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SUPPORTS FOR THE SOLID-PHASE SYNTHESIS OF OLIGONUCLEOTIDES WITH A 5'-TERMINAL PHOSPHATE GROUP*

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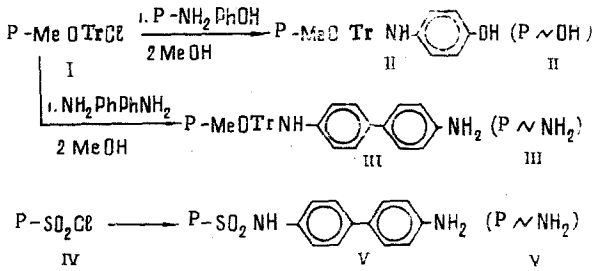
The advances achieved in the synthesis of five- to seven-membered oligodeoxyribonucleotides by the solid-phase method [1-3] are, at the present time, permitting this approach to be regarded as promising for the rapid preparation of oligonucleotides of a given composition. However, practically none of the oligodeoxyribonucleotides synthesized have contained a 5'-terminal phosphate group, since the supports used for the synthesis were modified by the introduction of trityl [2, 3] or carboxy [4, 5] anchor groups. The methods of modifying polymers suitable for the production of 5'-nucleotides described in the literature are, as a rule, extremely laborious.

In the present paper we consider the synthesis of supports containing aromatic hydroxy and amino groups as the centers for the addition of a 5'-nucleotide. The introduction of such anchor groups in the polymers was carried out by the following schemes:



In the first case, a methoxytritylated polymeric support (I) that is commonly used in the solid-phase synthesis of oligonucleotides [3] was subjected to modification and, therefore, the modification of the polymer amounted to the addition of p-aminophenol or benzidine, which enabled the synthesis of oligonucleotides with a phosphate group at either end of the chain to be carried out on the same initial support (I).

*The following abbreviations are adopted in this paper: P, polymeric support; TPS, 1,3,5-triisopropylbenzenesulfonyl chloride, C.A., condensing agent; pN(N), deoxynucleoside 5'-phosphate (deoxynucleoside protected at an exocyclic amino group).

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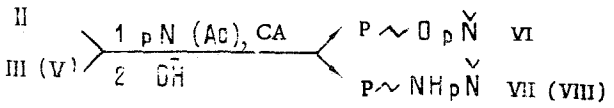
Polystyrenesulfonyl chloride (IV), which we have used previously for the solid-phase activation of the nucleotides [8], is also an accessible reagent, and the high activity of the sulfonyl chloride groups permits the introduction of benzidine residues with an efficiency even higher than in the first case. All the reactions were carried out by treating polymers (I) and (IV) with a 10-fold excess of a solution of the corresponding base in pyridine, the yield depending only on the degree of drying of the solution of the base and varying between 70 and 90%. The total capacity of the supports obtained, determined by the spectrophotometry of the solutions and the bases after their separation from a weighed sample of support (λ_{max} 230 nm, ε_{max} 6300 for p-aminophenol, and λ_{max} 330 nm, ε_{max} 31,000 for benzidine in the form of Congo Red), amounted to 0.2-0.3 meq/g.

To determine the optimum conditions for the addition of the first nucleotide to the supports, a comparative study was made of the efficiency of various condensing agents.



It was established that the use in this reaction of dicyclohexylcarbodiimide permits only 40% addition for polymer (II) and for the amino polymers (III) and (V) the yield falls to 15%, which corresponds to the results obtained by E. Ohtsuka [9] for an amino polymer with a low degree of cross-linking.

It proved to be more sucessful to use TPS as the condensing agent. For all the polymers, the yields of addition products (VI-VIII) rose to 70-75% provided that the preliminary activity of the nucleotide was carried out with a minimum (1.5-fold) excess of TPS for 1.5-2 h.

However, the most effective agent for performing this reaction proved to be polystyrenesulfonyl chloride [8], the use of which enabled the mononucleotide to be fixed to the support with a yield of about 90%. In this process, the mononucleotide was activated by being kept above a twofold excess of polystyrenesulfonyl chloride, after which the solution of active derivative was filtered directly onto the polymer-support.

