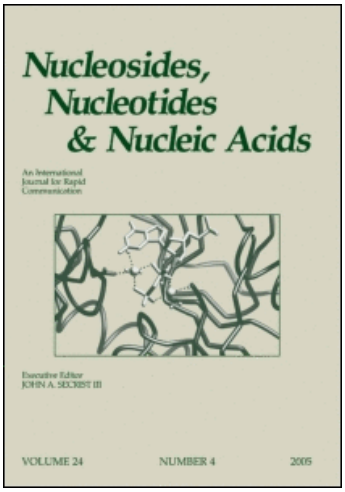

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 30 November 2004

To cite this Article Buck, Henk M.(2004) 'The Chemical and Biochemical Properties of Methylphosphotriester DNA', Nucleosides, Nucleotides and Nucleic Acids, 23: 12, 1833 — 1847
To link to this Article: DOI: 10.1081/NCN-200040620
URL: http://dx.doi.org/10.1081/NCN-200040620

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, No. 12, pp. 1833–1847, 2004

The Chemical and Biochemical Properties of Methylphosphotriester DNA

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ABSTRACT

Methylphosphotriester DNA shows a number of interesting bio-organic properties. Its behavior is quite different from selected modified DNAs as the related methylphosphonate oligonucleotides.

Key Words: Methylphosphotriester DNA; Antiparallel DNA; Parallel DNA; Left-handed DNA; Methyl transfer.

INTRODUCTION

With respect to the transfer of genetic information, it is of importance to understand the mechanistic aspects of stabilization and destabilization of the DNA duplex. From the many factors which play a role in these processes we focused our attention on the negatively charged phosphate groups in the two intertwined backbones. Neutralization of the charge of the phosphates by specific methylation resulted in methylphosphotriester DNAs with very exclusive (bio)chemical properties. Through methylation chirality at phosphorus is introduced which will be of importance for intra- and intermolecular dynamics.

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DOI: 10.1081/NCN-200040620
1525-7770 (Print); 1532-2335 (Online)

RESULTS AND DISCUSSION

Right-Handed Methylphosphotriester B-DNA

The stepwise non-automated preparation of methylphosphotriester DNA following the method of Caruthers et al.[1] was succesful for short fragments with 2–5 bases. 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.[2] Most of the protection of the bases A, C, and G was carried out with 9-fluorenylmethoxycarbonyl (Fmoc).[3,4] After isolation of the phosphites, the base-protected triester was obtained through oxidation with *t*-butyl hydroperoxide. For the removal of the Fmoc groups triethylamine was used. Strong hybridization affinity of these methylphosphotriester DNAs for complementary natural DNAs resulted in a right-handed antiparallel B-DNA duplex.[5,6] From hybridization studies for the complementary base ratio 1:1 in d($[C_P(O)OMe]_{n-1}$C).poly(dG) and d($[A_P(O)OMe]_{n-1}$ A).poly (dT) a linear relationship between the melting temperature (T_m) and the number of bases of methylphosphotriester DNA was established. The ratio of the slopes approaches the expectation value of 1.5, i.e., the ratio of the three pairs of hydrogen bonds in CG, and the two pairs of hydrogen bonds in AT. From the linear relationship of T_m vs. the number of hydrogen bridges (m) it was shown that $4 \leq m \leq 15$ corresponds with $30° \leq T_m \leq 90–100°C$.[7] Furthermore it could be shown that duplex stability of methylphosphotriester DNA with complementary natural DNA strongly depends on the length of the natural DNA. From the experimental results for the natural templates dG_n: dG_{10}, dG_{18}, dG_{30}, and poly (dG) the approximate T_m values of 12n, 13n, 15 n, and 20 n (°C), respectively, can be abstracted. The thermodynamic expression for the influence of every hybridization on a continuing adaptation of the geometry of the template has been described by the introduction of an additional entropy term. The importance of the cooperative process is that even short fragments of methylphosphotriester DNA in succession on the natural template not only give rise to an increase in duplex stability, but that these short fragments should also be more sensitive for mismatches.[5,6]

Hybridization experiments with complementary natural RNA as template showed a maximum in duplex stability for n = 2 in d($[C_P(O)OMe]_{n-1}$C).poly (rG) with T_m of 28°C; ref. T_m value for n = 3 is 13°C.[5,6] This means that for selective inhibition on the RNA level methylphosphotriester DNA is a poor candidate. 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 values of $n \geq 3$. Biological support for this behavior of methylphosphotriester DNA has been demonstrated with *short* well-characterized fragments. As an example we could demonstrate that addition of 10^{-5} M methylphosphotriester d($[A_P(O)OMe]_2$A) to rat fibroblast cells reduces DNA replication (monitored by ^{3}H-thymidine uptake) whereas protein synthesis is found to be essentially unaffected (monitored by ^{35}S-methionine uptake).[8] During the replication the leading strand is synthesized continuously whereas the lagging strand is synthesized in the form of short fragments known as Okazaki fragments. Due to these fragments, stretches of single-stranded DNA are present which can form stable miniduplexes with methyl-phosphotriester DNA which may be able to influence DNA replication by

