

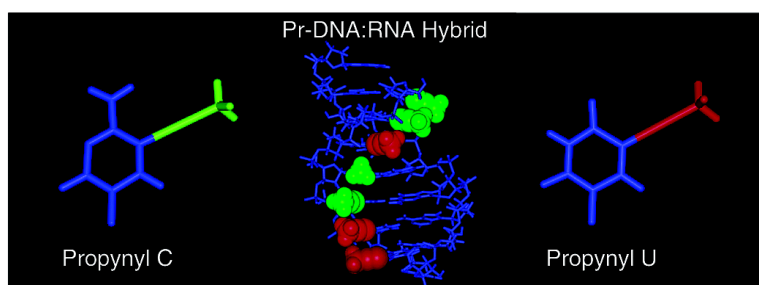
Article

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*J. Am. Chem. Soc.*, **2003**, 125 (20), 6090-6097 • DOI: 10.1021/ja021285d • Publication Date (Web): 24 April 2003

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## NMR Studies of DNA Single Strands and DNA:RNA Hybrids with and without 1-Propynylation at C5 of Oligopyrimidines<sup>‡,§</sup>

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**Abstract:** The 1-propynylation at C5 of consecutive pyrimidines in DNA can enhance DNA:RNA hybrid stability at 37 °C by over 1 kcal/mol of substitution [Barnes, T. W., III; Turner, D. H. *J. Am. Chem. Soc.* **2001**, *123*, 4107–4118]. To provide information on the structural consequences of propynylation, two-dimensional NMR spectroscopy was used to study the structures of several oligonucleotides. Intraresidual nuclear Overhauser effect spectroscopy cross peaks were observed at 30 °C and a 200 ms mixing time in the H6–H1' region for 5'(dC<sup>CP</sup>U<sup>CP</sup>C<sup>CP</sup>U<sup>CP</sup>U<sup>CP</sup>) (ssPrODN) but not for 5'(dCCUCCUU) (ssODN), suggesting preorganization of the propynylated single strand. NMR structures of the duplexes 5'(dC<sup>CP</sup>U<sup>CP</sup>C<sup>CP</sup>U<sup>CP</sup>U<sup>CP</sup>)-3':3'(rGAGGAGGAAAU)5' (PrODN:RNA), 5'(dCC<sup>UP</sup>U<sup>UP</sup>C<sup>UP</sup>U<sup>UP</sup>U<sup>UP</sup>)-3':3'(rGAGGAGGAAAU)5' (sPrODN1:RNA), and 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5' (ODN:RNA) indicate that their global structures are almost identical. The NMR data, however, suggest that the 5'-end of sPrODN1:RNA is more dynamic than that of PrODN:RNA. In the propynylated duplexes, the propyne group stacks on the aromatic ring of the 5'-base and extends into the major groove. The results suggest that the increased stability of the propynylated duplexes is caused by preorganization of the propynylated single strand and different interactions in the double strand. The propynyl group provides volume exclusion, enhanced stacking, and possibly different solvation.

### Introduction

Advances in synthetic nucleic acid chemistry have provided modifications to the backbone, sugars, and bases of nucleic acids to improve the affinity, chemical stability, cellular penetration, and potency of antisense oligonucleotides.<sup>1–10</sup> One modification that increases antisense target affinity is the substitution of

1-propyne for hydrogen at the C5 position of cytidine and uridine, resulting in 5-(1-propynyl)-2'-deoxycytidine (C<sup>P</sup>) and 5-(1-propynyl)-2'-deoxyuridine (U<sup>P</sup>)<sup>11,12</sup> (Figure 1). Propynylated oligonucleotides can provide activation of RNase H cleavage and inhibition of gene expression.<sup>3,13–16</sup>

Thermodynamic studies of C5-(1-propynyl) substitutions on pyrimidines have shown an increase in the melting temperature of DNA:RNA hybrids by 0.9–4.0 °C per modification.<sup>2,10,17</sup> More specifically, full propynylation of the DNA strand of 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5' (ODN:RNA) to give 5'(dC<sup>CP</sup>U<sup>CP</sup>C<sup>CP</sup>U<sup>CP</sup>U<sup>CP</sup>)-3':3'(rGAGGAGGAAAU)5' (PrODN:

<sup>‡</sup> Protein Data Bank entries INTQ, INTS, and INTT.

<sup>#</sup> Abbreviations: C<sup>P</sup>, 5-(1-propynyl)-2'-deoxycytidine; DNA:RNA, general hybrid of DNA and RNA; HETCOR, heteronuclear correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation spectroscopy; ODN:RNA, 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5'; PrODN, propynyl-containing oligodeoxynucleotide; PrODN:RNA, 5'(dC<sup>CP</sup>U<sup>CP</sup>C<sup>CP</sup>U<sup>CP</sup>U<sup>CP</sup>)-3':3'(rGAGGAGGAAAU)5'; rMD, restrained molecular dynamics; rmsd, root-mean-squared deviation; sPrODN1:RNA, 5'(dCC<sup>UP</sup>U<sup>UP</sup>C<sup>UP</sup>U<sup>UP</sup>U<sup>UP</sup>)-3':3'(rGAGGAGGAAAU)5'; ssDNA, general single-stranded DNA; ssODN, 5'(dCCUCCUU); ssPrODN, 5'(dC<sup>CP</sup>U<sup>CP</sup>C<sup>CP</sup>U<sup>CP</sup>U<sup>CP</sup>); ss-sPrODN1, 5'(dCC<sup>UP</sup>U<sup>UP</sup>C<sup>UP</sup>U<sup>UP</sup>U<sup>UP</sup>); TOCSY, total correlation spectroscopy; U<sup>P</sup>, 5-(1-propynyl)-2'-deoxyuridine.

