Hybridization of phosphate-methylated DNA and natural oligonucleotides : implications for protein-induced DNA duplex destabilization

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Hybridization of phosphate-methylated DNA and natural oligonucleotides. Implications for protein-induced DNA duplex destabilization

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Abstract. Duplex stabilities have been determined for several hybrids of phosphate-methylated oligonucleotides and natural DNA or RNA using UV hyperchromicity experiments. These hybrids have a higher stability than the corresponding natural duplexes, due to the absence of interstrand electrostatic repulsions. Comparison with stability data for hybrids of other neutral oligonucleotides (with phosphate-ethylated and methyl phosphonate linkages) and natural DNA or RNA revealed that differences in stability could be attributed mainly to steric and stereoelectronic factors. For phosphate-ethylated oligonucleotides, hybridization with a natural strand is strongly influenced by steric interactions of the ethyl group. Hybridization with RNA, which requires a tight A-type conformation, is therefore difficult and the ethyl orientation (inward or outward, depending on the phosphorus configuration) determines the strength of the association with natural DNA. In methyl phosphonate systems, it appears that the presence of a P-C bond disturbs the helix conformation for stereoelectronic reasons. This leads to a weaker hybridization with DNA and RNA for longer strands. Phosphate-methylated oligonucleotides are found to have an optimal combination of steric and stereoelectronic factors and form the strongest hybrids with natural DNA. Absence of intrastrand phosphate-phosphate repulsions causes a slightly different conformation for phosphate-methylated DNA, which is evident in the cooperative character of the hybridization with natural DNA. A thermodynamic model for this cooperativity is presented and the model studies are extended to protein-DNA complexes in which phosphate charges are also shielded. The preliminary results suggest that protein association can destabilize a DNA duplex, thus providing a possible mechanism for the action of DNA unwinding enzymes.

Introduction

Synthetic DNA oligomers with backbone modifications are useful as model compounds for studying DNA structure and dynamics¹ and as probes for elucidating the stereochemistry in enzymatic cleavage of oligonucleotide strands². Systems in which the charge of the phosphate groups is eliminated, *e.g.* phosphate triesters and phosphonates, have received particular attention, because of their resistance to breakdown by nucleases³ and their easy transport through cell membranes as a result of their lipophilic character⁴. The absence of the negative phosphate charge also minimizes electrostatic repulsions between the strands in a DNA duplex, thus raising the duplex stability⁵. These properties have led to the suggestion that neutral DNA analogues are well-suited for anti-sense hybridization techniques, where a specific genomic sequence is blocked by strong association with a complementary probe⁶.

In recent years, we have studied phosphate-methylated DNA systems, which are now easily available via new synthetic routes^{5,7}. It has already been demonstrated that methylation of the phosphate groups in oligothymidine

strands gives rise to a new duplex structure, based on thymine-thymine pairing⁸. In this duplex, the 5'→3' vectors run in the same direction, *i.e.* parallel, due to the symmetrical base pairing. Since pyrimidine-pyrimidine association forces the backbones into close proximity, phosphate charge shielding is essential for the formation of the parallel duplex. Very recently, this parallel duplex was also observed with cytosine-cytosine base pairing in the phosphate-methylated dinucleotide d(Cp(OMe)C) exclusively for the *S_p* configuration on phosphorus^{9a}. It is of interest to note that complexation of the phosphate groups with a polycationic protein (*e.g.* polylysine or polyornithine) can accomplish the same effect as methylation^{9a,10}. This provides the possibility of extrapolating the results with phosphate-methylated systems to protein-DNA interactions, which are important in understanding the action of specific enzymes which destabilize the DNA duplex.

In this paper, we present our results on the hybridization of phosphate-methylated oligomers and natural DNA or RNA. The duplex stabilities will be compared to those found for other types of neutral DNA. The discussion will focus on phosphate-ethylated and methyl phosphonate oli-

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Results

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gonucleotides, which have been extensively studied by Miller et al.^{3a,6c,11} and by Pless and Ts'o¹². For duplexes between phosphate-methylated and natural DNA, a more detailed study has been carried out, which provides insight into the structural properties of the phosphate-methylated strand.

Results

The various types of neutral DNA oligomers are identified using the following notation: phosphate-methylated, p(OMe); phosphate-ethylated, p(OEt); methyl phosphonate, p(Me). The synthesis of the phosphate-methylated

Fig. 1. ¹H NMR chemical shift of the imino protons in the system d(Ap(OMe)Ap(OMe)A) · poly(dT) vs. temperature.

ration. It will be shown (*vide infra*) that the phosphorus chirality in phosphate-methylated DNA does not influence the stability of hybrids with natural systems. Table I lists the T_m values of the systems studied, and the natural reference duplex d(A₃) · poly(dT) has a T_m of 20°C.

From the difference of 21°C in T_m value between this reference system and the corresponding phosphate-methylated system, it is obvious that a stabilization of the duplex structure indeed occurs upon neutralization. It can also be clearly seen that formation of G–C base pairs gives rise to a much higher duplex stability than found with A–T base pairs. Table II contains the stability data of the hybrids between methyl phosphonate and natural DNA or RNA, as reported by Miller et al. in several publications^{6c,11}. The results obtained on hybrids of phosphate-ethylated oligomers and natural systems shown in Table II come mainly from the work of Miller et al.^{3a} and Pless and Ts'o¹².

As a consequence of the independence of ionic strength (*vide supra*), the results given in Tables I and II can be compared to abstract the various factors which determine the duplex stabilities of the neutral/natural systems.

Table I T_m values for hybrids between phosphate-methylated oligonucleotides and natural DNA or RNA.

