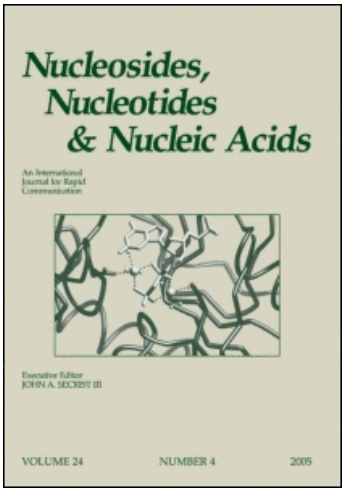

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Buck, Henk M.(2007) 'The Chemical and Biochemical Properties of Methylphosphotriester DNA and RNA in Comparison with Their Corresponding Methylphosphonates. A Dynamic Model Description', Nucleosides, Nucleotides and Nucleic Acids, 26: 2, 205 — 222
To link to this Article: DOI: 10.1080/15257770601112812
URL: http://dx.doi.org/10.1080/15257770601112812

Nucleosides, Nucleotides, and Nucleic Acids, 26:205–222, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770601112812

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THE CHEMICAL AND BIOCHEMICAL PROPERTIES OF METHYLPHOSPHOTRIESTER DNA AND RNA IN COMPARISON WITH THEIR CORRESPONDING METHYLPHOSPHONATES. A DYNAMIC MODEL DESCRIPTION

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□ *Methylphosphotriester DNA and RNA are of great interest to investigate their hybridization affinity with natural DNA and RNA with respect to their physical and biological properties. The results are compared with related modified oligonucleotides. Specific attention will be given to the development of recent antiretroviral nucleosides focused on their molecular conformation and the mechanistic aspects based on the physical properties of phosphorus in a trigonal bipyramidal configuration corresponding with in vitro and in vivo kinetics.*

Keywords: Methylphosphotriester DNA and RNA; parallel RNA; methylphosphonates; kinetics

INTRODUCTION

Recently we described the unique properties of methylphosphotriester DNA as expressed in their strong hybridization affinity toward natural DNA, the formation of parallel duplexes, and self-complementary left-handed Z-DNA.[1] We also briefly mentioned the synthesis and biochemical properties of methylphosphotriester RNA. These modified duplex RNAs may be of interest because of their ability to inhibit gene expression known as RNA interference.[2] With 2′-*O*Me methylphosphotriester RNA dimers we established the formation of parallel and self-complementary duplexes. The study of the latter complex formation was directed on the presence of a left- or right-handed duplex of S_P and R_P r[(2′-*O*Me)C_PG]$_2$ with 2′-*O*Me cytidine. The UV hyperchromicity, the melting curves with ^{1}H NMR, the ^{1}H NMR structural characterization, and the CD spectra focused on the molecular ellipticity, the conformation of the ribose rings and the bases reveal that there is only a preference for a right-handed helical duplex

Received 7 July 2006; accepted 20 October 2006.
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structure.[3,4] This is in sharp contrast with the methylphosphotriester DNA dimers S_P, R_P d(C_PG) and d(G_PC). For the C-G sequence a left-handed Z-DNA duplex is found whereas for the inverted sequence a right-handed B-DNA duplex is established.

The differences in conformational behavior will be discussed in relation to their natural counterparts in combination with the impact of the methyltriester under biological conditions. Based on the structural conformation of the sugar moiety we also focus on (recent) developments in the field of related modified oligonucleotides in comparison with the true-to-nature modified methylphosphotriester DNAs. Furthermore, we give specific attention to the development of anti-retroviral nucleosides especially focused on the dynamics and structural aspects corresponding with in vitro and in vivo kinetics.

RESULTS AND DISCUSSION

Self-Complementary Methylphosphotriester RNA. Formation of Antiparallel Miniduplexes

With a 600 MHz ^{1}H NMR conformational analysis directed on relevant ^{1}H-^{1}H NMR coupling constants measured in D_2O at 4°C we were able to calculate the time-averaged populations of the C_2'-*endo* puckered form x(C_2'-*endo*) and the C_4'–C_5' rotamers x(γ) of methylphosphotriester S_P, R_P d(C_PG)$_2$ and d(G_PC)$_2$. The formation of the duplexes was investigated with UV hyperchromicity and variable-temperature 300 and 400 MHz ^{1}H NMR experiments for the H_6 (dC) and H_1' (dG) protons in S_P, R_P d(C_PG)$_2$ and for the H_5(dC) and H_6(dC) protons in S_P, R_P d(G_PC)$_2$. The CD spectra for S_P, R_P d(G_PC)$_2$ showed the presence of a right-handed duplex whereas for S_P, R_P d(C_PG)$_2$ an inversed spectrum is measured corresponding with the left-handed Z duplex. The results of the 600 MHz ^{1}H NMR conformational analysis are given in Table 1.[5]

TABLE 1 Conformational analysis of C_2'-*endo* puckered form x(C_2'-*endo*) and the C_4'-C_5' rotamers x(γ) expressed as time-averaged population fractions in D_2O (4°C) for the methylphosphotriester of S_P, R_P d(C_PG)$_2$ and for S_P, R_P d(G_PC)$_2$

