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Synthesis of oligonucleotides on cellulose by a phosphotriester method

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ABSTRACT

The synthesis of oligothymidilic acids, $(dT)_m$ (where m = 4, 7, 10, 13, 16, 19, 22, and 25), was carried out using a solid phase approach in combination with the modified phosphotriester methodology developed in solution. Cellulose was used as the solid support after its functionalization with a specially featured dinucleoside diphosphate, 5'-O-p-chlorophenylphospho-2'(3')-O-acetyluridilyl-[2'(3')-3']-5'-O-dimethoxytritylthymidine p-chlorophenylester. The fully protected trideoxynucleoside triphosphate containing only thymidine was repeatedly used to elongate the oligonucleotide chain in the 3'-5' direction. Individual coupling yields ranged from 45% to 75%. The total time needed to prepare $(dT)_{25}$ was four days. Similarly, the tridecanucleotide d(AGAAGGTACTTTT) was synthesized in good yield. The results show that this approach can be used for a fast and economic way to synthesize oligodeoxynucleotides.

INTRODUCTION

The synthesis in solution of oligodeoxynucleotides of defined sequence by a modified phosphotriester method has been shown to be very successful[1] and its effectiveness demonstrated in the chemical synthesis of genes for human insulin[2]. The use of protected trideoxynucleotides as building blocks[2,3] and the application of high performance liquid chromatography (h.p.l.c.) as a purification tool have dramatically increased the speed by which the synthesis of DNA fragments, 12-15 bases long, can be carried out. However, the synthesis of the trinucleotide building blocks on a large scale, required to maintain a complete "codon" library, is a time consuming and costly process. Therefore a method which allows us to reduce the amount of trimer used in each elongation step is highly desirable. A way to reduce the losses of nucleotidic material and avoid the time consuming purification of intermediates, would be the synthesis of the oligonucleotides on a solid polymer. Several approaches to the synthesis of DNA fragments on a solid support have been published (for review see 4,5). Although a wide range of polymers have been used,

chemical synthesis of oligodeoxynucleotides has been accomplished mainly with the phosphodiester method[6,7]. Attempts to synthesize oligodeoxynucleotides by the phosphotriester method on solid-phase have been published[8], and recently a method to synthesize $d(T)_{19}$ was reported.[9] We describe here the synthesis of homo- and hetero-oligodeoxynucleotides on cellulose using the modified phosphotriester methodology and trideoxynucleotides as building blocks.[18]

RESULTS AND DISCUSSION

Cellulose was chosen as the solid support because of its inherent polarity, its well known swelling properties in polar solvents (i.e. pyridine) and its mechanical stability. The polymer was functionalized by reacting the cellulose with the dimer 5'-0-p-chlorophenylphospho-2'(3')-0-acetyluridilyl-[2'(3')-3']-5'-0-dimethoxytritylthymidine p-chlorophenyl-ester (7b).
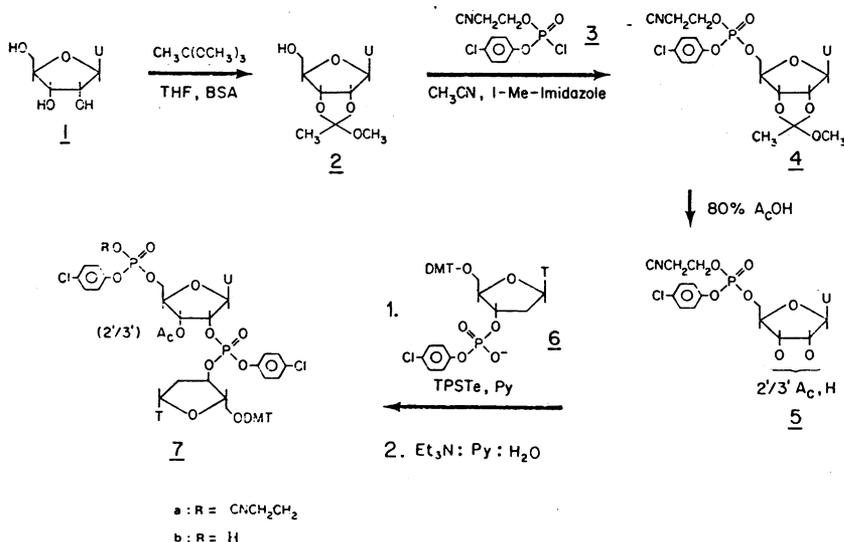
SCHEME I

The synthesis of (7b) is outlined in Scheme 1 and its features provide the following advantages:
A) It can be synthesized from easily available starting materials.
B) Coupling of the phosphodiester function to the cellulose with TPSTe[10] provide the attachment of (7b) to the polymer (see experimental part).
C) Deblocking of dimethoxytrityl group (DMT) from the deoxy- moiety in

acidic medium affords the corresponding 5'-hydroxyl derivative for the subsequent coupling step; therefore chain extension of the oligomer on the cellulose occurs in the more favorable 3'-5' direction.