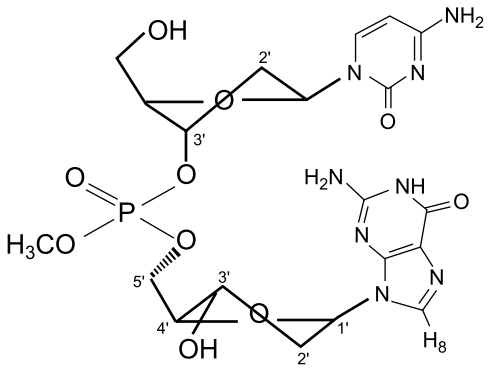


Figure 1. Schematic representation of the primary and secondary structure of methylphosphotriester d([C_P(O)OMe]G) in the left-handed Z geometry.

blocking the action of DNA polymerase in the lagging strand of the replication fork. Repeating sequences on this strand will contribute to an increase in duplex stability with short methylphosphotriester DNAs. If such a regularity is absent a mixture of short fragments methylphosphotriester DNAs is necessary.

In the protein synthesis a DNA strand is transcribed into mRNA which is hardly affected by (short) methylphosphotriester DNA in its translation into protein, vide supra. Apparently short methylphosphotriester DNA is unable to form a complex with the DNA strand which is synthesized continuously on the parental DNA of the leading strand.

From these results it is clear that the methylphosphotriester DNA mimics the behavior of natural DNA in the *absence* of stabilizing factors as salts, proteins and medium factors. On the other hand the duplex formation with natural RNA is hindered by steric constraints.

Left-Handed Methylphosphotriester Z-DNA

Phosphate methylation also gave the possibility to synthesize a self-complementary left-handed Z-DNA miniduplex d([C_P(O)OMe]G)$_2$.[9][a] With ^{1}H NMR and CD measurements the structure could be fully characterized. A schematic representation of the primary and secondary structure of methylphosphotriester d([C_P(O)OMe]G) in the left-handed Z geometry is shown in Fig. 1. The formation of the miniduplex is thermodynamically controlled by the effective phosphate shielding ($\Delta H^\circ <$) and the entropy gain in connection with the flexibility of the duplex ($\Delta S^\circ >$).

[a]A miniduplex d([T_P(O)OMe]A)$_2$ was found for sample temperatures lower than 15°C. This conclusion is based on the fact of an imino-proton signal at 12.5 ppm. NOE contacts show that H_8 of A resides in the vicinity of $H_{1'}$ and $H_{2'}$. The couplings $J_{1'2'}$ and $J_{1'2''}$ are approximately 6.5 Hz which points to a south conformation of the sugar ring in the dT and dA residues. However for Z-DNA it is to be expected that the dA furanose ring is North, while the dT furanose ring is South. This miniduplex shows a marked flexibility which allows syn$\leftrightarrow$anti-equilibration of A.

From the ^{1}H-^{1}H NMR coupling constants measured in D_2O at 4°C, the population densities of the $C_{2'}$- endo puckered 2′-deoxyribose rings and the $C_{4'}-C_{5'}$ rotamers γ^+ and γ^t can be abstracted resulting in the structural differences between the miniduplexes d([C_P(O)OMe]G)$_2$ and d([G_P(O)OMe]C)$_2$. For R_P and S_P d([G_P(O)OMe]C)$_2$ there is a preference for the $C_{2'}$-endo puckered form in both sugar rings accompanied with a γ^+ conformation of the $C_{4'}-C_{5'}$ bond and an anti orientation of the C and G bases which results in a right-handed B-DNA duplex. R_P and S_P d([C_P(O)OMe]G)$_2$ adopt the left-handed Z-DNA geometry. The $C_{2'}$-endo and $C_{3'}$-endo puckered sugar rings are found for the dC and dG nucleotide units, respectively, together with a preference for the γ^t conformation of the $C_{4'}-C_{5'}$ backbone in the dG residue. NOE contacts point to a syn orientation of the G base and an anti orientation of the C base.

Variable-temperature ^{1}H NMR and UV hyperchromicity measurements show T_m values of 9 and 13°C for the left-handed R_P and S_P diastereoisomers, respectively. For the right-handed diastereoisomers a T_m value of 27°C is found.