System	T_m (°C)	System	T_m (°C)
d(Ap(OMe)A) · poly(dT)	30	d(Ap(OMe)A) · poly(U)	13
d(Ap(OMe)Ap(OMe)A) · poly(dT)	41	d(Ap(OMe)Ap(OMe)A) · poly(U)	< 10
d([Ap(OMe)] ₃ A) · poly(dT)	57		
d(Cp(OMe)C) · poly(dG)	45		
d(Cp(OMe)Cp(OMe)C) · poly(dG)	55	d(Cp(OMe)Cp(OMe)C) · poly(rG)	12

Table II T_m values for hybrids between methyl phosphonate and phosphate-ethylated oligonucleotides and natural DNA or RNA.

System	T_m (°C)	System	T_m (°C)
d([Tp(Me)] ₈ T) · poly(dA)	12 ^a	d([Tp(Me)] ₈ T) · poly(rA)	< 0
d(Ap(Me)A) · poly(dT)	19	d(Ap(Me)A) · poly(U)	17
d(Ap(Me)Ap(Me)A) · poly(dT)	37	d(Ap(Me)Ap(Me)A) · poly(U)	33
d([Ap(Me)] ₃ A) · poly(dT)	45	d([Ap(Me)] ₃ A) · poly(U)	43
d([Tp(OEt)] ₇ T) · poly(dA)	9 ^b	d(T[p(OEt)] ₇ T) · poly(rA)	– 11 ^b
		d(Ap(OEt)A) · poly(U)	12

^a Range from 0–25°C. ^b Temperature of 90% dissociation into single strand ($T_{0.9}$).

Discussion

Steric factors

It is obvious from molecular models that the various alkyl groups in the neutral DNA systems, whether directly linked to phosphorus or attached to a phosphate oxygen, cause additional steric interactions compared to natural DNA. This is most obvious when comparing duplexes with either natural DNA or natural RNA, since in the former duplex a B-type helical geometry is found, while in the latter an A conformation is required¹⁷. Steric interactions are of higher importance in a duplex with an A geometry, since intrastrand phosphate-phosphate distances are strongly diminished¹⁸, while substituents on phosphorus are in closer contact with other parts of the DNA¹². In accordance with these facts, all systems listed in Tables I and II display a stronger hybridization with DNA than with RNA. The difference in T_m value is expected to follow the steric requirements of the phosphorus substituents. The increase in steric size on going from methyl phosphonate to phosphate-methylated to phosphate-ethylated is indeed reflected in the T_m values of the adenosine dinucleotides hybridized with poly(U): d(Ap(Me)A), 17°C; d(Ap(OMe)A), 13°C; d(Ap(OEt)A), 12°C. In these systems, no interactions between neighbouring phosphate groups are present. For trinucleotides, these become more important and cause stronger differences in T_m value: d(Ap(Me)Ap(Me)A) hybridizes with poly(U) and has a T_m value of 33°C, while no duplex between d(Ap(OMe)Ap(OMe)A) and poly(U) is found above 10°C.

Another factor that can influence steric interactions is the chirality of phosphorus which is present in all the phosphate-modified systems presented here. From a molecular model abstracted from a B DNA duplex, it follows that two orientations of the phosphorus substituents are possible, designated outwards and inwards, corresponding with the two chiral forms of the phosphorus atom. The *Cahn-Ingold-Prelog* R/S notation can be applied to phosphorus¹⁹, but gives a different label to spatially identical orientations of the alkyl substituent for the three systems. Model studies show that the inwards orientation can cause stronger steric repulsions, and hence a lower duplex stability is present for this phosphorus configuration. Experimental data on this effect are limited, since it requires separation of the diastereomeric forms of the neutral oligonucleotides. However, for phosphate-methylated DNA it has been found that the phosphorus configuration does not affect its ability to hybridize with natural DNA. This is most evident for the trinucleotide d(Ap(OMe)Ap(OMe)A), which consists of four diastereomeric forms. Hybridization with poly(dT) gives rise to a single sharp melting transition at 41°C in both UV and NMR experiments, indicating that all diastereomers form an equally stable duplex. A similar behaviour was found for all the diastereomeric mixtures shown in Table I. A more indirect proof of the similarity of the various diastereomeric forms was provided by stability studies on the parallel duplexes formed by phosphate-methylated oligothymidines (*vide supra*). Even for double-stranded d([Tp(OMe)]₅T), which contains five chiral centres in each strand, a well-defined melting transition at 65°C was found in both UV⁸ and ¹H NMR studies^{8a}.

For phosphate-ethylated triester systems, a clear difference has been found for the duplex stability of oligonucleotides containing one modified phosphate with a specific chirality. The self-complementary octamer d(GpGpApAp(OEt)-TpTpCpC) forms a duplex with a T_m value of 31°C for the outwards orientation of the ethyl group and already melts at 25°C when an inward orientation is present²⁰. Evidently,

the large ethyl group has a much stronger effect on the duplex stability in comparison with a methyl group. In the case of methyl phosphonate DNA, one would not expect, on the basis of steric factors, to find a difference between the two methyl orientations, since methyl is sterically even less demanding than methoxy (*vide supra*). For small fragments (up to four nucleotides), this is indeed the case, however longer fragments of methyl phosphonate DNA exhibit strong variations in their hybridization ability. For example, the hybrid of the decamer d(Tp(Me)-[TpTp(Me)]₄T), with alternating phosphate and methyl phosphonate groups and poly(dA), has a T_m value of 34°C when one chirality is present and dissociates at 2°C for the other chirality^{1b}. A similar behaviour is found for the hybridization of this decamer with the RNA poly(A): $T_m = 20^\circ\text{C}$ for the outward form and $T_m = 0^\circ\text{C}$ for the inward form. Furthermore, the nonamer d([Tp(Me)]₈T) shows a broad range of T_m values (0–25°C) upon hybridization with poly(dA) (see Table II), which demonstrates the importance of the diastereomeric composition of the oligomer for duplex stability¹². Evidently, other influences besides steric hindrance must cause a difference between the isomers of the chiral methyl phosphonate group. An explanation for this behaviour can be given, on the basis of the stereoelectronic effects in phosphate and methyl phosphonate groups.

