SOLID-PHASE TRIESTER SYNTHESIS OF MODIFIED TRIURIDYLATES USING AN EFFECTIVE CONDENSING AGENT

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Triuridylates containing 2'-deoxyuridine, 5-bromouridine, and 6-azauridine in various positions of the sequence have been synthesized by the use of an O-nucleophilic nucleotide condensation catalyst — 4-ethoxypyridine N-oxide — under the conditions of the solid-phase triester method.

Modified oligoribonucleotides are of interest as model codons in the study of the structural-functional organization of the ternary translation complex in cell-free protein-synthesizing systems [1, 2]. By virtue of the structural features of the sugar phosphate backbone, triuridylates containing 2'-deoxyuridine in various positions of the chain can be used in experiments to evaluate the contributions of individual nucleotides to the stabilization of complexes; codon—anticodon or codon—ribosome. In view of the spatial structure of 5-bromuridine (U^{Br}) and of 6-azauridine (aU), it could be assumed that oligoribonucleotides modified by residues of these compounds will also find use as model codons in the study of translation mechanisms.

The synthesis of triuridylates containing 2'-deoxyuridine and 5-bromouridine residues by the triester method in solution is associated with a number of difficulties in the isolation of the products at each state [3]. The solid-phase variant of nucleotide condensation is distinguished by numerous advantages in the preparation of even short trinucleotides (a decrease in the time of growth of the oligomer chain by eliminating chromatographic purifications in the various stages). Moreover, by the solid-phase method the syntheses of a number of combinations of triuridylates on a polymeric support with one and the same immobilized nucleoside becomes possible.

The use of a mixture of triisopropylbenzenesulfonyl chloride (TPS) and N-methylimidazole (MeIm) as condensing agent in the synthesis of oligodeoxynucleotides substantially shortens the time of condensation (15-20 min) and, with a successful choice of reaction conditions, almost completely excludes the side reactions of sulfonylation of the nucleosides [4, 5].

In nucleotide condensations with a 3'-P ribocomponent in the presence of TPS and MeIm an increase in the time of formation of the phosphotriester (25-30 min) was observed which led to a considerable modification of the ribonucleotide material [3]. Consequently, the rate of the main process depends directly on the ease of activation of of the 3'-O-phosphodiester grouping on its steric screening by the voluminous 2'-O-tetrahydropyranyl substituent.

In a number of syntheses of deoxynucleotides, including the solid-phase method, the efficacy of the use of a mixture of TPS and 4-substituted pyridine N-oxidine N-oxides (EPNOs) as condensing agents has been shown [8]. When the mechanism of nucleotide condensation with the participation of 4-substituted pyridine N-oxides and their low basicities (pK_a EPNO ~ 2) [6] are taken into consideration, it might be expected that with a decrease in the time of condensation the side reaction of the sulfonylation of the oligoribonucleotides will be insignificant.

The initial supports for the solid-phase synthesis of tryuridylates were obtained from Silochrome C-250 (S_1) and CPG-170 porous glass (S_2), the grafting-on of the anchoring succinate (Suc) groups and of the 5'-O-dimethoxytritylnucleosides being performed by a known procedure [9]. The free hydroxy groups of the ribonucleosides were blocked by acetylation.