D) The release of the synthesized oligonucleotide from the polymer is simple, fast, and essentially complete under the conditions used for the removal of the base-labile protecting groups of the oligonucleotidic chain. The aqueous concentrated ammonia hydrolyzes the acetyl group of the uridine used as anchoring group[11]. Subsequent nucleophilic attack of the free 2'(3')-hydroxyl group on the phosphate moiety triggers the release of the oligonucleotidic chain with a free 3' end (scheme 2).

SCHEME 2

Uridine was protected at its 2', 3'-diol group by treatment with tri-methyl orthoacetate[13] in THF in the presence of benzenesulfonic acid (BSA) as catalyst. The 2', 3'-cyclic derivative (2) was phosphorylated with the monofunctional phosphorylating agent 2-cyanoethyl-p-chlorophenyl-phosphorochloridate[2,14] (3, 2 equivalents) in acetonitrile and 1-methyl-imidazole (4 equivalents). The resulting compound (4) was treated with 80% aqueous acetic acid (10 minutes) and the 2'(3')-O-acetyl derivatives (5) isolated by silica gel column chromatography as a mixture of isomers. Finally, this material (5) was reacted with (6), 5'-O-dimethoxytrityl-3'-O-p-chlorophenylthymidilyl phosphate, (1.4 equivalents) in pyridine with TPSTe (2.8 equivalents) as coupling agent. The dimer (7a) so obtained was purified on silica gel and precipitated from petroleum ether (32% yield). The efficiency of the release mechanism was tested by treatment of (7a, 10 mg) with concentrated ammonia. After 30 minutes at 50 °C, the starting material (7a) had completely degraded into a base-line material and 5'-dimethoxytritylthymidine (TLC analysis)[12].

Coupling of the dinucleotide to cellulose was accomplished in the fol-lowing way: the 2-cyanoethyl group was selectively removed from the fully protected dimer (7a, 0.25 mmole) by treatment with a solution of triethyl-amine-pyridine-H_2O (1:3:1 v/v)[2] for 25 minutes at room temperature. Under these conditions the acetyl group was completely stable. The phosphodiester derivative (7b) so obtained was dried with pyridine and reacted with cellulose (Whatman CC31, 0.7 g) in anhydrous pyridine in the presence of TPSTe (0.9 mmole). The attachment of (7b) to the polymer was followed by monitoring the release of DMT residue[15] at 494 nm after acidic treatment of a small sample of polymer (see experimental). An average incorporation of ca. 100 μmole dimer/g cellulose was obtained.

Elongation of the nucleotidic chain was carried out with the trideoxy-nucleotide (9); the synthesis of this compound has previously been de-scribed[3]. The functionalized cellulose (8) (100 mg, 9.7 μmole of dimer) was first treated with 2% benzenesulfonic acid (BSA) in $CH_3OH/CHCl_3$ at 0°C in order to make the 5'-hydroxyl group of the thymidine available for the chain extension. After several coevaporations with pyridine, the dried polymer was reacted with (9) (50 μmoles) in pyridine containing TPSTe (220 μmole). TPSTe is known to be a very powerful condensing agent[2,10]; therefore the coupling reaction was stopped after two hours. The solid support was collected by centrifugation and the supernatant saved for the recovery of unreacted trimer (9) (see below). The cellulose was washed

with pyridine and then partially acetylated in order to block the un-
reacted 5'-hydroxyl groups. The resulting cellulose was washed (MeOH),
treated with 2% BSA, and then coupled again with the same quantity of
trimer (9). Eight such cycles were repeated under the same conditions in
four days. Table 1 lists the operations per coupling cycle.

After each cycle a sample (ca. 10 mg) of the polymer was treated with
conc. aq. NH_3 followed by 80% aq. AcOH to remove all protecting groups.
The unblocked oligonucleotidic material was then analyzed to calculate the
yields per coupling. For this purpose we used two different methods:
a) the release of DMT residue in acidic solution (494 nm)[15], which gave
a semiquantitative estimate of the amount of trimer (9) reacted, and
b) h.p.l.c. analysis[2], which allowed the accurate calculation of the
yields per coupling derived from the peak areas (254 nm) (figs. 1 and 2).
Table 2 shows the yields based on h.p.l.c. analysis.

