4940–4945 *Nucleic Acids Research, 1996, Vol. 24, No. 24*

Isoguanine quartets formed by d(T_4isoG$_4T_4$): tetraplex identification and stability

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Received August 20, 1996; Revised and Accepted November 4, 1996

ABSTRACT

The self-aggregation of the oligonucleotide d(T_4isoG$_4T_4$) (1) is investigated. Based on ion exchange HPLC experiments and CD spectroscopy, a tetrameric structure is identified. This structure was formed in the presence of sodium ions and shows almost the same chromatographic mobility on ion exchange HPLC as d($T_4G_4T_4$) (2). The ratio of aggregate versus monomer is temperature dependent and the tetraplex of [d(T_4isoG$_4T_4$)]$_4$ is more stable than that of [d($T_4G_4T_4$)]$_4$. A mixture of d(T_4isoG$_4T_4$) and d($T_4G_4T_4$) forms mixed tetraplexes containing strands of d(T_4isoG$_4T_4$) and d($T_4G_4T_4$).

INTRODUCTION

Oligodeoxynucleotides containing short runs of guanines, such as d(TG$_4$T), d(T_4G_4) or d(T_4G_4T), exhibit a tendency to aggregate in aqueous solution (1). The tetrameric structures are formed by Hoogsteen-type base pairs, the strands show parallel orientation and cations are bound in the center of the structure (Fig. 1a). These tetrameric aggregates, which are naturally occurring in telomeres (2), are normally identified by their strongly retarded mobility in gel electrophoresis. However, the protocol of electrophoresis is time consuming and short DNA fragments with <12 bases are difficult to separate. Furthermore, oligonucleotides with an identical number of charges but showing structural differences are difficult to resolve.

High performance anion exchange chromatography has successfully been used for the preparative and analytical separation of synthetic oligonucleotides (3). Oligonucleotides of different length can be separated effectively by ion exchange chromatography according to the increasing number of negative charges (4). The use of this method can provide '*n*' from '*n* – 1' chain length resolution, particularly for samples of *n* < 30 bases in length (5). As the separation of molecules depends on the number of phosphodiester charges, it can be used to separate oligonucleotide aggregates from single-stranded species. This manuscript reports the identification of tetraplexes formed by oligodeoxyribonucleotides containing consecutive isoG$_d$ residues and describes their separation from single-stranded molecules. In this context, the first quartet structure of the isoguanine-containing oligodeoxynucleotide d(T_4isoG$_4T_4$) (**1**) is established. Also, the formation of mixed quartets containing dG and isoG$_d$ residues will be discussed.

RESULTS AND DISCUSSION

Recently, our laboratory as well as others have reported on the synthesis and properties of oligoribo- and oligodeoxyribonucleotides containing isoguanine as a base (6–11). These compounds show unusual properties with respect to chemical reactivity (12) and to base pairing. It was observed that isoguanine forms stable duplexes with isocytosine within oligonucleotides of antiparallel chain orientation (7,13). Base pairing is also observed between isoguanine and cytosine within oligonucleotide duplexes having parallel chains (11,12,14). Apart from the pairing models with complementary bases, poly(isoguanylic acid) has been shown to form aggregates by self-association (15). Aggregation of an isoguanine ribonucleoside was also detected and a tetrameric structure (Fig. 1b) has been proposed different from that of guanosine (16). Nevertheless, defined species were neither detected nor separated in the case of either deoxynucleosides or oligonucleotides.

