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Reactivity of Nucleoside 5′-O-Phosphates, -phosphorothioates, -methanephosphonates, and -methanephosphonothioates toward Activated *Xylo*nucleosides

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ABSTRACT: *Alkylation of ambident thymidine 5′-O-(O-alkyl phosphorothioate) anions by means of 3′-O-sulfonylated xylothymidine occurs at both O- and S-nucleophilic centers, and formation of an internucleotide bond is accompanied by the process of elimination. Lack of chemoselectivity and low yields of products discriminate against such an approach as an effective method of stereocontrolled synthesis of P-chiral oligo(nucleoside phosphorothioate)s.* © 1999 John Wiley & Sons, Inc. Heteroatom Chem 10: 91–104, 1999

INTRODUCTION

Reaction of 5′-O-protected 3′-O-sulfonylated xylothymidine (**1**) with sodium thiobenzoate has been described by Cosstick and Vyle [1] as an effective route to 5′-O-protected 3′-deoxy-3′-S-benzoylthymidine, which upon removal of the benzoyl group gave 5′-O-protected 3′-deoxy-3′-mercaptothymidine. This reaction was performed in DMF solution at 90°C, and the yield of final product was above 90%. In light of these findings, it was tempting to study the reaction of **1** with a 3′-protected nucleoside 5′-O-alkyl phosphorothioate anion (**2**) as a direct route to sugar-phosphate backbone-modified oligonucleotides with the 3′-oxygen replaced by a sulfur atom. Supposition that an ambident O,O-dialkyl phosphorothioate anion will react *via* an attack of sulfur on an activated sp^3-carbon has been justified not only by analogy between ambident thiobenzoate and O,O-dialkyl phosphorothioate anions but also by the results of numerous studies indicating predominant formation of S-alkylated products obtained in reactions of O,O-dialkyl phosphorothioates with C-electrophiles [2–10].

Therefore, S-alkylation of ambident O,O-dialkyl phosphorothioate anion by 5′-O-protected 3′-O-sulfonylated xylothymidine (**1**) could provide a simplified route to 3′-deoxy-3′-mercaptothymidine, while reaction with a 3′-protected nucleoside 5′-O-alkyl phosphorothioate anion (**2**) would offer a simple access to modified oligonucleotides where the 3′-oxygen is replaced by sulfur. However, if the reaction of **2** with **1** involves an O-alkylation process, the possibility of separation of P-chiral **2**, such as a nucleoside 5′-O-(O-alkyl phosphorothioate), into diastereomeric species would offer a new route to stereospecific syntheses of oligo(nucleoside phosphorothioate)s. Such an opportunity has been presented recently in the patent literature [11,12]. If feasible, reaction of **1** with **2** could comprise an alternative route (Scheme 1) to the stereocontrolled

This article is dedicated to the late Academician Martin Israilevitch Kabachnik.

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synthesis of oligo(nucleoside phosphorothioate)s developed recently in this laboratory [13].

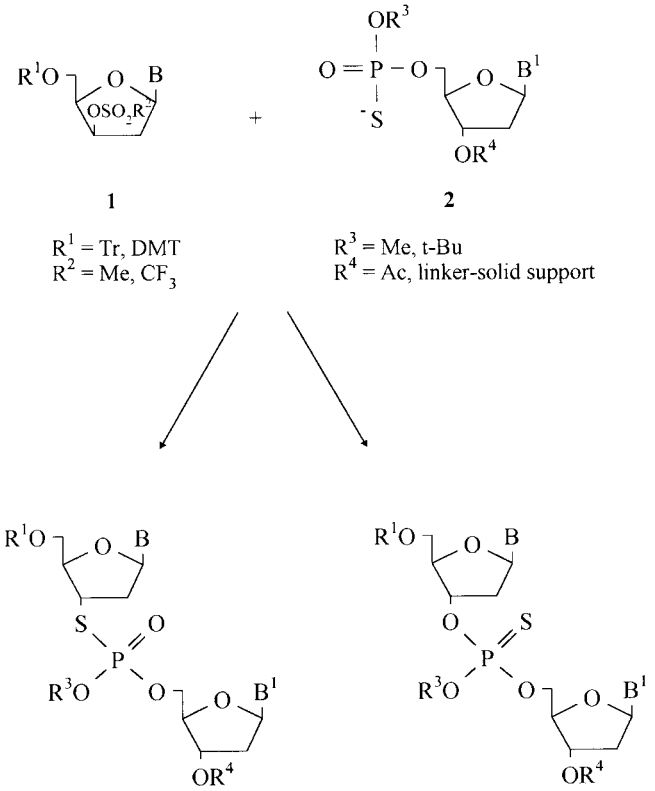
It was of great interest to examine which one of the above-mentioned synthetic possibilities would take place in reactions of **1** with **2**. In the same course of reactions, we also analyzed the reactivity of nucleoside 5′-O-(O-alkyl phosphate)s, -methanephosphonates, and -methanephosphonothioates toward activated xylonucleosides.

RESULTS

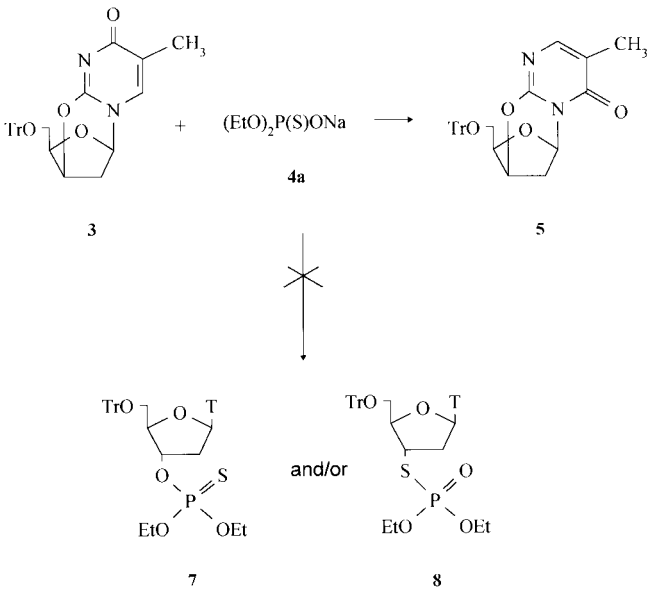
The first model system for reaction of activated xylonucleoside with ambident O,O-dialkyl phosphorothioate anion 5′-O-trityl-O²,3′-cycloanhydrothymidine (**3**) and sodium O,O-diethyl phosphorothioate (**4a**) has been chosen. Cycloanhydride **3** is broadly used for reaction with lithium azide as the route to production of 3′-deoxy-3′-azidothymidine (AZT) [14]. 5′-O-trityl-O²,3′-cycloanhydrothymidine (**3**) did not change when heated in DMF solution in a presence of **4a** at 100°C for 8 hours. However, if reaction of **3** with **4a** was performed in DMF solution and the process was maintained at 150°C for 28 hours, the only product of this reaction was 5′-O-trityl-O²,3′-cycloanhydro-N³-thymidine (**5**) isomeric with **3**, the product of its intramolecular $N^1 \rightarrow N^3$ rearrangement (Scheme 2).

This product was isolated in 23% yield, and its structure has been proved by ^{1}H- and ^{13}C-NMR and mass spectrometry. A similar result was obtained when **3** was heated in DMF solution with sodium O,O-diethyl phosphate (**6a**). Two-dimensional NMR spectroscopy and X-ray structure analysis of **5** and a discussion of the mechanism of its formation will be published elsewhere. Interestingly, reaction of **3** with a 2.5-fold molar excess of hydrogen O,O-diethyl phosphorothioate (**4b**) at 63°C in DMF solution provided, after 35 hours, a 50% yield (^{31}P-NMR assay) of a mixture of 5′-O-tritylthymidine 3′-O-(O,O-diethyl phosphorothioate) (**7**, ^{31}P-NMR 67.8) and 5′-O-tritylthymidine 3′-S-(O,O-diethyl phosphorothioate) (**8**, ^{31}P-NMR 24.6) in the ratio 54:46, respectively. The striking difference in the reactivity of the cycloanhydride **3** toward phosphorothioates **4a** and **4b** can be explained in terms of acid catalysis caused by **4b**.

The low reactivity of **4a** and **6a** toward **3** prompted us to prepare 5′-O-trityl-3′-O-methanesulfonylxylothymidine (**9**) [15] and its trifluoromethanesulfonyl analog **10** [16]. They were prepared by conversion of 5′-O-tritylthymidine into 5′-O-tritylxylothymidine (**11**) followed by further treatment with the corresponding sulfonating reagent, preferably the appropriate chloride or anhydride. Trifluoromethanosulfonyl chloride cannot be used in the synthesis of **10** because it produces in the reaction with **11** only 5′-O-trityl-3′-chloro-3′-deoxythymidine (**12**) (synthesis of **12** and its X-ray structure analysis will



SCHEME 1

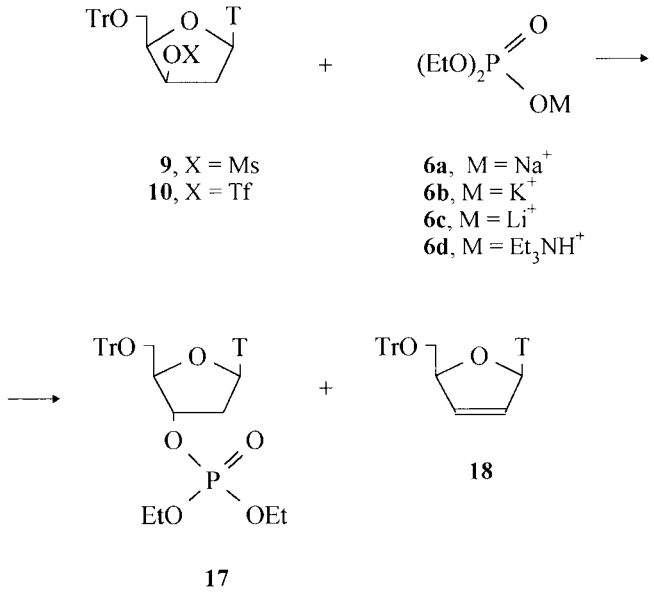


SCHEME 2

be published elsewhere). Such a course of reaction can be explained by the reactivity of **10** toward chloride ion present in the reaction mixture. It was found that the triflate derivative **10** partly decomposes during purification on silica gel. Therefore, **10** was prepared each time *in situ* just before the next reaction.