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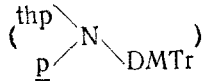
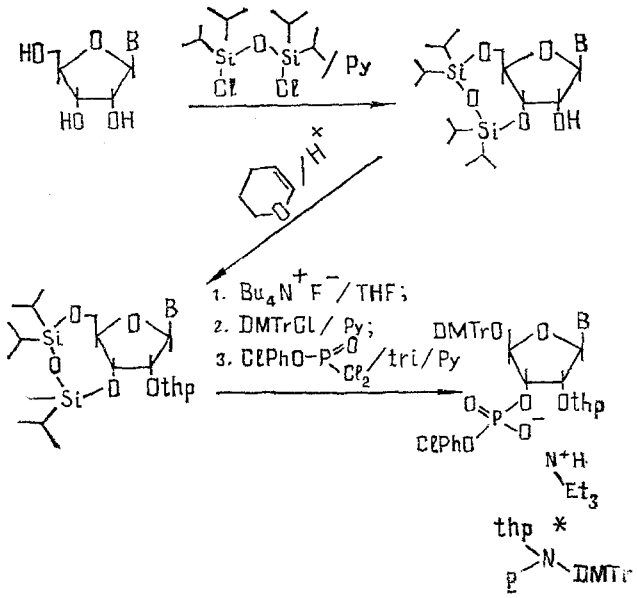
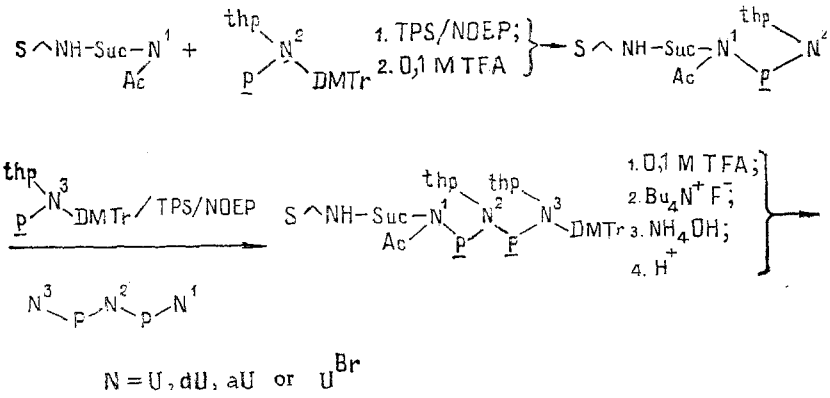
5'-O-Dimethoxytrityl-2'-O-tetrahydropyranylribonucleoside 3'-(p-chlorophenyl phosphate)s () were obtained according to developed methods [10, 11] using tetraisopropyldisiloxane protection for the selective blocking of the 3',5'-hydroxyls of the ribofuranose residue (scheme 1).



Scheme 1. B — U, aU, A^{Bz}, G^{iBu}; thp — tetrahydropyranyl group; p — p-chlorophenyl phosphate group; $Bu_4N^+F^-$ — tetrabutylammonium fluoride; tri — 1, 2, 4-triazole; * — triethylammonium salts of 5'-O-methoxy-2'-O-tetrahydropyranylribonucleoside 3'-(p-chlorophenylphosphate)s.

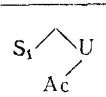
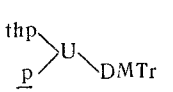
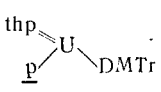
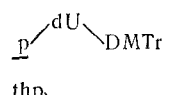
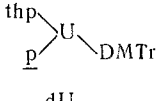
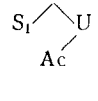
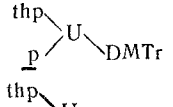
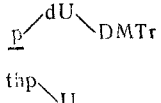
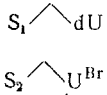
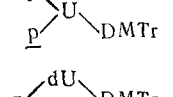
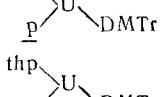
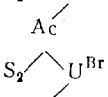
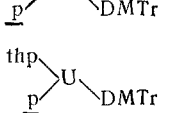
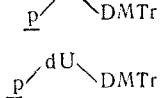
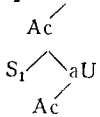
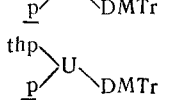
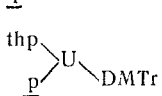
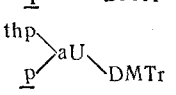
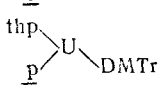
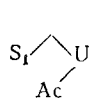
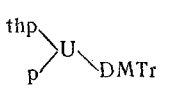
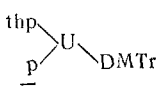
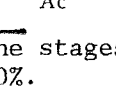
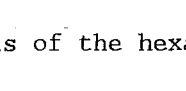
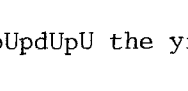
A cycle of growth of the nucleotide chain consisted of the operations of condensation, eliminating the dimethoxytrityl protection, and intermediate numerous washings of the polymeric support (Scheme 2). The amount of P-component grafted on in each stage was determined spectrophotometrically from the $(MeO)_2Tr$ cation split out.



