Solid support synthesis of oligothymidylates using phosphorochloridates and 1-alkylimidazoles

Reynaldo C. Pless and Robert L. Letsinger,

Department of Chemistry, Northwestern University, Evanston, Illinois 60201, USA

ABSTRACT

A study of the synthesis of oligothymidylates via phosphotriester inter-mediates on a polystyrene support is described. The sequence involves condensation of a phenyl nucleoside-3'-phosphorochloridate with the 5'-hydroxyl group of the carrier bound oligonucleotide derivative in the presence of 1-methylimidazole. Conditions for preparation of the phenyl nucleo-side phosphorochloridate as well as for the condensation on the support are discussed. d-TpTpTpT was obtained in 31% overall yield from carrier bound thymidine in one series of experiments, and d-TpTpTpTpT was ob-tained in 9% yield in another. The cycle for addition of one nucleotide unit can be completed in about six hours.
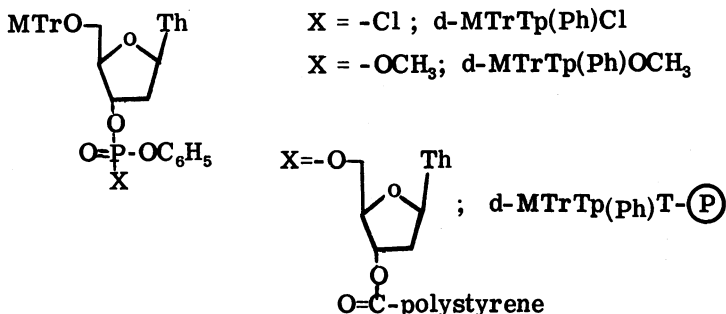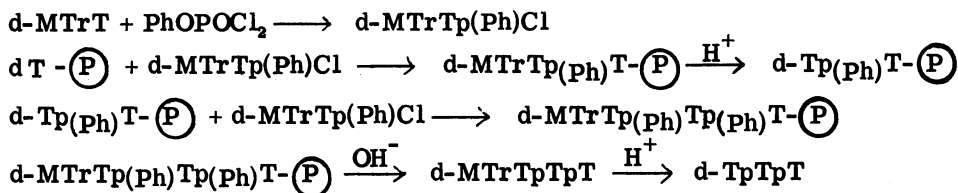
INTRODUCTION

Although early examples of oligonucleotide synthesis on an insoluble carrier utilized phosphotriester intermediates[1], most of the recent work has employed phosphodiester coupling methods[2]. An advantage of the latter route for polymer support work has been that the phosphorylating interme-diates are more reactive than those leading to phosphotriesters.

In a series describing the synthesis of oligoribonucleotide phosphotri-esters in solution, Reese et al.[3] reported that the condensation of phosphoro-monochloridates and phosphorodichloridates with nucleosides is greatly accelerated by 1-alkylimidazoles. On the basis of this observation it appeared that an oligonucleotide synthesis might be developed that pro-ceeded rapidly on a polymer support and also offered protection for the internucleotide links as phosphotriesters. We report in the present paper a study of an adaptation of the Reese sequence to the synthesis of oligo-thymidylates on a polystyrene carrier.

The synthetic approach is outlined in scheme 1, where dT-Ⓟ repre-sents thymidine linked through the 3'-OH via an ester bond to an insoluble

copolymer of styrene and p-vinylbenzoic acid, and MTr- represents the mono-p-methoxytrityl group. Other abbreviations used in the text are defined by the formulas in scheme 1.

d-MTrT + PhOPOCl$_2$ $\longrightarrow$ d-MTrTp(Ph)Cl

dT -Ⓟ + d-MTrTp(Ph)Cl $\longrightarrow$ d-MTrTp$_{(Ph)}$T-Ⓟ $\xrightarrow{H^+}$ d-Tp$_{(Ph)}$T-Ⓟ

d-Tp$_{(Ph)}$T-Ⓟ + d-MTrTp(Ph)Cl $\longrightarrow$ d-MTrTp$_{(Ph)}$Tp$_{(Ph)}$T-Ⓟ

d-MTrTp(Ph)Tp(Ph)T-Ⓟ $\xrightarrow{OH^-}$ d-MTrTpTpT $\xrightarrow{H^+}$ d-TpTpT

MTrO—⟨O⟩—Th
O=P-OC$_6$H$_5$
X

X = -Cl ; d-MTrTp(Ph)Cl
X = -OCH$_3$; d-MTrTp(Ph)OCH$_3$

X=-O—⟨O⟩—Th
O=C-polystyrene

; d-MTrTp(Ph)T-Ⓟ

Scheme 1.

