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Synthesis of Triazole-Linked Oligonucleotides with High Affinity to DNA Complements and an Analysis of Their Compatibility with Biosystems

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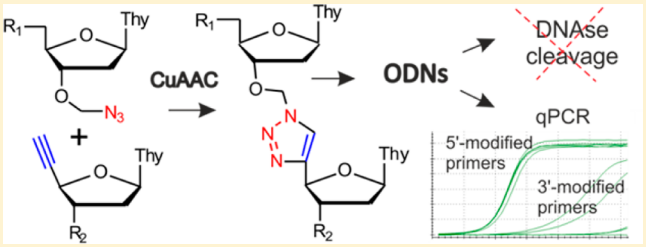
Supporting Information

ABSTRACT: New oligonucleotide analogues with triazole internucleotide linkages were synthesized, and their hybridization properties were studied. The analogues demonstrated DNA binding affinities similar to those of unmodified oligonucleotides. The modification was shown to protect the oligonucleotides from nuclease hydrolysis. The modified oligonucleotides were tested as PCR primers. Modifications remote from the 3′-terminus were tolerated by polymerases. Our results suggest that these new oligonucleotide analogues are among the most promising triazole DNA mimics characterized to date.



INTRODUCTION

Oligonucleotide (ON) analogues with triazole internucleotide linkages have shown great promise in the field of antisense technology and as DNA hybridization probes.[1−4] These analogues can be obtained by the iterative click ligation of azide- and alkyne-bearing nucleoside derivatives[5,6] or by automated phosphoramidite synthesis with modified dinucleoside blocks.[7] Although a significant number of triazole-linked ONs have been described to date,[5−9] few have demonstrated hybridization properties that meet the criteria for duplex stability. Isobe et al. reported a triazole modification that is particularly favorable for duplex stabilization.[9] Unfortunately, this stabilization was later shown to be sequence-dependent.[10] Nevertheless, triazole-linked ONs remain an intensively studied class of DNA mimics, primarily due to their potential for biocompatibility.[11,12] Unlike most internucleotide modifications, some triazole modifications can be tolerated by polymerases,[13,14] which makes triazole-linked ONs attractive as candidate diagnostic tools and therapeutic agents. Several promising types of triazole internucleotide modifications are shown in Scheme 1.

This paper introduces a new triazole internucleotide modification. The triazole linkage described herein is an isomer of a previously reported one (Scheme 1C).[6,13] A series of ON analogues bearing the triazole modification were obtained by automated solid-phase synthesis with a modified dinucleoside phosphoramidite block. The properties of the triazole-linked ONs are described.

RESULTS AND DISCUSSION

The triazole-linked dithymidine block used for incorporation into ONs was obtained by click-ligation of two nucleoside components (Scheme 2). The azido component was synthesized from 5′-*O*-benzoylthymidine **1**. An *O*-azidomethyl group was introduced into the 3′-position of **1** in two steps using a modified version of a method reported by Lonnberg and co-workers.[15] The first step was methylthiomethylation of the 3′-hydroxide group, which could be performed either by a modified Pummerer rearrangement[15] or by treatment with DMSO and benzoyl peroxide.[16,17] Both approaches are rather efficient and afford 3′-*O*-methylthiomethyl deoxynucleoside derivatives in comparable yields.[15,16,18] We used the modified Pummerer rearrangement. Nucleoside **1** was treated with a mixture of dimethyl sulfoxide and acetic anhydride in the presence of acetic acid. The 3′-*O*-methylthiomethyl derivative **2** was obtained in 80% yield.