	S_P d(C_PG)$_2$		R_P d(C_PG)$_2$		S_P d(G_PC)$_2$		R_P d(G_PC)$_2$	
	dC	pdG	dC	pdG	dG	pdC	dG	pdC
x (C_2'-*endo*)	0.86	0.04	0.86	0.07	0.88	0.61	0.86	0.59
x (γ^+)	0.61	0.00	0.70	0.00	0.61	0.77	0.63	0.79
x (γ^t)	0.25	0.81	0.26	0.77	0.23	0.17	0.22	0.14
x (γ^-)	0.14	0.19	0.04	0.23	0.16	0.06	0.15	0.05

TABLE 2 Conformational analysis of C_2'-*endo* puckered form x(C_2'-*endo*) and the C_4'-C_5' rotamers x(γ) expressed as time-averaged population fractions in D_2O (2°C) for the methylphosphotriester of S_P, R_P r [(2′-*O*Me)C_PG]$_2$

	S_P r[(2′-*O*Me)C_PG]$_2$		R_P r[(2′-*O*Me)C_PG]$_2$	
	r(2′-*O*Me)C	prG	r(2′-*O*Me)C	prG
x (C_2'-*endo*)	0.42	0.53	0.44	0.56
x (γ^+)	0.74	0.66	0.72	0.66
x (γ^t)	0.22	0.34	0.20	0.34
x (γ^-)	0.04	0.00	0.08	0.00

From Table 1 it is clearly shown that for the duplexes methylphosphotriester S_P and R_P d(C_PG)$_2$ C_2'-*endo* and C_3'-*endo* puckered deoxyribose rings are found for the dC and pdG residues respectively. This results in a *syn* conformation for the pdG base and an *anti* conformation for the dC base which is in correspondence with NOE contacts. This conformational behavior is well known for the left-handed Z geometry of the corresponding natural duplex. In the case of methylphosphotriester S_P and R_P d (G_PC)$_2$ there is a preference for the C_2'-*endo* puckered form. This means an *anti* conformation for dG and pdC related to the right-handed B geometry of the corresponding duplex.

The conformational behavior of the duplex of methylphosphotriester S_P and R_P r[(2′-*O*Me)C_PG]$_2$ is quite different from the corresponding methylphosphotriester d(C_PG)$_2$. The results are shown in Table 2. They are obtained with the same procedure as used for the deoxy compounds.[4–6]

There is a distinct difference between the conformations of the methylphosphotriesters S_P, R_P d(G_PC)$_2$, and S_P, R_P r[(2′-*O*Me)C_PG]$_2$. Although these corresponding triesters form a right-handed duplex, the differences in population of the C_2'-*endo* puckered ring form of the ribose rings in comparison with the deoxyribose rings show revealing figures. In the former case the formation of a B-DNA like structure is preferred based on the strongly pronounced C_2'-*endo* population whereas in the latter case this preference is reduced in which x(C_2'-*endo*) for r(2′-*O*Me)C_P is a little smaller than for prG. So there is an indication for an increased C_3'-*endo* pucker. This conformation is related to the A form of a natural DNA duplex. From model studies it can be clearly shown that ribose rings do not fit into a helix of the B-DNA type because there is not enough room for the 2′-*O*-methyl group. Even the 2′-*O*H group of the natural form brings the RNA duplex in the A form. With the tight conformational fixation caused by the 2′-*O*-methyl group a transition into a Z form is impossible. For such a conversion the C_3'-*endo* pucker must be changed into the C_2'-*endo* of r(2′-*O*Me)C_P and the C_3'-*endo* of the prG pucker, which is accompanied with an *anti* and *syn* conformation of C and G respectively. Recently this explanation

has been confirmed by a study of Popenda et al. on the high salt solution structure of a natural left-handed Z-RNA double helix with the alternating C-G sequence.[7] Their results show that r[(2′-*O*Me) $C_PG]_3$ containing 2′-*O*-methylated cytidine residues does not undergo a transformation into the Z form even under high salt condition. This is in excellent correspondence with the results of the methylphosphotriester miniduplexes. Introduction of *only* 2′-*O*-methyl guanosines in the natural RNA with alternating C-G sequences results under high salt condition into a left-handed double helix because of its favored C_3'-*endo* pucker accompanied by the *syn* orientation of the guanosine residues. These observations were based on ^{31}P NMR measurements.[7] Apparently the conversion C_3'-*endo* into C_2'-*endo* with cytidine in the *anti* conformation is the decisive step in the A → Z transition.

It has been established that the 2′-*O*-methyl groups in the double helix are directed toward the minor groove which results into its narrowing. An additional aspect of this study is the stereoselective binding with *S* 2-methyl-2,4-pentanediol (MPD) in the minor groove.[8] This molecular interaction was obtained by crystallization experiments of the duplex with the *R*,*S*-MPD mixture. This complexation will be helpful in the interpretation of the stereochemical configuration at phosphorus in the parallel duplex formation of methylphosphotriester RNA.

