Modified purine nucleosides as dangling ends of DNA duplexes: the effect of the nucleobase polarizability on stacking interactions

Helmut Rosemeyer and Frank Seela *

Laboratorium für Organische und Bioorganische Chemie, Institut für Chemie, Fachbereich Biologie/Chemie, Universität Osnabrück, Barbarastr. 7, D-49069 Osnabrück, Germany. E-mail: Frank.Seela@uni-osnabrueck.de

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Base-modified nucleotide residues have been appended to the 5′-terminus of the self-complementary oligo-2′-deoxynucleotide duplex [5′-d(CGCGCG)]$_2$ as dangling ends. Temperature-dependent UV measurements on the resulting oligomers indicate generally higher thermal stabilities (T_m) compared to that without an overhanging end. The duplex stabilization (ΔT_m) was correlated with the molecular polarizability (α_m) of the base of the pendant nucleoside showing that: the higher the molecular polarizability α_m of a dangling nucleobase, the higher the thermal stability of the DNA duplex.

Introduction

Unpaired terminal nucleotides (dangling ends) increase the thermal stability of oligo(β-D-ribo)- and oligo(2′-deoxy-β-D-ribonucleotide) duplexes.[1–9] It has been suggested that this effect is important in determining the stability of codon–anticodon associations.[10] Oligonucleotides with overhanging nucleotides were used to study the determinants of stacking interactions with respect to nucleic acid structure and stability.[7] Recently, octamers tailed with 1-(2-deoxy-β-D-ribofuranosyl)-5-nitroindol were designed as primers for cycle sequencing.[11] Moreover, studies of oligonucleotide hybridization of oligomers with dangling ends on a solid support have been accomplished showing the stabilizing effect of overhanging nucleoside residues on an array.[12] This analysis was performed to assess simultaneously the effects of differing bases at both 5′ and 3′-ends of oligonucleotide duplexes formed under identical hybridization conditions. Very recently a programmable and autonomous computing device fulfilling the requirements of a Turing machine (finite automata) was realized using double-stranded DNA molecules as both, 'software' and 'input data'.[13] One essential feature of the double strands used are overhanging sticky ends which allow the selective joining of data- and software-DNA to be processed by the restriction endonuclease *Fok*I and T4 DNA ligase ('hardware'). It was stated that the complexity of such a system is closely bound to the size and stability of the sticky ends.

For all these reasons it is important to know what specific interactions contribute to base stacking and how these might be changed. Three strategies have been followed to enhance intra- and interstrand stacking: (i) increasing the surface area of the nucleobases by adding extra rings but without disrupting their ability to form hydrogen bonds has been reported to increase duplex thermal stability;[7,14] (ii) the incorporation of dangling non-polar DNA base analogues such as phenanthrene or pyrene nucleosides at the end of a helix has been shown to stabilize a duplex, even when the 'bases' in question do not undergo pairing;[13,15] (iii) the addition of simple substituents to DNA bases has been shown to enhance base stacking.[16–18] In this vein are studies which clearly show that C(5)-methylated (dT, **1**), -halogenated (Br^{5}U$_d$, **2a**; I^{5}U$_d$, **2b**; Scheme 1), or propynylated pyrimidine nucleosides such as pry^{5}U$_d$ (**2c**, Scheme 1) create nucleic acid helices which are more stable than those without the substituents.[19–21]

In an influencing manuscript Sowers *et al.*[22] proposed that, rather than hydrophobic forces, it is the increased polarizability of methylated pyrimidine bases (which enhance van der Waals interactions with neighboring bases) that promotes stacking. The molecular polarizability (α_m) is a measure for the ease with which a dipole moment may be induced in the molecule. Nowadays, α_m-values are usually calculated from published increments. Very recently, Kool and co-workers[23] stated that the surface area as well as the polarizability of dangling aromatics but neither their dipole moment nor log *P* values are correlated to their stacking energy.