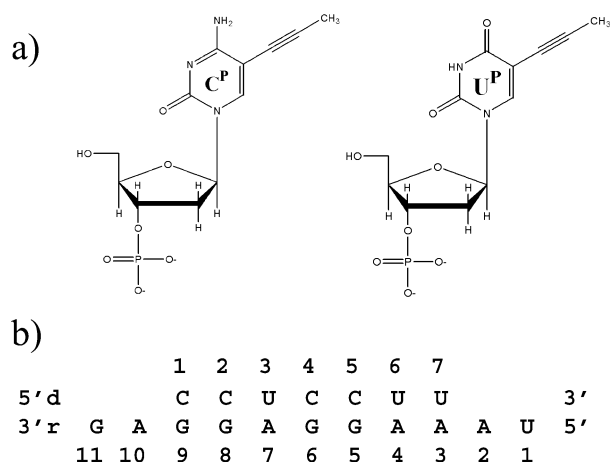
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**Figure 1.** (a) Structures of 5-(1-propynyl)-2'-deoxycytidine ( $C^P$ ) and 5-(1-propynyl)-2'-deoxyuridine ( $U^P$ ) and (b) the numbering scheme and sequence of the duplex investigated by NMR.

RNA) results in an 8.8 kcal/mol stabilization at 37 °C, in comparison to the unpropynylated duplex.<sup>17</sup> Interestingly, removal of the propynyl group from the 5' C of 5'(dC<sup>P</sup>C<sup>P</sup>.U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5' to give 5'(dCC<sup>P</sup>.U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5' (sPrODN1:RNA) destabilizes the duplex by 3 kcal/mol at 37 °C. Propynylation of the 5' C of 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5', however, to give 5'(dC<sup>P</sup>C<sup>P</sup>UCCUU)3':3'(rGAGGAGGAAAU)5' does not change the duplex stability at 37 °C.<sup>17</sup>

To provide structural insights into the thermodynamic properties of oligonucleotides containing propynyl substitutions, we compare the NMR spectra of the single strands, 5'(dC<sup>P</sup>C<sup>P</sup>.U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3' (ssPrODN), 5'(dCC<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3' (ss-sPrODN1), and 5'(dCCUCCUU)3' (ssODN) and report the NMR solution structures of the duplexes, 5'(dC<sup>P</sup>C<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5', 5'(dCC<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5', and 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5' (Figure 1). This propynylated heptamer sequence was chosen because it is a potent and specific antisense agent against the mRNA of the SV40 large T antigen gene.<sup>18</sup> The NMR data suggest that ssPrODN is partially structured below 50 °C but ssODN is not. In addition, the duplex structures generated from NMR-derived distance restraints and restrained molecular dynamics suggest that all three duplexes form almost-identical structures. The results suggest that the increased stability of the duplexes containing PrODNs may be explained by preorganization of ssPrODN and by different interactions in the double strand. The propynyl group provides volume exclusion, enhanced stacking interactions, and possibly different solvation.

## Experimental Section

**Sample Preparation for NMR.** The synthesis and purification of oligonucleotides used in this study are described elsewhere.<sup>17</sup> The ssODN, ssPrODN, and ss-sPrODN1 strands for the single-stranded samples were lyophilized and redissolved in ~300 μL of 100 mM KCl, 10 mM phosphate buffer, 0.5 mM Na<sub>2</sub>EDTA, pH = 7.0 to give strand concentrations of ~3 mM. Duplex samples were prepared by mixing equivalent amounts of the appropriate strands in ~300 μL of buffer to give duplex concentrations of ~2.5 mM. For the nonexchangeable

proton samples, D<sub>2</sub>O exchange was performed with lyophilization from 99.96% D<sub>2</sub>O. The samples were redissolved in ~300 μL of 99.996% D<sub>2</sub>O (Cambridge Isotope Laboratories). NMR spectra of the exchangeable protons were recorded in 100 mM KCl, 10 mM phosphate buffer, 0.5 mM K<sub>2</sub>EDTA, pH = 7.0 in 90:10 H<sub>2</sub>O/D<sub>2</sub>O.

**NMR Spectroscopy.** All spectra were acquired on a Varian model Inova 500 MHz spectrometer. One-dimensional (1D) imino proton spectra were acquired with a binomial pulse sequence.<sup>19</sup> Offset delays were selected to maximize the peak intensity at 12.5 ppm.

Nuclear Overhauser effect spectroscopy (NOESY) spectra of samples in D<sub>2</sub>O were acquired with mixing times in the range of 50–600 ms and temperatures in the range of 10–50 °C. FIDs were acquired over 256–512  $t_1$  increments, each having at least 2K complex points with at least 32 scans per FID. Two-dimensional (2D) NOESY spectra of samples dissolved in 90:10 (v:v) H<sub>2</sub>O:D<sub>2</sub>O were recorded with a mixing time of 150 ms. The spectra were acquired with at least 1K complex points and a spectral width of 10 kHz at 0 °C. The DQF-COSY spectra for sugar assignments were acquired at 10–30 °C with 512  $t_1$  increments, each having 2K complex data points using a spectral width of 4000 Hz centered on the HDO resonance. For each FID, 64 scans were collected with a recycle delay of 2.5 s. The <sup>1</sup>H–<sup>31</sup>P HETCOR experiments followed the method of Sklenar et al.<sup>20</sup> The spectra were acquired with a spectral width of 1400 Hz in the direct (<sup>1</sup>H) dimension and 1000 Hz in the indirect (<sup>31</sup>P) dimension. FIDs were acquired over 256  $t_1$  intervals, each having 1K complex points per FID. Total correlation spectroscopy (TOCSY) spectra were acquired with a mixing time of 210 ms, 4K data points, and a spectral width of 4000 Hz in both dimensions.

