Fluorescent Guanine

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8-Vinyl-2′-deoxyguanosine as a Fluorescent 2′-Deoxyguanosine Mimic for Investigating DNA Hybridization and Topology**

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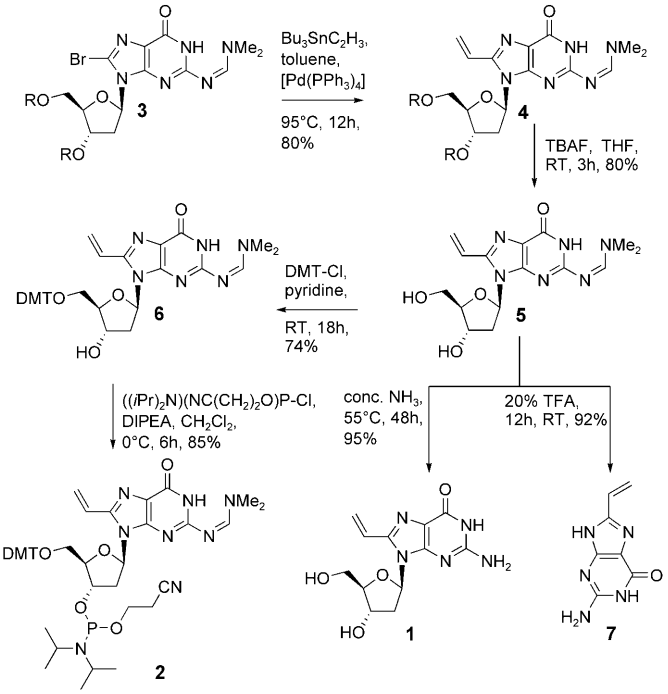
Fluorescent analogues of the natural nucleobases are widely used as molecular probes, for example, for investigating oligonucleotide conformation, detecting DNA hybridization, or single nucleotide polymorphisms.[1] Typical approaches are fluorophore-base conjugates[2] in which common fluorophores are tethered to the nucleobases, or expanded nucleobases[3] or nucleotides bearing a fluorescent aromatic moiety instead of a natural nucleobase.[4] Molecular probes for investigating oligonucleotide conformation and dynamics, however, should perfectly mimic the natural nucleobases to minimize structural disturbances and thus resemble the natural nucleobases with respect to their molecular shape and hydrogen-bonding pattern.[5] The earliest known and extensively used adenine analogue 2-aminopurine (AP) is the most prominent example of this class of molecules.[6] AP was used in a number of applications including investigation of base stacking,[7] extruded nucleobases,[8] metal-ion coordination,[9] and protein-induced conformational changes of DNA secondary structure.[10] Other fluorescent nucleobase analogues closely resemble their parent nucleobases, mostly pyrimidine and adenine mimics which include the title compound 8-vinyl-adenine, an adenine analogue.[11] There are few examples for guanine analogues that match the requirements for a suitable fluorescent probe, mainly isoxanthopterins, 8-azaguanine which exhibits anion fluorescence at higher pH values, 8-furyl-guanine which has yet to be incorporated into oligonucleotides, and very recently 8-(2-pyridinyl)-guanine.[11d,12] Our approach is directed towards the synthesis of a guanine analogue that maintains the nucleobase core while being made fluorescent in its neutral form by minimal substitution. Furthermore, the fluorescent nucleoside should be able to adopt both the nucleoside *syn* and *anti* conformation, which is a prerequisite for the formation of various G-quadruplex structures and the left-handed Z-DNA helix.