To establish experimental conditions for reactions of **9** and **10** with 3′-O-acetylthymidine 5′-O-(O-methyl phosphate) (**13**), 5′-O-(O-methyl phosphorothioate) (**14**), -methanephosphonate (**15**), -methanephosphonothioate (**16**), and model nucleophiles, O,O-diethyl phosphate (**6**) and O,O-diethyl phosphorothioate (**4**) have been used. Reaction of 5′-O-trityl-3′-O-methanesulfonylxylothymidine (**9**) with the potassium salt of **6** in DMF solution required heating to 100°C. The reaction progress was monitored by TLC and ^{31}P-NMR spectroscopy. After 7.5 hours, the reaction mixture was cooled, and, after silica gel chromatography, 5′-O-tritylthymidine3′-O-(O,O-diethyl phosphate) (**17**) and 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**) were isolated in yields of 17% and 44%, respectively. This result clearly indicated that the nucleophilic substitution process is accompanied by elimination (Scheme 3).

Changing the counterion in **6** to sodium, lithium, or triethylammonium did not suppress elimination. The ratios of **17** to **18** in the resulting reaction mixtures were analyzed by Normal-phase HPLC (NP-HPLC) and were in the range of 20–40% to 80–60%, respectively. Other modifications of reaction conditions such as using **6b** (K^+) in the presence of 18-crown-6 ether or using DMSO or pyridine as solvents were also unsuccessful with respect to suppression of the elimination process. Because it was reported, in recent work [16] on the preparation of 3′-hydroperoxy-3′-deoxythymidine *via* the mesylate ester **9**, that the extended reaction time led to an increase of the content of the elimination product **18**, suggesting that the elimination process follows substitution, phosphate **17** was refluxed in DMF solution with and without the presence of potassium O,O-diethyl phosphate (**6b**) for 3 hours. NP-HPLC analysis showed that elimination did not occur. This experiment proved that, in the reaction of **9** with phosphates **6**, the elimination process does not follow substitution; these two reactions are running in parallel. The more reactive triflate ester **10** was treated with phosphate **6b** in DMF at 0°C for 0.5 hour and then at ambient temperature overnight. The product of substitution, **17**, was isolated in an 11% yield. NP-HPLC showed also the presence of the elimination product **18** in the reaction mixture. Predominant elimination in the reaction of **9** or **10** with **6** reflects the poor nucleophilicity of the O,O-diethyl phosphate monoanion, but it also illustrates that the *trans* relationship of the 2′-hydrogen and the 3′-sulfonyloxy-group in **9** and **10** favors elimination.



SCHEME 3

Sodium O,O-diethyl phosphorothioate (**4a**) was reacted with activated xylothymidine derivatives **9** and **10** under similar conditions as employed for the phosphate **6**. Careful inspection of the reaction mixtures by TLC, ^{31}P-NMR spectroscopy, and reversed-phase HPLC (RP-HPLC) revealed the presence of three major products: the product of O-substitution **7**, the product of S-substitution **8**, and the product of elimination **18**. It was found that treatment of the mesylate ester **9** with the phosphorothioate **4a** resulted in predominant formation of the product of elimination **18**, similarly to the reaction of **9** with phosphates **6** mentioned earlier. In the reaction of the triflate ester **10**, the amount of elimination product **18** was greatly reduced. The products of O-substitution, **7**, and of S-substitution, **8**, were formed in a nearly equimolar ratio, and they were isolated from a reaction mixture by silica-gel chromatography in 23% and 12% yields, respectively.

The results concerning the reactivity of **4a** (sodium salt) with O-sulfonated xylothymidine **9** and **10** were compared with the results of alkylation of the same substrate with methyl iodide, isopropyl iodide, O-methyl triflate, and O-isopropyl triflate. Reactions were performed at ambient temperature to avoid thiono-thiolo rearrangement in DMF/CH_2Cl_2 solution. The ratio of the products of S- and O-alkylation were assigned from integrated ^{31}P-NMR spectra. Re-

action of the ambident phosphorothioate **4a** with methyl and isopropyl iodide and with the O-methyl triflate led to exclusive or predominant (>90%) S-alkylation, while alkylation with O-isopropyl triflate led to 37% O-alkylation. It is worth while to emphasize that the yields of triesters were satisfactory in the cases of methyl iodide and methyl triflate, whereas isopropylation, besides being more differentiated with respect to chemoselectivity, gave products in much lower yields.

Results of the reactions of mesylate **9** and triflate **10** with phosphate **6** and phosphorothioate **4** allowed us to select the optimum reaction conditions when nucleoside 5′-O-phosphates and their P-analogs were used as nucleophiles. Only triflate **10** was chosen as an electrophilic substrate because the studies mentioned earlier proved that substitution in the mesylate **9** requires harsh conditions, and predominant elimination was observed. Nucleophilic substrates 3′-O-acetylthymidine 5′-O-(O-methyl phosphate) (**13**) and the corresponding 5′-O-(O-methyl phosphorothioate) (**14**) were obtained by the phosphoramidite method depicted in Scheme 4.

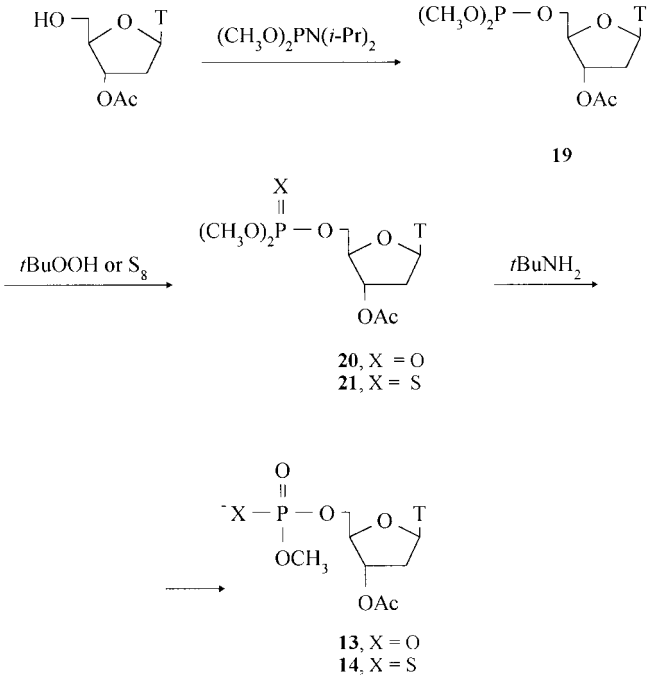
3′-O-Acetylthymidine was phosphitylated with N,N-diisopropylamino O,O-dimethyl phosphite to give the P-III intermediate **19**, which was oxidized in situ with *tert*-butyl hydroperoxide or sulfurized with elemental sulfur. The isolated phosphate **20** and phosphorothioate **21** were demethylated with *tert*-butylamine [17] to give the desired methyl-*tert*-butylammonium salts of **13** and **14** in excellent yields. 3′-O-Acetylthymidine 5′-O-methanephosphonate (**15**) and -methanephosphonothioate (**16**) were obtained by the method presented in Scheme 5.

3′-O-Acetylthymidine was phosphorylated with P,P-dichloromethanephosphonate or P,P-dichloromethanephosphonothioate to give **22** and **23**, respectively, which, without isolation, were reacted with methanol. The isolated compounds **24** and **25** were demethylated with *tert*-butylamine, and the desired products **15** and **16** were obtained in 30% and 64% total yields, respectively.

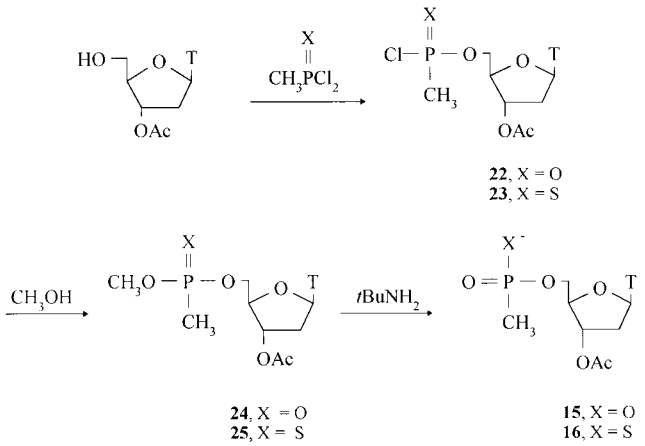
The obtained methyl-*tert*-butylammonium salts of 3′-O-acetylthymidine 5′-O-(O-methyl phosphate) (**13**), 5′-O-(O-methyl phosphorothioate) (**14**), 5′-O-methanephosphonate (**15**), and 5′-O-methanephosphonothioate (**16**) were reacted with 5′-O-trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) under the same conditions as established in the model studies (0°C, 0.5–1 hour, and then allowed to stand at ambient temperature overnight) (Scheme 6).