Stereoelectronic factors

The difference between the hybridization properties of methyl phosphonate and phosphate-methylated DNA with natural DNA may be due to the fact that the methyl phosphonate strands cannot readily adopt the right-handed backbone conformation which is needed for the formation

Fig. 2. Newman projections of the α^- rotamer and the ζ^- and ζ^+ rotamers for the methyl phosphonate group with the S_P configuration.

of a stable duplex. In particular, this concerns the backbone angles $\zeta(\text{C}(3')\text{--O}(3')\text{--P--O}(5'))$ and $\alpha(\text{O}(3')\text{--P--O}(5')\text{--C}(5'))$, which must reside in the ζ^- and α^- domains for right-handed DNA²¹. In the case of a methyl phosphonate linkage, it can be shown that the combination $\{\alpha^-, \zeta^-\}$ is energetically unfavoured. Fig. 2 depicts the Newman projections of the α^- and ζ^- rotamers for the S_P configuration of phosphorus. Clearly, both lone pairs on O(5') have a favourable *trans* orientation with respect to a phosphorus–oxygen bond²² in the α^- rotamer. The ζ^- rotamer shows an undesirable *trans* location of the phosphorus–carbon bond and one of the lone pairs on O(3'), which can be

Fig. 3. Newman projections of the α^- and α^+ rotamers and the ζ^- rotamer for the methyl phosphonate group with the R_P configuration.

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relieved by a rotation toward the ζ^+ domain. Fig. 3 shows the Newman projections of α^- and ζ^- for the R_P configuration. Now, it appears that the ζ^- rotamer corresponds with the advantageous *trans* orientation of the lone pairs on O(3') with respect to a phosphorus-oxygen bond. Clearly, the unfavoured *trans* location of one of the lone pairs on O(5') and the phosphorus-carbon bond drives a rotation from α^- to α^+ . It should be noted that the above reasoning cannot be verified on the basis of NMR-conformational analysis of the methyl phosphonate oligonucleotides in solution, since it is impossible to determine the conformational equilibria around the α and ζ linkages from the available set of vicinal proton-proton and proton-phosphorus coupling constants²³. However, the crystal structure of the methyl phosphonate dinucleotide d(Ap(Me)T) with the S_P configuration in the phosphonate moiety²⁴ indeed clearly shows the distorted backbone geometry which is realized by a rotation around the P-O(3') bond from ζ^- to ζ^+ (see Fig. 4). We feel that the crystal structure of

Fig. 4. Left: Standard $\{\alpha^-, \zeta^-\}$ conformation as observed in the X-ray crystal structure of natural r(AU)²⁵. Right: Distorted $\{\alpha^-, \zeta^+\}$ conformation which is found in the X-ray crystal structure of d(Ap(Me)T) with the S_P configuration²⁴.

d(Ap(Me)T) strongly supports our argument that phosphonate internucleotide linkages correspond with a backbone geometry which does not intrinsically match the right-handed DNA conformation. Notwithstanding, it can be concluded that short methyl phosphonate oligonucleotides may adopt the unfavourable $\{\alpha^-, \zeta^-\}$ conformation, since stable hybrids with natural DNAs are formed (*vide supra*). For longer strands of methyl phosphonate DNA, the right-handed backbone becomes highly unfavoured, which explains why only poor hybridization is found in these systems. In contrast, the $\{\alpha^-, \zeta^-\}$ conformation is readily available for phosphate-methylated DNA, since *trans* location of the lone pairs on either O(5') or O(3') with a phosphorus-carbon bond is excluded. In agreement with this explanation is the fact that the phosphate-methylated systems d(Ap(OMe)A), d(Ap(OMe)Ap(OMe)A) and d([Ap(OMe)]₃A) form more stable hybrids with poly(dT) (T_m values 30, 41 and 57°C) than do the corresponding methyl phosphonate systems d(Ap(Me)A), d(Ap(Me)Ap(Me)A) and d([Ap(Me)]₃A) (T_m values 19, 37 and 45°C).

Cooperative effects

The present results clearly show that phosphate-methylated oligonucleotides hybridize more strongly with natural DNA than do either methyl phosphonate or phosphate-ethylated systems. Nevertheless, it must be assumed that the phosphate-methylated strand adopts a somewhat different

geometry than natural DNA, since all *intrastrand* phosphate-phosphate repulsions are eliminated. The situation may be similar to the complexation between DNA and histones (basic proteins), where a one-sided shielding of the phosphate charges causes a bending of the DNA duplex around the protein²⁶.

We examined the effect of an altered geometry for the phosphate-methylated DNA on the hybridization process of the trinucleotide d(Ap(OMe)Ap(OMe)A) with natural oligo- and poly(dT). The T_m value of the hybrid was measured for various lengths of the natural strand and different degrees of occupancy. The results of these experiments (see Table III) clearly show that a certain number of phosphate-methylated trimers must be present to realize a stable duplex, suggesting a cooperative behaviour in the hybridization between phosphate-methylated and natural DNA.

Table III T_m values for hybrids between d(Ap(OMe)Ap(OMe)A) and natural oligo- and poly(dT).