The isoguanine-containing oligonucleotide d(T_4isoG$_4T_4$) (**1**) has been prepared by solid phase synthesis using the diphenylcarbamoyl residue for protection of the 2-oxo group (14,17). As it aggregates (14) in aqueous solution elucidation of the aggregate structure is of interest. The oligonucleotide d($T_4G_4T_4$) (**2**) was prepared for comparison. Formation of the aggregate was achieved by preheating the samples to 90°C followed by a 15 min storage at –20°C in a refrigerator (see Materials and Methods). All oligonucleotide samples were treated in the same manner and then applied to an ion exchange HPLC on a NucleoPac PA-100 column. Elution was performed with Tris–HCl buffer, pH 8.0, by increasing the NaCl concentration from 0.2 to 0.8 M. The temperature was controlled by an HPLC oven. The ion exchange chromatography profile of d(T_4isoG$_4T_4$) shows two well-separated peaks with greatly different retention times (Fig. 2). Both peaks contain the same oligonucleotide **1**. The fast migrating zone contains single-stranded oligomer **1**, while the slow migrating peak represents an aggregate. The intensity of the peaks is temperature dependent. The peak of the aggregate becomes smaller when the temperature is raised from 30 to 90°C. At the same time the peak of the monomer becomes larger (Fig. 2 and Table 1). The sum of the peak areas is nearly constant. We have proven that equillibration of the species taking place on the column is comparatively slow. This explains the formation of sharp peaks. Temperature-dependent decay of the tetraplex occurs in the injection coil, which is located in the oven, before entry of the sample into the column.

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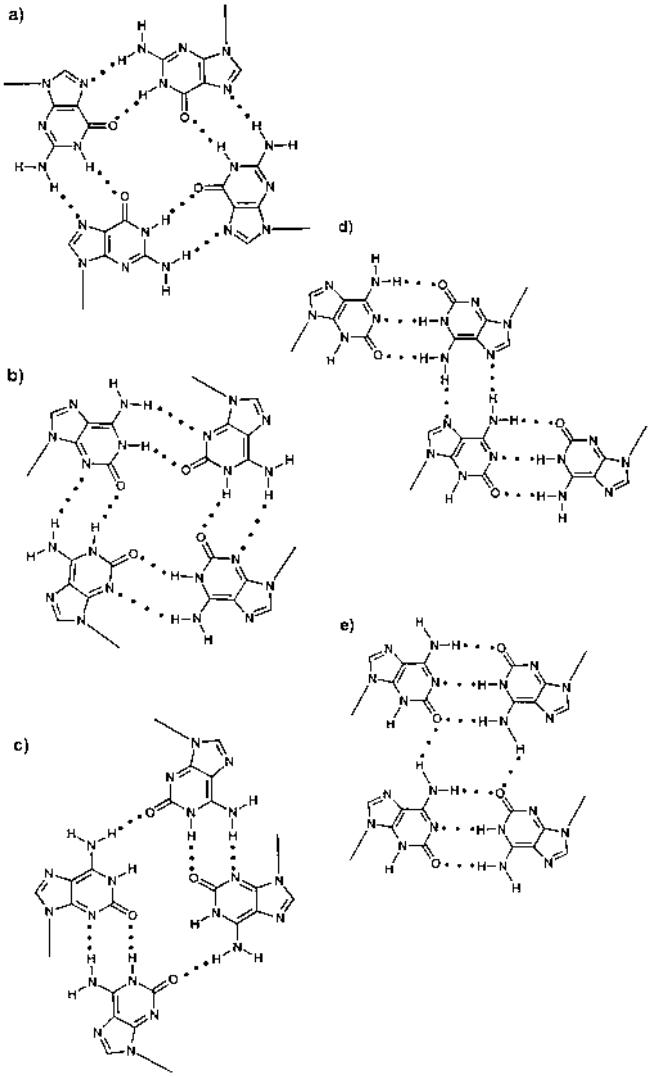

Figure 1. Tetrameric structures of 2′-deoxyguanosine (**a**) (1), isoguanosine (**b**) (16), poly(isoG) (**c**) (15) and the aggregate structure of isoguanine, pyranosyl-RNA (**d**, **e**) (22).