The obtained reaction mixtures were analyzed by TLC, ^{31}P-NMR spectroscopy, and RP-HPLC. Due to their complexity, it was not possible to isolate products by silica-gel chromatography, but the RP-HPLC technique proved to be effective to isolate the dinucleotides **26–31**. Retention times, ^{31}P-NMR and MS data, and yields of **26–31** are presented in Table 1.

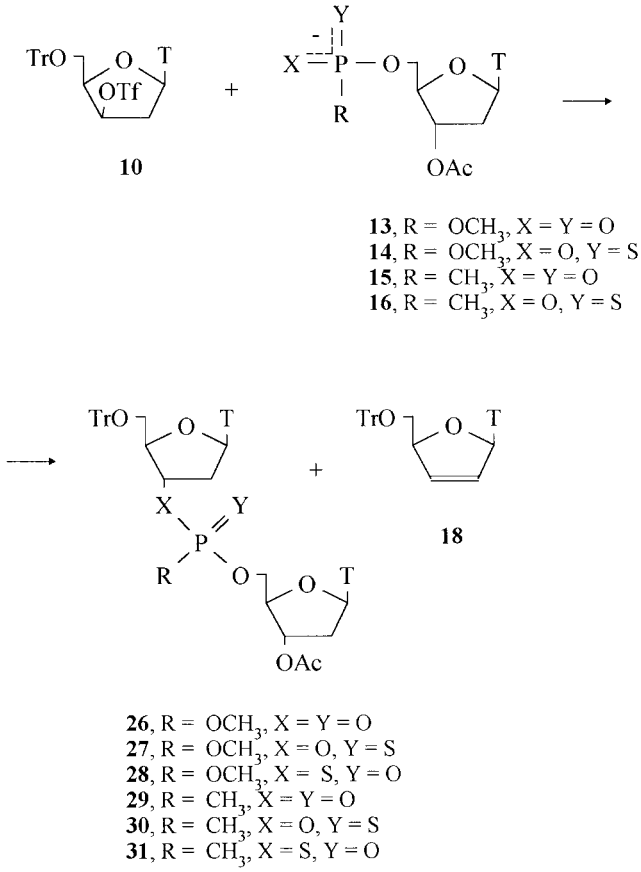
RP-HPLC analysis of the reaction mixtures also proved that, in all cases, nucleophilic substitution was accompanied by elimination. Similarly, as found in the model studies with O,O-diethyl phosphorothioate (**4**), also in reactions of phosphorothioate **14** and methanephosphonothioate **16**, substantial amounts of O-alkylation products were detected. ^{31}P-NMR analyses of appropriate reaction mixtures also



SCHEME 4



SCHEME 5



SCHEME 6

showed that O-substitution prevails in the reactions of **14**. On the other hand, nearly an equimolar ratio of **30** and **31** was formed in the reaction of the methanephosphonothioate **16**. ^{31}P-NMR and RP-HPLC analyses of reaction mixtures also proved that, in products **26–31**, both diastereomers were formed in nearly equimolar amounts.

DISCUSSION

Early attempts at formation of an internucleoside bond to synthesize oligonucleotides included reactions of activated nucleosides with nucleoside phosphates either by (a) an attack of nucleoside 3′-O-phosphate on a 5′-activated nucleoside [18–20] or (b) by an attack of a nucleoside 5′-O-phosphate at the 3′-activated carbon atom of the sugar part of the nucleoside [20–22]. The use of this latter approach to synthesize oligonucleotides possessing a modified phosphate group (e.g., phosphorothioate) was mentioned only recently in the patent literature [11, 12]. It was claimed that reactions of nucleoside 5′-O-(O-alkyl phosphorothioate)s and -methanephosphonothioates with 3′-O-activated xylonucleosides occur smoothly involving exclusively an attack of an oxygen atom of the phosphoro(no)thioate anion at the 3′-carbon only, and this methodology could be applied for a stereospecific synthesis of pure diastereomers of appropriate oligonucleotide analogs.

These claims were in sharp contrast with numerous literature reports [2–10] indicating that nucleophilic attack of ambident O,O-dialkyl phosphorothioate anions on sp^3 carbon atoms gives, in most cases, predominant formation of S-alkylated products. As early as in 1955, Kabachnik and Mastryukova [2] proved that, in the reaction of O,O-diethyl phosphorothioate with *n*-butyl bromide, only S-butyl-O,O-diethyl phosphorothioate was formed. Later extensive studies on ambident reactivity of phosphorothioate anions performed by Russian workers [3–5] and others [6–10] proved that the ratio and the yields of S- and O-substitution products depend on four factors: (1) the nature of substituents at phosphorus, electron-donating groups raising the ratio of O-alkylation to S-alkylation; (2) the nature of the electrophilic center, secondary carbon atoms favoring O-substitution; (3) the "hard" and "soft" character of the leaving group in the electrophile, the "hard" electrophiles favoring O-alkylation; and (4) polarity of the medium, the highly polar solvents favoring O-substitution.

The long-term efforts of this Laboratory are continuously focused upon the design of efficient methods of stereocontrolled methods of synthesis of oligo(nucleoside phosphorothioate)s [13] and oligo(nucleoside methanephosphonate)s [23] of predetermined sense of chirality at each internucleotide phosphorus atom. Because, in the patents [11, 12] mentioned earlier, there were no experimental data concerning conditions under which stereoregular oligonucleotides are formed, we reinvestigated the reactivity of anionic thymidine 5′-O-(O-methyl phosphate (**13**), -O-methyl phosphorothioate (**14**), -methanephosphonate (**15**), and -methanephosphonotioate (**16**) toward activated xylonucleosides **9** and **10**. These studies were preceded by investigations of model reactions of O,O-diethyl phosphate (**6**) and -phosphorothioate (**4**) anions. With the analysis of products formed in these model reactions, we were able to establish experimental conditions for substitution at the 3′-carbon atom of 3′-O-activated xylothymidine. Corresponding reactions were performed as previously presented. The expected products of nucleophilic substitution at the 3′-carbon in compounds **9** and **10** were formed only in miserable yields, and substitution was always accompanied by the elimination process. To achieve substitution of the mesyl group in **9**, harsh reaction

TABLE 1 RP-HPLC, ^{31}P-NMR, MS, and Yield Data of Obtained Dinucleotides **26–31**

Dinucleotide	*RP-HPLC t_r (min)*	*^{31}P-NMR δ (ppm)*	*MS M − 1*	*Yield of Isolated Compounds (%)*
Tr-$T_{OP(O)(OCH3)}$T-Ac **26**	13.40, 13.89	0.45, 0.01	843	11.8
Tr-$T_{OP(S)(OCH3)}$T-Ac **27**	20.25, 21.87	70.07, 69.64	859	6.8
Tr-$T_{SP(O)(OCH3)}$T-Ac **28**	14.28, 14.77	28.24, 27.92	859	3.8
Tr-$T_{OP(O)(CH3)}$T-Ac **29**	11.65, 12.33	33.30, 32.67	827	13.5
Tr-$T_{OP(S)(CH3)}$T-Ac **30**	18.72, 21.03	99.58, 98.13	843	6.6
Tr-$T_{SP(O)(CH3)}$T-Ac **31**	13.28, 14.08	54.08, 54.06	843	7.7

conditions (temperature at 100°C) were required. However, the most important observation discriminating against the preparative value of the approach under investigation was the lack of chemoselectivity and the formation of both possible products of substitution, namely, the formation of both 3′-O- and 3′-S phosphorothioates, **7** and **8**, respectively. A relatively high ratio of O-substitution to S-substitution in the reactions of **9** and **10** with the phosphorothioate anion, as proved in these studies, is not surprising since Mastryukova et al. [4] demonstrated in their studies on ambident reactivity of O,O-dialkyl phosphorothioates that the ratio of O- and S-alkylation strongly depends on the character of the leaving group in the electrophilic molecule. They also found that a "harder" character of the leaving group (e.g., tosyl) favored O-substitution. Michalska et al. [6] observed predominant O-alkylation in a reaction of α-D-glucopyranosyl bromide with silver salts of phosphorothioate diesters. A substantial ratio of O-alkylation to S-alkylation was also observed in our model reaction between phosphorothioate **4a** and O-isopropyl triflate.

The conclusions from discouraging results obtained with model O,O-diethyl phosphorothioates reacting with activated xylothymidines **9** and **10** were later reconfirmed by experimental evidence gained from reactions of **10** with 3′-O-acetylthymidine 5′-O-(O-methyl phosphate) **13** and its analogs such as 3′-O-acetylthymidine 5′-O-(O-methyl phosphorothioate) (**14**), -methanephosphonate (**15**), and -methanephosphonothioates (**16**). Results of these reactions are depicted in Scheme 6 and summarized in Table 1. An inspection of this table allows for the generalization that a low efficiency of substitution and formation of both O- and S-substitution products **27** and **28**, and **30** and **31**, respectively, discriminate against this approach for the synthesis of modified internucleotide linkages *via* alkylation of nucleoside P-achiral (O-methyl phosphate)s and -methanephosphonates or nucleoside P-chiral (O-methyl phosphorothioate)s and -methanephosphonothioates, with 3′-O-xylothymidine sulfonates. In light of these findings, attempts at the stereocontrolled synthesis of P-chiral analogs of oligonucleotides, requiring separated diastereomers of **14** or **16**, have been abandoned.

