5'-PHOSPHOURIDINE AS A SOURCE OF TERMINAL PHOSPHATE GROUPS IN OLIGONUCLEOTIDES

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A universal key component is proposed for the preparation of oligonucleotides with 3'- and 5'-terminal phosphate groups — 2',3'-dibenzoyluridin-5'-yl (4-chlorophenyl-phosphate) (pU(Bz)$_2$), which is a potential source of the phosphate group. The condensation of pU(Bz)$_2$ with the 5'-OH or the 3'-OH group of a protected oligonucleotide leads to the formation of oligodeoxyribonucleotides with 5'- or 3'-terminal uridine, respectively. The oxidation of the 2',3'-cis-glycol group of the terminal uridine unit followed by β-elimination forms oligodeoxyribonucleotides with terminal phosphate groups.

In one of the stages, the synthesis of extended DNA duplexes includes the assembly of oligodeoxyribonucleotides containing terminal phosphate groups [1-3]. In the present paper a universal key component for obtaining oligonucleotides with 3'- or 5'-terminal phosphate groups is proposed — 2',3'-O-dibenzoyluridin-5'-yl (4-chlorophenyl phosphate) (pU(Bz)$_2$). which is a potential source of the phosphate group. The condensation of pU(Bz)$_2$ with the 5'-OH or the 3'-OH group of the protected oligonucleotide leads to oligodeoxyribonucleotides with 5'- and 3'-terminal uridine residues, respectively. The oxidation of the 2',3'-cis-glycol group of the terminal uridine unit followed by β-elimination [4] forms oligodeoxyribonucleotides with terminal phosphate groups.

At the present time, in oligonucleotide synthesis, ribonucleosides are used as terminal protective units potentially bearing a phosphate grouping [5]. However, the protective groups of such ribonucleotides proposed previously [4, 5] are different for introduction into the 5'- and 3'-positions of an oligodeoxyribonucleotide chain. This complicates synthesis and increases the number of initial protected monomers, particularly in the preparation of oligonucleotide 3',5'-diphosphates.

In the work of J. G. Nadeau et al. [5], 2',3'-O-di(4,4'-dimethoxytrityl)uridin-5'-yl (4-chlorophenyl phosphate) (pU(DMeOTr)$_2$) was used for obtaining 5'-phosphorylated oligonucleotides. However, this compound cannot be used in the preparation of a 3'-phosphorylated oligonucleotides, since, in the triester method of synthesis, before the growth of the next block the elimination of the 5'-terminal acid-labile group in the nucleoside component, which includes pU(DMeOTr)$_2$ is carried out, which leads to the deblocking of the 2',3'-hydroxy groups of the uridine. In the synthesis of oligonucleotide 3'-phosphates using the standard triester method [6], 2',3'-di-O-benzoyluridine is used as the terminal block [4]. In order to standardize the method of obtaining oligonucleotides with terminal phosphate groups it is proposed to use 2',3'-di-O-benzoyluridine-5'-yl (4-chlorophenyl phosphate), obtained by the following scheme:

$$\underset{\text{I}}{\text{U}} \xrightarrow{\text{MeOTrCl}} \underset{\text{II}}{(\text{MeOTr})\text{U}} \xrightarrow{(C_6H_5CO)_2O} \underset{\text{III}}{(\text{MeOTr})\text{U}(\text{Bz})_2} \xrightarrow{H^+} \underset{\text{IV}}{\text{U}(\text{Bz})_2} \xrightarrow[2.\,HOCH_2CH_2CN]{1.\,ClC_6H_5O\overset{O}{\overset{\|}{P}}(Trl)_2} (\text{ClPh, CNEt})\,\underset{\text{V}}{\text{pU}(\text{Bz})_2} \xrightarrow{(C_2H_5)_3N} \underset{\text{VI}}{(\text{ClPh})\text{pU}(\text{Bz})_2}$$

Tri — 1, 2, 4-triazolide.

It must be pointed out that the benzoyl groupings in (V) are not eliminated under the conditions for eliminating the cyanoethyl group (pyridine—triethylamine—water (3:1:1)); this was confirmed by the resistance of the compound obtained to oxidation by $NaIO_4$. On the condensation of pU(Bz)$_2$ with a 5'-O-monomethoxytrityl-N-benzoylnucleoside, a 3'-terminal

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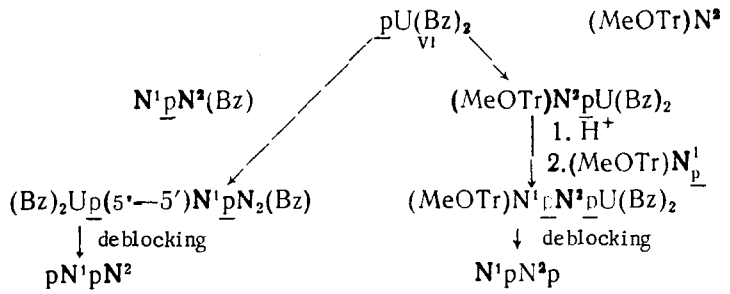
block is obtained that is a potential source of a 3'-terminal phosphate group. The interaction of pU(Bz)$_2$ with a 5'-hydroxy-N-benzoylnucleosid(oligonucleotid)-3'-yl (4-chlorophenyl β-cyanoethyl phosphate) leads to an oligonucleotide with 5'-terminal uridine. As a result of this reaction, a 5'–5'-phosphodiester bond is formed, and the block obtained in this way can be considered as a source of a 5'-terminal phosphate group:



