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# Hydroxymethylphosphonate: Novel Oligonucleotide Analogue

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Thymidine(3',5')thymidine hydroxymethylphosphonate **(10)**, the first oligonucleotide bearing hydroxymethylphosphonate function, and *t*-butylammonium 5'-*O*-monomethoxytritylthymidine 3'-*O*-acetoxymethylphosphonate **(7)**, oligonucleotide monomer, were synthesized using *t*-butylammonium *O*-methylacetoxymethylphosphonate **(4)** as a novel phosphonylating agent. Hydroxymethylphosphonate internucleotide linkage is stable at physiological pH and resistant to 3'- and 5'-exonucleases. While retaining the neutral character of the phosphonate modification, the presence of hydroxymethyl function increases hydrophilicity of hydroxymethylphosphonate oligonucleotide and its solubility in water compared to the parent methylphosphonate analogue. Therefore, hydroxymethylphosphonate modification can be used to fine-tune the physicochemical properties of antisense oligonucleotides.


## INTRODUCTION

Oligonucleotides—short, usually synthetic nucleic acids—are a new class of prospective therapeutics with a promising and diversified future in medical applications, known as antisense oligonucleotide therapy (AOT) (*1–4*). Malignancies and viral infections, including AIDS, are prominent examples of disease for which the antisense gene inhibition approach is being actively pursued (*5, 6*).

Several requirements for antisense agents as modulators of gene expression were defined, among which the most important are *selectivity*, *stability*, and *transportability* (*4*). Unmodified oligonucleotides are readily available and have low toxicity; however, they do not satisfy most of the requirements for antisense agents, and hence oligonucleotide analogues are often used (*3*). The most frequently applied modifications are those within the internucleotide phosphodiester residue leading to chiral at phosphorus phosphorothioate and methylphosphonate oligonucleotide analogues (*2, 3, 7, 8*). By virtue of phosphorus chirality and nonstereoselectivity of standard methods of oligonucleotide synthesis, phosphorus modifications are usually produced as a random mixture of $2^n$ diastereoisomers

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(*n*, number of P-chiral internucleotide groups). The absolute configuration at the P-chiral internucleotide linkage affects the physicochemical and biological properties of modified at phosphorus oligonucleotides such as resistance to nucleases (*2, 3*) and melting temperatures ($T_m$) of the duplexes formed with complementary sequences (*9–11*).

Methylphosphonate oligonucleotides are among the most promising antisense agents; however, in longer oligomers they suffer from low solubility in water (*2*) and decreasing stability of duplexes. The first phenomenon is a result of the presence of uncharged, hydrophobic methylphosphonate groups; the second may be caused by P-diastereoisomeric unhomogeneity of methylphosphonate oligonucleotide (*9*). Therefore, it is interesting to note that water solubility of stereoregular $S_PS_PS_P$ tetra(thymidine methylphosphonate) is lower than those of $R_PR_PR_P$ isomer (*12*), and that $S_PS_PS_P$ oligomer forms much less stable double-stranded structures with pentadecadeoxyadenylic acid than $R_PR_PR_P$ oligomer (*11*). The observations are consistent with recent work on molecular modeling of P-stereoregular methylphosphonates and phosphorothioate oligonucleotide analogues, which suggests a strong effect of phosphorus stereochemistry upon their hydration pattern (*13, 14*) and electrostatic potential surface (*15, 16*). This is especially true for methylphosphonate analogues in which an anionic oxygen atom of internucleotide phosphodiester residues is replaced by a neutral methyl group (*13*). The hydration pattern may in turn affect the physicochemical and biological properties of P-modified oligonucleotides. This may be of importance for future practical applications of methylphosphonate oligonucleotides, especially as it seems that the stereochemistry of internucleotide methylphosphonate function influences not only $T_m$, but also to some extent the efficacy of modified oligonucleotide cellular uptake (*17*).

Herein, we describe the synthesis of hydroxymethylphosphonate oligonucleotide, a novel type of oligonucleotide modification which, while retaining the stability to nucleases, is more hydrophilic than original methylphosphonate and therefore is more soluble in water. Additionally, phosphoryl oxygen, as a hydroxyl group of hydroxymethyl residue in **10**, contrary to methyl group of internucleotide methylphosphonate modification, can both participate in the hydration network. This may decrease the effect of stereochemistry at phosphorus on the oligonucleotide physicochemical properties, at least in regard to the phenomenon of hydration, and help in preventing the deterioration of modified oligonucleotide properties caused by stereochemical unhomogeneity.

