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Synthesis and DNA Incorporation of an Ethynyl-Bridged Cytosine *C*-Nucleoside as Guanosine Surrogate

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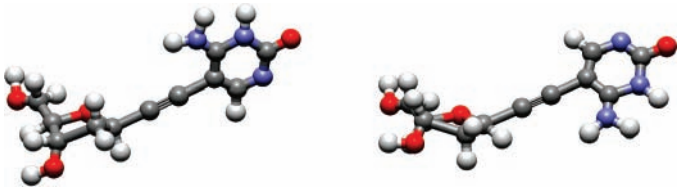
ABSTRACT



As a guanosine mimic that lacks the preference for syn or anti conformation a cytosine *C*-nucleoside was synthesized connecting the nucleobase at the anomeric center by an ethynyl linker. The key step was a Sonogashira cross coupling of 5-iodocytosine with 1′-ethynyl-2′-deoxyribose. The new *C*-nucleoside incorporated into G/C-alternating oligonucleotides emerged as guanosine substitute, however, with reduced duplex stability. B-Form DNA was strongly stabilized by the new surrogate even in typically Z-DNA forming sequences and in Z-form inducing environment.

Properties and recognition potential of large biomolecules like oligonucleotides are not only determined by the primary structure but also strongly depend on parameters like solvent, temperature, and salt concentration.[1] Conversion between DNA helix topologies like the right-handed A- and B-DNA or the left-handed Z-DNA double helix can be induced by changing the environment. The various DNA-forms encode information since they are specifically recognized by small molecules[2] or proteins[3] on the basis of hydrogen bonding or shape recognition in the duplex grooves. Therefore, it is of interest to specifically enforce one kind of DNA topology or to stabilize dsDNA by ribosyl or nucleobase modifications to preferentially obtain a defined DNA-form. Initially, it was our intention to stabilize Z-DNA providing a guanosine surrogate that resembles the required but energetically less favored syn conformation. Nucleoside modifications known to favor Z-DNA either address the required 2′-endo ribosyl conformation[4] or introduce substituents at C8 of purines[5] or at C5 of pyrimidines[6] to sterically enhance the likelihood

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for a syn nucleotide with respect to the thermodynamically favored anti conformation (Figure 1).

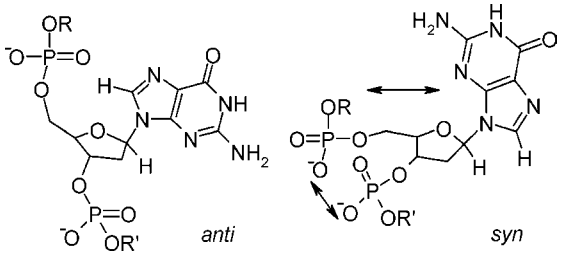


Figure 1. Guanosine in anti and syn conformation: The sterically disfavored syn conformation also suffers from stronger phosphodiester repulsion.

Our approach for a new syn guanosine surrogate is based on a linkage of the recognition unit at the anomeric center without any conformational preference for the respective torsion angle given by an ethynyl spacer combined with a hydrogen-bonding pattern comparable to guanosine (Figure 2). Rotation around the ethynyl linker axis in cytosinyl

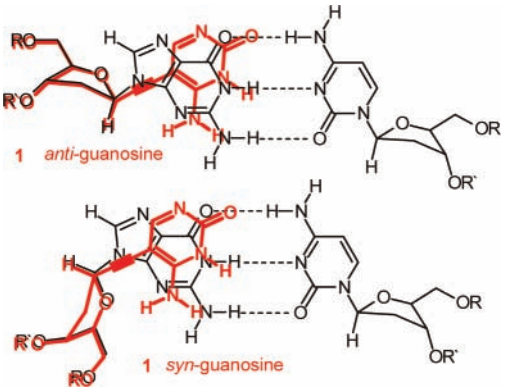


Figure 2. β-Ethynyl cytosine *C*-nucleoside **1** as surrogate for guanosine in anti and syn conformation.

nucleoside **1** should facilitate the conversion between the right- and left-handed DNA-forms. In addition, the ethynyl linked cytosine nucleoside **1** might be of interest within the field of artificial nucleosides and nucleobases in dsDNA.[7,8] It has the potential to function as a versatile nucleobase with respect to order and orientation of hydrogen-bonding recognition.