Recently, Seela and co-workers have shown that the incorporation of 7-substituted 7-deazapurine[24,25] as well as 7-substituted 8-aza-7-deazapurine nucleosides[26] into oligo(2′-deoxy-β-D-ribonucleotides) can lead to significantly enhanced duplex stability. In this communication we extend the above described results by studying a series of base-modified nucleosides as appending aromatics and shed light on how far their molecular polarizability (α_m) exerts influence on the DNA duplex stability.

Results and discussion

To separate stacking from hydrogen bond interactions we placed a series of regular and base-modified purine nucleoside residues in a dangling position—without a pairing partner—at the 5′-end of the duplex [5′-d(CGCGCG)]$_2$ (**10·10**). The sequence [5′-d(CGCGCG)]$_2$, showing a T_m value of 46 °C, allows a T_m increase induced by dangling nucleosides to be easily observed. It is known that this duplex forms a B-DNA structure at NaCl concentrations below 2.5 M. Furthermore, oligonucleotide duplexes with a random nucleotide composition have to be longer in order to show such a melting temperature. This results in a smaller and not so easily observable dangling end effect. Apart from thymidine all dangling nucleosides are purine analogues covering part of the $(n - 1)$ cytidine residue. The resulting thermal stabilization of the duplex by the overhanging nucleoside (ΔT_m) is measured by thermal denaturation experiments, with comparison to the duplex lacking the dangling nucleoside.

Scheme 1 presents the nucleosides and their abbreviations which have been appended to the duplex **10·10**.

All self-complementary oligo(2′-deoxy-β-D-ribonucleotides) (**10**–**20**) except **17** have been prepared by automated solid-phase

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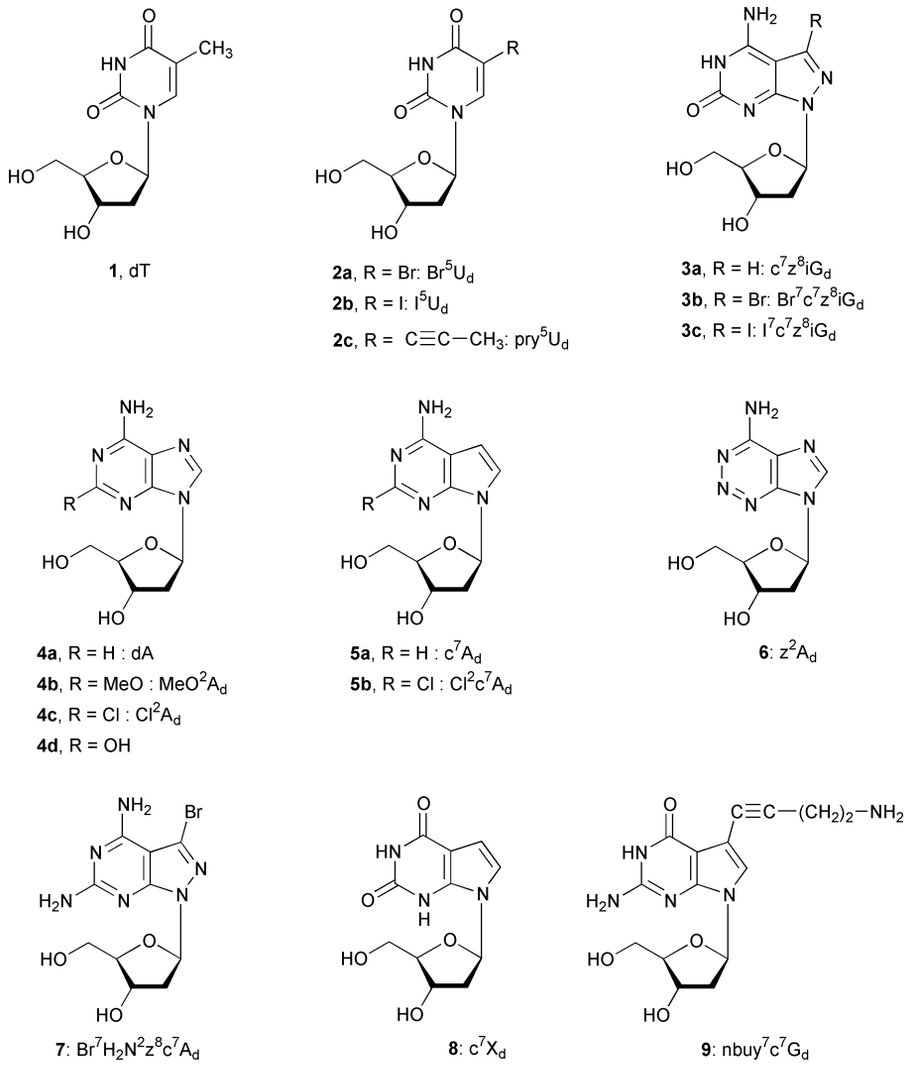

