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Synthesis and Thermal Denaturation Studies of Conformationally Restricted 3′-*C*-Ethynyl-3′-*O*,4′-*C*-methyleneribonucleotides

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Dedicated to Professor Wojciech J. Stec in celebration of his 65th birthday

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Incorporation of conformationally restricted 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleotide **X** monomers into oligodeoxyribonucleotides results in large decreases in affinity towards complementary DNA, whereas significantly more stable duplexes are formed with RNA complements. RNA selectivity becomes more pronounced upon multiple incorporations of **X** monomers. Increased thermal stability is observed upon positioning of two **X** monomers in a +1 zipper interstrand motif.

Introduction

A plethora of modified oligonucleotides has been synthesized during the last two decades,[1–2] stimulated by the potential of the antisense strategy to achieve specific control of gene expression. Among these, 2′,5′-linked nucleic acids have received marked attention.[3–5] Interesting physical properties of 2′,5′-linked nucleic acids include:[6–10] 1) their propensity to form duplexes with normal 3′,5′-linked RNA that are only slightly less stable than normal DNA:RNA or RNA:RNA duplexes, 2) a remarkable preference for complexation with complementary RNA, and 3) high resistance to nuclease digestion. The preferred furanose ring conformation for 2′,5′-linked RNA is C2′-*endo* (South-type),[10–12] whereas it is C3′-*endo* (North-type) for 2′,5′-linked DNA strands against normal DNA or RNA.[11,13] In contrast, the furanose rings of normal 3′,5′-linked RNA or DNA strands preferentially adopt C3′-*endo* and C2′-*endo* conformations, respectively.[14] Modification of normal DNA strands with conformationally restricted nucleosides such as LNAs[15–17] in which the furanose ring is locked in a C3′-*endo* conformation has proven to be a very powerful strategy for achieving strong hybridization with normal DNA or RNA complements. Correspondingly, it would be interesting to synthesize a nucleoside analog with a conformationally restricted South-type furanose ring that allows modification of 2′,5′-linked nucleic acids with the aim of achieving increased duplex stability with normal RNA. Recently, the synthesis of such analogs, i.e., 3′-*O*,4′-*C*-methyleneribonucleotides **A** (Figure 1), has been described.[18–19] Normal DNA strands containing incorporations of monomer **A** bind stronger to RNA complements than DNA complements and moreover display enhanced nuclease stability.[18–19] Stimulated by this and our interest[20–21] in analogs of the anticancer nucleoside 3′-*C*-ethynyluridine (EUrd),[22] we set out to synthesize the 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleotide monomer **X** (Figure 1). It has previously been shown that the furanose rings of 3′-*O*,4′-*C*-methyleneribonucleosides as well as 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleosides are conformationally biased towards similar South-type conformations (C1′-*exo*/C2′-*endo*).[19,21] However, the additional C3′-ethynyl group of monomer **X** is anticipated to make North-type conformations energetically even more unfavorable than for

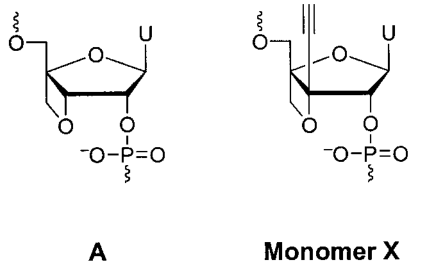

Figure 1. Structure of previously reported monomer **A**[18–19] and of 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleotide monomer **X** presented herein.

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monomer **A** due to the steric clash between the C3′-ethynyl group and the nucleobase moiety, in which both are positioned in *quasi*-axial positions in North-type conformations (Figure 2).

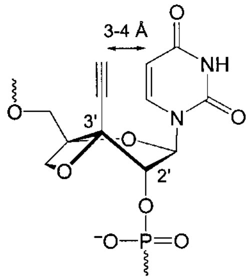


Figure 2. Illustration of the steric clash between the C3′-ethynyl group and the nucleobase of monomer **X** in a North-type conformation.