RESULTS

In the implementation of this scheme, two initial reactions were examined in some detail: the preparation of d-MTrTp(Ph)Cl and the formation of the triesters on the solid support.

The reaction of 5'-O-mono-p-methoxytritylthymidine(d-MTrT) with phenyl phosphorodichloridate was monitored conveniently by thin layer chromatography of the products of hydrolysis and methanolysis. The hydrolytic products afford a measure of the extent of reaction of d-MTrT (R_f of MTrT on silica gel with ethyl acetate, 0.35; the hydrolyzed phosphorylated products remain at the origin in this system) and the products of methanolysis indicate the amount of active d-MTrTp(Ph)Cl in solution just prior to methanolysis (R_f for d-MTrTp(Ph)OCH$_3$ 0.41).

With the aid of this test it was found that the reaction of d-MTrT (0.31 M) with phenyl phosphorodichloridate (0.26 M) and 1-methylimidazole (0.52 M) in acetonitrile-dioxane (1:1, v/v) proceeded rapidly; however, only about half of the phosphorylated nucleoside remained active for triester formation after

an hour period. At a higher ratio of 1-methylimidazole to the dichloridate, 5:1, a further reaction occurred leading to a strongly fluorescent product[4]. The best conversion to active d-MTrTp(Ph)Cl was obtained when the ratio of 1-methylimidazole to dichloridate was 1:1; however, even in this case, some deactivation of the initially formed monochloridate was evident.

The less reactive base, 5-chloro-1-ethyl-2-methyl-imidazole, introduced by Reese as a catalyst in forming phosphotriesters,[5] was superior to 1-methyl-imidazole in this step. Thus, d-MTrT (0.21 M) reacted within an hour with phenyl phosphorodichloridate (0.11 M) in the presence of excess 5-chloro-1-ethyl-2-methylimidazole (0.83 M) in dioxane-acetonitrile (1:1) with no attendant deactivation of the resulting phosphorylating reagent or modification of the thymine ring. When the solvent was dioxane, 5-chloro-1-ethyl-2-methyl-imidazolium chloride precipitated in semicrystalline form and d-MTrTp(Ph)Cl remained in solution. The reaction (0.30 M d-MTrT; 0.20 M phenyl phospho-dichloridate, and 0.30 M 5-chloro-1-ethyl-2-methylimidazole) proceeded within an hour, and there was no indication of deactivation of the reagent over a period of 72 hours.

At the triester forming stage, 1-methylimidazole appeared preferable to 5-chloro-1-ethyl-2-methylimidazole as a catalyst. In a model reaction with d-MTrTp(Ph)Cl (0.125 M), 1-methylimidazole (0.33 M), and 3'-O-acetylthymidine (0.25 M), the phosphorochloridate was converted to fully blocked dinucleoside phosphate (d-MTrTp(Ph)T(OAc)) in 80% yield in 10 minutes. When 5-chloro-1-ethyl-2-methylimidazole was used in place of 1-methylimidazole, several hours were required to obtain the same conversion.

Data on the use of d-MTrTp(Ph)Cl in synthesizing d-TpT in polymer support reactions are given in Table 1. In these cases d-MTrTp(Ph)Cl was prepared in dioxane solution, and the polymer reactions were carried out with 1:1 dioxane-acetonitrile (to avoid precipitation of the imidazolium salts in the insoluble polymer system). Products were cleaved from the carrier by sodium hydroxide in dioxane-water, and the methoxytrityl group was removed from d-MTr-TpT by hot aqueous acetic acid.

The results (exp. 1, 2) indicate that the maximum yield (73%) achieved with 1-methylimidazole as catalyst is reached within 1.5 hours. Recycling the polymer once or twice with fresh d-MTrTp(Ph)Cl solution (exp. 1b and 2) did not

Table 1. Synthesis of dTpT on Polymer Support

Exp.	d-MTrTp(Ph)Cl (moles/l.)	Mole ratio d-MTrTp(Ph)Cl to dT on polymer	Catalyst (moles/l.)	Time hr.	% d-TpT
1[a]	.07	5.4	MI[d](.09)	1.5	73
1[b]	.07	5.4	MI (.09)	4.5	73
2[c]	..07	5.4	MI (.09)	2.3	73
3	.20	10.	CI[e] (0.6)	1.5	51
4	.20	10.	CI (0.6)	3.8	63

(a) A portion of the polymer was analyzed after 1.5 hr.   (b) The remaining polymer was recycled with fresh reagents at the same initial concentration for two additional periods of 1.5 hr each.   (c) The polymer was exposed to fresh reagents after 45 min and after 90 min of reaction.   (d) 1-Methyl-imidazole.   (e) 5-Chloro-1-ethyl-2-methylimidazole.

further increase the yield. It may be noted (exp. 3,4) that the condensations catalyzed by 5-chloro-1-ethyl-2-methylimidazole were relatively slow even when large excesses of d-MTrTp(Ph)Cl and the imidazole were employed.

