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Parallel-Stranded Oligonucleotide Duplexes Containing 5-Methylisocytosine-Guanine and Isoguanine-Cytosine Base Pairs

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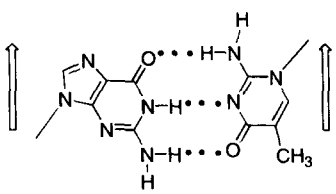
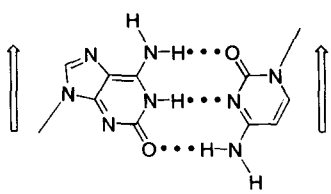
Abstract: Parallel-stranded oligonucleotides containing 5-methylisocytosine-guanine and/or isoguanine-cytosine base pairs were prepared. The coupling efficiency was higher than 99% when the phosphoramidites of 2'-deoxy-5-methylisocytidine (**2a**) and of 2'-deoxyisoguanosine (**3**) were employed in solid-phase synthesis. The glycosylic bond of 2'-deoxy-5-methylisocytidine (**1a**) is stabilized against acid in the case of the amidine derivatives **4a, b**. Depyrimidination occurs upon extended exposure to ammonia. The orientation of the parallel-stranded (ps) duplexes was unambiguously determined using the pyrene excimer fluorescence. The antiparallel (aps) duplex $d(A_{12})•d(T_{12})$ (**25•26**) turned into the parallel orientation when two central adenine-thymine base pairs were replaced by isoguanine-cytosine pairs. The thermal stability of ps-duplexes containing 5-methylisocytosine-guanine base pairs was found to be significantly lower than that of duplexes with isoguanine-cytosine pairs. According to the CD spectra, ps-DNA-DNA or DNA-RNA hybrids show differences in their secondary structures compared to their aps counterparts.

INTRODUCTION

Parallel-stranded DNA is formed when the guanine-cytosine base pairs of a nucleic acid duplex are replaced by guanine-isocytosine (or 5-methylisocytosine) and/or cytosine-isoguanine pairs (Motifs I and II).[1] As the adenine-thymine base pair is ambiguous with regard to the chain orientation, any single-stranded DNA or RNA can be hybridized with a base-modified oligonucleotide containing the 4 bases: adenine, thymine (uracil), isoguanine and isocytosine, in a parallel mode.[2] Although a number of oligonucleotides forming these duplexes have been synthesized in our laboratory, an unambiguous proof of the chain orientation of such parallel duplexes has not yet been made. Only recently an NMR investigation was performed on a particular duplex structure with parallel chain orientation.[3]



Guanine - 5-Methylisocytosine (ps)
Motif I



Isoguanine - Cytosine (ps)
Motif II

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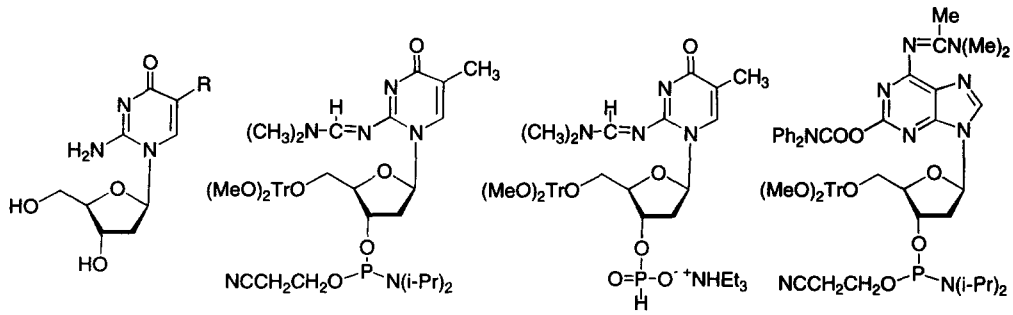
PII: S0040-4020(99)00511-6

This manuscript reports on parallel-stranded oligonucleotides containing the base pairs I and/or II. Furthermore, it describes experiments on oligomers labelled at their 5'-sites with a pyrene moiety. This moiety shows excimer fluorescence when two of those residues are in close proximity. This property [4-7] will be used to determine the chain orientation of the duplex **11•12** which is used in our laboratory as a reference compound for parallel stranded DNA.[8, 9] The oligonucleotides were prepared employing the base-modified phosphoramidites **2a** and **3** as well as the phosphonate **2b** and the phosphonate of 2'-deoxyisoguanosine. Apart from this work the stability of the 5-methylisocytosine-guanine base pair (I) vs that of isoguanine-cytosine (II) will be investigated and the thermodynamic data will be determined. Furthermore, investigations are undertaken resulting from the low glycosylic bond stability of 2'-deoxy-5-methylisocytidine (m^5iC_d) against acid and base which are directed on the selection of the most suitable protecting group.



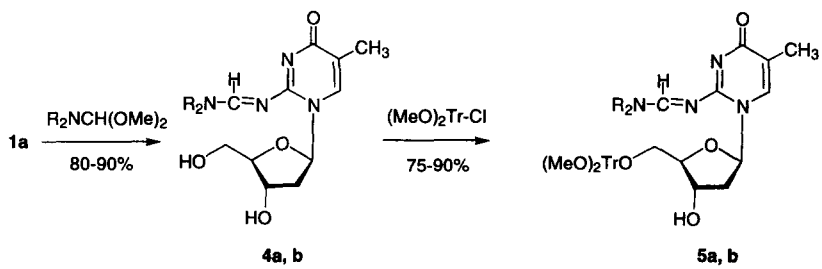
1a R = CH_3 **b** R = H **2a** **2b** **3**

RESULTS AND DISCUSSION

Monomers

It has been reported that 2'-deoxyisocytidine (**1b**) is a rather labile nucleoside. Therefore, the 2'-deoxy-5-methylisocytidine (**1a**) has been used as a substitute.[10] The 5-methyl group reduces the lability against base.[11, 12] However, a lower stability against acid is now observed.[11] The synthesis of 2'-deoxy-5-methylisocytidine (**1a**) has been described.[12] The starting material thymidine was converted into the 2,5'-anhydro derivative[13] which was treated with ammonia in MeOH to give compound **1a**.

Scheme 1



4a, b **5a, b**

a R = Me; **b** R = n-Bu

Regarding the amino group protection the literature reports on the accelerated alkaline deamination of the acylated 2'-deoxy-5-methylisocytidine.[14, 15] Therefore, amidine residues were chosen for the amino group

protection of **1a**.[10, 16, 17] As the rather low glycosylic bond stability of 2'-deoxyisoguanosine as well as of 2'-deoxy-5-methylisocytidine is a problem during the synthesis of parallel DNA (acidic conditions are neccessary for the detritylation), the influence of the amidine groups on the hydrolytic stability of the nucleoside (**1a**) was compared. Two different amidines were selected, the dimethylaminomethylidene (dmf) derivative **4a** and the di(n-butyl)aminomethylidene (dbf) derivative **4b**. They were prepared in methanolic solution and were isolated in more than 80% yield (Scheme 1).

Prior to the hydrolysis of the amidines, the stability of the nucleoside **1a** was investigated. Compound **1a** was hydrolyzed in 0.1 M HCl at 40°C and the reaction was followed by reverse-phase HPLC (conditions see Experimental Part). Fig. 1a shows the HPLC profile of samples taken after 15 min. Two peaks were detected at 15 min. The one with the faster mobility was identified as that of the base 5-methylisocytosine (**6**, m^5iCyt), and the other one as the free nucleoside **1a** (m^5iC_d). After 90 min, the nucleoside was completely hydrolyzed to 5-methylisocytosine. The half-life was determined to be 17 min under those conditions (Table 1). Moreover, according to the kinetic measurement, the hydrolysis of **1a** follows a first order kinetics and is pH independent between 0.5-0.001 M aq. HCl. Compared to m^5iC_d (**1a**), the other nucleoside (iG_d), which was necessary to form parallel duplexes, is hydrolyzed much faster and pH dependent. Fig. 1b shows that after 15 min of treatment nearly all the iG_d is hydrolyzed to give the free base. Compared to 2'-deoxyadenosine, which is the most acid-labile constituent of natural DNA (depurination), the stability of the glycosylic bond of **1a** is similar to that of 2'-deoxyadenosine under the stronger acidic condition (0.1 M HCl). However, **1a** is even much more acid labile than 2'-deoxyadenosine in an aq. HCl concentration below 0.01 M with the result of depyrimidination. This is shown in Fig. 1c, d, which present data of the hydrolysis after 90 min at 40°C in 0.01 M aq. HCl. In the case of 0.001 M aq. HCl the situation is the same. Also 2'-deoxyisoguanosine is more labile than 2'-deoxyadenosine in diluted acid (0.01 M HCl) which is indicated by Fig. 1e. Kinetic data of 2'-deoxyisoguanosine hydrolysis have been determined earlier.[18] Compared to 2'-deoxy-5-methylisocytidine, the 2'-deoxycytidine is a rather stable compound and is not significantly hydrolyzed in 0.1 M aq. HCl (data not shown).

