SOLID-PHASE SYNTHESIS OF OLIGONUCLEOTIDES REPRESENTING FRAGMENTS OF trp PROMOTERS

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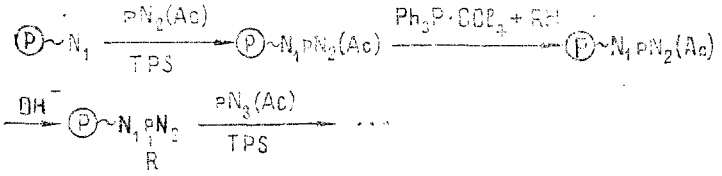
The self-complementary oligonucleotides AACTAGTT and AGTTAACT, which are homologs of functionally important sections of trp promoters of three related bacteria, have been synthesized by the solid-phase method. A new block variant of synthesis on a polymer has been worked out and has been used for obtaining the octanucleotides AACTAGTTp and AGTTAACTp.

DNA-like polymers with repeated oligonucleotide sequences are convenient model systems for studying the mechanism of nucleic acid–protein interactions [1]. Such polymers can easily be obtained by the chemical [2] or enzymatic ligation of one or more synthetic oligonucleotides. As a model for investigating the molecular mechanism of the transcription process performed by *E. coli* RNA polymerase we propose one of the polymers shown which contain repeated fragments homologous to the –5 to –19 section in the promoters of the trp operons of three related bacteria: *E. coli* [3], *S. typhimurium* [4], and *S. marcescens* [5].

	–19 –5	
(5′–3′)	AACTAGTTAACTAGTA*	E. coli
(5′–3′)	AACTAGTTAACTAGTA	S. typhimurium
(5′–3′)	AACCAGTTAACTAGTA	S. marcescens
(5′–3′)	...AACTAGTTAACTAGTTAACTAGTT...	
(3′–5′)	...TTGATCAATTGATCAATTGATCAATTGATCAA...	

The first step in the creation of such a structure is the chemical synthesis of the self-complementary octanucleotides AACTAGTT (I) and AGTTAACT (II). The synthesis of these oligonucleotides was effected by the solid-phase method using a styrene polymeric support of the grafted type in the semiautomatic apparatus described previously [6], the stepwise growth of the oligonucleotide chain taking place from the 5'- to the 3'-end by means of N- and O-protected mononucleotides with the stagewise blocking of the internucleotide phosphate group by aniline.



where Ⓟ~ represents the polymeric support;

N_1 and $pN_2(Ac)$ represent protected nucleosides and nucleotides; TPS represents triisopropylbenzenesulfonyl chloride; and R represents an anilino group.

*For convenience of writing, the symbol d (deoxy) has been omitted from all the formulas of the oligodeoxyribonucleotides.

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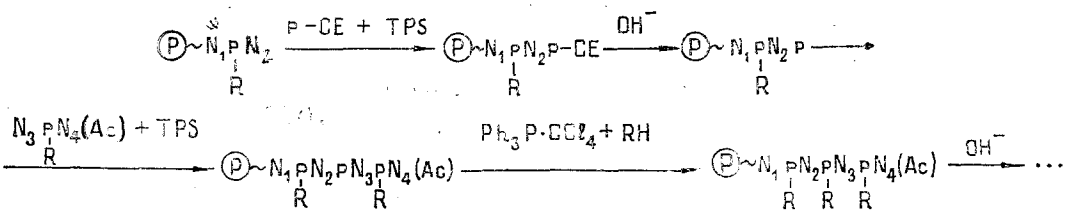
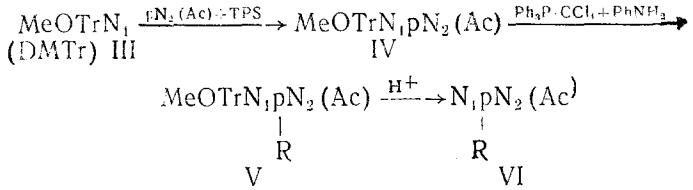
However, as we have reported previously [6, 7], the lengths of the oligonucleotides synthesized by this method cannot exceed 8-10 units because of difficulty in the preparative isolation of the desired product from the reaction mixture with the aid of the chromatographic sorbents usually employed. Such difficulties can be overcome by growing the oligonucleotide chain in blocks. One of the variants of the block synthesis is proposed in the present paper for obtaining the octanucleotides AACTAGTTp (Ia) and AGTTAACTp (IIa), in accordance with the scheme:



where CE represents a cyanoethyl group.