H.p.l.c. analysis of the products showed that the major co-products
were due to incomplete chain elongation. Small quantities of side products
that could not be accounted for are probably due to direct reaction of the
trimer (9) with the hydroxyl groups of the cellulose or chain degradation

TABLE 1

STEP	OPERATION	CHEMICAL USED	VOL.**	TIME (min.)
1	Removal of DMT	2% BSA in $CHCl_3$/MeOH*	10	10
2	Washing	MeOH, Pyridine	50,20	5
3	Drying	Pyridine	10	10
4	Coupling	Trimer, TPSTe, Py.	1	120
5	Washing	Pyridine	2	5
6	Acetylation	Ac_2O/Pyridine	1	30
7	Washing	Pyridine	8	10
		Methanol	30	10

* At 0 °C (ice-water bath). ** Milliliters per 100 mg polymer.
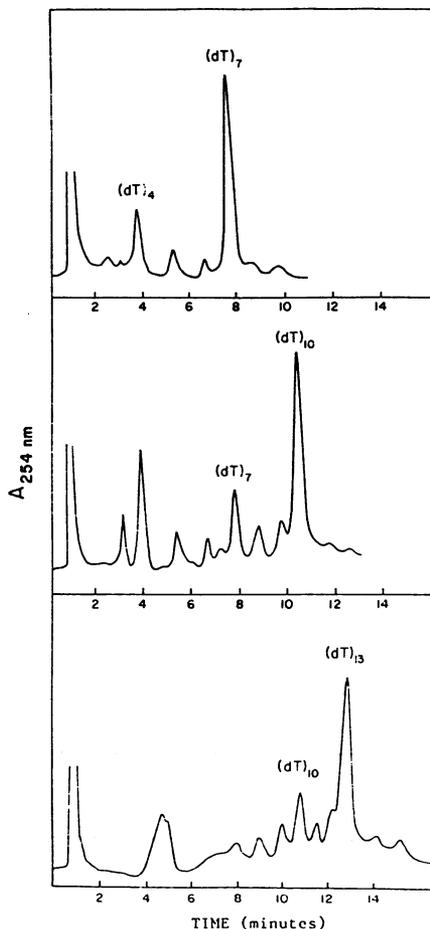
Fig.1. H.p.l.c. analysis of the oligothymidilic acids obtained after two (n=2), three (n=3), and four (n=4) coupling cycles.

during deblocking.   A method to block reactive hydroxyl groups after functionalization (i.e. coupling of (7b) to the cellulose) without changing the mechanical properties of the polymer support is currently under investigation.

Purification of the products was performed by h.p.l.c. on a strong anionic exchange resin (Permaphase AAX) by collecting the material corresponding to the last eluted peak.   The pooled solution (ca. 1.0 O.D. unit at 254 nm) was concentrated and the nucleotidic material desalted on
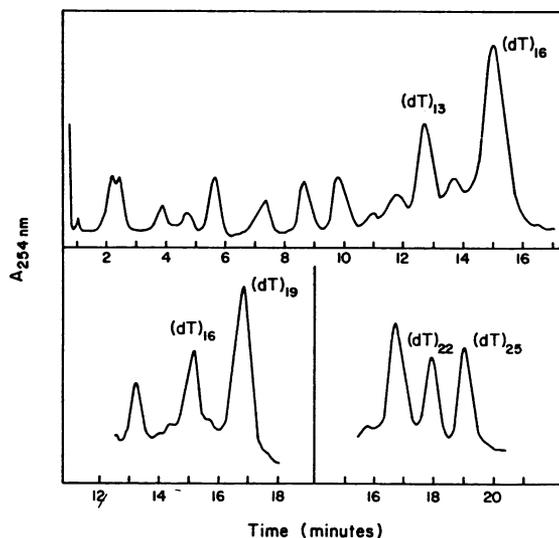
Fig.2. H.p.l.c. analysis of dT_m obtained after five (n=5), six (n=6), and eight (n=8) coupling cycles.

Bio-gel P-2. The size of each compound was confirmed by gel electrophoretic analysis (fig. 3). Two-dimensional sequence analysis of $(dT)_{13}$ showed the expected pattern for this compound (results not shown).

The synthesis in solution of oligonucleotides of defined sequence using the trinucleotide block approach[2] required the use of most of the 64 possible trinucleotide triphosphates. In order to investigate the reactivity of trimers with different base composition, we synthesized the following

TABLE 2

Cycle No.	Compound	Yield %
2	$(dT)_7$	73
3	$(dT)_{10}$	70
4	$(dT)_{13}$	62
5	$(dT)_{16}$	60
6	$(dT)_{19}$	57
7	$(dT)_{22}$	46
8	$(dT)_{25}$	48

oligonucleotide: d(AGAAGGTACTTTT) ($\underline{16}$). This oligomer, 13-bases long, was selected because its synthesis requires the use of four trimers, each having a different base at their 3'-phosphate end (see scheme 3).