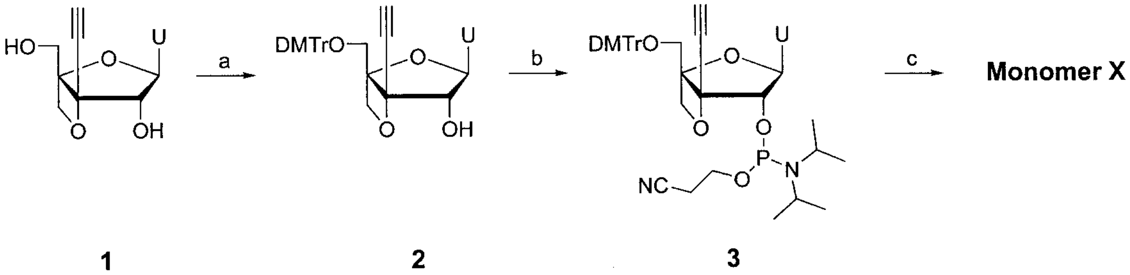
Herein we present the synthesis and thermal denaturation studies of oligodeoxyribonucleotides (ONs, 3′,5′-linked) containing incorporations of 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleotide monomer **X** (2′,5′-linked).

Results and Discussion

Selective O5′-tritylation of the known 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleoside **1**[21] to give nucleoside **2** in 60% yield (Scheme 1) was followed by standard O2′-phosphitylation affording amidite **3** in a satisfactory 86% yield. Phosphoramidite **3** was used as a building block for incorporation of monomer **X** into ONs using an automated DNA synthesizer. Standard procedures, except for extended coupling times for amidite **3** (15 min, using 1*H*-tetrazole as catalyst), resulted in excellent step-wise coupling yields (>98%) of phosphoramidite **3**. The composition and purity (>80%) of the synthesized oligonucleotides was verified by MALDI-MS analysis (Table S1) and ion-exchange HPLC, respectively.

The effect on duplex stability upon incorporation of monomer **X** into ONs was evaluated by recording thermal denaturation temperatures (T_m) in a medium salt buffer ($[Na^+]$ = 110 mM) (Table 1). In all cases the UV denaturation curves displayed smooth sigmoidal monophasic transitions with a shape similar to curves of unmodified reference duplexes (see Figure S1 in the Supporting Information). Monomer **X** was first incorporated in a thymidine rich 12-mer ON, previously used to study the properties of 3′-*O*,4′-*C*-methyleneribonucleotides in 3′,5′-DNA.[18] Incorporation of single monomer **X** induces a rather large decrease in duplex stability with complementary DNA, whereas only a slight destabilization is observed with RNA complements (**ON1**, ΔT_m = –5.0 °C and –1.0 °C, respectively). Incorporation of two **X** monomers results in synergistic decreases in duplex stability towards DNA or RNA complements (ΔT_m/mod ca. –7.0 °C or ca. –2.5 °C, respectively, data for **ON2**–**ON5**), regardless of whether two **X** monomers are sequentially incorporated or separated by one, two or three thymidines. The decreases in thermal stability of duplexes with complementary DNA/RNA are even more pronounced upon multiple incorporations of **X** monomers (data for **ON6**–**ON8**), although RNA selectivity is not compromised. It is noteworthy that quadruple modified **ON7** and **ON8** do not display transitions with DNA complements whereas hybridization with RNA complements is maintained. In general, the ΔT_m per modification values for **ON1**–**ON8** are ca. 2 °C lower than for ONs modified with 3′-*O*,4′-*C*-methyleneribonucleotides **A**.[18] Inspection of molecular modeling structures reveals that the ethynyl groups of monomer **X** are in close proximity to the 3′-phosphate group (closest C–P distance ca. 3–4 Å, Figure S2). Thus the slightly decreased thermal stability of the ONs containing monomer **X**, as compared to those containing monomer **A**, may result from an energetically unfavorable interaction of the additional C3′-ethynyl substituent with the hydration shell of the phosphate group, as suggested for other ethynyl or alkyl-substituted ONs.[23–24]