Table 1. Retention times of the oligonucleotides on ion exchange HPLC[a]

Oligomer	*T* (°C)	t_R (min) monomer/tetramer	Area (ratio) monomer/tetramer
$d(T_4isoG_4T_4)$	30	14/25	1/2
	90	16/32	2/1
$d(T_4G_4T_4)$	30	14/25	5/4
	90	16/–	8/0

[a]The solvent system of 25 mM Tris–HCl containing 1 mM EDTA buffer, pH 8.0, MeCN 90:10 (A) and A containing 1.0 M NaCl (B) was used with the following gradient: 30 min 20–80% B in A, 5 min 80% B in A, 5 min 80–20% B in A, with a flow rate of 0.75 ml/min. The chromatogram was recorded at 260 nm.

The same experiments as described for **1** have been performed with the guanine-containing oligomer **2**. Ion exchange HPLC at 30°C also revealed one fast and one slow migrating zone (Fig. 3a). Decay of this aggregate under the conditions as described above is also temperature dependent. However, in contrast to **1**, the slow

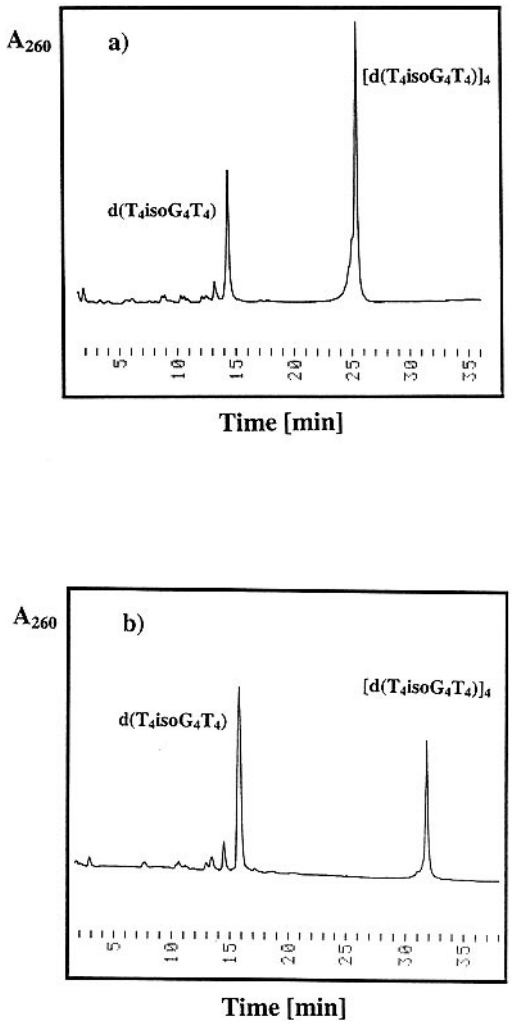

Figure 2. Ion exchange HPLC elution profile of 5′-$d(T_4isoG_4T_4)$ at 30°C (**a**) and at 90°C (**b**). For the gradient see Materials and Methods.

migrating peak disappears on increasing the temperature of the oven to 90°C (Fig. 3b). This indicates that the $d(T_4isoG_4T_4)$ aggregate is more stable than that of $d(T_4G_4T_4)$. This difference can be explained by a higher electron density of the 2-oxo group of isoguanine compared with the 6-oxo group of guanine, leading to a more favorable co-ordination complex (12). The almost identical retention times of the aggregates of $d(T_4G_4T_4)$ and $d(T_4isoG_4T_4)$ (Table 1) point to similar structures with the same number of charges. As both compounds are isomers, the complexes must have the same stoichiometry. As a result, a quartet structure is suggested for $d(T_4isoG_4T_4)$ (Fig. 4a), which has the same symmetry as $d(T_4G_4T_4)$ (18).