CONCLUSIONS

3′-O-Activated xylothymidine undergoes reactions with nucleoside 5′-O-phosphate and its P-analogs yielding mixtures of products of competitive substitution and elimination processes. The cycloanhydronucleoside did not give any substitution or elimination products when reacted with sodium O,O-dialkyl phosphate or phosphorothioate. Under prolonged heating at 150°C, only the $N^1 \rightarrow N^3$ rearrangement occurred. Activation of the 3′-hydroxyl group in a nucleoside via mesylate derivative is not sufficient to create an internucleotide bond because nucleophilic substitution of this group by O,O-dialkyl phosphate or phosphorothioate anions requires harsh conditions. Triflate activation is more effective, but reactions of nucleophilic substitution are always accompanied by elimination, and, in effect, yields of formed dinucleotides are only moderate. When ambident $>P(O)S^-$ nucleophiles were used, both S- and O-substitution products were obtained. There was no stereoselectivity in the formation of diastereomers of corresponding dinucleotides. Results presented here on the reactivity of thymidine 5′-O-(O-methylphosphate),-O-methylphosphorothioate, -methanephosphonate, and -methanephosphonothioate toward activated xylonucleosides cast serious doubts on the results disclosed in the patent literature [11, 12].

EXPERIMENTAL

Pyridine, triethylamine, dichloromethane, and benzene were dried by distillation from calcium hydride. N,N-Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were dried by distillation under reduced pressure. 5′-O-Trityl-O², 3′-cycloanhydrothymidine (**3**) was obtained from 5′-O-tritylthymidine [24]. Sodium O,O-diethyl phosphorothioate (**4a**) and hydrogen O,O-diethyl phosphorothioate were prepared from O,O-diethyl phosphite by the method of Foss [25]. Sodium O,O-diethyl phosphate (**6a**), potassium (**6b**), lithium (**6c**), and triethylammonium (**6d**) salts were obtained by neutralization of O,O-diethyl phosphoric acid (prepared by oxidation of O,O-diethylphosphite with potassium permanganate [26]) with appropriate hydroxides.

Progress of each reaction was monitored by TLC. HPLC analyses were performed on LDC Analytical using Econosil C-18 reverse-phase column (flow 1 mL/min) and a self-made normal-phase column filled with Lichrosorb Si 60 (flow 1.25 mL/min). The following gradients were used: system A, *n*-hexane (a), isopropyl alcohol (b), 0 min − 8% b, 30 min − 35% b; system B, water (a), acetonitrile (b), 0 min − 45% (b), 30 min − 85% (b); system C, water (a), a mixture of acetonitrile and water [4:1 (v/v)] (b), 0 min − 5% b, 1.5 min − 15% b, 3 min − 55% b, 5 min − 75% b, 30 min − 95% b. ^{1}H- and ^{31}P-NMR spectra were recorded on Bruker AC-200 (200 MHz) and DRX-500 (500 MHz) instruments. Mass spectrometry (MS) analyses were performed with a Finnigan MAT 95 apparatus.

5′-O-Trityl-O²,3′-cycloanhydro-N³-thymidine (**5**)

To a stirred solution of 5′-O-trityl-O²,3′-anhydrothymidine (**3**) (466 mg, 1 mmol) in anhydrous DMF (5 mL) was added sodium O,O-diethyl phosphorothioate **4a** (866 mg, 4.92 mmol). The stirred reaction mixture was maintained at reflux temperature for 28 hours. After having been cooled to room temperature, the brown solution was concentrated in vacuo to dryness. The residue was suspended in chloroform (150 mL), and the resulting solution was washed with water (3 × 30 mL), dried with anhydrous $MgSO_4$, and the solvent was evaporated to dryness. The crude product was purified by silica gel column chromatography (silica gel, 230–400 mesh, 10 g). The eluting system: CH_2Cl_2 (50 mL), CH_2Cl_2:$CHCl_3$ [1:1, v/v, (50 mL)], $CHCl_3$ (50 mL), $CHCl_3$ containing 0–5% MeOH. Appropriate fractions [TLC control, R_f 0.46, (chloroform:ethanol, 19:1, v/v)] were combined and evaporated to dryness, providing 107.2 mg of the product **5** as a colorless solid in 23% yield. M.p. 223–224°C. Elemental analysis: found C, 74.40%; H, 5.67%; N, 6.08%; required C, 74.72; H, 5.62; N, 6.01%. EIMS (high-resolution mass spectrum) for $C_{29}H_{26}O_4N_2$: calcd 466.1893, found 466.1876; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.96 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH_3), 2.33 (dd, 1H, $J_{2'\beta,2'\alpha}$ = 12.9, $J_{2'\beta,3'}$ = 1.4, 2′$_\beta$-H), 2.45 (ddd, 1H, $J_{2'\alpha,2'\beta}$ = 12.9, $J_{2'\alpha,1'}$ = 2.45, $J_{2'\alpha,3'}$ = 2.6, 2′$_\alpha$-H), 3.34 (ddd, 2H, $J_{5',5''}$ = 12.0 $J_{5',4'}$ = 6.8, 5′,5″-H), 4.26 (td, 1H, $J_{4',5'}$ = 6.8, $J_{4',3'}$ = 2.4, 4′-H), 5.17 (m, 1H, 3′-H), 6.75 (d, 1H, $J_{1',2'\alpha}$ = 4.0, 1′-H), 7.45 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.1, 6-H), 7.20–7.50 (m, 15H, Tr-H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 12.96 (5-CH_3), 32.80 (2′-C), 62.01 (5′-C), 76.87 (3′-C), 77.91 (1′-C), 84.38 (4′-C), 87.18 (Tr-C-O), 117.07 (5-C), 127.03 (Tr), 127.80 (Tr), 128.54 (Tr), 143.33 (Tr), 150.48 (6-C), 152.20 (2-C), 160.93 (4-C).

Reaction of 5′-O-Trityl-O²,3′-cycloanhydrothymidine (**3**) *with Hydrogen O,O-Diethyl Phosphorothioate* (**4b**)

To a stirred solution of 5′-O-trityl-O²,3′-cycloanhydrothymidine (**3**) (89 mg, 0.19 mmol) in anhydrous DMF (0.5 mL) was added hydrogen O,O-diethyl phosphorothioate (**4b**) (85 mg, 0.5 mmol). The reaction mixture was maintained at 63°C for 35 hours. Then the reaction mixture was analyzed by ^{31}P-NMR (81 MHz, DMSO-d_6) spectroscopy. Two major (50% yield) products were observed: 5′-O-tritylthymidine 3′-O-(O,O-diethyl phosphorothioate) (**7**, δ_P = 67.8) and 5′-O-tritylthymidine 3′-S-(O,O-diethyl phosphorothioate) (**8**, δ_P = 24.6) in the ratio 54:46, respectively. Structures of **7** and **8** were additionally proved by adding to the reaction mixtures authentic samples made by independent syntheses (vide infra).

5′-O-Trityl-3′-O-methanesulfonylxylothymidine (**9**)

5′-O-Tritylxylothymidine [24] (**11**) (7.2 g, 14.8 mmol) was dried by coevaporation with anhydrous pyridine (2 × 30 mL), resuspended in a fresh portion of anhydrous pyridine (50.0 mL), and treated at 0°C with methanesulfonyl chloride (3.6 mL, 46.5 mmol). The reaction mixture was stirred for 3 hours at 0°C and then at room temperature overnight. Then solvents were evaporated, and the residue was dissolved in 300 mL of ethyl acetate. The solution was washed with 3 × 50 mL of water, and combined water washings were extracted with 2 × 50 mL of ethyl acetate. Organic layers were combined and dried over anhydrous $MgSO_4$, and the solvent was removed to dryness. Crude product (8.1 g) was purified by column chromatography (silica-gel 230–400 mesh, 100 g, a gradient of 0–2% methanol in chloroform as eluting system). The title compound (4.35 g, 52% of yield)

was obtained as a colorless solid: m.p. 108–111°C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 1.80 (s, 3H, 5-CH_3), 2.46 (m, 1H, 2′$_\beta$-H), 2.76 (s, 3H, Ms-CH_3), 2.79 (m, 1H, 2′$_\alpha$-H), 3.49 (m, 2H, 5′,5″-H), 4.21 (m, 1H, 4′-H), 5.27 (m, 1H, 3′-H), 6.28 (dd, 1H, $J_{1',2'\beta} = 7.8$, $J_{1',2'\alpha} = 3.2$, 1′-H), 7.20–7.50 (m, 15H, Ar-H), 9.50 (s, 1H, 6-H).

5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**)

To a stirred solution of 5′-O-tritylxylothymidine (**11**) (110.0 mg, 0.23 mmol) in anhydrous dichloromethane (3.0 mL) and anhydrous pyridine (73.5 μL), trifluoromethanesulfonyl anhydride (96 μL, 0.57 mmol) was added dropwise during 5 minutes. After 10 minutes at −30°C, the reaction mixture was allowed to warm to −5–0°C, and stirring was continued for 1 hour. Attempts to purify crude **10** by silica-gel chromatography led to its substantial decomposition.