Starting from the polymer ($\underline{10}$) (n=1, 120 mg), the synthesis of the fully protected oligonucleotide derivative was accomplished by sequential addition of the three trimers (50 µmoles each): $DMT\text{-}Tp_oA^{Bz}p_oC^{Bz}p_o$ ($\underline{12}$),

SCHEME 3

$$\underline{10} \ (\ n=1\ )$$

2% BSA $\downarrow$

$$HO\text{-}Tp_oTp_oTp_oTp_o^{Ac}Up_o\text{-}Cellulose$$

1) $DMT\text{-}Tp_oA^{Bz}p_oC^{Bz}p_o$ ($\underline{12}$)
2) Ac_2O/Py (40%)
3) 2% BSA

$$HO\text{-}Tp_oA^{Bz}\ p_oC^{Bz}\ p_oTp_oTp_oTp_oTp_o^{Ac}Up_o\text{-}Cellulose$$

1) $DMT\text{-}A^{Bz}\ p_oG^{iBu}\ p_oG^{iBu}\ p_o$ ($\underline{13}$)
2) Ac_2O/Py (42%)
3) 2% BSA

$$HO\text{-}A^{Bz}\ p_oG^{iBu}\ p_oG^{iBu}\ p_oTp_oA^{Bz}\ p_oC^{Bz}\ p_oTp_oTp_oTp_oTp_o^{Ac}Up_o\text{-}Cellulose$$

$DMT\text{-}A^{Bz}\ p_oG^{iBu}\ p_oA^{Bz}\ p_o$ ($\underline{14}$)   (45%)

$$DMT\text{-}A^{Bz}\ p_oG^{iBu}\ p_oA^{Bz}\ p_oA^{Bz}\ p_oG^{iBu}\ p_oG^{iBu}\ p_oTp_oA^{Bz}\ p_oC^{Bz}\ p_oTp_oTp_oTp_oTp_o^{Ac}Up_o\text{-}Cellulose$$
$$(\underline{15})$$

1) conc. NH_3

2) 80% AcOH

$$HO\text{-}ApGpApApGpGpTpApCpTpTpTpT\text{-}OH$$
$$(\underline{16})$$

Fig. 3. Gel electrophoretic analysis on a 20% polyacrilamide slab, after incubation with $[\gamma - ^{32}P]$ ATP and polynucleotide kinase.

DMT-A$^{Bz}_{p_o}$G$^{iBu}_{p_o}$G$^{iBu}_{p_o}$ (13) and DMT-A$^{Bz}_{p_o}$G$^{iBu}_{p_o}$A$^{Bz}_{p_o}$ (14), where p_o = p-chlorophenyl-phosphate.

The trimers (12), (13), and (14) were prepared from the corresponding fully protected derivatives[2,3] by removal of the cyanoethyl group from the 3'-terminal phosphotriesters with Et_3N-Py-H_2O (1:3:1 v/v).

The three cycles were carried out under the same conditions as described in the synthesis of oligothymidilic acids. Scheme 3 outlines the synthesis of (16) and the yields per coupling calculated from h.p.l.c. analysis (see fig. 5) are shown in parentheses.

The final polymer (15, 85 mg) was treated with base (conc. aq. NH_3) and acid (80% aq. AcOH), the cellulose residue was pelleted off and the supernatant evaporated to dryness. The residue, dissolved in 4% aq. NH_3 (2 ml), was washed with ether and used for the isolation of (16). This was accomplished by repeated runs on h.p.l.c.; the material corresponding to the last eluted peak was collected and pooled. After desalting on Bio-Gel P2, the product (16) was analyzed as described for the oligothymidilic acids. From 0.5 ml of crude solution ca. 10 $A_{254 \; nm}$ units of pure product (16) was obtained. Gel electrophoretic analysis showed that (16)
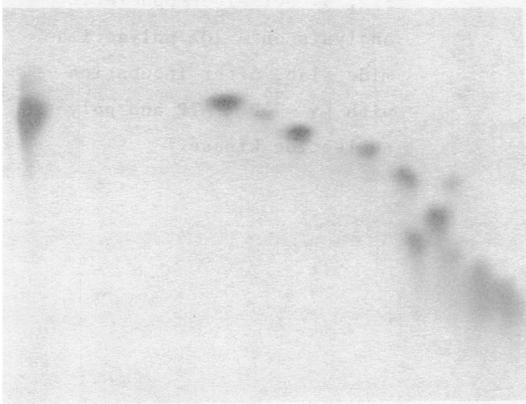
Fig. 4.   Two–dimen-
sional analysis of a partial
venom phosphodiesterase digest
of d(AGAAGGTACTTTT).Dimension 1,
electrophoresis on cellulose
acetate strip at pH 3.5, dimen-
sion 2, homochromatography on
DEAE cellulose thin layer plate.
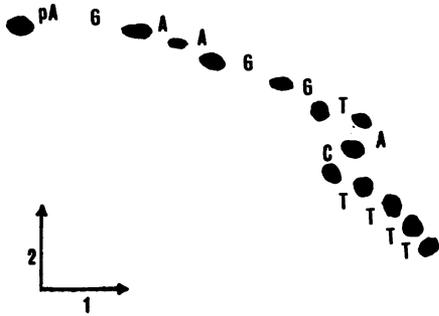
has the same mobility as $(dT)_{13}$ (see fig. 5) and two-dimensional sequence
analysis of the partial venom phosphodiesterase digest[16] confirmed that
(16) had the expected sequence (fig. 4).