Nucleoside **2** was reacted with molecular bromine followed by lithium azide in dry CH_3CN to produce the 3′-*O*-azidomethyl derivative **3** in 83% yield. The treatment of **3** with a K_2CO_3 suspension in dry methanol resulted in the removal of the benzoyl protecting group. A dimethoxytrityl protecting group was introduced by treatment with dimethoxytrityl chloride in dry pyridine. The resulting azide **5** was coupled with alkyne **6**[19] in the presence of $CuSO_4 \cdot 5H_2O$ and

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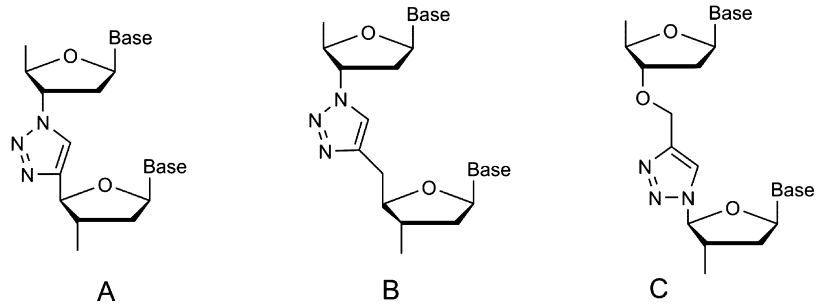
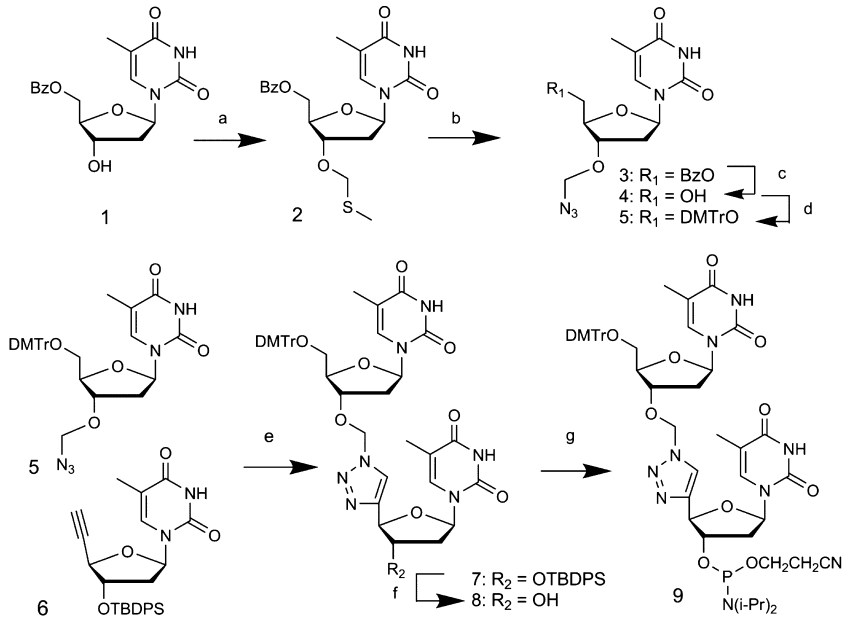
Scheme 1. Fragments of ON Analogues with Three Different Types of Triazole Internucleotide Modifications[6,9,19]



Scheme 2. Synthesis of the Phosphoramidite Dinucleoside Block with a Triazole Internucleoside Linkage[a]



[a]Reagents and conditions: (a) DMSO, Ac_2O, AcOH; (b) Br_2, LiN_3, CH_3CN; (c) K_2CO_3, MeOH; (d) DMTrCl, pyridine; (e) $CuSO_4$, sodium ascorbate, CH_2Cl_2/H_2O; (f) TBAF, THF; (g) $NCCH_2CH_2OP(N\text{-}i\text{-}Pr_2)_2$, 1*H*-tetrazole, pyridine, CH_2Cl_2.

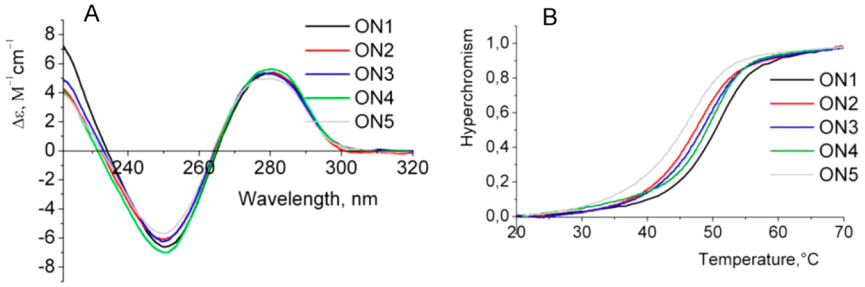


Figure 1. CD spectra (A) and melting curves (B) of the modified duplexes.

sodium ascorbate to give dinucleoside **7** with a yield of 88%. The silyl protection was removed with 0.5 M TBAF in THF. The treatment of dinucleoside **8**, bearing a free hydroxyl, with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite in the presence of 1*H*-tetrazole and pyridine in dry CH_2Cl_2 afforded the target phosphoramidite **9** in 74% yield.