The CD spectra which are shown in Fig. 2 clearly demonstrate the inversed spectrum for the left-handed Z-DNA structure of d([C_P(O)OMe]G)$_2$ in comparison with the right-handed B-DNA of d([G_P(O)OMe]C)$_2$.

The complete atomic resolution of the left-handed Z-DNA of the natural hexamer d($C_PG_PC_PG_PC_PG$) was given by Wang et al.[10] The crystallization was carried out under high-salt conditions necessary for the B–Z transition. Interestingly the corresponding tetramers also showed the same general form of left-handed Z-DNA.[11] From the work of Wang et al. it is clear that this study is representative for more general conclusions concerning the B–Z transition because the crystal structure represents twelve residues (six dimer pairs) which means one turn of the helix. The correspondence between phosphate methylation and high salt seems to be that methylation results in a complete neutralization of the anionic phosphate whereas high-salt condition results in the formation of intimate ion pairs with the phosphate backbone. The significance of

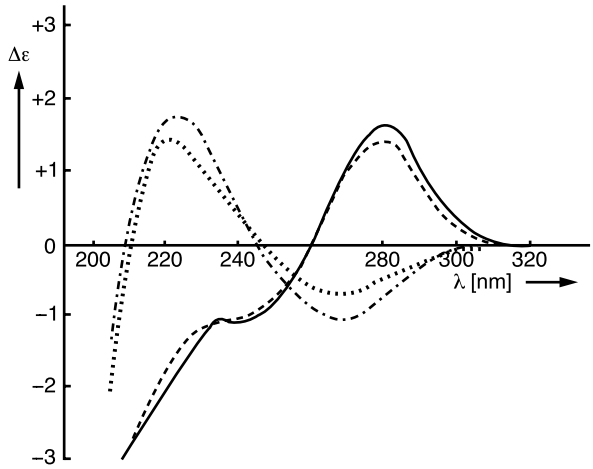


Figure 2. CD spectra of methylphosphotriester R_P d([C_P(O)OMe]G)$_2$ (–.–.–), S_P d([C_P(O)OMe]G)$_2$ (. . . . .), R_P d([G_P(O)OMe]C)$_2$ (———), and S_P d([G_P(O)OMe]C)$_2$ (--------) in water at 4°C.

the close ion-pair formation follows from the atomic resolution which shows that the phosphate groups are closer to each other on two sides of the minor groove in Z-DNA than in B-DNA. However the phosphate-methylated dimer and the natural tetramer are examples in which closest approach does not come up. So the impact of the high-salt concentration must be found in the intramolecular dynamics in the duplex stucture i.e. the change in the glycosidic torsion angle and the suger pucker for dC and dG necessary for the B–Z transition. Activation of phosphorus from a tetrahedral P(IV) into a trigonal bipyramidal (TBP) P(V) geometry can be enhanced by complex formation with solvated metal ions, making the phosphorus more susceptible to a fifth ligand, e.g. water, basic solvents or a protein site. It seems that only a symmetrical generation of P(V)TBP intermediates within d_PC fragments in which $O_{5'}$ is situated in the axial position of the TBP leads to a Z-DNA structure.[12] Axial location of $O_{5'}$ results in an increase of the electron density at $O_{5'}$ .This initiates a rotation of the 2′-deoxyribose ring around the $C_{4'}$–$C_{5'}$ bond, caused by the increased repulsion between the net electron density on $O_{5'}$ and $O_{1'}$ of the 2′-deoxyribose ring, which will be also accompanied by a rotation around the C_1'-N bond. Propagation of these rotations is effected by a synchronous rotation of the equatorial ligands around the principal axis of the TBP through which they become more accessible for shielding by the metal ions. In contrast to pyrimidine bases purine bases readily accommodate a syn position. Therefore the base of residue dC remains orientated within the anti domain throughout the entire B–Z transition. The effect of conformational transmission going from P(IV) to P(V) TBP has been put forward by us in a number of papers demonstrating the significantly greater population of the γ^- conformation for axially situated tetrahydrofurfuryl around the $C_{4'}$–$C_{5'}$ bond in 5'-P(V)TBP tetrahydrofurfuryls with respect to their related P(IV) compounds which show dominant γ^+ and γ^t conformation.[13–20] Further studies of longer methylphosphotriester DNA with alternating CG sequences showed that perfect shielding of the phosphate linkages not always result in a B–Z transition. The *complete* methylation of the tetramer was based on the method using Fmoc as base-protecting group in a non-automated preparation, vide supra.[21] Since there are three chiral phosphate-methylated groups we are dealing with eight diastereoisomeric compounds. The T_m curve shows three melting transitions at 18°, 30°, and 42°C. The CD spectrum of the diastereoisomeric mixture shows the characteristics of B-DNA. Apparently the conflicting structural preferences of the individual $d(C_PG)_2$ and $d(G_PC)_2$ segments for Z-and B-DNA respectively are best accommodated in an overall right-handed duplex structure. Our conclusion is that stabilization of the Z form must be the result of site-specific shielding of the phosphate groups in the $d(C_PG)$ units exclusively. This is in excellent agreement with ^{31}P NMR measurements of the natural hexamer at different concentrations of Mg^{2+}: $\delta(C_PG)_2$: −0.31 ppm and $\delta(G_PC)_2$: −0.08 ppm (ratio 3:2).[9]