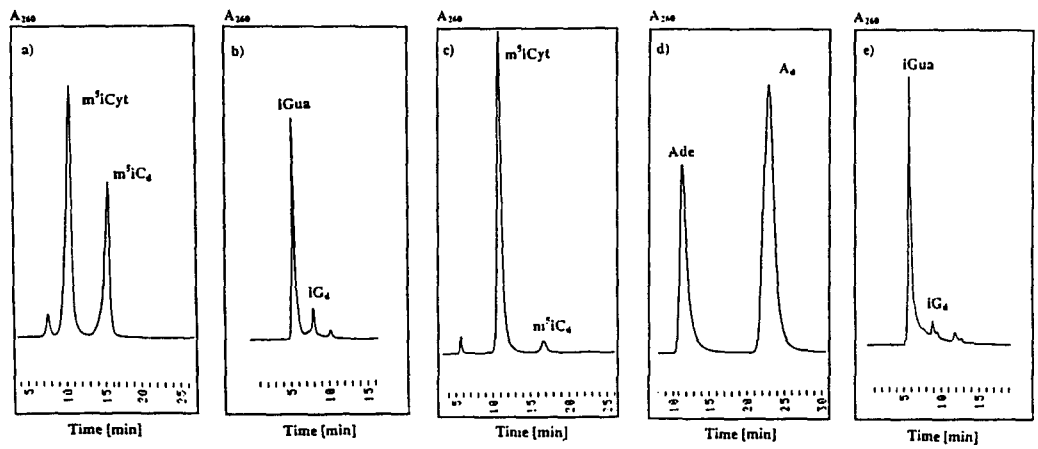
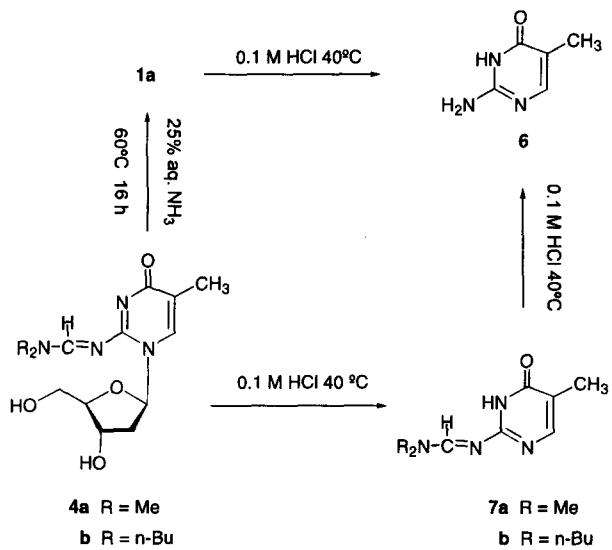


Fig. 1. HPLC profiles of the hydrolysis products (0.1 M HCl, 40°C) of **1a** and 2'-deoxyisoguanosine. a) Compound **1a** (15 min): 2% MeCN in 0.1 M $(Et_3NH)OAc$, pH 7.0, 1.0 mL/min; b) 2'-deoxyisoguanosine (15 min): 5% MeCN in 0.1 M $(Et_3NH)OAC$, pH 7.0, 0.6 mL/min. Hydrolysis (0.01 M HCl, 40°C) of c) compound **1a** (90 min): gradient see a); d) 2'-deoxyadenosine (90 min): gradient see b); e) 2'-deoxyisoguanosine (90 min): gradient see b).

The same experiments as performed with compound **1a** were carried out with the amidines **4a** and **4b**. In the case of **4a** three peaks were detected after 15 min of hydrolysis in 0.1 M aq. HCl at 40°C, which represent the free base **6** (m^5iCyt), the protected nucleoside **4a** ($dmf^2m^5iC_d$) and the protected base **7a** (dmf^2m^5iCyt), respectively (Fig. 2a). As the starting material **4a** was still present even after 90 min of hydrolysis (Fig. 2b) it is obvious that the glycosylic bond of the protected nucleoside **4a** is stabilized by the amidine function. The experiments were also performed with the di(n-butyl)formamidine **4b**. The hydrolysis of **4b** in 0.1 M aq. HCl gives only two peaks in the HPLC profile, the protected nucleoside **4b** ($dbf^2m^5iC_d$) and the protected base **7b** (dbf^2m^5iCyt) (Fig. 2c, d). Compared to compound **4a**, the di(n-butyl)aminomethylidene (dbf) group is much more stable against acid. However, the dbf group is also cleaved when the exposure is prolonged under acidic conditions, which also leads to the unprotected base as in the dimethylformamidine case. The process is illustrated in Scheme 2.

Scheme 2



When the hydrolysis was followed spectrophotometrically at 285 nm the glycosylic bond hydrolysis of compound **4b** can be determined without a significant cleavage of the protecting group. In the case of the dimethylamidine derivative **4a**, a partial removal of the protecting group was observed. Therefore, the kinetics determined UV spectrophotometrically at 285 nm results in a half-life (Table 1) which relates to the N-glycosylic bond hydrolysis in the case of **4b**; the half-life of **4a** describes N-glycosylic bond hydrolysis and to a small extent also the removal of the protecting group. Regarding the glycosylic bond stability, the di(n-butyl)aminomethylidene derivative **4b** should be selected from this point of view. These results and the corresponding finding of Benner[19] who has investigated the diisobutylformamidine derivative under conditions of DMT removal are in fairly good agreement. However, this group shows drawbacks upon storing under alkaline condition (see next section).

Table 1. Half-life values of 2'-deoxy-5-methylisocytidine and derivatives under acidic (0.1 M HCl) and alkaline (25% aq. ammonia) conditions, measured at 40°C.

Compd.	Half-life (τ) [min]		
	0.1 M [a])	0.5 M [a])	25% NH_3 [b])
m^5iC_d (**1a**)	17	17	[d])
$dmf^2m^5iC_d$ (**4a**)	28 [c])		7
$dbf^2m^5iC_d$ (**4b**)	83		176

[a]) Hydrolysis in aq. HCl was followed UV-spectrophotometrically at 242 nm for **1a** and 285 nm for **4a** and **4b**; the half-lives refer to the glycosylic bond cleavage. [b]) Hydrolysis in conc. aq. NH_3 was followed at 285 nm for **4a** and **4b**, the half-lives refer to the cleavage of the protecting groups. [c]) Due to protecting group removal the value might be slightly higher. [d]) Stable within 16 h at 60°C.

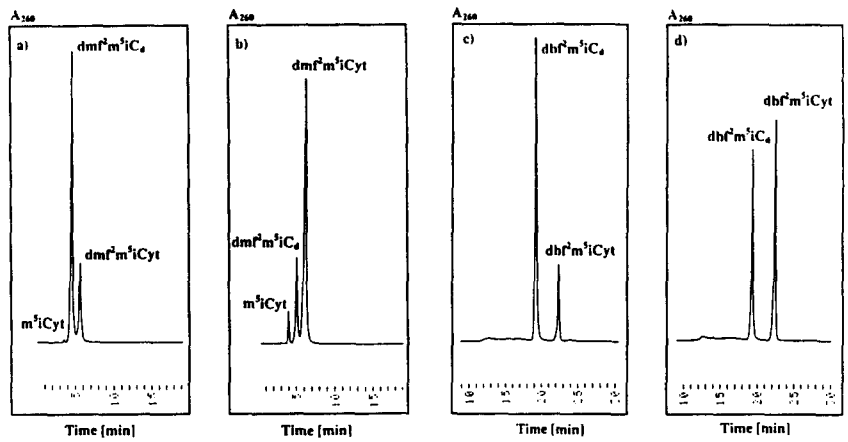


Fig. 2. HPLC profiles of the hydrolysis products (0.1 M HCl, 40°C) of **4a** and **4b** after 15 and 90 min of hydrolysis. a) Compound **4a** (15 min): 15% MeCN in 0.1 M $(Et_3NH)OAc$, pH 7.0, 0.8 mL/min; b) compound **4a** (90 min): gradient see a); c) compound **4b** (15 min): MeCN (A) and 0.1 M $(Et_3NH)OAC$, pH 7.0 (B), gradient: 5 min 5% A in B, 15 min 5-65% A in B, 5 min 65-5% A in B, 1 mL/min; d) compound **4b** (90 min): gradient see c).

Next, similar experiments as described for the hydrolysis in acidic solution were performed under alkaline conditions. In the case of **1a**, the formation of thymidine or the liberation of 5-methylisocytosine (**6**) was not observed when compound **1a** was stored in 25% aq. NH_3 solution at 60°C for not more than 16 h. However. a 20% formation of 5-methylisocytosine (**6**) was detected when the hydrolysis was prolonged to 72 h. This indicates that the glycosylic bond is not significantly cleaved in alkaline medium when deprotection conditions are chosen which do not last longer than 16 h at 60°C. According to this, the time of exposure of oligonucleotides containing 2'-deoxy-5-methylisocytidine to aq. NH_3 solution has to be limited. As the time of exposure to aq. NH_3 depends on the stability of the protecting groups, the half-life of the deprotection was measured for the

amidines **4a** and **4b**. The kinetics were followed by HPLC. It was observed that **4a** undergoes the deprotection via an intermediate, most likely a formyl derivative (Fig. 3a, b). However, there is no intermediate formed during the deprotection of compound **4b** (Fig. 3c, d). Only the deprotected nucleoside **1a** is formed within 16 h in both cases. Furthermore, it was observed that an increase of the time of treatment in 25% aq. NH_3 soln. at 60°C (72 h) results in the formation of about 20% of 5-methylisocytosine (**6**) (Fig. 3e). The deprotection in 25% aq. NH_3 can be also followed UV-spectrophotometrically (Table 1). Compound **4a** gave a half-life value of 7 min for the deprotection whereas the half-life of the di(n-butyl)formamidine **4b** was much longer (176 min). A significant formation of thymidine (deamination) was not detected. Consequently, the dimethylaminomethylidene derivative **4a** is suitable to be used in oligonucleotide synthesis, whereas the di(n-butyl)aminomethylidene residue is too stable considering a period of 10-fold half-lives for the complete deprotection of an oligonucleotide.

