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8-Vinylguanine Nucleo Amino Acid: A Fluorescent PNA Building Block

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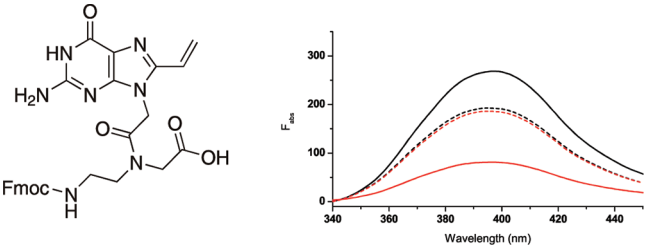
ABSTRACT



Attachment of a vinyl group at guanine position 8 provides fluorescent properties of the nucleobase. Therefore, 8-vinylguanine was introduced as a 2-aminoethylglycine peptide nucleic acid (PNA) building block. Incorporation of the guanine analog in short PNA sequences by Fmoc solid phase peptide synthesis allowed the differentiation between hybridization states of specific double strands with DNA, RNA, and PNA as well as quadruplex forming RNA/PNA oligomers based on fluorescence intensity.

Oligonucleotides use base pair recognition for the formation of various double strands, tetrades and other folds in multiple topologies. The incorporation of fluorescent nucleobase analogs allows the detection of these kinds of hybridization states and oligonucleotide topologies.[1] Typical approaches are the preparation of fluorophore-nucleobase conjugates, expanded nucleobases or nucleotides bearing a fluorescent aromatic moiety instead of a purine or pyrimidine.[2] Fluorescent analogs should closely resemble their parent nucleobases to minimize the influence of the modification with respect to recognition, conformation, and the micro environment.

Recently, we reported on 8-vinyl-2′-deoxyguanosine as a fluorescent 2′-deoxyguanosine mimic for the investigation of DNA hybridization and topology.[3] This only slightly modified guanosine analog maintains the nucleobase core, hydrogen bonding options, and resembles the torsion angles of DNA complexes while being fluorescent. 2-Aminoethylglycine peptide nucleic acids (PNA) attend broad interest as uncharged and achiral DNA/RNA mimic manifolds applied in diagnostics and as oligonucleotide analogs.[4] High binding affinity to complementary DNA and RNA as well as resistance to nucleases and proteases makes PNA an ideal tool for clinical diagnostics or biomolecular probes.[5,6] To take advantage of the fluorescent properties of 8-vinylguanine also in PNA oligomers, an 8-vinylguanine PNA building block was synthesized, incorporated into PNA oligomers, and investigated in PNA/PNA, PNA/oligonucleotide duplex formation and within a RNA/PNA quadruplex fold.

Synthesis of the 8-vinylguanine PNA building block **1** was provided by bromination at C8 of the guaninyl acetic acid **2**, attachment to the aminoethylglycine backbone followed by palladium catalyzed vinylation. Starting from 2-amino-6-chloro-purine **3**, the guaninyl acetic acid **2** was accessible following literature protocols (Scheme 1).[7]