Scheme 1

synthesis under standard conditions using appropriately protected phosphoramidites.[8,26,27] The synthesis of the still unpublished DNA building blocks of 7-deaza-2′-deoxyxanthosine (**8**, c^7X_d), 2-methoxy-2′-deoxyadenosine (**4b**, MeO^2A_d), 2-chloro-7-deaza-2′-deoxyadenosine (**5b**, $Cl^2c^7A_d$) and 7-(4-aminobut-1-ynyl)-7-deaza-2′-deoxyguanosine (**9**, $nbuy^7c^7G_d$) will be reported elsewhere.[28a–e] The oligomer **17** was synthesized using the appropriate phosphonate.[29] The oligonucleotides were removed from the solid support and base deprotected (conc. aq. NH_3). The resulting 5′-(DMT)-derivatives (DMT = 4,4'-dimethoxytrityl) were purified by reversed-phase RP-18 HPLC, detritylated, again purified by RP-18 HPLC and desalted (RP-18 HPLC) as described (see Experimental section). They were characterized by MALDI-TOF mass spectra, and their base composition was confirmed by enzymatic hydrolysis (see Experimental section). Their thermal stability (T_m) was determined by temperature-dependent UV-melting profiles in two different buffer systems (Table 1), and the thermodynamic data of duplex formation were calculated from each individual melting curve according to a two-state model (Table 1).[30]

According to Table 1 it is apparent that adenine stacks more strongly in the duplex than thymine does. The $\Delta\Delta G^\circ_{310}$ values {$\Delta\Delta G^\circ_{310} = \Delta G^\circ_{310}$[5′-d(**D**CGCGCG)]$_2$ − ΔG°_{310}[5′-d(CGCGCG)]$_2$ with **D** as dangling nucleoside} of the oligomers **10·10**, **11·11** and **14·14** (1.0–1.9 kcal mol^{-1}) are in sufficient agreement with data reported by Kool and co-workers.[23] Moreover, it can be seen that the overhanging nucleosides carrying modified nucleobases exhibit graduated stabilizing stacking effects ($\Delta T_m = T_m$[5′-d(**D**CGCGCG)]$_2$ − T_m[5′-d(CGCGCG)]$_2$, Table 2). The highest T_m value is reached by the duplex **19·19** carrying pendant 8-aza-7-bromo-7-deazapurine-2,6-diamine 2′-deoxy-β-D-ribofuranoside residues (**7**, $Br^7H_2N^2z^8c^7A_d$)[26] on both termini as well as by the duplex **20·20** carrying two 7-(4-aminobut-1-ynyl)-7-deaza-2′-deoxyguanosine (**9**, $nbuy^7c^7G_d$) residues.[28d,e] The first nucleoside has recently been shown to exhibit extraordinary stabilizing properties (4.5 °C per modification) also on DNA duplexes with a random sequence[27] while the second exhibits only marginal stabilizing effects.[28e]

Table 2 presents the stabilizing effect of the two overhanging nucleoside residues on the core duplex (ΔT_m) in two different buffer systems together with the molecular polarizability ($\alpha_m/10^{-24}$ cm^3)† of the corresponding nucleobase and the retention time (t_R/min) of the dangling nucleoside on a reversed-phase RP-18 HPLC column (5 × 250 mm). The latter is taken as a measure of the hydrophobicity of the nucleoside.