Good results for the introduction of the first nucleotide link into the amino and hydroxy polymers were obtained by means of the reaction that we have used previously [10] for the performance of oligonucleotide synthesis without condensing agents.

In the first stage, the polymer is phosphorylated at the hydroxy or amino groups with an excess of phosphorus oxychloride, and after the support has been washed with absolute pyridine the corresponding nucleotide is added to it at the phosphorochloridate group without the participation of a condensing agent. The total yield of polymer-nucleotide (VI-VIII) under these conditions amounts to 70-85%. The approach appears extremely promising in view of the mildness of the performance of both stages and the possibility of using nucleotides without protection at the 3'-hydroxy groups, which enables the following stage of the synthesis to be carried out without alkaline treatment. Furthermore, this variant is ideally included in the scheme of triester synthesis, since it permits a fully protected phosphate group to be obtained directly if, for phosphorylation, $POCl_3$ is replaced by esters of phosphorodichloridic acid. Preliminary experiments have shown that on the use of, for example, p-chlorophenyl phosphorodichloridate the yield of the corresponding triester of thymidylic acid for polymer (II) is 70-75%.

A nucleotide added to all the types of support that have been described is stable under the conditions of alkaline treatment and, thus, the further growth of the oligonucleotide chain can be carried out by the usual diester scheme [1-3]. The splitting off of the nucleotide material of a support with an N-trityl-p-aminophenol group (VI) is achieved by treatment with iodine [9] in dimethylformamide—1 M ammonium acetate (4:1) solution or with a mixture of dimethyl sulfoxide and acetic anhydride (1:1), and from the amino polymers (VII) and (VIII) by the action of isoamyl nitrite.

A nucleotide can also be split off from the trityl-containing supports (VI) and (VII) by the action of acids. In this method of removal, the corresponding phospho-protective group remains attached to the mono- or oligonucleotide and can be separated from it in solution. It must be observed that the time necessary for separating the nucleotide material

TABLE 1

Type of support	Nucleotide component		Oligonucleotide obtained	Yield,* %	Time, h
	composition	excess			
VI	pT(Ac)	3	pTpT	62	20
	pTpacG(Ac)	3	pTpTpG	45	15
	pacG(Ac)	3	pTpG	73	20
VII	pbzA(Ac)	4	pTpA	74	5†
VIII	pT(Ac)	3	pTpT	68	5†
	pbzA(Ac)	5	pTpA	71	5†

*The yield was determined after removal from the support and the complete deblocking of the oligonucleotide by concentrated ammonia, chromatographic separation on paper, and measurement of the optical densities of the eluates corresponding to the UV-absorbing zones.
†The mononucleotide was preactivated for 1.5 h.

from the support on treatment with iodine or isoamyl nitrite is several times longer than the analogous reactions in solution [9], amounting to 15-24 h, and the yield does not exceed 85-90%.

The synthesis of a number of di- and trinucleotides performed on polymeric supports has shown that the introduction of hydroxy or amino groups into the initial polymer (I) does not change the working characteristics of the supports and their yields at the stage of the addition of a nucleoside 5'-phosphate or dinucleotide with a 5'-terminal phosphate group in the diester variant under the use of TPS as condensing agent scarcely differ from the corresponding figures for the initial polymers [3]. The results of the synthesis are given in Table 1.

To confirm the structures of the di- and trinucleotides synthesized we compared their UV spectra with those constructed theoretically for the given compositions. The presence of a 5'-terminal phosphate group was shown by the decrease in electrophoretic mobility after treatment with alkaline phosphatase. For the trinucleotide pTpTpC we determined the T : pT : pC ratio (1:0.9:0.95) after dephosphorylation and hydrolysis with snake venom phosphodiesterase.

EXPERIMENTAL

The work was carried out with deoxynucleotides produced experimentally by Glavmikrobioprom, and with thymidine in the form of a commercial preparation of the firm "Reanal." The synthesis of pacG(Ac) and of pbzA(Ac) were carried out by the methods of Khorana et al. [11, 12]. Absolute pyridine was obtained by keeping pyridine over P_2O_5, distilling it over BaO, and storing it in columns over 4A molecular sieves. Paper chromatography (PC) was performed on FN-1 paper in the following systems: 1) isopropanol—concentrated ammonia—water (7:1:2); 2) butan-1-ol—acetic acid—water (5:2:3); and 3) ethanol—1 M ammonium acetate (7:3), pH 7.5.