The completely protected dinucleoside phosphate blocks necessary for this synthesis were obtained in solution by the general scheme:



The first stage was the preparation of compounds of type (IV) — carried out by the method developed by Khorana [8], i.e., by condensing the nucleoside and nucleotide components in the presence of TPS followed by the isolation of the product by extraction. The blocking of the internucleotide phosphate group with aniline in the presence of the condensing agent Ph_3P—CCl_4 was carried out as described in [9, 10]. According to the results of thin-layer chromatography, the reaction took place quantitatively in 4 h. The monomethoxytrityl group was eliminated by treatment with a 2% solution of trifluoroacetic acid in anhydrous methylene chloride, and the dimethoxytrityl group with a 1% solution of hydrogen chloride in anhydrous dioxane. The completely protected dinucleotide phosphate (VI) obtained was purified by chromatography on silica gel in chloroform containing methanol or by two or three reprecipitations from chloroform with a 7:3 mixture of petroleum ether and diethyl ether.

The blocks bzApbzA(Ac), bzApbzG(Ac), anCpT(Ac) and TpT(Ac) were obtained by this scheme with yields of 54, 65, 70, and 72%, respectively, calculated on the initial nucleoside component (III). We have worked out the conditions for adding such blocks to a polymeric support in the presence of γ-dimethylaminopyridine (DMAP). The amount of nucleotide material added to 1 g of polymer was 0.02-0.025 mole. The oligonucleotide chain was grown with the aid of the phosphorylation of the oligonucleotide attached to the polymer and subsequent condensation with the following oligonucleotide of type (IV). As the phosphorylating agent we chose β-cyanoethyl phosphate in the presence of TPS, which has already been used for the phosphorylation of the 3'-hydroxy groups of oligonucleotides both in solution [11] and on a polymer [12]. The reaction took place practically quantitatively, and no formation of the products of the "linkage" of the oligonucleotide material with an unnatural 3'—3' bond was observed, as took place in the case of polyfunctional phosphorylating agents of the type of $POCl_3$, $POIm_3$, etc.

The β-cyanoethyl group was eliminated by mild alkaline hydrolysis and the nucleotide component obtained in this way was introduced into the condensation reaction with a five- or sixfold excess of the block (VI) synthesized in solution.

After the next stage of block condensation, the polymer was treated with aniline in the presence of the complex Ph_3P—CCl_4 for blocking both the internucleotide phosphate group and also the 3'-terminal phosphate group that had not reacted; this prevented the subsequent growth of low-molecular-weight side chains, i.e., it decreased the relative amount of im-

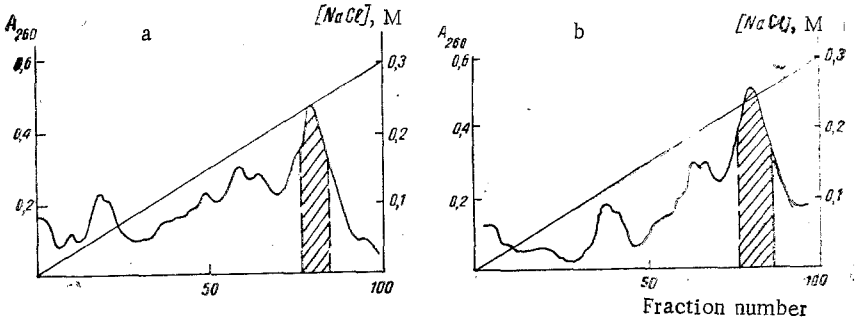


Fig. 1. Chromatography on a column (1 × 500 mm) of DEAE-cellulose in a linear concentration gradient of NaCl in 7 M urea: a) 120 OU_{260} of an oligonucleotide mixture containing AACTAGTTp (0.02 M Tris-HCl, pH 7.0; 3-ml fractions/7 min); b) 120 OU_{260} of an oligonucleotide mixture containing AGTTAACTp (0.2 M Tris-HCl, pH 7.0; 3-ml fractions/7 min).