Parallel Methylphosphotriester RNA. Formation of Miniduplexes

The methylphosphotriester S_P and R_P r[(2′-*O*Me)C_PU] show duplex formation only for the S_P configuration.[3] The conformation of the backbone and the ribose rings was deduced as demonstrated before. In the duplex structure both ribose rings predominantly populate a C_3'-*endo* conformation, that is, a standard A-RNA geometry. The NMR data based on the S_P duplex point to a parallel miniduplex structure based on one C-C base pair with two equivalent N_4-H–N_3 hydrogen bonds, and one U-U base pair with two equivalent O_4–H-N_3 hydrogen bonds. This structure shows a twofold rotational symmetry. The parallel duplex model confirmed by the high field ^1H NMR spectrum shows that the two backbone strands reside in magnetically equivalent environments. Thus the intrinsic symmetry results in the formation of two identical grooves. The exclusive stereochemical preference for S_P in the parallel C-C duplex leads to the conclusion that an inward location of the methyl group (R_P configuration) can not be accommodated in the duplex. The exclusive narrowing of the minor groove as is established in the double helix of r[(2′-*O*Me)$C_PG]_3$ which results in a stereoselective complexation with *S*-MPD, vide supra, will intensify the stereochemical preference for the S_P configuration in the parallel duplex.

The unique difference in duplex stability of the parallel RNA miniduplex and the corresponding DNA complexes has been determined using

well-known thermodynamic relations for the melting temperature (T_m) of the complexes. For that reason the UV hyperchromicity technique was used to monitor the T_m value as a function of c_T, the total strand concentration ($1/T_m$ vs ln c_T, slope $-(n-1)R/\Delta H^\circ$ where ΔH° is related to the association enthalpy, and n is the number of strands that associate to form an n-mer complex) and to determine the first derivative of the fraction (f) of the single strands as a function of T, the temperature of the relevant traject ($(\delta f/\delta T)_{T=T_m}$ versus T, slope $\Delta H^\circ/(2n+2)RT_m^2$). It was found that n varies from 2.04 (RNA) to 2.23 (DNA), that is, duplexes are formed. The stability of the RNA duplexes is substantially lower than for the corresponding DNA duplexes. The stability ratio for RNA/DNA is about 0.38.[3,9] The explanation for this reduced ratio stability of the RNA duplexes can be explained by the introduction of the 2′-*O*-methyl group that results in a diminishing of the base stacking in the RNA complexes.

The role of the methylphosphotriester linkages in stabilizing RNA duplexes is similar to its behavior in DNA duplexes. As a consequence of the phosphate methylation the interstrand phosphate repulsion is absent. Furthermore, there is a stability toward *exo*- and *endo* nucleases and a facile transport through membranes. However, the selectivity for DNA or RNA hybridization is mainly based on the difference in the deoxyribose and ribose ring pucker. Hybridization experiments of methylphosphotriester DNA with natural RNA as template showed a *maximum* in duplex stability for, for example, d(C_PC). poly (rG) with a T_m value of 28°C whereas d(C_PC_PC). poly (rG) has a T_m value of 12°C, vide infra. This means that for selective inhibition on the RNA level methylphosphotriester DNA is a poor candidate.[1] Apparently the constraints which methylphosphotriester DNA perceives in duplex formation with the natural DNA of the B type becomes more to expression in a duplex with natural RNA of the A type. For hybridization of the natural DNA or RNA template with the complementary methylphosphotriester DNA a cooperative effect is necessary for an optimal matching. Because methylphosphotriester DNA adopts the B geometry hybridization with natural RNA is hindered. In contrast with the modified DNA, natural DNA adopts the B *and* A geometry enabling complexation with natural DNA and RNA. Thus in spite of the strong and selective hybridization of methylphosphotriester DNA with natural DNA the triester can not accommodate the A geometry of RNA. The conformational selectivity results in a strong preference of methylphosphotriester DNA for hybrization with natural DNA in comparison with natural RNA. This selectivity in combination with a cooperative effect based on a continuing adaptation of the DNA template for short fragments of methylphosphotriester DNA has been demonstrated with hybridization and biological experiments concerning duplexes d(A_PA).poly(dT), $T_m = 30$°C and d(A_PA_PA).poly(dT), $T_m = 41$°C. In contrast with RNA as template we

found for d(ApA).poly (U), $T_m = 13°C$ and for d(ApApA).poly (U), $T_m < 10°C$, vide supra.[10] Biological support for this different behavior has been demonstrated for the methyltriester of d(ApApA).[11] A small but significant inhibition (15–20%) of cell growth of human ovarium malign cells has been induced by this trimer in a concentration of 1.5×10^{-4}M. This unique *selectivity* has been exclusively proven with the trimer as regard on DNA and protein synthesis in rat fibroblast cells. It was found that the DNA synthesis is markedly retarded with 80% inhibition while the protein synthesis remained essentially unaffected for a concentration of 10^{-5}M. During the replication the lagging strand is synthesized as Okazaki fragments. Due to these fragments stretches of single-stranded DNA are present which can form stable miniduplexes with the triester. Repeating sequences on this strand will contribute to an increase in duplex stability. Summarizing, the results based on the methylphosphotriester 2′-*O*-methyl RNA dimers which show parallel self-association and antiparallel self-complementary duplexes indicate that there will be no specific hybridization preference for natural RNA or DNA.

