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Enzymatic synthesis of 8-vinyl- and 8-styryl-2′-deoxyguanosine modified DNA—novel fluorescent molecular probes

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ABSTRACT

Fluorescent analogs of the natural nucleobases are widely used as molecular probes for investigating DNA hybridization and topology. In this study the guanosine analogs 8-vinyl- and 8-styryl-2′-deoxyguanosine were synthesized and converted into the corresponding 5′-triphosphates. These C8 modified nucleotides were processed by various DNA polymerases to create fluorescent DNA. Whereas the 8-styryl modified nucleotide somewhat hampers DNA synthesis 8-vinyl-2′-deoxyguanosine is processed by DNA polymerases emphasizing the broad applicability as a molecular probe for fluorescence spectroscopy.

Fluorescence spectroscopy is a powerful tool for investigating oligonucleotide conformation and dynamics. The molecular probes for this technique are fluorescent analogs of the natural nucleobases, for example, for detecting DNA hybridization or to discriminate between single nucleotide polymorphisms.[1] Typically, these probes are based on fluorophore-base conjugates in which common fluorophores are tethered to the nucleobases.[2] Other methods utilize expanded nucleobases[3] or nucleotides bearing a fluorescent aromatic moiety instead of the natural nucleobase.[4] Molecular probes for investigating oligonucleotide conformation and dynamics should mimic the natural nucleobases to minimize structural disturbances.[5] The most prominent example of this class of molecules is the adenine analog 2-aminopurine.[6] Other fluorescent nucleobase analogs closely resemble their parent nucleobase, mostly pyrimidine and adenine mimics. There are only a few examples for guanine analogs that match the requirements for a suitable fluorescent probe.[7]

Recently, we reported the efficient synthesis of the 8-vinyl-2′-deoxyguanosine phosphoramidite building block for solid-phase DNA synthesis and the use of 8-vinyl-2′-deoxyguanosine as a fluorescent probe for investigating DNA base pairing and the formation of different topologies.[8] As the emission properties of 8-vinyl-2′-deoxyguanosine are very sensitive towards changes within its microenvironment, the newly introduced fluorescent 2′-deoxyguanosine mimic is considered to have a potential comparable to 2′-aminopurine. To further expand the scope of applications for such fluorescent nucleotides by overcoming the inherent restrictions of chemical synthesis on solid-phase, DNA polymerases are promising tools to modify DNA in a site-specific and efficient manner.[9] Thereby, researchers have not only utilized wild-type enzymes but made also use of engineered DNA polymerases to accept modified 2′-deoxynucleoside-5′-triphosphates (dNTPs).[10] Among 8-substituted purines, especially dATP analogs have been used to synthesize functionalized DNA, whereby some of them were rather poor substrates for DNA polymerases.[11]

In this study, the authors have synthesized the corresponding triphosphate of 8-vinyl-2′-deoxyguanosine **1** for enzymatic synthesis of fluorescent DNA. Additionally, we have investigated the enzymatic acceptance of 8-styryl-2′-deoxyguanosine-5′-triphosphate **2**. This fluorescent analog of 2′-deoxyguanosine has already been introduced into DNA by solid-phase synthesis[12] and has been investigated additionally on nucleoside basis concerning its fluorescent properties.[13] It turned out that despite the increased steric size of the guanosine analog, the B-form structure of DNA is almost unaltered.[12] As the styryl modification underlies efficient and reversible *cis–trans* photoisomerization[13] it has also been shown that photochemical and physical properties can be controlled by external light stimuli. The conformational switching leads to dramatic changes in fluorescence intensities,[13] the thermal stability of duplex DNA[12] and guanosine self-assembling capability[14] reflecting the potential of 8-styryl-2′-deoxyguanosine to photochemically control nucleic acid structures.