The conflicting matter of interest in the helix consisting of alternating CG sequences makes that a site-specific interaction is necessary for the presence of a Z form. Because of the dynamic possibilities of ionic shielding and/or hydrogen bridging Nature is in a better position compared to modified DNAs as methylphosphotriester DNA. The impact of duplexes containing alternating CG sequences inserted in a random B-DNA will have a strong impact on the conformational behavior of the overall duplex formation. Formation of the local Z form within the duplex by site-specific hydrogen bonding of the enzymes initiates unwinding which may be of importance for transcription processes.[22]

Parallel Methylphosphotriester DNA

X-ray crystallographic studies on acetylated DNA or RNA nucleosides show modes of hydrogen-bonded pairing which differ from the antiparallel coupling. It was established that 3′,5′-di-*O*-acetylthymidine crystallizes in a parallel based-paired conformation with two equivalent O_4-H-N_3 hydrogen bonds which displays a two-fold rotational symmetry.[23,24] Similar observations were made for methylphosphotriester DNA showing parallel T=T O_4-H-N_3 pairing, and C=C N_4-H-N_3 pairing only for S_P chirality.[25,26] This exclusive methylation resulted in an extended investigation of the methylphosphotriester DNA of the T hexamer, the C hexamer, and the mixed dodecamer. These oligonucleotides were prepared following a different method.

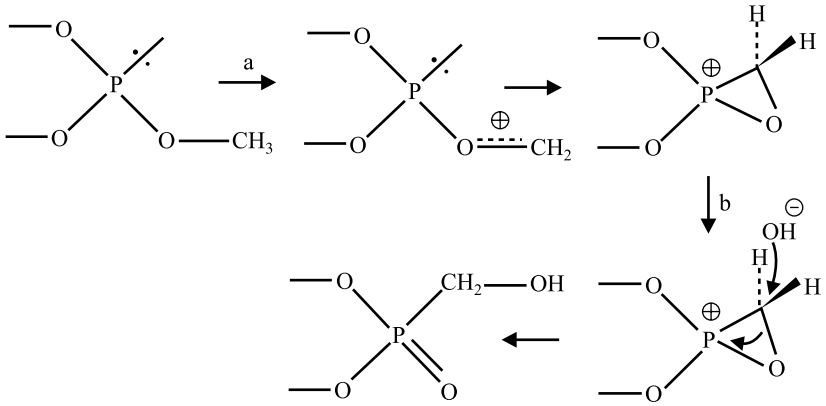
We started with the natural compounds which were prepared with an automatic procedure following a standard phosphite triester synthesis protocol. Phosphate methylation of the T hexamer could be performed with methyl methanesulfonate.[25] In the case of the C hexamer and the mixed CT dodecamer, base-protecting was carried out with benzoyl chloride followed by addition of methyl methanesulfonate. Base deprotection was carried out with hydrazine. The methylphosphophosphotriester DNAs were purified by means of short-column chromatography. The methylphosphotriester T hexamer shows the characteristics of a parallel duplex. The resulting duplex has a T_m value of approximately 67°C as was established with UV hyperchromicity for a substrate concentration of $1.3*10^{-5}$ M in water. Based on the ^1H NMR subspectra of the imino- and base-methyl protons the transitions have the same T_m value of 64°C for a substrate concentration of 10 mM.

A molecular model of methylphosphotriester T hexamer was constructed using the structural information of 3′, 5′-di-*O*-acetylthymidine and the detailed ^1H NMR data of the R_P and S_P diastereoisomers of the methylphosphotriesters of $d([T_P(O)OMe]T)_2$. We found that the parallel T=T base pairs indeed fit in a right-handed double helix with γ^+ and β^t conformations around the $C_{4'}-C_{5'}$ and $C_{5'}-O_{5'}$ bonds respectively with a south conformation of the 2′-deoxyribose rings, and an anti conformation of the T bases. The intrinsic symmetry results in the formation of two identical grooves instead of the major and minor grooves in B-DNA. The combination of the results leads to a structure with eight residues per turn, and a rise per base pair of 3.6 Å. The helix diameter is 15 Å whereas the purine-pyrimidine base pairs in B-DNA have a corresponding diameter of 21 Å.