The newly introduced fluorescent 2′-deoxyguanosine mimic 8-vinyl-2′-deoxyguanosine (VdG, **1**) matches these requirements while its emission properties are very sensitive towards changes within its microenvironment, such as the formation of different quadruplex structures. 2′-Deoxyguanosine was used as a starting material in the synthesis of the VdG phosphoramidite building block **2**. Bromination at the C8 position and subsequent protection of the exocyclic amino group and the hydroxy groups yielded compound **3** (Scheme 1).[13] Nucleoside **3** was subjected to Stille coupling



Scheme 1. Synthesis of the VdG phosphoramidite building block **2**, nucleoside **1** and nucleobase **7**. R = TBDMS. DIPEA = *N*,*N*-diisopropylethylamine, DMT = dimethoxytrityl, TBDMS = *tert*-butyldimethylsilyl, TBAF = tetrabutylammoniumfluoride, TFA = trifluoracetic acid.

with tributylvinyltin to give the vinyl-substituted 2′-deoxyguanosine derivative **4** in good yields. TBAF deprotection of the TBDMS groups yielded nucleoside **5** which was converted into the 5′-DMT protected compound **6**.[14] The VdG phosphoramidite building block **2** was generated using an established method.[15] In addition, nucleoside **1** and the respective nucleobase **7** were prepared from the protected nucleoside **5**.

An extinction coefficient of $(7.2 \pm 0.03)\ \mathrm{L\,mmol^{-1}\,cm^{-1}}$ at 260 nm was determined for VdG, which because of a red-shifted absorption maximum, is distinctly lower than the corresponding value of 2′-deoxyguanosine ($12.18\ \mathrm{L\,mmol^{-1}\,cm^{-1}}$)[16]. VdG exhibits the highest emission

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when irradiated at 277 nm but excitation at wavelengths up to 325 nm is also feasible allowing for site-specific probing of larger oligonucleotides. The quantum yield of VdG was determined relative to the respective AP nucleoside and found to be 0.72 ± 0.03 (see Supporting Information). The fluorescence properties of nucleoside **1** were examined at different pH values. The fluorescence intensity appeared to be unaffected by changes of pH value within the physiological relevant region between pH 5–9. The fluorescence intensity at lower and higher pH values is affected by protonation or deprotonation of the vinylguanine moiety (Supporting Information). A partial depurination was observed upon prolonged exposure to acidic solvents (3 days at pH 3).

The base pairing of VdG incorporated in double stranded DNA (dsDNA) was investigated by temperature dependent UV spectroscopy. The duplex stability of 5′-d(GATCGCTAG) + 5′-d(CTAGCGATC) (melting temperature T_m = 32 °C) was compared to the stabilities of an oligomer that contains VdG in the central position 5′-d(GATCVdGCTAG) (**O1**) together with the complementary strands 5′-d(CTAGXGATC) (X = dC, dT, dG, dA, abasic site) varying with respect to the opposed nucleobase position (Figure 1). The double strand with VdG–dC base pair turned out to be only 4 °C less stable, whereas mismatch pairing with dG or dT led to a drop of stability of about 14 °C. Stable duplexes were not even detected for dA and abasic site mismatches.

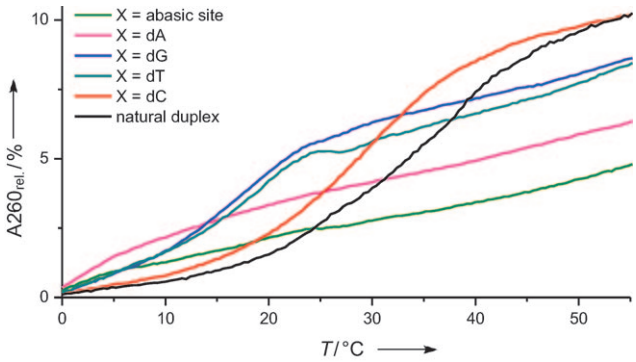

Figure 1. UV melting curves of duplexes formed between 5′-d(GATCVdGCTAG) (**O1**) and the respective complementary strand 5′-d(CTAGXGATC) (X = dC, dA, dG, dT, abasic site) and for comparison the natural dsDNA 5′-d(GATCGCTAG) + 5′-d(CTAGCGATC) (2.5 μM, 10 mM phosphate buffer pH 7, 100 mM NaCl, 260 nm).

