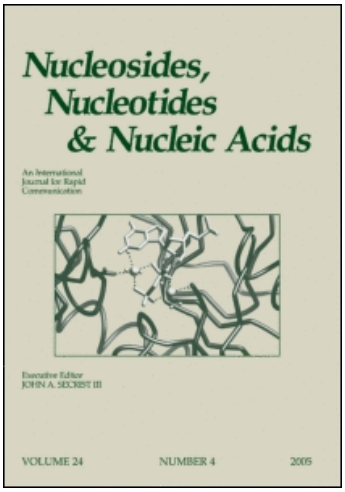

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OLIGODEOXYRIBONUCLEOTIDE-3'-PHOSPHATE SYNTHESIS BY SELECTIVE CLEAVAGE OF 3'-TERMINAL URIDINE

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Abstract. A universal method is evolved for synthesizing oligodeoxyribonucleotides containing a 3'-terminal phosphate group. This method consists of obtaining oligodeoxyribonucleotides containing a 3'-terminal uridine (incorporated by enzyme TdT-catalyzed reaction or by routine triester technique), in selective oxidation of the 3'-terminal ribose of uridine and subsequent β-elimination of the phosphate residue.

Studying modified inter-nucleotide bonds (pyrophosphate bonds in particular [1]) and oligodeoxyribonucleotide assembly into DNA-like duplexes (chemical ligation [2]) requires a universal method for synthesizing oligodeoxyribonucleotides with 3'-terminal phosphate groups. The synthesis method for oligodeoxyribonucleotide 3'-phosphates described recently in the literature [3] is based on the synthesis of oligonucleotides with 3'-terminal ribocytidine, subsequent oxidation of the cis-glycol group upon treatment by $NaIO_4$ and β-elimination of the oligonucleotide-3'-phosphate upon treatment by L-methionine. We propose to use for this purpose oligodeoxyribonucleotides containing 3'-terminal uridine. On the one hand, the application of uridine reduces one step of chemical synthesis,

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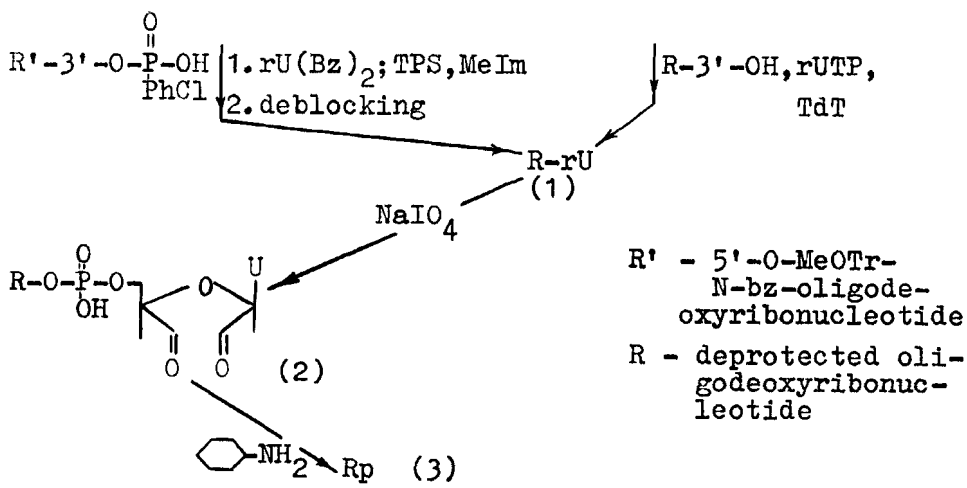
namely the blocking of NH_2-group in a heterocyclic base. On the other hand it is possible to use the terminal deoxyribonucleotidyltransferase [4] and rUTP for enzymatic addition of rU to the 3'-end of the deprotected oligodeoxyribonucleotide.

In this paper we propose simple general procedures for obtaining oligodeoxyribonucleotides with the 3'-terminal phosphate group.