System	Base ratio (A/T)	T_m (°C)
d(Ap(OMe)Ap(OMe)A) · poly(dT)	1	41
d(Ap(OMe)Ap(OMe)A) · d(T ₃₀)	1/2	39
	1	27
	1/2	23
d(Ap(OMe)Ap(OMe)A) · d(T ₁₀)	1	< 10
d(Ap(OMe)Ap(OMe)A) · d(T ₅)	1	< 10

Since the length of the natural DNA is of importance, the T_m value cannot solely be determined by the changes in enthalpy and entropy caused by base pair formation (ΔH_n^0 and ΔS_n^0 for n base pairs). We have therefore²⁷ previously suggested that the natural DNA strand must adapt its conformation prior to hybridization, introducing an extra loss of entropy $\Delta S_k^{0'}$ (for a length of k nucleotides). The cooperative behaviour can be incorporated via a parameter α , ranging from 0 to 1, which decreases the impact of the extra entropy loss by successive multiplications (yielding $\alpha^i \cdot \Delta S_k^{0'}$ for the i^{th} hybridization). On summing all contributions for p phosphate-methylated n -mers hybridized with a natural k -mer, it is found that:

$$T_m = \frac{\Delta H_n^0}{\Delta S_n^0 + \frac{1 - \alpha^p}{p \cdot (1 - \alpha)} \cdot \Delta S_k^{0'}}$$

Since ΔH_3^0 has been previously determined⁵ to be -23.4 kcal/mol for A-T base pairs, an analysis of the results in Table III in the light of this model can now be performed. It is found that the values $\alpha = 0.89$, $\Delta S_3^0 = -74.1$ cal · mol⁻¹ · K⁻¹, $\Delta S_{30}^{0'} = -6.2$ cal · mol⁻¹ · K⁻¹ and $\Delta S_{\text{poly}}^{0'} = -4.4$ cal · mol⁻¹ · K⁻¹ are consistent with the observations, while $\Delta S_5^{0'}$ and $\Delta S_{10}^{0'}$ must be lower than -8 cal · mol⁻¹ · K⁻¹.

From this model it appears that modification of the phosphates in one strand has two consequences, viz. (i) stabilization of the duplex due to elimination of electrostatic repulsions, giving a larger ΔH_n^0 and (ii) destabilization of the duplex due to structural changes in the modified strand, leading to a larger total entropy change. The balance of these effects has been found to be in favour of stronger duplex formation for the results presented on phosphate-methylated/natural systems (*vide supra*). A similar shielding of the phosphate groups in one strand can also occur in

protein-DNA complexes where cationic amino-acid side-chains associate with the negative phosphate groups (*vide supra*). In most earlier studies, proteins were chosen which simultaneously complex with both DNA strands²⁸, so that only the stabilizing effect is present, since any structural modifications are identical in both strands. However, on extrapolating the above results on hybrids formed between phosphate-methylated and natural DNA, one would expect a different result from protein complexation with the phosphate groups in *one* strand only. A structural difference between the strands will occur and the enthalpy and entropy of hybridization will be different from the phosphate-methylated/natural systems, which may lead to destabilization of the DNA duplex.

In order to study whether this type of duplex destabilization can occur, we have performed thermodynamic model studies on a DNA duplex containing A-T base pairs which is complexed with protein in only one strand²⁹. For these calculations, we adapted the model of Breslauer et al.³⁰ for determining the enthalpy and free energy of hybridization in natural DNA duplexes. They found that summing fixed contributions (Δh and Δg) for all dinucleotide units in a duplex gave correct values for these thermodynamic quantities. Using different dimer contributions, $\Delta h^* = \beta \cdot \Delta h$ and $\Delta g^* = \delta \cdot \Delta g$, for the A-T base pairs in our model duplex, and the abovementioned formula for the T_m value of shielded duplexes, we could evaluate the stability of a unilaterally protein-shielded duplex. The results of the calculations show²⁹ that the effect of protein shielding on the duplex stability depends mainly on the ratio δ/β . For $\delta/\beta > 1$, additional stabilization of the duplex is always found, independent of the number of shielded phosphates. When $\delta/\beta < 1$, an increase in the length of unilateral shielding results in a lower T_m value for the duplex.

This latter process may be of interest in understanding the action of enzymes, which can open a DNA duplex for replication or transcription. Several structural features known for these proteins coincide with our model assumptions. For enzymes which unwind the DNA duplex completely for replication (helicases), it is known that only a short stretch (*ca.* 8 base pairs) of the DNA is covered by the protein³¹. More specifically, the so-called gene 5 protein of phage fd is known to have a high affinity for a single strand of DNA and to associate primarily with 5 adjacent phosphate groups *via* lysine and arginine residues³². For the RNA polymerase enzymes which partially unwind a DNA duplex for transcription, it is established that *E. coli* RNA polymerase, for example, always remains associated with one of the DNA strands over a length of 14 base pairs³³. For many other RNA polymerases, it has been reported that lengths of 17³⁴ up to 70 base pairs³⁵ of the DNA duplex are covered by the protein. Furthermore, the interaction between DNA and protein in this case is thought to be mainly electrostatic³⁶. Our results, in good agreement with the above facts, show²⁹ that destabilization of a DNA duplex by phosphate shielding in the interior of the sequence (as in transcription) requires a modified stretch, which is almost twice as long, compared to a similar duplex opening at the end of the sequence (as in replication of linear DNA). It therefore may be possible that unilateral protein complexation serves as an initiating step for processes which involve the dissociation of the DNA duplex.