Electrophoresis was carried out on a Shandon instrument at a voltage of 5000 V in 0.05 N ammonium bicarbonate buffer, and the mobilities were calculated relative to that of pT. The molar extinctions were taken as 9600 for T, 20,700 for bzA, and 16,700 for acG [11, 12]. The initial polymeric matrices (I) and (IV) were synthesized by published methods [3, 8].

Synthesis of the Supports. a) Polymers (II) and (III). In a pear-shaped flask with a cock, a solution in 10 ml of pyridine of 10 mmole of the base (1.1 g of p-aminophenol or 1.8 g of benzidine) that had been recrystallized in an atmosphere of argon was dried by three distillations to dryness in vacuum. Then it was dissolved in 25 ml of absolute pyridine and after 3-5 ml of the solvent had been distilled off in vacuum, 5 g of the vacuum-dried methoxy tritylated support (I) containing 0.2 mmole of chlorine per gram of polymer was rapidly added in a dry chamber. The flask was pumped out stringently to degas the polymer and was shaken for a day, after which it was heated in the water bath at 50°C for 5-6 h, 1 ml of absolute methanol was rapidly poured in, and it was heated for another hour. Then the polymer was filtered off and was washed on the filter with pyridine (3 × 10 ml), 50% aqueous pyridine

(3 × 15 ml), ethanol (4 × 10 ml), and ether (4 × 10 ml). The amount of p-aminophenol or benzidine added was 0.15-0.17 mmole/g of support.

b) Polymer (V). A solution of 1.8 g (10 mmole) of benzidine in 10 ml of absolute pyridine that had been dried as described above was added in a dry chamber to 3 g of polystyrene sulfonyl chloride (0.3 mmole/g) previously swollen in 3 ml of absolute pyridine. The mixture was stirred on a shaking machine for 12 h, after which the polymer was washed as described above. The capacity of the support was 0.25 mmole per gram of polymer.

Addition of the Nucleotides. a) A pyridine solution of 0.6 mmole of a protected deoxynucleotide in 5 ml of absolute pyridine that had been dried by distilling off absolute pyridine three times was treated with 1 mmole (303 mg) of TPS and the mixture was kept in the dark for 2 h. Then 1 g of the appropriate support that had been dried in vacuum was added to the flask and the suspension was stirred for 5-6 h. The polymer was filtered off and washed on the filter as described above.

b) A reactor with a jacket and a filter was charged with 2 g of the support (II, III, or V), which was dried by the slow passage through it of 15-20 ml of absolute pyridine, and a mixture of 0.5 ml (5.5 mmole) of freshly distilled $POCl_3$ in 5 ml of absolute pyridine was added at a temperature of 0-5°C. The suspension was stirred for 5 min and then the support was filtered off and was washed with absolute pyridine (3 × 5 ml), and a solution of 2 mmole (0.5-0.7 g) of carefully dried nucleoside in 5 ml of absolute pyridine was added. The mixture was stirred for 12-15 h, after which the polymer was washed by the standard scheme. The excess of nucleoside after the evaporation of the pyridine and passage through D-1 × 2 (OH) was chromatographicallly pure and suitable for use without additional purification.

Synthesis of Dinucleotides. a) General Procedure. A flask with a cock was charged with 1 g of a support (VI-VIII) and this was dried by the distillation off of absolute pyridine in vacuum three times. In another flask 1 mmole (fivefold excess) of the corresponding protected mononucleotide was dried similarly and after the addition to the solution (4 ml) of 600 mg (2 mmole) of TPS the mixture was kept in the dark at 20°C for 1.5 h. The resulting solution of activated mononucleotide was transformed with the aid of a dry argon to the polymeric support and the mixture was stirred for 4 h, after which the polymer was washed on the filter with pyridine (5 × 5 ml), aqueous pyridine (3 × 5 ml), ethanol (5 × 5 ml), and ether (3 × 5 ml) and was dried in vacuum. The dinucleotides synthesized had the following characteristics:

pTpT λ_{max} 267 nm	RpT 0.86 (system 3)	E_f 1.0
pTpC λ_{max} 254 nm	R_f 0.21 (system 1), 0.25 (system 3)	E_f 0.9
pTpA λ_{max} 261 nm	R_f 0.25 (system 1)	E_f 0.92