purities. This circumstance may be regarded as one of the advantages of the proposed scheme of oligonucleotide synthesis as compared with the stepwise variant, where in each stage of condensation elongation not only of the oligonucleotide with the desired sequence but also of the shorter chains that are always formed in solid-phase synthesis takes place. The degree of conversion in the stage of forming the internucleotide bond averaged 80%, with the exception of the last stage (about 50%).

Chromatography on DEAE-cellulose in a sodium chloride gradient at pH 7.5 (Fig. 1a-b) and rechromatography on the sorbent amino-Silochrome AS_{III}-100 in a sodium phosphate gradient permitted the fairly rapid and efficient isolation of the oligonucleotides AACTAGTTp and AGTTAACTp in their homogeneous state according to the results of microcolumn chromatography.

Thus, the proposed block scheme of synthesis may be regarded as promising if one takes into account the relative simplicity of the preparation of the starting materials.

The quantitative characteristics of the solid-phase synthesis and the yields of the oligonucleotides are given below; the primary structures of the substances obtained were shown by the nucleotide map method [13] (Fig. 2a-d):

Oligonucleotide	Polymer, mg	Nucleotide component, μmole	Oligonucleotide isolated, μmole	Yield, %
I. AACTAGTT	100	2,8	0,13	4,6
II. AGTTAACT	200	5,6	0,10	1,8
Ia. AACTAGTTp	100	2,8	0,044	1,6
IIa. AGTTAACTp	150	2,4	0,03	1,2

Preliminary physicochemical results indicate that the octanucleotides synthesized are capable of forming complementary complexes under the conditions of the working of DNA ligase, which will subsequently permit DNA duplexes with the structure shown above to be obtained.

EXPERIMENTAL

Nucleoside nucleotides from Calbiochem (USA) and Novosibirsk, Merck TPS, Chemapol (Czechoslovakia) triphenylphosphine, and Soyuzreaktiv (Novosibirsk) amino-Silochrome AS_{III}-100 were used.

Paper chromatography was performed in system 1) ethanol—1 M ammonium acetate (6:3), and thin-layer chromatography on silica gel plates (Eastman Kodak No. 6060) in systems 2) water—acetonitrile (18:85) and 3) chloroform—methanol (9:1).

Analytical chromatography was performed on a MSFP-3 instrument [14] using microcolumns (1 × 50 mm) containing DEAE-cellulose in the Tomlinson—Tener system [15] and with amino-Silochrome in a gradient of Na phosphate buffer at pH 7.0 with a rate of elution of 360 μl/h.

N- and O-Substituted mononucleotides were obtained as described by Kolobushka and Florent'ev [16] using benzoyl as the N-blocking group. Additional purification of the pro-