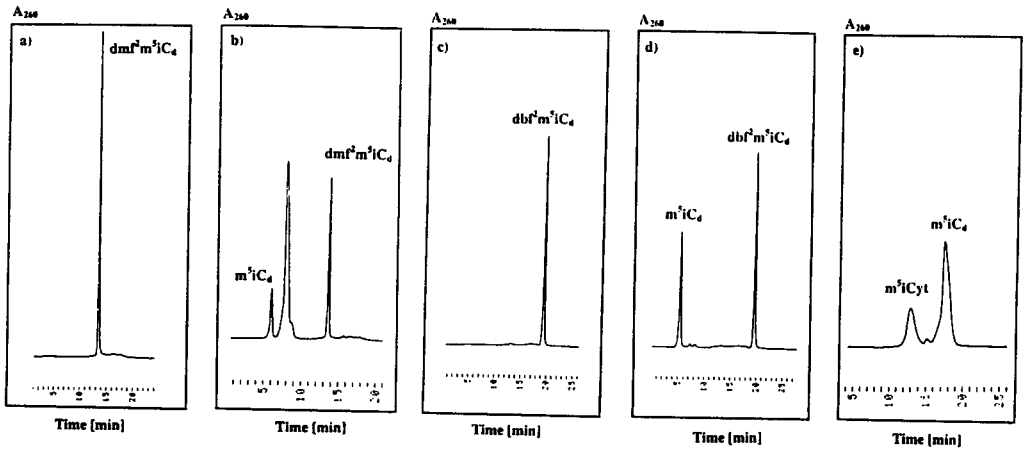


Fig. 3. HPLC profiles of **4a** and **4b** in water (3a,c). MeCN (A) and 0.1 M $(Et_3NH)OAc$, pH 7.0 (B), gradient: 5 min 5% A in B, 15 min 5-65% A in B, 5 min 65-5% A in B, 1 mL/min. Profiles of **4a** and **4b** in 25% aq. NH_3 solution at 60°C (3b,d,e). b) Compound **4a** (5 min of hydrolysis): gradient see a); d) compound **4b** (75 min): gradient see a); e) compound **4b** (72 h): 2 % MeCN in 0.1 M $(Et_3NH)OAc$, pH 7.0, 1 mL/min.

Then, compounds **4a** and **4b** were converted into the 5'-*O*-dimethoxytrityl derivatives **5a** (74%) and **5b** (89%) using standard conditions (Scheme 1). The phosphoramidite **2a** was prepared from compound **5a** (75%). Alternatively, the phosphonate **2b** was synthesized by the reaction of **5a** with PCl_3/*N*-methylmorpholine/1,2,4-triazole in CH_2Cl_2. Both compounds were purified chromatographically and isolated as solid foams. The building blocks, **2a**, **b**, **3** and the phosphonate of 2'-deoxyisoguanosine[18] were employed in the solid-phase oligonucleotide synthesis. For the synthesis of oligonucleotides with 3'-terminal 2'-deoxyisoguanosine (iG_d) the nucleoside was immobilized on amino-functionalized Fractosil. For this purpose compound **8** was succinylated giving compound **9** (88%). The latter was activated *via* the 4-nitrophenyl ester and linked to amino-functionalized Fractosil furnishing the polymer support **10** (Scheme 3). The ligand concentration was found to be 70 μmol/g of solid support.

Scheme 3

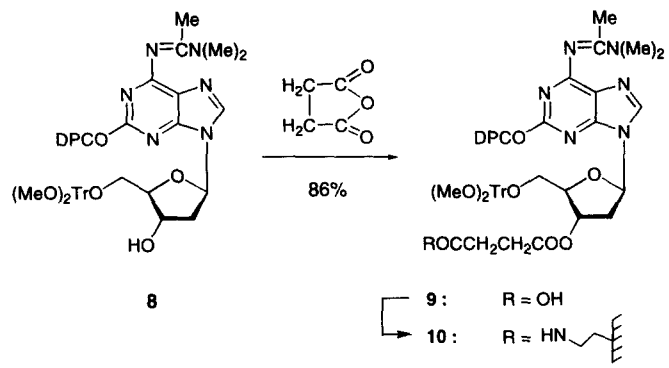


Table 2. ^{13}C NMR chemical shifts of 2'-deoxy-5-methylisocytidine and derivatives in (D_6)DMSO at 25°C.

	C(2)	C(4)	C(5)	C(6)	Me	C=N	Me_2N	C(1')	C(2')	C(3')	C(4')	C(5')	MeO
1a [a])	154.0	170.3	114.2	133.9	13.5			88.0	[b])	69.8	86.7	60.7	
4a [a])	156.9	170.5	116.2	133.8	13.8	158.0	35.3	85.5	[b])	70.3	87.3	61.3	
4b	156.8	170.5	116.1	133.6	13.5	157.5		85.7	[b])	70.0	87.3	61.1	
5a [a])	157.1	170.3	116.4	133.9	13.3	158.4	34.8	85.5	[b])	70.4	84.9	63.6	55.0
5b	156.8	170.4	116.3	133.2	13.4	158.1		85.8	40.8	70.2	85.6	63.4	55.0
7a	157.9	163.7	115.1	151.5	12.5	158.4	34.5						
7b	157.5	163.7	115.1	151.5	12.5	158.5							
2b	157.0	170.3	116.4	133.2	13.2	158.1	34.8	85.9	40.7	72.1	84.6	63.3	55.0

[a]) Assigned from gated-decoupled spectra. [b]) Superimposed by DMSO.

The compounds described above were characterized by ^{13}C (Table 2), ^{1}H NMR and UV spectra as well as elemental analysis (see Experimental Part). The carbon chemical shifts were assigned according to gated-decoupled ^{13}C NMR spectra. When the exocyclic amino group was protected by an amidine group, the chemical shift of C(2) of the heterocycle is shifted downfield (about 3 ppm). Also the chemical shift of C(4) of the protected bases **7a, b** is about 7 ppm shifted upfield compared with that of the protected nucleosides **4a, b**. The chemical shift of C(6) of the protected bases **7a** and **7b** is about 18 ppm shifted downfield. These differences are due to a N(3)-H tautomeric structure of the bases whereas the nucleosides represent the N(1)-H tautomeric state due to the attachment position of the deoxy-β-D-ribofuranosyl moiety.

Oligonucleotides

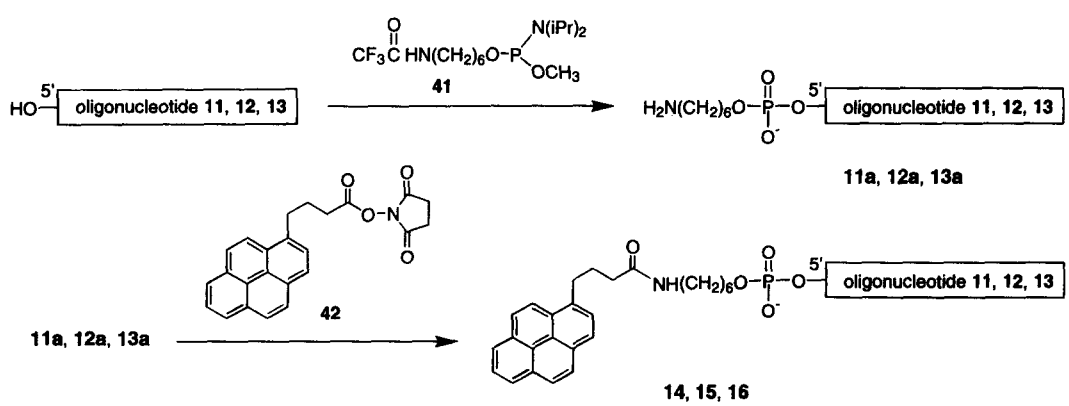
Synthesis. The oligonucleotide synthesis was performed on an ABI 392-08 synthesizer (phosphoramidite chemistry) or an ABI 380B synthesizer (H-phosphonate chemistry) using standard conditions. Compounds **2a** or **2b** were used for the incorporation of 2'-deoxy-5-methylisocytidine and 2'-deoxyisoguanosine residues were incorporated into oligonucleotides using the **3** or phosphonate of 2'-deoxyisoguanosine. The phosphoramidites

2a and **3** give the same coupling yields as the regular phosphoramidites (99%) during solid-phase synthesis. Also no prolonged coupling time[19] is required.[20] The oligonucleotides were deprotected in concentrated ammonia solution at 60°C for 16 h and purified by RP-HPLC. The nucleoside composition of oligonucleotides was determined by enzymatic hydrolysis using snake-venom phosphodiesterase followed by alkaline phosphatase and analyzed by RP-HPLC.

Determination of the chain orientation by pyrene monomer-excimer fluorescence. Earlier, the chain orientation of parallel duplexes has been studied on oligonucleotides containing adenine-thymine base pairs.[21] In this case the fluorescence resonance energy transfer (FRET) employing fluorescein as donor and tetramethylrhodamin as acceptor was used. A parallel duplex structure has been also determined for the self-complementary purine duplex $d(G\text{-}A)_{15}$ using the pyrene fluorophore as a structural probe.[7] The pyrene residues show excimer fluorescence when two pyrene moieties come into close proximity. The excited state dimer (excimer) shows a broad fluorescence around 430-600 nm being separated from the emission of the monomer (381, 398 nm). The pyrene moieties do not interact in the ground state, therefore, they do not influence the monomer-duplex equilibrium. In order to investigate the chain orientation of the duplex **11•12**, the oligonucleotides **14-16** (Table 3) were synthesized which carry a pyrene label at the 5'-site (Scheme 4). The synthesis of these compounds was performed by solid-phase chemistry by the use of the phosphoramidite of aminolink 2 (**41**) coupled to oligonucleotides **11-13** in the last coupling step. Regarding the work up of the compounds **11a-13a** see Experimental Part. The amino function of oligonucleotides was then derivatized with succinimidyl-1-pyrenebutyrate (**42**) furnishing the pyrene-labelled oligomers **14-16** (Scheme 4). Purification of the oligonucleotides **14-16** was performed on reverse-phase HPLC (see Experimental part).