Next, monomer **X** was evaluated in 13-mer AT-rich sequence contexts (Table 1, **ON9**–**ON16**). Incorporation of a single **X** monomer in this sequence context generally results in large decreases in duplex stability with DNA complements, while virtually unchanged duplex stability with RNA complements is observed (ΔT_m = –9.5 °C to –4.5 °C and ΔT_m = –1.5 °C to +1.5 °C, respectively, data for **ON9**–**ON11** and **ON13**). The markedly higher duplex stability of **ON12** with DNA or RNA complements (ΔT_m = –1.5 °C or +3.5 °C, respectively) is intriguing and indicates a sequence specific effect. Incorporation of two **X** monomers, separated by an adenosine, generally results in synergistic decreases in duplex stability towards DNA and RNA complements (**ON14**–**ON16**), in agreement with the observations in the



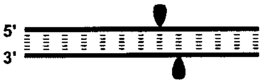
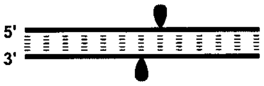
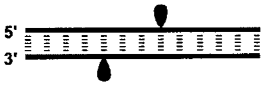
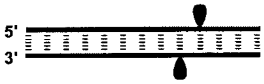
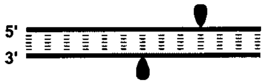
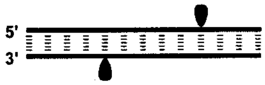
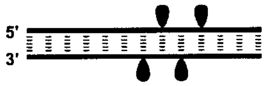
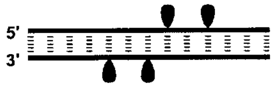
Scheme 1. Reagents and conditions: a) DMTrCl, DMAP, pyridine, room temp., 60%; b) $NC(CH_2)_2OP(Cl)N(iPr)_2$, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp., 86%; c) DNA-synthesizer; U = uracil-1-yl.

Table 1. Thermal denaturation temperatures (T_m values) for duplexes of **ON1**–**ON19** and DNA/RNA complements.[a]

		T_m (ΔT_m/mod)/°C	
		DNA	**RNA**
ON1	5′-GCG TTT TT**X** GCT	41.5 (–5.0)	42.5 (–1.0)
ON2	5′-GCG TTT T**XX** GCT	32.5 (–7.0)	38.5 (–2.5)
ON3	5′-GCG TTT **X**T**X** GCT	31.5 (–7.5)	37.5 (–3.0)
ON4	5′-GCG TT**X** TT**X** GCT	32.0 (–7.3)	39.0 (–2.3)
ON5	5′-GCG T**X**T TT**X** GCT	31.5 (–7.5)	40.0 (–1.8)
ON6	5′-GCG T**X**T **X**T**X** GCT	20.0 (–8.8)	33.0 (–3.5)
ON7	5′-GCG **XX**T T**XX** GCT	<10 (<–9.1)	29.5 (–3.5)
ON8	5′-GCG T**XX** **XX**T GCT	<10 (<–9.1)	27.5 (–4.0)
ON9	5′-CGT GAT A**X**A TAA A	25.0 (–6.5)	25.0 (–1.0)
ON10	5′-CGT GAT ATA **X**AA A	23.5 (–8.0)	24.5 (–1.5)
ON11	5′-TTT A**X**A TAT CAC G	27.0 (–4.5)	30.0 (+1.5)
ON12	5′-TTT ATA **X**AT CAC G	30.0 (–1.5)	32.0 (+3.5)
ON13	5′-TTT ATA TA**X** CAC G	22.0 (–9.5)	28.0 (–0.5)
ON14	5′-CGT GAT A**X**A **X**AA A	24.5 (–3.5)	21.5 (–2.3)
ON15	5′-TTT A**X**A **X**AT CAC G	20.5 (–5.5)	29.5 (+0.5)
ON16	5′-TTT ATA **X**A**X** CAC G	15.5 (–8.0)	26.0 (–1.3)
ON17	5′-GCG TTT TTT GCT	46.5	43.5
ON18	5′-CGT GAT ATA TAA A	31.5[b]	26.0
ON19	5′-TTT ATA TAT CAC G	31.5[b]	28.5

[a] Thermal denaturation temperatures [T_m values/°C (ΔT_m/mod = change in T_m value per **X** modification calculated relative to DNA:DNA or DNA:RNA reference duplex)] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature) recorded in a medium salt buffer ($[Na^+]$ = 110 mM, $[Cl^-]$ = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)), using 1.0 μM concentrations of the two complementary strands. T_m values are averages of at least two measurements; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer; see Scheme 1 for structure of monomer **X** (the symbol "**X**" has been bolded and underlined in the Table for clarity). [b] **ON18** and **ON19** are complementary.