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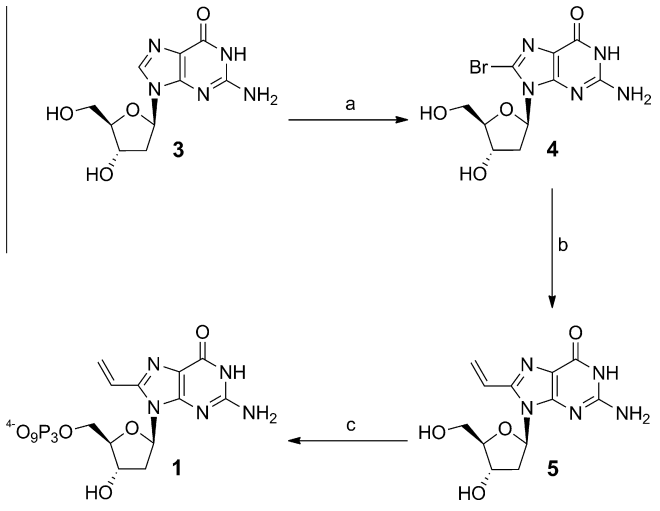
The synthesis of 8-vinyl-2′-deoxyguanosine-5′-triphosphate (VdGTP) **1** (Scheme 1) was carried out starting with bromination at the C8 position of 2′-deoxyguanosine **3** with NBS and precipitation in acetone as an efficient purification step.[15] Nucleoside **4** was subjected to Stille coupling with tributyl–vinyltin to give the vinyl-substituted derivative **5** in good yields.[16] The modified triphosphate **1** was generated using an established method.[17]

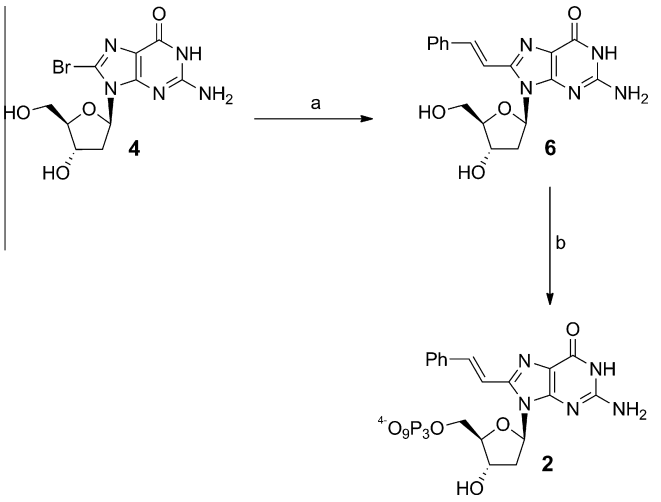
8-Styryl-2′-deoxyguanosine-5′-triphosphate (SdGTP) **2** was also synthesized in three steps from commercially available 2′-deoxyguanosine (Scheme 2). Bromination with NBS at position C8 followed by Suzuki–Miyaura cross-coupling with *trans*-2-phenylvinylboronic acid yielded compound **6** in good yields.[13,18] The corresponding triphosphate **2** was synthesized using a protection-free one-pot synthesis protocol introduced by Huang.[19]

To investigate the acceptance of VdGTP **1** and SdGTP **2** by DNA polymerases we carried out primer extension reactions (Fig. 1). Thereby, we used a radioactively 5′-^{32}P labelled DNA primer and a DNA template containing three cytosines that call for the incorporation of either dGMP, VdGMP or SdGMP, respectively (Fig. 1a). After incubation in presence of dNTPs and DNA polymerase reactions were analyzed by denaturing polyacrylamide gelelectrophoresis (PAGE) and visualized by phosphor imaging. We used the large fragment of the thermophilic *Taq* DNA polymerase (KlenTaq) from family A and the two B-family DNA polymerases KOD (exo$^-$) and Therminator (A485L mutant of *Thermococcus* species 9°N-7 DNA polymerase (exo$^-$)).

As expected, if only dATP, dTTP, and dCTP but neither natural dGTP nor any modified guanosine analog are present (Fig. 1; −dGTP) the primer strand cannot be elongated to the full length of 35 nucleotides (nt) by any of the DNA polymerases. Interestingly, KlenTaq DNA polymerase stops primer elongation mainly by the first cytosine displaying strong termination bands at position 24 and 25. As DNA synthesis is also inhibited after incorporating a mismatched nucleotide opposite the templating cytosine primer fragments with 28 nt length are prominent as well. In contrast, under the applied conditions primer elongation catalyzed by KOD and Therminator DNA polymerase is mainly paused by reaching the second cytosine at position 30. However, in presence of all four natural nucleotides (Fig. 1; +dGTP) all DNA polymerases are able to extend the primer to full-length oligonucleotide. The incorporation of an additional nucleotide in a non-template directed manner leading to 36 nt long products has been observed before using 3′–5′ exonuclease-deficient DNA polymerases.[20] Notably, the use of Therminator DNA polymerase resulted in additional, slower migrating products probably caused by different annealing sites and further elongation under the applied reaction conditions that are too excessive for simple DNA synthesis in presence of natural dNTPs. By replacing natural dGTP with VdGTP **1** we observed the formation of full-length products for all tested DNA polymerases (Fig. 1b; +VdGTP). This clearly demonstrates that VdGTP is not only processed by DNA polymerases but it also shows