## RESULTS AND DISCUSSION

The key intermediate in the synthesis of thymidine(3′,5′)thymidine hydroxymethylphosphonate **(10)** (Fig. 1) is the fully protected monomer 5′-*O*-monomethoxytritylthymidine 3′-*O*-(*O*-methylacetoxymethylphosphonate) **(6)**. Compound **6** was obtained in the reaction of 5′-*O*-monomethoxytritylthymidine **(5)** and *t*-butylammonium *O*-methylacetoxymethylphosphonate **(4)**, a new phosphonylating agent bearing the protected hydroxymethyl group. The phosphonylation reac-

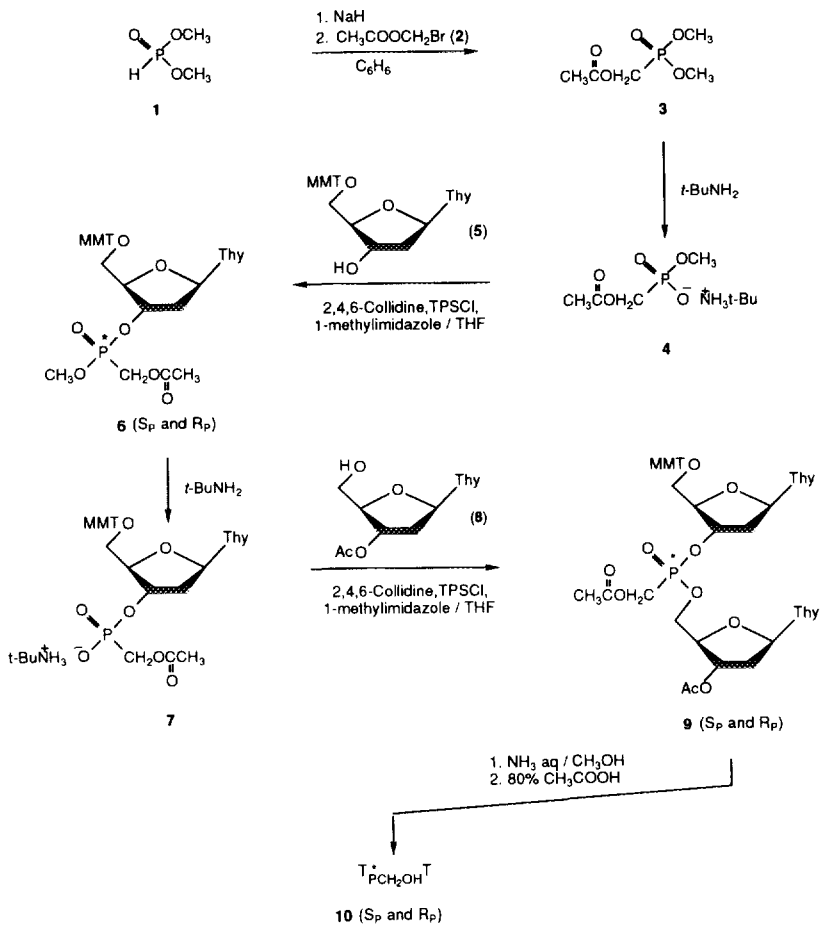


FIG. 1. Synthesis of thymidine(3',5')thymidine hydroxymethylphosphonate.

tion was performed in the presence of 4-toluenesulfonyl chloride as activating agent.

*O,O*-Dimethylacetoxymethylphosphonate **(3),** a precursor for synthesis of **4,** was obtained according to the method described in the literature (*18*). An approach to the synthesis of **3** via sodium salt of dimethylphosphite (*19*) and via the Michaelis–Arbuzov reaction (*20*) was tested. Unlike the literature data, we have found that the first approach provides product **3** in a higher yield than the synthesis with the Michaelis–Arbuzov method involving reaction of **2** and trimethylphosphite. The major by-product isolated from the former reaction (on the basis of $^{1}H$, $^{31}P$ NMR, and MS characteristics) (*21*) is *O,O*-dimethylmethylphosphonate. This may suggest that, simultaneously with the **3** formation, a fast competitive reaction between trimethylphosphite and methyl bromide generated from the original reaction between trimethylphosphite and **2** occurs. Additionally, several other by-products absorbing in the range 19–25 and 31–34 ppm were observed by $^{31}P$ NMR.

Selective demethylation of **3** leads to phosphonylating agent **4.** Three approaches were tested. Treatment of **3** with a mixture of thiophenol and triethylamine in dioxane solution (*22*) or 2-mercaptobenzotiazole in the presence of diisopropylamine (*23*) leads to near quantitative formation of **4** after 2 h. The third approach involves reaction of **3** with *t*-butylamine heated under reflux for 4 h (*24*). Despite a prolonged reaction time, the last procedure was chosen due to the ease of product **4** isolation. The procedure required only evaporation of *t*-butylamine and washing of resultant pure, crystalline product with hexane and diisopropyl ether and drying.

Monomer **6** is conveniently prepared from 5′-protected nucleoside **5** treated with phosphonylating agent **4** in the presence of 4-toluenesulfonyl chloride as activating agent and 2,4,6-collidine. 1-Methylimidazole is used as the nucleophilic catalyst and anhydrous tetrahydrofuran as the solvent. The yield of pure **6** isolated as a mixture of two diastereoisomers is 60%.

Conversion of **6** into *t*-butylammonium 5′-*O*-monomethoxytritylthymidine 3′-*O*-acetoxymethylphosphonate **(7)** occurs efficiently under treatment with *t*-butylamine under the same conditions as those described for synthesis of **4.** The yield of crude **7** is 88% and its purity is 97% as measured by $^{31}P$ NMR. This allows the use of **7** in oligonucleotide synthesis without further purification.