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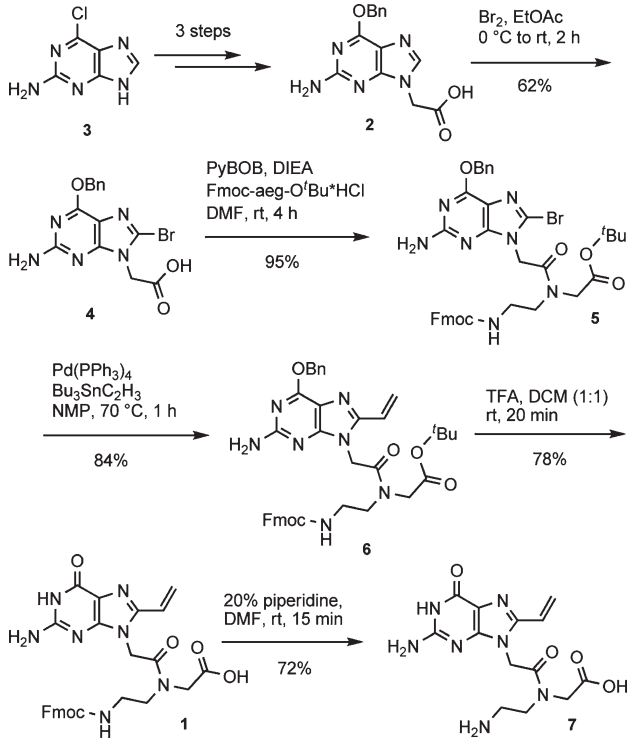
The acetic acid was regioselectively introduced at position N9 using benzyl 2-bromoacetate followed by saponification.[7a] An O6-benzyl-group was advantageous regarding solubility, purification, and coupling yields.[8] Bromination at C8 was performed in EtOAc with bromine to give **4** which was coupled to the backbone of Fmoc protected *tert*-butylester of *N*-(2-aminoethyl)glycine using PyBOB as activation reagent. Nucleo amino acid **5** was subjected to Stille coupling with tributylvinyltin to provide the vinylguanine derivative **6** in good yields. After treatment with trifluoroacetic acid in DCM the building block **1** (**Vg**) was obtained by precipitation from diethyl ether ready to be used in SPPS. To determine the extinction coefficient and the quantum yield of building block **1** (**Vg**) (Supporting Information), *N*-terminal deprotection was performed with 20% piperidine in DMF providing amino acid **7**.

PNA oligomers (Table 1) were synthesized via manual Fmoc SPPS considering reduced deprotection periods and capping cycles[9] as well as double couplings with shorter coupling times for the guaninyl nucleo amino acid[10] and the incorporation of the 8-vinlyguanine building block **1** (**Vg**). Furthermore, self-capping by acetylated nucleobase species during the neutralization and deprotection was avoided by an NMP/DIEA washing step after coupling and capping cycles.[11] Due to potential acetylation of the exocyclic amine during SPPS, no capping was applied after incorporation of **1** (**Vg**).[8] However, byproducts were not detected apart from oligomers lacking **Vg** in cases where single coupling was applied for the fluorescence building block. To enhance water solubility of PNA oligomers a lysine residue was attached to the *C*-terminus.[4] After cleavage from the resin, the PNA oligomers were purified using reversed phase HPLC and characterized by ESI−MS (Supporting Information).

Scheme 1. Syntheses of 8-Vinylguanine PNA Amino Acid **Vg**[a]



[a] Abbreviations: OBn, *O*-benzyl; DIEA, diisopropylamine; PyBOB, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; Fmoc, 9-fluoroenylmethoxycarbonyl; aeg, aminoethylglycine; TFA, trifluoracetic acid.

Table 1. Hybridization Stabilities of PNA/PNA, PNA/DNA, and PNA/RNA Hybrids based on UV Melting Curves

sequence	complementary strand	ratio	T_m (°C)
H-(gc[**Vg**]tgg)Lys-NH_2 (**PNA1**)	**PNA3**	1:1	36.0
H-(gcgtgg)Lys-NH_2 (**PNA2**)	**PNA3**	1:1	37.2
5′-CCACGC-3′ (**DNA1**)	**PNA1**	1:1	36.8
H-(g[**Vg**]gtcgg)Lys-NH_2 (**PVgH**)	**RQ**	2:1	71.6
H-(gggtcgg)Lys-NH_2 (**PgH**)	**RQ**	2:1	74.0
H-(ccca[**Vg**]cc)Lys-NH_2 (**PVgC**)	**RQ**	1:1	59.4
H-(cccagcc)Lys-NH_2 (**PgC**)	**RQ**	1:1	62.6