Two-dimensional spectra were processed with the Felix software package (Molecular Simulations Inc.). Proton spectra were referenced to H<sub>2</sub>O or HDO at 4.74 ppm at 30 °C. Phosphorus resonances were referenced to the phosphate peak at 0.0 ppm at 30 °C (pH = 7.0).

**Restraint Generation.** NOESY cross-peak volumes were determined by measuring the volume of interest with the Felix software package. Distance restraints for 5'(dC<sup>P</sup>C<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5' were generated from 50- and 150-ms-mixing-time NOE volumes with H1'–H2' cross peaks as a reference (2.80 Å). Other proton–proton distances were calculated from the two-spin approximation by scaling by 1/ $r^6$ . Error bars of ±30% of the calculated values were assigned to each distance, to reduce the potential for errors due to spin diffusion, phasing, baseline abnormalities, and noise. A total of 160 interproton distance restraints (102 intranucleotide and 58 internucleotide), 18 hydrogen-bond restraints for seven Watson–Crick pairs (included as distance restraints of 1.8–2.5 Å), and 59 dihedral angle restraints were included. Because coupling in the DQF-COSY and HETCOR experiments suggested typical A-form geometry for the RNA strand, parameters for the dihedral angles were loosely restrained to those observed in canonical A-form RNA duplexes for all RNA residues in each duplex. These restraints were as follows:  $\alpha$  (0° ± 150°),  $\beta$  (180° ± 30°),  $\gamma$  (60° ± 30°),  $\delta$  (90° ± 20°),  $\epsilon$  (–135° ± 35°),  $\zeta$  (0° ± 150°), and  $\chi$  (180° ± 30°).<sup>21</sup> The torsion angles for the PrODN strand were not restrained, to allow conformational freedom of the propynylated nucleotides.

Distance restraints for 5'(dCC<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rGAGGAGGAAAU)5' were generated from 150-ms-mixing-time NOE volumes with the dC1 H5–H6 cross peak as a reference (2.45 Å). Error bars of ±30% of the calculated values were assigned to each distance. A total of 149 interproton distance restraints (86 intranucleotide and 63 internucleotide), 18 hydrogen-bond restraints for seven Watson–Crick pairs (included as distance restraints of 1.8–2.5 Å), and 59 RNA dihedral angle restraints were included. The torsion angles for the

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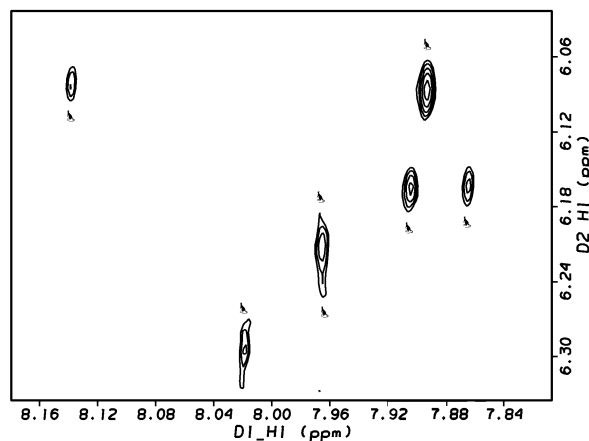
sPrODN1 strand were not restrained, to allow conformational freedom of the propynylated nucleotides.

Distance restraints for 5'(dCCUCCUU)3':3'(rGAGGAGGAAU)-5' were generated from 300-ms-mixing-time NOE volumes with pyrimidine H5–H6 cross peaks as a reference (2.45 Å). Error bars of  $\pm 40\%$  of the calculated values were assigned to each distance. A total of 140 interproton distance restraints (96 intranucleotide and 44 internucleotide), 18 hydrogen-bond restraints for seven Watson–Crick pairs (included as distance restraints of 1.8–2.5 Å), and 104 dihedral angle restraints were included. Generous angle restraints were applied to the ODN strand of the ODN:RNA duplex. These restraints were:  $\alpha$  ( $60^\circ \pm 30^\circ$ ,  $180^\circ \pm 30^\circ$ ),  $\beta$  ( $180^\circ \pm 90^\circ$ ),  $\gamma$  ( $60^\circ \pm 30^\circ$ ,  $-120^\circ \pm 90^\circ$ ),  $\delta$  ( $120^\circ \pm 60^\circ$ ),  $\epsilon$  ( $-135^\circ \pm 45^\circ$ ),  $\zeta$  ( $60^\circ \pm 30^\circ$ ,  $180^\circ \pm 30^\circ$ ), and  $\chi$  ( $30^\circ \pm 30^\circ$ ,  $-150^\circ \pm 30^\circ$ ). These values were chosen to include most torsion angles observed in nucleic acids in the Cambridge Structural Database and the Nucleic Acid Database.<sup>22</sup>

**Simulated Annealing.** Models of the duplexes consistent with NMR data were derived from restrained energy minimization and simulated annealing using the Discover 95 package on a Silicon Graphics computer. The modeling of the duplexes was done without the 5'rU1 and 3'rG11 dangling ends, because there are few cross peaks for these residues and they are not near the area of interest. A-form duplexes generated with Biosym InsightII software were used as starting structures. Calculations used the CVFF force field<sup>23,24</sup> in addition to flat-bottom restraint potentials with force constants of  $25 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$  for distance restraints and  $50 \text{ kcal mol}^{-1} \text{ rad}^{-2}$  for torsion angle restraints. The molecular dynamics protocol is described elsewhere.<sup>21,25</sup>