Fully protected dinucleotide 5′-*O*-monomethoxytritylthymidine(3′,5′)3′-*O*-acetylthymidine acetoxymethylphosphonate **(9)** was synthesized by reacting 3′-*O*-acetylthymidine **(8)** with triisopropylbenzenesulfonyl chloride activated **6,** in the presence of 2,4,6-collidine. Dry tetrahydrofuran or acetonitrile was used as reaction medium and 1-methylimidazole was added as a catalyst. The yield of **9** after chromatographic purification was 52%.

Deprotection of dinucleotide **9** was achieved in two steps. First, acetyl groups protecting of 3′-hydroxyl and hydroxymethylphosphonate residues were removed in the reaction with a solution of ammonia in water containing methyl alcohol (*25*). Both acetyl groups were removed at the similar rate since, during the reaction, both intermediates—partially deprotected 5′-*O*-monomethoxytritylthymidine (3′,5′)3′-*O*-acetylthymidine hydroxymethylphosphonate and 5′-*O*-monomethoxytritylthymidine (3′,5′)thymidine acetoxymethylphosphonate—were observed in near 1 : 1 ratio by TLC. In the second step, the 5′-*O*-monomethoxytrityl group was removed under standard conditions using 80% acetic acid (*26*). The yield of chromatographically purified dinucleotide **10** was 69%.

The reverse-phase-high-performance liquid chromatography (RP-HPLC) analysis of **10** revealed two well-resolved peaks corresponding to two P-epimeric diastereoisomers. The retention time ($R_t$) of **10** was compared with that of thymidine (3′,5′)thymidine methylphosphonate **(11)** and unmodified dinucleotide, thymidine (3′,5′)thymidine phosphate **(12).** The $R_t$ value increased in the following order: **12** (11.05 min) < **10** (13.16 and 14.48 min) < **11** (15.73 and 16.57 min), which reflects the anticipated higher hydrophilicity of **10** as compared to methylphosphonate analogue **11** (*27*).

CD measurements have shown a moderate effect of the modification at the phosphorus on the value of molecular ellipticity (Θ) (Fig. 2) (*28, 29*). CD spectrum of **10** was compared with those of **11** and **12.** Dinucleotides **10** and **11** were used as a mixture of two diastereoisomers. The shapes of the spectra are the same for

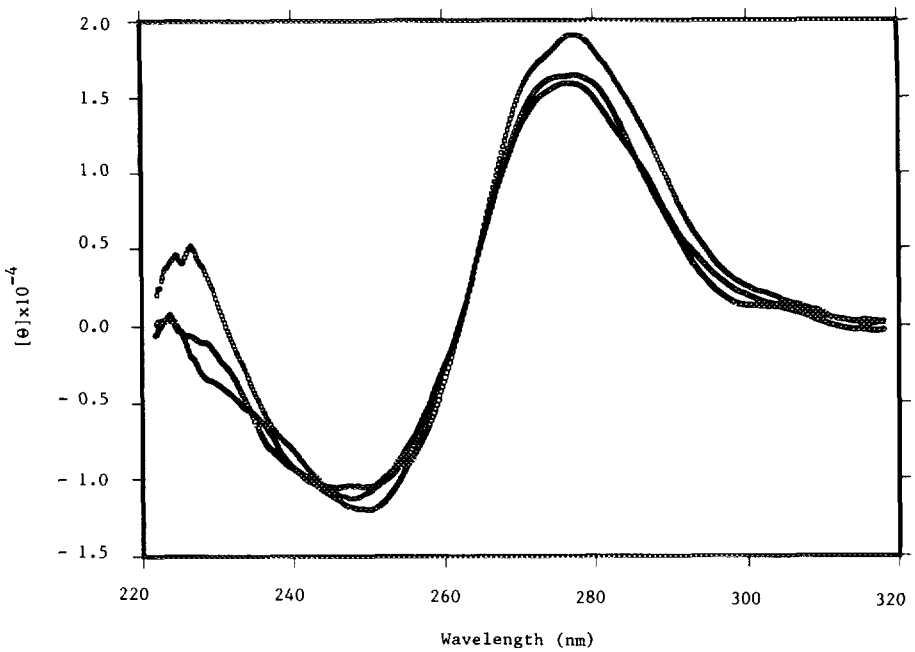


FIG. 2. Circular dichroism spectra of d($T_{PCH_2OH}T$) **(10)** (●), d($T_{PCH_3}T$) **(11)**, (○), and d($T_pT$) **(12)** (□). The molar ellipticity (Θ) is given per base residue.

all three dinucleotides, with the maximum of the peaks at 275.5 nm. The molecular ellipticity decreases in the order of **10** ≈ **11** < **12.** Similar values of Θ for **10** and **11** indicate the same phosphonate character for internucleotide linkage modification. Additionally, taking into account that molecular ellipticity is sensitive to base stacking, one can infer that hydroxymethylphosphonate modification perturbs the conformation of **10** not more than the methylphosphonate moiety in **11.**