The product from experiment 2 (d-MTrTp$_{(Ph)}$T-(P)) was used to study step-wise chain extension to the tetranucleotide stage. Details are provided in the experimental section, and data on the product distribution at each stage are summarized in Table 2. In this series demethoxytritylation of the polymer bound oligomers was effected with 80% aqueous acetic acid. The overall yields of d-TpT (stage 1), d-TpTpT (stage 2), and d-TpTpTpT (stage 3) from polymer bound thymidine were 73%, 35% and 31% respectively (calculated as mole per-cent of the nucleotidic products removed from the polymer carrier). These data correspond to an efficiency of chain elongation of 73%, 49% and 89% in the successive stages of synthesis. The cause for the low conversion in the second cycle is not known. Values for the release of methoxytritanol from the polymers indicated that the condensation at stage 2 had proceeded almost as well as stage 1 (80% of the theoretical amount of methoxytritanol, based on the amount of thymidine serving as the anchor to the support, was released from d-MTrTp(Ph)Tp(Ph)T-(P); the corresponding value at the d-MTrTp(Ph)T-(P) stage was 90%).

Table 2. Analysis of d-MTr[Tp(Ph)]$_n$-T-(P)

Stage	Band	R_f	O.D.$_{266}$ Units	Assignment[a]	Mole %
I; n=1		Solvent H			
	1	0.03	17.1	d-TpT	73
	2	0.18	1.2	d-PhpT[b]	8
	3	0.25	0.62	d-TpPh[b]	4
	4	0.32	0.28	?	(2)[c]
	5	0.55	1.52	dT	12
II; n=2		Solvent A			
	1	0.02	0.17	?	(1)[d]
	2	0.15	4.5	d-TpTpT	35
	3	0.36	4.2	d-TpT+(d-PhpT)[e]	45 + (7)[e]
	4	0.66	0.96	dT	12
III; n=3		DEAE Paper			
	1	0.08	7.0	d-TpTpTpT	31
	2	0.19	4.5	d-TpTpT	26
	3	0.45	3.2	d-TpT+(d-PhpT)[e]	24 + (4)[e]
	4	0.76	0.93	dT	15

(a) See experimental section. (b) Assigned on basis of comparisons with known samples in solvent H. (c) Monomeric species assumed. (d) Tetrameric species assumed. (e) The products in fraction 3 were separated by electrophoresis and by chromatography in solvent H, and the quantities estimated from the intensities of the spots.

Independent studies with d-MTrT-(P) showed that 80% acetic acid under standard conditions (3 hours at 100°) released only 92-94% of the amount of methoxytritanol that could be liberated by action of 5% trifluoracetic acid in benzene at room temperature. As another test of the synthetic sequence, d-TpTpTpTpT was prepared from   dT-(P), with the difference that the methoxytrityl groups were cleaved from the polymer bound oligothymidylate derivatives with 5% trifluoracetic acid in benzene rather than with aqueous acetic acid. At the various stages in this synthesis the overall yields of the desired species based on total material eluted were: (stage I) d-TpT,