Figs. 1 and 2 display the results graphically. According to Fig. 1, a linear trend exists between the stabilizing effect of the dangling nucleosides and the polarizability of its nucleobase: the more easily a dipole moment is induced within the overhanging nucleobase, the higher the ΔT_m value. From Fig. 2 it is also evident that a rough (non-linear) correlation does exist between the hydrophobicity of most of the dangling nucleosides (expressed in terms of t_R in RP-18 HPLC) and the stabilizing effect upon the duplex **10·10**,. An important

† The units of polarizability given, cm^3, should strictly be $4\pi\varepsilon_0$ cm^3 throughout the article.

Table 1 T_m values and thermodynamic data of duplex formation of the oligomer [5′-d(CGCGCG)]$_2$ carrying dangling units[a]

Oligonucleotide	T_m/°C	$\Delta H°$/kcal mol^{-1}[b]	$\Delta S°$/cal K^{-1} mol^{-1}[b]	$\Delta G°_{310}$/kcal mol^{-1}
5′-d(CGCGCG) **10** 3′-d(GCGCGC) **10**	47; 46	−52.1; −52.5	−140.9; −143.0	−8.4; −8.2
5′-d(**T**CGCGCG) **11** 3′-d(GCGCGC**T**) **11**	51; 53	−57.7; −56.0	−155.7; −149.7	−9.4; −9.6
5′-d(**c^{7}X**CGCGCG) **12** 3′-d(GCGCGC**c^{7}X**) **12**	52; 54	−67.0; −48.0	−183.0; −122.0	−10.3; −10.2
5′-d(**z^{2}A**CGCGCG) **13** 3′-d(GCGCGC**z^{2}A**) **13**	52; 53	−60.9; −59.0	−165.0; −158.0	−9.8; −10.0
5′-d(**A**CGCGCG) **14** 3′-d(GCGCGC**A**) **14**	55; 55	−60.7; −55.3	−163.6; −145.7	−10.0; −10.1
5′-d(**c^{7}A**CGCGCG) **15** 3′-d(GCGCGC**c^{7}A**) **15**	56; 57	−59.0; −58.0	−160.3; −154.4	−9.3; −10.1
5′-d(**MeO^{2}A**CGCGCG) **16** 3′-d(GCGCGC**MeO^{2}A**) **16**	56; 58	−62.6; −55.8	−169.5; −147.8	−10.1; −10.0
5′-d(**Cl^{2}A**CGCGCG) **17** 3′-d(GCGCGC**Cl^{2}A**) **17**	57; 59	−65.4; −55.3	−177.5; −145.8	−10.4; −10.1
5′-d(**Cl^{2}c^{7}A**CGCGCG) **18** 3′-d(GCGCGC**Cl^{2}c^{7}A**) **18**	58; 59	−63.6; −61.0	−170.0; −163.0	−10.9; −10.5
5′-d(**Br^{7}NH$_2$^{2}c^{7}z^{8}A$_d$**CGCGCG) **19** 3′-d(GCGCGC**Br^{7}NH$_2$^{2}c^{7}z^{8}A$_d$**) **19**	59; 60	−71.4; −59.8	−193.6; −158.2	−11.4; −10.7
5′-d(**nbuy^{7}c^{7}G**CGCGCG) **20** 3′-d(GCGCGC**nbuy^{7}c^{7}G**) **20**	60; nd	−59.5; nd	−157.0; nd	−10.8; nd

[a] First entries refer to measurements in 10 mM Na-cacodylate, 10 mM $MgCl_2$, 100 mM NaCl (pH 7); second entries refer to measurements in 10 mM Na_2HPO_4, 1 M NaCl (pH 7); concentration: 5 + 5 μM of single strands; nd: not detected. [b] Data within ± 15%.