Experimental

¹H NMR spectra were recorded in the FT mode at 200, 500 or 600 MHz³⁷ on Bruker spectrometers. Measurements in water refer to an 85/15 (v/v) mixture of H₂O and D₂O, in which the deuterium

provided the field-frequency lock. The strong water signal was suppressed using the technique described by Haasnoot et al.³⁸. Proton chemical shifts were referenced against HDO (δ 4.68 ppm). ³¹P NMR spectra were recorded in the FT mode at 80.9 MHz on a Bruker AC-200 spectrometer. Chemical shifts are relative to 85% H₃PO₄; they are designated positive if downfield from the standard. The UV hyperchromicity measurements were performed on a Perkin-Elmer 124 spectrophotometer using 10-mm cuvettes and a wavelength of 260 nm. The natural oligonucleotides d(T₅), d(T₁₀) and d(T₃₀) were prepared via the standard phosphorus amidite protocol¹³ on an Applied Biosystems 381A DNA synthesizer. Purification was carried out using alcohol precipitation³⁹.

d(Ap(OMe)A), *d*(Ap(OMe)Ap(OMe)A) and *d*([Ap(OMe)]₃A)

Starting compound 3'-O-acetyl-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine. 5'-O-(4,4'-Dimethoxytrityl)-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine¹⁴ (5 mmol, 3.2 g) was coevaporated three times with pyridine to remove water and dissolved in 15 ml pyridine. Acetic anhydride (12 mmol, 12.2 g) was added and the solution was stirred overnight. Pyridine was then removed by coevaporation with water and the yellow residue was dissolved in 50 ml chloroform. To eliminate excess acetic anhydride, this solution was treated with a 5% aqueous sodium bicarbonate solution and extracted with three 25 ml portions of water. Concentration of the organic layer afforded a white foam, which was dissolved in 30 ml of nitromethane/methanol (95/5 v/v) and treated with anhydrous zinc bromide (31.1 mmol, 7.19 g) for 1 h. To the red solution, 150 ml of a 5% aqueous ammonium acetate solution was added, followed by extraction with four portions of 50 ml dichloromethane. The organic layer was dried over sodium sulfate and concentrated. The brown residue was purified by column chromatography on a Woelm silica gel column. Impurities were first eluted with dichloromethane, after which the product was removed from the column using dichloromethane/methanol (95/5 v/v) as eluent (*R_f* 0.16). The product was obtained as a white foam in 78% yield (2.25 g). ¹H NMR (CDCl₃): 2.11 (3H, s, N=C-CH₃), 2.16 (3H, s, CH₃ acetyl), 2.42 (1H, m, H(2'')), 3.15 (6H, s, N(CH₃)₂), 3.25 (1H, m, H(2')), 3.94 (2H, m, H(5')/H(5'')), 4.27 (1H, m, H(4')), 5.58 (1H, m, H(3')), 6.30 (1H, dd, H(1')), 7.98 (1H, s, H(2)), 8.58 (1H, s, H(8)).

Coupling compound 3'-O-[(diisopropylamino)methoxyphosphino]-5'-O-(4,4'-dimethoxytrityl)-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine⁴⁰. 5'-O-(4,4'-Dimethoxytrityl)-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine¹⁴ (19.62 mmol, 12.2 g) and diisopropylethylamine (80 mmol, 14 ml) were dissolved in 60 ml chloroform under an argon atmosphere and chloro(diisopropylamino)methoxyphosphine⁴⁰ (21.6 mmol, 4.32 ml) was added over 90 s with stirring. After 1 h, the mixture was diluted with 500 ml ethyl acetate (pre-washed with sodium bicarbonate) and washed with four 200-ml portions of a saturated sodium chloride solution and 75 ml water. The solution was dried over sodium sulfate and concentrated. The resulting foam was chromatographed on a Woelm silica gel column, using dichloromethane/hexane/triethylamine (5/4/1 v/v/v) as eluent (*R_f* 0.40). The product was obtained as a yellowish foam in 70% yield (10.76 g) and stored under argon. ¹H NMR (CD₃CN): δ 1.12 (12H, m, CH₃ isopropyl), 2.01 (3H, s, N=C-CH₃), 2.53 (1H, m, H(2'')), 3.01 (1H, m, H(2')), 3.10 (12H, s, N(CH₃)₂), 3.29 (2H, d, H(5')/H(5'')), 3.26 (3H, d, POCH₃, *J* 13.2 Hz), 3.36 (3H, d, POCH₃, *J* 13.2 Hz), 3.55 (2H, m, H isopropyl), 3.73 (6H, s, OCH₃), 4.17 (1H, m, H(4')), 4.83 (1H, m, H(3')), 6.38 (1H, dd, H(1')), 6.73-7.40 (13H, m, trityl), 8.05 (1H, s, H(2)), 8.37 (1H, s, H(8)). ³¹P NMR (CD₃CN): δ 154.40 and 154.66.

Coupling reaction, oxidation and detritylation to form 3'-O-acetyl-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine-5'-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine-3' methyl phosphate¹³. The starting compound 3'-O-acetyl-6-N-[1-(dimethylamino)ethylidene]-2'-deoxyadenosine (1 mmol, 0.36 g) and the coupling compound (1 mmol, 0.78 g) were each coevaporated three times with pyridine and then dissolved in pyridine. To initiate the reaction, 1*H*-tetrazole (5 mmol, 0.35 g) was added. The solution was then stirred for 2 h and concentrated. ³¹P NMR (CD₃CN): δ 146.13 and 145.91 (ratio 1.08:1).