**Optical Spectroscopy.** The oligoribonucleotide concentration was calculated from high-temperature absorbance at 280 nm with predicted single-strand extinction coefficients.<sup>26,27</sup> The ssODN concentration was determined from high-temperature absorbance at 260 nm on the basis of monomer extinction coefficients.<sup>28</sup> The ssPrODN concentration was calculated from high-temperature absorbance at 260 nm, on the basis of monomer extinction coefficients of  $3200$  and  $5000 \text{ M}^{-1} \text{ cm}^{-1}$  for U<sup>p</sup> and C<sup>p</sup>, respectively (provided by M. D. Matteucci and B. C. Froehler). These were also used in conjunction with the DNA monomer extinction coefficient of C at 260 nm to estimate the concentration of ss-sPrODN1. Appropriate strands were mixed at 1:1 concentration ratios, denatured for 1 min at  $90^\circ \text{C}$ , and reannealed by slow cooling. Duplex extinction coefficients at high temperature were approximated by adding the extinction coefficients of the two single strands. The single strands and duplexes were lyophilized and redissolved in  $\sim 400 \mu\text{L}$  of 1 M NaCl, 20 mM sodium cacodylate, 0.5 mM Na<sub>2</sub>EDTA, pH = 7.0, resulting in final concentrations of  $1.6 \times 10^{-4}$ ,  $1.1 \times 10^{-4}$ ,  $6.0 \times 10^{-5}$ ,  $7.2 \times 10^{-5}$ ,  $5.2 \times 10^{-5}$ , and  $4.0 \times 10^{-5}$  M for ssODN, ssPrODN, ss-sPrODN1, ODN:RNA, PrODN:RNA, and sPrODN1:RNA, respectively.

Absorbance spectra of single-strand and duplex solutions used for circular dichroism (CD) were measured on a Beckman Coulter model DU-640C spectrophotometer with a high-performance temperature controller in a cell with a path length of  $L = 0.1 \text{ cm}$ . Scans were collected at 25 and  $80^\circ \text{C}$  over wavelengths from 205 to 320 nm to ensure that absorbances in this wavelength range were between 0.1 and 1.0 for  $L = 0.1 \text{ cm}$ . CD spectra of single strands and duplexes were measured on a Jasco model J-710 spectropolarimeter in a cell



**Figure 2.** The 200 ms NOESY walk region of 5'(dC<sup>p</sup>C<sup>p</sup>U<sup>p</sup>C<sup>p</sup>C<sup>p</sup>U<sup>p</sup>U<sup>p</sup>)3' at  $10^\circ \text{C}$ . The seven intraresidue cross peaks are labeled with an asterisk. Spectral widths were chosen to include only the H6 resonances in the first dimension and only the H1' resonances in the second dimension. An equivalent spectrum for 5'(dCCUCCUU)3' shows no cross peaks. (See Supporting Information for 1D spectra of the single-strand samples.)

with  $L = 0.1 \text{ cm}$ . Data were collected at 0.5 nm intervals, at a scan speed of 100 nm/min. Four scans of the samples at room temperature were collected and averaged. The molar ellipticity,  $[\theta]$ , was calculated from the observed ellipticity,  $\theta$ , and concentration,  $c$ :

$$[\theta] = \frac{\theta}{cL} \quad (1)$$

## Results

**Assignment of Nonexchangeable Proton and Phosphorus Resonances.** NMR resonances were assigned essentially as described by Varani et al.<sup>29,30</sup> In the H6–H1' region of the 200-ms NOESY spectrum of ssODN at  $10^\circ \text{C}$ , no NOESY cross peaks are observed. Spectra of ssODN also lack chemical-shift dispersion. For example, the seven H6 protons all resonate within  $\sim 0.1 \text{ ppm}$ , as do the seven H1' protons. The base and H1' resonances are more dispersed in ssPrODN and ss-sPrODN1 (see Supporting Information), although definitive chemical-shift assignments cannot be made. The TOCSY spectrum, however, provides enough information to determine which H1', H2', H3', and H4' resonances belong to a single sugar. Seven and eight cross peaks are observed in the H6–H1' region of the 200-ms NOESY spectra of ssPrODN (Figure 2) and ss-sPrODN1 at  $10^\circ \text{C}$ , respectively. The ssPrODN and ss-sPrODN1 are likely flexible; therefore, most of these cross peaks are more likely intraresidue than interresidue peaks. These cross peaks allow the assignment of base protons to their respective sugar protons. No clearly interresidue cross peaks were observed, however, in any region of the ssPrODN or ss-sPrODN1 spectra, preventing assignments to particular nucleotides. At higher temperatures, the cross peaks in the H6–H1' region of the NOESY spectra of ssPrODN and ss-sPrODN1 begin to disappear. By  $50^\circ \text{C}$ , no such cross peaks are observed.