RESULTS AND DISCUSSION

According to our method, the overall strategy of 3'-phosphooligodeoxyribonucleotide synthesis includes:

1. The synthesis of oligodeoxyribonucleotides containing 3'-terminal uridine;
2. The oxidative splitting of the ribose ring cis-glycol group with the formation of a dialdehyde fragment;
3. β-elimination of the oligodeoxyribonucleotide-3'-phosphate resulting in 3'-end internucleotide bond cleavage:



In this paper we describe two methods for the synthesis of an oligodeoxyribonucleotide with 3'-terminal uridine: enzymatic addition of prU to the 3'-end of the deprotected oligodeoxyribonucleotide or conventional procedures of the triester synthesis of oligodeoxyribonucleotides [5], including the application of 2',3'-O-dibenzoyluridine for introducing 3'-terminal ribose [6]. Uridine, unlike other ribonucleosides [3], excludes introduction of protective groups to heterocyclic bases.

A single ribonucleotide addition to the 3'-end of a deblocked oligodeoxyribonucleotide was studied in detail. The reaction was carried out in buffer A containing 1 M potassium cacodylate, pH 7.6; 10 mM $CoCl_2$ and 2 mM dithiothreitol [4]. One of the following synthetic deprotected oligodeoxyribonucleotides d(ACTCTGTTC) (I), d(CCTGGAATT) (II), d(CACTATCAATAAGT) (III) and one of the four ribonucleoside-5'-triphosphates were substrates in the terminal deoxyribonucleotidyltransferase (TdT) - catalyzed reaction. Taken in an equimolar amount to the oligonucleotide, rUTP or rCTP revealed a single ribonucleotide incorporation into the 3'-end of the oligodeoxyribonucleotide, yielding 80-85% of the desired product. Purine ribonucleoside-5'-triphosphates formed multiple rather than single addition products. Thus, in the presense of rGTP, compound I forms d(ACTCTGTTC)rG (50%) and d(ACTCTGTTC)rG_2 (40%). So the conditions chosen for a single ribonucleotide addition were as follows: the reaction mixture (V_{total} = 10 µl) contained buffer A: 1.5×10^{-4}M oligodeoxyribonucleotide; $1,5 \times 10^{-4}$M rUTP; 25 u.a. * TdT.

The optimal conditions for ribose oxidation and cleavage were assayed on the model compound dTprU. In fact, the oxidation of dTprU (0.013 M) by sodium periodate [7] (0.025

M) is completed in 30 minutes at room temperature (TLC - control, system B).

The β-elimination reaction (the cleavage of the $C^{5'}$-O bond in compound 2, see scheme) was carried out in the presence of various amines[8]. The results are summarized in TABLE 1.

As seen from TABLE 1, complete cleavage of the $C^{5'}$-O bond in compound 2 (see scheme) was achieved by using 0.3 M aqueous benzylamine or 0.3 M aqueous cyclohexylamine for 1 hr. at 20°C. In further experiments the treatment by 0.3 M aqueous cyclohexylamine was used for the β-elimination reaction.

TABLE 1.

Conditions for the β-elimination reaction in oxidized dinucleotide dTprU (1.3×10^{-2} M) upon treatment with amines

Amine, M	pKa	pH	Time (min)	Temperature, °C	Cleavage %
Aniline 0.033	4.63	5.4	30	90	80
Piperidine 0.05-2.0	11.22	8.5	30	90	50
Benzylamine 0.3	9.33	9.0	60	20	100
Cyclohexylamine 0.3	10.66	10.0	60	20	100

*One unit is the enzyme activity that catalyzes the incorporation of 1 nmol of dTMP into acid-precipitable fraction within 60 min under assay conditions.

Thus, the synthesis of oligodeoxyribonucleotides with 3'-terminal phosphate groups from oligodeoxyribonucleotide-3'-rU amounts to a consecutive treatment of the oligonucleotide with a 25 mM solution of $NaIO_4$ (0.5 hr;, 20°C) and 0.3 M solution of cyclohexylamine (1 hr., 20°C).

This method was successfully applied for synthesizing some oligodeoxyribonucleotide-3'-phosphates (see TABLE 2).

Reaction mixture analysis and isolation of oligodeoxyribonucleotide-3'-phosphates were achieved by ion-exchange HPLC, with Lichrosorb-NH_2 as support.

