SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING A 3'-TERMINAL PHOSPHATE GROUP

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Two methods for synthesizing oligodeoxyribonucleotides containing 3'-terminal phosphosphate groups have been developed. The first consists in the synthesis and the introduction into the oligonucleotides of a block containing a 3'-terminal bis(2-cyanoethyl) phosphate group and the second in the use of the transesterification reaction of oligonucleotides with 2-cyanoethanol in the presence of cesium fluoride. Both methods permit the fairly effective synthesis of oligodeoxyribonucleotide 3'-phosphates.

Chemical ligation (alternative to enzymatic ligation) [1] permits fairly long DNA duplexes containing both natural phosphodiester and also modified internucleotide bonds to be obtained [2]. In this process, the oligodeoxyribonucleotides used in the synthesis may contain either 5'- or 3'-phosphate groups. However, chemical ligation takes place more effectively when oligonucleotide 3'-phosphates are used [2, 3]. It was therefore necessary within the framework of the triester synthesis of oligonucleotides, which has well recommended itself [4], to develop a method for obtaining oligodeoxyribonucleotide 3'-phosphates.

In developing the general strategy of the synthesis of such compounds we endeavored to avoid protective groupings capable of elimination only under conditions differing from the traditional ones (NH_3, AcOH). Such conditions are satisfied by the 2-cyanoethyl protective group, which is eliminated on treatment of an oligonucleotide with ammonia (under the conditions for deblocking an exocyclic amino group).

We have previously described the synthesis of a number of oligonucleotides [5, 6] using a nucleoside 3'-[bis(2-cyanoethyl) phosphate] as the monomeric nucleoside unit for the growth of the oligonucleotide chain. In spite of the fact that the yields in the condensation of a nucleotide component with this compound are fairly high, averaging 75-85% both in solution [5] and on a polymeric support [6], its stability is extremely low, and one of the two terminal 2-cyanoethyl groups can easily be split out in the presence of aqueous pyridine. This complicates both the preparation of the nucleoside 3'-[bis(2-cyanoethyl)phosphate] and their isolation on a silica gel column.

The same anomalous lability of the 2-cyanoethyl group has also been reported in the case of a nucleoside 3'-(2-cyanoethyl phosphate), the splitting out of which took place an order of magnitude faster than in the case of 2-cyanoethyl phosphate itself [7].

At the same time, N-benzoyl-5'-O-monomethoxytrityldeoxynucleoside 3'-[bis(2-cyanoethyl)-phosphate] and also nucleoside 5'-[bis(2-cyanoethyl)phosphate]s represent completely stable compounds. All this permits the assumption that the 5'-hydroxy group of the nucleotide is involved in the splitting out of one of the 2-cyanoethyl groups in a nucleoside 3'-[bis(2-cyanoethyl)phsophate]. The construction of a model of a nucleoside 3'-[bis(2-cyanoethyl)-phosphate] showed that the 5'-hydroxy group is actually close to the 3'-phosphate group and can promote β-elimination.

In view of this, we used the following scheme for the synthesis of an oligonucleotide and for the phosphorylation of 5'-O-monomethoxytrityldinucleoside monophosphate:

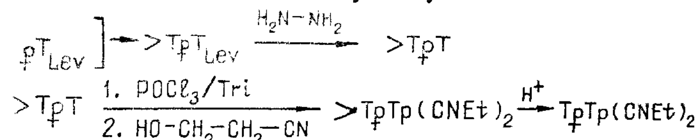

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TABLE 1. Conditions for the Synthesis of the Nonanucleotide d(C-C-T-G-G-A-A-T-Tp)*

Oligonucleotide	Starting material, umole		Yield, %
	¯P component	OH component	
1. >ČpTp(ClPh, CNEt)	>Čp(ClPh) (150)	Tp(ClPh, CNEt) (100)	89
2. >ČpČpTp(ClPh,CNEt)	>Čp(ClPh) (60)	ČpTp(ClPh,CNEt) (40)	78
3. >ČpČpTpǦpǦp(ClPh,CNEt)	>ČpČpTp(ClPh)(40)	ǦpǦp(ClPh,CNEt) (27)	72
4. >TpT$_{Lev}$	(ClPh)pT$_{Lev}$ (900)	>T (600)	86
5. >ǍpǍpTpTp(CNEt)$_2$	>ǍpǍp(ClPh)(375)	TpTp(CNEt)$_2$ (250)	80
6. >ČpČpTpǦpǦpǍpǍpTpTp	>ČpČpTpǦpǦp(ClPh) (10)	ǍpǍpTpTp(CNEt)$_2$ (7)	64

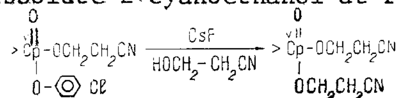
*The symbols recommended by the IUPAC-IUB nomenclature are used, the symbol d being omitted throughout for the sake of brevity. Other abbreviations: ClPh — p-chlorophenyl; CNEt — 2-cyanoethyl; Lev — $CH_3COCH_2CH_2COO$; >Ňp — N-benzoyl-5'-O-monomethoxytritylnucleoside 3'-(p-chlorphenylcyanoethyl phosphate); Tri — 1,2,4-triazole; MeIm — 1-methylimidazole.