The presence of interresidue cross peaks and a greater dispersion of chemical shifts allowed for the assignment of resonances for the duplexes. Table S1 (Supporting Information) summarizes chemical-shift assignments for the duplexes at  $30^\circ \text{C}$ . The H5' and H5'' assignments are not stereospecific,

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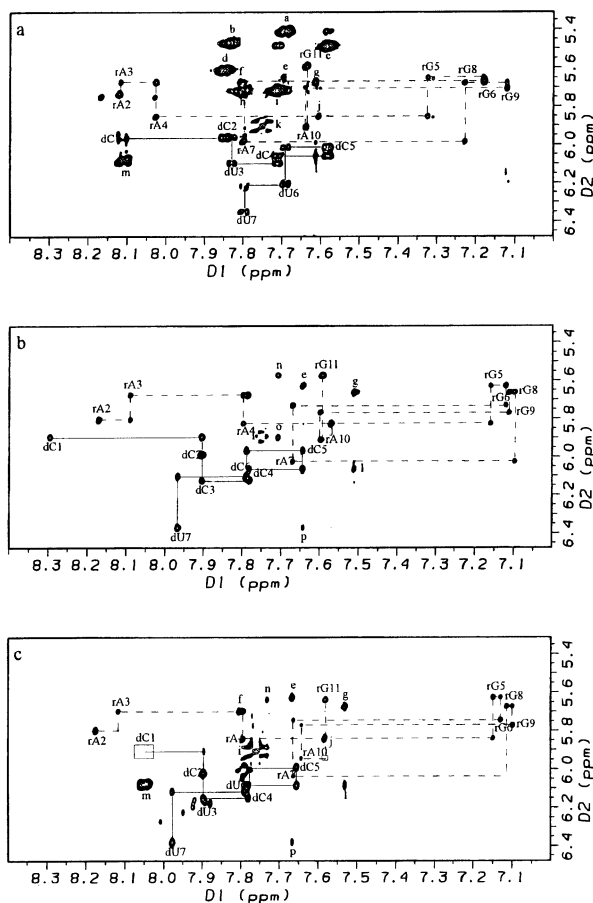
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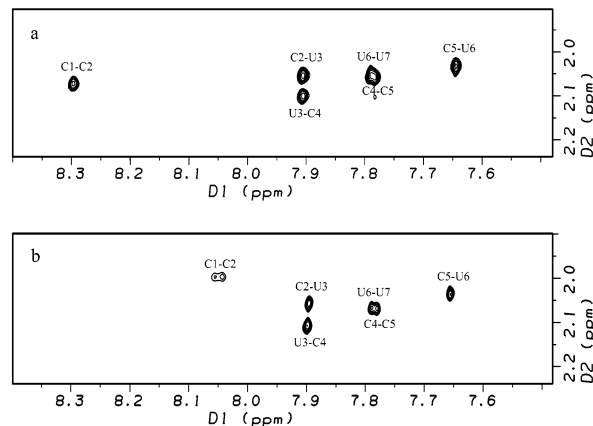


**Figure 3.** (a) The 300 ms NOESY walk region of 5'(dCCUCCUU)3':3'(rGAGGAGGAAAU)5', (b) 150 ms NOESY walk region of 5'(dCPCPUPUP)3':3'(rGAGGAGGAAAU)5', and (c) 150 ms NOESY walk region of 5'(dCCPUPCPCPUPUP)3':3'(rGAGGAGGAAAU)5' at 30 °C. The NOESY walk cross peaks of the DNA and RNA strands are connected by a solid line and a dotted line, respectively. Intrastrand cross peaks are labeled with the sugar type, residue type, and residue number. Other labeled cross peaks are as follows: a, U6H6–U6H5; b, U3H6–U3H5; c, C5H6–C5H5; d, C2H6–C2H5; e, A4H2–G5H1'; f, A2H2–A3H1'; g, A7H2–G8H1'; h, U7H6–U7H5; i, C4H6–C4H5; j, A3H2–A4H1'; k, U1H6–U1H5; l, A7H2–C4H1'; m, C1H6–C1H5; n, A10H2–G11H1', o, A10H2–C1H1', and p A4H2–U7H1'. The 300 ms NOESY spectrum shown for ODN:RNA was used for modeling because the 150 ms NOESY spectrum had many less-intense and/or missing cross peaks in comparison to the PrODN:RNA and sPrODN1:RNA spectra. The boxes in the spectrum shown in panel c represent missing cross peaks. The resonance assignments for these protons are based on observable cross peaks in other regions of the NOESY spectrum.

although the H5' protons are expected to resonate downfield from the H5'' protons because of the negative charge on the phosphate.<sup>29</sup> A comparison of the chemical-shift data in Table S1 shows that substitution of the propynyl group at the C5 position of pyrimidines does not result in chemical-shift changes of >0.2 ppm for base and sugar protons, except for dC1H6 in sPrODN1:RNA versus PrODN:RNA.

NOESY spectra at 30 °C are shown in Figures 3 and 4. From dC1 to dU7 and from rA2 to rG11, assignments in the base (H8/H6/H2)–(H1'/H5) regions follow standard connectivity pathways for both the DNA and RNA strands.

Assignment of H2' resonances follows from strong cross peaks to H1' resonances in the 100-ms-mixing-time NOESY spectra and from weak cross peaks in the DQF–COSY spectra. The H2' assignments are confirmed by strong H8/H6(*n*) to H2'–(*n* – 1) cross peaks in the base-to-sugar region of the short-



**Figure 4.** Propynyl–H6 region of the 150 ms NOESY spectra of the (a) PrODN:RNA and (b) sPrODN1:RNA duplexes at 30 °C. The cross peaks are labeled as (*n*)H6–(*n* + 1)HPr.

mixing-time NOESY spectra, as typically observed for A-form conformations.