Table 2. Thermal denaturation data and schematic illustrations of duplexes with monomer **X** positioned in various interstrand zipper motifs.[a]

Duplex	T_m (ΔT_m/mod)/ °C	Schematic illustration	Constitution
ON9:**ON11**	35.0 (+1.8)		+1 zipper
ON9:**ON12**	25.0 (–3.3)		–1 zipper
ON9:**ON13**	16.5 (–7.5)		–3 zipper
ON10:**ON11**	21.5 (–5.0)		–1 zipper
ON10:**ON12**	22.0 (–4.8)		–3 zipper
ON10:**ON13**	13.5 (–9.0)		–5 zipper
ON14:**ON15**	23.0 (–2.1)		–
ON14:**ON16**	< 10 (< –5.4)		–

[a] For conditions of thermal denaturation experiments see Table 1. Black droplets illustrate the ethynyl moiety of monomer **X**.

thymidine rich 12-mer sequence context (**ON2**–**ON5**). A noticeable exception is **ON14**:**DNA** for which a rather stable duplex is observed (compare T_m values **ON9**:**DNA**, **ON10**:**DNA** and **ON14**:**DNA**).

To evaluate possible interstrand effects, duplexes of two strands, each containing monomers **X**, were studied. Positioning of two **X** monomers in –1, –3 or –5 interstrand zipper arrangements (Table 2, see schematic illustrations) results in merely additive decreases in duplex stability, suggesting that two **X** monomers exert limited influence on each other. Intriguingly, a +1 zipper arrangement of **X** monomers leads to formation of a very stable duplex (compare T_m values of, e.g., **ON9**:**DNA**, **ON11**:**DNA** and **ON9**:**ON11**). This remarkable directional effect on duplex stability suggests that an energetically more favorable local helix geometry (increased nucleobase stacking, improved solvation, etc.) is only adopted upon positioning of **X** monomers in +1 zipper motifs. The distinct behavior of the +1 zipper arranged **X** monomers is also partially reflected in **ON14**:**ON15**, which may be regarded as a dual –1 zipper arrangement of monomers **X**, or as the fairly high T_m value (23.0 °C) rather suggests, as a single +1 zipper plus two isolated **X** monomers. However, further studies of additional +1 zipper arrangements of **X** monomers in other sequence contexts are needed to determine whether the observed directional effect is general.

In summary, incorporation of conformationally restricted 3′-*C*-ethynyl-3′-*O*,4′-*C*-methyleneribonucleotide monomer **X** into oligodeoxyribonucleotides results in large decreases in affinity towards DNA complements whereas approximately unaltered affinity towards RNA complements is observed.

Experimental Section

General Remarks: Solvents and reagents were of analytical quality and obtained from commercial suppliers and used without further purification with the exception of dichloromethane, which was distilled prior to use. Petroleum ether of the distillation range 60–80 °C was used. 1,2-Dichloroethane was dried through storage over activated 4-Å molecular sieves. A Karl-Fischer apparatus was used to check the water content of the anhydrous solvents. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with fluorescence indicator (SiO_2-60, F-254) which were visualized a) under UV light, b) by dipping in 5% conc. sulfuric acid in absolute ethanol (v/v) followed by heating or c) by dipping in a solution of molybdato-phosphoric acid (12.5 g/L) and cerium(IV)sulfate (5 g/L) in 3% conc. sulfuric acid in water (v/v) followed by heating. Silica gel column chromatography using moderate pressure (pressure ball) was performed with Silica gel 60 (particle size 0.040–0.063 mm, Merck). Evaporation of solvents was carried out under reduced pressure with a temperature not exceeding 50 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried under high vacuum for at least 12 h to give the products in high purity (>95%). ^{13}C NMR or ^{31}P NMR spectroscopy ascertained the sample purity. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded at 300 MHz, 75.5 MHz and 121.5 MHz, respectively. Chemical shifts are reported in parts per million (ppm) relative to deuterated solvent as the internal standard (δ_H: [D$_6$]DMSO 2.50 ppm; δ_C: [D$_6$]DMSO 39.43 ppm). Exchangeable (ex) protons were detected by disappearance of peaks on D_2O addition. Assignments of NMR spectra were based on 2D spectra (HETCOR, COSY) and follow standard nucleoside nomenclature. The carbon atom of C4 substituents is numbered C-5′′. Similar conventions apply for the corresponding hydrogen atoms. Quaternary carbons were not assigned in the ^{13}C NMR spectra. Traces of solvents in NMR spectra were identified by reference to published data.[25] MALDI-HRMS were recorded in positive ion mode using an IonSpec Fourier transform mass spectrometer.