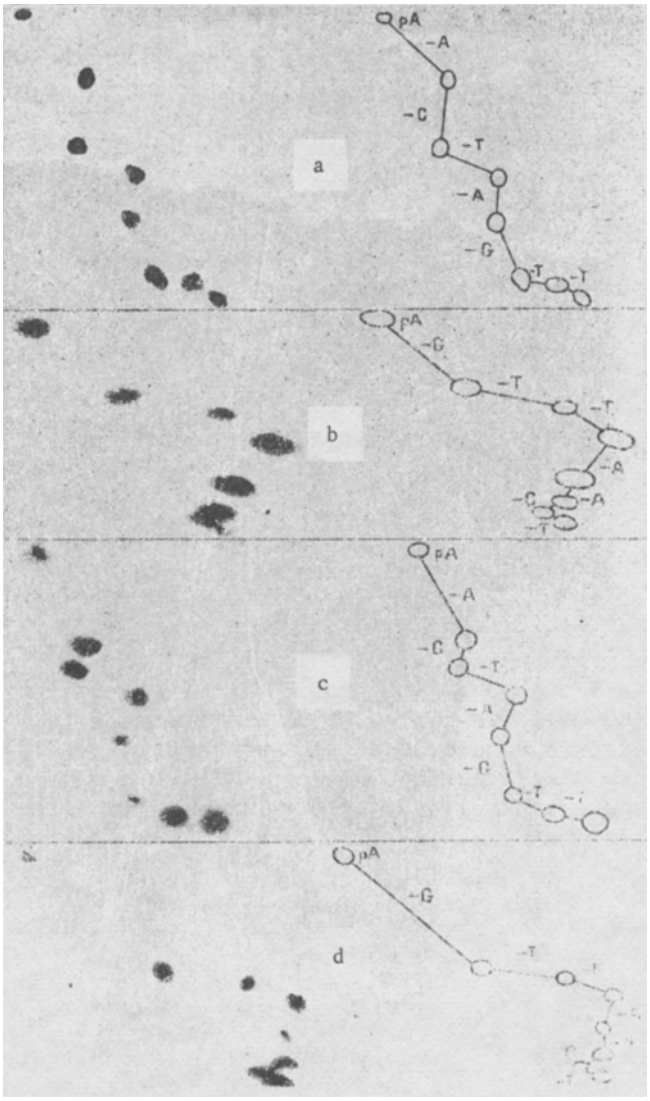


Fig. 2. Two-dimensional separation of the products of the partial hydrolysis with snake venom phosphodiesterase of the 5'-^{32}P-phosphorylated octanucleotides: a) AACTAGTT; b) AGTTAACT (obtained by the stepwise method); c) AACTAGTT; d) AGTTAACT (obtained by the block method and previously dephosphorylated). Directions: E — electrophoresis in acetylcellulose in pyridine-acetate buffer (pH 7.5) at 90 V/cm; H — homochromatography on PEI-cellulose at pH 7.5.

tected nucleotides was performed by gel filtration on Sephadex LH-20 in the water—pyridine (1:49) system [7] or by washing an aqueous pyridine solution of the nucleotide with a 3:1 mixture of methylene chloride and butanol [17].

The synthesis of the protected nucleosides (III) and the blocks (IV) was carried out by the method of Khorana [18, 19].

The blockage of the intermediate phosphate groups was effected by analogy with method of synthesizing mononucleotide dianilides described in [9]. The reaction was performed at 20°C for 4 h, 7.5 mmole of CCl_4, 7.5 mmole of triphenylphosphine, and 15 mmole of aniline being added to 1.5 mmole of a compound (IV). The yields of the type (V) compounds were quantitative.

Elimination of a 5'-O-MeOTr Group. With cooling to −20°C, 60 ml of a 2% solution of trifluoroacetic acid in anhydrous CH_2Cl_2 was added to a solution of 1.3 mmole of MeOTranCpT(Ac), (R) in 60 ml of anhydrous CH_2Cl_2. The mixture was kept for 15 min, and then, with stirring, 60 ml of saturated aqueous $KHCO_3$ solution was added. The organic layer was separated off and washed once more with 40 ml of $KHCO_3$ solution and then with water (2 × 20 ml) and was evaporated; the residue was dried by azeotropic distillation with benzene and was then dissolved in 15 ml of CH_2Cl_2 and the solution was added dropwise to a 7:3 mixture of petroleum ether and diethyl ether. The last precipitation procedure was repeated twice. Finer purification of the substance was achieved by chromatography on silica gel. The anCpT(Ac) (R) was obtained with a yield of 95% (1.22 mmole), R_f 0.7 (system 3). The block bzApbzG(Ac) (R) was obtained similarly with a yield of 90% (1.1 mmole). R_f 0.6 (system 3).