Table 2 Polarizability of nucleobases, T_m enhancements of duplexes carrying corresponding dangling nucleotides and retention times (min) of corresponding nucleosides in reversed-phase RP-18 HPLC

Dangling nucleoside	Polarizability (a_m/10^{-24} cm^3) of corresponding base	ΔT_m ± 0.5/°C[a]	t_R/min[b]
dT, **1**	11.77 ± 0.5	+4; +7	9.8 ± 0.2
c^{7}X$_d$, **8**	13.95 ± 0.5	+5; +8	9.9 ± 0.2
z^{2}A$_d$, **6**	13.92 ± 0.5	+5; +7	10.6 ± 0.3
dA, **4a**	14.68 ± 0.5	+8; +9	16.7 ± 0.4
c^{7}A$_d$, **5a**	15.43 ± 0.5	+9; +11	22.7 ± 0.8
MeO^{2}A$_d$, **4b**	17.33 ± 0.5	+9; +12	37.5 ± 1.7
Cl^{2}A$_d$, **4c**	16.62 ± 0.5	+10; +13	49.1 ± 0.6
Cl^{2}c^{7}A$_d$, **5b**	17.38 ± 0.5	+11; +13	63.3 ± 1.9
Br^{7}H$_2$N^{2}z^{8}c^{7}A$_d$, **7**	19.41 ± 0.5	+12; +14	80.4 ± 2.5
nbuy^{7}c^{7}G$_d$, **9**	22.74 ± 0.5	+13; nd	11.3 ± 0.3

[a] First entries refer to measurements in 10 mM Na-cacodylate, 100 mM NaCl, 10 mM $MgCl_2$ (pH 7), second entries to measurements in 10 mM Na_2HPO_4, 1 M NaCl (pH 7). [b] 95% $Et_3NH^+OAc^-$–5% MeCN (flow rate: 0.7 ml min^{-1}), column: RP-18, 250 × 10 mm; each compound was injected separately to avoid any aggregation of different nucleosides.

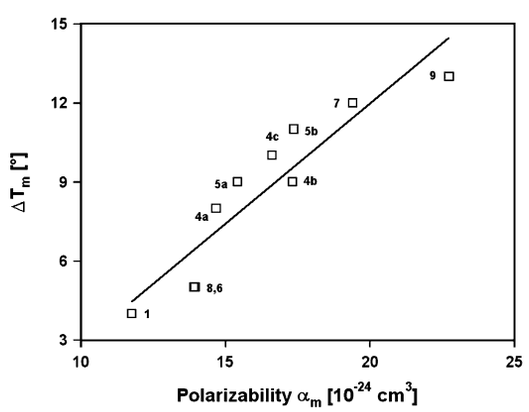

Fig. 1 $\Delta T_m = T_m$[5′-d(**D**CGCGCG)]$_2$ − T_m[5′-d(CGCGCG)]$_2$ with **D** as dangling nucleoside (Table 1) as a function of the molecular polarizability a_m of the base of **D**. The numbers at the data points indicate the overhanging nucleosides according to Scheme 1.