In general, separation of DNA fragments occurs on ion exchange resins according to their number of charges. Nevertheless, elution is also influenced by the relative base composition of the fragments (19). Abnormal elution patterns were observed for DNA fragments of the same length but differing in sequence-directed conformation. It was observed that two DNA fragments of the same length which co-migrate in agarose gels have slightly different chromatographic mobility on ion exchange HPLC and can be separated efficiently (4). From several chromatographic runs of

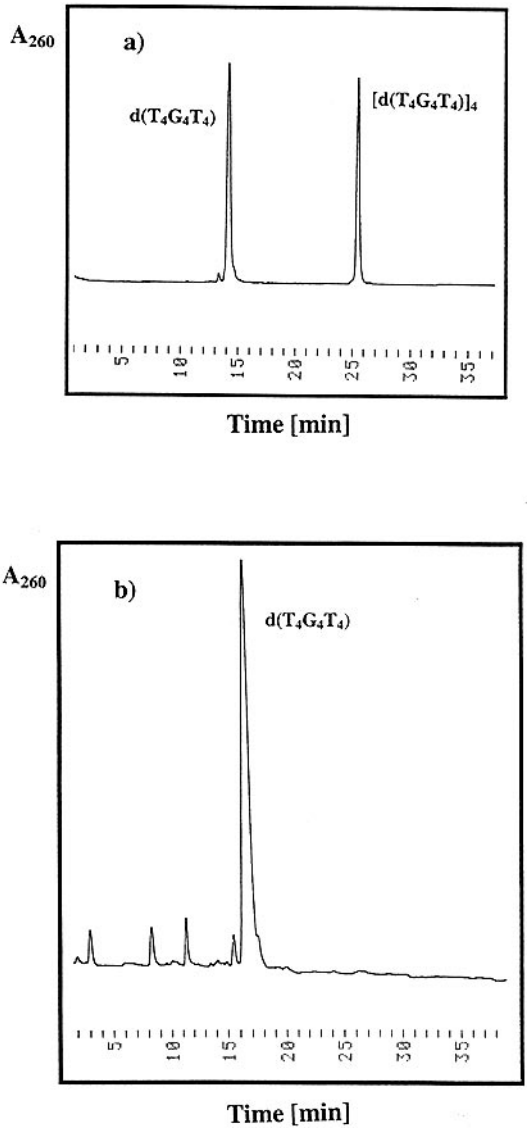

Figure 3. Ion exchange HPLC elution profile of 5′-d($T_4G_4T_4$) at 30°C (**a**) and at 90°C (**b**). For the gradient see Materials and Methods.