According to our data and those of others on methylphosphonate oligonucleotides, an effect of absolute configuration of internucleotide hydroxymethylphosphonate moiety on conformation of oligonucleotides of type **10,** and in turn on Θ value (*27, 28*), can be anticipated. On the other hand, due to the ability of hydroxyl function of hydroxymethylphosphonate residue to participate in hydration interactions, one can expect a decreased influence of stereochemistry at phosphorus of hydroxymethylphosphonate oligonucleotides on the physicochemical properties of these compounds. This aspect will be addressed in future studies on stereocontrolled synthesis of oligonucleotides such as **10** (*8*).

Since some chemical degradation in the internucleotide linkage for methylphosphonate oligonucleotide analogues, especially under basic conditions, was reported in the literature (*30*), the stability of dinucleotide **10** at different pHs was tested. It was found that **10** is stable in the pH range of 3–8, although traces of degradation were detected after 72 h, even at pH 7. Thymidine, and most probably thymidine 5′-*O*-(hydroxymethylphosphonate), were observed as degradation products. At pH 12, **10** is hydrolyzed completely after 1 h.

It is assumed that 3'-exonuclease activity is responsible for most of the unmodified antisense oligonucleotide degradation in serum (*31*). Since oligonucleotide methylphosphonates are resistant against nucleases (*32*), similar resistance can be expected for **10.** However, it was recently shown that fluorophosphate oligonucleotide can be cleaved by some nucleolytic enzymes (*33*). This is most probably due to the polar character of F–P linkage which could mimic the P–OH function of internucleotide phosphate residue in its interactions with the active center of enzymes (*34, 35*). This finding prompted us to test the resistance of (3',5')hydroxymethylphosphonate internucleotide linkage of dinucleotide **10** to 5'- and 3'-exonucleases. The enzymes from *Crotalus durissus terrificus* venom and from bovine spleen were used. No degradation due to the enzyme action was observed. In the case of venom 5'-exonuclease, the pH of the buffer used for enzymatic reaction was 8.9. Some hydrolysis of **10** was detected after 5 h. This can be ascribed to chemical degradation under increased pH conditions rather than to enzymatic digestion, since the same level of hydrolysis was observed for a blank experiment without enzyme addition. It thus seems that hydroxymethylphosphonate-modified internucleotide linkage, similarly to methylphosphonate, is resistant to 3'- as well as 5'-exonuclease activity.

In conclusion, the method for synthesis of hydroxymethylphosphonate, a novel type of oligonucleotide modification, was developed. Hydroxymethylphosphonate internucleotide linkage is stable at physiological pH, resistant against 3'- and 5'-exonucleases, and more hydrophilic than parent methylphosphonate residue, increasing oligonucleotide solubility in water. Hydroxymethylphosphonate modification can thus be used to fine-tune the physicochemical properties of antisense oligonucleotides.

## EXPERIMENTAL

Column chromatography and TLC were performed on silica gel 230–400 mesh and F254 plates, respectively (Merck, Philadelphia, PA). Methyl bromoacetate was purchased from Aldrich (Milwaukee, WI). Phosphodiesterase I (EC 3.1.4.1) type VIII from *C. durissus terrificus* venom and phosphodiesterase II (EC 3.1.16.1) from bovine spleen were purchased from Sigma (St. Louis, MO). Ultraviolet spectra were recorded with a Uvikon 860 spectrometer (Kontron Instruments AG, Berlin, Germany). $^{1}H$, $^{13}C$, and $^{31}P$ NMR spectra were recorded with a Bruker MSL 300 spectrometer operating at 300.13, 75.48, and 121.47 MHz, respectively, with TMS for $^{1}H$ and $^{13}C$ and 85% $H_3PO_4$ for $^{31}P$ as external standards. Positive chemical shift values were assigned for compounds resonating at lower fields than standards. LSIMS mass spectra were recorded on a Finnigan MAT 95 with $Cs^+$ gun operating at 13 keV (glycerin matrix). CD spectra were recorded on a Jasco J-600 spectropolarimeter. RP-HPLC was performed with the Hewlett–Packard 1050 system, using a Whatman Partisphere C18 5 $\mu$m 4.7 × 235-mm column. The elution conditions are given below.