Elimination of the 5'-O-DMTr Group. With cooling to −20°C, 50 ml of a 1% solution of HCl in anhydrous dioxane was added to a solution of 2 mmole of DMTrbzApbzA(Ac), (R) in 50 ml of anhydrous CH_2Cl_2. The mixture was kept for 1 min, and 15 ml of pyridine (anhydrous) was added. The pyridine was eliminated by azeotropic distillation with benzene, the residue was dissolved in chloroform (60 ml), and the solution was washed with water (3 × 40 ml). The organic layer was evaporated in vacuum, and the residue was dried by the distillation of CH_2Cl_2 from it and was then dissolved in 60 ml of CH_2Cl_2 and reprecipitated with a 7:3 mixture of petroleum ether and diethyl ether. The resulting precipitate was reprecipitated twice. The yield of bzApbzA(Ac) (R) was 59% (1.2 mmole). The precipitation of TpT(Ac) (R) was performed similarly, giving an 80% yield (3.2 mmole). The R_f value in system 3 for bzApbzA(Ac) (R) was 0.58 and for TpT(Ac) (R) 0.68.

Addition of the Blocks (VI) to the Polymer. On a glass filter, 3 g of polymeric support [polymer-(trityl carbinol)] was washed with dry methylene chloride (5 × 10 ml), and then a saturated solution of PCl_5 in methylene chloride was added and the mixture was allowed to stand for 5 min. The solution was filtered and the polymer residue was washed with CH_2Cl_2 (5 × 30 ml) and, without being brought to dryness, was transferred to a flask. A mixture of 0.5 g (0.55 mmole) of bzApbzG(Ac), (R) 1.5 mg (0.015 mmole) of DMAP and 0.075 ml (1.5 mmole) of triethylamine (abs.) was dissolved in 3 ml of CH_2Cl_2 and was added to the polymer-(trityl chloride). The mixture was left at 20°C for 24 h, then 0.5 ml of methanol was added, and after 30 min the polymeric support was washed free from an excess of the reagents successively with methylene chloride, ethanol, and ether. The specific loading of the polymer amounted to 0.025 mmole/g. The block bzApbzG(Ac), (R) was added similarly, giving a loading of 0.020 mmole/g.

To perform all the synthetic operations on the polymer we used a laboratory synthesizer with a reactor of the column type working in the semiautomatic regime [6]. The condensation reaction, the blockage of the internucleotide phosphate group the elimination of the N-, O-, and phosphate-blocking groups, and the splitting off of the nucleotide material from the polymer were carried out by a procedure described previously [6].

Phosphorylation with β-Cyanoethyl Phosphate. A solution in 2-3 ml of anhydrous pyridine of 1 mmole of β-cyanoethyl phosphate that had previously been dried by repeated distillation with pyridine was treated with 2 mmole of TPS. The mixture was allowed to stand for 1.5 h and was then transferred to the reactor containing the polymer which had previously

been washed with 10 ml of anhydrous pyridine. Phosphorylation was carried out for 4 h with the pumping of the solution in a closed cycle. The polymer was washed successively with anhydrous pyridine and with pyridine–water (7:3).

Elimination of the Cyanoethyl Group. After the phosphorylation reaction, the polymer, which had been washed with 70% aqueous pyridine, was covered with a 0.5 M solution of Et_4NOH in 70% aqueous pyridine and the mixture was left to stand at 20°C for 30 min. The polymer was washed successively with 70% aqueous pyridine, anhydrous pyridine, a 2% solution of acetic acid in pyridine, and anhydrous pyridine again.

Block Condensation. A solution in 1.0 ml of anhydrous pyridine of 0.1 mmole of a block (VI) that had previously been dried by the repeated distillation of pyridine was treated with 0.3 mmole of TPS. The resulting mixture was poured onto the polymer that had previously been washed with anhydrous pyridine, and the mixture was kept at 20°C for 12 h. Then the polymer was washed successively with anhydrous pyridine and with 70% aqueous pyridine.

The synthesis of AACTAGTT (I) was carried out on 2 g of polymeric support containing 56 μmole of bzA. After each stage of condensation the 3'-hydroxy groups that had not taken part in the reaction were blocked with phenyl isocyanate [20]. After the synthesis had been completed and all the protective groups had been split off from the polymer (100 mg), 150 OU_{260} of nucleotide material was obtained. Chromatography on DEAE-cellulose and amino-Silochrome led to the isolation of 11.3 OU_{260} (0.13 μmole) of AACTAGTT. The yield was 4.6% in relation to the initial nucleoside.