Phosphorus assignments were made directly from the strong H3'–P correlations in the <sup>1</sup>H–<sup>31</sup>P HETCOR spectra (data not shown). All observed <sup>31</sup>P resonances are within a 1.5 ppm range, suggesting that the duplex does not adopt an unusual conformation at these positions.<sup>31</sup>

**Assignments of Exchangeable Proton Resonances.** The imino (10–15 ppm, Supporting Information) and amino (6–9 ppm) regions of the <sup>1</sup>H NMR spectra recorded in an H<sub>2</sub>O solution provide information concerning hydrogen bonding between base pairs. As expected for a duplex with four GC and three AU pairs,<sup>32</sup> four G imino resonances are observed between 11.9 and 12.9 ppm and three U imino resonances are observed between 13.5 and 14.2 ppm for each duplex. The terminal rU1 and rG11 imino peaks are broad or missing in all three duplexes. By recording 1D NOE difference spectra and 2D NOESY WATERGATE spectra, the G and U imino resonances and C amino resonances were tentatively assigned (data not shown).

**Sugar Puckers.** In general, the conformations of the deoxyribose rings determine the overall structure of a deoxyribose strand. Strands in A-type conformations contain nucleotides with C3'-endo sugar puckers, whereas those in B-type conformations contain nucleotides with C2'-endo sugar puckers.<sup>33,34</sup> Analysis of the fine structure of cross peaks in a DQF–COSY spectrum offers a reliable estimation of sugar conformations.<sup>35</sup> An H1'–H2'' DQF–COSY cross peak of a deoxysugar with a C2'-endo pucker has a fine structure that splits the cross peak into 16 components, whereas the H1'–H2' cross peak has a much simpler fine structure. For a deoxysugar with a C3'-endo sugar pucker, the H1'–H2'' cross peak has a simple fine structure, whereas the H1'–H2' cross peak is either of low intensity or totally absent because of cancellation of antiphase peaks. The ssPrODN spectrum displays evidence of a mixture of C2'-endo

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and C3'-endo sugar puckers. Four H1'-H2'' DQF-COSY cross peaks exhibit fine structure with split peaks whereas others do not. The H1'-H2' DQF-COSY cross peaks are medium intensity, suggesting C2'-endo or a combination of C2'- and C3'-endo sugar puckers. The H1', H2', and H2'' resonances of ssPrODN have significant dispersion; thus, quantitative determination of the coupling constants is possible. The sugar puckers were determined by measuring the total splitting of the H1' protons, i.e.,  $\Sigma H1'$ , in the 2D DQF-COSY spectrum. Then, the quantity  $\Sigma H1'$  can be used to find the fraction of time,  $f_s$ , that the sugar of each nucleotide spends in the C2'-endo conformation:<sup>36</sup>

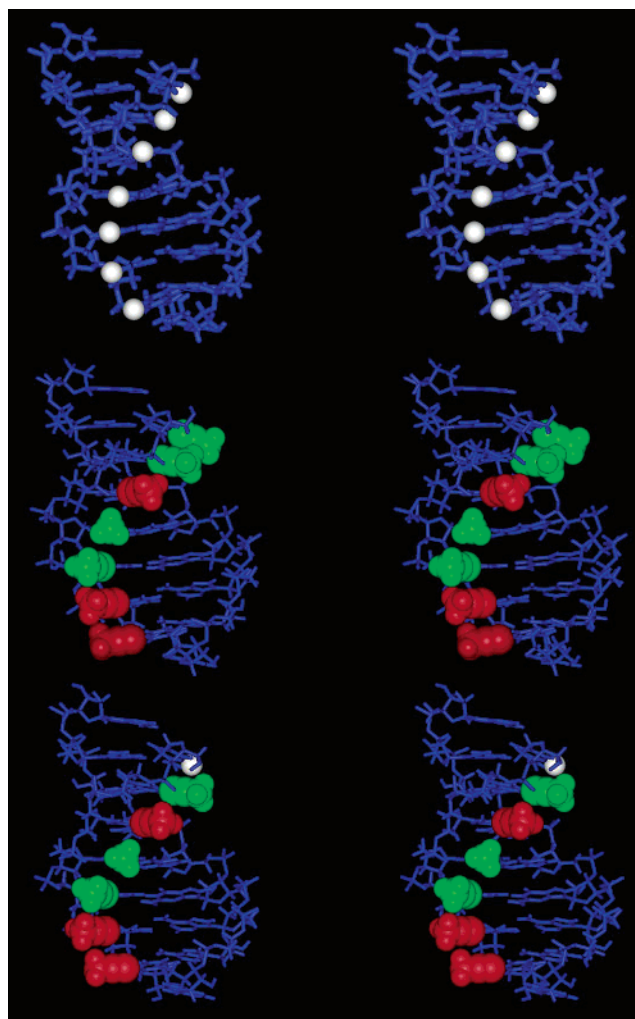
$$f_s = \frac{\Sigma H1' - 9.8}{5.9} \quad (2)$$

where  $\Sigma H1' = J_{1'2'} + J_{1'2''}$ . The average  $f_s$  value for ssPrODN is  $69\% \pm 15\%$ . Similar  $f_s$  values for DNA dimers, trimers, and tetramers containing single-stranded pyrimidine steps have been reported previously.<sup>37-41</sup> The H1', H2', and H2'' resonances of the ssODN are so overlapped that coupling constants could not be calculated and fine structure of the cross peaks could not be investigated. The average  $f_s$  values are 49%, 52%, and 52% for the DNA strands of PrODN:RNA, sPrODN1:RNA, and ODN:RNA, respectively.

**Structure Determination.** Distance restraints for structure determination are listed in Table S2 (Supporting Information). Structures derived in the absence of solvent and structures derived with water both displayed similar structural features and overall geometry. Because there are no apparent differences, further discussion is based on 30 structures derived in vacuo for each duplex.