Reaction of 5′-O-Trityl-3′-O-methanesulfonylxylothymidine (**9**) *with Potassium O,O-Diethyl Phosphate* (**6b**)

To a solution of **9** (28 mg, 0.05 mmol) in anhydrous DMF (0.5 mL), **6b** (28.8 mg, 0.15 mmol) was added, and the resulting solution was heated at 100°C for 7.5 hours. Then the solution was cooled to room temperature, DMF was removed under reduced pressure, and the residue was dissolved in 10 mL of chloroform. The chloroform solution was washed with 2 × 4 mL of water, dried with anhydrous $MgSO_4$, and the solvent was evaporated to dryness. TLC analysis [developing system: acetone:petroleum ether, 1:3 (v/v)] of the reaction mixture revealed the presence of two products (R_f 0.2 and 0.8) that were purified by preparative TLC. The less polar product was identified as 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**), 10.2 mg (43.8%): ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.25 (d, 3H, $J_{5\text{-}CH3,6\text{-}H} = 1.2$, 5-$CH_3$), 3.39 (m, 2H, 5′,5″-H), 4.98 (m, 1H, 4′-H), 5.90 (ddd, 1H, 2′-H), 6.37 (ddd, 1H, 3′-H), 7.07 (m, 1H, 1′-H), 7.20–7.50 (m, 16H, Ar-H), 8.91 (br.s, 1H, = N^3-H); MS: FAB −ve, *m/z* 465 [(M − 1)$^-$]. The more polar product was identified as 5′-O-tritylthymidine 3′-O-(O,O-diethyl phosphate) (**17**), 5.5 mg (17.3%): ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.32 (m, 6H, Et-CH_3), 1.39 (s, 3H, 5-CH_3), 2.41 (m, 1H, 2′$_\alpha$-H), 2.63 (m, 1H, 2′$_\beta$-H), 3.38 (dd, 1H, $J_{5',5''} = 10.6$, $J_{5',4'} = 2.6$, 5′-H), 3.52 (dd, 1H, $J_{5'',5'} = 10.6$, $J_{5'',4'} = 2.7$, 5″-H), 4.07 (m, 4H, Et-OCH_2-), 4.28 (m, 1H, 4′-H), 5.14 (m, 1H, 3′-H), 6.45 (dd, 1H, $J_{1',2'\beta} = 8.6$, $J_{1',2'\alpha} = 5.5$, 1′-H), 7.20–7.50 (m, 15H, Ar-H), 7.57 (s, 1H, 6-H), 8.52 (br.s, 1H, = N^3-H); ^{31}P-NMR (81 MHz, $CDCl_3$) $\delta_P = -1.33$; MS: FAB +ve, *m/z* 621 (M + 1)$^+$; FAB −ve, *m/z* 619 (M − 1)$^-$.

The structure of **17** was additionally confirmed by its independent synthesis starting from 5′-O-tritylthymidine and O,O-diethyl phosphite via the Atherton-Todd reaction. **17** was obtained in 46% yield; ^{1}H-NMR and MS data vide supra.

Reaction of 5′-O-Trityl-3′-O-methanesulfonylxylothymidine (**9**) *with Different Salts of O,O-Diethyl Phosphate* (**6a–d**)*: General Procedure*

To a solution of **9** (0.05 mmol) in anhydrous DMF (or DMSO, or pyridine) (0.5 mL) at room temperature, each corresponding **6a–d** (0.15 mmol) was added, and this mixture was kept for several hours at 80°C (or 100°C); reaction progress was monitored by means of TLC. Analysis of the final reaction mixture was performed by means of NP-HPLC (system A). Two products **17** (t_r = 17.95 min) and **18** (t_r = 12.97 min) were observed.

Reaction of 5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) *with Potassium O,O-Diethyl Phosphate* (**6b**)

To a stirred solution of **10** (0.23 mmole) in dichloromethane prepared in situ, a solution of **6b** (199 mg, 1.04 mmol) in dry DMF (4.0 mL) was added dropwise during 10 minutes at −35°C. The reaction temperature was maintained at −5°C for 1 hour, then it was allowed to warm to room temperature and left for 12 hours. Solvents were removed under reduced pressure, the residue was extracted with 50 mL of chloroform, and this extract was washed with 3 × 10 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. Separation by silica gel column chromatography (silica-gel 230–400 mesh, 10.0 g, eluting system: 0–1% of methanol in chloroform) provided 5′-O-tritylthymidine 3′-O-(O,O-diethyl phosphate) (**17**) (15.2 mg, 10.8%) as a colorless solid (analytical data as shown earlier).

Reaction of 5′-O-Trityl-3′-O-methanesulfonylxylothymidine (**9**) *with Sodium O,O-Diethyl Phosphorothioate* (**4a**)

To a stirred solution of **9** (21.5 mg, 0.0382 mmol) in anhydrous DMF (4.0 mL), **4a** was added (38 mg, 0.167 mmol) at room temperature. The reaction mix-

ture was warmed to 100 ± 4°C, and stirring was continued for 22.5 hours. Then it was cooled, and the solvent was evaporated to dryness. The ^{31}P-NMR (81 MHz, $CDCl_3$) spectrum for the mixture indicated the presence of 5′-O-tritylthymidine 3′-O(O,O-diethyl phosphorothioate) (**7**) (δ_P = 67.60). RP-HPLC analysis (system B) showed the presence of the following products: (a) 5′-O-trityl-2′,3′-didehydrothymidine (**18**) (t_r = 16.88 min, 59.1%), (b) **7** (t_r = 30.72 min, 15.8%), (c) 5′-O-tritylthymidine 3′-S-(O,O-diethyl phosphorothiolate) (**8**) (t_r = 18.63 min, 1.5%), (d) unreacted **9** (t_r = 14.63 min, 5.3%), and (e) unidentified compounds (t_r = 16.08, 20.15, and 21.65 min, 18.3% total).

Reaction of 5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) *with Sodium O,O-Diethyl Phosphorothioate* (**4a**)

To a stirred solution of **10** (0.20 mmole) prepared in situ, in dichloromethane (2 mL) a solution of **4a** (173.8 mg, 0.91 mmol) in dry DMF (2.0 mL) was added dropwise during 10 minutes. After 1.5 hours of stirring below 0°C, the mixture was allowed to warm gradually to room temperature, and the reaction mixture was maintained at this temperature overnight. Solvents were removed under reduced pressure, and the residue was dissolved in 50 mL of chloroform, and this solution was washed with 3 × 10 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. Purification by means of silica-gel column chromatography (silica-gel 60H, 10.0 g, eluted with chloroform containing 0–5% of methanol) provided the following compounds: (a) 5′-O-tritylthymidine 3′-O-(O,O-diethyl phosphorothioate) (**7**) [28.9 mg (23%) as a colorless foam; ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 67.79; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.29 (m, 6H, Et-CH_3), 1.43 (s, 3H, 5-CH_3), 2.3–2.7 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.45 (m, 2H, 5′,5″-H), 3.90–4.20 (m, 4H, Et-CH_2-), 4.27 (m, 1H, 4′-H), 5.38 (m, 1H, 3′-H), 6.44 (q, 1H, $J_{1',2'\alpha}$ = 5.6, $J_{1',2'\beta}$ = 8.7, 1′-H), 7.20–7.60 (m, 16H, Ar-H), 8.80 (br.s, 1H, = N^3-H): MS: FAB −ve, *m/z* 635 (M − 1)$^-$, 607 (M-Et)$^-$]; (b) 5′-O-trityl-3′-deoxythymidine 3′-S-(O,O-diethyl phosphorothiolate) (**8**), [14.9 mg (11.8%), ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 24.52; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.24–1.34 (m, 6H, Et-CH_3), 1.43 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.2, 5-CH_3), 2.61 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.45 (m, 2H, 5′,5″-H), 3.71–4.20 (m, 6H, 3′, 4′, Et-CH_2-), 6.23 (dd, 1H, $J_{1',2'\alpha}$ = 4.9, $J_{1',2'\beta}$ = 6.6, 1′-H), 7.15–7.60 (m, 16H, Ar-H), 10.17 (br.s, 1H, = N^3-H); MS: FAB +ve, *m/z* 659 (M + Na)$^+$, −ve, *m/z* 635 (M − 1)$^-$]; (c) 5′-O-tritylxylothymidine (**11**) (12.2 mg, 12.8%); and (d) 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**) (12.2 mg, 13.3%).

Reaction of Sodium O,O-Diethyl Phosphorothioate (**4a**) *with Methyl Iodide, Isopropyl Iodide, O-Methyl triflate, and O-Isopropyl triflate: General Procedure*

To a solution of each appropriate alkylating agent (0.04 mmole) in a mixture DMF/CH_2Cl_2 [1:1 (v/v), 0.4 mL], **4a** (0.01 mmole) was added. The reaction mixtures were maintained at room temperature for 12 hours [15 days for reaction of **4a** with isopropyl iodide, but even after such a long time, the reaction was not completed (^{31}P-NMR assay)]. The ratio of products of S- and O-alkylation were assigned from integrated ^{31}P-NMR spectra. Depending upon the type of alkylating agent used, the following yields (^{31}P-NMR) of products were obtained: 100% (methyl iodide), 96% (O-methyl triflate), 25% (isopropyl iodide), and 28% (O-isopropyl triflate).