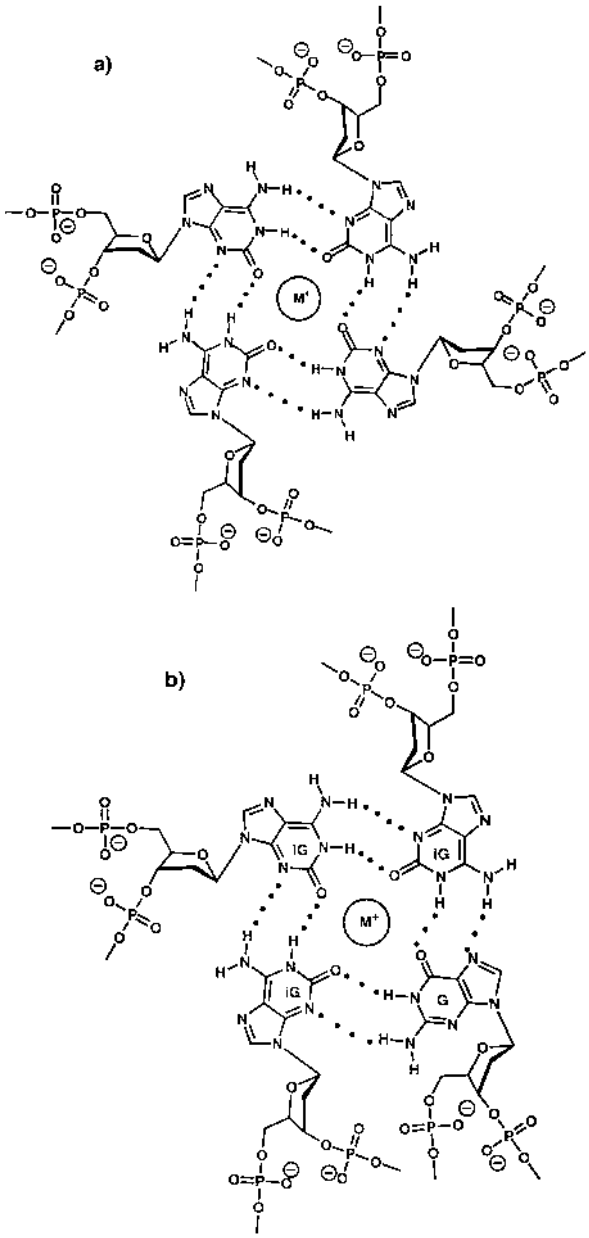

Figure 4. Proposed quartet structures of 5′-d(T_4isoG$_4T_4$) (**a**) and one of the possible quartet structures of mixed aggregates formed by a mixture of d($T_4G_4T_4$) and d(T_4isoG$_4T_4$) (**b**).

oligonucleotides **1** and **2** it was observed that the tetraplex of d(T_4isoG$_4T_4$) migrates slightly faster than that of d($T_4G_4T_4$). This enables us to observe if a strand of the d(T_4isoG$_4T_4$) tetraplex can be replaced by one of d($T_4G_4T_4$) and *vice versa*. These mixed aggregates (heterotetraplexes) should be resolvable on the ion exchange column. For this purpose a 1:1 mixture of oligonucleotides **1** and **2** was prepared and analyzed by ion exchange HPLC. At least four slow migrating peaks appeared in the elution profile (Fig. 5), whereas the monomeric compounds show only one peak. The appearance of these assemblies can be explained by the formation of heterotetramers which consist of single strands of d(T_4isoG$_4T_4$) and d($T_4G_4T_4$). As the retention time of the mixed aggregates is very similar to the pure oligomer quartets, they must have been formed by the same number of strands, namely four (see for example Fig. 4b).

Self-assembled quartets of guanine-rich oligonucleotides have already been studied in detail and tetraplex structures were established by single crystal X-ray analysis (20,21) as well as by NMR spectroscopy (21). The tetrameric structure is formed by hydrogen bonds with the participation of the ring nitrogens N-1 and N-7, as well as of the 2-amino- and 6-oxo substituents, according to Figure 1a. Based on the substituent pattern of isoguanine and guanine, the quartets of d(T_4isoG$_4T_4$) and d($T_4G_4T_4$) must have different structures. Earlier, an isoG quartet structure was proposed by D.Shugar for poly(isoG) (15). This structure has a 2-fold symmetry and contains two sets of hydrogen bonds (Fig. 1c). The bases are held together using the ring nitrogens N-1 and N-3 as well as the 6-amino and 2-oxo groups. Recently, another structure with a C_4 symmetry was proposed for the ribonucleoside isoguanosine (16). In this case

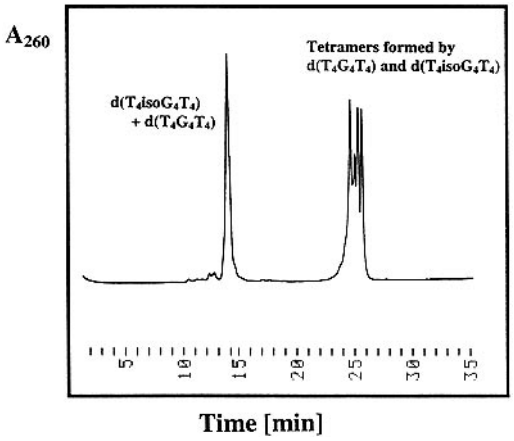

Figure 5. Ion exchange HPLC elution profile of the mixture of 5′-d($T_4G_4T_4$) and 5′-d(T_4isoG$_4T_4$) at 30°C. For the gradient see Materials and Methods.