*O,O-Dimethylacetoxymethylphosphonate* (**3**) (*18*)

A solution of methanol (1.0 ml, 0.025 mol) in anhydrous benzene (12.5 ml) was added dropwise to a stirred suspension of sodium hydride (0.92 g, 0.025 mol) in benzene (12.5 ml). After stirring for 20 min, the mixture was cooled in an ice-water bath and a solution of dimethylphosphite (2.5 ml, 0.025 mol) in benzene (5.0 ml) was added dropwise. Stirring was continued for 20 min, and the resulting mixture was added dropwise to a stirred solution of acetoxymethyl bromide (4.1 g, 0.027 mol) in benzene (12.5 ml). The reaction mixture was then stirred overnight at room temperature, and the sodium bromide filtered off and washed with benzene (12.5 ml). The filtrate and washings were mixed and benzene evaporated. The oily residue was then distilled under vacuum. The fraction boiling at 80–90°C/ 0.5–0.6 mmHg was collected, affording **3** with 70% (3.2 g) yield. $\delta^{31}$P NMR ($C_6D_6$) 22.30; $\delta^{1}$H NMR ($C_6D_6$) 1.63 (s, 3H, $CH_3CO$), 3.42 (d, 6H, $J_{PH}$ = 10.8 Hz, $POCH_3$), 4.31 (d, 2H, $J_{PH}$ = 8.56 Hz, $PCH_2$); $\delta^{13}$C NMR ($C_6D_6$) 20.55 (s, $CH_3CO$), 53.26 (d, $J_{PC}$ = 6.48 Hz, $POCH_3$), 56.90 (d, $J_{PC}$ = 167.35 Hz, $PCH_2$), 170.07 (s, $CH_3CO$).

*t-Butylammonium O-methylacetoxymethylphosphate* (**4**)

*O,O*-Dimethylacetoxymethylphosphonate (0.7 g, 3.8 mmol) and *t*-butylamine (10.0 ml) were mixed and heated to reflux for 4 h and then left overnight at room temperature. *t*-Butylamine was evaporated under reduced pressure, yielding oily residue which crystalized after evaporation under high vacuum. The resultant crystalline solid was washed first with *n*-hexane (10.0 ml), and then with diisopropyl ether (10.0 ml), affording **4** with 80% (0.74 g) yield and purity 95%, according to $^{31}$P NMR analysis. $\delta^{31}$P NMR ($D_2O$) 17.04; $\delta^{1}$H NMR ($D_2O$) 1.36 (s, 9H, $CH_3$ : *t*-butylammonium cation); 2.13 (s, 3H, $CH_3CO$), 3.61 (d, 3H, $J_{PH}$ = 10.44 Hz, $POCH_3$), 4.24 (d, 2H, $J_{PH}$ = 8.41, $PCH_2$); $\delta^{13}$C NMR ($D_2O$) 22.21 (s, $CH_3CO$), 28.82 (s, $CH_3$; *t*-butyl-ammonium cation), 53.76 (s, tertiary carbon of *t*-butylammonium cation), 54.17 (d, $J_{PC}$ = 5.4 Hz, $POCH_3$), 60.01 (d, $J_{PC}$ = 158.62 Hz, $PCH_2$), 175.67 (d, $J_{PC}$ = 8.3 Hz, $CH_3CO$); MS/FAB(+VE) 242 [M + H].

*5'-O-Monomethoxytritylthymidine 3'-O-(O-methylacetoxymethylphosphonate)* (**6**)

To a solution of **4** (0.48 g, 2.0 mmol) in dry tetrahydrofuran (4.0 ml), 2,4,6-collidine (0.36 g, 3.0 mmol) and solution of 4-toluenesulfonyl chloride (0.57 g, 3.0 mmol) in tetrahydrofuran (1.0 ml) were added. The mixture was stirred for 15 min at room temperature, and then 5'-*O*-monomethoxytritylthymidine (0.72 g, 1.4 mmol) in dry tetrahydrofuran (1.0 ml) and 1-methylimidazole (0.33 g, 4.0 mmol) were added. The reaction mixture was stirred for 4 h at room temperature, and then the solvent was evaporated under reduced pressure. The oily residue was dissolved in $CHCl_3$ (15.0 ml). The resulting solution was washed with water (3 × 10.0 ml) and dried over magnesium sulfate, and the solvent was evaporated under reduced pressure. The crude product was redissolved in toluene and precipitated with hexanes and the collected precipitate purified by silica gel column chromatog-