FIGURES 1 and 2 show a chromatographic elution profile obtained from an aliquot after(FIG. 1)rUMP incorporation into the 3'-end of oligodeoxyribonucleotide I and (FIG. 2) selective removal of the 3'-ribose moiety from d(ACGGA)rU.

TABLE 2.

Synthesized oligodeoxyribonucleotide-3'-phosphates

Oligonucleotide-3'-phosphate	Yield (%)	Quantity (μmol)
A - via triester method		
d(ACGGAp)	85	0.35
d(CACTATCAp)	75	0.50
d(TCGACCATAAAAp)	75	1.0
B - via TdT-catalyzed rU-addition		
d(ACTCTGTTCp)	80	0.1
d(CACTATCAATAAGTp)	80	0.1
d(TGGAGTCATTACCGp)	75	0.15

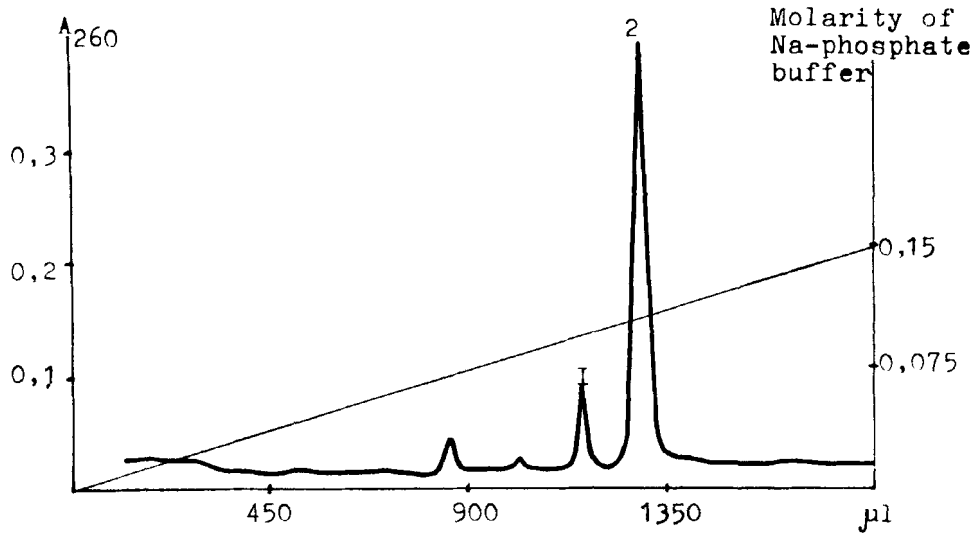


FIG. 1. Chromatography on Lichrosorb-NH_2 (in a sodium phosphate concentration gradient 0 →0.15 M, pH 7.0, in 7 M urea) of the reaction mixture obtained after rU-incorporation by TdT-catalyzed reaction into the 3'-end of d(ACTCTGTTC).
I. d(ACTCTGTTC)
2. d(ACTCTGTTC)rU.

The presence of the 3'-phosphate group in synthesized oligodeoxyribonucleotide-3'-phosphates was confirmed by enzymatic dephosphorylation. The dephosphorylated oligonucleotides formed in hydrolysis are readily separated from oligonucleotide-3'-phosphates by ion-exchange chromatography due to a lower total charge. Synthesized oligodeoxyribonucleotide-3'-phosphates were completely degradable by the simultaneous action of alkaline phosphatase and snake venom phosphodiesterase [9].