The recovery of the excess trimers was accomplished by one of the
following procedures: a) purification of the trimer 3'-phosphodiester by
gel exclusion column chromatography on Sephadex LH-60[17], or b) reuse of
the filtrate solution for further condensation either on polymer support or
in solution. From the data obtained so far we calculated that 35-70% of
the excess trimer could be recovered.

CONCLUSIONS

This study demonstrates that a solid phase approach combined with the
trinucleotide phosphotriester methodology has several advantages over the
synthesis of oligonucleotides in solution[2]. Only one kind of

Fig.5. (a) Synthesis of
d(AGAAGGTACTTTT) monitored by
h.p.l.c. after the second, third,
and fourth coupling cycle.

(b) Gel electrophoretic analysis
of the product (P) purified by
h.p.l.c. References are $d(T)_{13}$
and a dodecamer (Ref).
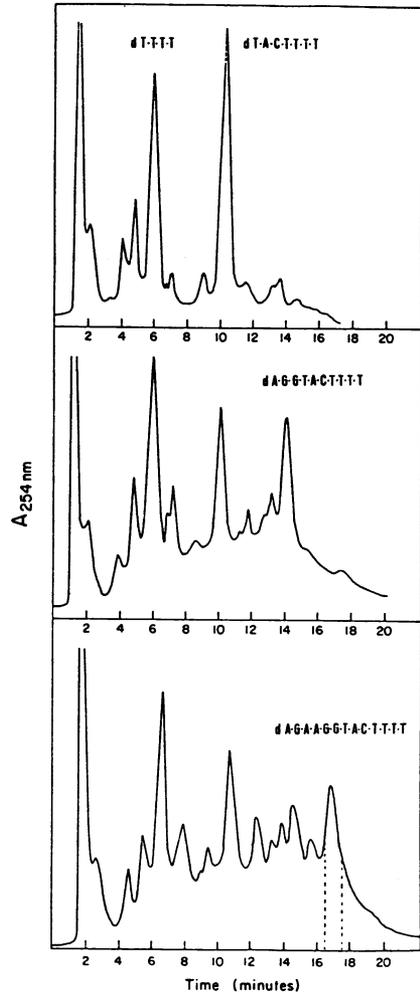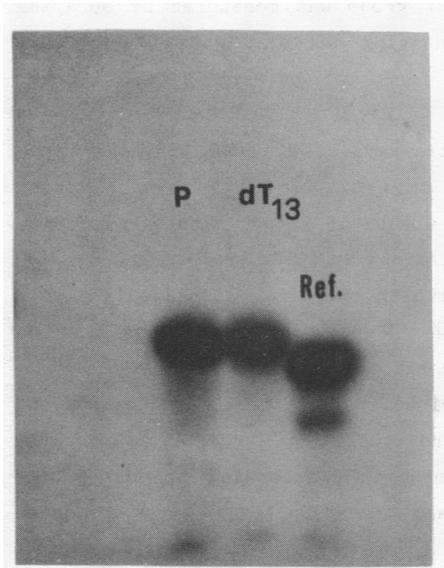
trinucleotide building blocks (3'-phosphate) is necessary for the chain
elongation. The synthesis is faster, because washing procedures substitute
column chromatography on silica gel. This also reduces the loss of
oligonucleotidic material. Because of the recovery of the unreacted
trinucleotide at the end of each coupling step, and the reduced scale of
synthesis on the cellulose, the amount of trimer used per coupling is
significantly reduced. Functionalization of cellulose with a ribo-
deoxyribo dinucleoside diphosphate containing uridine and one of the four

deoxyribonucleosides provides the starting polymer necessary for the solid-phase synthesis of DNA fragments with different bases at the 3'-end.

In the synthesis of an oligonucleotide 13 bases long with trinucleotides of different sequence, the yields per coupling were lower than the ones in the synthesis of homopolymers $(dT)_m$. However, no base specificity has so far been observed.

MATERIALS AND GENERAL PROCEDURES

Analytical grade reagents were used throughout this synthetic study. Pyridine was dried over KOH, distilled, and stored over molecular sieves (type 4A). Thin layer chromatography (TLC) was performed on silica gel plates (Merck, Silica Gel 60 F254) with methanol/chloroform (1:9 v/v) as solvent system. The presence of the DMT group was monitored by spraying the TLC plate with 10% aq. H_2SO_4 and heating the plate to 60°C. Purification by column chromatography was performed on silica gel (Merck, Silica Gel 60H).

H.p.l.c. was performed with a Spectra-Physics SP 8000 Liquid Chromatograph on Permaphase AAX (du Pont) (50 cm x 0.4 cm I.D.) using a linear gradient of water to 1M KCl, pH 4.5, at a rate of 3% per minute. The elution was performed at constant flow (3 ml/min.) at 60°C.