b) pTpTpC. In a flask, 100 mg of the support (VI) (0.025 mmole of pT) and 80 mg (0.08 mmole) of pTpacG(Ac) were dried by distilling off absolute pyridine four times and when on the last occasion the reaction mixture had been evaporated to a volume of 0.5 ml 100 mg (0.33 mmole) of TPS was added. The mixture was kept in the dark at 20°C for 15 h. Then the polymer was washed by the usual method. After elimination from the support, deblocking, and chromatography on paper, the trinucleotide had the following characteristics: λ_{max} 261 nm, λ_{min} 233 nm, R_f 0.2 (system 2) and 0.16 (system 3). The T : pT : pC ratio was 1:0.9:0.95 after hydrolysis with phosphomonoesterase and snake venom phosphodiesterase.

Isolation of the Oligonucleotides. a) A 100-mg sample of support (VI) with a synthesized oligonucleotide was shaken at 20°C with 2 ml of a 20% solution of freshly sublimed iodine in DMF—1 M ammonium acetate (4:1) for a day. Then the polymer was washed with pyridine (3 × 2 ml), 50% aqueous pyridine (3 × 2 ml), and ethanol (4 × 2 ml). The combined filtrate was evaporated, the residue was dissolved in 8 ml of water, and the iodine was extracted with 3 × 15 ml of ether. The aqueous layer was evaporated to dryness and was kept for a day at 20°C and for 3 h at 50°C with 3 ml of concentrated ammonia and, after evaporation, was chromatographed in the appropriate system.

b) A 100-mg sample of support (VI) was shaken at 20°C with 3 ml of DMSO—acetic anhydride (1:1) for 1.5 days. The polymer was washed and the oligonucleotide was isolated by the method described above.

c) The nucleotide material was removed from the amino polymers (VII and VIII) by treating 100 mg of the support with 1.5 ml of a solution of 0.7 ml of isoamyl nitrite in 1.4 ml of

pyridine—acetic acid (1:1) at 20°C for 24 h. The polymer was washed by method (a) and after three evaporations with aqueous and then with absolute pyridine the nucleotide material was precipitated with ether. The precipitate was dissolved in concentrated ammonia solution, kept for a day at 20°C and for 3 h at 50°C, and chromatographed on paper.

SUMMARY

A simple method is proposed for obtaining two kinds of polymeric supports of the polystyrene type for the solid-phase synthesis of oligonucleotides containing 5'-terminal phosphate groups. For both kinds of supports the optimum conditions of adding the first nucleotide and of splitting out the nucleotide material from the polymer have been worked out and the synthesis of a number of di- and trinucleotides of the deoxy series has been performed.

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SYNTHESIS OF A FRAGMENT OF HISTONE H2B WITH THE AMINO ACID SEQUENCE 30-39 AND ITS $Lys^{31,33}$ ANALOG

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Recently, great attention has been devoted to the study of the structure and function of histones, which affect the template activity of DNA. It is assumed that DNA is capable of transmitting genetic information only if it is not bound to histones.

On the phosphorylation of the histone fraction H2B, of the fairly large number of serine residues only Ser^{14} and Ser^{36} are phosphorylated by phosphokinase. This shows a high specificity of the elements of the primary structure of the histones in relation to such processes as phosphorylation, acetylation, etc. But the process of phosphorylation of histones is directly connected with the stability of the DNP complexes and, consequently, with the transmission of genetic information. Upon what factors does such a high specificity of the enzyme depend? Is it explained by the immediate environment of the serine residue or does it depend on the secondary structure of the molecule as a whole?

In order to answer these questions, we have synthesized a fragment of the histone H2B with the sequence Lys-Arg-Ser-Arg-Lys-Glu-Ser-Tyr-Ser-Val and its $Lys^{31,33}$ analog. This fragment is interesting because, in addition to Ser^{36}, it contains two serine residues which do not undergo phosphorylation by phosphokinase in the natural material.

Fragment 30-39 was synthesized by condensing two pentapeptides by the carbodiimide method with the addition of N-hydroxysuccinimide. The synthesis of its $Lys^{31,33}$ analog was also

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