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# Complete Template-Directed Enzymatic Synthesis of a Potential Antisense DNA Containing 42 Methylphosphonodiester Bonds

M. A. Dineva,[a] S. Chakurov,[b] E. K. Bratovanova,[a] I. Devedjiev[c] and D. D. Petkov[a]*
[a]*Laboratory of Biocatalysis, Institute of Organic Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[b]*Department of Biochemistry, Biological Faculty, Sofia University, "St. Kl. Ohridsky", 1421 Sofia, Bulgaria* [c]*Labotaroy of Polycondensation Processes, Institute of Polymers, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.*

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**Abstract**—$P^{\alpha}$-Methyl thymidine triphosphate was prepared through the pyrophosphorolysis of $P^{\alpha}$-methyl thymidine diphosphate $P^{\beta}$-diphenyl ester and tested as an alternative substrate for *E. coli* DNA polymerase 1 (Klenow fragment) using several template–primer systems requiring the formation of 1 to 42 methylphosphono diester bonds. The enzyme catalyzes the incorporation of a P-methyl thymidylic residue with (**Sp**)-configuration at a single site in a recessive 3'-end as well as at multiple sites along a growing 167 nucleotide long chain. The synthesis of a full length product, containing 42 sites of methylphosphonate incorporation was observed.

Recent development in antisense techniques have been directed towards the synthesis of modified oligonucleotides with increased nuclease resistance and plasma membrane permeability.[1] A substitution of some or all phosphodiester bonds by methylphosphonodiester bonds yields oligonucleotide analogues that meet these requirements.[2] Conventional chemical synthesis of these modified nucleic acids is not stereospecific and yields a mixture of **Rp** and **Sp** configurations at each phosphorus chiral center.[3] The resulting diastereomers demonstrate different hybridization stability and nuclease resistance.[4,5]

Experiments with terminal deoxynucleotidyltransferase, however, suggest the formation of a homochiral product.[6] Recently Victorova *et al.*[7] reported the ability of several DNA polymerases to incorporate P-methylated thymidylic residue into the 3' -end of a DNA chain. Moreover, using HIV reverse transcriptase, they observed DNA chain elongation beyond the modified nucleotide.

Here we report a two-step chemical synthesis of $P^{\alpha}$-methyl thymidine triphosphate ($TTP_{Me}$) and its Klenow fragment (KF)-catalyzed copolymerization to yield specific fragments containing 1–42 methylphosphono diester bonds.

## Results and Discussion

$P^{\alpha}$-Methyl thymidine triphosphate ($TTP_{Me}$) was obtained by the pyrophosphorolysis of $P^{\alpha}$-methyl thymidine diphosphate $P^{\beta}$-diphenyl ester. The activated methylphosphonate was prepared through the phosphorylation of 5'-methylphosphonate ($TMP_{Me}$) by diphenyl phosphorochloridate (Scheme I). The pyrophosphorolysis reaction is completed within 15 min and the chromatographic purification produces HPLC-pure product with physical characteristics presented in Table 1. Analytical data (Table 1) suggest that $TTP_{Me}$ is not contaminated by TTP. Product chirality (**Sp** or **Rp**) was not assigned.

The incorporation of methylphosphonate thymidylic residues ($T_m$) into the 3'-end of a DNA chain (*Eco*RI/*Bam*HI fragment) should proceed as follows:

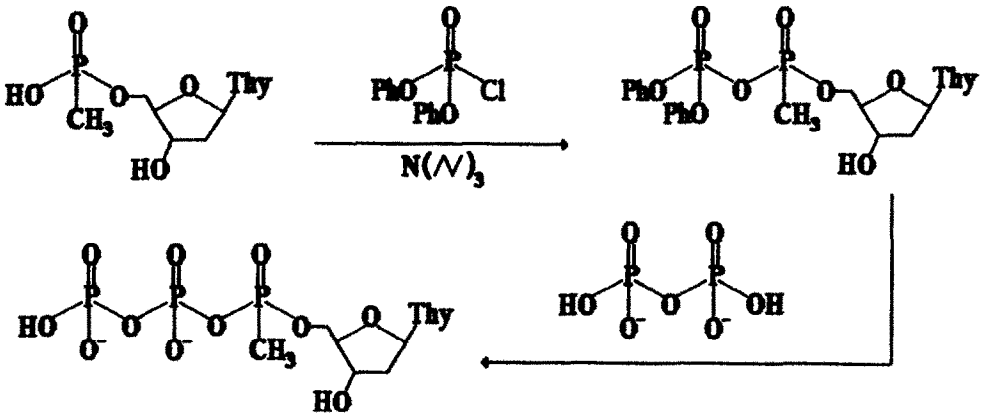
AATTC———G +dATP +dCTP* KF A A T T C———GGA C*
G———CCTAG +dGTP +dTTP$_{Me}$ → $T_{Me}T_{Me}$AAG———CCT$_{Me}$G

The separation of reaction products by denaturing polyacrylamide electrophoresis yields a radioactive band corresponding to the $T_{Me}$ containing fragment (Figure 1A). Considering the initial hydrolysis rate to be insignificant compared to the initial synthetic rate (Figure 1B) this band can be assigned to the 3'-$T_{Me}$ containing product.