The phosphite was then oxidized by addition of a solution of iodine (1 mmol, 0.26 g) in acetonitrile/lutidine/water (3/2/1 v/v/v). After

5 min, and ex solution concentrat Woelm v/v) as (CDCl₃). The pr v/v) an then a tration yield (CD₃O

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5 min, the brown solution was diluted with 100 ml ethyl acetate and extracted with 20 ml of a 1% aqueous sodium bisulfate solution. The organic layer was dried over sodium sulfate and concentrated. The resulting viscous oil was chromatographed on a Woelm silica gel column, using dichloromethane/methanol (90/10 v/v) as eluent (R_f 0.40). The yield was 30% (0.32 g). ^{31}P NMR (CDCl_3): δ 0.05 and -0.33 (ratio 1.09:1).

The product was then dissolved in 10 ml of acetic acid/water (4/1 v/v) and the solution heated to 50°C for 2 h; 25 ml cold water was then added to precipitate the 4,4'-dimethoxytrityl alcohol. Filtration and coevaporation with water yielded the dinucleotide in a yield of 81% (0.18 g). R_f ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$) 0.26. ^{31}P NMR (CD_3OD): δ 3.34 and 3.29.

$d(\text{Ap}(\text{OMe})\text{A})$

The final acetylation of the 5'-hydroxyl group was performed as described for the starting compound. The N(6)-protecting groups were then removed¹⁴ by dissolving the material at 40°C in 1,2-ethanediamine/phenol/water (2/8/1 v/v/v). After 1 h, the mixture was coevaporated with water and the product isolated using two-dimensional preparative thin-layer chromatography: R_f 0.23 (eluent dichloromethane/methanol 70/30 v/v). ^1H NMR (D_2O): δ 2.08 (3H, s, CH_3 acetyl), 2.12 (3H, s, CH_3 acetyl), 2.33 (1H, m, $\text{H}(2'')$ of dAp), 2.44 (1H, m, $\text{H}(2')$ of dAp), 2.62 (1H, m, $\text{H}(2'')$ of pdA), 2.84 (1H, m, $\text{H}(2')$ of pdA), 3.49 (2H, m, $\text{H}(5'')/\text{H}(5')$ of dAp), 3.76 (3H, d, POCH_3 , J 11.4 Hz), 4.22 (1H, m, $\text{H}(4')$ of dAp), 4.39 (3H, m, $\text{H}(4'')/\text{H}(5'')/\text{H}(5')$ of pdA), 5.00 (1H, $\text{H}(3')$ of dAp), 5.45 (1H, m, $\text{H}(3')$ of pdA), 6.03 (1H, dd, $\text{H}(1')$ of dAp), 6.26 (1H, dd, $\text{H}(1')$ of pdA), 7.88 (1H, s, $\text{H}(2)$ of dAp), 7.96 (1H, s, $\text{H}(2)$ of pdA), 8.02 (1H, s, $\text{H}(8)$ of dAp), 8.12 (1H, s, $\text{H}(8)$ of pdA). ^{31}P NMR (D_2O): δ 1.62 and 1.72. UV: λ_{max} 257 nm, A_{250}/A_{260} 0.83, A_{280}/A_{260} 0.10.

$d(\text{Ap}(\text{OMe})\text{Ap}(\text{OMe})\text{A})$

The sequence of coupling with the amidite, oxidation and detritylation was repeated to add another residue. Coupling gave: ^{31}P NMR (CD_3CN): δ 146.34 and 145.68 (ratio 1.17:1) for the phosphite linkage. Oxidation: ^{31}P NMR (CD_3CN): δ 4.43 and 4.33. After detritylation, the free 5'-hydroxyl group was acetylated as described for the starting compound. The N(6)-protecting groups were then removed using the procedure described for the dinucleotide. The product was isolated using two-dimensional preparative thin-layer chromatography, with dichloromethane/methanol (70/30 v/v) as eluent (R_f 0.12). ^{31}P NMR (D_2O): δ 3.87. UV: λ_{max} 257 nm, A_{250}/A_{260} 0.94, A_{280}/A_{260} 0.12. The ^1H NMR spectrum is too crowded to allow a complete assignment.

$d[\text{Ap}(\text{OMe})]_3\text{A}$

After another coupling protocol, the tetranucleotide was obtained using two-dimensional thin layer chromatography in a minute quantity, with the result that no NMR data are available (R_f 0.07, eluent dichloromethane/methanol 70/30 v/v). UV: λ_{max} 260 nm, A_{250}/A_{260} 0.83, A_{280}/A_{260} 0.16.

$d(\text{Cp}(\text{OMe})\text{C})$ and $d(\text{Cp}(\text{OMe})\text{Cp}(\text{OMe})\text{C})$

4-N-[(9-Fluorenyl)methoxycarbonyl]-2'-deoxycytidine. 2'-Deoxycytidine (4.54 g, 20 mmol) was dried by coevaporation with dry pyridine and subsequently suspended in 100 ml of dry pyridine. 13.0 ml (100 mmol) of trimethylchlorosilane was added dropwise and the mixture was stirred for 15 min⁴¹. 5.8 g (22 mmol) of (9-fluorenyl)methoxycarbonyl chloride was then added and the reaction mixture was stirred for 2 h at room temperature, yielding a white suspension in the yellow solution. Hydrolysis of the trimethylsilyl groups and unreacted chlorides was effected by addition of 20 ml water. After stirring for 15 min, a clear yellow solution was obtained, which was evaporated to near dryness by coevaporation with toluene. Upon addition of 300 ml water to the resulting oil, a white precipitate appeared. The mixture was shaken vigorously until no more yellow oil was visible. After addition of 150 ml of ethyl acetate and shaking, a precipitate formed on the separation layer, which was isolated by filtration and washed with ethyl acetate. After drying *in vacuo*, the product was obtained as a white solid (8.71 g, 97%). ^1H NMR (acetone- d_6): δ 2.40 (1H, m, $\text{H}(2'')$), 2.59 (1H, m, $\text{H}(2'')$), 3.77 (2H, m, $\text{H}(5'')/\text{H}(5')$), 4.03 (1H, m, $\text{H}(4')$), 4.33 (1H, t, CH Fmoc), 4.49

(3H, m, $\text{H}(3'')/\text{CH}_2$ Fmoc), 6.23 (1H, t, $\text{H}(1'')$), 7.12 (1H, d, $\text{H}(5)$), 7.37 (4H, m, aromatic Fmoc), 7.85 (4H, m, aromatic Fmoc), 8.50 (1H, d, $\text{H}(6)$).