As the condensing agent we used a mixture of triisopropylbenzenesulfonyl chloride and N-methylimidazole [8] (P component:TPS:MeIm = 1:3:6). The conditions for synthesizing a nonanucleotide are given in Table 1. The splitting out of the levulinyl protection was carried out by a method similar to that described by Van Boom and Burgers [9], although not in pyridine but in acetonitrile. As was expected, the dinucleoside phosphate (I) synthesized proved to be completely stable under the conditions of deblocking the monomethoxytrityl protection and separation on silica gel.

After the elimination of the N,P-protective groups, (NH_3, 50°C, 12 h) and the 5'-O-monomethoxytrityl group (80% aqueous solution of CH_3COOH for 30 min), the desired nonanucleotide was isolated by ion-exchange chromatography on DEAE-cellulose (pH 7,5 and 3.5). The homogeneity of the nonanucleotide synthesized was confirmed by high-performance chromatography (Fig. 1) and its primary structure by the Maxam—Gilbert method [10].

However, the preparation of oligodeoxyribonucleotides by the scheme described above presupposes the production of the nonstandard block (I) containing a 3'-terminal bis(2-cyanoethyl phosphate) group. We therefore developed a general scheme for obtaining oligodeoxyribonucleoside 3'-phosphates from completely protected oligonucleotides containing a 3'-terminal p-chlorophenyl 2-cyanoethyl phosphate group. For this purpose we made use of the transesterification of triesters of oligonucleotides with alcohols in the presence of cesium fluoride [11].

The transesterification of the mono- and oligonucleotides was carried out in a saturated solution of cesium fluoride in absolute 2-cyanoethanol at room temperature for 10-14 h.



After the end of the reaction and the elimination of the N-, P-, and 5'-O-protective groups, the mixture was subjected to analysis by microcolumn chromatography on DEAE-cellulose or Lichrosorb-NH_2 (Fig. 2). The yields of products after the isolation of the mono- and oligonucleotide 3'-phosphates are given below:

Mono- or oligonucleotide	Yield, %
1. >Ǧp(ClPh, CNEt)	80
2. >ǦpǍpTpǍp(ClPh,CNEt)	68
3. ČpTp(ClPh, CNEt)	73
4. >ǦpČpǍpTp(ClPh, CNEt)	60
5. >ǦpǍpTpČpČpǍp(ClPh,CNEt)	44

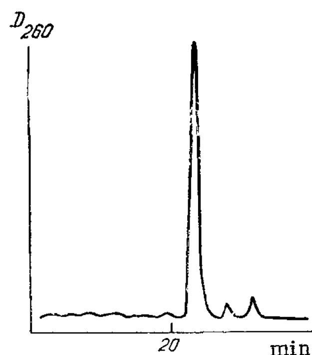


Fig. 1. Analytical chromatography of the nonanucleotide d(CCTGAATTp) on a Zorbax C-8 column (Du Pont, USA). The analysis was performed on a Tracor chromatograph (Netherlands) in a linear concentration gradient of methanol (5-35%) in 0.1 M ammonium acetate 45°C.

The low solubility of cesium fluoride and of the oligonucleotide in 2-cyanoethanol must be pointed out. Consequently, the use of the more soluble tetrabutylammonium fluoride will apparently permit the yield of oligonucleotide 3'-phosphates to be raised.

The presence of a 3'-terminal phsophate group in each of the oligonucleotides was shown by the change in their chromatographic mobilities on DEAE-cellulose after treatment with alkaline phosphatase, and also by the stability of the oligonucleotide 3'-phosphates to the action of snake venom phosphodiesterase. At the same time, the separation of the reaction mixture after transesterification in the case of 10- to 15-membered oligonucleotides may represent a serious problem, since the desired and the initial oligonucleotides differ by a single charge.

The preparation of such a type of mixture can be substantially facilitated by previous treatment with snake venom phosphodiesterase, which cleaves the initial oligonucleotide with a terminal p(ClPh) protective group and does not affect the 3'-phosphorylated oligonucleotide.

EXPERIMENTAL

We used deoxyribonucleosides, 3'-O-levulinylthymidine 5'-(p-chlorophenyl phosphate), and 2,4,6-triisopropylbenzenesulfonyl chloride from the Novosibirsk Institute of Organic Chemistry of the Siberian Branch of the Academy of Sciences of the USSR, ethylene cyanohydrin and 1-methylimidazole from Merck (GFR), 1,2,4-triazole from Fluka (Switzerland), and DEAE-cellulose DE-32 from Whatman (United Kingdom).

Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates (Merck, GFR), and column chromatography on silica gel L 40/100 (Chemapol, Czechoslovakia).

The N-benzoylnucleosides were synthesized with the use of the intermediate protection of the hydroxy groups by chlorotrimethylsilane [12]. The monomethoxytritylation and phosphorylation of the N-benzoylnucleosides was performed by a standard procedure [4].