For the methylphosphotriester C hexamer and the mixed CT dodecamer no duplex formation could be detected. Because the S_P chirality is a condition for duplex formation in the case of C=C duplexes, the mixed chirality during the synthesis cancels this possibility.[26] Different from our approach oxalyl-CPG was used as a labile support for the synthesis of the methylphosphotriester T hexamer. The FAB mass spectroscopic numbers and the UV hyperchromicity data revealed that no duplex formation was found.[27] Standard methyl phosphoramidite chemistry was employed in the coupling and *t*-butyl hydroperoxide was used to oxidize the phosphite intermediates to the methylphosphotriester T hexamer. Cleavage from the support was effectuated with ammonium hydroxide in methanol resulting in full methylation of the phosphate linkages. However, in the stepwise non-automated preparation of methylphosphotriester DNAs we also used *t*-butyl hydroperoxide in order to oxidize the phosphite into the

methylphosphotriester. As was shown the phosphate-methylated R_P, S_P d([T_P(O)OMe]T)$_2$, S_P d([T_P(O)OMe]C)$_2$, and S_P d([C_P(O)OMe]C)$_2$ exhibit parallel miniduplexes with T_m values of 30°, 26°, and 33°C, respectively as was established with UV hyperchromicity and ^{1}H NMR. In a recent publication we mentioned that in the case of a methoxyl group at P(III) one deals with a very reactive primary carbon as methyl resulting in competition with P(III) toward *t*-butyl hydroperoxide.[28] If the reaction is carried out on a *solid support* the methyl group is more accessible than phosphorus. In this situation the phosphorus is indirectly involved in the oxidation process. These kind of interactions are generally known as neighboring-group participation. Via an intramolecular S_N2 mechanism the phosphorus site donates its electron pair to the primary carbon. The hydrogen which is then located in the axis of the TBP C(V), reacts as *hydride* in a concerted way with the oxygen of the OH-group of *t*-BuOOH under formation of a three-membered ring-intermediate as shown in Scheme 1. In the next step the nucleophilic attack of OH^- on the secondary carbon results under ring opening in hydroxymethylphosphonate with *t*-butyl alcohol. The driving force for this conversion comes from the ring opening accompanied with the formation of a P=O bond. On the other hand in *solution* the phosphorus is readily accessible for oxidation. With all data available as UV hyperchromicity, the melting curves with ^{1}H NMR, the FAB mass spectroscopic numbers, and the ^{1}H NMR characterization it is clear that in the case we are dealing with the oxalyl-CPG support, hydroxymethylphosphonate T hexamer is formed which is an isomer of methylphosphotriester T hexamer.

Hydroxymethylphosphonate shows resemblance with methylphosphonate. However, methylphosphonate DNAs are strongly hindered by their backbone structure for duplex formation,vide infra.[5,29] This explains the absence of self-association of the hydroxymethylphosphonate derivative d([T_P(O)CH_2OH]$_5$T). Recently the significance of parallel duplex formation has been found in the ends of chromosomes capped by telomers consisting of a GGGG tetrad alignment in the presence of K^+ ions with a fourfold rotational symmetry in consequence of N_2–H–N_7 and N_1–H–O_6 hydrogen bonding.[30] The latter mode is exclusively found in the parallel formation of T=T pairing.



Scheme 1. The reaction mechanism for the conversion of methylphosphitetriester in hydroxymethylphosphonate: a = *t*-BuOOH/CH_2Cl_2; b = OH^-.

Parallel Natural DNA

There is a growing interest in studies for the synthesis of peptide-oligonucleotide conjugates focused on the transport of oligonucleotides into cells. The increase of duplex stability with or without covalent peptide conjugation is a challenging therapeutic goal. So it was found that a synthetic amphiphilic α-helix peptide could bind with double or triple stranded DNA.[31] The enhanced stabilization was significant for cationic α-helix peptides which demonstrates the importance of electrostatic interaction of the positive charge of the peptide and the negatively charged phosphodiester linkages of DNA.[32] An exclusive example in this area was demonstrated by us for the formation of a parallel right-handed duplex as shown by T=T and C=C interactions accompanied with two equivalent hydrogen bonds O_4—H–N_3 and N_4–H—N_3 respectively.[33] It was shown that the natural oligomer dT_{10} forms a parallel duplex in the presence of cationic oligopeptides as octadeca (L-lysine)(Lys_{18}) and octadeca (L-ornithine)(Orn_{18}). Complexation of the ε- and δ- ammonium groups, respectively on their side chains of four and three methylene groups, with the anionic phosphate linkages diminishes the electrostatic phosphate-phosphate repulsion. In oligomers as dC_{10}, $d(C_6T_6)$, and $d(T_6C_2T_2)$ parallel duplex formation occurs in the presence of Lys_{18} and *not* in the presence of Orn_{18}. Model studies showed that in the case of Orn_{18} complexation must occur with one of the non-bonded oxygens in the phosphate groups with unfavorable O_R *pro*chirality thereby precluding C=C base formation. By varying the ratio of peptide/DNA it was found that the largest UV hyperchromicity effect occurs when one peptide molecule is present for two strands. This indicates a geometry in which an oligopeptide strand is located in one of the identical grooves of the DNA duplex. These triple helices are examples of self-assembly of T=T and C=C base pairing in which the peptide and the duplex accommodate their foldings which results in a stereospecific recognition in the latter case.