The effect of $P^{\alpha}$-methyl modification in TTP on the kinetics of the phosphodiester bond formation is seen from the time dependence of [$^3$H]dCTP incorporation subsequent to the 3'-end of a DNA strand (Figure 1B).

Burgers and Eckstein have shown that DNA polymerase I specifically incorporates the $P^{\alpha}$-thiotriphosphates **Sp** diastereomer in the presence of the **Rp** diastereomer to yield a **Rp** configuration.[8] In respect to the identical substrate stereospecificity of KF and terminal transferase for phosphorothioates as well as the identical stereospecificity of terminal transferase for methylphosphonates and phosphorothioates,[6] it is reasonable to assume that KF should incorporate the **Rp** diastereomer to yield $T_{Me}$ with **Sp** configuration.

Provided chemical synthesis is non-stereospecific, $TTP_{Me}$ should be a mixture of **Sp** and **Rp** diastereoisomers. Therefore, the initial rate of $T_{Me}$ incorporation is expected to be approximately twice as low as the incorporation rate of non-modified T. These rates differ by a factor of 5 (Figure 1B). Hence, KF utilizes the reactive diastereomer of $TTP_{Me}$ less efficiently than non-modified TTP.



Scheme I.

Table 1. Physical characteristics of $TTP_{Me}$, its synthetic precursor $TMP_{Me}$ and unmodified TTP

| Compound | $^1$HNMR($CH_3$ – P) (ppm) | $^2$J(Hz) | TLC $R_f$(A) | $R_f$(B) | HPLC RT(min) |
|---|---|---|---|---|---|
| $TTP_{Me}$ | 1.79d | 18 | 0.42 | 0.49 | 1.10 |
| $TMP_{Me}$ | 1.42d | 17 | 0.63 | 0.68 | 1.54 |
| TTP | — | — | 0.17 | 0.19 | 0.68 |