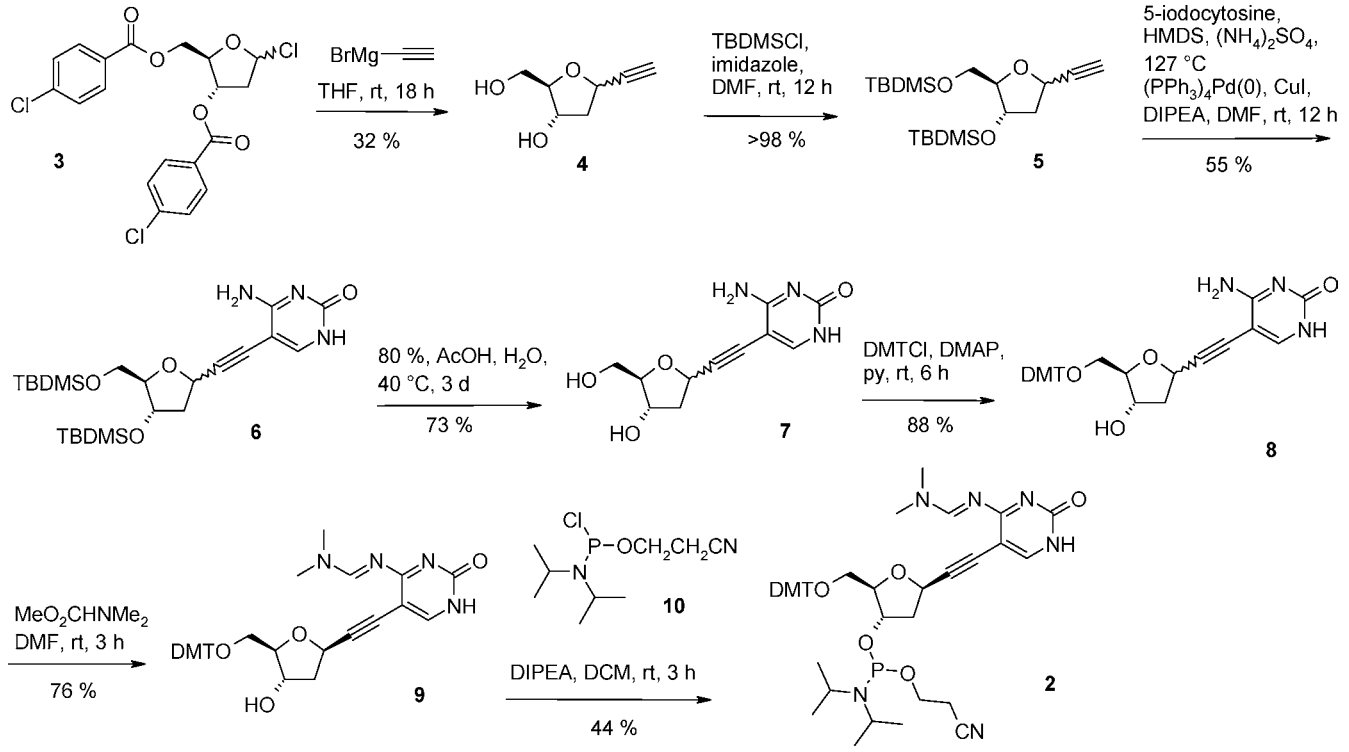
The design of the artificial nucleoside **1** is based on the resemblance of the hydrogen-bonding pattern with the Watson−Crick site of guanine (Figure 2) in combination with the purine distance and the orientation of donor/acceptor functionalities. Free rotation around the ethynyl linker should allow equally well imitation of syn and anti conformation. Therefore, nucleoside **1** with a cytosine linked at C5 to the anomeric center in β-configuration by an ethynyl spacer fulfills the requirements of a guanosine surrogate with proper hydrogen-bonding pattern. For incorporation in oligonucleotides by solid-phase synthesis, the preparation of phosphoramidite **2** was required that is amidine protected at the nucleobase and equipped with the temporary dimethoxytrityl protection at 5′-OH.

The key step of the synthesis of phosphoramidite **2** was the Sonogashira cross coupling[9] to connect the 5-iodo cytosine with a 1′-ethynyl-2′-deoxyribosyl derivative (Scheme 1). The ethynyl group was introduced in analogy to other alkyl groups[10] at the anomeric center by Grignard reaction of ethynyl magnesium bromide with 1-chloro-3,5-di-*O*-(*p*-chlorobenzoyl)-2-deoxy-D-ribofuranose (**3**). Since the chlorobenzoyl protecting groups were cleaved in the substitution step, 12 equiv of Grignard reagent were required to obtain the resulting *C*-nucleoside **4**. Silyl protection of the ethynyl deoxyribose leads to ethynyl deoxyribose **5** ready for Sonogashira cross coupling. 5-Iodocytidine was persilylated with hexamethyldisilazane (HMDS) before it was used as reagent for the coupling with ethynyl deoxyribose **5** catalyzed by tetrakis(triphenylphosphine)palladium(0) and copper iodide to yield *C*-nucleoside **6**. Deprotection of the hydroxy groups was accomplished with 80% acetic acid in water at 40 °C since the standard reagent TBAF lead to separation problems. Therefore, protection of the exocyclic amino group of cytosine had to be done after desilysation owing to hydrolysis sensitivity of the dimethyl amino methylene group under the acetic acid cleavage conditions used. Previous to nucleobase protection the tritylation needed to be done since the dimethyl amino methylene group was completely cleaved under tritylation conditions. Therefore, the fully deprotected nucleoside **7** was treated with dimethoxytrityl-chloride in pyridine and a catalytic amount of DMAP to yield the DMT-protected nucleoside **8**. In a first attempt to prepare the ribosyl phosphoramidite with ethynyl-bridged cytosine, benzoyl protection of the nucleobase was applied. A mixture obtained of up to three times protected *C*-nucleosides turned out to be disadvantageous. Therefore, protection of the exocyclic amino group was done by treatment of nucleoside **8** with dimethoxy amino methylene in DMF at room-temperature providing nucleoside **9** in 76% yield as a pure β-anomer. Remarkably, the high yield was obtained, although separation from the α-anomer by column chromatography was accomplished at this stage.