Next, fluorescence measurements of the oligonucleotides **14-16** were performed. The single strands of the oligomers **14**, **15**, and **16** show only the typical monomer fluorescence in the range of the pyrene residue (Fig. 4a). Also the antiparallel hybrid **15•16** (Fig. 4b) show a similar fluorescence, as an excimer cannot be formed. However, the excimer fluorescence can occur only on the parallel-stranded duplex **14•15** (Fig. 4b). Indeed, this duplex having the labels in close proximity shows excimer fluorescence. This confirms a parallel chain orientation for this particular duplex, as well as for related duplexes containing 5-methylisocytosine-guanine, isoguanine-cytosine and adenine-thymine base pairs.

Scheme 4



11: 5'-d(TiCATAAiCTiGiGAT), **12**: 5'-d(AGTATTGACCTA), **13**: 5'-(TAGGTCAATACT) (see also Table 4)

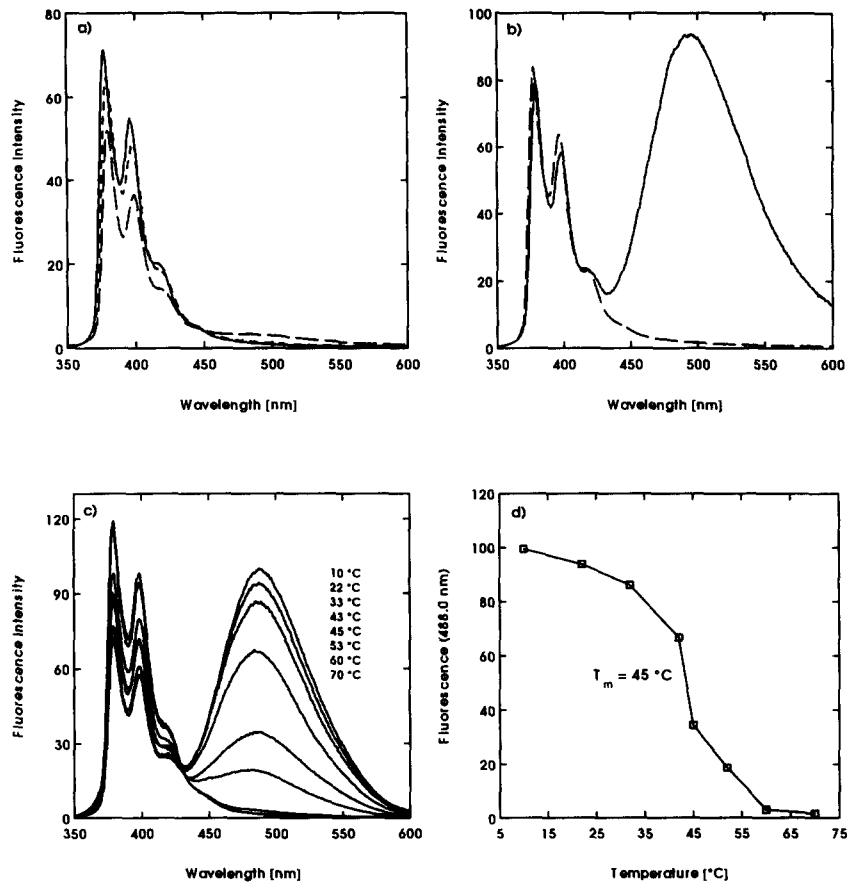


Fig. 4. Fluorescence emission spectra of pyrene-labelled oligonucleotides measured in 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Na-cacodylate buffer, pH 7.0. a) Single-stranded oligomers: **14** (long dashed line); **15** (solid line) and **16** (short dashed line) at 7°C; b) the aps-hybrid **15•16** (dashed line) and ps-hybrid **14•15** (solid line) at 7°C; c) the ps-hybrid **14•15** measured at various temperatures; d) temperature-dependent fluorescence intensities of the ps-hybrid **14•15** at 488 nm.

Moreover, the temperature-dependent fluorescence spectra were measured (Fig. 4c). The magnitude of the excimer fluorescence decreases with increasing temperature. A sigmoidal temperature-dependent fluorescence curve of ps-**14•15** is observed leading to a T_m-value of 45°C (Fig. 4d). The T_m-value obtained from UV-measurements is 47°C and that of the unlabelled duplex is 42°C. The T_m-value difference of 5°C between the duplexes **11•12** and **14•15** is due to the additional stacking interaction of pyrene residues. The T_m-values and thermodynamic data for pyrene labelled and unlabelled oligonucleotides with parallel or antiparallel chain orientation are shown in Table 3.

Table 3. T_m-values and thermodynamic data of oligonucleotide and pyrene-labelled derivatives.[a])

Duplexes [b]) [c])	T_m [°C]	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol·K]	$\Delta G°_{298}$ [kcal/mol]
5'-d(TiCATAAiCTiGiGAT) (**11**) 5'-d(A GTATT GA C C TA) (**12**)	42	-84	-240	-9.8
5'-P-d(TiCATAAiCTiGiGAT) (**14**) 5'-P-d(A GTATT GA C C TA) (**15**)	47	-74	-204	-10.7
3'-d(TCATAACTGGAT) (**13**) 5'-d(AGTATTGACCTA) (**12**)	47	-82	-230	-10.4
3'-d(TCATAACTGGAT)-P (**16**) 5'-P-d(AGTATT GACCTA) (**15**)	52	-82	-225	-12.3

[a])Measured UV-spectrophotometrically in 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Na-cacodylate buffer, pH 7.0. [b]) see Table 4. [c]) P: pyrene residue linked to the 5'-end of the oligomer.

Stability of the 5-methylisocytosine-guanine vs the isoguanine-cytosine base pair. Semiempirical calculation of the stability of the base pairs isoguanine-cytosine and 5-methylisocytosine-guanine were performed recently. It was reported that ΔH° for the 5-methylisocytosine-guanine base pair is 12.0 kcal/mol; the value for the isoguanine-cytosine pair is -13.7 kcal/mol.[2] However, no experimental data are available. In order to investigate this matter, the oligonucleotides **17-21** have been synthesized containing 5-methylisocytosine-guanine or isoguanine-cytosine base pairs but no adenine-thymine pairs. The T_m-values were determined in 1 M NaCl and the thermal data were determined by the shape analysis of the melting profiles using the program MeltWin.[22] The data of duplex melting are summarized in Table 4. A rather stable hybrid is formed when compound **17** is hybridized with **18** (Table 4). This hybrid is considered as a fully matched duplex with 6 5-methylisocytosine-guanine base pairs with parallel chain orientation. It surprises us that the T_m value of this duplex is 11°C lower than that of the parallel duplex **19•20** with 6 isoguanine-cytosine pairs. The decrease of the 5-methylisocytosine-guanine might be caused by the influence of the 5-methyl group and by better overlap of the nucleobases. The same T_m-value as found for the parallel duplex **17•18** was also found for the antiparallel duplex **21•21** containing 6 guanine-cytosine base pairs. Also oligonucleotides with alternating 5-methylisocytosine-guanine and isoguanine-cytosine pairs were investigated. These duplexes can form only 5 base pairs when arranged in a parallel manner. In this case the selfcomplementary duplex 5'-d(iCGiCGiCG) (**22•22**) shows also a lower T_m-value than that of the oligomer 5'-d(CiGCiGCiG) (**23•23**). However, the aps-duplex 5'-d(CGCGCG) (**24•24**) is much more stable than the ps one as one more base pair is present.

Regarding the ΔH° values *vs* the amount of the base pairs (one base pair at the end is excluded) a rough calculation can be made. From this calculation, the values of ΔH° per base pair are -9.8 kcal for 5-methylisocytosine-guanine, -12.8 kcal for isoguanine-cytosine and -9.2 kcal for guanine-cytosine. These data show the same tendency as that obtained by PM3 semiempirical calculations but are somewhat lower.[2] Apparently, the results indicate that an isoguanine-cytosine base pair is significantly more stable than a 5-

methylisocytosine-guanine pair. The latter can show a similar stability than that of guanine-cytosine formed in an antiparallel duplex (Table 4). However, as these oligonucleotide duplexes are autonomous molecules it is not surprising that the nearest neighbour influence within those parallel DNAs is different to that of a regular DNAs. This implicates that a base pair stability adjustment is possible in oligonucleotide duplexes with parallel chain orientation different from that with antiparallel chains.

Table 4. T_m-values and thermodynamic data of oligonucleotides. [a])

Duplexes [b])	T_m [°C]	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol·K]	$\Delta G°_{298}$ [kcal/mol]
5'-d(iCiCiC G G G) (**17**) 5'-d(G G G iCiCiC) (**18**)	35	-49	-132	-7.8
5'-d(C C C iGiGiG) (**19**) 5'-d(iGiGiG C C C) (**20**)	46	-64	-174	-9.8
5'-d(C C C G G G) (**21**) 3'-d(G G G C C C) (**21**)	35	-46	-125	-6.7
5'-d(iCGiC GiC G) (**22**) 5'-d(iCG iCG iCG) (**22**)	21	[c])	[c])	[c])
5'-d(CiG CiG CiG) (**23**) 5'-d(CiG CiG CiG) (**23**)	31	-39	-106	-6.3
5'-d(C G C G C G) (**24**) 3'-d(G C G C G C) (**24**)	44	-51	-139	-7.7

[a]) Measured UV-spectrophotometrically in 1 M NaCl, 100 mM $MgCl_2$, 60 mM Na-cacodylate (pH 7.0) buffer; the oligomer concentration is 9 μM. [b]) d(iC) = 2'-deoxy-5-methylisocytidine. [c]) Not calculated due to the imperfect melting curve.