also the ring nitrogens N-1 and N-3 participate in hydrogen bonding (Fig. 1b), but not N-1 and N-7, as observed in the G quartet. Nevertheless, other structures can be discussed. Very recently, two possible symmetrical structures for aggregates that contain the 1-H and 3-H tautomers of isoguanine pyranosides were proposed by Eschenmoser (22), illustrated in Figure 1d and e.

Our chromatographic experiments cannot discriminate between those structures. However, as the isoG tetrad of Figure 1b contains cavities similar to those found for the G quartet, this structure is very likely. It forms a similar inner shell of 16 atoms with the same number of hydrogen bonds. Therefore, the central cavity of the isoG quartet is ideal for selective metal ion binding, e.g. for Na^+ coordination, and has been shown that isoguanosine self-assembly and ion coordination are highly cation dependent. It is difficult to envisage how the other structures shown in Figure 1d and e can form those cavities. Regarding self-assembly of the oligonucleotide d(T_4isoG$_4T_4$), a tetraplex structure as shown in Figure 4a with parallel strand orientation is very probable. Nevertheless, from model building of an aggregate according to the structure of Figure 4a it is apparent that this structure might show steric strain. This structure is also adaptable to the heterotetraplexes formed by a mixture of **1** and **2**. As shown in Figure 4a and c, the size of the ion cavities is similar in the cases of [d(T_4isoG$_4T_4$)]$_4$ and [d(T_4isoG$_4T_4$)]$_3$[d($T_4G_4T_4$)]. Furthermore, the formation of hetero-tetraplexes implies that base pairing of guanine and isoguanine is strong. Guanine–isoguanine base pairing has recently been observed in the case of pyranosyl-RNA (22).

It has been reported that G quartets formed by oligonucleotides show very characteristic CD spectra which are temperature dependent (23). The CD spectrum of d($T_4G_4T_4$) (Fig. 6b) is characterized by a positive band with a high ellipticity around 270 nm with a trough at 240 nm. The relatively broad peak around 270 nm is shifted to a higher wavelength in temperature-dependent experiments. The conservative spectrum is the typical signature of a G tetraplex. The CD spectrum of d(T_4isoG$_4T_4$) (Fig. 6a) is different. It shows a strong ellipticity at 305 nm and only a slightly positive band at 275 nm, as well as a trough at 240 nm. The band at 305 nm is comparable with that at 270 nm in the case of the G tetraplex. The differences depend on the characteristics of the UV spectrum of 2′-deoxyisoguanosine, which exhibits maxima at 247 and 292 nm (H_2O). As the CD band at 305 nm is exclusively