N^1, N^2 — are deoxyribonucleosides protected at the amino groups

The yields of protected oligonucleotides obtained by the proposed scheme are given below:

Oligonucleotide	Yield, %
(MeOTr) bzdApdTprU $(Bz)_2$	80
(MeOTr) dTprU $(Bz)_2$	60
(MeOTr)dbzGpdTpbzdApdTprU $(Bz)_2$	80
2', 3'-$(Bz)_2$rU5'p5'bzdGp (ClPh, CNEt)	70
2', 3'-$(Bz)_2$rU5'p5'bzdGpdTpbzdApdTprU $(Bz)_2$	70
2', 3'-$(Bz)_2$rU5'p5'bzdGpdTpbzdApdTpbzdGpbzdCpbzdC (Bz)	60
(MeOTr) dTpbzdGpdTpbzdGpdTpbzdApdTprU $(Bz)_2$	70

The synthesis of protected oligodeoxyribonucleotides with 3'- or 5'-terminal uridine by the proposed procedure can be performed in the triester variant both in solution and on a polymeric support. After the elimination of all the protective groupings the oligonucleotides containing terminal uridine units were isolated by HPLC. The selective splitting out of the iridine was performed with 0.025 M sodium periodate followed by β-elimination in the presence of cyclohexylamine [4]. The presence of a 3'- (or 5'-)-phosphate group was confirmed by analysis of the products of the hydrolysis of the oligonucleotides by alkaline phosphatase. The oligodeoxyribonucleotides obtained containing 5'- or 3'-terminal phosphate groups — pd(GTATGCC), d(GTAT)p, and d(TGTGTAT)p — were used in chemical ligation; the oligonucleotide containing 3',5'-diphosphate groups — pd(GTAT)p — was used as the substrate for T4 RNA ligase (phosphate group donor).

EXPERIMENTAL

We used deoxyribonocleosides produced by the Omutninsk chemical factory, and uridine from Reanal (Hungary).

Thin-layer chromatography (TLC) was performed on plates coated with silica gel (Eastman Kodak) in the chloroform–ethanol (9:1) system and column chromatography in a column (4 × 6 cm) containing silica gel 40/100 μ (Chemapol), Czechoslovakia).

HPLC was performed on a Tracor chromatograph (Holland):

a) ion-exchange variant — on a column (4.6 × 250 mm) with Polisil SA (5 μ) in a linear concentration gradient of KH_2PO_4 (0-0.3 M), pH 6.5, in 30% MeOH at a rate of elution of 1.5 ml/min;

b) reversed-phase variant — on a column (4.6 × 250 mm) with Zorbax C8 (5 μ) in a linear concentration gradient of methanol (0-35%) in 0.1 M ammonium acetate at a rate of elution of 1 ml/min, 40°C.

Hydrolysis of Oligonucleotides with Terminal Phosphate Groups by *E. coli* Alkaline Phosphatase. A solution of 0.1-0.5 OU of an oligonucleotide in 10 μl 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 mixture was kept at 37°C for 1.5 h and was then separated by HPLC (ion-exchange variant).

The synthesis of the 5'-monomethoxytrityl-N-benzoylnucleosid-3'-yl (4-chlorophenyl β-cyanoethyl phosphate) was carried out by a standard procedure [6].

Synthesis of 2',3'-O-Dibenzoyluridine. Uridine was converted into 5'-monomethoxytrityl-2',3'-O-dibenzoyluridine as described in [7]; yield 80%. The monomethoxytrityl protection was removed from the 5'-hydroxy group with a 20% solution of trichloroacetic acid in chloroform [6]. The product was isolated by chromatography on silica gel in a linear gradient of concentrations of methanol in chloroform (0-10%).

Synthesis of 2',3'-O-Dibenzoyluridin-5'-yl (4-Chlorophenyl β-Cyanoethyl Phosphate). To prepare the phosphorylating agent, 240 mg (3.48 mmole) of triazole and 0.4 ml (2.88 mmole) of absolute triethylamine were dissolved in 4 ml of absolute dioxane. To the resulting solution was added 0.24 ml (1.5 mmole) of p-chlorophenyl phosphorodichloridate and the mixture was kept for 20 min. The precipitate that had deposited was filtered off rapidly, and the filtrate was added to a solution of 454 mg (1 mmole) of 2',3'-dibenzoyluridine in 10 ml of absolute pyridine. The reaction mixture was kept for 1 h. After the end of the reaction (monitoring by TLC in the chloroform–ethanol (95:5) system), 0.35 ml (5 mmole) of β-cyanoethanol was added. The reaction time was 1.5 h. The mixture was decomposed with water and was worked up by a standard method [6]. The desired substance was isolated by column chromatography on silica gel in a linear gradient of ethanol in chloroform (0-5%). The yield of $pU(Bz)_2$ was 60%.

