucleotide 3'-phosphates permits the whole process to be based on standard components of the triester method of synthesizing oligonucleotides:

Oligodeoxyribonucleotide-3'-uridine	Yield of 3'-phosphate,%
d(ACGGAp)*r*U	85
d(CACTATCAp)*r*U	75
d(TCGACCATAAAAp)rU	75
d(ACCTACCp)*r*U	95

Furthermore, oligodeoxyribonucleotides containing 5'-terminal uridine residues (with a 5' → 5' uridine—oligodeoxyribonucleotide bond) synthesized in a similar way can be used for obtaining 5'-phosphorylated oligonucleotides [11]. Thus, synthetic oligodeoxyribonucleotides having both 3'- and 5'-terminal uridine units are intermediate compounds in the chemical synthesis of DNA fragments with terminal phosphate groups.

EXPERIMENTAL

Deoxyribonucleosides, 3'-O-levulinylnucleoside 5'-(p-chlorophenyl phosphate)s, and 2,4,6-triisopropylbenzenesulfonyl chloride produced by the Novosibirsk Institute of Organic Chemistry of the Siberian Branch of the USSR Academy of Sciences were used, together with ethylene cyanohydrin, 1-methylimidazole, LiChrosorb-NH_2, and 1,2,4-triazole from Merck and DEAE-cellulose DE-32 from Whatman.

Chromatography on FN-1 paper was performed in systems 1) ethanol–1 M ammonium acetate, pH 7.5 (7:3), and 2) n-propanol–conc. ammonia–water (65:10:35); thin-layer chromatography (TLC) on silica gel plates from Eastman Kodak in system 3) chloroform–methanol (9:1); and column chromatography, (column 4 × 6 cm) with silica gel 40/100 μ (Chemapol, Czechoslovakia).

High-performance ion-exchange chromatography (HPLC) was performed on a column (1 × 30 mm) containing LiChrosorb-NH_2 (5 μ) in a linear concentration gradient of Na phosphate buffer, pH 7.0 at a rate of elution of 3 ml/h using a Millichrom chromatograph.

Hydrolysis of Oligonucleotides with 3'-Phosphate Groups by E. coli Alkaline Phosphatase. A solution of 0.1-0.5 OU of oligonucleotide in 10 ml of double-distilled water was treated with 0.1 unit/ml of alkaline phosphatase (EC 3.1.3.1) in a buffer containing 0.02 M NH_4HCO_3, 0.04 M $MgCl_2$, pH 8.5. The hydrolysis products were deposited on a column (1 × 30 mm) of LiChrosorb-NH_2 and were separated in a linear concentration gradient of sodium phosphate buffer (0 → 0.12 M), pH 7.0, in 7 M urea.

The synthesis of the N-benzoylnucleosides was performed with the use of intermediate protection of the hydroxy groups by chlorotrimethylsilane [12]. The introduction of a monomethoxy trityl group and the phosphorylation of the N-benzoylnucleosides was performed by a standard procedure [6].

Synthesis of 2',3'-Dibenzoyl-5'-O-monomethoxytrityluridine. A solution of 1 mmole of 5'-O-monomethoxytrityluridine in 10 ml of absolute pyridine was treated with 2.6 mmole of benzoic anhydride and 160 mg of γ-dimethylaminopyridine. After 1-1.5 h, the completeness of the reaction was checked by TLC. The reaction mixture was worked up and the reaction product was isolated as described in [6]. The yield of 2',3'-dibenzoyl-5'-O-monomethoxytrityluridine was 80%.

General Procedure for Eliminating Monomethoxytrityl Protection from a 5'-Hydroxy Group (Preparation of an OH Component). A solution of 1 mmole of a mono- or oligonucleotide in 6 ml of chloroform was cooled to 0°C, and 10 ml of a saturated solution of sodium bicarbonate, pH 8.0, was added. The organic layer was separated off and the aqueous layer was extracted with chloroform (3 × 20 ml). The combined extract was washed with water (2 × 20 ml) and dried with anhydrous sodium sulfate. The desired product was isolated by chromatography on silica gel in a linear concentration gradient of ethanol in chloroform (0 → 10%). In the detritylation of oligonucleotides with more than three units, the oligonucleotide was used in condensation without previous separation on silica gel.

