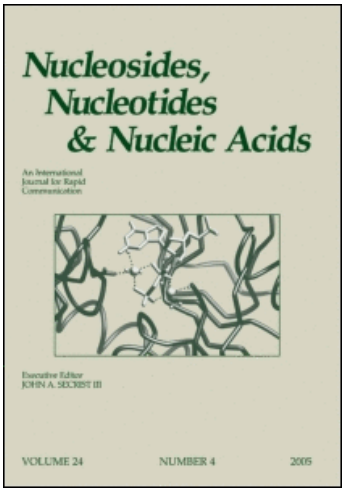


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Modification of Oligo (Poly) Nucleotide Phosphomonoester Groups in Aqueous Solutions

M. G. Ivanovskaya[a]; M. B. Gottikh[a]; Z. A. Shabarova[a]
[a] Department of Chemistry, and A.N. Belozersky Laboratory of molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow, USSR

To cite this Article Ivanovskaya, M. G. , Gottikh, M. B. and Shabarova, Z. A.(1987) 'Modification of Oligo (Poly) Nucleotide Phosphomonoester Groups in Aqueous Solutions', Nucleosides, Nucleotides and Nucleic Acids, 6: 5, 913 — 934
To link to this Article: DOI: 10.1080/15257778708073437
URL: http://dx.doi.org/10.1080/15257778708073437

NUCLEOSIDES & NUCLEOTIDES, 6(5), 913-934 (1987)

MODIFICATION OF OLIGO(POLY)NUCLEOTIDE PHOSPHOMONOESTER GROUPS IN AQUEOUS SOLUTIONS

M.G. Ivanovskaya, M.B. Gottikh and Z.A. Shabarova

Department of Chemistry, and A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, U S S R

ABSTRACT. Selective modification of oligo(poly)nucleotide phosphomonoester groups in an aqueous medium by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide in the presence of various nucleophilic agents has been investigated. Optimal conditions of the modification by amino- and hydroxycompounds have been found. Based on these studies a general efficient method for preparation of oligo(poly)nucleotide phosphoamidates and phosphodiesters in an aqueous solution has been developed. The method allows to prepare both oligodeoxyribonucleotide derivatives at 3'- and 5'-terminal phosphate groups and oligoribonucleotide derivatives at 5'-terminal phosphate groups with 80-100% yields.

INTRODUCTION

Oligonucleotide derivatives, containing various non-nucleotide groups covalently bound to terminal phosphate groups, are finding ever wider application as chemical probes [1], markers [2], affinity reagents [3], agents for chemical ligation [4] and for termination of transcription [5]. For extensive application of the above oligonucleotide derivatives in different molecular biology investigations it is necessary to elaborate an efficient and rather simple method for introduction of various non-nucleotide residues into oligonucleotides. The crucial step in preparing oligonucleotide derivatives at the terminal phosphate consists in selective activation of phosphomonoester groups of oligo(poly)nucleotides.

The carbodiimide method, evolved by H. Khorana [6], appears to be the most suitable procedure for modification of phosphomonoester groups. Khorana's methodology was based on utilization of dicyclohexylcarbodiimide in anhydrous or slightly aqueous solutions [7-9]. The use of an anhydrous medium limited the applicability of the method because unprotected oligo- and polynucleotides, DNAs, RNAs do not dissolve in organic solvents. Furthermore, such side processes as alkylation of heterocyclic

bases [10], cleavage and izomerization of internucleotide bonds in RNA [11] take place in an anhydrous medium. To solve these problems it appeared tempting to evolve a carbodiimide activation strategy for modification of phosphomonoesters in an aqueous medium.

In the present communication a thorough investigation of oligonucleotide phosphomonoester groups modification, induced by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), in the presence of different nucleophilic agents is described. This investigation has made it possible to suggest a general and effective method for synthesizing a wide range of oligonucleotide derivatives in an aqueous medium.

MATERIALS AND METHODS

Reagents and enzymes. Nucleoside-5'-phosphates were obtained from "Sigma"; d(TGGCCAAGCTp), d(TTGAATGp) were kindly provided by T.S. Oretskaya (Moscow State University); $(pU)_5$, $(pA)_{11}$, $(pU)_{30}$ were from SRCTI (SD AS USSR). EDC, MES, MeIm [12], Lichrosorb-NH_2 (10 μm); aniline, imidazole, morpholine, benzylamine, n-butylamine, ethylenediamine and 4-nitrophenol were obtained from "Merck"; 2-hydroxypyridine - from "Aldrich"; glycine methyl ester, serine methyl ester, tyrosine methyl ester, cysteine methyl ester - from "Koch-Light"; N-hydroxybenzotriazole, 3-hydroxypropionitrile, Dans-chloride [12] - from "Fluka", Nucleosil C18 (5 μm) - from "Chemapol"; methanol, ethanol, n-propanol, 2-aminoethanol, ethyleneglycol - from "Soyuzkhimreaktiv", USSR. 2-(N-Dans)-aminoethanol was synthesized as described earlier [13]. Bacterial alkaline phosphatase and snake venom phosphodiesterase (PDE) were obtained from "Worthington Biochemicals Corporation".

Chromatography and electrophoresis. Electrophoresis was performed on FN-1 paper at 900-1000 v in 0.05 M triethylammonium bicarbonate buffer, pH 7.5, for 1.5-2 h using "Labor" apparatus. Chromatography was carried out on Lichrosorb-NH_2 columns (1x50 mm) in a linear gradient of sodium phosphate, pH 7.5, in 7 M urea, and on Nucleosil C18 columns (2x62 mm) in a linear gradient of methanol in 0.1 M ammonium acetate, pH 6.0, using HPL chromatograph "Milichrom", USSR. Gel-filtration was accomplished on Biogel P-2 (200-400 mesh, "Bio Rad"). Paper chromatography was carried out on FN1 paper in the systems: ethanol-1 M ammonium acetate, pH 7.5 (7:3 v/v) (System A) and ethanol-1 M ammonium acetate, pH 7.5 (8:2 v/v) (System B).

Buffers. To maintain stable pH values during modification procedures the following buffers were used: pH 1- 0.1 N aqueous HCl; pH 2 - 0.01 M aqueous HCl; pH 3-6 - 0.4 M MES-buffers, pH 7-9 - 0.4 M MeIm-buffer.

General procedure for preparation of mononucleotide phosphoamidates. An aqueous solution of amine (100 µl of 3 M solution) pretitrated to pH 4.5-5.5 by 6 N aqueous HCl was added to a water-soluble salt of the mononucleotide (1-5 µmol). Then 9.6 mg (50 µmol) of EDC was added and the reaction mixture was stored at 20°C for the time given in Table 1. The phosphoamidates were isolated by paper electrophoresis or by paper chromatography in system A. The yields of pA phosphoamidates are given in Table 1.