In comparable hybridization and biological experiments of Miller et al. it could be shown that methyl*phosphonate* DNAs show the *reverse* properties. For longer fragments methylphosphonate DNA there is a strong *decrease* in hybridization affinity for natural DNA whereas the translation of mRNA and the corresponding protein synthesis is hampered.[12] These modified systems show a substantial difference with the true-to-nature methylphosphotriester DNAs in their geometrical arrangement. Methylphosphonate DNA can not readily adopt a right-handed backbone conformation which is needed for a stable duplex with natural DNA. The presence of a P-C bond disturbs this helix structure for stereoelectronic reasons. Apparently this P-C linkage shows a better accommodation for RNA folding.[1]

In the past years different oligonucleotides have been prepared that also show high-affinity recognition of RNA. An interesting example has been demonstrated by Wengel et al. with a locked nucleic acid (LNA) or α-L-*ribo* configured LNA (α-L-LNA) containing 2′-*O*- or 2′-*N*H-, 4′-*C* -methylene-β-D-ribofuranosyl and 2′-*O*- or 2′-*N*H-, 4′-*C*-methylene-α-L-ribofuranosyl monomers with high stability against nucleases.[13,14] The dominant geometrical role of LNA has been established for LNA-DNA hybrids. Here the A character or North type conformation of the sugar ring of the LNA monomers influences the sugar conformation of neighboring monomers. The high-affinity binding with complementary RNA has been demonstrated by Brown et al. with very short LNAs inserted in relatively long 2′-*O*Me RNAs. As to be expected these mixmer oligonucleotides were used to interrupt the life cycle of human immuno deficiency virus type 1 (HIV-1).[15] They showed a site-specific inhibition of viral replication and transcription targeted at the TAR region. *A similar procedure has been followed by us in an earlier study using random phosphate methylation accompanied with a low degree of methylation directed at HIV-1 RNA loops, namely TAR, PBS, NEF,*

and VIF constructs.[1,16–19]* It should be noticed that *complete* phosphate-methylated DNA is unable to inhibit RNA transcription.†

Phosphonylmethyl Nucleosides as Chain Terminator in DNA Synthesis. A Mechanistic View

It is well-known that single-stranded HIV RNA replicates through double-helical DNA intermediates. DNA complementary to viral RNA is synthesized by reverse transcriptase which means that reverse transcriptase is an RNA-directed DNA polymerase. For that reason nucleosides have been synthesized especially focused on the intervention of the HIV virus replication. An important aspect in the development of these precursors which under metabolic conditions are converted in triphosphates is the preference of the reverse transcriptase for a North pucker of the sugar moiety. This conformation corresponds with the A-DNA and RNA structures whereas a South pucker adapts to B-DNA. As triphosphate they are incorporated into the nascent chain via S_N2 displacement of the pyrophosphate by the 3′-hydroxyl of the preceding nucleotide unit under chain termination.[24]

Therefore, it is of interest to mention the recent work of Wu et al. on deoxythreosyl phosphonate nucleosides as selective anti-HIV compounds.[25] One of the important properties of these phosphonates is the stability against enzymatic degradation, because of its P-C bond, vide supra. There are two new compounds which show a very high anti-HIV-1 inhibition in comparison with other related compounds synthesized as the sodium salts of 1′-(adenin-9-yl)-2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose and 1′-(thymin-1-yl)-2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose. Their structures are given in Figure 1.

For the sugar conformation of both compounds we suppose the favored North structure with the interaction of reverse transcriptase. This

*Related research has been shown that TAR-oligonucleotides inhibit the expression of a CAT gene that is regulated by the HTLV III HX10-LTR which contains the selected TAR region in comparison with an arbitrary sequence of the same number of bases.[20]

†For the preparation of partially and complete phosphate-methylated DNAs based on a solid-phase pro-cedure of Kuijpers et al. and of Alul et al.,[21,22] the obtained results are in sharp contrast with our stepwise non-automated preparation.[1,23] If the reaction is carried out on a solid support the methyl group is more accessible than phosphorus in the oxidation with *t*-butyl hydroperoxide. After abstraction of a hydride anion of the methyl group accompanied with the formation of a three-membered ring, nucleophilic attack of OH^- on the secondary carbon results under ring opening in hydroxymethylphosphonate DNA. The driving force comes from the ring opening with the formation of a P═O bond. Since hydroxymethylphosphonate DNA shows resemblance with methylphosphonate DNA, the formation of duplexes on the DNA level is hindered by the unfavorable helix backbone in the phosphonate. In the case of the preparation of partially phosphate-methylated DNAs using a solid support a 400 MHz ^1H NMR spectrum is available.[21] The interpretation was based on the intensity of the methyl-phosphate resonances relative to the other proton resonances as H_5'/H_5'' of the 5′-end nucleotide. However this selection has been taken place arbitrarily. Moreover no duplex formation has been established.[22]

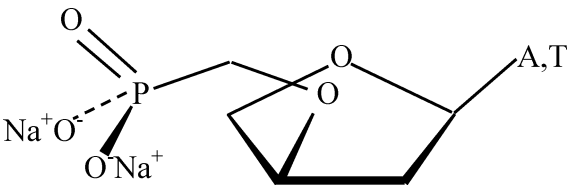


FIGURE 1 Structures of the sodium salts of 1′-(adenin-9-yl)-2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose and 1′-(thymin-1-yl)-2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose.