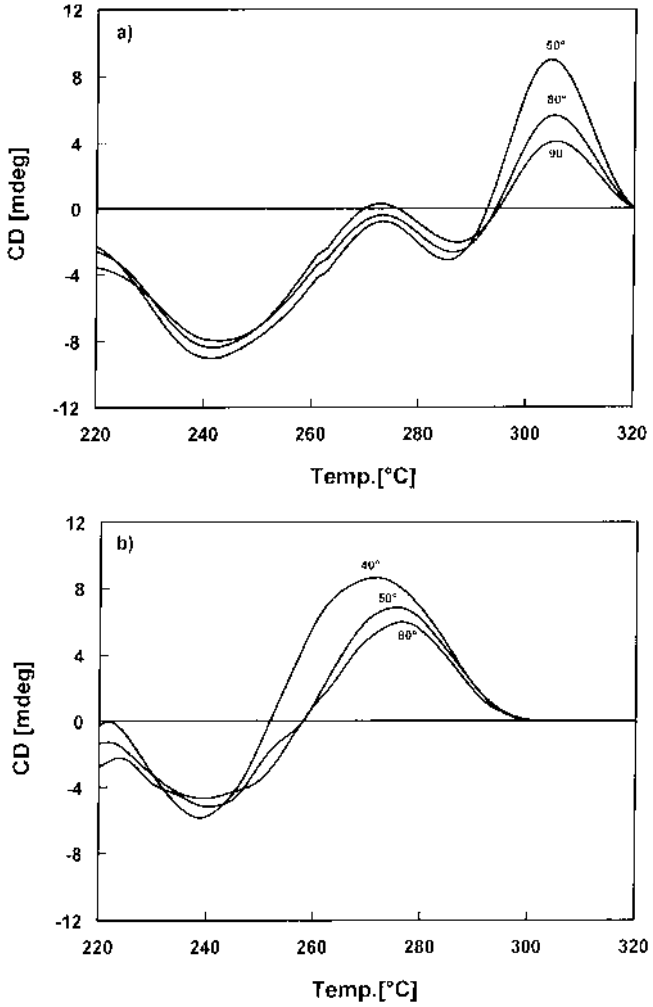

Figure 6. CD spectra of oligonucleotide **1** at 50, 80 and 90°C (**a**) and oligonucleotide **2** at 40, 50 and 80°C (**b**) in 1 M NaCl, 10 mM $MgCl_2$, 10 mM Na cacodylate (pH 7). Oligomer concentration 5.0 µM.

caused by the isoguanine residues of d(T_4isoG$_4T_4$), the temperature-dependent changes in this band (Fig. 6a) can be related to the amount of tetraplex species present in solution. For structural details of isoG quartet structures single crystal X-ray analysis of oligonucleotides is necessary, similar to compounds containing a G quartet (20).

MATERIALS AND METHODS

General

Reverse phase HPLC was carried out on a 4 × 250 mm RP-18 (10 µm) LiChrosorb column (Merck) with a Merck-Hitachi HPLC pump (model 655 A-12) connected to a variable wavelength monitor (model 655-A), a controller (model L-5000) and an integrator (model D-2000). The solid phase synthesis of oligo-nucleotides was carried out on an automated DNA synthesizer (Applied Biosystems model ABI 380 B for H-phosphonate synthesis). The phosphonates of dG and dT were purchased from Sigma (St Louis, MO) and the CPG (controlled pore glass; 30–50 µmol immobilized protected 2′-deoxynucleoside/g solid support) from Milligene (Eschborn, Germany). Snake venom

phosphodiesterase (EC 3.1.4.1, *Crotallus durissus*) and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli*) were products of Boehringer Mannheim (Mannheim, Germany). All other reagents are commercially available and were used as received. The solvents were purified and dried according to standard procedures.

RP-18 HPLC separation of oligonucleotides

HPLC was carried out as reported (6). The following solvent gradients were used: 0.1 M Et_3NHOAc, pH 7.0, MeCN 95:5 (A) and CH_3CN (B). They were used in the following order: gradient I, 3 min 15% B in A, 12 min 15–40% B in A, 5 min 40–15% B in A with a flow rate of 1.0 ml/min; gradient II, 20 min 0–20% B in A with a flow rate of 1.0 ml/min; gradient III, 20 min 100% A with a flow rate of 0.6 ml/min.

CD spectra

The CD spectra were measured in 1 cm cuvettes using a Jasco 600 spectropolarimeter (Tokyo, Japan) connected to a temperature controller (RCS 6; Lauda, Germany) and a bath (RK 20; Lauda, Germany). CD spectra of the oligonucleotides were measured in 1 M NaCl, 10 mM $MgCl_2$, 10 mM Na cacodylate, pH 7.0 (oligomer concentration 5.0 µM). The spectra were recorded from 220 to 320 nm and the data were treated using DP-J600/PC System V.1.31.