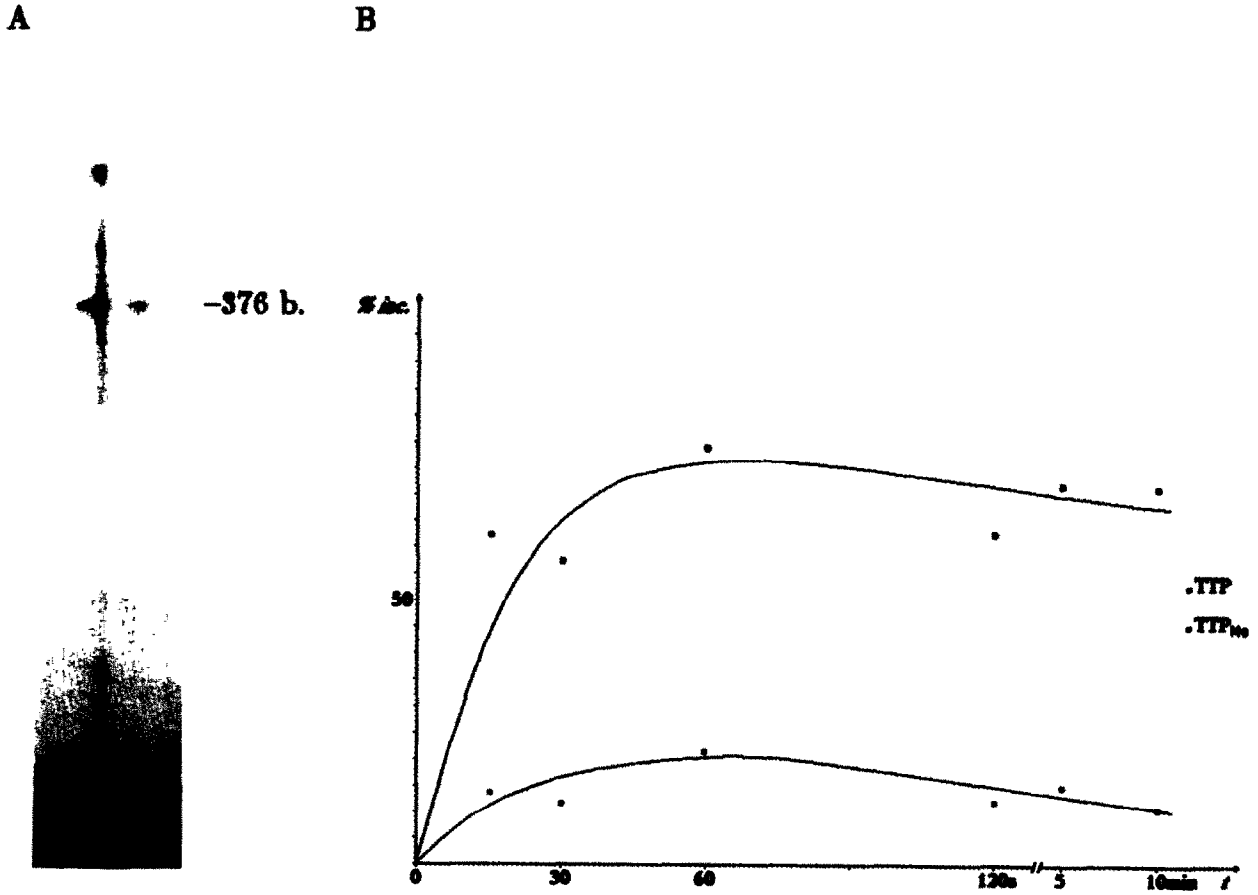



Figure 1. KF Catalyzed 3'-recessive end extension in the presense of $TTP_{Me}$: (A) Electrophoretic analysis. [α -$^{32}$P]dCTP incorporation subsequent to: 1—$TTP_{Me}$; C—TTP: (B) Time course of [$^3$H]dCTP incorporation subsequent to TTP or $TTP_{Me}$

Multiple incorporation of $T_{Me}$ was established through the KF-catalyzed extension of a 28 nucleotide long primer on a 167 template. Electrophoretic analysis results are presented on Figure 2. In contrast to shorter methylphosphonate containing nucleotides[9] the electrophoretic mobilities of modified and non-modified DNA appear to be similar. The analysis of the reaction products produced after 10 min and after 1 h does not reveal significant differences in electrophoretic patterns. Therefore KF efficiently copies the template polydeoxyribonucleotide when $TTP_{Me}$ is substituted for TTP to yield a full length product containing 42 methylphosphonodiester out of 166 internucleotide bonds. Furthermore, as seen from the nucleotide sequence, this result is possible if and only if two (7 times) and three (4 times) consecutive methylphosphonodiester bonds are formed.

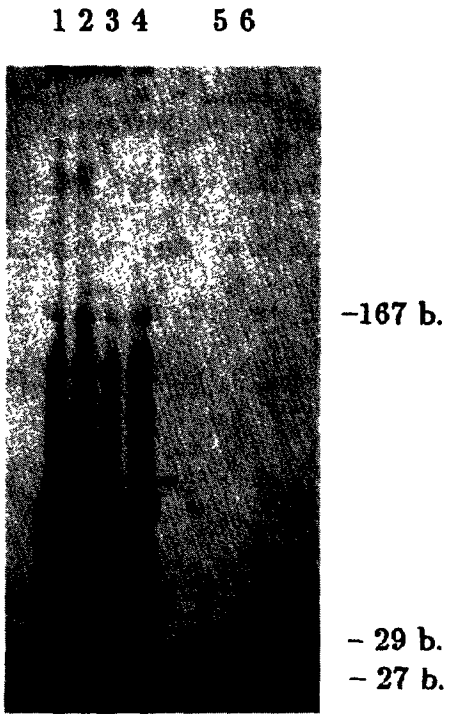



**Figure 2.** PAGE separation of KF-catalyzed primer extension products. Reaction products using: 1—TTP (10 min); 2—$TTP_{Me}$ (10 min); 3—TTP (1 h); 4—$TTP_{Me}$ (1 h); oligonucleotide markers: 5—27-mer; 6—29-mer

To conclude, we have shown that a rather large antisense DNA, containing methylphosphonodiester bonds can be obtained by KF-catalyzed sequence-specific synthesis. The incorporation of consecutive modified thymidilic residues implies that similar approach may be applicable to the synthesis of all P-methyl DNA derivatives.

## Materials and Methods

*Klenow fragment* (E. coli *DNA polymerase I large fragment)*

This was obtained from Amersham and used without further purification.

[$\gamma$-$^{32}$P]dATP, [$\alpha$-$^{32}$P]dCTP, [$^3$H]dCTP and canonical dNTPs were purchased from Amersham.

*Primer oligodeoxyribonucleotide*

GGG TAA ACA AGC GGT TGA AGT CAA TTT G was synthesized on a Milligen 7500 DNA synthesizer using phosphoramidite chemistry and purified by reverse phase chromatography.