General procedure for preparation of mononucleotide derivatives with simple alcohols. An alcohol solution (100 µl of 3-6 M solution) in 0.4 M MES buffer (pH 4.5-5.5) was added to water-soluble salt of the mononucleotide (1-5 µmol). Then 9.6 mg (50 µmol) of EDC was added and the reaction mixture was stored at 20°C for the time given in Table 1. The mononucleotide phosphodiesters were isolated by paper electrophoresis. The yields of pA phosphodiesters are given in Table 1.

Preparation of 2-(N-Dans)-aminoethyl esters of mononucleotides. 44 mg (0.15 mmol) of 2-(N-Dans)-aminoethanol, dissolved in 30 µl of DMFA [12], was adjusted to pH 3.0 by adding 20 µl of 6 N aqueous HCl. This solution was added to a water-soluble salt of a mononucleotide (1-5 µmol) in 40 µl of 0.4 M MES buffer, pH 5.5. The resultant solution (pH 4.5-5.0) was supplemented with 9.6 mg (50 µmol) of EDC and incubated at 10°C for 24 hours. The 2-(N-Dans)-aminoethyl esters of mononucleotides were isolated by paper chromatography in system B. For 2-(N-Dans)-aminoethyl ester of pA R_f was 0.79, and the yield - 40%.

Preparation of 4-nitrophenyl esters of mononucleotides. The solution of 4-nitrophenol (50 µl of 6 M solution in DMFA-water (1:1, v/v), pretitrated to pH 6.5-7.0 by 6 N aqueous HCl, was added dropwise to a water-soluble salt of a mononucleotide (1-5 µmol) dissolved in 50 µl of DMFA-water (1:1, v/v). The resulting mixture was supplemented with 9.6 mg (50 µmol) of EDC and allowed to stay at 10°C for 20-24 h. 4-Nitrophenyl esters of pA and pC were isolated by paper chromatography in System B. The yields were 95-100%. When the reaction of pU, dpT and pG with 4-nitrophenol was over, the excess of 4-nitrophenol was extracted by chloroform and the water fraction was evaporated in vacuum, dissolved in 0.2 M of Na_2CO_3 (pH 10.5) and incubated for

TABLE 1.

PREPARATION OF pA PHOSPHOAMIDATES AND PHOSPHODIESTERS UNDER OPTIMAL REACTION CONDITIONS (CONCENTRATION OF pA - 0.02 M, EDC - 0.5 M)

Nucleophilic agent	pH	Nucleophile concentration, M	T°C	Process time, hours	Yield of pA derivative, %
aniline	4.5	3.0	20	1	95
imidazole	6.0	3.0	20	1	90
glycine methyl ester	4.5	3.0	20	1	85
morpholine	5.0	3.0	20	2	90
benzylamine	4.5	3.0	20	4	85
butylamine	5.0	3.0	20	6	80
methanol	4.5	6.0	20	2	80
ethanol	4.5	3.0	20	2	85
n-propanol	4.5	3.0	20	2	80
3-hydroxypropinonitrile	4.5	4.0	20	4	90
2-(N-Dans)-aminoethanol	4.5	1.5	10	24	40
4-nitrophenol	6.5	3.0	10	24	95
N-hydroxybenzo triazole	4.5	3.0	5	4	95
2-hydroxypyridine	5.0	3.0	5	2	90
2-hydroxyethyl ester of bromoacetic acid	4.5	3.0	10	6	90

24 h at 20°C (or for 6-10 h at 37°C). 4-Nitrophenyl esters of pU, pT and pG were isolated by paper chromatography in system B. The yields were 75-80%.

Preparation of N-hydroxybenzotriazole esters of mononucleotides. 40 mg (0.3 mmol) of N-hydroxybezotriazole was dissolved in 50 µl of DMFA-water mixture (4:1, v/v). Then 6 µl of 2 N NaOH was added under stirring to adjust pH of the solution to 4.0-4.5. This mixture was added to a solution of a water-soluble salt of the nucleotide (1-5 µmol) dissolved in 40 µl DMFA-water (3:1, v/v). The reaction mixture was shaked, supplemented with EDC (9.6 mg, 50 µmol) and allowed to stay at 5°C for 3.5-4.0 hours. N-hydroxybenzotriazole esters of mononucleotides were isolated by paper chromatography in System B. The yields were 90-95%.

General procedure for preparation of oligo(poly)nucleotide phosphoamidates. A water solution of amine (50 µl of a 3 M solution) pretitrated by 6 N aqueous HCl to pH 4.5-5.5 was added to a water-soluble salt of the oligo(poly)nucleotide (0.001-0.01 µmol). The reaction mixture was supplemented with 4.8 mg (25 µmol) of EDC and allowed to stay at 4°C for the time indicated in Table 2. The excess of the reagents was separated by gel-filtration on Biogel P-2, and the oligonucleotide phosphoamidates were isolated by HPLC on nucleosil C18 columns. Conversion of phosphomonoester groups of oligonucleotides is presented in Table 2.

General procedures for preparation of oligonucleotide derivatives with simple alcohols. A water-soluble salt of oligo(poly)nucleotide (0.001-0.1 µmol) was supplemented with 50 µl of a 3-6 M alcohol solution in 0.4 M MES-buffer, containing 2 M $MgCl_2$, pH 4.5-5.5, and 4.8 mg (25 µmol) of EDC. The reaction mixture was incubated at 4°C for the time indicated in Table 2. After gel-filtration on Biogel P-2, the oligonucleotide phosphodiesters were isolated by HPLC on Nucleosil C18. Conversion of phosphomonoester groups of oligonucleotides is presented in Table 2.

Preparation of 2-(N-Dans)-aminoethyl ester of d(TGGCCAAGCTp). d(TGGCCAAGCTp) (0.03 µmol) was dissolved in 40 µl of 0.4 M MES-buffer, containing 2 M $MgCl_2$, pH 4.0-4.5. The reagents were added as described above for 2-(N-Dans)-aminoethyl ester of mononucleotides. The reaction mixture was incubated at 4°C for 24 h. The reaction product was isolated by gel-filtration on Biogel P-2 followed by HPLC on Nucleosil C18. The yield was 30%.

TABLE 2.