conclusion is based on the North conformation of the corresponding 9-(2′-deoxy-β-D-*threo*-ribofuranosyl)-adenine, that is, 3′-*O*H has the same orientation as 3′-*O*CH_2- in the phosphonomethyl compounds.[26] We found over a large temperature range from $-40°$ to 95°C a population density of the North conformer of 1.00–0.86. This has been established with variable-temperature 300 and 500 MHz ^{1}H NMR measurements. In the experiments at sample temperatures higher than 0°C, we used D_2O as the medium; all other experiments refer to the solvent DMF-d_6. In *both* cases the North conformation is associated with a favorable axial orientation of the adenine (thymine) base (anomeric effect) as well as a favourable axial orientation of the 3′-*O*H group and the corresponding 3′-*O*CH_2-group in the phosphonomethyl compounds. It must be noticed that Wu et al. constructed a model to analyze the interaction between the incorporated nucleotide and reverse transcriptase. They found that in this model the sugar is in the North conformation.[25]

An aspect of the phosphonate nucleosides which has not obtained the interest about what it deserves is based on the promoting effect of the methylene carbon compared to oxygen in generating a trigonal bipyramidal (TBP)P(V) geometry as one of the crucial steps in chain elongation.

In an earlier study of Castelijns et al. we described the low-temperature ^{1}H, ^{13}C, and ^{31}P NMR measurements of the reaction of several pentavalent oxyphosphoranes with FSO_3H in CH_2Cl_2 at low temperature.[27] Rapid equilibria between neutral oxyphosphoranes and the enol phosphonium ions can be obtained by implying certain structural constraints on the systems. This is illustrated in Figure 2.

With the observed phenomena we determined the activation parameters for the equilibrium $P^+(IV)^* + P(V) \leftrightarrows P(V)^* + P^+(IV)$ from line-broadening experiments. The results are summarized in Table 3.

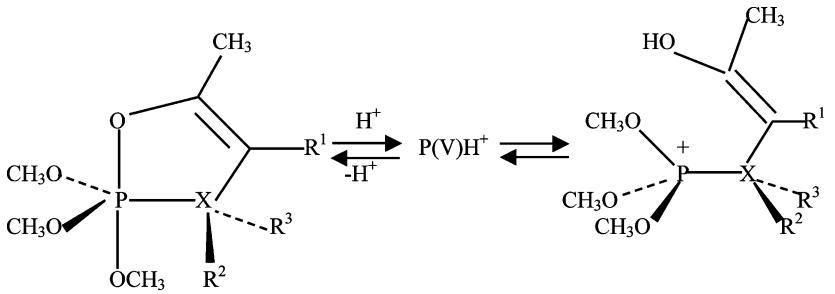
The large negative values for $\Delta S^{\neq}$ of these reactions indicate a rate determining bimolecular process which involves the proton-transfer step.[28] This finding is in excellent correspondence with a recent characterization of the transition state of the well-known biochemical intramolecular phosphorylation, viz., the RNase A catalyzed hydrolysis of single-stranded RNA.[29] The values for **a** correspond with that found for oxonium-water proton exchange processes.[28] The $\Delta H^{\neq}$ value for **b** is also comparable.

TABLE 3 Activation parameters ($\Delta G^{\neq}$,$\Delta H^{\neq}$, and $\Delta S^{\neq}$) and rate constants k for the equilibria between oxyphosphoranes and their enol phosphonium ions. [a]

Compd	$\Delta H^{\neq}$ kJ/mol	$\Delta S^{\neq}$ J/(deg mol)	$\Delta G^{\neq}$ kJ/mol	k, L mol^{-1} s^{-1} (−25°C)
a	12.40	−75.6	31.5	3.2×10^6
b	11.5	−155.4	51.2	5.7×10^2

[a]The line width (Δ) of the methoxy doublets below the coalescence temperature was measured at half-height. The line broadening $\Delta\nu$ was determined from a plot of ln Δ vs $1/T$. From the plot of $\ln(\Delta\nu)$ versus $1/T$, the E_A and the exchange rate, $1/\tau$ (at 248 K), were determined, which were used to calculate the activation parameters according to the following: $\Delta H^{\neq} = E_A$—RT; $\ln(1/\tau) = \ln(RT/Nh) + \Delta S^{\neq}/R$—$\Delta H^{\neq}/RT$; $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$.

However, its $\Delta S^{\neq}$ value is anomalously large and negative. Apparently the bond-making and bond-breaking processes for proton transfer in both compounds are very similar ($\Delta H^{\neq}$), whereas the structural factors to attain the transition state differ significantly ($\Delta S^{\neq}$). Since the starting materials and products are identical, a symmetric transition state is expected which shows the features of the protonated *apical* ring-oxygen oxyphosphorane (P(V)H$^+$). It will be obvious that whenever the enol phosphonium ions which are involved in the equilibria possess more degrees of freedom, more entropy has to be suspended to attain the stage of the enol form which resembles the protonated oxyphosphorane. Thus, the rigidity of the enol phosphonium ion, in which the attacking OH-group results in an axial P—O bond of the P(V)TBP, is more manifest in **a** than in **b**. These experiments clearly show the positive effect of the equatorial >*C*H-P group in comparison with the corresponding -*O*-P group on the formation



P(V) P$^+$(IV)

a: X = C, R^1 = C(O)CH$_3$, R^2 = H, and R^3 = C$_6$H$_4$ *p*-Cl

b: X = O, R^1 = CH$_3$, R^2 = l.p., and R^3 = l.p.