raphy. The eluting solvent system used was 50–60% ethyl acetate in hexane followed by 5% $CH_3OH$ in $CH_2Cl_2$. The yield of **6** obtained as a mixture of two diastereoisomers was 57% (0.53 g). TLC ($CHCl_3$ : $CH_3OH$, 9 : 1) $R_f$ 0.63 (ethyl acetate : hexane, 8 : 2) $R_f$ 0.08; UV (96% $C_2H_5OH$) $\lambda_{max}$264.0, $\lambda_{min}$ 249.0; $\delta^{31}$P NMR ($C_6D_6$) 21.50 and 21.80; $\delta^1$H NMR ($C_6D_6$) 1.54 (s, 3H, $CH_3CO$), 1.57 and 1.69 [s, s, 3H, $CH_3$(5)], 2.19–2.31 (m, 1H, H2'), 2.40–2.51(m, 1H, H2''), 3.27 (d, 3H, $J_{PH}$ = 11.0 Hz, $POCH_3$) and 3.40 (d, 3H, $J_{PH}$ = 11.0 Hz, $POCH_3$), 3.30 and 3.31 (s, s, 3H, $CH_3O$: monomethoxytrityl), 3.45 (d, 1/2H, $J_{H4'5'}$ = 3.2 Hz, H5') and 3.48 (d, 1/2H, $J_{H4'5'}$ = 3.0 Hz, H5'), 4.20 (d, 1H, $J_{PH}$ = 8.4 Hz, $PCH_2$) and 4.21 (d, 1H, $J_{PH}$ = 8.5 Hz, $PCH_2$), 4.23 (d, 1H, $J_{H4'5'}$ = 3.0 Hz, H4'), 4.28 (d, 1/2H, $J_{H4'5'}$ = 3.3 Hz, H5') and 4.29 (d, 1/2H, $J_{H4'5'}$ = 3.3 Hz, H5'), 5.36–5.44 (m, 1H, H3'), 6.50–6.60 (m, 1H, H1'), 6.70–7.45 (14H, aromatic : monomethoxytrityl group), 7.50 and 7.53 (s, s, 1H, H6), 9.90 and 9.92 (s, s, 1H, H3); $\delta^{13}$C NMR ($C_6H_6$) 12.83 [s, $CH_3$ (5)], 20.54 (s, $CH_3CO$), 39.97 and 40.39 (s, s, C2'), 53.16 (s, $POCH_3$), 57.57 (d, $J_{PC}$ = 167.79 Hz, $PCH_2$), ~78 (s, C3'), 85.64 (s, C1'), 88.50 (s, C4'), 112.03, 128.21, 128.38, 128.70, 129.02, 129.60, 131.52, 135.54, 160.24 (aromatic : monomethoxytrityl), 114.50 (s, C5), 136.04 (s, C6), 151.22 (s, C2), 164.01 (s, C4), 170.20 (s, $CH_3CO$); MS/FAB (+VE) 664 [M].

*Note:* Diastereoisomeric **6** can be separated into individual species by means of semipreparative TLC using ethyl acetate : heptane (8 : 2) as a developing solvent system (fourfold developing of the plate). $\delta^{31}$P NMR ($C_6H_6$) **6**-FAST 21.80, **6**-SLOW 21.50.

### *t-Butylammonium 5'-O-monomethoxytritylthymidine 3'-O-acetoxymethylphosphonate* (**7**)

Monomer **6** (0.43 g, 0.65 mmol) and *t*-butylamine (4.0 ml) were mixed and heated to reflux for 4 h and left overnight at room temperature, and *t*-butylamine was evaporated under reduced pressure. This yielded a white, solid foam which was dissolved in $CH_2Cl_2$ and precipitated with diisopropyl ether. The precipitate was filtered and dried under vacuum. The yield of chromatographically homogeneous **7** (97% pure according to $^{31}$P NMR measurement) was 88% (0.42 g). TLC ($CH_3CN$ : $H_2O$, 9 : 1) $R_f$ 0.22; UV (96% $C_2H_5OH$) $\lambda_{max}$266.0, $\lambda_{min}$249.0; $\delta^{31}$P ($C_6D_6$) 12.73; $\delta^1$H NMR (DMSO-d6) 1.17 (s, 9H, $CH_3$: *t*-butylammonium cation), 1.31 (s, 3H, $CH_3CO$), 1.8 [s, 3H, $CH_3$ (5)], 2.20–2.30 (m, 2H, H2'), 3.10–3.18 (m, 2H, H5'), 3.68 (s, 3H, $CH_3O$ : monomethoxytrityl), 3.78 (d, 2H, $J_{PH}$ = 8.4 Hz, $PCH_2$), 4.00–4.07 (b s, 1H, H4'), 4.74–4.82 (b s, 1H, H3'), 6.15 (t, 1H, $J_{H1'2'}$ = 7.5 Hz, H1'), 6.83, 6.86, 7.16–7.40 (14H, aromatic : monomethoxytrityl group), 7.45 (s, 1H, H6); δ13C NMR ($CDCl_3$) 11.61 [s, $CH_3$(5)], 20.72 (s, $CH_3CO$), 27.74 (s, $CH_3$: *t*-butylammonium cation), 40.01 (s, C2'), 51.22 (s, tertiary carbon of *t*-butylammonium cation), 55.21 (s, $CH_3O$: monomethoxytrityl), 59.22 (d, $J_{PC}$ = 153.2 HZ, $PCH_2$), 63.92 (s, C5'), 76.24 (s, C3'), 84.69 (s, C1'), 87.29 (s, C4'), 113.27 (s, C5), 130.41 (s, C6), 111.27, 127.25, 128.44, 134.81, 135.65, 143.72, 143.82, 158.80 (s, s, aromatic : monomethoxytrityl), 150.74 (s, C2), 164.08 (s, C4), 170.52 (s, $CH_3CO$); MS/FAB(+VE) 724 [M + H].