PREPARATION OF OLIGO(POLY)NUCLEOTIDE PHOSPHOAMIDATES AND PHOSPHODIESTERS UNDER OPTIMAL REACTION CONDITIONS (CONCENTRATION OF EDC - 0.5 M, TEMPERATURE -4°C)

Oligo- or polynucleotide	Nucleophilic agent	pH	Nucleotide concentration (per monomeric union), mM	Nucleophile concentration, M	Process time, hours	Conversion of phosphomonoester groups, %
d(TTGAATGp)	aniline	4.5	0.2	3.0	2	95
	imidazole	6.0	0.2	3.0	4	90
	morpholine	5.0	0.2	3.0	6	95
	1,4-diaminobutane	4.5	0.2	3.0	8	90
	2-hydroxyethyl ester of bromoacetic acid	4.5	0.2	3.0	6	80
d(TGGCCAACGTp)	imidazole	6.0	0.2	3.0	4	90
	ethylenediamine	4.5	0.1	3.0	2	95
	ethanol	4.5	0.1	6.0	6	98
	ethyleneglycol	4.5	0.1	6.0	6	95
	2-(N-Dans)amino-ethanol	4.0	0.1	1.5	24	30
	N-hydroxybenzotriazole	4.5	0.1	3.0	4	95
$(pU)_5$	N-hydroxybensotriazole	4.5	0.2	3.0	4	95
	ethylenediamine	4.5	0.2	3.0	2	100
$(pA)_{11}$	4-nitrophenol	6.5	0.1	3.0	24	70
	ethylenediamine	4.5	0.1	3.0	2	95
$(pU)_{30}$	ethylenediamine	4.5	0.03	3.0	2	90

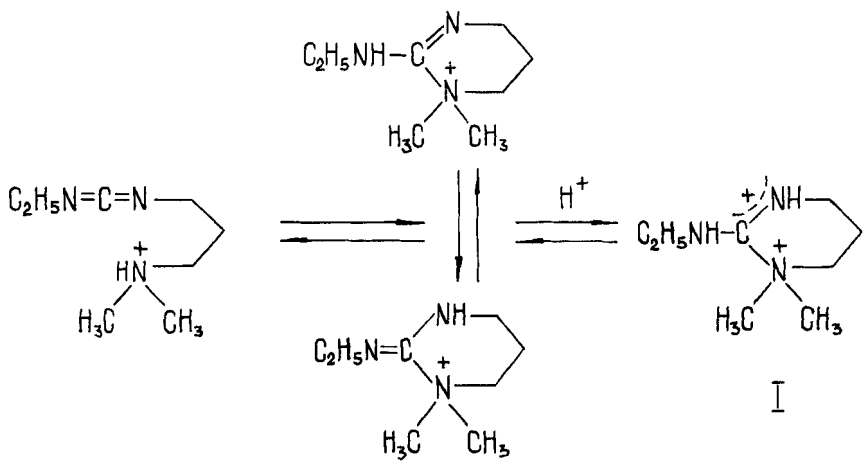
Preparation of N-hydroxybenzotriazole esters of $(pU)_5$ and d(TGGCCAAGCTp). 0.01-0.02 μmol of the oligonucleotide was dissolved in 40 μl of DMFA - 0.4 M MES-buffer, containing 2 M $MgCl_2$ (pH 4.5-5.5) 3:1 (v/v). The reagents were added as described above for hydroxybenzotriazole ester of mononucleotides. The reaction mixture was incubated at 4°C for 3 h. The products were isolated by gel-filtration on Biogel P-2 with 90% yields.

RESULTS AND DISCUSSION

Reaction of mononucleotides with amino- and hydroxycompounds

Mononucleotide pA was selected as an object for a detailed study of the EDC-induced modification of phosphomonoester groups. The heterocyclic base of pA is not modified by carbodiimides and therefore it becomes possible to investigate only the conversions of the phosphomonoester group.

The characteristic feature of EDC is its ability to exist in different tautometric forms depending on pH of the reaction medium [14]:



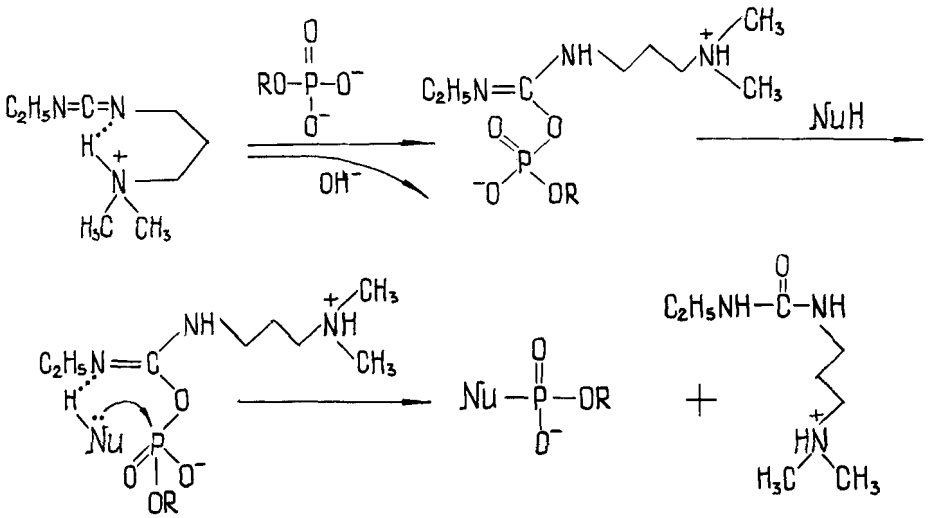
For this reason EDC-induced reactions are usually pH-dependent [15]. We have found pH-dependence of phosphomonoester group modification to be individual for each class of nucleophilic agents (amines, alcohols, phenols). In the case of EDC-induced modification of pA by amines we established the forming of phosphoamidates to take place in a wide pH-range (from 2 to 10, Table 3). The maximal efficiency and rate of modification for amines of different basicity were observed at pH from 3 to 5 (Table 3). Under these conditions EDC predominantly exists in the most reactive form I. The modification efficiency sharply decreases if pH is brought down to 1 and below, because a phos-

TABLE 3

EFFICIENCY OF EDC-INDUCED MODIFICATION OF pA BY AMINES AT VARIOUS pH VALUES (CONCENTRATION OF pA - 0.03 M, AMINE - 3 M, EDC - 0.5 M, temperature - 20°C)