General Procedure for Eliminating 2-Cyanoethyl Protection from a 3'-Terminal Phosphate Group (Preparation of a P-Component) [6]. A solution of 1 mmole of a mono- or oligonucleotide 3'-(2-cyanoethyl phosphate) in 10 ml of pyridine–triethylamine–water (3:1:1 by volume) was kept at room temperature for 15-20 min (monitoring by TLC).

General Procedure for Performing Internucleotide Condensation. A mixture of 1 mmole of a P-component and 0.5-0.8 mmole of a OH-component was dried by evaporation with absolute pyridine (3 × 5 ml). In the last step, evaporation was carried out to a volume at which the total concentration of the components with allowance for the 6 mmole of N-methylimidazole added later was 0.1 M. Then 3 mmole of triisopropylbenzenesulfonyl chloride was added to the reaction mixture and it was left at room temperature with the course of the reaction being monitored by TLC. The reaction time was 15-30 min. The reaction mixture was decomposed with water and was worked up by the standard procedure [6]. The desired oligonucleotide was isolated by column chromatography on silica gel. The yields in the condensation stage were 70-90%.

General Procedure for Eliminating a Levulinyl Protective Group from the 3'-Hydroxyl of a Mono- or Oligonucleotide.* To 0.5 ml of hydrazine hydrate were added 7 ml of aceto-

* The procedure for eliminating a levulinyl protection in acetonitrile was developed by E. V. Yarmolinskaya (Novosibirsk Institute of Organic Chemistry, Siberian Branch, Academy of Sciences of the USSR).

nitrile and, in drops, 1.5-2 ml of acetic acid until the solution had become clear and it was made up with acetonitrile to 10 ml. A solution of 0.5 mmole of d[> Tp T(Lev)] in 5 ml of this solution was kept at room temperature for 6-8 min, after which an excess (1 ml) of acetylacetone was added. The mixture was left for 10-15 min and was then evaporated, and the residue was subjected to column chromatography on silica gel. Yield, 75-80%.

General Procedure for the Phosphorylation of a 3'-OH Group in a Mono- or Oligonucleotide. Ia) A solution of 6 mmole of 1,2,4-triazole in 5 ml of absolute pyridine was cooled to 10°C, and 2 mmole of phosphorus oxychloride was added. The resulting precipitate (pyridine hydrochloride) was filtered off, and the filtrate was added to a solution of 1 mmole of d[-(Me<OTr)TpT] in 5 ml of absolute pyridine. After 3 min (monitoring by TLC), the reaction mixture was worked up by the standard procedure [3]. The oigonucleotide 3'-(bis-2-cyanothyl phosphate) was isolated by column chromatography on silica gel. Yield 74%. The two 2-cyanoethyl phosphate in the pyridinium form (1.5 mmole) and TPS (3 mmole) were dissolved in 10 ml of absolute pyridine, and the mixture was kept for 1 h. The resulting solution was added dropwise over 3 min to a 1 mM solution of a dinucleoside phosphate in absolute pyridine. The mixture was kept for 40 min and then, with cooling, 3 ml of a 1 M solution of triethylammonium bicarbonate (TEAB) was added and it was evaporated. The residue was re-evaporated with absolute pyridine (2 × 5 ml), and 2-cyanoethanol (3 mmole), N-methylimidazole (9 mmole), and TPS (3 mmole) were added. The mixture was kept for 10 min (with monitoring by TLC) and then 10 ml of 0.1 M TEAB was added and the whole was dissolved in 150 ml of chloroform; this solution was washed with 0.1 M TEAB (2 × 30 ml) and with water (2 × 39 ml), dried over sodium sulfate, and concentrated in vacuum. The desired product was isolated by column chromatography on silica gel. Yield 85%.

General Procedure for Transesterification of a 3'-Terminal Chlorophenyl Ester of a Mono- or Oligonucleotide. IIa) A monomethoxytritylated mono- or oligonucleotide was dissolved in the minimum volume of a saturated solution of cesium fluoride in absolute ethylene cyanohydrin. The mixture was kept at room temperature for 10-14 h and was then treated with an excess of concentrated aqueous ammonia at 50°C for 12 h. The reaction mixture was evaporated, the monomethoxytrityl group was eliminated by the action of an 80% solution of acetic acid, and the desired oligonucleotide was isolated by ion-exchange chromatography on DEAE-cellulose in a linear concentration gradient of sodium chloride.