64%; (stage II) d-TpTpT, 38%; and (stage IV) d-TpTpTpTpT, 9%. The polymer was not analyzed at the tetranucleotide stage. Figures 1 and 2 show the elution profiles on DEAE cellulose of the base hydrolysates, after demethoxytritylation, at the tri- and pentanucleotide stages of synthesis. Major peaks 1, 2, 4 and 5 in fig. 1 were identified as pyridine, dT, d-TpT, and d-TpTpT, respectively. At the penta stage the mixture was considerably more complex. The major peaks were: 1, pyridine; 2, thymidine; 5, d-TpTpT; 6, d-TpTpTpT + a small amount of an unidentified nucleotidic material; and 7, d-TpTpTpTpT + a minor component that was subsequently removed by electrophoresis.
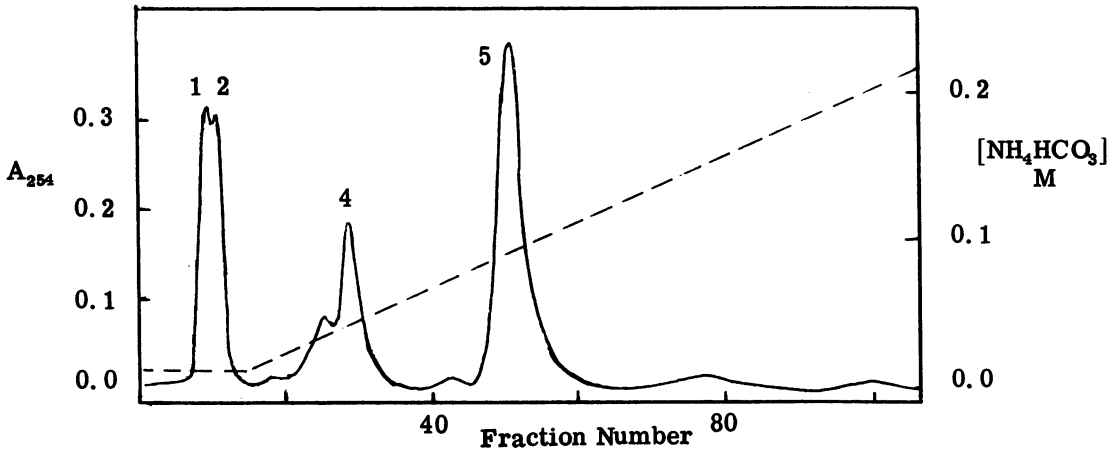

Fig. 1: Fractionation on DEAE-Cellulose of Products
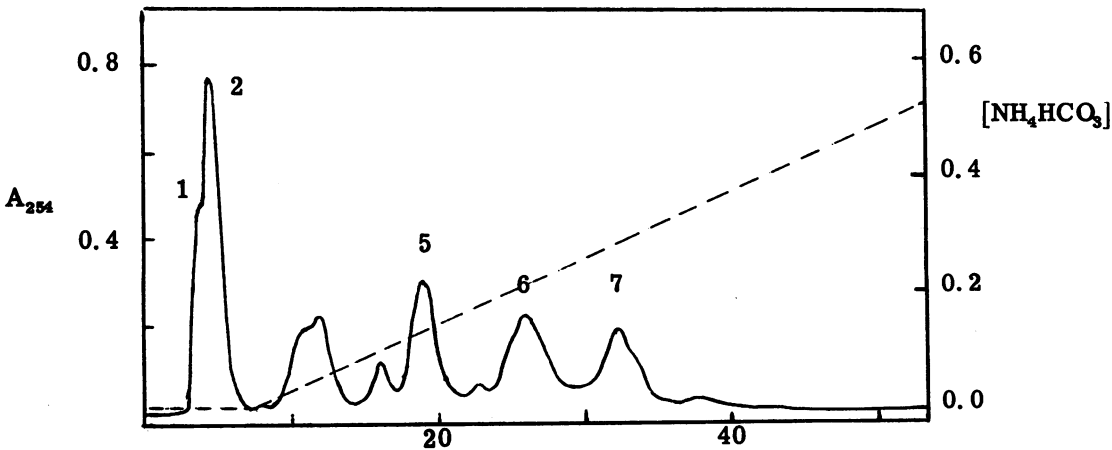from "Trimer" Stage of Synthesis

Fig. 2: Fractionation on DEAE-Cellulose of Products
from "Pentamer" Stage of Synthesis

DISCUSSION

This study shows that a triester type synthesis employing an aryl phosphorodichloridate can be used conveniently to construct short chain oligothymidylates on an insoluble support. The manipulations are simple and the procedure is rapid. When trifluoroacetic acid is used for demethoxytritylation, the complete cycle for addition of one nucleotide unit can be accomplished in less than six hours. This represents a great advantage in time relative to previous triester type syntheses, which require several days for coupling a single nucleotide unit to the chain. The procedure is somewhat more rapid than those employing a diester synthetic route and the yields are comparable.

The work also points up a number of problems to be solved if long chains are to be made routinely. The methoxytrityl group is useful for exploratory studies since it provides a convenient means for assaying the extent of reaction on the polymer support. On the other hand, it is difficult to remove completely from polymer bound nucleotides with aqueous acetic acid, and recent model studies on cleavage with 5% trifluoracetic acid in benzene indicate that the terminal 5'-OH of a nucleotide phosphotriester derivative is partially capped off and made inaccessible to phosphorylating agents when this strong acid is employed. Better protecting groups are needed for both the 5'-OH and for the phosphoryl group. Recent work by Van Boom et al. has shown that phenyl esters at 3'-5' internucleotide links do not hydrolyze selectively when treated with alkali; some 3'-5' internucleotide cleavage occurs as well as the desired hydrolysis to a phosphodiester[6]. Nonselective hydrolysis could account for the appearance of 5'-terminal phenyl esters (e.g. d-PhpT, table 2) in the product mixtures[7] and would be expected to reduce the yield at the d-TpTpTpT stage by ~20%[6]. Ultimately, for long chain syntheses on supports, phosphorylation procedures are needed that are both rapid and highly selective for the terminal hydroxyl group of the nucleotide chain.