Fluorescence of VdG is likely to depend on the neighboring nucleobases. Therefore, a series of oligonucleotides with the general sequence 5′-d(GATXVdGXTAG) (X = dC (**O1**), dT (**O2**), dA (**O3**), dG (**O4**)) was investigated. A pronounced 9–12-fold reduction of emission intensity was observed upon incorporating VdG (**1**) into single-stranded DNA (ssDNA) oligonucleotides that do not adopt distinct conformations (Supporting Information). VdG fluorescence was quenched most effectively by neighboring guanine or thymine bases whereas adjacent cytosine and adenine moieties led to higher residual emission (Supporting Information). Since quenching caused by neighboring bases can be monitored most meaningful within a uniform topology, fluorescence was determined also after formation of B-DNA double strands by addition of the respective complementary strands 5′-d(CTAXCXATC) (X = dG (**O5**), dA (**O6**), dT (**O7**), dC (**O8**)).

Quenching of VdG fluorescence in dsDNA turned out to be comparable but more pronounced than single-strand results. Neighboring guanine and thymine nucleobases led to highest quenching of VdG fluorescence whereas adjacent cytosine and especially adenine were less influential (Figure 2).

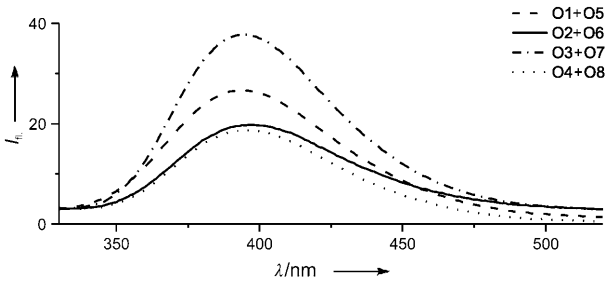

Figure 2. Emission spectra of duplexes **O1** + **O5**, **O2** + **O6**, **O3** + **O7**, **O4** + **O8** (duplex concentration: 500 nM, 5 °C, 10 mM phosphate buffer pH 7, 100 mM NaCl, excitation wavelength: 277 nm).

DNA double-strand formation can be monitored by VdG incorporation as indicated by decreasing emission upon formation of B-form helices. This was indicated by comparing the fluorescence spectra of the oligomers **O1** + **O5**, **O2** + **O6**, **O3** + **O7**, **O4** + **O8**, detected as single-stranded oligomers at temperatures above the melting temperature (40 °C/50 °C) and duplexes at 5 °C. The intensity of VdG emission was found to be virtually identical with samples containing only the single-stranded oligomers **O1**, **O2**, **O3**, and **O4** at elevated temperatures whereas significant fluorescence quenching was observed at temperatures below the respective melting temperatures of the duplexes (Figure 3 a,b and Supporting Information).

Duplex formation with B-DNA topology was confirmed by comparison of circular dichroism (CD) spectra and UV melting curves of VdG functionalized duplexes (**O1** + **O5**, **O2** + **O6**, **O3** + **O7**, **O4** + **O8**) with the respective guanine-containing duplexes **O9** + **O5**, **O10** + **O6**, **O11** + **O7**, **O12** + **O8** (5′-d(GATXGXTAG), X = dC (**O9**), dT (**O10**), dA (**O11**), dG (**O12**)). VdG incorporation into dsDNA appeared to have only negligible effects on the formation of a B-form DNA helix and on the double-strand stabilities (ΔT_m = 1–5 °C) (Figure 3 c,d and Supporting Information).

In a second application, the VdG fluorophore was used as sensor for different DNA G-quadruplex topologies. VdG was incorporated into human telomeric DNA 5′-d[AGGG-(TTAGGG)$_3$T] (**O13**). In the presence of its complementary strand a regular B-DNA duplex is formed, whereas the single-stranded telomeric DNA folds into quadruplex structures with topologies that depend on temperature, pH value, and the respective metal ion.[17] In aqueous NaCl solution a quadruplex structure is predominant with two edgewise and one diagonal connecting TTA loops between the respective