FIGURE 2 Protonation of the *apical* ring-oxygen of oxyphosphoranes (**a** and **b**) resulting their corresponding enol phosphonium ions.

of the P(V)TBP geometry. In some aspects this mechanistic view is in correspondence with a recent report of Lin et al. concerning the mechanistic investigations of the Staudinger ligation of azides and phosphines in which an equilibrium is proposed between a P(V)TBP and a phosphonium ion.[30] Under the conditions of our model experiments no protonation of the *exo* oxygen in the *apical* position takes place resulting under loss of methanol. When in compound **b** the *apical* methoxy group has been replaced by dimethoxyphosphate then addition of FSO_3H in CH_2Cl_2 at low temperature results in *exo* cleavage of dimethyl phosphoric acid and the phosphonium ion under conservation of its ring structure. The reaction is reversible.

A further support for this mechanistic view has been found by Kuijpers et al. in the synthesis of methylphosphonate DNAs using 2-(acetoxymethyl) benzoyl (AMB) as base-protecting group. Using methanolic K_2CO_3 as deprotecting reagent chain cleavage occurs, most like through nucleophilic substitution of CH_3O^- at phosphorus. *For the corresponding methylphosphotriester no cleavage was established.*[31,32] The modified DNAs with AMB as protecting group under investigation were prepared as dimers via a stepwise nonautomated procedure. Using saturated ammonia solution in methanol as an alternative reagent for deprotection, a significant improvement of the synthesis of methylphosphonate DNAs was realized.[32] The best explanation which may be given is based on the formation of a P(V)TBP with the CH_3-group in an equatorial position, resulting in displacement of the 5′- and 3′-*O*-protonated groups along two routes at a relatively high concentration of CH_3O^-. At lower concentrations only the deprotection of the base takes place. In the case of the methylphosphotriesters the deprotection is accompanied with a *small* amount of phosphate-demethylation under *both* conditions with maintenance of the backbone structure. So methylphosphonate DNA and methylphosphotriester DNA show a quite different behavior against nucleophilic attack which may result in backbone cleavage and methylphosphate demethylation respectively. Therefore, it is of interest to suggest that in vivo stability of both modified DNAs is influenced by different mechanisms. Methylphosphonate DNA may follow the fundamental pathway for backbone hydrolysis of natural DNA chains with the assistance of DNA polymerases. In the case of methylphosphotriester DNA demethylation finds its counterpart in the *N*-terminal domain of *E.coli* which reacts exclusively with the S_P configuration by direct methyl transfer to one of the cysteine residues. This transfer results in an irreversible loss of enzyme activity.[33–35]

This brings us back to the exclusive anti-HIV effect of the deoxythreosyl phosphonate derivatives as shown in Figure 1. Therefore it is of interest to consider the in vitro experiments of Wu et al., vide supra, for the kinetic parameters of dATP and the diphosphate of 1′-(adenin-9-yl)- 2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose incorporated in a DNA hybrid using HIV

reverse transcriptase. The results show an *increase* in K_M for the modified nucleoside and a *decrease* in k_{cat} for dATP.[25] Besides the lower affinity of the modified DNA for the reverse transcriptase, the incorporation of the diphosphate of the modified nucleoside in the DNA chain seems more effective than the incorporation of dATP under loss of pyrophosphate. The latter result is in good correspondence with our data of the offered model systems and their corresponding reactivity as shown in Table 3. Interestingly Wu et al. also have established that the aforementioned diphosphate of 1′-(adenin-9-yl)- 2′-deoxy-3′-*O*-(phosphonomethyl)-L-threose compared with dATP is a very poor substrate for DNA polymerase α. Apparently the DNA polymerase α prefers the South conformation of the sugar ring. The model compound 2′-dA shows the conformational behavior needed for the interaction with the reverse transcriptase and DNA polymerase α. With variable-temperature 300 and 500 MHz ^{1}H NMR a rapid North/South equilibrium is found with a slight preference for the South conformation.[26]

Summarizing the kinetics focused on k_{cat} of the in vitro experiments of Wu et al. show that there is a good correspondence with our kinetic data of the equilibrium between selected oxyphosphoranes and their enol phosphonium ions.

CONCLUDING COMMENTS

Our interest on modified nucleotides is focused on the nature of the backbone which has been changed from anionic to uncharged. The latter group of nucleotides has been investigated extensively, that is, methylphosphotriester at the interface of chemistry and molecular biology. Especially the methylphosphotriester RNAs are of interest because they will show genetic inhibition on the DNA *and* the RNA level of hybridization. In contrast methylphosphotriester DNA exhibits only hybridization on the DNA level. Special attention is given to the conformation of the sugar ring and the chirality of phosphorus which play an important role in DNA and RNA recognition. It is to be expected that relatively long 2′-*O*Me methylphosphotriester RNA oligomers will show the same conformational behavior as the dimers in the present work. This reasoning is based on the excellent accommodation of very short LNAs inserted in relatively long 2′-*O*Me RNAs.[13–15] Therefore, we think that the methylphosphotriester RNAs will hybridize easily with natural RNA taking profit from the absence of interstrand phosphate repulsion, stability towards nucleases, and a facile transport through membrames in combination with an efficient conformational match. The choice for 2′-*O*Me methylphosphotriester RNA as antisense oligoribonucleotide has the advantage that it can interfere with (pre)-mRNA by blocking a specific location resulting in a compositional change of the translated protein or in reduction of the translation ability

of mRNA into the corresponding protein. Because of the A conformation of RNA a similar result is obtainable only with *partial* phosphate-methylated DNA, vide supra.[36]