EXPERIMENTAL SECTION

Materials and Methods. Phenyl phosphorodichloridate was distilled at reduced pressure and stored in sealed glass ampules. Dioxane was rendered anhydrous by refluxing over lithium aluminum hydride and distilling

onto Linde Molecular sieves 4A. Acetonitrile was first refluxed with po-
tassium permanganate, then with sodium hydride, and finally distilled from
concentrated sulfuric acid onto Linde Molecular sieves 3A. 1-Methyl-
imidazole and 5-chloro-1-ethyl-2-methylimidazole[5] were vacuum distilled
and stored over Molecular Sieves 4A.

Electrophoresis was carried out on Whatman 3MM paper in 0.05 M
sodium phosphate, pH 7.2, using a Savant flat plate electrophoretic cham-
ber and a Savant Model HV 2000 volt power source operated at 2000 volts.
Mobilities are defined relative to thymidine-5'-phosphate, as R_m^{dpT} values.
Eastman Chromagram 6060 silicagel plates were used for thin layer chroma-
tography (tlc). Paper chromatography was performed on Whatman 3MM in
the following systems: solvent A: 2-propanol-concentrated aqueous ammonia-
water (7:1:2); solvent F: 1-propanol-concentrated aqueous ammonia-water
(55:10:35), and Solvent H: 1-butanol-water (86:14). Fractionation on DEAE
cellulose was conducted on Whatman DE 81 sheets or on Cellex-D (Biorad
Laboratories) columns.

For quantitative evaluation of paper chromatograms, the bands were
eluted with water and their absorbance was measured against the eluates
from the appropriate paper blanks. Molar amounts were calculated using
extinction coefficients of 9650, 18500, 25800, 34000, and 42500 $M^{-1}cm^{-1}$
for dT, d-TpT, d-TpTpT, d-TpTpTpT, and d-TpTpTpTpT, respectively[8].

General Procedures. For the carrier reactions the polymer was placed
in a plastic syringe provided with a plug of porous polypropylene at the hub,
and the phosphorylating mixture was periodically drawn into the syringe from
a septum covered vial and expelled from the syringe. This action served to
mix the polymer-carrier components with reactants and to compress the
polymer to expel residual solvent. After reaction, the polymer was washed
by repeatedly drawing in and expelling fresh solvent.

For detritylation with 5% trifluoroacetic acid in benzene, the polymer
was similarly subjected to cycles consisting of 5 min exposure to the acidic
medium followed by washing with benzene, acetonitrile, and benzene. Three
to six such cycles were generally required to effect complete cleavage of the
protecting group, as indicated by lack of yellow color in the acid wash solu-
tion. Detritylation with 80% aqueous acetic acid was carried out in a small

glass vessel at 100°, following which the polymer was filtered off and washed. The amount of methoxytritanol was determined quantitatively from the absorption spectrum in 20% trifluoracetic acid in benzene; λ_{max} 478 nm, ϵ_{max} 60,000 $M^{-1} cm^{-1}$, in good agreement with values found in acetic acid-hydrochloric acid (λ_{max} 480 nm, ϵ_{max} 60,300 $M^{-1} cm^{-1}$)[9].

Carrier-bound material was released by suspending the polymer in 0.5 M NaOH in dioxane-water (1:1, v/v) at room temperature for 20 hr ("standard conditions"). The mixture was neutralized with Dowex 50W-X8 (pyridinium form); then the resin was filtered off and washed repeatedly with dioxane-water (1:1), dioxane, and water. The filtrate and the washings were combined, lyophilized, and redissolved in water for analysis. With the low cross-linked polymer used in the model syntheses of d-MTrTp(Ph)T-(P) (0.05% p-divinylbenzene), alkaline hydrolysis for 20 hr regularly gave 80% to 100% recovery of carrier bound species. The extended oligonucleotide syntheses were conducted on a more highly cross-linked support (0.2% p-divinylbenzene). Alkaline cleavage from this polymer was more sluggish. Treatment with 0.5 M sodium hydroxide in dioxane-water (1:1) for 72 hr released 90% of the total polymer bound thymidine in dT-P, determined from quantitative detritylation of the precursor, d-MTrT-(P). The standard 20 hr hydrolysis liberated 50% to 75% of the carrier bound species. All recovery data have been calculated for dT-(P), i.e. correcting for the theoretical weight increase imparted by the synthesized material. The nucleotidic products were routinely characterized by direct comparison with standards by paper chromatography in solvents A and H and electrophoresis at pH 7.2.