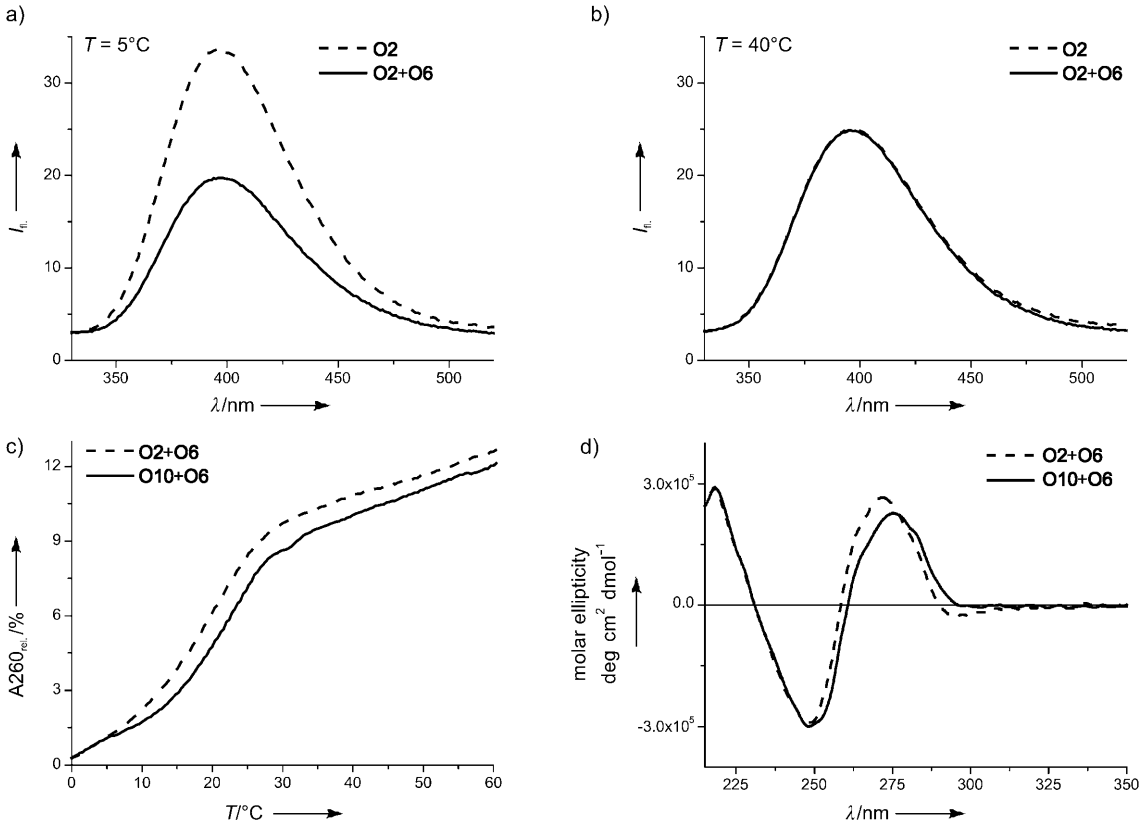

Figure 3. Fluorescence quenching upon double-helix formation, for **O2** and **O2**+**O6**. Emission spectra of **O2** and **O2**+**O6** at a) 5 °C. b) 40 °C. (500 nM, 10 mM phosphate buffer pH 7, 100 mM NaCl, excitation wavelength: 277 nm). c) Melting curves of VdG modified duplex **O2**+**O6** and guanine duplex **O10**+**O6** (2.5 μM, 10 mM phosphate buffer pH 7, 100 mM NaCl, 260 nm). d) CD spectra of VdG modified duplex **O2**+**O6** and guanine duplex **O10**+**O6** (5 μM, 10 mM phosphate buffer pH 7, 100 mM NaCl).

triple-G repeats (Figure 4).[17a–c] In aqueous KCl solution, however, the predominant quadruplex forms are characterized by a double chain reversal connecting TTA loop spanning one flank of the quadruplex core (Figure 4b).[17c] To examine VdG as a fluorescence sensor with different roles in the quadruplex (central/terminal position and *syn*/anti conformation) the telomeric sequence 5′-d[AGGG-(TTAGGG)$_3$T] (**O13**) was modified with the VdG nucleotide at positions G3, G4, or G15, respectively. The emission properties of the VdG-functionalized oligonucleotides 5′-d[AGVdGG(TTAGGG)$_3$T] (**O14**), 5′-d[AGGVdG-(TTAGGG)$_3$T] (**O15**), and 5′-d[A-(GGGTTA)$_2$GVdGGTTAGGGT] (**O16**) were investigated in aqueous KCl and NaCl solution.