N,N-Diisopropylamino O,O-Dimethyl Phosphite [27]

To a vigorously stirred solution of phosphorus trichloride (13.9 mL, 159.3 mmol) in anhydrous benzene (100 mL), cooled with an external ice-water bath, was added dropwise (20 drops per minute) a solution of N,N-diisopropylamine (49.9 mL, 379.7 mmol) in anhydrous benzene (100 mL). Then the reaction mixture was stirred at ambient temperature for 2 hours. The amine hydrochloride was removed by filtration and washed with benzene. To the solution of N,N-diisopropylphosphoramidodichloridite, a mixture of methanol (12.9 mL, 318.6 mmol) and triethylamine (44.2 mL, 318.6 mmol) was added dropwise at 0°C. The reaction mixture was maintained at 0°C for 0.5 hour and at ambient temperature for another 4 hours. Precipitated triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was distilled in vacuo providing pure product 19.9 g (yield 64.6%), b.p. 38–40°C (0.45 mmHg), ^{31}P-NMR (81 MHz, C_6D_6) δ_P = 150.7, ^{1}H-NMR (200 MHz, C_6D_6) δ_H: 1.17 (d, 12H, *i*-Pr-CH_3), 3.33 (d, 6H, $J_{OCH3,P}$ = 12.8, P-OCH_3), 3.65 [m, 2H, N-$CH(CH_3)_2$].

3′-O-Acetylthymidine 5′-O-(*O,O-Dimethyl Phosphate*) (**20**)

To a stirred solution of 3′-O-acetylthymidine (284 mg, 1 mmol) in anhydrous dichloromethane (8 mL),

tetrazole (78.1 mg, 1.1 mmol) and then N,N-diisopropylamino O,O-dimethyl phosphite (0.77 mL, 4 mmole) were added. Stirring of the reaction mixture was continued at room temperature for 4 days. To this reaction mixture, a solution of *tert*-butyl hydroperoxide in benzene was added until no phosphite intermediate **19** (^{31}P-NMR δ_P = 141.50 in benzene) was observed (TLC assay). Solvents were removed under reduced pressure, and the resulting solid residue was extracted with 50 mL of chloroform. This extract was washed with 3 × 5 mL of water and evaporated to dryness. Purification of the product was achieved by means of column chromatography [silica gel: 230–400 mesh, 4 g, gradient of 2–5% ethanol in chloroform as an eluting system; purity controlled with TLC, R_f 0.31 (chloroform:ethanol 19:1, v/v)]. Title compound was obtained in the yield of 94.9% (380 mg) [^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 1.9; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.90 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH_3), 2.08 (s, 3H, Ac-CH_3), 2.25 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.79 (dd, 6H, $J_{H,P}$ = 11.2, J = 2.1, P-OCH_3), 4.12 (m, 1H, 4′-H), 4.27 (m, 2H, 5′, 5″-H), 5.23 (m, 1H, 3′-H), 6.36 (dd, 1H, $J_{1',2'\alpha}$ = 5.5, $J_{1',2'\beta}$ = 9.4, 1′-H), 7.44 (d, 1H, $J_{H\text{-}6,5\text{-}CH3}$ = 1.1, 6-H), 9.91 (br.s, 1H, = N^3-H); MS: FAB +ve, *m/z* 351 (M + 1)$^+$, 701 (2M + 1)$^+$, FAB −ve, *m/z* 349 (M − 1)$^-$, 699 (2M − 1)$^-$].

3′-O-Acetylthymidine 5′-O-(O,O-Dimethyl phosphorothioate) (**21**)

Into a stirred solution of 3′-O-acetylthymidine (2.8 g, 10 mmol) in anhydrous dichloromethane (100 mL) at room temperature was added tetrazole (771 mg, 11 mmol) followed by N,N-diisopropylamino O,O-dimethyl phosphite (4.4 mL, 23 mmol). The reaction mixture was left for 48 hours at ambient temperature. The solvent was evaporated under reduced pressure, and the remaining foam (intermediate **19** as proven by ^{31}P-NMR) was dissolved in ca. 100 mL of anhydrous benzene. This solution was treated with 855 mg (27 mmol) of elemental sulfur and stirred at room temperature for 24 hours. The ^{31}P-NMR spectra indicated that the reaction had been completed. An excess of sulfur was filtered off, benzene was removed by evaporation, and 25 mL of acetonitrile was added. The precipitated sulfur was filtered off again, and the solvent was evaporated. Purification of the crude product by column chromatography (silica gel: 230–400 mesh, 70.0 g; gradient of 1–2% methanol in chloroform as an eluting system) gave the pure title compound (1.20 g, 94.8%). ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 72.4; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.96 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.2, 5-CH3), 2.10 (s, 3H, Ac-CH_3), 2.25 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.78 (dd, 6H, $J_{H,P}$ = 13.7, J = 1.0, P-OCH_3), 4.20–4.50 (m, 3H, 4′, 5′, 5″-H), 5.27 (m, 1H, 3′-H), 6.41 (dd, 1H, $J_{1',2'\alpha}$ = 5.3, $J_{1',2'\beta}$ = 9.2, 1′-H), 7.49 (d, 1H, $J_{H\text{-}6,5\text{-}CH3}$ = 1.1, 6-H), 9.12 (br.s, 1H, = N^3-H); MS: EI *m/z* 408 (M)$^+$, 409 (M + 1)$^+$.

N-Methyl-tert-butylammonium 3′-O-Acetylthymidine 5′-O-(O-Methyl phosphate) (**13**)

Compound **20** (13.0 mg, 0.03 mmol) was suspended in *tert*-butylamine (1 mL, 9.5 mmol), and the resulting mixture was stirred at room temperature for 24 hours. The solvent was evaporated to dryness leaving the desired **13** in a quantitative yield as a colorless solid. R_f 0.13 [developing system: chloroform:ethanol (19:1, v/v) containing 1% triethylamine]; ^{31}P-NMR (81 MHz, CD_3OD) $-\delta_P$ = 2.45; ^{1}H-NMR (200 MHz, CD_3OD) δ_H: 1.24 (s, 9H, *t*-Bu-CH_3), 1.94 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH3), 2.05 (S, 3H, Ac-CH_3), 2.40 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.60 (d, 6H, $J_{H,P}$ = 10.9, P-OCH_3), 4.15–4.25 (m, 3H, 4′, 5′, 5″-H), 5.39 (m, 1H, 3′-H), 6.35 (dd, 1H, $J_{1',2'\alpha}$ = 5.9, $J_{1',2'\beta}$ = 8.8, 1′-H), 7.85 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.1, 6-H); MS: FAB +ve, *m/z* 466 (M + 1)$^+$; FAB −ve, *m/z* 377 [M-(*t*-$BuNH_2CH_3$)]$^-$.

N-Methyl-tert-butylammonium 3′-O-Acetylthymidine 5′-O-(O-Methyl phosphorothioate) (**14**)

3′-O-Acetylthymidine 5′-O-(O,O-dimethyl phosphorothioate) (**21**) (22.0 mg, 0.05 mmol) was suspended in *tert*-butylamine (2.0 mL, 19.0 mmol), and the reaction mixture was maintained at ambient temperature for the following 15 hours. Solvent was removed by evaporation to dryness, and the product, the N-methyl *tert*-butylammonium salt of **14**, was obtained in a quantitative yield. R_f 0.47 [chloroform:ethanol (19:1, v/v) containing 1% triethylamine). ^{31}P-NMR (81 MHz, CD_3OD) δ_P = 60.22 (46.44%), 60.16 (53.56%); ^{1}H-NMR (200 MHz, CD_3OD) δ_H: 1.22 (s, 2 × 9H, *t*-Bu-CH_3), 1.96–1.99 (m, 2 × 3H, 5-CH_3), 2.01–2.20 (s, 2 × 3H, Ac-CH_3), 2.30–2.50 (m, 2 × 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.50–3.70 (m, 2 × 3H, P-OCH_3), 4.00–4.30 (m, 2 × 3H, 4′, 5′,5″-H), 5.35–5.45 (m, 2 × 1H, 3′-H), 6.35–6.45 (m, 2 × 1H, 1′-H), 7.90–8.00 (m, 2 × 1H, 6-H); MS: FAB −ve, *m/z* 393 [M-(*t*-$BuNH_2CH_3$)]$^-$.

3′-O-Acetylthymidine 5′-O-(O-Methyl methanephosphonate) (**24**)

To a stirred solution of methanephosphonodichloridate [28] (1.33 g, 10 mmol) in anhydrous pyridine

(5 mL), a solution of 3′-O-acetylthymidine (1.42 g, 5 mmol) in anhydrous pyridine (5 mL) was added dropwise over a period of 20 minutes at −10°C. Then the reaction mixture was gradually warmed to room temperature and left for 1 hour. After that time, 2 mL of anhydrous methanol (49 mmole) was added dropwise at 0°C, and stirring was continued for 1 hour. Solvent was removed under reduced pressure, and the residue was extracted with 150 mL of chloroform and then washed with 3 × 35 mL of water. The combined water layer was washed with 2 × 10 mL of chloroform. The combined organic layer was dried with anhydrous $MgSO_4$. The solvent was removed by evaporation, and the product was purified by silica-gel column chromatography (silica-gel, 230–400 mesh, 50 g, eluted with a gradient of 0–2% methanol in chloroform). The collected fractions [controlled by TLC, R_f 0.28, chloroform:ethanol (19:1, v/v)] gave 1.05 g of title compound. The yield was 56.1%. MS: EI, *m/z* 376 (M^+), 377 $(M + 1)^+$; ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 33.72 (57.4%), 33.16 (42.6%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.50 (d, 2 × 3H, $J_{H,P}$ = 17.5, P-CH_3), 1.89 (d, 2 × 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.2, 5-CH_3), 2.07 (s, 2 × 3H, Ac-CH_3), 2.00–2.25 (m, 2 × 2H, 2′$_\alpha$, 2′$_\beta$-H), 3.73 (d, 2 × 3H, $J_{H,P}$ = 11.1, P-OCH_3), 4.15–4.27 (m, 2 × 3H, 4′,5′,5″-H), 5.22 (m, 2 × 1H, 3′-H), 6.33 (dd, 2 × 1H, $J_{1',2'\alpha}$ = 5.0, $J_{1',2'\beta}$ = 8.1, 1′-H), 7.43 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.2, 6-H), 7.49 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.2, 6-H), 9.87 (br.s, 2 × 1H, = N^3-H).