Preparation of d-MTrTp(Ph)Cl Solution. 5'-O-Mono-p-methoxytrityl-thymidine (667 mg, 1.30 mmol) was dried by azeotropic distillation of two 5 ml portions of dioxane. A solution of phenyl phosphorodichloridate (140 μl, 0.87 mmol) and 5-chloro-1-ethyl-2-methylimidazole (165 μl, 1.30 mmol) in anhydrous dioxane (6 ml) was added and the mixture was stirred at room temperature for 24 hr. The precipitate was allowed to settle, and the supernatant liquid was removed for polymer reactions and analysis. Hydrolysis of a sample in 0.5 M 5-chloro-1-ethyl-2-methylimidazole in dioxane-water (1:1) for 30 min gave rise to two spots on tlc (silica gel, ethyl acetate); R_f 0.39 (d-MTrT) and R_f 0.0 (d-MTrTpPh), the ratio of intensities being approx-

imately 1:2. After solvolysis of the supernatant in 0.5 M methanolic 5-chloro-1-ethyl-2-methylimidazole for 30 min, a strong spot at R_f(EtOAc) 0.42 (d-MTrTp(Ph)OCH$_3$ and d-MTrT) and a faint trace at the origin were observed. Spraying with 10% perchloric acid gave a positive methoxytritanol test for all uv-absorbing bands.

dT-(P). An insoluble copolymer prepared from styrene, p-vinylbenzoic acid, and p-divinylbenzene in a molar ratio of 475:25:1[1], was extracted with hot dioxane and benzene for several hours to remove low molecular weight material. The polymer (5.17 g) was treated with 50 ml of benzene-thionyl chloride (1:1, v/v) for 5 hr at reflux, washed extensively with anhydrous benzene and ether, and dried to yield 4.72 g of the acid chloride. Infrared analysis indicated approximately 90% conversion of the acid groups (5.9 μ) to active acyl groups (5.65 μ and 5.75 μ).

For the synthesis of d-MTrT-(P), this acid chloride (2.536 g, ~1.1 m-equiv. of -COCl) was treated with 5'-O-mono-p-methoxytritylthymidine (3.10 g, 6 mmol) in anhydrous pyridine (30 ml) at room temperature for 40 hr. The polymer was recovered by filtration and washed with anhydrous pyridine. Residual acid groups were converted to the inert amide form by the following procedure[10]. The polymer was treated with 0.7 M p-nitrophenol in anhydrous pyridine for 16 hr, washed with pyridine-pyrrolidine (9:1, v/v) for 2 hr, then treated with p-nitrophenol and N, N'-dicyclohexylcarbodiimide (1.0 M and 1.1 M, respectively) in pyridine for 36 hr, and again washed with pyridine-pyrrolidine for 2 hr. After filtration, extensive washing with pyridine, benzene, ethanol, and ether, and drying, 2.352 g of d-MTrT-(P) was obtained.

d-MTrT-(P) (796.2 mg) was heated in 80% aqueous acetic acid (10 ml) at 100° for 3 hr. This treatment released 0.175 μmol of mono-p-methoxytritanol per mg of dT-(P) and yielded 764.2 mg of polymer. Treatment of a sample with 5% trifluoracetic acid in benzene released some additional methoxytritanol (0.012 μmol/mg of dT-(P)).

A sample of dT-(P)(11.5 mg) was subjected to standard alkaline hydrolysis for 20 hr. The hydrolysate chromatographed on paper in solvent A as a single uv-absorbing band (R_f = 0.66; 10.3 O.D.$_{267}$ units), correponding to 0.093 μmol of thymidine per mg of dT-(P). Standard alkaline treatment for 72 hr released 0.157 μmol of thymidine per mg of dT-(P)

d-Tp(Ph)T-$\circled{P}$: An aliquot of d-MTrTp(Ph)Cl solution (0.6 ml, 75 μmol of d-MTrTp(Ph)Cl) was diluted with anhydrous acetonitrile (0.6 ml). 1-Methylimidazole (9 μl, 113 μmol) was added, and the solution was immediately brought in contact with 102.0 mg of dT-$\circled{P}$ (approximately 18 μmol of carrier bound thymidine). After 45 min at room temperature, the polymer was filtered off and washed briefly with dioxane-acetonitrile (1:1). This cycle was repeated twice, using fresh aliquots of d-MTrTp(Ph)Cl solution (thus exposing the polymer to a total of 225 μmoles of d-MTrTp(Ph)Cl over a 2.25 hr period). After extensive washing with acetonitrile, dioxane, benzene, and ether, and drying in vacuo, 108.4 mg of d-MTrTp(Ph)T-$\circled{P}$ was obtained.