The synthesis of AGTTAACT (II) was performed on 1.5 g of polymer containing 42 μmole of bzA. After the completion of the synthesis, 160 OU_{260} of nucleotide material was split off from the polymer (200 mg). As a result of chromatography on DEAE-cellulose and amino-Silochrome, 9.0 OU_{260} (0.1 μmole) of AGTTACT was isolated. Yield 1.8%.

The synthesis of AACTAGTTp (Ia) was carried out on 0.2 g of polymer containing 5.6 μmole of bzApbzA. After the completion of the synthesis, 120 OU_{260} of nucleotide material

R

was split off from the polymer (100 mg). Chromatography on DEAE-cellulose and amino-Silochrome led to the isolation of 4.0 OU_{260} (0.44 μmole) of AACTAGTTp. Yield 1.6%.

The synthesis of AGTTAACTp (IIa) was carried out on 0.25 g of polymer containing 4.0 μmole of bzApbzG. After the completion of the synthesis, 120 OU_{260} of nucleotide material was split off from 150 mg of the polymer. As the result of chromatography on DEAE-cellulose and amino-Silochrome, 3.0 OU_{260} (0.03 μmole) of AGTTAACTp was isolated. Yield 1.2%.

CONCLUSION

1. The octanucleotides AACTAGTT and AGTTAACT containing the sequences of the conservative sections of trp promotors have been obtained by the solid-phase methods using stepwise growth of the chain.

2. A block variant of the solid-phase synthesis has been developed and has been used for the preparation of the 3'-phosphorylated octanucleotides with the same primary structures: AACTAGTTp and AGTTAACTp.

V. L. Drutse took part in the proof of the structures of the oligonucleotides by the nucleotide map method.

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SYNTHESIS OF TRIACONTAN-1-OL FROM DODECANEDIOIC ACID

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A method has been developed for obtaining triacontan-1-ol — a natural plant growth stimulator. Synthesized triacontan-1-ol has an appreciable influence on the growth and crop yield of tomatoes and on the flowering of asters.

Triacontan-1-ol (I) is an effective natural plant growth stimulator [1, 2]. The influence on the growth activity of homologues of (I) differing in their chain lengths (C_{16}-C_{32}) has been investigated, and not one of them causes an increase in the growth of plants [3]. A number of complex syntheses of the alcohol (I) have been published [4-7]. We have developed a simpler method of obtaining the alcohol (I) from the readily available dodecanediodic acid (II) and 1-bromooctadecane, by the following scheme:

$$\underset{\text{II}}{HOOC(CH_2)_{10}COOH} \rightarrow \underset{\text{III}}{OH(CH_2)_{12}OH} \rightarrow \underset{\text{IV}}{Cl(CH_2)_{12}OH} \rightarrow$$

$$\xrightarrow[2.\ Mg,\ THF]{1.\ n\text{-}PrMgCl} \underset{\text{V}}{ClMg(CH_2)_{12}OMgCl} \xrightarrow[2.\ H_3O^{\oplus}]{1.\ n\text{-}C_{14}H_{29}Br} \underset{\text{I}}{CH_3(CH_2)_{29}OH}$$

Dodecanedioic acid (II) was converted through its diester by a known method [8] into dodecane-1,12-diol (III), which, on treatment with hydrochloric acid by a modified method [8], gave 12-chlorododecan-1-ol (IV) in high yield. Using the method of Cahier et al. [9], the Grignard reagent (V) was obtained from the chlorohydrin (IV) by treating it with an equimolecular amount of n-C_3H_7-MgCl in tetrahydrofuran solution followed by the treatment of the magnesium alcoholate formed with magnesium. The reaction of (V) at −5 to 0°C with 1-bromooctadecane in the presence of catalytic amounts of Li_2CuCl_4 formed the magnesium derivative of the alcohol (I) which, on treatment with hydrochloric acid, gave the alcohol (I). The total yield of the alcohol (I) calculated on the initial acid (II) was 68%. The

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