The developed precedure was applied for introduction of the ^{32}P -3'-terminal phosphate group into oligodeoxyribonuc-

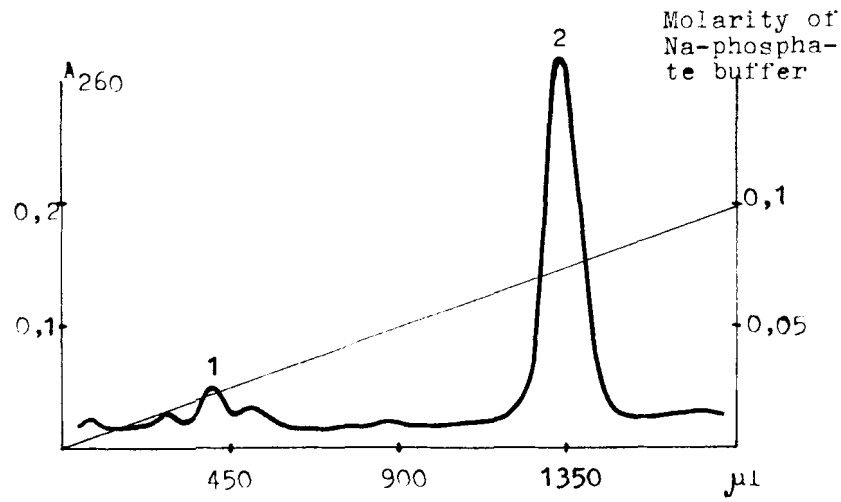


FIG. 2. Chromatography on Lichrosorb-NH_2 (in a sodium-phosphate concentration gradient 0 → 0.1 M, pH 7.0, in 7 M urea) of the reaction mixture obtained after d(ACGGA)rU cleavage.
1. d(ACGGAp)rU;
2. d(ACGGA)p.

leotides. It is in this way that ^{32}P-d(CCTGGAATT$\overset{*}{p}$) was obtained. The identity of the compound synthesized was confirmed by polyacrylamide gel electrophoresis.

The method developed and described in this paper can be easily applied for rapid and effective synthesis of oligodeoxyribonucleotide containing 3'-terminal rU (either by the enzyme TdT-catalyzed reaction of the oligodeoxyribonucleotide with rUTP or by the routine triester technique). Subsequent chemical cleavage by $NaIO_4$/amine treatment produced oligodeoxyribonucleotide-3'-phosphates obtained in good yields. The method can be readily applied to the synthesis of oligodeoxyribonucleotide-3'-phosphates despite the length and the primary structure of the starting oligodeoxy-

ribonucleotides. Another possibility is the synthesis of an oligodeoxyribonucleotide, containing the ^{32}P -3'-terminal labelled phosphate group.

EXPERIMENTAL

General. Used in the work were: deoxyribonucleotides, alkaline phosphatase (EC 3.1.3.1), snake venom phosphodiesterase (EC 3.1.4.1), deoxynucleotidyltransferase from calf thymus (EC 2.7.7.31) (Novosibirsk, USSR); 1,3,5-triisopropylbenzenesylphonyl chloride (TPS), N-methylimidazole (Merck, FRG), rATP, rCTP, rGTP, rUTP (Serva, FRG).

Paper chromatography on FN-1 (Filtrak, GDR) and thin-layer chromatography (TLC) on cellulose disks (Eastman Kodak, USA) were performed in the following systems of solvents: A - ethanol + 1 M ammonium acetate, pH 7.5 (7:3 v/v); B - propanol + ammonia + Water (55:10:35 v/v); TLC was performed on kieselgel 60 (Merck, FRG) in a system C-chloroform-ethanol (9:1 v/v).

High performance liquid chromatography (HPLC) was carried out by using "Millichrom" (Novosibirsk, USSR) equipped with a Lichrosorb-NH_2 ("Merck", FRG) column (1x30 mm, 5 μm) in a sodium-phosphate linear gradient (0 → 0.2 M), pH 7.0 in 7 M urea.

Hydrolysis of the oligonucleotides by alkalinephosphatase from E.coli. 0.1-0.5 o.u. of the oligonucleotide was dissolved in 10 μl of distilled water, and 0.1 u.a./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 was added. The hydrolysis products were loaded on a column (1x30 mm) with Lichrosorb-NH_2 and were separated in a linear Na-phosphate buffer concentration gradient (0 → 0.12 M), pH 7.0, in 7 M urea.

Hydrolysis of oligodeoxyribonucleotides by snake venom phosphodiesterase was carried out according to Khorana[9].