5'-O-(4-Methoxytrityl)-4-N-[(9-fluorenyl)methoxycarbonyl]-2'-deoxycytidine. Base-protected 2'-deoxycytidine (8.55 g, 19 mmol) was dried by coevaporation with dry pyridine and subsequently suspended in 200 ml of dry pyridine. After addition of 6.5 g (21 mmol) of 4-methoxytrityl chloride, the solution was stirred for 15 h. The mixture was then poured into a saturated aqueous sodium bicarbonate solution (250 ml) and extracted three times with dichloromethane. The combined organic layers were washed with a saturated aqueous sodium bicarbonate solution and dried over magnesium sulfate. After filtration, the solution was concentrated *in vacuo*. Removal of all pyridine from the brownish oil was accomplished by coevaporation with toluene (twice) and 2-butanone. The resulting brown foam was purified by column chromatography on Woelm silica gel using 2-butanone as eluent (R_f 0.37), yielding 8.90 g (65%) white solid product. ^1H NMR (CDCl_3): δ 2.25 (1H, m, $\text{H}(2'')$), 2.71 (1H, m, $\text{H}(2'')$), 3.47 (2H, m, $\text{H}(5'')/\text{H}(5')$), 3.79 (3H, s, OCH_3 trityl), 4.14 (1H, m, $\text{H}(4')$), 4.29 (1H, t, CH Fmoc), 4.50 (3H, m, $\text{H}(3'')/\text{CH}_2$ Fmoc), 6.28 (1H, t, $\text{H}(1'')$), 6.85 (2H, m, trityl), 6.99 (1H, d, $\text{H}(5)$), 7.31 (12H, m, trityl), 7.41 (4H, m, aromatic Fmoc), 7.69 (4H, m, aromatic Fmoc), 8.22 (1H, d, $\text{H}(6)$).

Starting compound 3'-O-acetyl-4-N-[(9-fluorenyl)methoxycarbonyl]-2'-deoxycytidine. The 5'-tritylated, base-protected 2'-deoxycytidine (5.00 g, 7 mmol) was stirred for 15 min in a mixture of 50 ml of dry pyridine and 5 ml of acetic anhydride. The mixture was then poured into 50 ml of a saturated aqueous sodium bicarbonate solution and extracted twice with dichloromethane. The combined organic layers were dried over magnesium sulfate. After filtration, the solution was concentrated *in vacuo*. The resulting oil was purified by column chromatography on Woelm silica gel using 2-butanone as eluent (R_f 0.65), yielding a white solid. This was stirred for 15 h in a mixture of 160 ml acetic acid and 40 ml water. After addition of a further 120 ml water, the reaction mixture was concentrated *in vacuo* to near dryness. The excess acetic acid and water were removed by coevaporation with 2-butanone. Crystallization from 2-butanone yielded the product as a white solid (2.04 g, 57%). ^1H NMR ($\text{CDCl}_3/\text{CH}_3\text{OD}$ 9/1 v/v): δ 2.12 (3H, s, CH_3 acetyl), 2.27 (1H, m, $\text{H}(2'')$), 2.66 (1H, m, $\text{H}(2'')$), 3.86 (2H, m, $\text{H}(5'')/\text{H}(5')$), 4.20 (1H, m, $\text{H}(4')$), 4.31 (1H, t, CH Fmoc), 4.55 (2H, d, CH_2 Fmoc), 5.33 (1H, m, $\text{H}(3'')$), 6.29 (1H, t, $\text{H}(1'')$), 7.36 (5H, m, $\text{H}(5)$ /aromatic Fmoc), 7.74 (4H, m, aromatic Fmoc), 8.39 (1H, d, $\text{H}(6)$).

Bis(diisopropylamino)methoxyphosphine. 139.8 g (1.02 mol) phosphorus trichloride was cooled to -8°C and 32.53 g (1.02 mol) dry methanol was added dropwise over 1 h with stirring. The hydrochloric acid produced was absorbed in a gas trap containing water. Pure methoxydichlorophosphine was obtained by distillation at 100 mmHg (b.p. 35°C). ^{31}P NMR (CDCl_3): δ 181.5. A solution of diisopropylamine (162.1 g, 1.6 mol) in 500 ml dry diethyl ether was cooled to 0°C and a solution of methoxydichlorophosphine (53.37 g, 0.4 mol) in 20 ml dry diethyl ether was added dropwise over 1 h. The ammonium salt was then removed by filtration and the solution was concentrated *in vacuo*. Pure bis(diisopropylamino)methoxyphosphine was obtained by distillation of the residue at 1 mmHg (b.p. 115°C) in a yield of 20.9 g (20%). ^1H NMR (CDCl_3): δ 1.20 (24H, m, CH_3), 3.35 (3H, d, POCH_3 , J 11.2 Hz), 3.52 (4H, m, CH). ^{31}P NMR (CDCl_3): δ 131.7.