Unfortunately no x-ray crystal data are available for the definite molecular structures of the methylphosphotriester DNA and RNA duplexes. Most evidence for the anti-parallel base pairing and the self-association of parallel pyrimidine pairing has been obtained from NMR data and QM/MM calculations. One of the most intriguing questions which remains is the shielding of the phosphate linkages in DNA and RNA under "natural" conditions that may play an important role in the dynamics of the double helix resulting into a supercoiled form with rather little change in local structure, that is, conservation of the base-pair fidelity with exclusive binding motifs as shown for selected phosphate groups localized by binding to histones via positively charged ammonium sites of lysine residues.[37] However, under conditions of atomic resolution the shielding of the phosphates is presented mostly as a complexation with hydrated cations. This model has been presented for the different forms of Z-DNA in which phosphates are coordinated to a hydrated magnesium ion or simple coordinated to water.[38] With QM/MM calculations we described recently different models for phosphate shielding in DNA duplexes with ab initio and molecular mechanics calculations.[39] The outcome of this work is that correspondence with the actual situation will be reached by partial shielding of the phosphates by protons via hydrogen bridging. Recent investigations based on XPS studies show that for monolayers of single-stranded DNA formed from NaCl buffer and washed thoroughly no Na^+ signal is detected.[40] Since the XPS results indicate that the single-stranded layer is not charged, it was proposed that neutralization of DNA takes place by protons resulting in self-assembled monolayers with a free energy gain from base stacking and hydrogen bonding. Interestingly the absence of monovalent cations in x-ray studies of DNA crystals is in good correspondence with the XPS data. There is enough evidence to show the importance of proton shielding and the (*reversible*) methyl coordination with phosphates in DNA delivered by histone proteins which may play a crucial role in the field of epigenetics.[1,41–44]‡

In our opinion phosphate shielding as present in methylphosphotriester DNA has only obtained relative interest because of the (apparent) stability of methylphosphonate DNA, vide supra, under biological conditions. *Even* on

‡Epigenetic gene silencing is fundamental to cell determination and function. The epigenetic systems involved in repression of gene activity are based on histon methylation via the mono-, di-, or trimethylated cation state of lysine residues (DNA backbone interaction) and methylation of DNA bases. There are findings that methylation of histones and DNA bases are cooperative in gene-silencing networks in the cell.[44] Such a cooperative model has been shown for naïve model systems with alternating CpG sequences. The significance of shielding of the phosphates in combination with methylation of the bases for conformational changes in DNA has been theoretically described by van Lier et al.[45] Experimental evidence was given by Sugiyama et al.[46]

a one-level base-pair interaction the stability of the methylphosphotriester nucleotide has been demonstrated. Using the nucleoside diphosphate d(pTp), the formation of a T-T pair could only be obtained by methylation of the phosphate groups. The 500 MHz ^{1}H NMR spectrum shows an imino proton signal at a low field position of 13.45 ppm in comparison with the unlinked thymidine bases of 11.2 ppm. From the dimerization equilibrium under the NMR conditions which is below the primitive nucleotide concentration of 5 mM, it follows that the formation constant is more than 200 M^{-1}. In comparison with other data of T-T pairing on the dinucleotide and hexanucleotide level it could be clearly established that the duplexes are parallel.[47]

With the results of our research on the various configurations of phosphorus especially focused on a P(V)TBP geometry as stable compound, intermediate, and transition state we came into the field of chemistry and kinetics of methylphosphonate nucleosides. These nucleosides are converted in a diphosphate via a metabolic process or synthetic approach. Then they function as DNA-chain terminator.

A number of novel anti-HIV nucleosides have been synthesized and their kinetic parameters have been determined in vitro experiments incorporated in DNA for HIV reverse transcriptase by Wu et al. We compared our kinetic data based on an equilibrium between selected oxyphosphoranes and the corresponding enol phosphonium ions with the aforementioned results. There is a good agreement with the k_{cat} values obtained for dATP and the diphosphate of the methylphosphonate nucleosides focused on their differences in reactivity.