Amine	pH	Degree of pA modification, %					
		0.5 h	1 h	2 h	6 h	16 h	24 h
aniline	3.5	100	100	100	100	100	100
	4.5	90-95	100	100	100	100	100
pK_a = 4.68	6.0	-	85-87	90-93	100	100	100
ethylenediamine	4.5	-	100	100	100	100	100
	5.0	-	98	100	100	100	100
pK_a = 6.99	6.0	-	60-65	75-80	100	100	100
	7.0	-	25-30	35-50	60-65	90-95	100
glycine methyl ester	3.0	100	100	100	100	100	100
	4.5	90-95	100	100	100	100	100
pK_a = 7.73	6.5	-	40-45	60-65	90-95	100	100
	7.5	-	-	30-35	80-85	-	100
butylamine	1	-	-	5-10	-	-	5-10
pK_a = 10.6	2	-	-	60-70	-	-	80-85
	3	-	-	70-80	-	-	95-98
	4	-	-	90-95	-	-	100
	5	-	-	80-90	-	-	95-98
	6	-	-	80-85	-	-	95-98
	7	-	-	80-85	-	-	100
	8	-	-	50-55	-	-	75-80
	9	-	-	40-45	-	-	60-65
	10	-	-	15-20	-	-	40-45
	11	-	-	0	-	-	0

phomonoester group becames wholly undissociate (pK_1=1.0, pK_2 = 6.5) and thus unable to form an EDC-adduct. The distinguishing feature of EDC is its ability to induce phosphomonoester group modification at pH higher than 6 (Table 3). We suggest this ability may be explained by the presence of a strongly basic tertiary aminogroup (pK_a = 11.1 [14]) in its structure. This aminogroup is always protonated under modification conditions. Apparently at pH > 6 EDC can be activated in consequence of the intramolecular proton transfer from the tertiary nitrogen atom to the imide one. In this case the phosphomonoester modification appears to proceed according to the following scheme:



NuH - a nucleophilic agent

This assumption is confirmed by inhibition of the EDC-induced modification at pH>10 (Table 3) when the tertiary amine group becomes unprotonated. Besides, carbodiimides whose structure lacks the protonodonor group, for example, 1-cyclohexyl--3-(2-morpholinoethyl) carbodiimide metho-4-toluenesulfonate, do not induce phosphomonoester group modification at pH>6 [16]. The proposed scheme is in accordance with I.T. Ibrahim and A. Williams data about reactivity of the acyclic form of EDC in a basic medium [14].

An investigation of the pA reaction with hydroxycompounds in the presence of EDC has shown the optimal pH range to be dependent on the hydroxycompound nature.

In the case of simple alcohols, such as methanol, ethanol, n-propanol and others, the reaction proceeds at pH from 2 to 6 (Table 4) and phosphodiesters are formed. The increase of pH-value to 7 and higher results in pyrophosphate as the only reac-

TABLE 4

EFFICIENCY OF EDC-INDUCED MODIFICATION OF pA BY HYDROXY-COMPOUNDS AT VARIOUS pH VALUES (CONCENTRATION OF pA - 0.03 M, HYDROXYCOMPOUND - 3 M, EDC - 0.5 M, TEMPERATURE - 20°C)

Hydroxycompound	pH	Degree of pA modification, %				
		0.5 h	2 h	6 h	16 h	24 h
n-propanol*	1.0	0	0	0	0	0
	2.0	30-35	80-85	100	100	100
	3.2	30-35	95-100	100	100	100
	4.5	25-30	100	100	100	100
	5.5	15-20	95	100	100	100
	6.0	10-15	90-95	100	100	100
	7.0	0	0	0	3-5	5-10
	8.0	0	0	0	0	0
4-nitrophenol pK_a =7.15	3.5	-	-	-	3-5	5-10
	5.0	-	-	-	15-20	20-25
	6.5	-	-	-	90-95	100
	7.0	-	-	-	60-65	90-95
	8.0	-	-	-	45-50	60-65
	8.4	-	-	-	10-15	15-20
N-hydroxybenzo-triazole pK_a = 4.00	2.5	45-50	60-65	90-95	-	100
	4.0	-	-	90-95	-	100
	6.0	50-55	-	90-95	-	95-97
	7.0	5-10	15-20	35-40	-	35-40
	8.0	0	0	0	-	0
2-hydroxypyridine pK_a = 11.65	3.0	75-80	100	100	-	100
	5.0	65-70	100	100	-	100
	7.0	20-25	90-95	78-83	-	85-90
	8.0	10-15	70-75	30-35	-	47-52
	9.0	3-5	60-65	15-20	-	40-45

* A similar dependence is observed for methanol, ethanol and other simple alcohols.

tion product. This sharp alteration in the reaction course is caused by the appearance in the reaction medium of a dianion-phosphate which is a stronger nucleophile than alcohol and reacts first of all with the pA-carbodiimide adduct.

Phenols, unlike alcohols, are not nucleophilic in the non-ionized form. In contrast, the phenolate ion is one of the strongest nucleophilic agents and so the reaction with phenols becomes possible at pH $\geqslant pK_a$ of phenol. We succeeded in synthesizing 4-nitrophenyl ester of pA. The reaction proceeds most efficiently at pH 6.5-7.0 with no pyrophosphate being formed (Table 4). However, preparation of pA derivatives with phenol (pK_a=10) and N-Ac-Tyr methyl ester (pK_a = 10.5) has proved to be impos-

sible apparently as a result of EDC inactivation at pH $\geqslant$ 10. Moreover, the nucleophilic substitution in pA-EDC-adduct by phenolate anion is impeded by electrostatic repulsion of the same charged particles. This effect accounts for a decreased rate of the reaction of pA with 4-nitrophenol in comparison with amines and alcohols (Table 4).

EDC-induced phosphomonoester group modification in the presence of N-hydroxybenzotriazole and 2-hydroxypyridine proceeds with high yields in a wide pH-range (from 2 to 8, Table 4). The very high activity of the above compounds may be due to their specific electron structure.

An investigation of the dependence of modification efficiency on the concentration of reagents has revealed that a sufficiently high concentration of a nucleophilic agent (1-3 M) and EDC (0.5 M) is required (Table 5).

A rise in the temperature from 4°C to 50°C leads to an increase in the modification rate; yet the yields of the derivatives decrease owing to acceleration of EDC competitive hydrolysis.

We have found the modification of other deoxyribo- and ribonucleotides to occur the same as pA. However, it is necessary to take into account the possibility of the heterocyclic bases (U, T, G) modification by EDC. We have found no heterocyclic bases modification at pH$\leqslant$6 (48 h, 20°C). At pH > 6 this modification may be detected after 2 h incubation at 20°C. The above modification of U, T and G is reversible, and we recommend the following procedure for its elimination: treatment by 0.2 M Na_2CO_3 solution (pH 10.5) for 6-10 h at 37°C (or 24 h at 20°C).

Consequently, we have determined the optimal conditions for the modification of the phosphomonoester groups in mononucleotides. It is recommended to couple mononucleotides to amines, simple alcohols, N-hydroxybenzotriazole and 2-hydroxypyridine at pH of the reaction mixture from 4.5 to 5.5. Lower pH values are not advisable for the reaction of deoxyribonucleotides in view of their possible apurinization. The optimal concentrations are as follows: EDC - 0.5 M; nucleophilic agent - 3 M (for amines it is possible to decrease concentration to 1-0.5 M); and the optimal temperature is 4-20°C (Table 1). For the reaction with 4-nitrophenol the optimal pH value is 6.5-7.0, while other conditions are similar to those indicated above (Table 1).