The presence of the DNA duplexes was ascertained by ^{1}H NMR measurements on the dT_{10}-Lys_{18} triple helix. The imino protons involved in the hydrogen bonding correspond exactly with the position observed for the methylphosphotriester DNA in the parallel duplex of the T hexamer. The mechanism for the peptide-induced parallel duplex formation is based on complexation of one strand dT_{10} with the peptide via electrostatic attraction (proton shielding). This template is suitable for complexation with the other strand dT_{10}. The positive charge location in the cationic oligopeptides is of eminent importance. Since Lys_{18} and Orn_{18} form a triple helix with dT_{10} it was of interest to observe the effect of poly L-arginine. In that case the positive charge is strongly delocalized over carbon and nitrogens which are involved in the π-redistribution. Although there is complex formation in a 1:1 ratio thus affording a template for a triple helix no hyperchromicity is observed between 10°–70°C.

One can imagine that the formation of a peptide-induced parallel DNA duplex results in a temporary fixation of genetic information. Via pyrimidine-rich regions it is possible then that parallel duplexes interfere with various levels of the genetic information transfer.[34]

The results which are offered show the unusual hybridization affinity of natural DNA for parallel duplexes if escorted by peptides via well-defined local charge interactions. The stereopreference of phosphodiester linkages is of importance for base differentation.

The absence of major and minor grooves in parallel DNA duplexes has been for instance shown for DNA oligonucleotides with dA and dU residues in a trans AU base pairing (reverse Watson & Crick base pairing).[35] As is to be expected the nuclease

activity on this parallel duplex was found to be generally lower than on the corresponding antiparallel duplex and probably related to changes in the groove dimensions. This conclusion may be also of interest for parallel DNA duplexes in general.

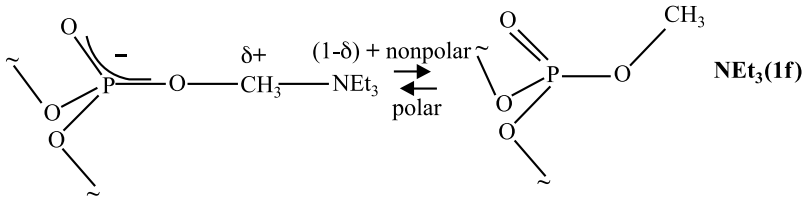
Selected Random Methylphosphotriester DNA

Selected random long methylphosphotriester DNAs used for site-specific inhibition were synthesized according to a three-step procedure: 1) base protection with Fmoc, vide supra, of an automatically synthesized natural DNA sequence; 2) methylation of the phosphate groups with *p*-toluenesulfonyl chloride and methanol;[36] and 3) removal of the Fmoc groups with triethylamine, vide supra. Although useful for short DNA fragments, it appeared that for long fragments introduction of the Fmoc group results in an almost insoluble product which causes low yields in the next steps.[37,38] Analysis of the long fragments methylphosphotriester DNA prepared in this way show that we are dealing with the natural oligomers complexed with organic cations as among others $MeNEt_3^+$.[38]b With the biochemical data available we come to the conclusion that this composition is a rather realistic substitute for the corresponding methylphosphotriester DNA.

This view is supported by the results of Moody et al. based on the hybridization of an 18-mer prepared in this manner with a specific location on the *E.coli pab B* gene (coding for p-aminobenzoate synthase) inserted in a single-stranded M13 phage.[40] There is a strong decrease in relative DNA synthesis activity for that specific location. A control experiment in which the complementarity is absent showed no decrease of the relative synthesis activity along the template strand.[41]c