Scheme 2.

Model experiments on the synthesis of a protected diuridylate on support S_1 with immobilized uridine showed that a ratio of the reacting substances 3'-P-component:TPS:EPNO = 1:2:4 is the optimum and the yield of dinucleoside phosphotriester amounts to 76-78% (10-fold excess of 3'-component to immobilized nucleoside). A decrease or an increase in the amount of TPS and/or EPNO lowered the yield in the first case and was ineffective in the second case. The time of condensation with the participation EPNO, unlike the catalyst based on MeIm used earlier [12], was shortened to 3-4 min.

TABLE 1. Degrees of Conversion in the Stages of the Synthesis of Modified Triuridylates*

Modified support	Capacity of the support, μmole of nucleoside/g	Grafting of nucleotide I			Grafting of nucleotide II			5-3-Trinucleotide obtained
		initial nucleotide	yield		initial nucleotide	yield		
			μmole/g	%		μmole/g	%	
	71		53	76		47	90	UpUpU
	71		42	60		37	82	UpdUpU
	71		53	76		45	90	dUpUpU
	90		51	68		42	81	UpUpdU
	86		60	70		52	86	$UpdUpU^{Br}$
	86		43	50		42	98	$dUpUpU^{Br}$
	86		40	47		30	75	UpUpaU
	71		44	62		38	83	UpaUpU
	71		38	57		20	53	aUpUpU

*In the stages of the synthesis of the hexanucleotide ApUpGpUpdUpU the yields were 89, 64, 76, 82, and 60%.

The lowered yield of dinucleoside phosphotriesters observed in the first stage of the synthesis of the modified triuridylates (Table 1) is apparently explained by features of the spatial structure of the support and of the protected 3'-ribonucleotide and is in harmony with literature results [9, 13].

The splitting out of the 5'-O-dimethoxytrityl group with 0.1 M trifluoroacetic acid (TFA) after the first grafting may be accompanied by partial deblocking of the 2'-O-tetrahydropyranyl group. The absence of 2'-O-protection in the second grafted nucleoside on subsequent nucleotide condensation could lead to the formation of a branched tetranucleotide with 3'—5' and 2'—3' internucleotide bonds. No fragments with an unnatural internucleotide bond were detected by microcolumn HPLC of the products of complete enzymatic hydrolysis of the triuridylates obtained.

After the elimination of the P- and O-protective groups and the splitting of the trinucleotide from the support, it was purified with the aid of ion-exchange and reversed-phase chromatography. The modified triuridylates were isolated in amounts of 10-40 OU_{260}.

EXPERIMENTAL

We used ribonucleosides, 2,4,6-triisopropylbenzenesulfonyl chloride, and Silochrome C-250 silica gel from the Biolar Scientific Production Combine, Olaine; 5-bromouridine, N-methylimidazole, 1,2,4-triazole, 3,4-dihydro-2H-pyran, and CPG-170 porous glass from Fluka. 6-Azauridine was obtained by method [14] and 2'-deoxyuridine from uridine in acordance with the procedure of [15]. Tetraisopropyldisiloxane dichloride, p-chlorophenyl phosphorodichloridate, 4-ethoxypyridine N-oxide, and tetrabutylammonium fluoride were obtained by the procedures of [16, 17, 8, and 18], respectively.

Absolute solvents — pyridine, tetrahydrofuran, dioxane, chloroform, methylene chloride, and dichloroethane — were prepared as recommended in [17].

The protected ribonucleosides and 2-deoxyuridine were phosphorylated with the aid of p-chlorophenyl phosphorobistriazolidate under the conditions described in [1].