*5'-O-Monomethoxytritylthymidine (3'-5')3'-O-acetylthymidine acetoxymethylphosphonate* (**9**)

To a solution of **7** (0.036 g, 0.05 mmol), 4-toluenesulfonyl chloride (0.014 g, 0.075 mmol) and 2,4,6-collidine (0.009 g, 10 μl, 0.075 mmol) in dry tetrahydrofuran or acetonitrile (0.2 ml), a solution of 3'-*O*-acetylthymidine (0.014 g, 0.05 mmol) and 1-methylimidazole (0.008 g, 8 μl, 0.1 mmol) in dry tetrahydrofuran or acetonitrile (0.1 ml) was added. The reaction mixture was stirred ca. 4 h (TLC control, solvent system: $CHCl_3$ : $CH_3OH$, 9 : 1) at room temperature, and the solvent evaporated. The oily residue was dissolved in $CH_2Cl_2$ (2 ml) and the resultant solution extracted with $H_2O$ (3 × 1 ml). The organic fraction was dried over $MgSO_4$ and the solvent evaporated. The residue was redissolved in $CH_2Cl_2$ and precipitated with hexane. The crude product was purified by silica gel column chromatography using a gradient of 0–3% methyl alcohol in methylene chloride as eluent. The yield was 52% (0.024 g). TLC ($CHCl_3$ : $CH_3OH$, 9 : 1) $R_f$ 0.60; UV (96% $C_2H_5OH$) $\lambda_{max}$264.0, $\lambda_{min}$244.0; $\delta^{31}$P NMR [$(CD_3)_2CO$] 22.10 and 22.71; $\delta^1$H NMR [$(CD_3)_2CO$] 1.57 [d, $CH_3$(5): 3'-terminal, $J_{CH_3(5)H6}$ = 0.9 Hz], 1.61 [d, $CH_3$(5): 3'-terminal, $J_{CH_3(5)H6}$ = 0.9 Hz] and 2.03 [d, $CH_3$(5): 5'-terminal, $J_{CH_3(5)H6}$ = 1.1 Hz], 2.04 [d, $CH_3$(5): 5'-terminal, $J_{CH_3(5)H6}$ = 1.1 Hz] [1.57-2.04, 6H, $CH_3$(5)], 2.21 and 2.25 (s, s, 3H, $CH_3CO$: internucleotide), 2.26 and 2.27 (s, s, 3H, $CH_3CO$: 3'-terminal), 2.50–2.63 (m, 2H, H2'), 2.70–2.82 (m, 2H, H2''), 3.60–3.66 (m, 2H, H5'), 3.970 and 3.972 (s, 3H, $CH_3O$: monomethoxytrityl), 4.25–4.57, 4.57–4.66, 4.66–4.80 (m, m, 2H, H4', 2H, H5'), 4.70 (d, 2H, $J_{PH}$ = 8.4 Hz, $PCH_2$), 5.35–5.62 (m, m, 2H, H3'), 6.30–6.47 (m, 1H, H1'), 6.47–6.60 (m, 1H, H1'), 7.00–7.70 (16H, aromatic : monomethoxytrityl group), 7.766 and 7.772 (d, 3H, $J_{CH_3(5)H6}$ = 1.2 Hz, H6), 7.800, 7.807, and 7.815 (d, d ?, 1H, $J_{CH_3(5)H6}$ = 1.4 Hz, $H_6$; 1H, $J_{CH_3(5)H6}$ = 1.4 Hz, $H_6$); $\delta^{13}$C NMR [$(CD_3)_2CO$] 12.37 and 12.91 [s, $CH_3$(5)], 20.58 and 21.12 (s, $CH_3CO$), 37.85, 37.63 and 40.40 (s, C2'), 56.09 (s, $CH_3O$ : monomethoxytrityl), 58.76 (d, $J_{PC}$ = 134.0 Hz, $PCH_2$), 64.71 and 68.9 (s, C5'), 75.29, 79.45 and 84.36 (s, C3'), 86.26 (s, C1'); 86.26, 87.06 and 88.98 (s, C4'), 112.32 (s, C5), 114.65, 128.70, 129.38, 129.90, 129.96, 132.05, 136.23, 137.62, 145.56, 160.78 (aromatic : monomethoxytrityl), 137.93 and 137.96 (s, C6), 152.46 (s, C2), 166.46 (s, C4), 172.52 (s, $CH_3CO$); MS/FAB (-VE) 915 [M-H].

*Thymidine (3',5') Thymidine Hydroxymethylphosphonate* (**10**)

*Removal of acetyl groups.* 5'-*O*-Monomethoxytritylthymidine(3',5')3'-*O*-acetylthymidine acetoxymethylphosphonate (**9**) (0.024 g, 0.026 mmol) was dissolved in $CH_3OH$ (0.7 ml) and, to the resultant solution, concentrated ammonia solution in water (25%) was added (0.7 ml). The reaction mixture was maintained at room temperature for 45 min (TLC monitoring, solvent system $CH_2Cl_2$ : $CH_3OH$, 9 : 1), then degassed and evaporated to dryness, yielding chromatographically homogeneous 5'-*O*-monomethoxytritylthymidine(3',5')thymidine hydroxymethylphosphonate as a white solid.