In order to determine the strength of isoguanine-cytosine base pairs during the formation of parallel-stranded duplexes, the dA-dT base pair within the duplex formed by 5'd(T_{12}) (**25**) and 5'd(A_{12}) (**26**) was replaced stepwise by those of isoguanine-cytosine. The stability of the resulting duplexes was studied by T_m-measurements using temperature-dependent UV-absorbance at 260 nm. Data are shown in Table 5.

If only one adenine-thymine base pair was displaced by isoguanine-cytosine the T_m-value was reduced by 7°C (**25•26** → **27•28**). This indicates that isoguanine-cytosine destabilizes the duplex when both strands adopt an antiparallel chain orientation. However, when two adenine-thymine pairs were replaced by isoguanine-cytosine, the antiparallel chain was rearranged to a parallel orientation resulting in a 2°C increase of the T_m-value (**27•28** → **29•30**, Table 5). This demonstrates that the weak binding of a reverse Watson-Crick adenine-thymine base pair can be compensated by the reverse Watson-Crick isoguanine-cytosine base pair. A T_m-value increase of about 6°C is found for each additional base pair displacement (Table 5). Regarding the thermal data which were obtained by calculation based on the melting curves, it is apparent that the enthalpy is not favoured although the T_m value is increased. The loss of enthalpy is compensated by a more favourable entropy. There-

fore, the thermal stability of oligonucleotide duplexes is increased by the increase of isoguanine-cytosine base pairs vs adenine-thymine pair within duplexes showing parallel chain orientation (Table 5).

Table 5. T_m-values and thermodynamic data of homomeric ps- and aps-duplexes. [a])

Duplexes		T_m [°C]	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol·K]	$\Delta G°_{298}$ [kcal/mol]	h(%) [260nm]
5'd(T TT TTT TT TTTT) (**25**) 3'd(AAAAAAAAAAAA) (**26**)	aps	44	-84	-238	-9.8	22
5'd(TTTTTiGT TT TTT) (**27**) 3'd(AAAAACAAAAAA) (**28**)	aps	37	-74	-212	-8.1	26
5'd(TTTT TiGiGTTTTT) (**29**) 5'd(AAAAAC CAAAAA) (**30**)	ps	39	-71	-201	-8.8	15
5'd(TTTTiGiGiGTTTTT) (**31**) 5'd(AAAACC CAAAAA) (**32**)	ps	45	-68	-188	-9.9	14
5'd(TTTT CiGiGiGTTTT) (**33**) 5'd(AAAAiGCC CAAAA) (**34**)	ps	50	-58	-152	-10.6	14
5'd(TTTiGCiGiGiGTTTT) (**35**) 5'd(AAACiGC C CAAAA) (**36**)	ps	53	-56	-146	-10.8	12
5'd(AAACiGCiGiGiGAAA) (**37**) 5'd(TTTiGCiG C C C TTT) (**38**)	ps	61	-71	-186	-13.2	12

[a]) Measured UV-spectrophotometrically in 1 M NaCl, 100mM $MgCl_2$, 60 mM Na-cacodylate buffer, pH 7.0; the oligonucleotide concentration is 5 μM (single-strand).

Stability and CD spectra of DNA-RNA hybrids. The stability of DNA-RNA duplex was also investigated. For this purpose the oligodeoxyribonucleotide **11** was hybridized with the oligoribonucleotide **39** (Table 6). The T_m-value of the DNA-RNA hybrid (**11•39**) is only 2°C lower than that of its DNA-DNA counterpart (**11•12**, Table 6). The T_m-value difference of the corresponding aps-hydrids is 4°C (**13•39** vs **13•12**). This indicates that the nearest neighbour influence of DNA-RNA hybrids is different in the case of parallel and antiparallel duplexes. Nevertheless, the results show that parallel-hybridization can be successfully used in a similar way as antiparallel hybridization, not only in DNA duplexes but also in DNA-RNA hybrids. This can be of importance in the field of antisense therapeutics or hybridization techniques used in molecular biology.[23] The duplex with the highest T_m-value is always the aps-duplex with 5-methylisocytosine-isoguanine base pairs (aps-**11•40**) reflecting that the base pairs formed between isoguanine and 5-methylisocytosine are more stable than those of guanine with cytosine. The hypochromicity of melting is similar for ps and aps DNA (ps-**11•12** vs aps-**11•40**, see Fig. 5).

Table 6. T_m-values and thermodynamic data of chimeric oligonucleotide duplexes. [a])

Duplexes[b])	T_m [°C]	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol·K]	$\Delta G°_{298}$ [kcal/mol]
5'-d(TiCATAAiCTiGiGAT) (**11**) 5'-r(A GUAUU GA C CUA) (**39**)	42	-59	-161	-9.3
5'-d(TiCATAAiCTiGiGAT) (**11**) 5'-d(A GTATT GA C C TA) (**12**)	44	-85	-242	-10.3
3'-d(TCATAA CTGGAT) (**13**) 5'-r(AGUAUUGACCUA) (**39**)	47	-74	-205	-10.5
3'-d(TCATAACTGGAT) (**13**) 5'-d(AGTATTGACCTA) (**12**)	51	-90	-256	-11.4
5'-d(TiCATAAiCTiGiGAT) (**11**) 3'-d(AiGTATTiGAiCiCTA) (**40**)	60	-94	-257	-14.8
3'-d(TCATAACTGGAT) (**13**) 5'-d(AGTATTGACCTA) (**11**)	51	-91	-256	-11.4

For [a]) see Table 5. [b]) see Table 4.

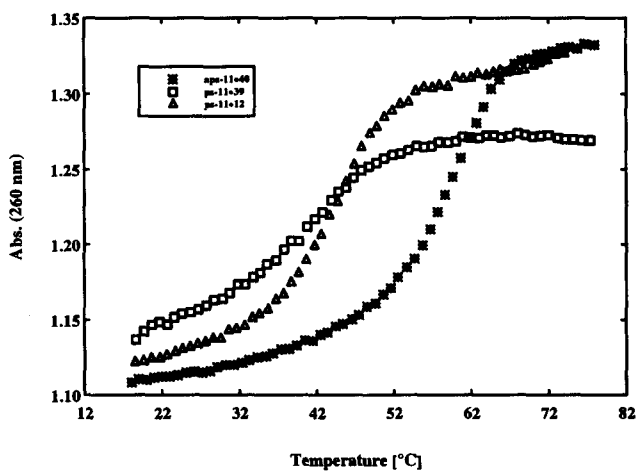


Fig. 5. Melting profiles of hybrids **11•12** (ps DNA-DNA), **11•39** (ps DNA-RNA), and **11•40** (aps DNA-DNA, see Table 6) measured at 260 nm, conditions see Table 6.

In order to investigate the conformational properties of ps-DNA in solution, the CD spectra of DNA-DNA hybrids of ps-**11•12**, aps-**11•40**, and aps-**12•13** as well as of DNA-RNA hybrids of ps-**11•39** and aps-**13•39** were examined in 1 M NaCl, 100 mM $MgCl_2$, 60 mM Na-cacodylate buffer (pH 7.0). The results are

shown in Fig. 6. The ps-oligonucleotides show negative lobes around 250 nm as it is observed for the aps-duplex **12•13**. However, compared to the CD spectrum of aps-**12•13** (B-DNA), the ps-duplexes **11•12** and **11•39** exhibit only weak positive bands near 275 nm. The CD spectra of iG_d- and m^5iC_d-containing ps-duplex (ps-**11•12** and ps-**11•39**) are similar to that of iG_d- and m^5iC_d-containing aps-DNA (aps-**11•40**) but apparently different from that of either unmodified B-DNA (aps-**12•13**) or A-type of DNA-RNA (aps-**13•39**). These differences are not only due to the change of the chain orientation but also result from the altered spectroscopic properties of the modified bases.

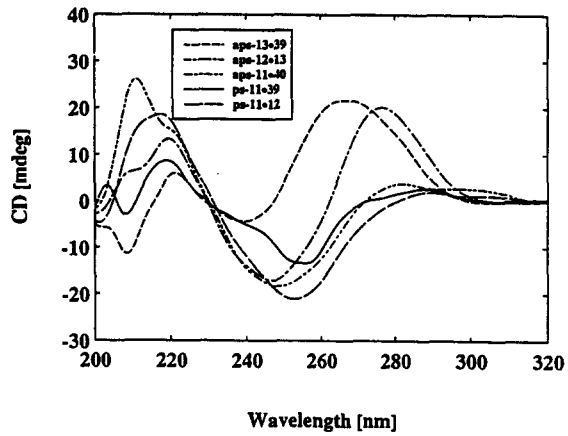


Fig. 6. CD spectra of the hybrids **11•12** (ps DNA-DNA), **11•39** (ps DNA-RNA), **11•40** (aps DNA-DNA), **12•13** (aps DNA-DNA), and **13•39** (aps DNA-RNA) at 5°C. Conditions see Table 6.

CONCLUSION

The 2'-deoxy-5-methylisocytidine (**1a**) is a rather labile nucleoside. The glycosylic bond stability of **1a** against acid is stabilized in the case of the amidine derivatives **4a, b.** These compounds are also cleaved at the glycosylic bond upon prolonged treatment with ammonia. Significant conversion of 2'-deoxy-5-methylisocytidine into thymidine is not observed, under neither alkaline nor acidic conditions. According to this, the time of ammonia deprotection of oligonucleotides containing **1a** has to be limited. The phosphoramitide **2a** carrying a dimethylaminomethylidene protecting group was found to be the most suitable derivative to be used in solid-phase oligonucleotide synthesis. It gives a coupling yield of 99%.[20] The phosphoramidite of 2'-deoxyisoguanosine (**3**) shows the same high coupling efficiency. Extended coupling times are not necessary. The oligonucleotides are rather stable when kept in aqueous solution under neutral conditions.