Reactions of mononucleotides with bifunctional agents

It was interesting to study EDC-induced phosphomonoester groups modification by bifunctional agents containing both similar and different functional groups. In case of reagents

TABLE 5

EFFICIENCY OF EDC-INDUCED MODIFICATION OF pA BY AMINES AND ALCOHOLS AT VARIOUS REAGENT CONCENTRATIONS AND TEMPERATURE (pA CONCENTRATION - 0.02 M, pH - 4.5)

Amine or alcohol	Amine or alcohol concentration, M	EDC concentration, M	T°C	Degree of pA modification, %				
				1 h	2 h	4 h	6 h	24 h
ethanol	3	0.5	4	30-35	42-47	90-95	100	100
	3	0.5	20	50-55	90-100	100	100	100
	3	0.5	50	70-75	70-75	70-75	70-75	70-75
	1	0.5	20	35-40	45-50	55-60	60-65	70-75
	1	0.5	50	45-50	47-52	50-52	55-60	55-60
	1	0.3	20	30-35	40-45	50-55	51-56	52-57
	1	0.3	50	35-40	36-41	36-42	40-45	43-48
	0.3	0.3	20	15-20	20-25	30-35	35-40	36-42
	0.3	0.1	20	0	0	0	0	0
aniline	3	0.5	20	100	100	100	100	100
	0.3	0.5	20	95	95	95	95	95
ethylenediamine	3	0.5	20	100	100	100	100	100
	0.3	0.5	20	80-85	90	90	90	90
benzylamine	3	0.5	20	75-80	85-90	100	100	100
	0.3	0.5	20	25-30	30-35	45-50	55-60	85-90
butylamine	3	0.5	20	30-35	60-65	80-85	95-100	100
	1	0.5	20	10-15	55-60	60-65	-	95
	0.3	0.5	20	5	45-50	50-55	-	80-85

with similar functional groups (diamines,glycols) only one functional group reacts, while the other remains intact (Table 6). The presence of an unsubstituted amino- or hydroxygroup in such derivatives allows to use them for further derivatization, for instance, acylation or alkylation. One should point to an unusual reaction course under pA coupling to ethylenglycol. In this case not only 2-hydroxy ethyl ester of pA (yield 80-85%) but also by-product - nucleoside A was formed (yield 15-20%). An analogous process of the dephosphorylation takes place also under preparation of oligonucleotide 2-hydroxyethylesters. At the same time isolated 2-hydroxyethyl esters of mono(oligo)-nucleotides are stable in neutral and slightly acidic solutions. The dephosphorylation seems to occur at the stage of interaction of ethylenglycol with EDC-pA-adduct.

As for bifunctional reagents containing different functional groups, the strongest nucleophilic group interacts first of all with a preactivated phosphomonoester group. For example, in an EDC-induced coupling of pA to tyrosine methyl ester or cysteine methyl ester, only phosphoamidates but not phosphodiester or phosphothiol are formed. No variations in modification conditions - pH values of the reaction mixtures or the reagent concentrations - altered the reaction course.

Isolation of mononucleotide derivatives, their structure

Phosphoamide and phosphodiester derivatives of pA were isolated by electrophoresis on paper at pH 7.5 or by paper chromatography. The anilide, imidazolide, morpholide, benzylamide, butylamide of pA; and methyl, ethyl, propyl esters of pA were found identical to compounds previously synthesized in our laboratory [17]. The presence of an unsubstituted amino group in aminoethylamide and aminobutylamide of pA was proved by a colour reaction with ninhydrin.

The structure of pA derivatives with amino acid methyl esters was proved by acidic hydrolysis followed by amino acid assay on an amino acid analyzer. The nucleotide amount was determined spectrophotometrically. The nucleotide - amino acid molar ratio was close to an equimolar one.

The structure of 2-cyanoethyl ester of pA was confirmed by alkaline hydrolysis (conc. ammonium hydroxyd, 37°C, 24h) and by snake venom PDE hydrolysis. The structure of 4-nitrophenyl, 2-(N-Dans)-aminoethyl esters of pA and also of pA derivatives with 2-hydroxypyridine and N-hydroxybenzotriazole was determined by snake venom PDE hydrolysis followed by separation of the hyd-

TABLE 6

EDC-INDUCED MODIFICATION OF pA IN THE PRESENCE OF BIFUNCTIONAL REAGENTS (CONCENTRATION OF pA - 0.02 M, EDC - 0.5 M, TEMPERATURE - 20°C)

Bifunctional reagent	Reagent concentration, M	pH	Process time, hours	Type of synthesized bond	Yield of pA derivative, %
ethylenediamine	3.0	4.5	1	P-N	97
1,4-diaminobutane	3.0	4.5	6	P-N	95
ethyleneglycol	6.0	5.0	6	P-O	85
serine methyl ester	3.0	2.0	2	P-N	95
		4.5	2	P-N	90
		6.0	6	P-N	95
tyrosine methyl ester	3.0	1.5	2	P-N	85
	1.5	5.0	24	P-N	30
cysteine methyl ester	3.0	2.0	2	P-N	95
		5.0	2	P-N	100
		8.0	6	P-N	70

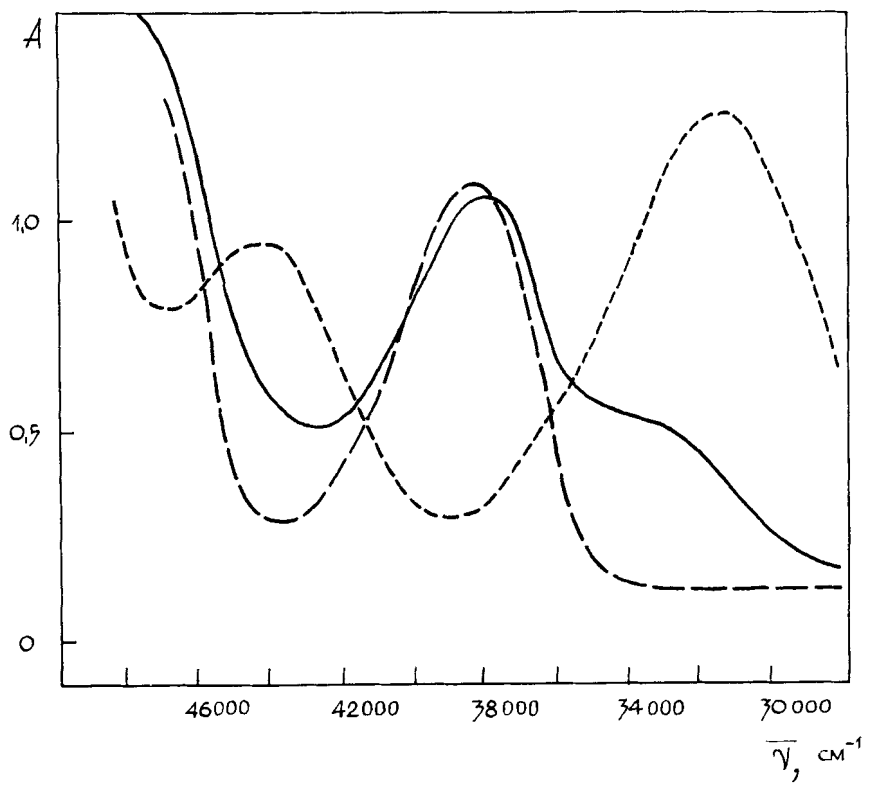


Figure 1. UV-spectra of 4-nitrophenyl ester of pA (———), starting pA (— — —) and 4-nitrophenol (- - - -).