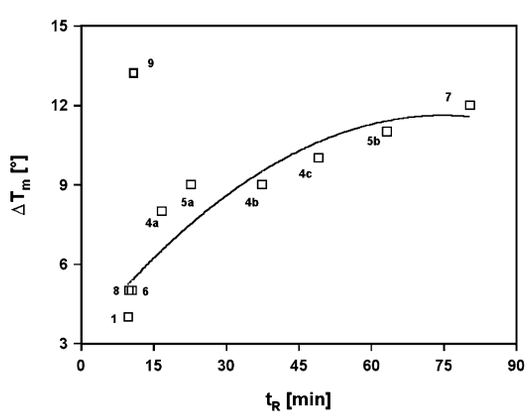

Fig. 2 $\Delta T_m = T_m$[5′-d(**D**CGCGCG)]$_2$ − T_m[5′-d(CGCGCG)]$_2$ with **D** as dangling nucleoside as a function of the retention time t_R of **D** in RP-18 HPLC. The numbers at the data points indicate the overhanging nucleosides according to Scheme 1.

exception is the duplex **20·20**. This carries a dangling 7-(4-aminobut-1-ynyl)-7-deaza-2′-deoxyguanosine (**9**, nbuy^{7}c^{7}G$_d$) residue which is obviously protonated at the amino group of the side chain. This can be deduced from the low t_R value (11.3 min) with RP-18 HPLC with elution by a buffer of pH 7. It is, therefore, obvious that the polarizability of the nucleobase, and not neccessarily solvation effects, is the important determinant for the stacking propensity and therewith for the ultimate thermal stability of an ordered nucleic acid single strand or a nucleic acid double helix.

Entirely in line with the results described above are findings described by Seela and co-workers on the stability of parallel oligonucleotide duplexes of the sequence 5′-d(iGCiGCiGC)·5′-d(iGCiGCiGC) (iG$_d$: 2′-deoxyisoguanosine, **4d**).[31] This duplex contains only five iG$_d$dC base pairs and one dangling nucleoside on each terminus (5′-end: iG$_d$; 3′-end: dC). It exhibits a T_m-value of 33 °C (60 mM Na-cacodylate, 100 mM $MgCl_2$, 1 M NaCl, pH 7).[31] Upon replacement of all iG$_d$ residues by either 8-aza-7-deaza-2′-deoxyisoguanosine (**3a**, c^{7}z^{8}iG$_d$, Scheme 1) or its 7-bromo- or 7-iodo-substituted derivatives (**3b**, Br^{7}c^{7}z^{8}iG$_d$; **3c**, I^{7}c^{7}z^{8}iG$_d$, Scheme 1) the T_m values of the corresponding duplexes are raised to 41, 57 or 62 °C, respectively (**21·21**, **22·22**, **23·23**, Table 3).

Table 3 T_m data of parallel stranded oligonucleotide duplexes and polarizability values (α_m) of modified nucleobases[a]

Oligonucleotide	T_m/°C	Polarizability ($\alpha_m/10^{-24}$ cm^3) of the corresponding base
5′-d(**3a**C**3a**C**3a**C) **21** 5′-d(**3a**C**3a**C**3a**C) **21**	41	14.06 ± 0.5
5′-d(**3b**C**3b**C**3b**C) **22** 5′-d(**3b**C**3b**C**3b**C) **22**	57	17.05 ± 0.5
5′-d(**3c**C**3c**C**3c**C) **23** 5′-d(**3c**C**3c**C**3c**C) **23**	62	19.07 ± 0.5
5′-d(TiCATAAiCT**3a3a**AT) **24** 5′-d(AGTATTGACCTA) **25**	43	14.06 ± 0.5
5′-d(TiCATAAiCT**3b3b**AT) **26** 5′-d(AGTATTGACCTA) **25**	47	17.05 ± 0.5
5′-d(TiCATAAiCT**3c3c**AT) **27** 5′-d(AGTATTGACCTA) **25**	49	19.07 ± 0.5

[a] Measurements were performed in 10 mM Na-cacodylate, 10 mM $MgCl_2$, 100 mM NaCl (pH 7) at 5 + 5 μM single strand concentration.