Coupling reaction, oxidation and detritylation to form 3'-O-acetyl-4-N-[(9-fluorenyl)methoxycarbonyl]-2'-deoxycytidine-5' 4-N-[(9-fluorenyl)methoxycarbonyl]-2'-deoxycytidine-3' methyl phosphate. 2.00 g (2.8 mmol) of 5'-tritylated, base-protected 2'-deoxycytidine was dried by coevaporation with dry pyridine and subsequently dissolved in 20 ml of dry pyridine. 0.10 g (1.4 mmol) of 1H-tetrazole and 0.86 g (3.3 mmol) bis(diisopropylamino)methoxyphosphine (dried by coevaporation with dry pyridine) were added and the reaction mixture was stirred at room temperature for 15 min. Formation of the phosphoramidite coupling compound *in situ* was evident from the ^{31}P NMR spectrum (CDCl_3 : δ 149.5 and 150.2). 1.65 g (3.4 mmol) of the starting compound 3'-O-acetyl-4-N-[(9-fluorenyl)methoxycarbonyl]-2'-deoxycytidine and 0.50 g (7 mmol) 1H-tetrazole (both dried by coevaporation with dry pyridine) were then added and the reaction mixture was stirred at room temperature for a further 2 h. The phosphite [^{31}P NMR (CDCl_3): δ 140.4

and 140.8] thus obtained was oxidized by addition of 1.5 ml of *tert*-butyl hydroperoxide⁴² to give the phosphate [³¹P NMR (CDCl₃): -0.33 and -0.44]. After stirring for 5 min, the mixture was concentrated *in vacuo* to near dryness and coevaporated twice with toluene and 2-butanone. The product was ditritylated by addition of a mixture of 100 ml acetic acid and 25 ml water. After stirring for 15 h, a further 75 ml water was added and the mixture was concentrated *in vacuo* to near dryness. The excess acetic acid and water were removed by coevaporation with 2-butanone and ethyl acetate. The product was purified by column chromatography on Woelm silica gel. The impurities were first eluted with ethyl acetate/dichloromethane (9/1 v/v); then the product was obtained using ethyl acetate/dichloromethane/ethanol (85/10/5 v/v/v) as eluent (*R_f* 0.25) with a yield of 1.25 g (44%). ¹H NMR (CDCl₃): δ 2.10 (3H, s, CH₃ acetyl), 2.43 (2H, m, H(2') of dCp and pdC), 2.77 (2H, m, H(2'') of dCp and pdC), 3.84 (3H, d, POCH₃, *J* 11.0 Hz), 3.91 (2H, m, H(5')/H(5'') of dCp), 4.2–4.5 (10H, m, H(4') of dCp/H(4'')/H(5')/H(5'') of pdC/CH/CH₂ Fmoc), 5.29 (2H, m, H(3') of dCp and pdC), 6.24 (2H, m, H(1') of dCp and pdC), 7.30 (10H, m, H(5) of dCp and pdC/aromatic Fmoc), 7.53 (4H, m, aromatic Fmoc), 7.77 (4H, m, aromatic Fmoc), 8.07 (1H, d, H(6) of dCp), 8.20 (1H, d, H(6) of pdC). ³¹P NMR (CDCl₃): δ -0.35 and 0.07 ppm.

d(Cp(OMe)C)

The base-protecting Fmoc groups were removed^{15b} by treatment with a mixture of triethylamine (20 mmol/mmol Fmoc) and of pyridine (10 ml/mmol Fmoc). After *ca.* 3 h, thin-layer chromatography showed the reaction to be complete. The reaction mixture was concentrated *in vacuo* and the excess pyridine and triethylamine were removed by coevaporating three times with toluene. Extraction of an aqueous solution of the residue with dichloromethane removed virtually all the split-off fluorene groups. ¹H NMR (D₂O): δ 2.06 (3H, s, CH₃ acetyl), 2.30 (2H, m, H(2') of dCp and pdC), 2.56 (2H, m, H(2'') of dCp and pdC), 3.70 (2H, m, H(5')/H(5'') of dCp), 3.78 (3H, d, POCH₃, *J* 11.4 Hz), 4.20 (1H, m, H(4') of dCp), 4.32 (3H, m, H(4'')/H(5')/H(5'') of pdC), 4.96 (1H, m, H(3') of dCp), 5.28 (1H, m, H(3'') of pdC), 5.98 (1H, d, H(5) of dCp), 5.99 (1H, d, H(5) of pdC), 6.15 (1H, dd, H(1') of dCp), 6.18 (1H, dd, H(1') of pdC), 7.69 (1H, d, H(6) of dCp), 7.73 (1H, d, H(6) of pdC), ³¹P NMR (D₂O): 1.96 (*S_p*) and 2.07 (*R_p*).

d(Cp(OMe)Cp(OMe)C)

The sequence of coupling with the amidite, oxidation and detritylation was repeated to add another residue. Coupling gave: ³¹P NMR (CDCl₃): δ 140.7, 0.65 and -0.40. Oxidation: ³¹P NMR (CDCl₃): δ -0.02, -0.05, -1.00 and -2.00. After detritylation, the product was purified by column chromatography on Woelm silica gel using chloroform/methanol (9/1 v/v) as eluent (*R_f* 0.11). ³¹P NMR (CDCl₃): δ -0.13 and -0.27. The base-protecting Fmoc groups were removed by treatment with pyridine and triethylamine^{15b}. Evaporation of the pyridine yielded a crude product, which was purified by extraction of an aqueous solution with dichloromethane. The ¹H NMR spectrum was too crowded to allow complete assignment. ³¹P NMR (D₂O): δ 1.9–2.3 and 3.2. UV: λ_{max} 268 nm, A₂₅₀/A₂₇₀ 0.76, A₂₉₀/A₂₇₀ 0.26.

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