rolyzate by paper electrophoresis. The pA - alcohol molar ratios determined spectrophotometrically were 1:0.97, 1:0.98, 1:0.87, 1:1.05 consequently. UV spectra of synthesized pA phosphodiesters are shown in Figures 1-4.

Reactions of oligonucleotides with amino- and hydroxycompounds

The chemical nature of phosphomonoester groups in mono- and oligonucleotides is identical, and therefore the results obtained for mononucleotides have been used as a basis for selective modification of oligo(poly)nucleotides. Reaction conditions determined for mononucleotides have proved to be wholly applicable to EDC-induced coupling of oligonucleotides to amines. A number of deoxyribo- and ribooligonucleotide phosphoamidates have been synthesized under above conditions (Table 2).

In contrast to the reaction with amines, the reaction with alcohols has proved to be more complex. Noticeable modification of

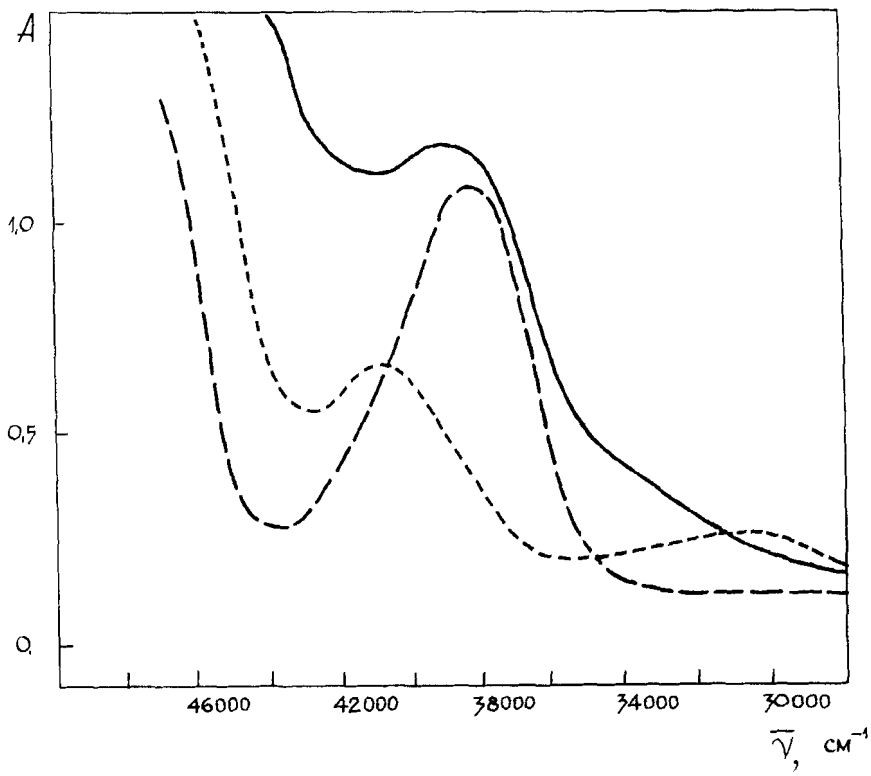


Figure 2. UV-spectra of 2-(N-Dans)-aminoethyl ester of pA (———), starting pA (— — —) and 2-(N-Dans)-aminoethanol (– – – –).

heterocyclic bases (U,T and G) in oligonucleotides by EDC takes place under conditions when these bases are not modified in mononucleotides (pH 5.5, 4°C, 16 h). We assume this increase of undesirable modification may be caused by the polyelectrolyte effect of a polyanion oligonucleotide. The cation reagent - EDC - owing to electrostatic interactions seems to be concentrated near the polyanion sugar-phosphate bone. The result is an increase in EDC local concentration near heterocyclic bases and modification acceleration. Remarkably, no such acceleration of heterocyclic bases modification is observed during oligonucleotide phosphoamidate preparation. Probably, protonated amine (its concentration exceeds 6-7 times the carbodiimide one) forms a polyelectrolyte layer near the oligonucleotide and protects it against modification.

To lower the degree of undesirable modification in the synthesis of oligonucleotide phosphodiesters we have used the property

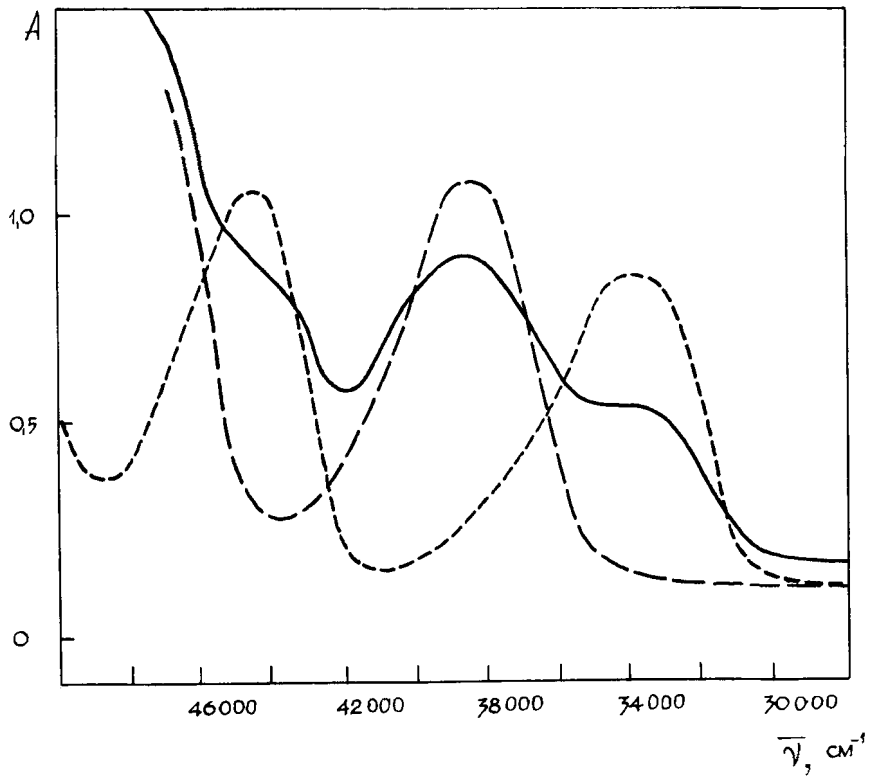


Figure 3. UV-spectra of pA derivative with 2-hydroxypyridine (———), starting pA (— — —) and 2-hydroxypyridine (- - - -).