The influence of VdG incorporation on the thermal stability of the respective quadruplex folds and the B-type duplexes formed by oligomers **O14**–**O16** with their complementary strand d[A(CCCTAA)$_4$T] (**O17**) was investigated by means of temperature-dependent UV spectroscopy. A slight rise in the thermal stability of the duplex ($\Delta T_m = +(1–2)$ °C) and quadruplex structures ($\Delta T_m = +(0–5)$ °C) was observed (see Supporting Information). Therefore, VdG incorporation does not significantly affect the stability of telomeric DNA topologies.

Furthermore, CD spectra of the respective oligomers **O14**–**O16** were compared to the spectra obtained from unmodified **O13** in NaCl and KCl solution. For duplex formation with **O17** the CD spectra were found to be almost identical (Figure 5, and Supporting Information). The CD spectra of all single-stranded oligomers **O13**–**O16** clearly indicated the formation of NaCl and KCl-forms irrespective of the VdG modification and were in agreement with published data (Figure 5, and Supporting Information).[19] Only slight deviations with respect to the intensity of the respective Cotton effects were observed in some cases when comparing the spectra of **O13** with the CD data of **O14**, **O15**, and **O16** (Figure 5, and Supporting Information). Three of the quadruplex structures require the VdG nucleotide in *syn* and three in *anti* conformation indicating that the VdG nucleotide can be incorporated almost equally well as a mimic for *anti*-dG and for *syn*-dG.

The fluorescence properties of the VdG modified oligomers **O14**–**O16** were closely correlated to the respective topology. The emission spectra of the duplexes **O14**–**O16** + **O17** exhibited emission maxima at 398 nm (Figure 6, and Supporting Information) as it was observed in the case of duplex formation of oligomers **O1**–**O4** (Figure 2); no salt dependency was indicated.

The emission maxima of the quadruplex forming oligomers **O14**–**O16** were blue shifted by approximately 4–8 nm with respect to the double-stranded oligomers (Figure 6, and Supporting Information). It can clearly be differentiated,

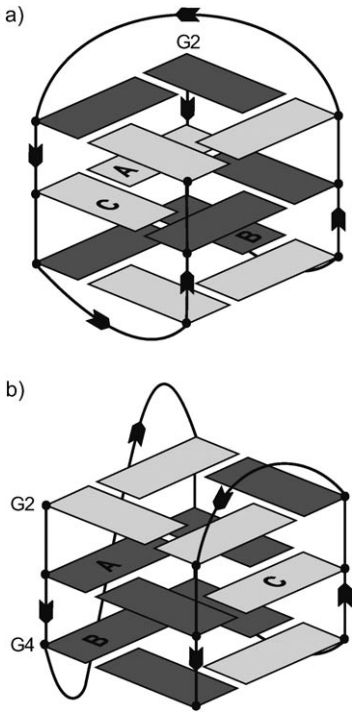

Figure 4. Schematic representation of quadruplex solution structures adopted by human telomeric DNA a) NaCl form. b) KCl form.[18] *Syn* dG residues are light gray, *anti* dG residues are dark gray, arrows indicate the strand orientation (5′ to 3′). The respective positions of the VdG substitution are indicated with capital letters A (**O14**), B (**O15**), C (**O16**).