Hybridization properties of **Vg** containing PNAs with complementary PNA and DNA oligomers were investigated by fluorescence spectroscopy and thermal denaturation studies. The stability of the double strand formed by **PNA1** (H-(gc[**Vg**]tgg)Lys-NH_2) containing the **Vg** moiety as guanine nucleo amino acid analog with the complementary sequence H-(cgcacc)Lys-NH_2 (**PNA3**) provided a comparable stability (ΔT_m = 1.2 °C) as the guanine analogous **PNA2** (H-(gcgtgg)Lys-NH_2)/**PNA3** complex (Supporting Information). Thermal denaturation studies of the respective **PNA1/DNA1** ($^{5'}$CCACGC$^{3'}$) duplex indicated a stability (T_m = 36.8 °C) in the extent of the PNA double strand. **PNA1**/**PNA3** duplex formation was also clearly indicated by fluorescence spectroscopy. Excitation of **Vg** at 277 nm provided fluorescence response for the **PNA1** single strand and the respective **PNA1**/**PNA3** duplex (Figure 1). Hybrid formation at 5 °C led to a 72% decrease in emission intensity. Heating the **PNA1**/**PNA3** duplex above the melting temperature to 70 °C provided a fluorescence spectrum that is almost identical to the **PNA1** single strand. The fluorescence curves obtained for the **PNA1**/**DNA1** duplex were similar to the spectra recorded for the respective PNA double strand. Therefore, incorporation of **Vg** in PNA oligomers allows the detection of PNA/PNA and PNA/DNA duplex formation.

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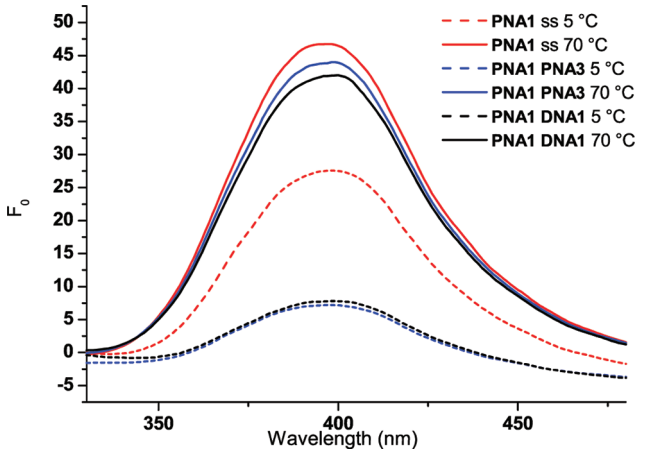

Figure 1. Fluorescence quenching upon double helix formation, shown for 1:1 mixture of **PNA1** in the presence and absence of complementary **PNA3** or **DNA1** (2.5 μM, 10 mM phosphate buffer pH 7, 100 mM NaCl, excitation wavelength: 277 nm).

For investigating the hybridization properties of RNA/PNA interacting oligomers by fluorescence spectroscopy, a quadruplex forming RNA (**RQ**) was prepared as introduced by Armitage et al.[12] **RQ** is a truncated version of RDQ, an RNA aptamer originally selected for binding to the Fragile X mental retardation protein.[13] With complementary and homologous PNA probes Armitage et al. were able to invade the RNA guanine quadruplex and form stable 1:1 duplexes (complementary probe) or 1:2 quadruplex (homologous probe) PNA-RNA hybrids (Figure 2).[12,14]