Of the 30 structures of the ODN:RNA, PrODN:RNA, and sPrODN1:RNA duplexes generated by the rMD protocol, 20, 21, and 22, respectively, have similar simulated total energies. Superposition reveals that the overall structure and local features are well determined (Supporting Information). The average rmsd values for the all-atom pairwise superposition of these structures are 0.56, 0.62, and 0.60 Å for ODN:RNA, PrODN:RNA, and sPrODN1:RNA, respectively. These structures converged to satisfy all distance restraints within 0.13, 0.18, and 0.20 Å and all dihedral angle restraints within 3.4°, 2.8°, and 3.1° for ODN:RNA, PrODN:RNA, and sPrODN1:RNA, respectively. Stereo images of all three duplexes are shown in Figure 5.

**CD Spectra.** The NMR results show that the structures of the PrODN:RNA and sPrODN1:RNA duplexes are similar. Previous CD spectra, however, suggested that the structures are different.<sup>17</sup> Therefore, we remeasured the CD spectra. The spectra for the PrODN:RNA and ODN:RNA duplexes are within the experimental error of those reported previously. The CD spectrum of the sPrODN1:RNA duplex, however, differs from that previously reported but is very similar to the spectrum of



**Figure 5.** Stereo images of the major groove of (a) 5'(dCCUCCUU)3':3'(rAGGAGGAAA)5', (b) 5'(dC<sup>P</sup>C<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rAGGAGGAAA)5', and (c) 5'(dCC<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>)3':3'(rAGGAGGAAA)5'. White atoms are pyrimidine H5 protons, green atoms represent the propynyl group of C<sup>P</sup>, and red atoms represent the propynyl group of U<sup>P</sup>.

the PrODN:RNA duplex (see Supporting Information). The nucleic acid in the previous sample may have been partially degraded.

## Discussion

**Structural Analysis of ssODN, ss-sPrODN1, and ssPrODN.** NOESY cross peaks in the H6-H1' region are not observed for ssODN, and there is little dispersion in the base and sugar regions of the spectra, suggesting a lack of structure. For ssPrODN and ss-sPrODN1, intraresidue but not interresidue cross peaks are observed at 10 °C in the short-mixing-time NOESY spectra and the proton resonances are more dispersed. The lack of interresidue cross peaks suggests that the bases may not be rigidly stacked. The presence of the intraresidue cross peaks at 10 °C, however, suggests that ssPrODN and ss-sPrODN1 have some sort of structure not present in ssODN at 10 °C. The lack of intraresidue NOESY walk cross peaks at 50 °C suggests that the ssPrODN and ss-sPrODN1 strands are no longer preorganized at high temperature and may more closely resemble the ssODN strand.

**Structural Analysis of Duplexes.** The chemical-shift values obtained for the propynylated duplexes are similar to those

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obtained for the unmodified duplex, even for the protons adjacent to the propynylation site. The average deviation of proton chemical shifts between all three duplexes is only 0.04 ppm. The similarity in chemical shifts suggests that propynylation does not cause a significant global or local structural change (Figure 5). The similarity of the CD spectra for these oligomers also suggests that all the duplexes are in a similar conformation.

Several NOEs are crucial for structural determination of the duplexes. The presence of two cross-strand NOEs, rA4H2–dU7H1' and rA7H2–dC4H1', in short-mixing-time NOESY spectra suggests that the minor groove is narrower than that typically observed for the A-form conformation. Cross peaks involving the methyl protons of the propynyl groups are also important to determine their location in the helix (see Supporting Information). Most propynyl protons have a cross peak to H3' of the intrastrand 5'-neighboring residue. All the propynyl groups have an interstrand cross peak from the propynyl proton to the H6 of the 5'-neighboring residue in the 150-ms-mixing-time NOESY spectra (Figure 4). In longer-mixing-time spectra, some propynyl protons have a cross peak to their own H6.

The strength of the intranucleotide cross peaks between H3' and H6 is an indication of the pucker of the deoxyribose sugar. The NMR-derived distance between these two protons in the deoxyribose strand is, on average, 3.3, 3.1, and 3.4 Å for PrODN:RNA, sPrODN1:RNA, and ODN:RNA, respectively. These distances are more similar to typical distances found in the C2'-endo conformation (3.0 Å) than that found in the C3'-endo conformation (3.9 Å). The average fraction of C2'-endo sugar puckers of the deoxyribose sugars determined from the total splitting of the H1' protons in the 2D DQF–COSY spectra are 49%, 52%, and 52% for PrODN:RNA, sPrODN1:RNA, and ODN:RNA, respectively. This indicates that the sugar puckers of the deoxyribose sugars are a mixture of C2'-endo and C3'-endo conformations, with a smaller percentage of C2'-endo puckers than that observed for ssPrODN.

The sugar puckers were also calculated from the average duplex structures. Except for rU1 and rG11, all ribose sugar puckers for all the duplexes are C3'-endo. For the DNA strand, however, the sugar puckers range from C3'-endo to C2'-endo, with some residues in the C4'-exo and O4'-endo conformations. The precise conformation adopted by deoxyriboses in DNA:RNA hybrids has been an area of much debate. A dynamic equilibrium with a mixture of C2'-endo, O4'-endo, and C3'-endo<sup>42–45</sup> or a more rigid O4'-endo sugar pucker<sup>46–49</sup> are most often invoked to describe the deoxyribose sugars in DNA:RNA hybrids. There are three characteristics that distinguish between those two types of sugar puckers on the basis of NMR data.<sup>48</sup> For O4'-endo sugar puckers, H2''–H3' COSY cross peaks are observable, H1'–H4' NOESY cross peaks are stronger than

H1'–H2' NOESY cross peaks, and the NMR calculated distance for H1'–H4' is 2.6–2.8 Å. On the other hand, for the dynamic equilibrium between C2'-endo and C3'-endo conformations, H2''–H3' COSY cross peaks are not observed, H1'–H2' NOESY cross peaks are stronger than H1'–H4' cross peaks, and the NMR calculated distance of H1'–H4' is >3.2 Å. The NMR data of the three duplexes studied here exhibit all three characteristics of the dynamic equilibrium between C2'-endo and C3'-endo sugar puckers for the DNA strands.