[b]An 18-mer complexed in this way has been used to test its hybridization ability over its full length with $tRNA^{Phe}$ directed on the amino acid stem and TψC arm. However, the interpretation of the corresponding NMR spectrum given in Ref. [37] was not correct: 'The spectrum at 20°C part of the imino-proton region of an equimolar mixture of $tRNA^{Phe}$ and the 18-mer d(TGG. TGC. GAA. TTC. TGT. GGA.) complementary to the amino acid stem and the TψC arm (basis 59 to 76) of $tRNA^{Phe}$, shows new imino resonances at 12.4 (6 A–T), 13.3 and 13.6 (9 G–C) and 14.1 ppm (3 A–U). At 50°C no imino resonances are seen for $tRNA^{Phe}$ whereas the mixture shows resonances at 12.8, 13.4, and 13.9 ppm (peak areas 6:9:3), that is, these signals result from the duplex of the 18-mer with bases 59 to 76 of $tRNA^{Phe}$. The A–T and A–U base pairs correspond to signals at 13.9 and 12.8 ppm, respectively as is seen from the peak areas.' However, this *contradicts* with 12.8 (6 A–T), 13.4 (9 G–C), and 13.9 (3 A–U). After the retraction was published, study of the NMR spectra revealed that the observation is 12.8, 13.4, and 13.9 ppm (peak areas 3:9:6). This means that by interchange of numbers the interpretation of the NMR spectrum of the mixture at 20°C and 50°C is misleading. We cannot but recognize that the NMR spectrum of the suggested duplex structure is *only* the result of line-broadening and shifting in the NMR spectrum of the $tRNA^{Phe}$. Moreover the upfield part of the latter one (<12.5 ppm) which was not shown, had been used to demonstrate the appearance of the new A–T hybridization in the suggested duplex structure. It must be noticed that this NMR analysis differs completely from the explanation given in a Report of Eindhoven University (1990) quoted in Ref. [39].

[c]For the 14–31 region of the *pab B* gene a complementary natural 18-mer was used as the primer for the sequencing reaction. The reaction was performed with the Klenow fragment of DNA polymerase I and standard deoxy/dideoxy ratios. The −110– −93 region was used at the site-specific blocker. A comparison with a natural 18-mer of the complementary sequence of the latter region is not possible because this 18-mer would act as a primer for the Klenow fragment too.



Scheme 2. Phase-transfer induced methyl-transfer reactions.

A strong indication that we are dealing with an effective substitute for the corresponding methylphosphotriester DNA is found in a specifically inhibit transcription. Addition to *E. coli* cells of a suchlike 22-mer, complementary to the repressor binding site of the *lac* operon, strongly reduces the formation of β-galactosidase compared with a random sequence.[42,43] Recently a 21-mer, which was designed to form a triplex (TFO) also with the operator of the *lac* operon, was found to be able to specifically inhibit β-galactosidase within 15 minutes.[44] This is in exellent agreement with our experimental data. In contrast, the *natural* sense control was found to be incapable of inhibiting the β-galactosidase production. This suggests that the RNA polymerase is strong enough to replace the sense control when moving through the operator. However, as we know that methylphosphotriester DNA is less accessible for enzymatic recognition, vide infra, it is realistic to assume that under the experimental biological conditions a phase-transfer induced methyl transfer has taken place resulting in partial and/or complete shielding of the phosphate backbone.[34] This is shown in Scheme 2.

A similar migration of methyl groups was found between aliphatic amines in water. It has been shown that rate enhancements of methyl-transfer reactions will be effectuated by *desolvation* and approximation effects.[45] It is likely that the results of similar biological experiments focused on site-specific inhibition of genetic transfer[d] and the interruption of the life cycle of the human immunodeficiency virus type-1[37] may be explained by this mechanism.

[d]In a comparable experiment with *Salmonella Typhimurium* it had been shown that two sense control 21-mers, also complexed with organic cations as $MeNEt_3^+$ (From Ref. [38], Recently a biochemical application in favor of the introduction of a cationic-anionic interaction has been established by Wang et al. in their study of molecularly engineered biodegradable polymers used as protecting agents for DNA plasmids. This encapsulation of plasmid DNA, for instance encoding an antigenic sequence, provides a sustained release of DNA and subsequent production of encoded protein antigen for generating prolonged immune responses. Using an additional monomer as *N*-methyldiethanolamine, protonation under the biological conditions takes place which results in a positively charged tertiary amine which binds effectively with the phosphate linkages. The corresponding delay of the DNA release has been resulted in an enhanced immune response in vivo: Ref. [46]), vide supra, complementary with the active-site regions of the alanine racemase genes *alr* and *dad B* (bases 91 to 111 and 94 to 114, respectively) which only differ in 4 bases, exclusively inhibit the transcription of their corresponding target genes. This result is quoted in Ref. [37] under References and Notes(10): H.M. Moody, S.A.M. Biezen, H.J.M. Kocken, H.J.J. Sobczak, M.H.P. van Genderen, Buck, H.M.