The chain orientation of a set of oligonucleotide duplexes containing adenine-thymine and isoguanine-cytosine as well as 5-methylisocytosine-guanine base pairs was determined as an ambiguous proof. The excimer fluorescence of terminal pyrene residues was used to evaluate the chain orientation. The aps-homoduplex **25•26** turns into parallel, when two adenine-thymine base pairs (out of 12) are replaced by 2 isoguanine-cytosine pairs (Table 5). According to the thermal data which were determined from the melting profiles, the isoguanine-cytosine base pair was found to be stronger than that of 5-methylisocytosine-guanine. Parallel-stranded duplexes can be formed not only in the case of DNA-DNA but also DNA-RNA hybrids. The chimeric ps-RNA-DNA duplexes are less stable than their DNA-DNA counterparts. The CD spectra of ps-duplexes formed by 5-

methylisocytosine-guanine and isoguanine-cytosine base pairs exhibit a different secondary structure from the normal B-DNA or A-type structures of DNA-RNA duplexes.

EXPERIMENTAL

General. See lit.[18] The solid-phase synthesis of oligonucleotides was carried out on automated DNA synthesizers (Applied Biosystems, ABI 392-08 for phosphoramidite chemistry and ABI 380 B for H-phosphonate chemistry). All the reagents are commercially available and used as received. The solvents were purified and dried according to the standard procedures. Thin-layer chromatography (TLC): TLC aluminium sheets silica gel 60 F_{254} (0.2 mm, Merck, Germany). Reversed-phase HPLC: 4×250 mm RP-18 (5 μm)-LiChrosorb column (Merck, Germany) with a Merck-Hitachi HPLC pump (model 655 A-12), a variable-wavelength monitor (model 655-A), a controller (model L-5000), and an integrator (model D-2000). UV Spectra: U-3200 spectrometer (Hitachi, Japan); λ_{max} in nm, ε in M^{-1} cm^{-1}, Half-life values (τ) were measured using a U-3000 spectrometer (Hitachi, Japan), connected with a temperature controller (Lauda, Germany). Melting curves: Cary 1E UV/VIS spectrophotometer (Varian, Australia) equipped with a thermoelectrical controller. Evaluation of thermodynamic data from the melting curves according to a two-state model was performed using the program MeltWin (version 3.1). NMR Spectra: Avance DPX-250 and AMX-500 spectrometer (Bruker, Germany), δ values in ppm downfield from internal $SiMe_4$ (^1H, ^{13}C) or external 85% H_3PO_4 soln. (^{31}P).

Alkaline hydrolysis of nucleosides. The half-life of nucleoside was measured in 25% aq. ammonia soln. at 40°C. The reaction was followed UV-spectrophotometrically at 285 nm.

Acidic hydrolysis of nucleosides. The half-life of nucleoside was measured in aq. HCl at different concentrations at 40°C. The reaction was followed UV-spectrophotometrically at the wavelength indicated in Table 1.

Hydrolysis product analysis. The compounds produced by hydrolysis under alkaline or acidic conditions were identified by RP-HPLC with the gradient as indicated in Fig.1-3.

Composition analysis of oligonucleotides. The oligonucleotides (0.2 A_{260} units) were dissolved in 0.1 M Tris-HCl buffer (pH 8.3, 200 μL) and treated with snake-venom phosphodiesterase (3 μL) at 37°C for 45 min and then alkaline phosphatase (3 μL) at 37°C for 30 min. The mixture was analyzed on reversed-phase HPLC (RP-18, gradient III, at 260 nm). Quantification of the material was made on the basis of the peak areas, which were divided by the extinction coefficients of the nucleoside constituents (ε_{260}: dG11700, dT 8800, dA 15400, m^5iC_d 6100, iG_d 4300).

Fluorescence measurements. Fluorescence spectra were measured on a F-4500 spectrometer (Hitachi. Japan) connected with a temperature controller. Samples were prepared by dissolving 0.2 A_{260} units of the oligonucleotide in solution containing 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Na-cacodylate buffer, pH 7.0 (1 mL). The mixture was equilibrated at the temperature of the measurement for 10 min in 1 cm quartz cuvette. The excitation occurred at 340 nm and the emission was recorded in 1-nm steps from 350 to 600 nm.

CD spectra. The CD spectra were measured from 220 to 320 nm in 1-cm cuvettes using a Jasco 600 spectropolarimeter connected with a temperature controller and a Lauda-RCS-6 bath (Lauda, Germany). The oligonucleotides were dissolved in 1 M NaCl, 100 mM $MgCl_2$, 10 mM Na-cacodylate (pH 7.0). The concentration was 5.0 μM.

2-[(N,N-Dimethylamino)methylidene]amino-5-methyl-4(3H)-pyrimidinone (**7a**). To a suspension of 5-methylisocytosine (**6**)[24] (125 mg, 1 mmol) in MeOH (25 mL), *N,N*-dimethylformamide dimethyl acetal (0.3 mL, 2.2 mmol) was added. The reaction mixture was stirred at 40°C for 2 h (TLC monitoring, CH_2Cl_2/MeOH 8:1),

the mixture was evaporated to dryness. The colourless powder was dissolved in CH_2Cl_2/MeOH (8:1) and applied on FC (silica gel, 2.5×12 cm, CH_2Cl_2/MeOH 8:1). Evaporation of the main fraction gave **7a** as colourless powder (180 mg, 99%). TLC (CH_2Cl_2/MeOH 8:1): R_f 0.47. UV (MeOH): 236 (11200), 289 (17800). ^{1}H NMR ((D$_6$)DMSO): 1.81 (s, 3 H, Me-C(5)); 3.00 (s, 3 H, MeN); 3.12 (s, 3 H, MeN); 7.54 (s, 1 H, H-C(6)); 8.58 (s, 1 H, HC=N); 11.49 (s, 1 H, H-N(3)). Anal. calc. for $C_8H_{12}N_4O$ (180.21): C 53.32, H 6.71, N 31.09; found: C 53.29, H 6.60, N 30.98.

*2-[(*N,N-*Di(n-butyl)amino)methylidene]amino-5-methyl-4(3*H*)-pyrimidinone* (**7b**). To a suspension of compound **6** (125 mg, 1 mmol) in MeOH (25 mL), *N,N*-di(n-butyl)formamide dimethyl acetal (0.46 mL, 2.2 mmol) was added. The reaction mixture was stirred at 40°C. After the starting material had reacted completely (about 1 h. TLC monitoring with CH_2Cl_2/MeOH 8:1), the mixture was evaporated to dryness. The white powder was dissolved in CH_2Cl_2 and then applied on FC (silica gel, 2.5×12 cm, CH_2Cl_2/MeOH 98:2 and then 95:5). Evaporation of the main fraction gave **7b** as colourless powder (237 mg, 90%). TLC (CH_2Cl_2/MeOH 8:1): R_f 0.58. UV (MeOH): 237 (12400), 292 (21000). ^{1}H NMR ((D$_6$)DMSO): 0.89 (m, 6 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.26 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.52 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.81 (s, 3 H, Me-C(5)); 3.43 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 7.54 (s, 1 H, H-C(6)); 8.57 (s, 1 H, HC=N); 11.45 (s, 1 H, H-N(3)) Anal. calc. for $C_{14}H_{24}N_4O$ (264.37): C 63.61, H 9.15, N 21.19; found: C 63.55, H 9.05, N 21.20.

*1-(2-Deoxy-*β*-D-*erythro-*pentofuranosyl)-2-[(*N,N-*dimethylamino)methylidene]amino-5-methyl-4(1*H*)-pyrimidinone* (**4a**). To a suspension of compound **1a** (121 mg, 0.5 mmol) in MeOH (5 mL), *N,N*-dimethylformamide dimethyl acetal (0.18 mL, 1.37 mmol) was added. The reaction mixture was stirred at r. t. for 2 h (TLC monitoring, CH_2Cl_2/MeOH 5:1), the mixture was evaporated to dryness. The residue was applied on FC (silica gel, 2.5×12 cm, CH_2Cl_2/MeOH 5:1). Evaporation of the main fraction gave **4a** as colourless powder (134 mg, 90%). TLC (CH_2Cl_2/MeOH 5:1): R_f 0.39. UV (MeOH): 246 (23100), 282 (30800). ^{1}H NMR ((D$_6$)DMSO): 1.78 (s, 3 H, Me-C(5)); 2.10 (m, 2 H, H-C(2')); 3.04 (s, 3 H, MeN); 3.17 (s, 3 H, MeN); 3.59 (m, 2 H, H-C(5')); 3.79 (m, 1 H, H-C(4')); 4.24 (m, 1 H, H-C(3')); 5.04 (t, J = 5.2, 1 H, HO-C(5')); 5.24 (d, J = 4.2, 1 H, HO-C(3')); 6.65 (t, 1 H, J = 6.3, H-C(1')); 7.74 (s, 1 H, H-C(6)); 8.57 (s, 1 H, HC=N). Anal. calc. for $C_{13}H_{20}N_4O_4$ (296.33): C 52.69, H 6.80, N 18.91; found: C 52.49, H 6.85, N 18.71.