General Method for Eliminating the 2-Cyanoethyl Protection from the Terminal Phosphate Group (Preparation of the P-component and Condensation) [13]. A solution of 1 mmole of a mono- or oligonucleotide in 10 ml of pyridine—triethylamine—water (3:1:1 by volume) was kept at room temperature for 15-20 min (monitoring by TLC in the $CHCl_3$—EtOH (9:1) system).

General Procedure for Eliminating Monomethoxytrityl Protection from a 5'-Hydroxy Group (Preparation of the OH Component for Condensation). A solution 1 mmole of a mono- or oligonucleotide in 6 ml of chloroform was cooled to 0°C, and 6 ml of a cooled 20% solution of trichloroacetic acid in chloroform was added. The mixture was kept for 20 min (monitoring by TLC in the $CHCl_3$—EtOH (9:1) system). The trichloroacetic acid was neutralized with a

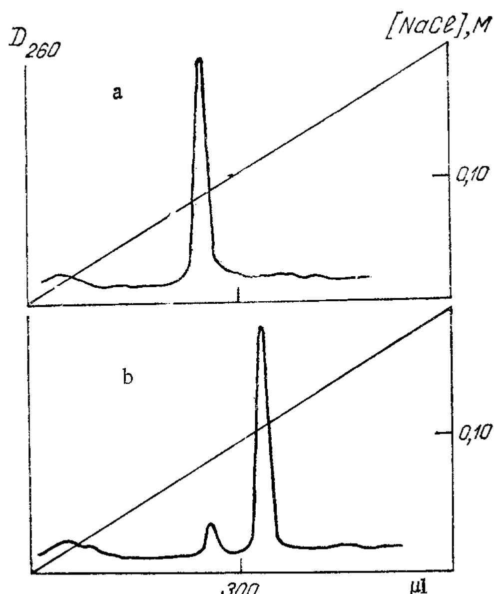


Fig. 2. Analytical chromatography on DEAE-cellulose of the N,O,P-deblocked dinucleotide d(CpTp): a) initial dinucleotide CpTp(ClPh); b) reaction mixture after treatment of the dinucleotide with a saturated solution of cesium fluoride in 2-cyanoethanol.

saturated solution of sodium bicarbonate (pH 8.0). 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 case of the detritylation of oligonucleotides with more than three links, the oligonucleotide was used in condensation without preliminary separation on silica gel.

General Procedure for Performing Internucleotide Condensation. A mixture of 1 mmole of the P component and 0.5-0.8 mmole of the OH component was dried by evaporation with absolute pyridine three times. In the final step, evaporation was carried out to a volume at which the total concentration of components taking into account the 6 mmole of 1-methylimidazole then added was 0.1 M. After this, 3 mmole of triisopropylbenzenesulfonyl chloride was added to the reaction mixture and it was left at room temperature with monitoring of the course of the reaction by means of TLC ($CHCl_3$–EtOH (9:1)). Reaction time 15-30 min. The reaction mixture was decomposed with water and treated by a standard method [4]. The desired substance was isolated by column chromatography on silica gel. The yields at the condensation stage were 70-90%.

General Procedure for the Eliminating the Levulinyl Protection from the 3'-Hydroxy Group of a Mono- or Oligonucleotide.* To 0.5 ml of hydrazine hydrate were added 7 ml of acetonitrile and, in drops, 1.5-2 ml of acetic acid until the solution had become clear, and the solution was then made up to 10 ml with acetonitrile. A solution of $>TPT_{Lev}$ in 5 ml of the resulting 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. The desired product was isolated by column chromatography on silica gel. Yield 75-80%.

General Procedure for the Introduction of a 3 -[Bis-(2-cyanoethyl) phosphate] Group into a Dinucleoside Phosphate. A solution of 6 mmole of 1,2,4-triazole in 5 ml absolute pyridine was cooled to 10°C, and 2 mmole of phosphorus oxychloride was added. The precipitate that deposited (pyridine hydrochloride) was filtered off, and the filtrate was added to a solution

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

of 1 mmole of >TpT in 5 ml of absolute pyridine. After 3 min (TLC in the $CHCl_3$—EtOH (9:1) system), an excess (20 mmole) of 2-cyanoethanol was added to the mixture. The reaction was complete after 1 h (TLC in the $CHCl_3$—EtOH (9:1) system). The reaction mixture was worked up by the standard method [4]. The desired product was isolated by column chromatography on silica gel, yield 74%.

General Method for the Transesterification of Mono- and Oligonucleotides. A 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, the desired oligonucleotide was isolated by ion-exchange chromatography on DEAE-cellulose in a linear concentration gradient of sodium chloride.

CONCLUSIONS

1. A method has been proposed for obtaining oligonucleotide 3'-phosphates which consists in the introduction into the synthesis of an oligonucleotide block containing a 3'-terminal bis(2-cyanoethyl phosphate) group.

2. A method for synthesizing oligonucleotide 3'-phosphates by using the transesterification of oligonucleotides in the presence of cesium fluoride and 2-cyanoethanol has also been proposed.

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