(1*S*,3*R*,4*R*,5*S*)-4-[2-Cyanoethoxy(diisopropylamino)phosphanyloxy]-4,4′-dimethoxytrityloxymethyl)-5-ethynyl-3-(uracil-1-yl)-2,6-dioxabicyclo[3.2.0]heptane (2): Diol **1**[21] (0.37 g, 1.32 mmol) was dried by coevaporation with anhydrous pyridine (5 mL) and redissolved in anhydrous pyridine (3 mL). DMTrCl (0.58 g, 1.72 mmol) and DMAP (32 mg, 0.26 mmol) were successively added to this and the reaction mixture was stirred for 3 h at room temp. under argon whereupon MeOH (1 mL) was added. The solution was diluted with EtOAc (30 mL) and washed with sat. aq. $NaHCO_3$ (2 × 10 mL). The organic phase was evaporated to dryness, coevaporated with toluene (3 × 10 mL) and the resulting residue purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v) to afford nucleoside **2** (0.46 g, 60%) as a white solid material. R_f = 0.5 (EtOAc). MALDI-HRMS: *m*/*z* = 605.1904 ([M + Na]$^+$, $C_{33}H_{30}N_2Na^+O_8$ calc. 605.1894). ^{1}H NMR ([D$_6$]DMSO): δ = 11.51 (br. s, 1 H, ex, NH), 7.73 (d, 1 H, *J* = 8.1 Hz, H-6), 6.87–7.43 (m, 13 H, DMT), 6.24 (d, 1 H, *J* = 8.1 Hz, H-1′), 6.12 (d, 1 H, ex, *J* = 7.0 Hz, 2′-OH), 5.50 (d, 1 H, *J* = 8.1 Hz, H-5), 4.66 (d, 1 H, *J* = 8.1 Hz, H-5′′), 4.35 (d, 1 H, *J* = 8.1 Hz, H-5′′), 4.18 (br. t, 1 H, H-2′), 4.06 (s, 1 H, HC≡C), 3.74 (s, 6 H, CH_3O), 3.45 (d, 1 H, *J* = 10.3 Hz, H-5′), 3.32 (H-5′ partial overlap with H_2O) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 162.7, 158.1, 150.6, 144.5, 140.5 (C-6), 135.1 (DMT), 135.0 (DMT), 129.7 (DMT), 127.8 (DMT), 127.6 (DMT), 126.7 (DMT), 113.1 (DMT), 102.4 (C-5), 85.8, 85.6, 85.3, 84.9, 82.2, 79.1, 77.3 (C-2′), 76.1, 62.3, 55.0 (CH_3O) ppm. A trace impurity of EtOAc was identified.