of oligonucleotides to form tight ionic pairs with Mg^{2+} ions [18]. If the reaction is carried out at pH $\leqslant$ 5.5, but in the presence of 2 M $MgCl_2$ and at 4°C, heterocyclic bases modification does not exceed 5-10% after 24 h. To synthesize 4-nitrophenyl esters of oligonucleotides the reaction has to be carried out at pH > 6 (see the mononucleotide reactions). In this case partial heterocyclic bases modification takes place. For oligodeoxyribonucleotides it is possible to eliminate this modification at conditions found for mononucleotides. Since oligoribonucleotides incubated at pH 10.5 are hydrolyzed, a different approach is needed for preparation of 4-nitrophenyl esters of oligoribonucleotides containing U and G residues.

The developed method is the only one for preparation of oligonucleotide phosphodiester in an aqueous medium. We have synthesized a wide number of oligonucleotide derivatives with different

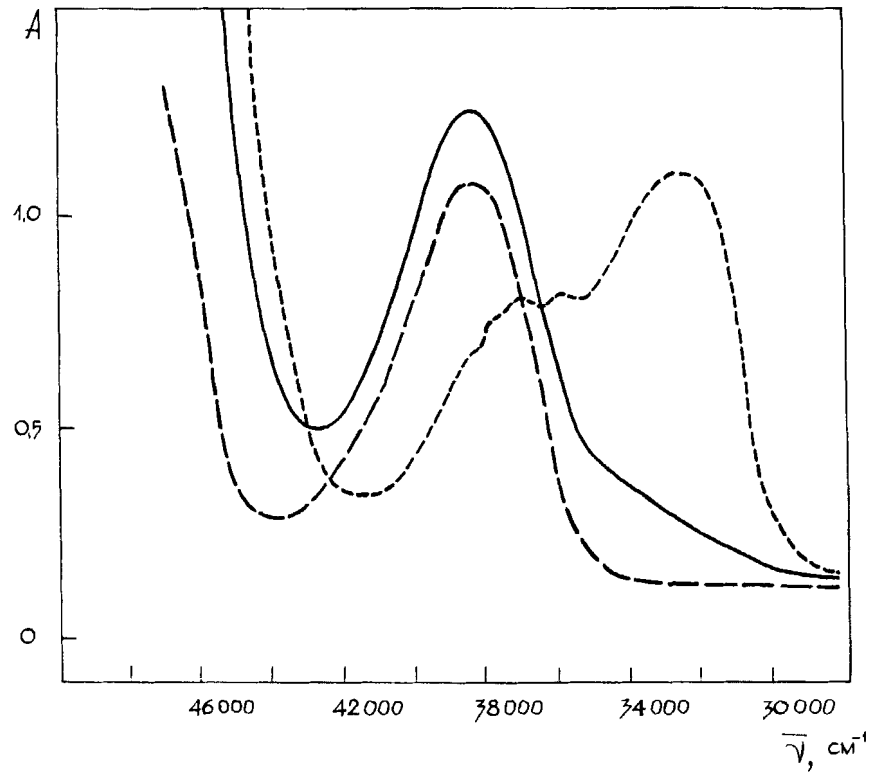


Figure 4. UV-spectra of pA derivative with N-hydroxybenzotriazole (——), starting pA (— — —) and N-hydroxybenzotriazole (- - - -).

type hydroxycompounds including formerly virtually inaccessible 4-nitrophenyl esters, N-hydroxybenzotriazole and 2-hydroxypyridine derivatives of unprotected oligonucleotides. Besides, an oligonucleotide affinity reagent, containing the bromoacetic acid residue, and fluorescent N-Dans-aminoethyl ester of the decanucleotide have been synthesized (Table 2).

Isolation of oligo(poly)nucleotide derivatives

Phosphoamidates and phosphodiesters of oligonucleotides were separated from the excess of reagents by gel-filtration on biogel P-2 followed by chromatography on Lichrosorb-NH_2 or Nucleosil C18. All synthesized derivatives are homogeneous in chromatography on ion-exchange and reversed-phase carriers. Chromatography of the reaction mixtures after synthesis of aminoethylamide and N-hydroxybenzotriazole ester of $(pU)_5$ is shown in Figure 5. The formation of phosphoamide or phosphodiester linkage at the terminal phos-