3′-O-Acetylthymidine 5′-O-(O-Methyl methanephosphonothioate) (**25**)

To a cold (0°C) stirred solution of methanephosphonothiodichloridate [29] (1.1 mL, 10 mmol) in dry pyridine (5 mL) was added dropwise a solution of 3′-O-acetylthymidine (1.42 g, 5 mmol) in anhydrous pyridine (5 mL). The reaction mixture was stirred for 15 minutes, then the reaction temperature was gradually elevated to room temperature, and stirring was continued for about 2 hours. Anhydrous methanol (2 mL) was added dropwise at 0°C. After 3 hours, the solvent was evaporated to dryness, the residue was twice coevaporated with toluene (2 × 5 mL), and the remaining oil was partitioned between distilled water (50 mL) and chloroform (150 mL). The chloroform layer was washed with distilled water (2 × 30 mL). The combined water fractions were washed with chloroform (2 × 15 mL). The combined organic fractions were dried with anhydrous $MgSO_4$ and concentrated. The residue was purified by silica-gel column chromatography [silica-gel: 230–400 mesh, 50.0 g, eluted with a gradient of methanol (0–2%) in chloroform]. The collected fractions [monitored by TLC, R_f 0.46, chloroform:ethanol (19:1, v/v)] gave the title compound (1.569 g). The yield was 80.0%. MS: EI, *m/z* 392 (M^+), 393 $(M + 1)^+$; ^{31}P-NMR (81 MHz, CD_3OD) δ_P = 101.14 (57.7%), 100.79 (42.3%); ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.81 (d, 3H, $J_{H,P}$ = 15.4, P-CH_3), 1.82 (d, 3H, $J_{H,P}$ = 15.4, P-CH_3), 1.92 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH_3), 1.93 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH_3), 2.08 (s, 2 × 3H, Ac-CH_3), 1.80–2.50 (m, 2 × 2H, 2′$_\alpha$, 2′$_\beta$-H), 4.18 (m, 2 × 3H, P-OCH_3), 4.15–4.47 (m, 2 × 3H, 4′,5′,5″-H), 5.22 (m, 2 × 1H, 3′-H), 6.34 (m, 2 × 1H, 1′-H), 7.44 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.1, 6-H), 7.45 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.1, 6-H), 9.77 (br.s, 2 × 1H, = N^3-H).

N-Methyl-tert-butylammonium 3′-O-Acetylthymidine 5′-O-Methanephosphonate (**16**)

3′-O-Acetylthymidine-5′-O-(O-methyl methanephosphonate) (**24**) (59.7 mg, 0.16 mmol) was suspended in *tert*-butylamine (1.0 mL, 9.5 mmol), and the solution was maintained at ambient temperature for 7 days. The solvent was removed by evaporation to dryness, and the N-methyl *tert*-butylammonium salt of 3′-O-acetylthymidine 5′-O-methanephosphonate was obtained in quantitative yield. ^{31}P-NMR: (81 MHz, $CDCl_3$) δ_P = 23.52; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.25 (d, 3H, $J_{CH3,P}$ = 13.9, P-CH_3), 1.33 (s, 9H, *t*-Bu-CH_3), 1.37 (s, 3H, N-CH_3), 1.88 (d, 3H, $J_{5\text{-}CH3,6\text{-}H}$ = 1.1, 5-CH_3), 2.01 (s, 3H, Ac-CH_3), 2.25 (m, 2H, 2′$_\alpha$, 2′$_\beta$-H), 4.09 (m, 3H, 4′, 5′, 5″-H), 5.31 (m, 1H, 3′-H), 6.34 (t, 1H, $J_{1',2'}$ = 7.9, 1′-H), 7.76 (d, 1H, $J_{6\text{-}H,5\text{-}CH3}$ = 1.1, 6-H); MS: FAB −ve, *m/z* 361 [(M-(*t*-$BuNH_2CH_3$)]$^-$.

N-Methyl-tert-butylammonium 3′-O-Acetylthymidine 5′-O-Methanephosphonothionate (**17**)

3′-O-Acetylthymidine 5′-O-(O-methyl methanephosphonothionate) (**25**) (78.0 mg, 0.2 mmol) was suspended in *tert*-butylamine (2.0 mL, 19 mmol), and the solution was maintained at ambient temperature for 14 days. TLC monitoring indicated that the starting material had been consumed. The solvent was removed by evaporation to dryness, and the N-methyl-*tert*-butylammonium salt of 3′-O-acetylthymidine 5′-O-methanephosphonothioate was obtained in quantitative yield. ^{31}P-NMR (81 MHz, CDCl3) δ_P = 77.29 (41.4%), 77.05 (58.6%); MS: FAB −ve, *m/z* 377 [M-(*t*-$BuNH_2CH_3$)]$^-$; ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.33 (s, 2 × 9H, *t*-Bu-CH_3), 1.37 (s, 2 × 3H, N-CH_3), 1.68 (d, 2 × 3H, $J_{H,P}$ = 14.3, P-CH_3), 1.87 (s, 2 × 3H, 5-CH_3), 2.08 (s, 2 × 3H, Ac-CH_3),

2.25–2.45 (m, 2 × 2H, $2'_{\alpha}$, $2'_{\beta}$-H), 4.20–4.45 (m, 2 × 3H, 4′, 5′, 5″-H), 5.75–5.50 (m, 2 × 1H, 3′-H), 6.25–6.40 (m, 2 × 1H), 7.69 (s, 2 × 1H, 6-H).

Preparation of 5′-Trityl-3′-O-acetyldithymidine-(3′,5′)-O-(O-methyl phosphate) (**26**)

Into a stirred solution of 3′-O-acetylthymidine (106.8 mg, 0.376 mmol) in anhydrous acetonitrile (3.0 mL) was added slowly a mixture of tetrazole (41.0 mg, 0.58 mmol) and 5′-O-tritylthymidine 3′-O-methyl-N,N-diisopropylphosphoramidite (276.8 mg, 0.43 mmol) (made by phosphitylation of 5′-O-tritylthymidine by methyl N,N-diisopropylaminochlorophosphoramidite in the presence of diisopropylethylamine [30]) in anhydrous acetonitrile (2.0 mL) over a period of 20 minutes under argon protection. Stirring was continued for about 20 minutes. To this reaction mixture was added a solution of *tert*-butyl hydroperoxide (45.06 mg, 0.5 mmol) in benzene. After 20 minutes, the solvents were evaporated to dryness, the products extracted by ethyl acetate (50 mL), washed with saturated sodium bicarbonate (30 mL), saturated sodium chloride (30 mL), and water (2 × 20 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated to dryness. Purification by silica-gel column chromatography (silica-gel: 230–400 mesh, 23.0 g; gradient of 0–2% methanol in chloroform as eluent) gave 185.3 mg of pure product **26** (yield 62.7%). MS: FAB −ve, *m/z* 843 [$(M - 1)^-$, 5%], 844 (M^-, 2%); ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = −0.05 (53.4%), −0.47 (46%).

Preparation of 5′-O-Trityl-3′-O-acetyldithymidine-(3′,5′)-O-(O-methyl phosphorothioate) (**27**)

Into a stirred solution of 3′-O-acetylthymidine (100.0 mg, 0.35 mmol) and tetrazole (37.2 mg, 0.53 mmol) in anhydrous acetonitrile (3.0 mL) was added slowly a solution of 5′-O-tritylthymidine 3′-O-methyl-N,N-diisopropylphosphoramidite (278.5 mg, 0.43 mmol) in anhydrous acetonitrile (2.0 mL) under argon protection over a period of 20 minutes. After 20 minutes, the solvent was removed to dryness by evaporation, then 10 mL of anhydrous benzene was added. To the resulting solution was added at room temperature elemental sulfur (18 mg, 0.56 mmol), stirring was continued for 1 hour, 20 mL of benzene was added, and washed with saturated sodium bicarbonate (15 mL), saturated sodium chloride (15 mL), and water (2 × 15 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness. The crude product, 305.0 mg, was purified by silica-gel column chromatography (silica-gel: 230–400 mesh, 40 g, eluted with a gradient of 0–1.5% methanol in chloroform). Collected fractions (R_f 0.38, chloroform:ethanol 19:1, v/v) were evaporated to give 189.8 mg of pure product (yield 63.1%). MS: FAB −ve, *m/e* 859 [$(M - 1)^-$, 3%], 860 (M^-, 2%); ^{31}P-NMR: (81 MHz, $CDCl_3$) δ_P = 70.068 (55.5%), 69.656 (44.5%).