*Eco*RI/*Bam*HI 376 b.p. fragment from pBR322 was isolated by low-melting agarose gel electrophoresis.

*Template polydeoxyribonucleotide*

TCG ACT TTC TGA AAG CAA CGC CGC AAA GAT GAA TGA CGA TGT TGA TGG TAT TGT ACG CAC CCC ACT CGC TGA ACT GTT AGA TGG GCA AGG ACA AAC AGC TGA TCA AGG TCC AGA AAG TGT GAA CTT CAA CTC TCC TGA CCA AAT TGA CTT CAA CCG CTT GTT TAC CC was obtained from *Mycoplasma pneumoniae*[10] by PCR amplification using the above mentioned primer and *Taq* polymerase.

*$TTP_{Me}$ Synthesis*

The pyridinium salt of $TMP_{Me}$ (0.2 mmol) was dissolved in anhydrous dioxane (2 ml) and reacted for 3 h with diphenyl phosphorochloridate (0.3 mmol) in the presence of triethylamine (0.4 mmol). The solvent was removed by evaporation and the residue was washed with ether/petroleum ether (5 ml/ 25 ml). The solvent was decanted and the remaining material was used as methylphosphonylating reagent. Dried tri-*n*-propylammonium pyrophosphate (2 mmol), was dissolved in anhydrous pyridine (5 ml) and added to the methylphosphonylating reagent. After the reaction was completed, the solvent was removed. The residue was purified by column chromatography on DEAE-32 cellulose (25 x 1.6 cm) with a linear concentration gradient (0.01–0.3 M) of triethylammonium bicarbonate (TEAB), pH 7.5. Fractions, eluted with 0.07–0.09 M TEAB, contained the product as judged by TLC analysis. They were pooled, diluted with an equal volume of ethanol and evaporated *in vacuo* to remove the buffer. Yield 28%.

*Analytical*

One spot in TLC on Silica sheets (Kieselgel 60) using two different systems: A—dioxane: water: 25% $NH_3$ (6:4:1, v/v), B—isopropanol: water: 25% $NH_3$ (7:2:1 v/v); one peak on HPLC using LKB solvent delivery system on RP 18 (5 µm) column (Merck) and isocratic elution with methanol/60mM $KH_2PO_4$ (5:95 v/v) pH 4.0; a downfield shift of the $CH_3$ – P group signal compared to $TMP_{Me}$ in $^1$H NMR (Table 1) (250 MHz, $D_2O$, trimethylsilylpropionic acid as internal standard).

*3'-Recessive end extension*

10 µM 376 b.p. *Eco*RI/*Bam*HI fragment of the plasmid pBR322, 50 µM dATP, dGTP, dCTP, TTP or 100 µM $TTP_{Me}$, 1.5 pmol [$\alpha$-$^{32}$P]dCTP and 1 unit of KF in 15 µl

of 10 mM Tris–HCl buffer, pH 7.9, containing 5 mM $MgCl_2$ and 0.4 mM dithiothreitol, were incubated for 15 min at 23 °C. The reaction products were analyzed on 12% denaturing polyacrylamide gel electrophoresis and autoradiography.

*Primer extension*

The template polydeoxyribonucleotide (0.3 pmol) was denatured at 95 °C (5 min) with a ten-fold excess of the primer oligodeoxyribonucleotide in KF buffer and allowed to renature at room temperature (20 min) to yield the primer: template complex. Then dATP (20 μM), dGTP (20 μM), dCTP(15 μM), [α -$^{32}$P]dCTP (1.5 pmol), TTP(20 μM) or $TTP_{Me}$ (500 μM) were added. Reaction mixtures were incubated with 1 unit KF for 10 min or 1 h. The reaction products were analyzed on 15% denaturing polyacrylamide gel electrophoresis.

*Kinetic studies*

Nucleotide incorporation beyond the P-methyl thymidylic residue was followed using the standard acid precipitation assay.[11] 10 μl Polymerase reaction mixture contained 5 ng of the pUC19 plasmid, linearized by *Bam*HI, 5 mmol $MgCl_2$, 10 mmol Tris–HCl (pH 7.5), 20 μM dATP, dGTP, dTTP or $TTP_{Me}$, 10 μMdCTP, 2 μM [$^3$H]dCTP and 1 unit of KF. Aliquots withdrawn at appropriate time intervals were placed onto Whatman GF/C filter disks. The disks were washed twice with washing solution (5% TCA/ 20% $Na_4P_2O_7$) and once with 70%$C_2H_5OH$, then dried and counted in a Beckman LC 1801 liquid scintillation counter.

## Acknowledgements

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