The configuration of the *C*-nucleosides at the anomeric center was determined by ^{1}H NMR spectroscopy. A pseudotriplet is typically found for the H-C1′ of β-nucleosides, whereas a doublet of doublets is representing the α-isomer. Furthermore, the chemical shift and the multiplicity of the H-*C*2′ protons are characteristic for the anomers.[11] Finally, treatment of nucleoside **9** with 2-cyanoethyl-*N*,*N*-diisopropylidene (**10**) and DIPEA in anhydrous DCM at room temperature lead to the corresponding phosphoramidite **2** in 44% yield recovering 30% of nucleoside **9**.

Phosphoramidite **2** was used in solid-phase synthesis of DNA oligomers d(CGCG*CG) (**11**) and d(CGCG*CGCGCG*CG) (**12**) with G* indicating the cytosinyl nucleoside **1** using

Scheme 1



standard protocols.[12] The constitutional integrity of oligomers **11** and **12** was indicated by high-resolution mass spectrometry. The sequences for incorporation of the newly synthesized nucleotide **10** were oriented on the assumption that the ethynyl linked cytosine **1** might be suitable as a syn guanosine mimic for Z-DNA induction. The respective natural DNA oligomers with alternating C/G-sequence $d(CG)_3$ (**13**) and $d(CG)_6$ (**14**) are easily converted into Z-form DNA with increasing NaCl concentration.[13]

The influence of guanosine surrogate **1** on the double helix stability was investigated by temperature-dependent UV spectroscopy. At 150 mM NaCl concentration both modified oligomers **11** (T_m < 5 °C, >4% hyperchromicity (H), 3.3 μM) and **12** (T_m = 50 °C, 12% H, 3.3 μM) containing two or four modifications in the double strand, respectively, provided significantly lower duplex stabilities compared to the C/G-counterparts **13** (T_m = 42 °C, 3% H, 3.3 μM) and **14** (T_m > 80 °C, >8% H, 3.3 μM) (Figure 3). The distinct loss of stability in double strands containing the modified nucleotide **1** compared to the C/G-sequences indicates that the surrogates are not perfectly placed as guanine mimetic. Induction of Z-form DNA with 4 M NaCl leads to a loss of stability for the C/G oligomers **13** (T_m = 27 °C, 8% H, 3.3 μM) and **14** (T_m = 71 °C, 17% H, 3.3 μM), whereas the modified oligomer **12** (T_m = 56 °C, 14% H, 3.3 μM) was even stabilized by high salt concentration (Supporting Informatioin). The *C*-nucleoside **1** seems to have either a strong stabilizing effect in Z-DNA functioning as a syn guanosine surrogate or to prohibit the conformational reorganization from B- to Z-form of the double helix.

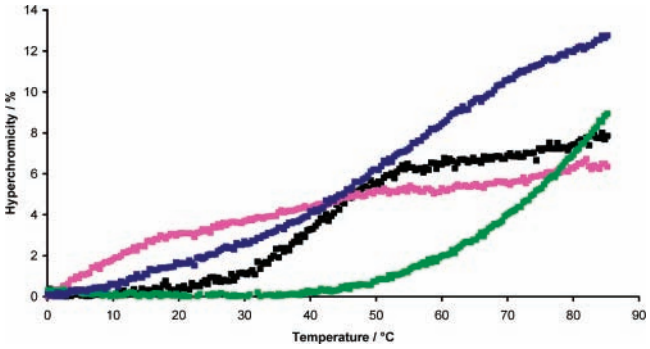


Figure 3. UV melting curves of the modified oligomers d(CGCG*CG) **11** (purple) and d(CGCG*CGCGCG*CG) **12** (blue) in comparison to the hexamer $d(CG)_3$ **13** (black) and the dodecamer $d(CG)_6$ **14** (green), each 3.3 μM, in 0.1 M NaH_2PO_4 buffer, pH 7, 150 mM NaCl.