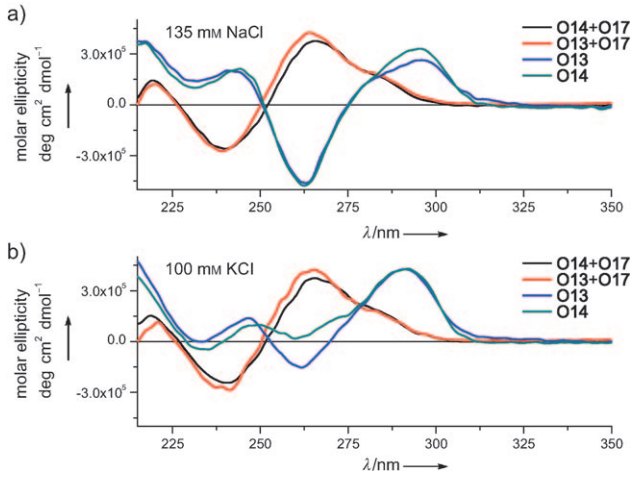

Figure 5. CD spectra of single-stranded human telomeric DNA oligonucleotides **O13** and **O14** and the respective duplexes with the complementary strand **O17** under a) NaCl conditions (4 μM, 5 °C in 10 mM phosphate buffer pH 7, 135 mM NaCl) and b) KCl conditions (4 μM, 5 °C, 10 mM phosphate buffer pH 7, 100 mM KCl).

whether VdG-labeled DNA oligomers form monomers, dimers, or a tetramer fold.

The differentiation of the NaCl and KCl quadruplex through the VdG nucleotide emission was demonstrated by an almost threefold higher emission for **O14** and **O16** in the respective NaCl form than the KCl form (Figure 6, Supporting Information). For this topological differentiation, VdG in

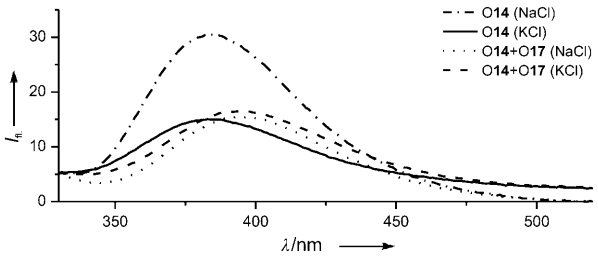

Figure 6. Emission spectra of single-stranded VdG modified human telomeric oligonucleotide **O14** and the duplex **O14** + **O17** in the presence of NaCl and KCl (500 nM, 10 mM phosphate buffer pH 7, 135 mM NaCl or 100 mM KCl, excitation wavelength: 277 nm).

a central G-tetrad layer is required, because the emission spectra of both quadruplex forms of oligomer **O15** with VdG in the terminal layer were indistinguishable. Nevertheless, quadruplex formation was still detectable for oligomer **O15** by a twofold higher emission intensity compared to the respective duplex. Overall, VdG can be incorporated in different kinds of DNA topologies. Fluorescence readout of VdG was found to be indicative for monomeric, double helical and quadruplex secondary structures, quadruplex topology, and position of the G-tetrad layer.

8-Vinyl-2′-deoxyguanosine (VdG) was developed as a novel fluorescent 2′-deoxyguanosine analogue, which resembles the guanine base pairing in DNA double strands and quadruplex structures with respect to hydrogen bonding and geometrical requirements. It thus offers a robust and sensitive fluorescence sensor for microenvironment detection. The nucleotide VdG is considered to have a potential comparable to 2-aminopurine that is widely used as fluorescent adenine analogue. We have shown a convenient synthesis of the VdG phosphoramidite building block for solid-phase DNA synthesis and the use of VdG as a fluorescence probe for DNA base pairing and formation of different topologies. A correlation of the fluorescence properties of VdG with the monomer–duplex transition, the type of neighboring nucleobase, and the respective conformation of the oligomer was found for VdG-containing double strands. VdG was further used to differentiate between topologies of G-quadruplexes. VdG was shown to be capable of adopting both the nucleotide *syn* and *anti* conformation required for distinct quadruplex structures. Thus VdG provides a sensitive fluorescent tool within nucleic acid chemistry.

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