Preparation of 5′-O-Trityl-3′-O-acetyldithymidine-(3′,5′)-O-(methanephosphonate) (**29**)

To a stirred solution of methanephosphonodichloridate (27.0 mg, 0.2 mmol) in dry pyridine (1.0 mL) at 0°C was added dropwise a solution of 5′-O-tritylthymidine (21.6 mg, 0.045 mmol) in dry pyridine (1.0 mL) over a period of 15 minutes. After 10 minutes at 0°C, the mixture was allowed to warm to room temperature, and stirring was continued for 1 hour. Then 3′-O-acetylthymidine (63 mg, 0.22 mmol) was added, and the resulting mixture was stirred for 3 hours. The solvent was evaporated to dryness, and the residue was extracted with chloroform (30 mL). The organic layer was washed with 3 × 10 mL of distilled water, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness. The ^{31}P-NMR spectrum recorded for this mixture (81 MHz, CH_3CN/DMSO-d_6 as an external lock) indicated that the title compound was formed: δ_P = 32.97, 32.36. The presence of some other by-products (^{31}P-NMR 32.84 corresponding to symmetrical diester) was also observed. The solvent was evaporated to give a crude compound **29**. MS: FAB +ve *m/z* 829 $(M + 1)^+$; −ve, *m/z* 827 $(M - 1)^-$.

Preparation of 5′-O-Trityl-3′-O-acetyldithymidine-(3′,5′)-O-(methanephosphonothioate) (**30**)

To a stirred solution of methanephosphonothiodichloridate (13 μL, 0.12 mmol) in dry pyridine (1.0 mL) maintained at ambient temperature was added dropwise a solution of 5′-O-trityl-2′-deoxythymidine (20.0 mg, 0.04 mmol) in dry pyridine (1.0 mL) over a period of 15 minutes. Stirring was continued for 1 hour. Then 3′-O-acetylthymidine (58 mg, 0.2 mmol) was added, and stirring of the reaction mixture was continued for 3 hours. The ^{31}P-NMR [(81 MHz, CH_3CN/DMSO-d_6 as an external lock), δ_P = 99.57, 99.34] showed that the title compound was formed as a mixture of two diastereomers in the ratio ca. 2:3. As previously described, also in this case the presence of symmetrical diester (^{31}P-NMR δ_P = 98.51) was noticed. The solvent was evaporated to dryness to give a crude compound **30**. MS: FAB −ve, *m/z* 843 $(M - 1)^-$.

Reaction of 5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) *with 3′-O-Acetylthymidine 5′-O-(O-Methyl phosphate)* (**13**)

To a stirred dichloromethane (4 mL) solution of **10** (0.04 mmol), prepared in situ (vide supra), the N-methyl-*tert*-butylammonium salt of 3′-O-acetylthymidine 5′-O-(O-methyl phosphate) (**13**) (143.6 mg, 0.31 mmol) in dry DMF (1.0 mL) was added dropwise during 3 minutes at −30°C. After 0.5 hour, the mixture was allowed to warm gradually to room temperature and left overnight. The solvents were removed under reduced pressure to dryness, the residue was extracted with 15 mL of chloroform, and this extract was washed with 3 × 5 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. Purification by RP-HPLC (system C) gave the following compounds: (a) 5′-O-trityl-3′-O-acetyldithymidine-(3′,5′)-O-(O-methyl phosphate) (**26**) (3.97 mg, yield 11.8%). ^{31}P-NMR (202 MHz, $CDCl_3$) δ_P = 0.45, 0.01; MS: FAB −ve, *m/z* 843 $(M - 1)^-$; t_r = 13.40 minutes, 13.89 minutes [structure of **26** was additionally confirmed by its comparison with a standard made by phosphoramidite method (vide supra)]; (b) 5′-O-tritylxylothymidine (**11**) [1.24 mg, 6.4% (recovered)], t_r = 17.28 minutes; (c) 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**) (0.08 mg, yield 0.5%), t_r = 21.92 minutes.

Reaction of 5′-O-Trityl-3′-trifluoromethanesulfonylxylothymidine (**10**) *with 3′-O-Acetylthymidine 5′-O-(O-methyl phosphorothioate)* (**14**)

To a stirred dichloromethane (4.0 mL) solution of **10** (0.04 mmole), prepared in situ (vide supra), the N-methyl-*tert*-butylammonium salt of 3′-O-acetylthymidine 5′-O-(O-methyl phosphorothioate) (**14**) (136 mg, 0.39 mmol) in dry DMF (1.0 mL) was added dropwise during 3 minutes at −30°C. The reaction mixture was maintained for 0.5 hour below 0°C, and then it was allowed gradually to warm to room temperature. ^{31}P-NMR (81 MHz, DMSO-d_6 as an external lock) spectrum recorded after 12 hours showed the presence of 5′-O-trityl-3′-acetyldithymidine-(3′-S,5′-O)-(O-methyl phosphorothioate) (**28**) (δ_P = 27.51, 27.35) and 5′-O-trityl-3′-O-acetyldithymidine-(3′,5′)-O-(O-methyl phosphorothioate) (**27**) (δ_P = 69.92, 69.72) in the ratio of 36:64, respectively, albeit in low yield. Solvents were removed under reduced pressure, and the solid residue was extracted with 15 mL of chloroform. This extract was washed with 3 × 5 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. Purification by RP-HPLC (System C) provided the following compounds: (a) **28** (1.4 mg, 3.8%). ^{31}P-NMR (202 MHz, $CDCl_3$) δ_P = 28.24, 27.92; MS: FAB −ve, *m/z* 859 $(M - 1)^-$ t_r = 14.28 minutes, 14.77; (b) **27** (2.5 mg, yield 6.8%); ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 70.07, 69.64; MS: FAB −ve, *m/z* 859 $(M - 1)^-$; t_r = 20.25, 21.87 minutes. The structure of **27** was additionally confirmed by its comparison with a standard made by the phosphoramidite method (vide supra). Analysis of chromatogram also revealed the presence in the reaction mixture of 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**) and 5′-O-tritylxylothymidine (**11**).

Reaction of 5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) *with 3′-O-Acetylthymidine 5′-O-Methanephosphonate* (**15**)

To a stirred dichloromethane (8.3 mL) solution of **10** (0.083 mmole), prepared in situ (vide supra), the N-methyl-*tert*-butylammonium salt of 3′-O-acetylthymidine 5′-O-methanephosphonate (**15**) (264.4 mg, 0.59 mmol) in dry DMF (2.0 mL) was added dropwise during 5 minutes at −30°C. After the reaction mixture had been maintained for 1 hour below 0°C, it was allowed to gradually warm to room temperature and kept overnight. Solvents were removed under reduced pressure to dryness, the residue was extracted with 30 mL of chloroform, and this extract was washed with 3 × 10 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. Purification by RP-HPLC (System C) provided the following compounds: (a) 5′-O-trityl-3′-O-acetyldithymidine-(3′,5′)-O-(methanephosphonate) (**29**) 7.0 mg, 13.5% yield. ^{31}P-NMR (202 MHz, $CDCl_3$) δ_P = 33.30, 32.67; MS: FAB −ve, *m/z* 827 $(M - 1)^-$; t_r = 11.65, 12.33 minutes [structure of **29** was additionally confirmed by its comparison with a standard made by condensation of 5′-O-tritylthymidine 3′-O-methanephosphonochloridate with 3′-O-acetylthymidine (vide supra)]; (b) 5′-O-tritylxylothymidine (**11**) 6.7 mg, 16.6%, t_r = 16.3 minutes; (c) 5′-O-trityl-2′,3′-didehydro-3′-deoxythymidine (**18**) 3.0 mg, 7.8%, t_r = 20.63 minutes.

Reaction of 5′-O-Trityl-3′-O-trifluoromethanesulfonylxylothymidine (**10**) *with 3′-O-Acetylthymidine 5′-O-Methanephosphonothioate* (**16**)

To a stirred dichloromethane (8.8 mL) solution of **10** (0.88 mmol), prepared in situ (vide supra), the N-methyl-*tert*-butylammonium salt of 3′-O-acetyl-

thymidine 5′-O-methanephosphonothioate (**16**) (334 mg, 0.72 mmol) in dry DMF (2.0 mL) was added dropwise during 5 minutes at −30°C. After the reaction mixture had been maintained for 1 hour below 0°C, the mixture was allowed gradually to warm to room temperature and left overnight. The solvents were removed under reduced pressure to dryness, the residue was extracted with 15 mL of chloroform, and this extract was washed with 3 × 5 mL of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. The ^{31}P-NMR (81 MHz, CH_3CN/DMSO-d_6 as an external lock) spectrum showed a presence in the reaction mixture of 5′-O-trityl-3′-O-acetyldithymidine-(3′-S,5′-O)-(methanephosphonothioate) (**31**) (δ_P = 54.61, 55.27) and 5′-O-trityl-3′-O-acetyldithymidine-(3′,5′)-O-(methanephosphonothionoate) (**30**) (δ_P = 100.17, 99.24) in the ratio 53.9:46.1. Separation of the reaction mixture by RP-HPLC (system C) provided the following compounds: (a) **31**, 5.7 mg, 7.7%, ^{31}P-NMR (202 MHz, $CDCl_3$) δ_P = 54.08, 54.06; MS: FAB −ve, *m/z* 843 (M − 1); t_r = 13.28, 14.08 minutes; (b) **30**, 4.9 mg, 6.6%, ^{31}P-NMR (81 MHz, $CDCl_3$) δ_P = 99.58, MS: FAB −ve, *m/z* 843 (M − 1), t_r = 18.72 minutes, t_r = 21.03 minutes [structure of **30** was additionally confirmed by its comparison with a standard made by a condensation of 5′-O-tritylthymidine 3′-O-methanephosphonothiochloridate with 3′-O-acetylthymidine (vide supra)]; (c) 5′-O-tritylxylothymidine (**11**), 5.6 mg, 13.2%, t_r = 16.35 minutes.

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