(1*S*,3*R*,4*R*,5*S*)-4-[2-Cyanoethoxy(diisopropylamino)phosphanyloxy]-1-(4,4′-dimethoxytrityloxymethyl)-5-ethynyl-3-(uracil-1-yl)-2,6-dioxabicyclo[3.2.0]heptane (3): Nucleoside **2** (140 mg, 0.24 mmol) was coevaporated with anhydrous 1,2-dichloroethane (3 × 5 mL) and dissolved in a mixture of anhydrous *N*,*N*′-diisopropylethylamine (0.3 mL) and anhydrous CH_2Cl_2 (1.2 mL). 2-Cyanoethyl *N*,*N*′-(diisopropyl)phosphoramidochloridite (111 µL, 0.50 mmol) was added to this, and the reaction mixture was stirred under argon at room temp. for 1 h. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with sat. aq. $NaHCO_3$ (15 mL). The aqueous phase was back-extracted with CH_2Cl_2 (25 mL) and the combined organic phase evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–45% EtOAc in petroleum ether, v/v) to afford amidite **3** (161 mg, 86%) as a white solid material. R_f = 0.7 (EtOAc). MALDI-HRMS: *m*/*z* = 805.2956 ([M + Na]$^+$, $C_{42}H_{47}N_4Na^+O_9P$ calc. 805.2973). ^{31}P NMR (CH_3CN + [D$_6$]DMSO): δ = 153.9, 152.3 ppm.

Synthesis of Modified Oligonucleotides: The synthesis of oligodeoxyribonucleotides (ONs) containing monomer **X** was performed on a 0.2 µmol scale with an automated DNA synthesizer. Standard procedures were used except for extended coupling times for amidite **3** (15 min, using 1*H*-tetrazole as catalyst), which resulted in stepwise coupling yields >98% of phosphoramidite **3** and >99% of unmodified deoxyribonucleotide phosphoramidites. Removal of nucleobase protecting groups and cleavage from solid support was effected using 32% aqueous ammonia for 12 h at 55 °C. The crude ONs were subsequently a) precipitated from abs. EtOH (–18 °C, 12 h) and washed with abs. EtOH or b) purified by ion-exchange HPLC with a LaChrom L-7000 system (VWR International) equipped with a Gen-Pak Fax: column (100 mm × 4.6 mm) using an isocratic hold of 95% A-buffer for 5 min followed by a linear gradient to 70% B-buffer over 41 min at a flow rate of 0.75 mL/min (A-buffer: 25 mM Tris-Cl, 1 mM EDTA, pH 8.0; B-buffer: 1 M NaCl), followed by desalting using commercial NAP-columns. The composition and purity (>80%) of the synthesized oligonucleotides was verified by MALDI-MS analysis and analytical ion-exchange HPLC, respectively.

Thermal Denaturation Studies: Concentrations of ONs were calculated using the following extinction coefficients (OD_{260}/µmol): G, 10.5; A, 13.9; T/U, 7.9; C, 6.6. Thermal denaturation temperatures (T_m values in °C) were measured with a Perkin–Elmer Lambda 35 UV/Vis spectrometer equipped with a PTP-6 Peltier temperature programmer and determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs. temperature) recorded in medium salt buffer (T_m-buffer; 100 mM NaCl, 0.1 mM EDTA and pH 7.0 adjusted with 10 mM NaH_2PO_4/5 mM Na_2HPO_4). ONs (1.0 µM each strand) were thoroughly mixed, denatured by heating and subsequently cooled to the starting temperature of the experiment. The absorbance was measured at 0.5 °C intervals using 1 mL quartz optical cells with a pathlength of 1.0 cm. The temperature of the denaturation experiments ranged from at least 15 °C below T_m to 15 °C above T_m (although not below 5 °C). A temperature ramp of 1.0 °C/min was used in all experiments. Reported thermal denaturation temperatures are an average of at least two measurements within ±1.0 °C.

Supporting Information (see also the footnote on the first page of this article): MALDI-MS of synthesized ONs (Table S1), representative thermal denaturation curves (Figure S1), lowest energy structure of **ON9**:**DNA** from molecular modeling (Figure S2), details of molecular modeling procedure, ^{13}C NMR spectrum of nucleoside **2** and ^{31}P NMR spectrum of phosphoramidite **3**.

Note Added in Proof (September 21, 2005): For a very recent application of 2′,5′-linked DNA as triplex forming oligonucleotides, see: S. Obika, A. Hiroto, O. Nakagawa, T. Imanishi, *Chem. Commun.* **2005**, 2793–2795.

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