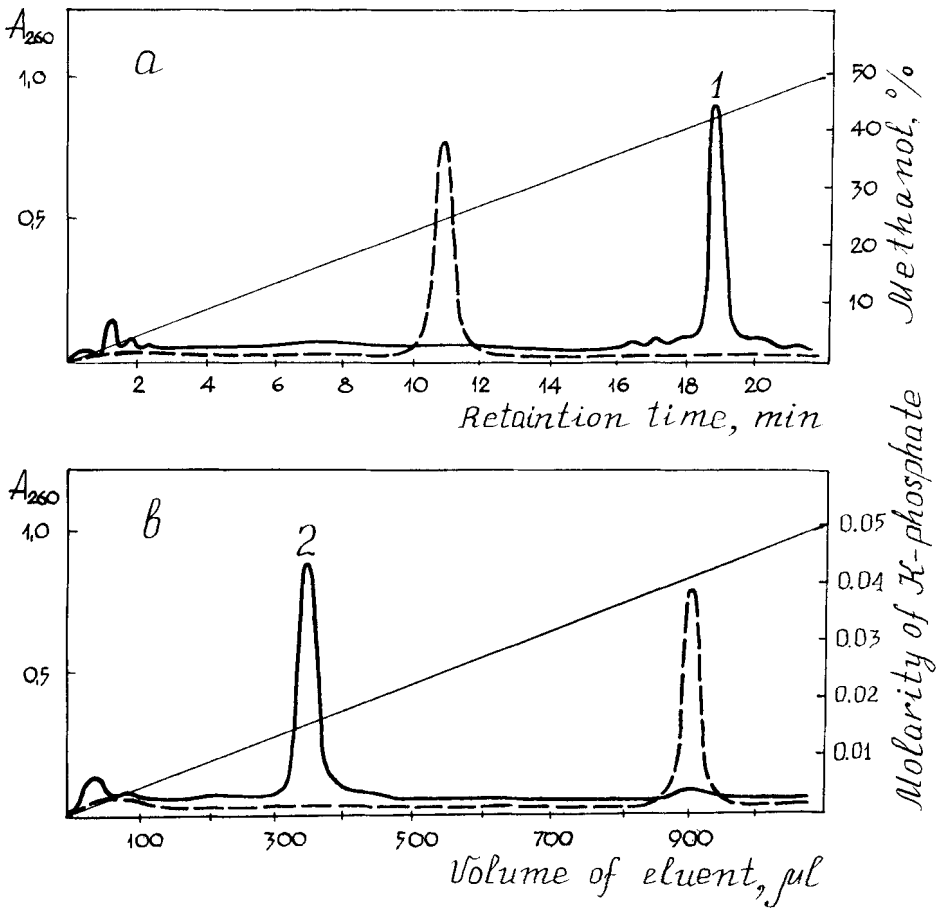


Figure 5. Chromatography of the reaction mixtures after synthesis of N-hydroxybenzotriazole ester of $(pU)_5$ on Nucleosil C18 (a) and aminoethylamide of $(pU)_5$ on Lichrosorb-NH_2 (b).
(– – –) chromatography of the starting $(pU)_5$;
(———) chromatography of the reaction mixtures,
peak 1 - N-hydroxybenzotriazole ester of $(pU)_5$,
peak 2 - aminoethylamide of $(pU)_5$.

phate of oligonucleotides was confirmed by the lack of hydrolysis of these substances by bacterial alkaline phosphatase.

CONCLUSION

In the present communication a general efficient method for modification of the phosphomonoester groups of unprotected oligonucleotides of practically any length and composition is proposed. The following modification characteristics have been found:

1) Modification proceeds at a higher rate in a wide pH interval 2-9. This is due to the nature of the carbodiimide used, containing a proton donor group.

2) The optimal pH range depends on the nature of a nucleophilic agent.

3) The strongest nucleophilic agent present in the reaction mixture is always involved in the condensation. All our data about reactivity of different nucleophilic agents allow us to arrange the investigated nucleophiles according to their ability to attack the EDC-phosphate adduct in the following order: amines, N-hydroxybenzotriazole, 2-hydroxypyridine > 4-nitrophenol > dianionphosphate > alcohol > water > monoanionphosphate. This reaction "sensitivity" to the strength of a nucleophile enables us to suggest a reaction proceeding according to the SN2 mechanism via a transition state of the trigonal bipyramid structure [19].

The developed method exhibits high selectivity of modification. Owing to the fact that internucleotide phosphates are not affected, not only 3'- and 5'-terminal phosphates of oligodeoxyribonucleotides but also 5'-terminal phosphates of oligoribonucleotides may be easily modified.

The simplicity and the one-step character of the method allow us to recommend it for derivatization of synthetic and natural DNAs and RNAs along with the method based on imidazolide intermedaites [20]. Moreover, the use of the water-soluble carbodiimide as a condensing agent in oligo- or polynucleotide chemistry seems to be more perspective, because it permits to prepare not only phosphoramidate but phosphodiester derivatives including derivatives with polymer supports [21,22].

REFERENCES

1. L.V. Mochalova, I.N. Shatsky, A.A. Bogdanov, V.D. Vasiliev, J. Mol. Biol. 159, 637-650 (1982).
2. A. Chollet, E.H. Kawashima, Nucl. Acids Res. 13, 1529-1541 (1985).

3. V.V. Vlassov, S.A. Gaidamakov, V.V. Gorn, S.A. Grachev, FEBS Lett. 182, 415-418 (1985).
4. Z.A. Shabarova, M.G. Ivanovskaya, M.G. Isaguliants, FEBS Lett. 154, 288-292 (1983).
5. G. Lancelot, U. Asseline, N.T. Thuong, C. Helene, Biochemistry 24, 2521-2529 (1985).
6. H.G,Khorana, Some Recent Development in the Chemistry of Phosphate Ester of Biological Interest, Ed. Z.A. Shabarova, Mir, Moscow, 1964, pp. 146-162.
7. O. Pongs, E. Lanka, in Methods in Enzymology, Eds. W.B. Jacoby, M. Wilchek, Academic Press, New York, 1977, 46, pp. 669-676.
8. M. Smith, H.G. Khorana, in Methods in Enzymology, Eds. S.P. Colowick, N.O. Kaplan, Academic Press, New York, 1963, 6, pp. 645-669.
9. H.G. Khorana, J. Amer. Chem. Soc. 81, 4657-4660 (1959).
10. R.K. Ralph, R.J. Young, H.G. Khorana, J. Amer. Chem. Soc. 85, 2002-2052 (1963).
11. V.F. Zarytova, V.K. Right, T.S. Chernikova, Bioorgan. Khimiya, 3, 1626-1631 (1977).
12. Abbreviations: EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; MES, 2-morpholinoethanesulfonate; MeIm, 1-methylimidazole; Dans, 5-dimethylaminonaphthalene-1--sulfonyl; DMFA, N,N'-dimethylformamide.
13. N.V. Bulychev, A.V. Lebedev, L.Z. Benimetskaya, A.L. Kozionov, Yu.E. Nesterikhin, S.Yu. Novozhilov, S.G. Raytian, M.J. Stockman, Bioorgan. Khimiya 10, 520-527 (1984).
14. J.T. Ibrahim, A. Williams, J. Am. Chem. Soc. 100, 7420-7421 (1978).
15. W.J. Rkzyzosiak, J. Biernat, J. Ciesioka, P. Gornicki, M.Wiewiorowski, Nucl. Acids Res. 7, 1663-1674 (1979).
16. G.A. Dobronravova, S.A. Zykov, A.A. Mustaev, G.A. Nevinski, Bioorgan. Khimiya 8, 1349-1357 (1982).
17. V.V. Shumyantseva, N.I. Sokolova, Z.A. Shabarova, Nucl. Acids Res. 3, 903-916 (1976).
18. D.M, Rose, G.F. Polnaszek, R.G. Bryant, Biopolymers 21, 653-664 (1982).
19. A.J. Kirby, S.G. Warren, The Organic Chemistry of Phosphorus, Ed. A.N. Pudowick, Mir, Moscow, 1971, pp. 381-387.
20. B.C.F. Chu, G.M, Wahl, L.E. Orgel, Nucl. Acids Res. 11, 6513-6529 (1983).

21. P.T. Gilham, Biochemistry 7, 2809-2813 (1968).
22. N.W.Y. Ho, R.E. Duncan, P.T. Gilham, Biochemistry 20, 64-67 (1981).

Received July 11, 1986.