Cellulose (Whatman CC 31) was washed with pyridine, rinsed extensively with methanol, and dried _in vacuo_ over P_2O_5. All reactions at the polymer level were carried out under magnetic stirring.

The fully protected mono- and trinucleotides were synthesized by following the procedures described previously[2,3]. 2,4,6-Triisopropyl-benzenesulfonyltetrazole (TPSTe) was synthesized according to the procedure reported in the literature[10] and recrystallized from benzene-petroleum ether.

2'(3')-0-Acetyluridine-5'-0-2-cyanoethyl-p-chlorophenylphosphate (5).

Uridine, (1), (61 mmole) was suspended in a solution of anhydrous THF (200 ml) containing trimethyl orthoacetate (125 mmole) and anhydrous benzenesulfonic acid (BSA) (0.6 g)[13]. After stirring at 25°C for ten minutes under exclusion of moisture, the solution had become clear. TLC showed complete conversion of uridine into a new compound with higher R_f. The reaction was quenched by addition of methanol saturated with NH_3 (5 ml) and the solvents removed under reduced pressure. The oily residue was dissolved in $CHCl_3$ (300 ml) and extracted with saturated aq. $NaHCO_3$

solution (150 ml) and water (150 ml). The organic layer was dried (MgSO$_4$) and evaporated to dryness.

Without further purification, 2',3'-O-methoxyethylideneuridine (2) (ca. 60 mmole) was dissolved in anhydrous acetonitrile (200 ml) containing 1-N-methylimidazole (200 mmole). The solution was chilled (ice-water) and freshly prepared 2-cyanoethyl-p-chlorophenylphosphorochloridate[2,14] (90 mmole) in CH$_3$CN (25 ml) was added dropwise over a 45 minute period. The solution was then left to warm up to room temperature. TLC analysis showed virtually complete phosphorylation of (2). The solvent was removed under reduced pressure, and the residual oil partitioned between CHCl$_3$ (200 ml) and aq. NaHCO$_3$ (150 ml). The CHCl$_3$ layer was washed with water (150 ml) and then concentrated to a small volume (50 ml). This solution was triturated twice with pentane (500 ml) to remove most of the 1-N-methylimidazole. The product obtained (3) was treated with 80% aqueous acetic acid (200 ml) at room temperature for 10 minutes. Finally, the acetic acid was removed under reduced pressure by coevaporation with absolute ethanol. TLC of the glass residue showed only one product. Purification of (5) was performed on a silica gel column (150 g). After washing with CHCl$_3$ the pure product was eluted with a solution of MeOH in CHCl$_3$ (1 to 4% v/v). The fractions containing the product were pooled and concentrated to a small volume. Precipitation of (5) from petroleum ether (35° - 60° C) gave a white solid (14.5 g; 50% yield based on uridine). R_f=0.31; UV (95% EtOH): λ_{max} 258 nm (ε 9,600), λ_{min} 226 nm (ε 1,800). Anal: C$_{20}$H$_{21}$N$_3$O$_{10}$PCl (529.5); calcd: C 45.32, H 3.96, Cl 6.70, N 5.90, P 7.90; found: C 45.2, H 4.09, Cl 6.91, N 5.88, P 7.98.

5'-O-2-Cyanoethyl-p-chlorophosphophenyl-2'(3')-O-acetyluridilyl-[2'(3')-3']-5'-O-dimethoxytritylthymidine p-chlorophenylester(7a).

5'-O-Dimethoxytritylthymidine-3'-O-2-cyanoethyl-p-chlorophenylphosphate (7.0 mmole) was treated with a solution of triethylamine/pyridine/water (1:3:1 v/v) (50 ml) at room temperature for 25 minutes. The phosphodiester derivative (6) so obtained was dried by coevaporation with anhydrous pyridine (3 x 25 ml). 2'(3')-O-Acetyluridine-5'-O-2-cyanoethyl-p-chlorophenylphosphate (5) (6.6 mmole) was added to derivative (6) and the mixture dried again with pyridine (30 ml). TPSTe (10.5 mmole) in anhydrous pyridine (50 ml) was added to this solution. The reaction mixture was kept in vacuo and protected from the light for 16 hours. The reaction was stopped by addition of water, and the solvents removed under reduced pressure. The

residual material in $CHCl_3$ (250 ml) was extracted with aqueous $NaHCO_3$ (100 ml), and then washed with water (100 ml). The combined $CHCl_3$ extracts were evaporated to dryness.

Chromatography of the product on silica gel (75 g) was performed using $MeOH/CHCl_3$ (1:99 v/v) as solvent system. The fractions containing the product were evaporated to a glass. Precipitation of this material from petroleum ether (35° - 60° C) gave a colorless solid (2.6 g, 32% yield). Its homogeneity was confirmed by TLC analysis (R_f=0.65).