In order to have a more quantitative control on the significance of the kinetic data and the corresponding activation parameters of our model systems as given in Figure 2, we compare the results with the kinetics of protein phosphorylation from literature. In the diverse family of protein kinases, the catalytic subunit of cyclic AMP-dependent protein kinase is the best characterized member. It catalyzes the transfer of γ-phosphate of ATP to serine or threonine residues of the substrate peptides under release of ADP.[48,49] The transition state is a P(V)TBP in which Asp 166 serves as the catalytic base to accept the proton of the substrate serine. The catalytic role of Lys 168 is demonstrated to keep ATP and the substrate peptide in the near-attack conformation necessary for the P(V)TBP transition. In fact our rigid model systems accompanied with an intermolecular proton transfer is in some way related to this phosphorylation reaction. For the enzymatic reaction it was experimentally found that $\Delta G^{\neq} = 57.7$ kJ/mol.[49] For the corresponding model compound **b** we found 51.2 kJ/mol (57.8 kJ/mol at 25°C) as given in Table 3. The close correspondence between these values may be based on the fact that the intramolecular reacting sites within the model system are already partially pre-organized to stabilize the transition state. In fact we are dealing with a combination of restraining the substrate's

(enol form) degrees of freedom with the assistance of an electric dipole evolution on the reaction coordinate. No enzymatic data are available for γ phosphonomethyl ADP. The expectation is that $\Delta G^{\neq}$ will be lower in correspondence with **a** for which $\Delta G^{\neq} = 31.5$ kJ/mol (34.9 kJ/mol at 25°C).

EXPERIMENTAL

The ^{1}H NMR spectra were recorded on Bruker AM 600, AM 500, AM 400, CXP 300, and AC 200 NMR spectrometers. TMS was used as internal standard for samples in organic solvents. For samples in aqueous solution (D_2O) the residual HDO peak was set at 4.68 ppm. ^{31}P NMR spectra were recorded at 81 MHz on the AC 200 instrument and referenced against 85% H_3PO_4 as external standard.

UV hyperchromicity experiments were performed on a Perkin Elmer 124 spectro-photometer at a wavelength of 260 nm. CD experiments were carried out on a Jobin Yvon Dichrographe III instrument using aqueous Tris/HCl buffer solutions (pH = 7.5).

Methylphosphotriester DNA

The stepwise nonautomated preparation of methylphosphotriester DNA following the method of Caruthers et al. was succesful for short fragments with 2–5 bases.[50] Initially, we have synthesized methylphosphotriester DNA containing A bases with the amidine 6-*N*-(1-(dimethylamino) ethylene) as protective group for the 6-NH_2 group of A.[51] Most of the protection of the bases A, C, and G was carried out with 9-fluorenylmethoxycarbonyl (Fmoc).[52,53] After isolation of the phosphites, the base protected triester was obtained through oxidation with *t*-butyl hydroperoxide. For the removal of the Fmoc-group triethylamine was used and finally reversed-phase HPLC separation of the S_P and R_P diastereoisomers was used in the case of dimers.

Methylphosphotriester RNA

For the synthesis of phosphate-methylated RNA dimers the compounds are stabilized by a 2′-*O*-methyl group in the 5′-terminal residue.[3,4] With the work on the synthesis of phosphate-methylated DNA fragments, we selected the Fmoc-group for the transient protection of the NH_2-groups of A, C, and G. 5′-Tritylation and introduction of the phosphoramidite function on O_3′ were performed according to standard protocols. For the coupling with the second RNA fragment we selected the levulinyl (Lev) group. Oxidation was performed with *t*-butyl hydroperoxide. The Fmoc-and Lev-groups were simultaneously removed with methanolic K_2CO_3. Subsequently,

detritylation was realized under acidic conditions. Separation of the S_P and R_P diastereoisomers was performed by reversed-phase HPLC.

Random Phosphate-Methylation of Long DNA Fragments with a Low Degree of Methylation

These oligonucleotides were synthesized according to a three-step procedure (i) base protection with Fmoc, vide supra, via an automatically synthesized natural DNA sequence; (ii) methylation of the phosphate groups with p-toluenesulfonyl chloride and methanol; and (iii) removal of the Fmoc-groups with triethylamine, vide supra. Although useful for short fragments, it appeared that for long oligonucleotides introduction of the Fmoc-groups results in an almost insoluble product which causes *low* yields in the next steps.

Pentavalent oxyphosphoranes and their corresponding tetravalent enol phosphonium ions

The compounds prepared for the mechanistic aspects of the dynamic equilibra between pentavalent protonated oxyphosphoranes and their isomeric tetravalent enol phosphonium ions via intermolecular proton transfer in relation to a mechanistic view on the phosphonylmethyl nucleosides as chain terminator in DNA synthesis are indicated as **a** and **b** in Figure 2:

a: 2,2,2-trimethoxy-3(*p*-chlorophenyl)-4-acetyl-5-methyl-2,2,2,3-tetrahydro-1,2-oxaphosphole, and

b: 2,2,2-trimethoxy-4,5-dimethyl-2,2-dihydro-1,3,2-dioxaphosphole.

For the synthetic details and the corresponding phosphonium ions after protonation of the *endo*-cyclic *apical* oxygen of the pentavalent oxyphosphoranes, and the determination of the activation parameters we refer to the experimental section of Castelijns et al.[27] It was mentioned that protonation of the *exo* oxygen in the *apical* position does not take place. However in the corresponding structure:

2,2-dimethoxy-2-(dimethylphosphato)-4-benzoyl-5-phenyl-2,2-dihydro-1,3,2-dioxaphosphole protonation takes place at the *exo* oxygen of the dimethyl phosphate group in the *apical* position. The reversible process under formation of dimethyl phosphoric acid and the phosphonium ion under ring conservation is also described in Castelijns et al.[27]

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