The deoxyribose sugars of propynylated nucleotides of PrODN:RNA and sPrODN1:RNA adopt a larger percentage of C3'-endo sugar pucker than deoxyribose sugars in single-stranded DNA, DNA duplexes, and ssPrODN. C2'-endo sugar puckers tend to increase the intrastrand phosphate separation, and therefore, B-DNA structures have a larger rise between base pairs and are longer overall than A-form helices.<sup>50</sup> The PrODN strand may increase the percentage of sugar puckers in the C3'-endo conformation to reduce the length of the PrODN strand so that the strand more resembles the A-form shape of the more rigid RNA strand. Also, this decreases the rise per base pair, which may increase the stacking interactions between the propynyl group and the adjacent bases.

One striking difference between the NMR spectra of the three duplexes is observed in the NOESY walk region of sPrODN1:RNA. In all the NOESY spectra acquired for sPrODN1:RNA, the dC1H1'–dC1H6 cross peak is missing and the dC1H1'–dC2H6 cross peak is weak. One possible explanation is that the unpropynylated dC1 in an otherwise fully propynylated DNA strand may be more dynamic than the dC1 residues of the fully propynylated and the unmodified duplexes. The rG9 imino proton resonance of sPrODN1:RNA is overlapped with other imino proton resonances. The area under the overlapped peaks integrates to three protons at 0 and 30 °C. This area and the chemical shift of this resonance suggest that the dC1–rG9 Watson–Crick pair is formed at 0 and 30 °C. It is unclear whether this Watson–Crick pair is formed all the time, however, because the NOESY spectra indicate that dC1 is dynamic. The dC1H5–dC1H6 cross peak, however, is strong and displays no evidence that dC1 is dynamic. It is possible that this nucleotide is in a conformation in which the dC1H1'–C1H6 and dC1H1'–dC2H6 distances are longer than typically expected, resulting in weaker NOESY cross peaks.

The NMR spectra of the duplexes exhibit characteristic features of DNA:RNA hybrids. Exchangeable <sup>1</sup>H NMR spectra show that base pairs are formed at 0 °C for all the Watson–Crick pairs in all the duplexes. The base-to-H1' region of the NOESY spectra indicates that all bases are in the anti conformation. For all residues A4–A10 of the RNA strand, weak or nonexistent cross peaks in the DQF–COSY spectra indicate H1'–H2' couplings of <2 Hz, which suggests predominantly C3'-endo sugar puckers. C3'-endo sugar puckers of the ribose sugars are confirmed by the large H3'–H4' couplings. Strong- or medium-intensity cross peaks are observed in the short-mixing-time NOESY spectra from base H8/H6 protons to 5'-neighboring H2' protons for all the residues, which is consistent with no or slight deviation from a typical A-form conformation. All observed resonances in the 1D <sup>31</sup>P spectrum of each duplex are within a 1.5 ppm range, suggesting that the propynyl groups

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**Aspects of Molecular Recognition.** The results described here reveal three important changes when introducing propynylated DNA nucleotides into a single strand or duplex. The propynyl group causes the ssPrODN to adopt a structure that is not observed in ssODN. This could result from volume exclusion and new interactions due to the propynyl groups. The result is that the ssPrODN strand is, to some degree, preorganized for duplex formation. Presumably, this contributes to the smaller unfavorable entropy change observed for duplex formation.<sup>17</sup> Also, the bulky, hydrophobic propynyl groups are positioned in the major groove. These functional groups may act as a hydrophobic spine and, consequently, a driving force for the folding and stability of the duplex. Last, the addition of the propynyl group may provide more-favorable stacking interactions, because the triple bond of the propynyl group is situated below the adjacent 5' base. In combination, these effects may explain the greater stability of propynylated duplexes in comparison to unmodified duplexes. The relatively modest differences in duplex conformations suggest that these hypotheses may be tested by computational approaches, particularly at temperatures  $>50$  °C, where all the single strands are relatively unstructured. PrODNs have unique properties that can be exploited for molecular recognition of nucleic acids. Understanding the interactions that provide these properties should

lead to design of additional novel approaches for recognizing nucleic acids.

**Acknowledgment.** This publication was supported by NIH Grant Nos. GM 22939 (to D.H.T.) and GM 53826 (to T.R.K.). We thank Drs. M. D. Matteuchi and B. C. Froehler for initially inspiring this study and for providing an initial sample of 5'-(dC<sup>P</sup>C<sup>P</sup>U<sup>P</sup>C<sup>P</sup>C<sup>P</sup>U<sup>P</sup>U<sup>P</sup>). We also thank K. Patel for assistance with the spectropolarimeter. T.W.B. was an Oral Cellular and Molecular Biology Training Scholar (PHS Grant No. DE07202-10).

**Supporting Information Available:** Chemical-shift assignments for the duplexes, distance restraints for structure determination of the duplexes, 1D NMR spectra showing the H1'–H5 and H6 regions of the single strands, 1D NMR spectra showing the imino spectra of the duplexes, 2D NOESY spectra showing the sugar–base, sugar–sugar, and base–base regions of the duplexes, superposition of derived structures, and absorbance and CD spectra for the single strands and duplexes (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA021285D