Anal: $C_{57}H_{55}N_5O_{19}P_2$ (1247); calcd: C 54.90, H 4.44, Cl 5.68, N 5.61, P 4.96; found C 54.61, H 4.44, Cl 5.92, N 5.91, P 5.31.

To confirm the release mechanism, this compound (7a, 10 mg) was treated with conc. aqueous ammonia/pyridine (9:1 v/v; 2 ml) for 30 minutes at 60° C TLC analysis showed a complete degradation of (7a) into a baseline material and a compound containing the DMT group. This latter compound was identified as 5'-DMT-thymidine (TLC). Removal of DMT with 2% BSA gave a compound identical to thymidine.

Functionalization of cellulose with (7b)

The fully protected dimer (7a) was treated with a solution of pyridine-triethylamine-water (3:1:1 v/v) (20 ml) at room temperature. After 25 minutes, TLC showed complete conversion of (7a) into its phosphodiester derivative (base-line material (7b)). The latter material was dried by coevaporation with pyridine (3 x 15 ml) and then reacted with dry cellulose (Whatman CC 31) (0.7 g) in anhydrous pyridine (3 ml) in the presence of TPSTe (0.28 g). The mixture was stirred overnight (16 hours) at room temperature. The polymer was then filtered (sintered glass filter M) and washed with dry pyridine. The filtrate was stored for the recovery of unreacted dimer (7b). The cellulose was washed extensively with MeOH (60 ml) and dried in a dessicator over KOH.

The extent of coupling of (7b) to the polymer was determined by treating a sample of cellulose (8, 10 mg) with 2% BSA in $CHCl_3$-MeOH (7:3 v/v) (2 ml) at 0°C for 10 minutes. After centrifugation of the solid particles, the absorbancy of the released DMT group was measured at 494 nm. A standard curve was obtained by using different amounts of 5'-O-dimethoxytrityl thymidine in 2% BSA stock solution. From the data obtained, we calculated an average incorporation of ca 100 μmoles dimer (7b) per g cellulose.

Another sample (8, 10 mg) was treated with conc. aqueous ammonia for 8 hours at 60°C. The polymer was centrifuged off and the supernatant concen-

trated to dryness. The residual material was dissolved in $CHCl_3$ (1 ml) and analyzed by TLC. The only product detected was 5'-O-dimethoxytrityl thymidine. Treatment of the chloroform solution with 4% BSA in CH_3OH-$CHCl_3$ (1 ml) gave thymidine and DMT residue. From the total amount of DMT (494 nm) we calculated that the ammonia treatment had released ca 95% of the 5'-O- DMT-thymidine from the polymer.

Synthesis of oligothymidilic acids, (dT)m (m=4,7,10,13,16,19,22, and 25).

The functionalized polymer (8, 100 mg; 9.7 μmole of dimer) was first treated with 2% BSA in $CHCl_3$/MeOH (7:3 v/v, 10 ml) at $0°C$ for 10 minutes as described above. After washing the polymer on the filter with methanol (50 ml) the cellulose was dried by flushing with anhydrous pyridine (20 ml) The trimer (9) (50 μmoles) was mixed with the polymer in a 10 ml round-bottom flask. The mixture was dried by coevaporation with pyridine (2 x 5 ml) and finally resuspended in anhydrous pyridine (1.0 ml). TPSTe (75 mg) was added quickly and the reaction mixture kept in vacuo. After two hours of continuous stirring, pyridine (2 ml) was added to the reaction vessel and the polymer recovered by centrifugation. The supernatant was collected separately for the recovery of excess trimer (9). The polymer so obtained was resuspended in a solution of pyridine-acetic anhydride (10:1 v/v, 1 ml) and stirred for 30 minutes. After washing the cellulose on a filter with pyridine (8 ml) and methanol (30 ml), the polymer was ready for the next coupling step (see Table 1).

The following cycle: a) deblocking of DMT group from the polymer with BSA, b) coupling of the polymer so obtained with trimer (9) in pyridine containing TPSTe, and c) acetylation of the resulting polymer with pyridine/Ac_2O was repeated seven more times under the same conditions.

Release of the oligonucleotides from the polymer and removal of protecting groups.