Dinucleoside block **9** was used directly for the solid-phase synthesis of modified ONs using standard phosphoramidite protocols. The coupling time was increased to 15 min for the modified phosphoramidite. No decrease in coupling efficiency was observed (98−99% stepwise coupling yields for both modified and unmodified amidites).

ONs bearing triazole fragments at the 5′-terminus, near the 3′-terminus, and/or in the middle of the chain were obtained and hybridized to complementary ssDNAs. The CD spectra of the duplexes (Figure 1A) suggest that the modification did not cause any significant changes in duplex geometry.

The impact of the modification on duplex stability was evaluated by UV-melting experiments (Figure 1B). The results of the UV-melting experiments are summarized in Table 1. As is evident from these data, the modification tends to destabilize duplexes slightly. However, the destabilization is minor (average ΔT_m per linkage −1.4 °C) compared to those of previously described isosequential duplexes bearing other

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Table 1. Sequences, MALDI-TOF MS Data, and Melting Temperatures of the Duplexes Formed by Modified ONs and Their Unmodified Complement[a]

code	sequence of a modified strand (5′→3′)[b]	*m/z*, found (calcd for $[M + H]^+$)	T_m, ±0.5 °C (ΔT_m, °C)[c]
ON1	TTAACTTCTTCACATTC	5072.2 (5071.3)	50.3
ON2	**X**AACTTCTTCACATTC	5059.3 (5058.4)	50.0 (−0.3)
ON3	TTAACTTCTTCACA**X**C	5059.1 (5058.4)	48.2 (−2.1)
ON4	TTAACTTC**X**CACATTC	5058.5 (5058.4)	49.1 (−1.2)
ON5	**X**AACTTC**X**CACA**X**C	5032.3 (5032.5)	45.4 (−4.9)

[a]Duplex concentration 5×10^{-6} M. [b]**X** denotes the triazole-linked dithymidine fragment. [c]T_m is the difference between modified and natural duplexes.

triazole modifications (average ΔT_m −5.3 °C for the triazole modification A (Scheme 1)[19] and −4.0 °C for the modification B (Scheme 1)[10]). Thus, the new ON analogues are among the most promising triazole-bearing DNA mimics in terms of hybridization properties.

Because the new modification has a relatively insignificant impact on duplex geometry, we hypothesized that it might be tolerated by ON-recognizing enzymes. Oligonucleotides with a triazole linkage isomeric with that described here ("isotriazole"; C in Scheme 1) are known to be recognized by DNA and RNA polymerases.[13,14] Plasmid constructs with such linkages have proven functional in *E. coli*.[11] Although the "isotriazole" modification appeared to reduce duplex stability (ΔT_m of approximately −8 °C per modification) and caused local duplex distortion,[12] it was tolerated in both primers and templates by Taq polymerase.[13] To investigate the compatibility of the new triazole linkage with the thermostable polymerases Taq and Pfu, we synthesized a series of PCR primers with modifications at various positions. PCR with the Taq polymerase was quite efficient with all but the 3′-modified primers, while the Pfu polymerase only tolerated modifications near the 5′-terminus (Figure 2A). The sensitivity of Taq to primer modification was evaluated more rigorously by qPCR (Figure 2B). Modifications at up to eight nucleotides from the 3′-end resulted in a substantial decrease in the product accumulation rate.