Scheme 1. Synthesis of 8-vinyl-2′-deoxyguanosine-5′-triphosphate (VdGTP) **1**. Reagents and conditions: (a) *N*-bromosuccinimide, acetonitrile/water 4:1, 1 h, rt, 91%; (b) tributyl(vinyl)stannane, tetrakis(triphenylphosphine)-palladium(0), *N*-methyl-2-pyrrolidone, 2 h, 110 °C, 80%; (c) 1,8-bis-(dimethylamino)naphthalene, phosphorus oxychloride, trimethyl phosphate then bis-tri-*n*-butylammonium pyrophosphate, *n*-tributylamine, 14%.



Scheme 2. Synthesis of 8-styryl-2′-deoxyguanosine-5′-triphosphate **2**. Reagents and conditions: (a) *trans*-2-phenylvinylboronic acid, sodium carbonate, tris(3-sulfophenyl)phosphine trisodium salt, palladium(II) acetate, water/acetonitrile 2:1, 16 h, 80 °C, 72%; (b) 2-chloro-4-H-1,2,3-benzodioxaphosphorin-4-one, bis-tri-*n*-butylammonium pyrophosphate, *n*-tributylamine, *N*,*N*-dimethylformamide then nucleoside **6**, iodine solution, 21%.

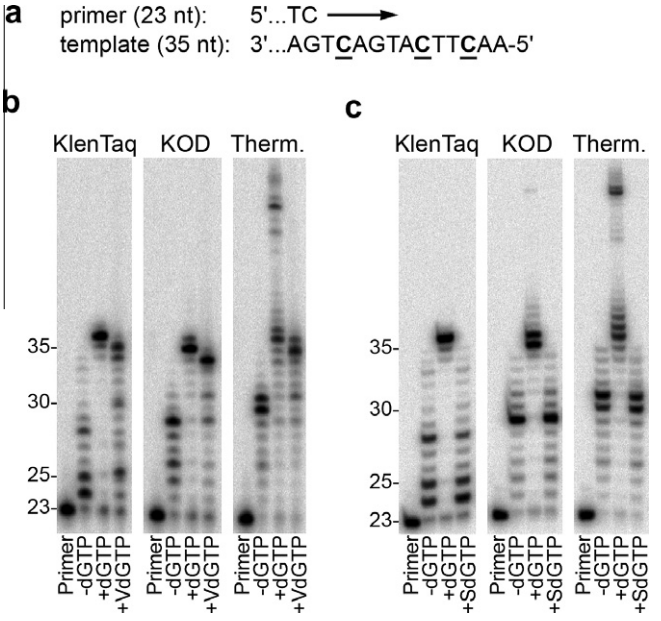


Figure 1. Primer extension studies: incorporation of (b) VdGMP and (c) SdGMP by KlenTaq, KOD, and Therminator (Therm.) DNA polymerase. (a) Partial primer template sequences employed. (b) and (c) Primer: primer only; −dGTP: primer extension in the presence of dATP, dTTP, and dCTP; +dGTP: as −dGTP but in the presence of dGTP; +VdGTP: as −dGTP but in the presence of VdGTP **1**; +SdGTP: as −dGTP but in the presence of SdGTP **2**.

that incorporated VdG units can be further elongated in primer extension reactions. Next, we studied the action of the even more size-demanding dGTP analog SdGTP **2** on DNA polymerases (Fig. 1c). Thereby, primer extension experiments in presence of dATP, dTTP, dCTP and SdGTP (+SdGTP) yielded DNA products similar to those that are formed when only the three natural dNTPs but neither dGTP nor SdGTP are present (−dGTP). This suggests that SdGTP is not accepted by the tested DNA polymerases.

To investigate the efficiency and selectivity of VdGMP incorporation in more detail, we carried out single incorporation experiments in presence of different concentrations of VdGTP and dGTP, respectively (Fig. 2). Therefore, we used four different primer template combinations displaying all four natural nucleotides in the template at the first position after the primer binding site (Fig. 2a). In case of correct nucleotide incorporation (N=C), reactions were incubated for 5 min (KlenTaq) and 10 min (KOD and Therminator). To induce mismatch incorporation (N=G, A, T) reactions were performed for 60 min. Doing so, two primer bands can be detected after PAGE analysis reflecting the given 23 nt long primer and the elongated one (24 nt).