The polymer (10, n=1-8, 10 mg) suspended in pyridine (0.5 ml) was treated with concentrated aqueous ammonia (4 ml) at $60°C$ for 8 hours. The solid polymer was removed by centrifugation and discarded. The supernatant was evaporated to dryness, and the residue treated with 80% aq. acetic acid at room temperature for 15 minutes. The acetic acid was removed by evaporation under reduced pressure and the solid residue redissolved in 4% aqueous ammonia (2 ml). After washing with ether (3 x 1 ml) the aqueous solution was analyzed on h.p.l.c. For each coupling step the chromatogram

showed the formation of a new product with higher retention time (see figs. 1 and 2). The yields per coupling were calculated from the peak areas and are based on the next shorter oligomer. For each elongation step the final product, after complete deblocking, was isolated by preparative h.p.l.c on Permaphase AAX. The fractions corresponding to the last peak were pooled (0.5 - 1.0 O.D. units at 254 nm), the solution concentrated and desalted on Bio-gel P-2. Gel electrophoretic analysis on a 20% acrylamide slab of the oligomers $(dT)_m$ (m=13,16,19, 22, and 25) after their incubation with $[\gamma-^{32}P]$-ATP and T_4 polynucleotide kinase, showed that each fragment released from the polymer had the expected size (fig. 3). Furthermore, $[^{32}P]$ $(dT)_{13}$ was analyzed by two dimensional sequence analysis of its partial venom phosphodiesterase digestion products[16]. The analysis confirmed the structure of this compound.

Synthesis of d(AGAAGGTACTTTT) (16).

The functionalized polymer (8, 125 mg) was treated with 2% BSA as described above and used for the synthesis of the 13-base long oligonucleotide of defined sequence (16). This was accomplished by using four different trimers: $DMT-Tp_oTp_oTp_o$ (9), $DMT-Tp_oA^{Bz}p_oC^{Bz}p_o$ (12), $DMT-A^{Bz}p_oG^{iBu}p_oG^{iBu}p_o$ (13), and $DMT-A^{Bz}p_oG^{iBu}p_oA^{Bz}p_o$ (14), where p_o = p-chlorophenylphosphate.

The coupling of these trimers (50 μmoles each) to the polymer was carried out in the sequential order shown in scheme 3, under the same conditions as reported above for the synthesis of oligothymidilates. The yields obtained for each coupling are shown in scheme 3 and were calculated by h.p.l.c. analysis based on the next shorter fragment.

The fully protected oligomer obtained after the fourth cycle was released from the polymer and deblocked with ammonia and acetic acid as described above. The isolated product (16) (h.p.l.c.) had the same mobility as $(dT)_{13}$ on 20% polyacrylamide slab (see fig. 5). Furthermore, two-dimensional sequence analysis of the partial venom phosphodiesterase digest of (16) confirmed the expected sequence (fig. 4).

Recovery of the excess trinucleotides

A. Chromatography on Sephadex LH-60.

The filtrate pyridine solution (3 ml) containing the excess trinucleotide $(DMT-Tp_oA^{Bz}p_oC^{Bz}p_o$, ca 40 μmole) and TPSTe was used for the recovery of this compound (12) by gel exclusion chromatography. Water (0.5

ml) was first added and the solution evaporated to dryness. The residue was dissolved in THF/MeOH (95:5 v/v, 4 ml) and applied to a column of Sephadex LH-60 (0.7 x 100 cm) preequilibrated in the same solvent. Isocratic elution of the product was obtained with THF/MeOH (95:5 v/v). Fractions of 4 ml were collected. The fractions containing product (fraction 38 to 50) (detected as DMT-positive baseline material) were pooled, concentrated and the trimer precipitated from petroleum ether (25 mg, 35% recovery yield).

B. Reuse of the excess trimer for synthesis in solution .

The pyridine solution containing the excess DMT-$Ap_o^{Bz}Ap_o^{Bz}Gp_o^{iBu}$ (ca 40 µmole) and TPSTe was dried by coevaporation with pyridine (2x2 ml). 3'-0-Anisoyl- 2-N-isobutrylguanosine (14 mg, 29 µmole) was added and the mixture dried by coevaporation with pyridine (2x2 ml). Additional TPSTe (50 mg) was added, and the reactants dissolved in pyridine (1 ml). The reaction mixture was kept in vacuo for 6 hours. TLC analysis showed complete conversion of the nucleoside into the product tetramer. This latter compound was purified on a silica gel column and obtained as a homogeneous solid after precipitation from petroleum ether (23 mg).

C. Reuse for solid-phase synthesis.

The pyridine solution (3 ml) recovered from a coupling step containing the trimer DMT-$Tp_oTp_oTp_o$ (9, ca 40 µmole), was reused to elongate the oligonucleotidic chain on cellulose. The trimer was added to the polymer (10) (n=1, 100 mg) and the suspension dried by coevaporation with pyridine. An additional portion of TPSTe (75 mg) was added and the reaction mixture suspended in pyridine (1 ml). The fully protected oligomer was released from the polymer and deblocked with aq. ammonia and aq. acetic acid as described above. H.p.l.c. analysis showed a coupling yield of 43% for $d(T)_7$ based on $d(T)_4$.

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Abbreviations: DMT = 4,4'-dimethoxytrityl, BSA = benzenesulfonic acid, TPSTe = 2,4,6,-Triisopropylbenzenesulfonyltetrazole, CE = 2-cyanoethyl, Bz = benzoyl, iBu = isobutryl, Ac = acetyl.

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