*Removal of 5'-O-monomethoxytrityl group.* Crude 5'-*O*-monomethoxytritylthymidine(3',5')thymidine hydroxymethylphosphonate (ca. 0.026 mmol) was dis-

solved in 80% acetic acid (1 ml) and heated to 60°C. After ca. 1 h (TLC control, solvent system $CH_2Cl_2$ : $CH_3OH$, 8 : 2), acetic acid was coevaporated with *n*-butyl alcohol. The crude product was dissolved in pyridine and, after precipitation with hexane, purified by means of silica gel column chromatography. As the eluting solvent system, 0–10% $CH_3OH$ in $CH_2Cl_2$ was used. Chromatographically homogeneous thymidine(3',5')thymidine hydroxymethylphosphonate was dissolved in $H_2O$ and lyophilized. The yield was 69% (0.010 g). TLC ($CH_2Cl_2$ : $CH_3OH$, 8 : 2) $R_f$ 0.29; HPLC (gradient from 5 to 25% $CH_3CN$ in 0.05 *M* TEAA (pH 7.0) during 25 min, 1.0 ml/min) $R_t$ = 13.16 min and 14.48 min; UV (96% $C_2H_5OH$) $\lambda_{max}$265.0, $\lambda_{min}$233.6; $\delta^{31}P$ 27.19 and 27.08; $\delta^{1}H$ ($D_2O$), 1.70–1.79 [m, 6H, $CH_3$(5)], 2.30–2.48 and 2.48–2.60 (m, m, 4H, H2'), 3.70–3.77, 4.10–4.16, 4.16–4.21, 4.27–4.45 (m, m, 2H, H4' and 4H, H5'), 4.04 (d, 1H, $J_{PH}$ = 6.0 Hz, $PCH_2$) and 4.05 (d, 1H, $J_{PH}$ = 6.1 Hz, $PCH_2$), 4.45–4.58 and 5.04–5.14 (m, m, 2H, H3'), 6.16–6.26 (m, 2H, H1'), 7.474, 7.501, 7.498, 7.57 (s, 3H, H6); $\delta^{13}C$ ($D_2O$) 11.61 and 11.66 [s, $CH_3$(5)], 37.60, 37.69, 38.28, 38.45 (s, C2'), 55.16 (d, $J_{PC}$ = 166.3 Hz, $PCH_2$), 60.49, 66.06, and 69.99 (s, C5'), 76.98 and 77.30 (s, C3'), 85.19, 85.30, and 85.38 (s, s, C1' and C4'), 111.52 and 111.67 (s, C5), 137.16, 137.31, and 137.41 (s, C6,); MS/FAB (+VE) 561 [M + 1].

*Note*: Under the same HPLC conditions, the $R_t$ of $T_{PMe}T$ **(11)** and $T_pT$ **(12)** were 15.13 and 16.57 and 11.05 min, respectively.

### *Circular Dichroism Measurements*

The CD spectra of thymidine(3',5')thymidine hydroxymethylphosphonate **(10)**, thymidine(3',5')thymidine methylphosphonate **(11)**, and thymidine(3',5')thymidine phosphate **(12)** were measured in 10 mM $Na_2HPO_4$, pH 7.0. The concentrations of **10, 11,** and **12** were $1.65 \times 10^{-5}$, and $1.08 \times 10^{-5}$ M, respectively, and the extinction coefficient used for all three dinucleotides was $\varepsilon_{265} = 8450\ M^{-1}\ cm^{-1}$ per base (*10*).

### *Stability of Thymidine (3',5') Thymidine Hydroxymethylphosphonate* (*10*) *in Water Solution*

Five samples (pH 2–12) were prepared by diluting a 20 mM water solution of **10** (10 μl) with 0.1 M Britton–Robinson buffer (50 μl) (*36*) and maintained at room temperature for 72 h. At the designated time intervals, aliquots of each solution were analyzed by TLC using $CH_2Cl_2$ : $CH_3OH$ (8 : 2) as eluting solvent system. Thymidine was used as one of the degradation products standards.

### *Resistance of Thymidine (3',5') Thymidine Hydroxymethylphosphonate to Exonucleases*

*Phosphodiesterase I (EC 3.1.4.1).* To 100 mM Tris–HCl buffer, pH 8.9 (100 μl) containing 20 mM $MgCl_2$, 20 mM solution of **10** (5 μl) and phosphodiesterase (10 μl, 0.03 U, Sigma, Lot 119F0730) were added. A blank reaction with no enzyme added and control reaction with $T_pT$ **(12)** was run simultaneously. Reactions were maintained at room temperature for 22 h. Aliquots of each reaction were analyzed

by means of TLC using $CH_2Cl_2$ : $CH_3OH$ (8 : 2) as eluting solvent system after 15 min, 2.5, 5, and 22 h.

*Phosphodiesterase II (EC 3.1.16.1).* To 100 mM NaOAc buffer, pH 4.8 (100 μl), containing 1 mM $ZnCl_2$, a 20 mM solution of 10 (5 μl) and phosphodiesterase (10 μl, 0.5 U, Sigma, Lot 101H9550) were added and the same procedure as that described above was followed.

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