Solid phase synthesis of oligonucleotides 1 and 2

Synthesis was performed on a DNA synthesizer (model 380 B; Applied Biosystems, Weiterstadt, Germany). The oligonucleotide d(T_4isoG$_4T_4$) was synthesized using the DPC-protected phosphonate (17) according to a modified protocol (17) and d($T_4G_4T_4$) was synthesized following the regular protocol for phosphonates (24). Deprotection of the oligonucleotides was performed in aqueous 25% NH_3 at 60°C for 18 h. The 5′-DMT-protected oligomers were purified by HPLC on a 250×4 mm RP-18 column (gradient I), isolated and the 4,4′-dimethoxytrityl residues were removed by treatment with 2.5% dichloroacetic acid, CH_2Cl_2 solution for 5 min at room temperature. The detritylated oligomers were purified by HPLC with gradient II. The oligomers were desalted on a 4 cm column (RP-18, silica gel) using H_2O for elution of the salt, while the oligomers were eluted with MeOH, H_2O (3:2). The oligonucleotides were lyophilized on a Speed-Vac evaporator to yield colorless solids which were dissolved in 100 µl H_2O and stored frozen at –18°C.

Ion exchange HPLC

The ion exchange chromatography was performed on a 4× 50 mm NucleoPac PA-100 column (P/N 043018; Dionex, USA) using a Merck-Hitachi HPLC apparatus with one pump (model 655 A-12) connected to a proportioning valve, a variable wavelength monitor (model 655-A) and a controller (model L-5000), which connected with an integrator (model D-2000). A column oven (model L-7350; Merck, Germany) was used to control the temperature of the ion exchange column as well as the injector loop. The oligonucleotide samples were prepared as follows. A sample of 0.15 A_{260} units was dissolved in H_2O (100 µl). The solution was heated to 90°C for 2 min, brought to room temperature (5 min) and kept in a refrigerator (–20°C) for 15 min to be frozen. Then the sample was brought to room temperature and injected into the system, which had been preheated to the required temperature. The column was eluted using the following systems: 25 mM Tris–HCl containing 1 mM EDTA buffer, pH 8.0, MeCN 90:10 (A); 25 mM Tris–HCl, 1.0 M NaCl containing 1 mM EDTA buffer, pH 8.0, MeCN 90:10 (B). The following gradient was used: 30 min 20–80% B in A, 5 min 80% B in A, 5 min 80–20% B in A, with a flow rate of 0.75 ml/min. The spectrum was recorded at 260 nm. For results see Figures 2 and 3 and Table 1.

Table 2. Retention times and analytical data of oligonucleotides

	d(T_4isoG$_4T_4$) (1)	d($T_4G_4T_4$) (2)
Retention time (min)[a]	19.6	18.2
Thermal hyperchromicity (%)[b]	15	9
Yield (A_{260} units)	32	42
Nucleoside composition	2:1 (dT/isoG$_d$)	2:1 (dT/dG)

[a]RP-18 HPLC with gradient II.
[b]At 260 nm.

Composition analysis of oligonucleotides

The oligonucleotides (0.2 A_{260} units) were dissolved in 200 µl 0.1 M Tris–HCl buffer, pH 8.3, and treated with snake venom phosphodiesterase (3 µl) at 37°C for 45 min and alkaline phosphatase (3 µl) at 37°C for 30 min. The mixture was analyzed by reversed phase HPLC (RP-18, gradient III, at 280 nm for **1** or 260 nm for **2**). 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} isoG$_d$ 4300, dG 11 700, dT 8800; ε_{280} isoG$_d$ 7300, dT 6337) (Table 2).

ACKNOWLEDGEMENTS

We thank Dr E.Uhlmann for helpful discussions. Financial support by Hoechst AG and generous gifts of enzymes by Boehringer Mannheim GmbH are gratefully acknowledged.

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