CD spectroscopy at various temperatures and NaCl concentrations were used to address the DNA topological preference and the conformational change between the helical forms. The modified oligomer **12** was used in comparison to C/G-dodecamer **14** to exemplary show the B- to Z-form transition induced by increasing

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the NaCl concentration (Figures 4 and 5). As known from literature the C/G-alternating dodecamer **14** turns from

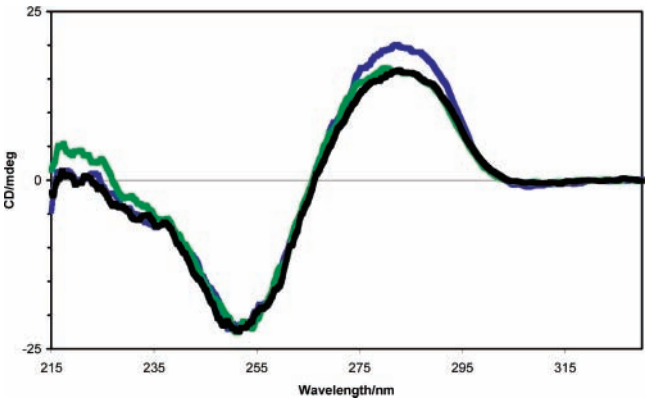

Figure 4. CD spectra of dodecamer d(CGCG*CGCGCG*CG) **12** (20 μM, in 0.1 M NaH_2PO_4 buffer, pH 7) at 20 °C at 0 M NaCl (black), 1 M NaCl (green), and 4 M NaCl (blue).

B-DNA at 0 and 1 M NaCl concentration to Z-DNA at 4 M NaCl.[1] In contrast, it was not possible to induce Z-DNA in the double strand of oligomer **12** that contained four *C*-nucleotide **1** modifications. The B-form DNA was predominant at all salt concentrations. It is quite remarkable that the guanosine surrogate **1** obviously fits well within the B-DNA duplex and additionally seems to prohibit the topological change to the otherwise favored Z-DNA. The influence of the temperature on the DNA helix topology was also investigated providing only a marginal loss in helix propensity with increasing temperature. No deviation from the B-form DNA was indicated (Supporting Information).

The results obtained with oligomer **12** were essentially confirmed by respective CD spectra of the self-pairing duplex of hexamer **11** that only contains two nucleoside surrogates **1**. Nevertheless, the overall lower stability of the duplex was recognized in lower helix propensity and the requirement to investigate the conformational analysis at lower temperatures (Supporting Information).

In conclusion, the synthesis of a guanosine surrogate was presented that has the unique feature of a β-ethynyl linked

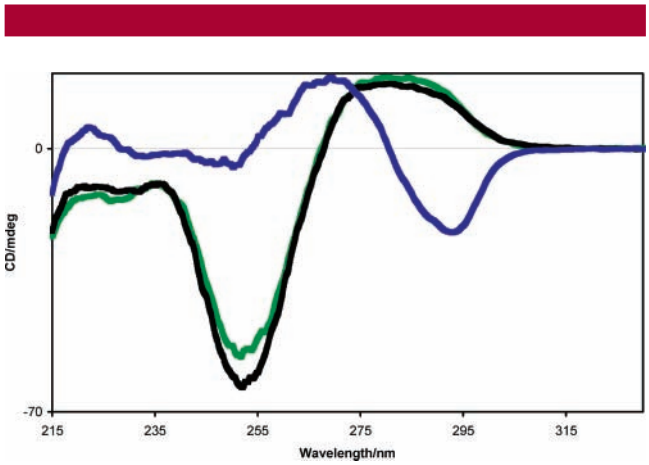

Figure 5. CD spectra of dodecamer $d(CG)_6$ **14** (20 μM, in 0.1 M NaH_2PO_4 buffer, pH 7 at 20 °C) at 0 M NaCl (black), 1 M NaCl (green), and 4 M (blue) NaCl concentration.

recognition unit at the anomeric center. This provides free rotation of the artificial nucleobase without preference to orientation in syn- or anti-like conformation. The hydrogen donor/acceptor pattern of the recognition unit resembles the guanine Watson−Crick site. The new surrogate turned out to stabilize the DNA duplex in its B-form when incorporated in DNA that is destined to form Z-DNA under high salt conditions. Studies toward the potential of the new *C*-nucleoside to differentiate between A-DNA and B-form DNA are currently under investigation.

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Supporting Information Available: Complete experimental details for the preparation and characterization of compounds **2** − **9**; analytical data of oligomers **11** and **12**; temperature-dependent UV and CD spectra at various NaCl concentrations and temperatures of oligomers **11**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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