Comparison with Other Backbone-Modified DNAs

Compared with other backbone-modified DNAs as methylphosphonate DNA, stability data on hybridization of methylphosphotriester DNA and methylphosphonate DNA show an equal stability at the level of 2, 3 or 4 base pairs. For longer fragments methylphosphonate DNA there is a strong decrease in hydridization affinity compared with methylphosphotriester DNA. The explanation is that methylphosphonate DNA can not readily adopt the right-handed backbone conformation which is needed for the formation of a stable duplex. In fact the presence of a P–C bond disturbs the helix conformation for stereoelectronic reasons.[5,29]

Generally an outward orientation of the substituent linked to phosphorus stabilizes the duplex formation. In the case of methylphosphotriester DNA with complementary natural DNA the difference between the outward orientation (S_P configuration) and the inward orientation (R_P configuration) is absent. It should be noticed that the configuration of phosphorus in the case of other backbone-modified DNAs plays an important role in their hybridization stability with natural DNA.

The potential use of phosphorothioates oligomers as site-specific inhibition agents is well-known.[7] An interesting aspect is that not all the information possessed by these oligomers resides solely in the base sequence which is the result of the pronounced electron density on sulphur (caused by the relatively small one-center electronic repulsion integral compared with oxygen). This leads to maintaining solubility in solvents with various polarity and presumably in enhancing compatibility with peptides. A nice application of these properties has been demonstrated for multiple C_PG motifs on a phosphorothioate backbone with palindromic sequences. Biological studies indicate that suchlike C_PG oligodeoxynucleotides similar to those of bacterial DNA are effective in modulating immune response.[47] Inverse of the C_PG motif eliminates the trigger for immune response which demonstrates the unique biochemical properties of these motifs, vide supra.

The influence of the chirality of phosphorus is also observable in the case of parallel duplex formation between methylphosphotriester DNAs. As was mentioned, vide supra, C=C pairing leads to a parallel duplex only in the case of the S_P configuration. Apparently the R_P configuration which is accommodated in the case of T=T pairing is unfavorable for the C=C pairing. Modeling studies revealed a larger propellor twist angle in C=C (41°) compared to T=T (25°) which explains the stereochemical preference. The larger propellor twist angle in the case of C=C pairing resulting in a narrowing of the helix groove disfavors accommodation of the methyl groups in the R_P configuration.

An important aspect of the backbone-modified DNAs is their nuclease resistance. Both methylphosphonate DNA and methylphosphotriester DNA are nuclease resistant because of their non-ionic backbone. Only the *N*-terminal domain of Ada of *E. coli* repairs exclusively the S_P configuration of methylphosphotriester DNA by direct methyl transfer of the methyl group to one of the cysteine residues. This transfer results in an irreversible loss of repair activity. In fact *E.coli* employs the Ada protein both to repair DNA alkylation products and to regulate the transcription of genes which encode for DNA alkylation repair proteins.[48,49] Recently it has been demonstrated that the DNA repair protein can be converted into a real enzyme by removal of its internal nucleophile by an external nucleophile as methyl acceptor.[50]

CONCLUSIONS

Methylphosphotriester DNA shows a number of (bio) chemical properties which are related to natural DNA. In fact methylation mimics a shielding of the negatively charged phosphate groups which under natural conditions is afforded by (cationic) peptides, metal ions, and solvents. In various experiments this specific correspondence is demonstrated. The stereochemical analogy between methylation and peptide complexation of the phosphodiester linkages is demonstrated in an explicit way for the S_P chirality and O_S *pro*chirality in the parallel duplex formation with C=C base pairs. For the exclusive formation of T=T base pairs there is no stereochemical prerequisite.

It is of interest to mention that methylphosphotriester RNA (2′ OH replaced by 2′ OMe) itself behaves as methylphosphotriester DNA in the formation of self-complementary right-handed antiparallel and parallel (U=U and C=C) duplexes although with lower stability in comparison with the corresponding methylphosphotriester DNA. In the presence of C=C pairs also S_P chirality is a prerequisite.[51]

At the end it may be of significance to state that the process of methylation and demethylation of histones is in some respect related to the behavior of methylphosphotriester DNA with regard to its replicational and transcriptional retardation ability. It is generally accepted that a methyl group cannot be removed from a histone via an active process.[52,53] This implies that it will remain on a promoter which results in inhibition of transcription (transcriptional silencing). The activated methyl groups are present as methylated lysines in a mono-, di-, or trimethylated state, and as mono- or dimethylated arginines. Methyl transfer initiated by the electrostatic interaction between the positively charged ε-methyl ammonium groups of the lysines and the negatively charged phosphate linkages is facilitated by the amphiphilic character of the methylated ammonium groups of the lysines. This is also meant for the methylated arginines. We suggest that this mechanistic view is closely related to the phase-transfer induced methyl-transfer mechanism, vide supra.

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Received May 5, 2004
Accepted August 5, 2004