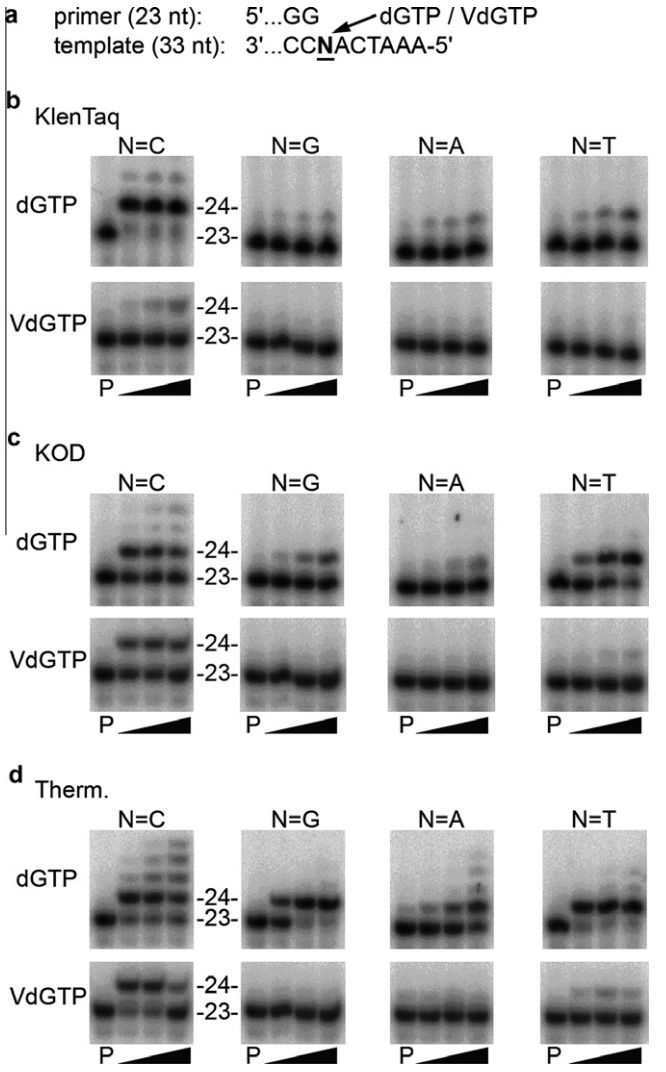


Figure 2. Single incorporation experiments in presence of differing nucleotide concentrations employing natural dGTP and VdGTP. P: primer only (23 nt). Reactions were carried out for 10 min (KlenTaq 5 min) in case of N=C or for 60 min for N=G, A, and T in presence of 10, 50, and 200 μM dGTP and VdGTP, respectively. (a) Partial primer template sequences employed. (b) Incorporation catalyzed by KlenTaq DNA polymerase. (c) Incorporation catalyzed by KOD DNA polymerase. (d) Incorporation catalyzed by Therminator (Therm.) DNA polymerase.

Considering the incorporation opposite matching cytosine, KOD and Therminator DNA polymerase were able to process VdGTP as efficiently as natural dGTP whereas KlenTaq DNA polymerase showed a drastically decreased incorporation efficiency in presence of VdGTP (Fig. 2b–d; N=C). Notably, higher concentrations of VdGTP inhibited the incorporation step catalyzed by KOD and Therminator DNA polymerase yielding less elongated primer products. Interestingly, we observed only opposite the mismatching thymidine (N=T) some incorporation of VdGMP. In contrast, natural dGMP was apparently better incorporated opposite all mismatching nucleotides (Fig. 2b–d; N=G, A, T). In case of KlenTaq DNA polymerase lacking VdGMP incorporation opposite the mismatching nucleotides might be due to a generally diminished acceptance of VdGTP. However, KOD and Therminator DNA polymerase process VdGTP as efficiently as dGTP in the match case (N=C) but show less incorporation opposite mismatching nucleotides compared to reactions in presence of natural dGTP. Thus, VdGTP is apparently more selectively processed by these DNA polymerases than dGTP. Nevertheless, VdGTP showed only poor efficiencies in PCR experiments using the above mentioned DNA polymerases (data not shown).