Synthesis of protected mononucleotides. N-Benzoylnucleosides were obtained by benzoyl chloride acting on nucleosides [5] with subsequent alkaline hydrolysis, and also by using trimethylchlorosilane[10] as an intermediate protector of nucleoside hydroxyl groups. The completely protected nucleoside-3'-phosphates were synthesized by a consecutive treatment of 5'-O-, N-protected nucleosides with p-chlorophenyl phosphobistriazolide and ethylenecyanohydrin. Removal of the monomethoxytrityl group was performed by a 10% solution of trichloracetic acid in chloroform at 0°C. The cyanoethyl group was removed by treatment with triethylamine-water-pyridine (1:1:3 v/v) for 15-20 min [5].

Overall procedures for internucleotide condensation. A mixture of 1.0 mmol nucleotide and 0.8 mmol nucleoside components was dried by three-fold evaporation in the presence of absolute pyridine. The final evaporation was performed at a volume when the total concentration of the components, taking into account the N-methylimidazole added subsequently should make up 0.1 M. Then the reaction mixture was treated with 3 mmol of TPS and incubated at room temperature for 10-30 min. (TLC-control, System C); it was treated according to the method already described [5]. The end product was isolated by column chromatography on silicagel in an ethanol gradient concentration in chloroform (0-10%). In accordance with these procedures, dTprU; d[(MeOTr)bzApbzCpbzGpbzGpbzAp]-rU(Bz)$_2$; d[(MeOTr)bzCpbzApbzCpTpbzApTpbzCpbzAp]rU(Bz)$_2$; d[(MeOTr)TpbzCpbzGpbzApbzCpbzCpbzApTpbzApbzApbzApbzAp]rU(Bz)$_2$ were synthesized with the corresponding yields of 90%, 80%, 70% and 65%. Z = p-chlorophenyl.

3'-terminal ribose residue addition to oligodeoxyribonucleotides with Terminal Transferase and rUTP. The reaction mixture (10 μl) contained 1 M Potassium cacodylate, pH 7.6, 10 mM $CoCl_2$, 2 mM dithiothreitol, 1.5×10^{-4} M oligodeoxyribonucleotide, 1.5×10^{-4} - 1.5×10^{-3} M rUTP and 10-25 u.a./10 μl TdT. After incubation for 1 hr. at 37°C the reaction was terminated by adding 5% trichloroacetic acid. The mixture was deproteinated by chloroform - i-AmOH extraction (24:1 v/v, 3x10 μl). The reaction mixture was applied onto a Silasorb C-18 column (3x5 mm) and washed (5% MeOH, 100 μl). The product was eluted by 30% MeOH (300 μl).

Synthesis of the oligodeoxyribonucleotide-3'-phosphate via ribose cleavage. The oligonucleotide with 3'-terminal rU was dissolved in water (a final concentration of 2-25 mM oligonucleotide was used) and supplemented with 0.1 M solution of $NaIO_4$ (a final concentration of 25 mM $NaIO_4$). The reaction mixture was incubated at room temperature in darkness for 0.5 hr. A 10% ethyleneglycol solution in water was added, and allowed to stand for 15 min. at room temperature. Then a 1 M cyclohexylamine solution in water was added (a final concentration of 0.3 M cyclohexylamine) and the reaction was carried out at room temperature for 1 hr. Immediately after that the oligodeoxyribonucleotide-3'-phosphate was isolated as described below.

Overall procedures of the oligodeoxyribonucleotide-3'-phosphate isolation. For di- and trinucleotide cleavage, the reaction mixture was separated by paper chromatography in system A. In the case of dN(Nn)p, where $n > 3$, the reaction mixture (24 μl) was loaded on a column (1x30 mm) with Lichrosorb-NH_2, and washed with 175 μl of 7 M urea; chromatography was performed in a sodium phosphate concentrati-

on gradient (0-0.2 M), pH 7.0, in 7 M urea. The volume of the eluate: 1800 μl, the rate - 50 μl/min.

Fractions containing oligodeoxyribonucleotide-3'-phosphate were applied onto a Silasorb C-18 column(3x10 mm) and washed with water (500 μl). The desalted product was eluted by 30% MeOH (500 μl).

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