HPLC was conducted on a chromatographic system assembled from domestic instruments (Nauchpribor Industrial Association, Orel) in 0.4 × 25 cm columns; reversed-phase chromatography on Silasorb-C_{18} (7 μm) (Chemapol) that had been treated with chlorotrimethylsilane, and microcolumn chromatography on a Milikhrom instrument in columns (2 × 62 mm) with Silasorb-C_{18} (5 μm) and Silasorb-NH_2 (7 μm). Ion-exchange chromatography was performed on 0.8 × 18 cm columns of Toyopearl DEAE-650 (Toyo Soda).

UV spectra were recorded on a Specord UV-Vis spectrometer.

General Procedure for Performing Internucleotide Condensation. A flow-through reactor was charged with 100 mg of support containing 71 μmole/g grafted nucleoside. The support was first dried by three evaporations with pyridine. Then it was mixed with 70 μmole of protected 3'nucleotide, 140 μmole of TPS, and 280 μmole of EPNO that had all been dried in vacuum, and 2 ml of methylene chloride was added. The reaction mixture was shaken for 3-4 min, and then 2 ml of ethanol was added, and after 5 min the solvent was eliminated by filtration. The support was washed successively (2 × 5 ml) with methylene chloride, dimethylformamide, ethanol, and methylene chloride.

The elimination of the dimethoxytrityl group with 0.1 M TFA in dichloroethane (2 × 1 ml) was performed each time for 5 sec, alternating with chlororoform washing (2 × 5 ml). The filtrates were evaporated, the residue was dissolved in 25 ml of a mixture of 42% perchloric acid and ethanol (3:2), and the amount of $(MeO)_2Tr$ cation split off was determined spectrophotometrically in an aliquot of the solution obtained.

Procedure for Eliminating p-Chlorophenyl Protection and Splitting off Triuridylates from the Supports. To 100 mg of support with a grafted-on triuridylate was added 0.2 ml of a 0.33 M solution of $Bu_4N^+F^-$ in 40% aqueous pyridine (pH 7), and the mixture was kept at 20°C for an hour. After being washed with aqueous pyridine (2 × 2 ml) and with ethanol (2 × 5 ml), the support was covered with 25% aqueous ammonia and was kept in it at 50°C for an hour. Then it was filtered off and was washed with water, and the filtrate was evaporated to dryness.

Procedure for Removing 2'-O-Tetrahydropyranyl Protection. The residue was dissolved in 2 ml of 0.01 N hydrochloric acid and the solution was kept at 50°C for 2.5 h and was then neutralized with 2 ml of 1 M ammonium bicarbonate, and it was evaporated to dryness in vacuum and was then co-evaporated with 15 ml of ethanol twice.

Chromatographic Purification of the Triuridylates. An aqueous solution of a triuridylate was deposited on a column of Toyopearl DEAE that had been washed beforehand with Tris-HCL (pH 7.3), and elution was performed with a gradient of sodium chloride (0–0.2 M). The triuridylates were desalted on a column of Silasorb-C_{18}, with the use of water as eluent.

Enzymatic Hydrolysis of the Triuridylates and Microcolumn Chromatography of the Products of Their Hydrolysis. To 3 OU_{260} of a triuridylate in 10 µl was added 5 µl of a solution of nuclease P_1 (from Penicillium citrinum) (0.5 act. units/ml), and the mixture was kept at 20°C for 10 min. A solution of the hydrolysate was deposited on a column of Silasorb-NH_2 and elution was carried out with 0.02 M KH_2PO_4 in 10% aqueous acetonitrile in the isocratic regime. Reversed-phase chromatography on a column with Silasorb-C_{18} was carried out in a stepwise gradient of acetonitrile (0.5–7%) in 0.1 M ammonium acetate.

SUMMARY

Triuridylates containing 2'deoxyuridine, 5-bromouridine, and 6-azauridine residues in various positions of the sequence have been synthesized under the conditions of the solid-phase triester method using an O-nucleophilic nucleotide condensation catalyst — 4-ethoxypyridine N-oxide. The optimum ratios of the P-component and the condensing agent have been found.

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