A sample of this polymer (22.5 mg) was subjected to alkaline treatment for 15 hr. The hydrolysate was detritylated with 80% aqueous acetic acid. Electrophoresis gave major bands at R_m^{dpT} -0.14 (thymidine); R_m^{dpT} 0.38 (d-TpT); R_m^{dpT} 0.52 (d-PhpT and d-TpPh), and a trace at R_m^{dpT} 0.64. Two-thirds of the total hydrolysate was analyzed by paper chromatography in solvent H (Table 2). The various species amount to 0.089 μmol per milligram of dT-$\circled{P}$.

d-MTrTp(Ph)T-$\circled{P}$ (83.6 mg) was heated at 100° in 8 ml of 80% aqueous acetic acid for 3.5 hr. This treatment released 0.141 μmol of mono-p-methoxytritanol per mg of dT-$\circled{P}$ and yielded after washing and drying 80.0 mg of d-Tp(Ph)T-$\circled{P}$.

d-Tp(Ph)Tp(Ph)T-$\circled{P}$. A sample of d-Tp(Ph)T-$\circled{P}$ (69.6 mg) was treated with d-MTrTp(Ph)Cl using the same conditions and relative amounts as in the synthesis of d-MTrTp(Ph)T-$\circled{P}$, except that the reaction time was 3x60 min. After washing and drying, the resulting polymer (d-MTrTp(Ph)Tp(Ph)T-$\circled{P}$) weighed 75 mg. Standard alkaline hydrolyses of a 6.2 mg sample and demethoxytritylation showed 0.115 μmol of methoxytritanol per mg of dT-$\circled{P}$. Paper chromatography in solvent H gave bands at R_f 0.00 (d-TpTpT); R_f 0.02 (d-TpT); R_f 0.14 (d-PhpT); and R_f 0.51 (thymidine). The electrophoretogram showed major bands at R_m^{dpT} -0.09 (dT); R_m^{dpT} 0.40 (d-TpT); R_m^{dpT} 0.52 (d-PhpT); R_m^{dpT} 0.64 (d-TpTpT); and a trace at R_m^{dpT} 0.77. Two-thirds of the total base hydrolysate was analyzed by paper chromatography in solvent A (see table 2). A total of 0.131 μmol of the nucleotidic species (per

mg dT-(P)) was liberated.

On heating with 80% aqueous acetic acid (100°, 3.5 hr) the polymer (68.5 mg) released 0.126 μmol of methoxytritanol per mg dT-(P) and yielded d-Tp$_{(Ph)}$Tp$_{(Ph)}$T-(P) (66.7 mg).

d-Tp$_{(Ph)}$Tp$_{(Ph)}$Tp$_{(Ph)}$T-(P). Treatment of a portion of the above polymer (55.2 mg) with d-MTrTp(Ph)Cl as before yielded 64.5 mg of the polymer deriv- ative. Standard alkaline hydrolysis of 21.6 mg of the product followed by de- methoxytritylation of the released nucleotides produced 0.061 μmol of methoxy- tritanol per mg of dT-(P). Analytical electrophoresis showed major bands at R_m^{dpT} -0.08 (thymidine); R_m^{dpT} 0.41 (d-TpT); R_m^{dpT} 0.55 (d-PhpT); R_m^{dpT} 0.65 (d-TpTpT); R_m^{dpT} 0.90 (d-TpTpTpT); and a trace at R_m^{dpT} 0.95. One third of the total hydrolysate was chromatographed on DEAE paper in 0.2 M ammonium bicarbonate in water-ethanol (9:1). Bands visible under ultra- violet light were cut out (along with the corresponding paper blanks) and eluted with 0.5 M ammonium bicarbonate in water-ethanol (9:1); O.D. data are summarized in table 2. The various components amount to 0.097 μmol per mg dT(P).

d-TpTpTpTpT. A synthesis leading from dT-(P) to the pentamer stage was carried out under essentially the same conditions except that 5% tri- fluoroacetic acid was used to cleave methoxytrityl from the carrier bound oligothymidine derivatives at each stage. The final polymer bound product (d-MTrTp$_{(Ph)}$Tp$_{(Ph)}$Tp$_{(Ph)}$Tp$_{(Ph)}$T-(P), 21.3 mg) was subjected to standard alka- line treatment. After demethoxytritylation of the released nucleotides with 80% aqueous acetic acid, the hydrolysate was fractionated on a column (1 cm x 30 cm) of DEAE cellulose using a linear gradient of 250 ml of 0.01 M ammonium bicarbonate and 250 ml of 1 M ammonium bicarbonate, pH 8.6 (fig. 2). The total nucleotide recovery is estimated to be 0.08 μmol per mg of initial dT-(P).