*1-(2-Deoxy-*β*-D-*erythro-*pentofuranosyl)-2-[(*N,N-*di(n-butyl)amino)methylidene]amino-5-methyl-4(1*H*)-pyrimidinone* (**4b**). To a suspension of compound **1a** (484 mg, 2 mmol) in MeOH (30 mL), *N,N*-di(n-butyl)formamide dimethyl acetal (1.1 mL, 5.4 mmol) was added. The reaction mixture was stirred at 40°C. After the reaction was complete (2 h, TLC monitoring, CH_2Cl_2/MeOH 8:1), the mixture was evaporated to dryness. The residue was applied on FC (silica gel, 2.5×12 cm, CH_2Cl_2/MeOH 8:1). Evaporation of the main fraction gave **4b** as colourless powder (610 mg, 80%). TLC (CH_2Cl_2/MeOH 8:1): R_f 0.40. UV (MeOH): 247 (21000), 285 (31000). ^{1}H NMR ((D$_6$)DMSO): 0.91 (m, 6 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.29 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.57 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 1.78 (s, 3 H, Me-C(5)); 2.09 (m, 2 H, H-C(2')); 3.46 (m, 4 H, ($CH_3CH_2CH_2CH_2)_2$N); 3.60 (m, 2 H, H-C(5')); 3.78 (m, 1 H, H-C(4')); 4.22 (m, 1 H, H-C(3')); 5.07 (t, J = 5.1, 1 H, HO-C(5')); 5.22 (d, J = 4.0, 1 H, HO-C(3')); 6.59 (t, J = 6.4, 1 H, H-C(1')); 7.77 (s, 1 H, H-C(6)); 8.58 (s, 1 H, HC=N). Anal. calc. for $C_{19}H_{32}N_4O_4$ (380.49): C 59.98, H 8.48, N 14.73; found: C 60.10, H 8.38, N 14.70.

*1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-*β*-D-*erythro-*pentofuranosyl]-2-[(*N,N-*dimethylamino)methylidene]amino-5-methyl-4(1*H*)-pyrimidinone* (**5a**). Compound **4a** (1.05 g, 3.53 mmol) was dried by co-evaporation with anh. pyridine (3×15 mL), and was then dissolved in dry pyridine (25 mL). 4,4'-Dimethoxytrityl chloride (1.66 g, 4.9 mmol) was added in three portions (each every 30 min) at 0°C under stirring. The mixture was brought up to r. t. and stirred for 12 h. After addition of MeOH (5 mL), the reaction mixture was evapo-

rated and the residue was dissolved in CH_2Cl_2 (70 mL). The solution was washed with 5% aq. $NaHCO_3$ (20 mL) and H_2O (20 mL), dried (Na_2SO_4) and the organic layer was evaporated to give an oil. It was applied to FC (silica gel, 4×12 cm). After washing with CH_2Cl_2/Me_2CO/Et_3N (70:30:0.1) compound **5a** was eluted with CH_2Cl_2/MeOH/Et_3N (93:7:0.1); colourless foam (1.57 g, 74%). TLC (CH_2Cl_2/MeOH 6:1): R_f 0.67. UV (MeOH): 237 (32200), 282 (29400). ^{1}H NMR ((D$_6$)DMSO): 1.46 (s, 3 H, Me-C(5)); 2.22 (m, 2 H, H-C(2')); 3.05 (s, 3 H, MeN); 3.18 (s, 3 H, MeN); 3.22 (m, 2 H, H-C(5')); 3.74 (s, 6 H, (*Me*O)$_2$Tr); 3.91 (m, 1 H, H-C(4')); 4.33 (m, 1 H, H-C(3')); 5.35 (d, *J* = 4.3, 1 H, HO-C(3')); 6.70 (t, *J* = 6.6, 1 H, H-C(1')); 6.88-7.31 (m, 13 H, (MeO)$_2$*Tr*); 7.69 (s, 1 H, H-C(6)); 8.59 (s, 1 H, HC=N). Anal. calc. for $C_{34}H_{38}N_4O_6$ (598.70): C 68.21, H 6.40, N 9.36; found: C 67.89, H 6.39, N 9.32.

*1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-2-{[*N,N*-Di(n-butyl)amino]methylidene}amino-5-methyl-4(1*H*)-pyrimidinone* (**5b**). Compound **5b** was prepared as described for **5a**. Compound **4b** (750 mg, 1.97 mmol), dry pyridine (20 mL), 4,4'-dimethoxytrityl chloride (1.02 g, 3 mmol), MeOH (5 mL), CH_2Cl_2 (35 mL), 5% aq. $NaHCO_3$ soln. (2×10 mL), and H_2O (20 mL). FC (silica gel, 4×12 cm). After washing with CH_2Cl_2/Me_2CO/Et_3N (70:30:0.1) compound **5b** was eluted with CH_2Cl_2/MeOH/Et_3N (93:7:0.1); colourless foam (1.2 g, 89%). TLC (CH_2Cl_2/MeOH 6:1): R_f 0.67. UV (MeOH): 237 (37700), 284 (32300). ^{1}H NMR ((D$_6$)DMSO): 0.91 (m, 6 H, (*CH*$_3$CH$_2$CH$_2$CH$_2$)$_2$N); 1.28 (m, 4 H, (CH$_3$*CH*$_2$CH$_2$CH$_2$)$_2$N); 1.45 (s, 3 H, Me-C(5)); 1.56 (m, 4 H, (CH$_3$CH$_2$*CH*$_2$CH$_2$)$_2$N); 2.20 (m, 2 H, H-C(2')); 3.22 (m, 2 H, H-C(5')); 3.44 (m, 4 H, (CH$_3$CH$_2$CH$_2$*CH*$_2$)$_2$N); 3.71 (m, 6 H, (*Me*O)$_2$Tr); 3.93 (m, 1 H, H-C(4')); 4.32 (m, 1 H, H-C(3')); 5.33 (d, *J* = 4.2, 1 H, HO-C(3')); 6.64 (t, 1 H, *J* = 6.4, H-C(1')); 6.81-7.41 (m, 13 H, (MeO)$_2$*Tr*); 7.62 (s, 1 H, H-C(6)); 8.60 (s, 1 H, HC=N).

*1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-2-[(*N,N*-dimethylamino)methylidene]amino-5-methyl-4(1*H*)-pyrimidinone 3'-*O*-(2-cyanoethyl diisopropylphosphoramidite)* (**2a**). To a stirring solution of compound **5a** (700 mg, 1.2 mmol) in dry CH_2Cl_2 (32 mL) was added *N*-ethyl-diisopropylamine (0.48 mL, 2.69 mmol) and chloro-(2-cyanoethoxy)-*N,N*-(diisopropylamino)phosphine (0.5 mL, 2.24 mmol) were added by a syringe under argon at r. t. The mixture was stirred for 2 h. Then a 5% aq. $NaHCO_3$ soln. (35 mL) was added, and the mixture was extracted with CH_2Cl_2 (3×15 mL). The extract was dried (Na_2SO_4) and evaporated to an oil. The residue was applied to FC (silica gel column, 2.5×10 cm, CH_2Cl_2/Me_2CO/Et_3N 70:30:0.1 and then CH_2Cl_2/MeOH/Et_3N 45:5:0.1), giving **2a** as colourless foam (720 mg, 75%). This was dissolved in CH_2Cl_2 (5 mL) and added to stirring hexane (500 mL) cooled to -30°C. The precipitate was isolated by filtration and the powder was dried under vacuum (570 mg). TLC (CH_2Cl_2/MeOH/Et_3N 90:10:0.2): R_f 0.46. UV (MeOH): 237 (37800), 282 (32300). ^{1}H NMR ((D$_6$)DMSO): 1.20 (m, 12 H, (*Me*$_2$CH)$_2$N); 1.67 (2 s, 3 H, Me-C(5)); 2.44 (m, 2 H, NC*H*$_2$CH$_2$); 2.63 (m, 2 H, H-C(2')); 3.10 (2 s, 3 H, MeN); 3.17 (2 s, 3 H, MeN); 3.53 (m, 4 H, H-C(5'), CNCH$_2$*CH*$_2$); 3.81 (m, 8 H, (*Me*O)$_2$Tr, (Me$_2$*CH*)$_2$N); 4.20 (m, 1 H, H-C(4')); 4.61 (m, 1 H, H-C(3')); 6.85-7.34 (m, H-C(1'), (MeO)$_2$*Tr*); 7.68 (2 s, 1 H, H-C(6)); 8.85 (s, 1 H, HC=N). ^{31}P-NMR ($CDCl_3$): 150.4, 149.7.

*1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-2-[(*N,N*-dimethylamino)methylidene]amino-5-methyl-4(1*H*)-pyrimidinone* 3'-*(triethylammonium phosphonate)* (**2b**). To a stirring solution of 1*H*-1,2,4-triazole (1.02 g,14.8 mmol) and *N*-methylmorpholine (4.5 mL) in anh. CH_2Cl_2 (25 mL) was added PCl_3 (0.42 mL, 4.7 mmol) at r. t.. After stirring for 30 min, the soln. was cooled to 0°C, and a solution of compound **5a** (494 mg, 0.83 mmol) in CH_2Cl_2 (20 mL) was added dropwise over 5 min under stirring. The stirring was continued for 1 h at r. t.. The mixture was poured into 1 M aq. (Et_3NH)HCO_3 (50 mL). The phases were separated and the aq. phase was extracted with CH_2Cl_2 (3×15 mL), and the combined org. layers were dried (Na_2SO_4) and evaporated to give an oil. The residue was dissolved in CH_2Cl_2, applied to FC (silica gel, 2.5×10

cm, CH_2Cl_2/MeOH/Et_3N 88:10:2), and the main zone giving a colourless foam. This foam was dissolved in CH_2Cl_2 (10 mL) and washed with 0.1 M aq. $(Et_3NH)HCO_3$ (5×20 mL). The aq. layer was extracted with CH_2Cl_2 (3×20 mL). The combined CH_2Cl_2 layers were dried (Na_2SO_4), filtered and evaporated to give **2b** as a colourless foam (259 mg, 41%). TLC (CH_2Cl_2/MeOH 6:1): R_f 0.48. UV (MeOH): 237 (32800), 282 (27200). ^{1}H NMR ((D$_6$)DMSO): 1.13 (t, J = 7.3, 9 H, (CH_3CH_2)$_3$N); 1.39 (s, 3 H, Me-C(5)); 2.25 (m, 2 H, H-C(2')); 2.97 (m, 6 H, (CH_3CH_2)$_3$N); 3.05 (s, 3 H, MeN); 3.18 (s, 3 H, MeN); 3.25 (m, 2 H, H-C(5')); 3.74 (s, 6 H, (*Me*O)$_2$Tr); 4.08 (m, 1 H, H-C(4')); 4.76 (m, 1 H, H-C(3')); 6.68 (t, 1 H, J = 6.6, H-C(1')); 6.87-7.41 (m, 13 H, (MeO)$_2$*Tr*); 7.60 (s, 1 H, H-C(6)); 8.58 (s, 1 H, HC=N). ^{31}P-NMR ((D$_6$)DMSO): 2.86 (^1J(P, H) = 578, ^3J(P, H) = 8.8).