This means that with increasing polarizability of the modified nucleotide residues incorporated (**3a–c**), the stability of the parallel oriented duplex is enhanced (Fig. 3). An analogous result is found for the parallel-stranded duplexes 5′-d(TiCATAAiCT**XX**AT)·5′-d(AGTATTGACCTA) with **X** being either **3a** (**24·25**), **3b** (**26·25**) or **3c** (**27·25**) and iC_d being 5-methyl-2′-deoxyisocytidine (Table 3, Fig. 3).[31]

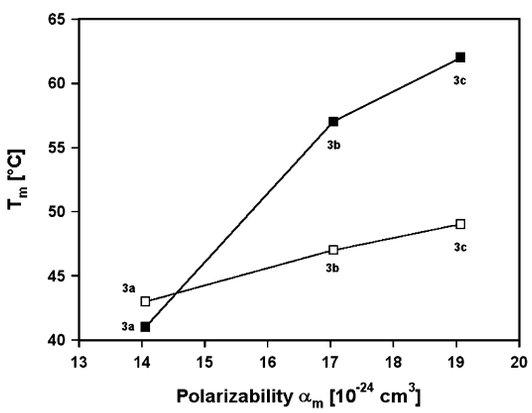

Fig. 3 T_m values of the parallel duplexes 5′-d(**X**C**X**C**X**C)·5′-d(**X**C**X**C**X**C) (■—■, **21·21**, **22·22**, **23·23**, Table 3) and 5′-d(TiCATAAiCT**XX**AT)·5′-d(AGTATTGACCTA) (□—□, **24·25**, **26·25**, **27·25**, Table 3) containing **X** = **3a**, **b** or **c** (Scheme 1) as a function of the molecular polarizability α_m of the base of **X**; iC_d = 5-methyl-2′-deoxyisocytidine.

Conclusion and perspective

It has been clearly demonstrated that enhancement of the molecular polarizability, α_m, of a pendant base-modified nucleotide exerts a stabilizing influence on the duplex stability. Based on the results described above it is also anticipated that the incorporation of a highly polarizable nucleotide residue into the inner part of a parallel or an antiparallel oligonucleotide with a random sequence will enhance duplex stability if the ability for hydrogen bond formation of the Watson–Crick motif is also retained. If this is abolished or at least significantly reduced, the T_m value of the resulting duplex will certainly decrease; this decrease of thermal stability, however, may be partly compensated if the modified nucleobase is highly polarizable.

Experimental

Solid-phase synthesis of oligonucleotides

The synthesis of the oligonucleotides was accomplished on a 1 μmol scale using the appropriate 3′-phosphoramidites of the base-modified 2′-deoxy-β-D-ribonucleosides[26,27,28a–e,29] as well as those of the regular 2′-deoxyribonucleosides being commercially available (Sigma, St. Louis, USA). The synthesis followed the regular protocol of the DNA synthesizer (Model 392 B, Applied Biosystems, Weiterstadt, Germany).[32] The oligonucleotides were recovered from the synthesizer as the 5′-dimethoxytritylated derivatives. After treatment with 25% aq. NH_3 for 12 h at 60 °C to cleave off the nucleobase protecting groups, the 5′-dimethoxytritylated oligomers were purified by reverse-phase HPLC (see below; RP-18 column, 250 × 4 mm, 7 μm, solvent system I). Detritylation was performed with 80% HOAc–H_2O for 2 min at room temperature. Detritylated oligomers were again purified by RP-18 HPLC (solvent system II). Oligonucleotides were desalted on a 4 × 25 mm HPLC cartridge (RP-18 silica gel). Inorganic material was eluted with H_2O (10 ml) while the oligomers were eluted with MeOH–H_2O (3 : 2, v/v). The oligomers were lyophilized on a Speed-Vac evaporator and stored frozen at −23 °C.