Thus, the new triazole-linked ONs are generally usable as PCR primers, but only some polymerases can tolerate the modifications, and the dependence of PCR efficiency on the modification position should be taken into account. Modified PCR primers may be useful for SNP-based allele detection should they demonstrate an enhanced sensitivity to mismatches. To assess the hybridization specificity of the new triazole-linked ONs, we synthesized a set of ON4 complements with mismatches opposite to the modified fragment of ON4. Melting temperatures of mismatched duplexes were obtained and compared with that of a fully matched duplex. Sequences of the mismatched duplexes and T_m data are given in the Supporting Information. Generally, the triazole modification appears to impart slightly increased sensitivity to mismatches opposing the nucleoside which precedes the triazole fragment.

An important aspect of compatibility with biosystems is stability in biological liquids. Non-natural internucleotide fragments are well-known to confer increased stability to fully modified ONs. We tested the effect of partial triazole modification on the rate of ON hydrolysis by DNase I. The triple modification of the 17-mer caused an approximately 1.5- to 2-fold increase in ON half-life. The previously reported triazole modification[10] (B in Scheme 1) had a similar effect on the rate of enzymatic hydrolysis (see the Supporting Information). It can be concluded that even the partial triazole modification of internucleotide linkages may substantially improve ON biostability.

CONCLUSION

A dinucleotide with a new triazole internucleotide modification was synthetized and incorporated into ONs. The ONs bearing triazole fragments demonstrated high hybridization affinity to DNA complements and increased resistance to nuclease digestion. ONs with 5′-modified termini were functional as PCR primers. Our results suggest that these new ON analogues are adequate mimics of native DNA with enhanced biostability and may be attractive tools for diagnostics or the investigation of DNA structure.

EXPERIMENTAL SECTION

All reagents were commercially available unless otherwise mentioned and used without further purification.1-(2,5,6-Trideoxy-3-*O*-(*tert*-butyldiphenylsilyl)-*β*-D-*erythro*-hex-5-ynofuranosyl)-5-methyluracil (**6**) was synthesized as previously described.[19] All solvents were purchased from Khimmed (Russia). Dichloromethane was distilled from phosphorus pentaoxide, pyridine was distilled from calcium hydride, and THF was distilled from lithium alumohydride prior to use. Column chromatography (CC) was performed on silica gel Kieselgel 60 (0.040−0.063 mm, Merck, Germany). TLC was performed on silica gel Kieselgel 60 F254 precoated plates (Merck) with detection by UV using the following solvent systems (compositions expressed as v/v): ethanol/methylene chloride 1/49 (A), 1/20 (B). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMXIII-400 NMR spectrometer (Germany). Chemical shifts are given in parts per million (ppm). The coupling constants (*J*) are given in Hz. Abbreviations used: dmT, 5′-deoxy-5′-methylenethymidine moiety; aT, 3′-*O*-aminomethylthymidine moiety. Spectral assignments were completed by COSY DQF and {^{1}H−^{13}C}HSQC experiments (see the Supporting Information). The NMR data were processed and presented using MestReNova version 7.0.3 (Mestrelab Research SL, Spain).

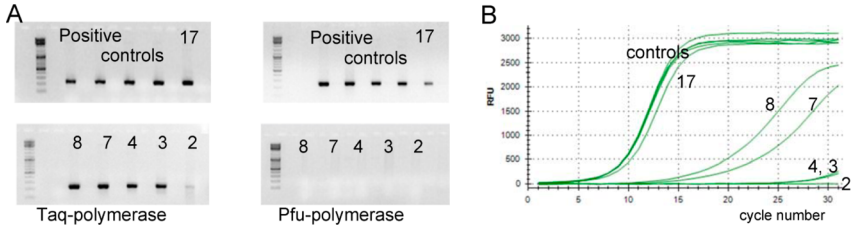

Figure 2. PCR with modified primers. (A) Relative sensitivity of the Taq (left) and Pfu (right) polymerases to primer modification. The first four lanes of the upper gels are the products of PCR with unmodified primers. The numbers represent the positions of the modifications (number of nucleotides from the 3′-terminus). For primer sequences, see the Supporting Information. (B) Accumulation kinetics curves obtained during qPCR with Taq polymerase.