*9-[2-Deoxy-5-*O*-(4,4'-dimethoxytriphenylmethyl)-*ß*-D-*erythro*-pentofuranosyl]-6-[1-(*N,N*-dimethylamino)ethylidene]amino-2-(diphenylcarbamoyl)oxy-9*H*-purine 3'-(3-carboxypropanoate)* (**9**). To a soln. of compound **8** (392 mg, 0.47 mmol) in anh. 1,2-dichloroethane (1.0 mL), 4-(dimethylamino)pyridine (29.9 mg, 0.24 mmol), succinic anhydride (78.6 mg, 1.03 mmol), and Et_3N (65.2 µL, 0.48 mmol) were added. The mixture was stirred for 1 h at 50°C. Then the soln. was diluted with 1,2-dichloroethane (15 mL), washed with ice-cold 10% aq. citric acid soln. (3×10 mL) and H_2O (2×10 mL). The org. layer was dried (Na_2SO_4), evaporated, and the residue submitted to FC (silica gel, column 10×2 cm, CH_2Cl_2/Me_2CO 80:20, then CH_2Cl_2/CH_3OH 95:5). Colourless foam (385 mg, 88%). TLC (CH_2Cl_2/CH_3OH 9:1): R_f 0.45. UV (MeOH): 234 (46300), 308 (16200). ^{1}H NMR ((D$_6$)DMSO): 2.07 (s, 3 H, CH_3-C); 2.54, 3.02 (2 m, 2 H, H-C(2')); 3.10 (s, 6 H, Me_2N); 3.22, 3.28 (2 s, 4 H, CH_2CH_2); 3.67 (s, 6 H, (*Me*O)$_2$Tr); 3.87 (m, 2 H, H-C(5')); 4.09 (m, 1 H, H-C(4')); 5.38 (m, 1 H, H-C(3')); 6.31 (t, 1 H, J = 7.0, H-C(1')); 6.74-7.39 (m, 23 H, Ph_2N, (MeO)$_2$*Tr*); 8.27 (s, 1 H, H-C(8)); 12.22 (s, 1 H, COOH). ^{13}C NMR ((D$_6$)DMSO): 157.9 (C(2)); 151.5 (C(4)); 113.1 (C(5)); 155.5 (C(6)); 140.8 (C(8)); 151.7 (C=O); 83.1 (C(1')); 74.5 (C(3')); 85.7 (C(4')); 63.8 (C(5')). Anal. calc. for $C_{52}H_{51}N_7O_{10}$ (934.02): C 66.87, H 5.50, N 10.50; found: C 66.76, H 5.52, N 10.40.

*9-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-*ß*-D-*erythro*-pentofuranosyl]-6-[1-(*N,N*-dimethylamino)ethylidene]amino-2-(diphenylcarbamoyl)oxy-9*H*-purine 3'-[3-(*N*-Fractosil-carbamoyl)propanoate]* (**10**). To a soln. of **9** (122 mg, 0.13 mmol) in 1,4-dioxane/pyridine (95:5, 1 mL), 4-nitrophenol (40 mg, 0.29 mmol) and *N,N*-dicyclohexylcarbodiimide (60 mg, 0.3 mmol) were added under stirring at r. t. After 2 h, dicyclohexylurea was removed by filtration. To the filtrate *Fractosil* 200 (450 µmol NH_2/g, dp 100 µm, 200 mg) and DMF (1 mL) was added. Then Et_3N (200 µL) was introduced, and the suspension was shaken for 4 h at r. t. Ac_2O (60 µL) was added and shaking was continued for another 30 min. The *Fractosil* derivative **10** was filtered, washed with DMF, EtOH, and Et_2O, and dried in *vacuo* (216 mg). The amount of silica-gel-bound nucleoside was determined by treatment of **10** (5 mg) with 0.1 M TsOH/MeCN (10 mL). From the absorbance (498 nm) of the supernatant, 70 µmol of linked **9**/g *Fractosil* was calculated (ε_{DMT} = 70000).

Solid-phase synthesis of the oligonucleotides **11-13**, **17-38** *and* **40**. The oligonucleotide synthesis was performed on a 1-µmol scale using the phosphoramidites **2a** and/or **3**.[18] A standard protocol was used on an ABI 392-08 synthesizer[25] and the deprotection was performed in 25% aq. NH_3 at 60°C for 16 h. The phosphoramidites of dA, dG, dT, dC and the CPG-columns were commercial products of PerSepetive Biosystems GmbH, Germany. The oligonucleotides **11**, **19**, **20**, and **23** were alternatively synthesized using the phosphonate **2b** together with that of dA, dT, and iG_d[18] on an ABI 380B synthesizer. The oligonucleotides with 3'-terminal m^5iC_d-residues were synthesized using Rainbow universal CPG (Clontech Laboratories, Inc., USA). For the oligonucleotides with 3'-terminal iG_d-residues the polymer resin **10** was used. The oligonucleotides with terminal amino function (**11a-13a**) were achieved in the last coupling step with the aminolink-2 phosphoramidite **41** (Applied Biosystems, Germany). Purification of the oligonucleotides were performed on reversed-phase HPLC

employing the following solvent systems: 0.1 M aq. $(Et_3NH)OAc$ (pH 7.0) (A), CH_3CN (B), and 0.1 M $(Et_3NH)OAc$ (pH 7.0)/MeCN 95:5 (C). Following gradients were used: (I) 3 min 15% B in C, 12 min 15-40% B in C , 5 min 40-15% B in C with flow rate of 1.0 mL/min; (II) 20 min 0-20% B in C with flow rate of 1.0 mL/min; (III) 30 min 100% C with flow rate of 0.6 mL/min; (IV) 30 min 10-40% B in A with flow rate of 1.0 mL/min.

The oligomers with 5'-DMT residues were purified by HPLC (250×4 mm, RP-18 column) using gradient I. The DMT residues were removed by treatment with 2.5% $Cl_2CHCOOH/CH_2Cl_2$ for 5 min at r. t. The detritylated oligomers were purified by HPLC (250×4 mm RP-18 column) with gradient II. Oligonucleotides containing an aminolink-2 residue[25] (**11a-13a**) were purified by HPLC (250×4 mm, RP-18 column) with gradient IV. The oligomers were separated by RP-HPLC, desalted on RP-18 column (125×4 mm) with H_2O, while the oligomers were eluted by $MeOH/H_2O$ (3:2). The purified oligonucleotides were lyophilized (Speed-Vac evaporator) to yield colourless solids which were dissolved in 100 μL of H_2O and stored frozen at -18°C. The oligonucleotides were characterized by enzymatic hydrolysis and by MALDI-TOF mass spectra. Selected data are summarized in Table 7.

Synthesis of the pyrene labelled oligonucleotides **14-16**. To a solution of the oligonucleotides **11a-13a** (30 A_{260} units) in H_2O (50 μL), 0.2 M Na-borate, pH 9.5 (50 μL) and dioxane (80 μL) was added a freshly prepared stock solution of succinimidyl-1-pyrenebutyrate (**42**) (10 mg/mL; Molecular Probes, USA) in dry DMF (200 μL). After the solution was stored in the darkness overnight, the mixtures were applied to HPLC (RP-18 column, 250×4) and eluted with gradient V (30 min 5%-50% B in A, 5 min 50-80% B in A, 5 min 80% B in A, flow rate 1.0 mL/min). The oligomers **14-16** were desalted as described and lyophilized on a Speed-Vac evaporator to yield colourless solids (approximately 15 A_{260} units). The nucleoside composition was determined by enzymatic digestion. The purity was checked by HPLC as described. The peak contents were identified by the UV-spectra. The A_{260}/A_{344} ratio was calculated to be 0.27 to 0.30, which indicates the attachment of 1 pyrene label onto one oligonucleotide.

Table 7. Molecular masses of selected oligonucleotides determined by MALDI-TOF mass spectra.

Oligomer	Retention time [min][a)]	M^+ (found) [Da]	M^+ (calc.) [Da]
5'-d(TiCATAAiCTiGiGAT) (**11**)	14	3671.4	3672.5
5'-d(iCiCiCGGG) (**17**)	17	1834.5	1835.3
5'-d(CCCiGiGiG) (**19**)	18	1793.7	1793.2
5'-d(iGiGiGCCC) (**20**)	18	1792.9	1793.2
5'-d(iCGiCGiCG) (**22**)	19	1835.4	1835.3
5'-d(TTTTTiGTTTTTT) (**27**)	19	3613.5	3613.4
5'-d(TTTTTiGiGTTTTT) (**29**)	18	3637.5	3638.4
5'-d(AAAAiGCCCAAAA) (**34**)	13	3639.3	3640.4
5'-d(TTTiGCiGiGiGTTTT) (**35**)	13	3674.1	3673.4
5'-d(AAACiGCCCAAAA) (**36**)	13	3617.4	3616.4
5'-d(ATiCiCAiGTTATiGA) (**40**)	14	3670.5	3672.5

[a)] The retention times refer to the gradient I for RP-HPLC.

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