To study multiple incorporation of the fluorescent nucleotides in a row we performed primer extension experiments using a DNA template that bears only cytosine residues after the primer binding site (Fig. 3). Thus, we were able to investigate whether multiple VdG or SdG nucleotides can be incorporated in a row. If only natural dGTP is present the primer strand is extended efficiently to DNA products that migrate markedly slower (Fig. 3; dGTP). As up to 46 guanidine moieties can be incorporated in a row we suggest that the smearing of the full-length band is caused by unspecific primer binding during catalysis at the applied temperature of 72 °C. Additionally, dG and dC rich DNA sequences are known to fold into stable secondary structures that may affect DNA mobility in PAGE as well.[21] In presence of VdGTP the incorporation of multiple fluorescent nucleotides can be observed for all studied DNA polymerases (Fig. 3b; VdGTP). Whereas in case of KlenTaq and KOD DNA polymerase (Fig. 3b; left and middle panel)

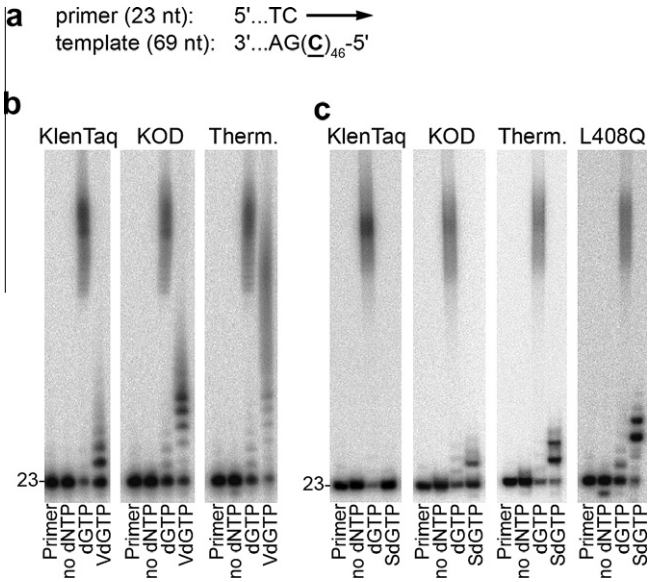


Figure 3. Multiple incorporation of the modified nucleotides (b) VdGMP and (c) SdGMP by KlenTaq, KOD, Therminator (Therm.), and Therminator L408Q (L408Q) DNA polymerase, respectively. (a) Partial primer template sequences employed. (b) and (c) Primer: primer only; no dNTP: incubation without any dNTP; dGTP: primer extension in the presence of dGTP only; VdGTP: primer extension in the presence of VdGTP **1** only; SdGTP: primer extension in the presence of SdGTP **2** only.

two and five nucleotides are introduced, respectively, Therminator DNA polymerase is capable of incorporating even more than 10 VdG units in a row (Fig. 3; right panel). Spiking the reaction mixtures with natural dGTP aside from VdGTP would probably allow the formation of full-length products. Interestingly, also in case of the sterical challenging SdGTP we observed the incorporation of one or two SdGMPs by KOD and Therminator DNA polymerase, respectively (Fig. 3c; SdGTP). In contrast, reactions catalyzed by KlenTaq DNA polymerase did not show any detectable primer elongation in presence of SdGTP (Fig. 3c; left panel). To further investigate the acceptance of SdGTP in primer extension experiments we used the single mutant L408Q of Therminator DNA polymerase (Fig. 3c; right panel). This enzyme has been developed by directed evolution by Staiger and Marx and displays an enhanced substrate spectra accepting ribonucleotides and nucleobase-modified dNTPs.[10b] Here, the L408Q mutation apparently enables Therminator DNA polymerase to incorporate up to three adjacent SdG units in a row resulting in an almost quantitative primer conversion yielding primer products labelled by at least two SdG moieties.

In summary, we report the synthesis of 8-vinyl- and 8-styryl-2′-deoxyguanosine-5′-triphosphates and attempts to enzymatically introduce the fluorescent nucleotides into DNA by various DNA polymerases. It clearly turned out that the vinyl-modified nucleotide VdGTP is suitable for the enzymatic synthesis of fluorescent DNA. It can be easily and specifically incorporated within a growing primer strand at single and even at multiple adjacent positions. In contrast, we observed that the enzymatic conversion of the styryl-modified nucleotide SdGTP is markedly inhibited. This is caused by the rather large modification at position C8 of the guanine nucleobase. Nevertheless, we have shown that up to three 8-styryl guanosines can be successfully attached to the 3′-end of a DNA primer strand. Interestingly, DNA polymerases behave differently by processing the fluorescent dGTPs showing less acceptance in case of KlenTaq DNA polymerase (A-family) but efficient catalysis if B-family DNA polymerases are used. Taken together, at least the fluorescent analog VdGTP is suitable for the enzymatic introduction into DNA emphasizing the broad applicability and potential of 8-vinyl-guanosine.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.056.

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