General Procedure for Cleaving the Ribose Unit in an Oligonucleotid-3'-yloridine. A solution of 0.16 μmole of an oligonucleotid-3'-yluridine in 9 μl of water was treated with 3 μl of 0.1 M $NaIO_4$. The reaction mixture was kept at room temperature in the dark for 0.5 h and then 1 μl of a 10% solution of ethylene glycol in water was added and it was kept for another 15 min at room temperature, after which 3 μl of water and 8 μl of 1 M aqueous cyclohexylamine were added and the reaction mixture was kept at room temperaure for 1 h. The oligonucleotide 3'-phosphate was isolated by ion-exchange chromatography in a linear concentration gradient of Na phosphate beffer, pH 7.0, in 7 M urea. The yield of oligonucleotide 3'-phosphate was 75-95%.

SUMMARY

1. A comparison has been made of methods for obtaining oligonucleotide 3'-phosphates based on the procedures of the triester synthesis. It has been shown that a universal method is the synthesis of an oligonucleotid-3'-yluridine with the subsequent selective cleavage of the ribonucleotide and the liberation of terminal 3'-phosphate group.

2. Penta-, hepta-, nona-, and dodecadeoxyribonucleotides containing 3'-terminal phosphate groups have been synthesized by the methods described and are being used at the present time for studying the chemical ligation reaction.

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CLEAVAGE OF THE NATURAL LIGNIN AND THE DIOXANE LIGNIN OF KENAF BY THIOACETIC ACID

G. N. Dalimova and Kh. A. Abduazimov

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The dioxane lignin and the natural lignin of kenaf undergo 37.62% and 94.6% cleavage, respectively. The combined monomeric degradation products have been studied by the GLC method. The presence of substances relating to three types of structural units has been established: p-coumaryl, gualacyl, and syringyl. It has been shown by chromatography on Sephadex LH-20 (with ethanol—water (9:1) as solvent and eluent) that the phenolic products of degradation extracted by ethyl acetate at pH 2 consist of five fractions: oligomers, tetramers, trimers, dimers, and monomers.

The chemical structures of natural and isolated lignins have been studied previously by the method of thioacetic acid cleavage [1-5].

Continuing a study of the chemical structures of the lignins of kenaf, we have performed the cleavage of the natural lignin and the dioxane lignin of kenaf (DLAK-1; fraction I according to its time of isolation [6]) by thioacetic acid. Of the DLAK-I 37.62% underwent cleavage, and of the natural lignin of the kenaf 94.6%. The phenolic cleavage products were extracted with ether (pH 8) and with ethyl acetate (pH 2), the amounts of these products as percentages of the total phenolic substances from the natural kenaf lignin being 42.66 and 52.00 and from the kenaf dioxane lignin 25.54 and 12.08, respectively.

Chromatography on an analytical column of Sephadex LH-20 (with ethanol—water (9:1) as solvent) showed that the phenolic cleavage products extracted by ethyl acetate at pH 2 consisted of fractions of oligomers, tetramers, trimers, dimers, and monomers in the following percentages of the total for the DLAK-I and the natural kenaf lignin, respectively: 7.9, 32.7, 14.0, 31.2, and 14.3; and 20.5, 34.6, 13.60, 17.0, and 14.30. The analytical column was calibrated in a manner similar to that described previously [4].

The total of the monomeric degradation products extracted by ether at pH 8 was studied by the GLC method under conditions similar to those given in [5]. The compositions and yields of the monomeric cleavage products were as follows:

Substance	DLAK-I % in the mixture	DLAK-I % on the lignin	Natural lignin % in mixture	Natural lignin % on the Komarov lignin
Phenol	2,49	0,64	1,08	0,46
Guaiacol	5,80	1,50	4,06	1,73
p-Hydroxyphenylethane	6,33	1,69	3,52	1,46
p-Hydroxyphenylpropane	—	—	2 03	0,88
Guaiacylethane	12,15	3,08	2,03	0,88
Vanillin	1,10	0,28	—	—
1-Guaiacylethanol	—	—	2,17	0,93
Guaiacylpropane	16,57	4,18	22,73	9,7
Syringylpropane	22,10	5,57	25,58	11,06
1-Guaiacylpropanol	—	—	4,87	2,1
3-Guaiacylpropanol	11,05	2,8	7,44	3,2
Unidentified	22,10	5,57	25,75	21,3
Ratio of the p-coumaryl to guaiacyl to syringyl units	0,2:1,0:0,47		0,15:1,0:0,6	

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