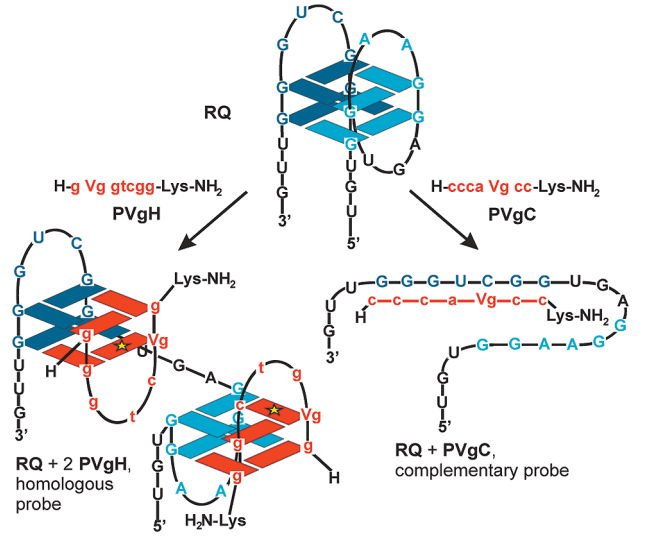

Figure 2. RNA quadruplex **RQ** opens to an RNA/PNA duplex adding PNA oligomer **PVgC** and leads to a dimer of quadruplexes adding PNA **PVgH** (only one of four possible quadruplexes shown).[12]

(12) Marin, V. L.; Armitage, B. A. *Biochemistry* **2006**, *45*, 1745–1754.

In analogy to the PNA hybridization experiments with **RQ** done by Armitage et al. PNA sequences were introduced containing **Vg** (Table 1) investigating the fluorescence readout for the various topologies.[12] UV melting curves of the tetrade complexes **RQ** + **PgH** and **RQ** + **PVgH** as well as the duplexes **RQ** + **PgC** and **RQ** + **PVgC** (ΔT_m = 3 °C) both turned out quite similar (Figure 3). The introduction of the guaninyl nucleo amino acid vinyl group has negligible influence on the complex stabilities.

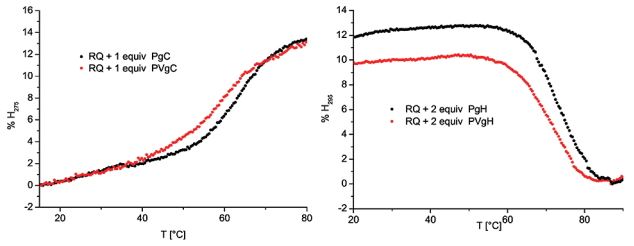

Figure 3. UV melting curves for (left) **RQ** duplex forming **PVgC** and **PgC** (275 nm) and (right) quadruplex inducing **PVgH** and **PgH** (295 nm, 275 nm, 2.0 μM, 10 mM Tris-HCl buffer pH 7, 100 mM KCl, 1 cm cuvette).

The guaninyl vinyl group also does not affect the duplex or quadruplex secondary structures as indicated by CD spectroscopy (Figure 4). In complex with **RQ** the PNA sequences **PVgH** and **PVgC** provided similar CD spectra as the complexes induced by **PgH** and **PgC**, respectively.

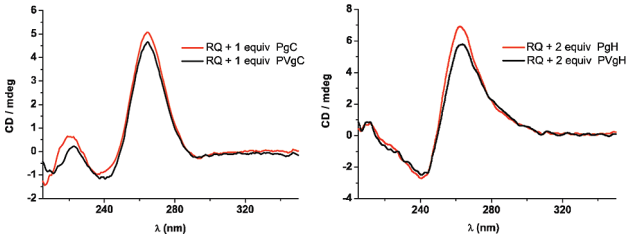

Figure 4. CD spectra of **RQ** with 1 equiv **PVgC** (black)/**PgC** (red) or 2 equiv **PVgH** (black)/**PgH** (red) (1:1 and 1:2 mixture, 1.0 μM **RQ**, 10 mM Tris-HCl buffer pH 7, 100 mM KCl, 22 °C).

The fluorescence response of the modified PNA oligomers **PVgH** and **PVgC** was evaluated upon hybridization to **RQ**. Duplex formation between RNA **RQ** and PNA **PVgC** was indicated by fluorescence response after excitation at 277 nm. The fluorescence intensity decreased by 70% upon duplex formation of **PVgC** with **RQ** at 22 °C (Figure 5). As a control experiment, the fluorescence spectrum of the equimolar mixture of **RQ** and **PVgC** was measured above the melting temperature (95 °C) and was found to be almost identical to the spectrum of single-stranded **PVgC**.