Elimination of the β-Cyanoethyl Protection from the Terminal Phosphate Group. A solution of 698 mg (1 mmole) of 2',3'-di-O-benzoyluridin-5'-yl (4-chlorophenyl β-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 in the chloroform–ethanol (9:1) system). The further treatment was as described in [6]. Internucleotide condensation was carried out by a standard method [7].

Synthesis of Oligonucleotide Blocks Containing Ribouridine at the 3'- or the 5'-End (General Procedure). A mixture of 1.0 mmole of $pU(Bz)_2$ and 0.8 mmole of the component donating the hydroxy group was carefully dried by evaporation with absolute pyridine (5 × 5 ml). Then 6 mmole of N-methylimidazole and 3 mmole of TPS (concentration of oligonucleotide 0.1 M) were added, and the reaction mixture was kept at 20°C for 10-15 min (monitoring by TLC). The mixture was decomposed and worked up as in [7]. The desired product was isolated by column chromatography on silica gel in a concentration gradient of ethanol in chloroform (0-10%). The yields after column chromatography amounted to 60-65%.

General Procedure for Splitting Out the Terminal Ribose Unit. An oligonucleotide with a terminal (3' or 5'-)-uridine residue was dissolved in water (final concentration 2-25 mM of oligonucleotide), and a 0.1 M solution of $NaIO_4$ was added (the final concentration of $NaIO_4$ being 25 mM). The reaction mixture was kept at room temperature for 0.5 h. Then ethylene glycol was added (the final concentration of ethylene glycol being 10%), the mixture was kept at room temperature for 15 min, and 1 M cyclohexylamine was added (final concentration of cyclohexylamine being 0.3 M). The new mixture was kept at room temperature for 1.5 h. The oligonucleotides containing 3'- (or 5'-)-terminal phosphate groups were isolated by HPLC (ion-exchange variant. The yields of oligodeoxyribonucleotid-3'(5')-yl phosphates were 75-95%.

CONCLUSIONS

1. A universal component has been proposed for obtaining oligodeoxyribonucleotid-3'(5')-yl phosphates.

2. A number of oligodeoxyribonucleotides containing 3'- or 5'-terminal phosphate groups has been synthesized with the use of $pU(Bz)_2$.

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HYDROGENOLYSIS OF RICE HUSK PROTOLIGNIN. II

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It has been established that the partial hydrogenolysis of rice husk protolignin (RHP) takes place at a temperature of 180°C and an initial hydrogen pressure of 10 atm in an alkaline medium. Conditions have been selected under which the greatest yield of low-molecular-weight products (91% on the Komarov lignin) is obtained: hydrogenolysis in an alkaline medium in the presence of an anthraquinone (AQ) catalyst. The addition of AQ increases the yield of low-molecular-weight products by a factor of 1.8. Semiempirical formulas have been derived for incompletely hydrolyzed lignin residues. A study of the molecular-weight distribution of these lignins has shown that they are polydisperse. It has been established that in the process of hydrogenolysis AQ promotes the demethoxylation of structural units with syringyl nuclei.

It is known that the presence of anthraquinone (AQ) in the alkaline hydrolysis of lignin intensifies the breakdown of the macromolecule to low-molecular-weight products [1, 2]. By catalyzing the cleavage of β-alkyl aryl ether bonds, AQ, in addition, is capable of interacting with certain structural fragments of the lignin (of the p-hydroxybenzyl alcohol type), thereby decreasing the process of condensation. However, in this case it behaves not as a catalyst but as a reagent that is consumed in the course of the reaction [3].

The presence of oxygen lowers the concentration of active reduced forms of AQ and has an adverse effect on the course of delignification [1]. Therefore, by excluding the action of oxygen and performing the reaction in hydrogen which, by exerting a reducing action, also prevents condensation, one may expect an increase in the yield of low-molecular-weight products [4]. A number of investigations devoted to the hydrogenolysis of lignins have been reported in the literature. Some of them were directed to studying the structure of lignin while others had an applied nature — the preparation of the products of the degradation of lignin that may be a source of liquid fuel, chemical reagents, biologically active substances, etc. [5].

The aim of the present work was to study the alkaline hydrogenolysis of rice husk lignin in the presence of AQ and without it in hydrogen in order to obtain information on the structure of this lignin and to reveal biologically active substances among the combined products.

The results obtained when the reaction was performed in an atmosphere of hydrogen in the presence of AQ (I) were compared with those of alkaline hydrolysis without AQ in hydrogen (II) and without AQ in air (III). The yields of low-molecular-weight lignin degradation products are given below (% on the Komarov lignin).

Conditions of extraction	Reaction conditions		
	I	*II*	*III*
pH 8, ethereal extract	34.1	19.5	8.3
pH 2, ethereal extract	26.5	27.9	34.5
pH 2, ethyl acetate extract	30.8	15.2	8.3
Total low-molecular-weight products	91.4	62.6	51.1

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