On electrophoresis, material from peak 9 gave a major band at R_m^{dpT} 0.85 and a minor band (approximately 15% of the total optical density) at R_m^{dpT} 1.0. The major band was desalted by paper chromatograph in solvent F (R_f 0.32, homogeneous). Enzyme degradation of this material was per- formed as previously described[11]. The digests were analyzed by high pres- sure chromatography on a pellicular anion exchange column in 0.05 M potas-

sium phosphate, pH 7.2. Digestion with bovine spleen phosphodiesterase produced thymidine and thymidine-3' phosphate in a molar ratio of 1:3.7, while treatment with snake venom phosphodiesterase afforded thymidine and thymidine-5' phosphate in a 1:3.9 proportion.

ACKNOWLEDGEMENT

REFERENCES

1. Letsinger, R. L., and Mahadevan, V. (1965) J. Amer. Chem. Soc., 87, 3526; (1966) ibid. 88, 5319. Letsinger, R. L. Caruthers, M. H., Miller, P. S., and Ogilvie, K. K. (1967) ibid. 89, 7146. Shimidzu, T., and Letsinger, R. L. (1968) J. Org. Chem. 33, 708; (1971) Bull. Chem. Soc. Japan 44, 1673. Ogilvie, K. K. and Kroeker, K. (1972) Canad. J. Chem. 50, 1211.

2. Melby, L. R., and Strobach, D. R. (1967) J. Am. Chem. Soc. 89, 450; (1969) J. Org. Chem. 34, 421, 427. Letsinger, R. L., Caruthers, M. H. and Jerina, D. M. (1967) Biochemistry 6, 1379. Blackburn, G. M., Brown, M. J., and Harris, M. R. (1967) J. Chem. Soc. C, 2438. Yip, K. F., and Tsou, K. C. (1971) J. Amer. Chem. Soc. 93, 3272. Zarytova V. F., Potapov, V. L., Shabarova, Z. A., and Knorre, D. G.,(1971) Dokl. Akad. Nauk. SSSR 199, 1072, Köster, H. (1972) Tetrahedron Letter 1527. Ohtsuka, E., Morioka, S., and Ikehara, M., (1972) J. Am. Chem. Soc. 94, 3229. Glaser, R., Sequin, V. and Tamm, Ch. (1973) Helv. Chim. Acta 56, 654. Köster, H., Pollak, A., and Cramer, F. (1974) Liebigs Ann. Chem. 949.

3. Reese, C. B. (1970) Colloq. Int. Centr. Nat. Rech. Sci. 182, 319. cf. Reese, C. B., and Saffhill (1968) Chem. Comm. 767.

4. The fluorescent material results from attack of an active phosphorylating species on the thymine ring, followed by displacement by 1-methyl-imidazole. The chemistry of the resulting imidazolium pyrimidone moiety will be discussed elsewhere.

5. van Boom, J. H., Burgers, P.M.J., Owen, G. R., Reese, C. B., and Saffhill, R. (1971) Chem. Comm. 869. van Boom, J. H. deRooy, J. F.M., and Reese (1973) J. Chem. Soc. Perkin I, 2513.

6. van Boom, J. H., Burgers, P.M.J., and van Deursen, P.H., Arentzen, R., and Reese, C. B. (1974) Tetrahedron Letters 3785.

7. 5'-Terminal phenyl esters could also arise if any phenyl phosphorodi-chloridate remains in the mixture containing d-MTrTp(Ph)Cl that is used in the polymer reaction.

8. Jacob, T. M., and Khorana, H. G. (1965), J. Amer. Chem. Soc. 87, 368, report values for d-Tr(Tp)$_n$T oligomers. For comparison with values for d-(pT)$_n$ oligomers see Naylor, R, and Gilham, P. T. (1966) Biochem. 5, 2726.

9. Brand, K. (1925) J. prakt. Chem., Neue Folge 109, 1.

10. Caruthers, M. H. (1968), Ph. D. Dissertation, Northwestern University, Evanston, Illinois.

11. Ralph, R. K., Connors, W. J., Schaller, H., Khorana, H. G. (1963) J. Amer. Chem. Soc. 85, 1983.