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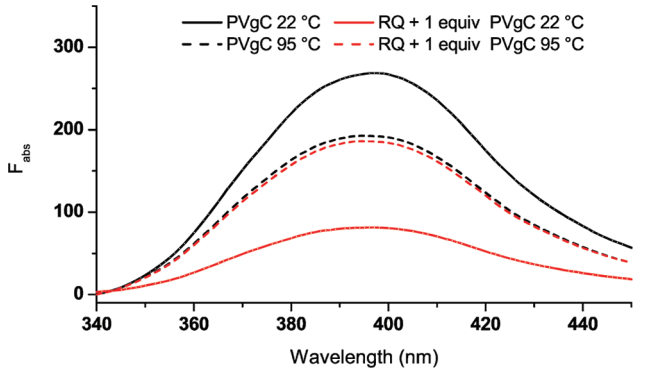

Figure 5. Fluorescence quenching upon double helix formation, shown for 1:1 mixture of **PVgC** in presence (red) and absence (black) of **RQ** (2.0 μM, 10 mM Tris-HCl buffer pH 7, 100 mM KCl, excitation wavelength: 277 nm).

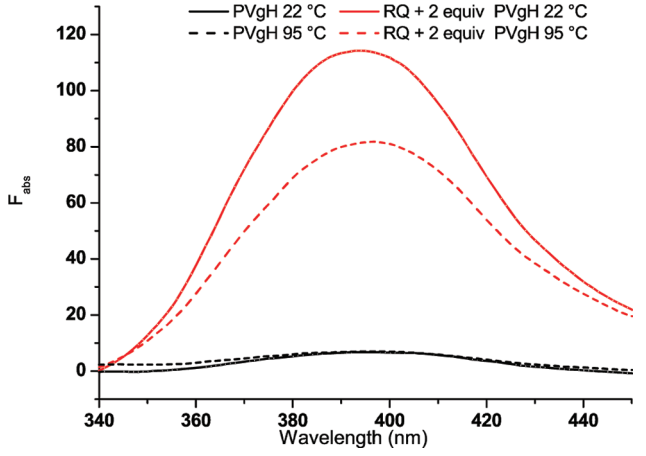

Figure 6. Fluorescence increase upon quadruplex formation, shown for 1:2 mixture **PVgH** in presence (red) and absence (black) of **RQ** (1.0 μM, 10 mM Tris-HCl buffer pH 7, 100 mM KCl, excitation wavelength: 277 nm).

PNA **PVgH** was expected to induce a quadruplex topology by interaction with RNA **RQ**. Therefore, the change in hydrogen bonding interaction and surrounding of the vinylguanine should be reflected in the fluorescence response. In absence of **RQ** quite a low fluorescence intensity of PNA **PVgH** was detected, that seems to result from quenching by neighboring guanine nucleobases (Figure 6). In the presence of **RQ**, the fluorescence intensity increased by about 93%, indicating the formation of the hybrid quadruplex at 22 °C. At 95 °C, the intensity was decreased due to melting of the quadruplex (Figure 6).

In conclusion, the synthesis of the fluorescent 8-vinylguaninyl peptide nucleic acid building block **Vg** was reported. Incorporation into PNA sequences by Fmoc-SPPS chemistry was provided under standard coupling and cleavage conditions. The fluorescent **Vg** label was used to probe the hybridization of PNA/PNA, PNA/DNA, PNA/RNA double strands and an RNA/PNA quadruplex. A clear differentiation between the PNA single strands and the pairing complexes with their respective topology was indicated by fluorescence intensity. The vinyl modification of the guaninyl nucleo amino acid was proven to have minimal influence on the complex stabilities and secondary structures. Vinylguanine does not interfere with a guanine-like hydrogen bonding pattern, adapts structurally well to the respective pairing topology, and allows a sensitive fluorescent readout of the topology and the direct environment of the nucleo amino acid **Vg**.

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Supporting Information Available. General methods, characterization data and experimental procedures for all new compounds as well as synthesized oligoamides. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.