For characterization of the oligomers enzymatic tandem hydrolysis was performed using snake-venom phosphodiesterase (EC 3.1.15.1, *Crotallus adamanteus*) and alkaline phosphatase (EC 3.1.3.1, *E. coli*) as described.[33] The reaction mixture was analyzed on reversed-phase HPLC (RP-18, solvent system III). Quantification of the resulting nucleosides was made on the basis of the peak areas which were divided by the absorption coefficients of the nucleoside constituents at λ = 260 nm.

HPLC separation

HPLC was carried out on a 250 × 4 mm PR-18 column (Merck, Germany) on a Merck-Hitachi HPLC apparatus with one pump (Model 655-A-12) connected with a proportioning valve, a variable wavelength monitor (Model 655 A), a controller (Model L-5000), and an integrator (Model D-2000). The solvent gradients, consisting of 0.1 M (Et_3HN)OAc (pH 7.0)–MeCN 95 : 5 (A) and MeCN (B), were used in the following order: gradient I, 3 min 15% B in A, 7 min 15–40% B in A, flow rate 1 ml min^{-1}; gradient II, 20 min 0–20% B in A, flow rate 1 ml min^{-1}; solvent system III: 20 min 100% A, flow rate 0.6 ml min^{-1}.

Melting experiments

The thermal dissociation–association of the oligomers was measured by temperature-dependent UV melting profiles using a Cary 1E UV/VIS spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller; the actual temperature was measured in the reference cell with a Pt-100 resistor. The thermodynamic data of duplex formation were calculated by curve fitting to a two-state-model using the program MeltWin according to ref. 30.

Mass spectrometry

MALDI-TOF mass spectra were run on a BIFLEX III instrument (Bruker Saxonia Analytik GmbH, Leipzig, Germany) in the reflector mode. The average power of the nitrogen laser (337.1 nm) at 20 Hz was 3–4 mW (150–200 μJ per pulse) with a delay time of 600 ns. All measurements were performed using the positive detection mode with the following parameters: dwell time: 1.00 ns, delay: 40000 ns; Uis1: 19.00 kV, Uis2: 15.80 kV, Urefl: 20.00 kV, Ulens: 9.35 kV. The spectra were obtained by overlaying 500–1000 single pulses with a cut-off mass

of 1000 Da. The spectrometer was calibrated using an oligonucleotide calibration standard (Bruker, Part No. 206200) containing a 12-mer (3645.44 Da), a 20-mer (6117.04 Da) and a 30-mer (9191.03 Da).

The sample preparation was performed on Scout MTP MALDI targets (Bruker) as follows: 1 μl of the supernatant of a saturated soln. of recrystallized 3-hydroxypicolinic acid (3-hydroxypyridine-2-carboxylic acid) in double distilled H_2O containing BioRad microbeads AG 50W-X8 (100–200 mesh, NH_4^+-form) was spotted on a target well. A suspension (1 μl) containing 15–20 microbeads in H_2O was added followed by 1 μl of an aq. oligonucleotide soln. (concentration: 0.1 A_{260} units per 10 μl H_2O). The mixture was carefully dried on the target, and the microbeads were removed mechanically with a tip. The following molecular masses (MH^+/Da) of modified oligo(2′-deoxy-β-D-ribonucleotides) were obtained. **10**: calc.: 1793; found:1794. **11**: calc.: 2097; found: 2095. **12**: calc.: 2122; found: 2123. **13**: calc.: 2106; found: 2109. **14**: calc.: 2107; found: 2109. **15**: calc.: 2106; found: 2107. **16**: calc.: 2135; found: 2136. **17**: calc.: 2139; found: 2140. **18**: calc: 2140; found: 2144. **19**: calc.: 2200; found: 2202. **20**: calc.: 2189; found: 2190.

Molecular polarizability

Molecular polarizability values (α_m) were calculated from polarizability increments (central atom and neighboring sphere considering the order and aromaticity of bonds) using the program ChemSketch (version 4.55, provided by Advanced Chemistry Developments Inc., Toronto, Canada; http://www.acdlabs.com).

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