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Mass Spectra. MALDI TOF mass spectra were acquired on a Bruker Microflex mass spectrometer (Bruker, Germany) in a linear mode (+20 kV). Each spectrum was accumulated using 200 laser shots (N_2 gas laser, 337 nm). A solution of 35 g/mL of 3-hydroxypicolinic acid with dibasic ammonium citrate was used as a matrix. ESI HR mass spectra were acquired on a Bruker microTOF II spectrometer (Germany) in a positive ion mode (capillary voltage −4500 V, end plate offset −500 V, interface capillary temperature 180 °C, nitrogen dry gas, 4.0 L/min, scanning range from m/z 50 to 3000 Da, scanning frequency 1 scan/s). Internal calibration was done using Electrospray Calibration Solution (Fluka).

Oligonucleotides. ONs were synthesized on an Applied Biosystems 3400 DNA synthesizer (USA) using standard phosphoramidite protocols and purified using preparative-scale reverse-phase HPLC on a 250 mm × 4.0 mm^2 Hypersil C18 column with detection at 260 nm. Chromatography of dimethoxytrityl protected ONs was performed using a 10−50% gradient of CH_3CN in 0.05 M TEAA. Detritylated oligonucleotides were further purified in a 0−25% gradient of CH_3CN in TEAA buffer.

UV Melting and CD. Melting curves of the duplexes were recorded on a Shimadzu UV 160-A spectrophotometer (Japan) using a thermostated cell in 20 mM sodium phosphate buffer, 100 mM NaCl, 01 mM EDTA, and pH 7.0, with the concentration of each duplex being 2.5×10^{-6} M. Samples were denatured at 95 °C for 5 min and slowly cooled to 20 °C prior to measurements. The duplex absorbance at 260 nm was measured as a function of temperature. It was registered every 0.5 °C from 20 to 70 °C. Thermodynamic parameters of duplex formation were obtained by performing nonlinear regression analysis using DataFit version 9.0.059 (Oakdale Engineering, USA). A calculation method taking into account the temperature dependence of UV absorbance of duplexes and single strands was applied. Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter at 20 °C using samples annealed in the same buffer under the same conditions as for the thermal denaturation studies. The CD values ($\Delta\varepsilon$) are given per mole of nucleotides.

PCR and qPCR. PCR products from 215-mer and 222-mer templates (fragments of a pGEM vector, Promega, US) were generated using 2.5 μL of 10× buffer in a total reaction volume of 25 μL with 1 ng of the DNA template, 0.5 μM of each primer, 0.2 mM dNTP, and 0.5 unit of Taq DNA polymerase (Lytech, Russia) or Pfu DNA polymerase (Fermentas, US). 10X Taq buffer: 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3; 10X Pfu buffer: 200 mM Tris-HCl (pH 8.8 at 25 °C), 100 mM KCl, 100 mM $(NH4)_2SO_4$, 20 mM $MgSO_4$, 1.0% Triton X-100, and 1 mg/mL nuclease-free BSA. PCR cycling conditions: 95 °C (initial denaturation) for 2 min then 35 cycles of 95 °C (denaturation) for 15 s, 55 °C (annealing) for 20 s and 72 °C (extension) for 30 s. The reaction mixture was loaded onto a 2% agarose gel in 1X TBE buffer. DNA bands were visualized by staining with ethidium bromide after electrophoresis. Accumulation kinetic curves were obtained by SYBR Green qPCR using the CFX C1000 real-time PCR detection systems (Bio-Rad Laboratories, Inc., USA). Data analysis and curve plotting were performed using the bundled software.

Nuclease Hydrolysis. ON (0.35 nmol) was dissolved in 15 μL of a reaction buffer (100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 1 mM $CaCl_2$). A 5 μL aliquot was taken as a control, and 1 unit of DNaseI (Fermentas, US) in 1 μL of storage buffer was added to the remaining solution. The mixture was incubated at 37 °C for 15 min, an aliquot was removed and stored in a freezer at −20 °C until the end of the reaction, and the mixture was incubated at 37 °C for another 15 min. Once all three samples were collected, 1 μL of formamide was added to each sample. The samples were heated to 95 °C and then cooled on ice and loaded onto a denaturing 20% polyacrylamide gel for electrophoresis. The gel was run in TBE buffer (90 mM Tris-Borate (pH 8) and 20 mM EDTA).

5′-*O*-Benzoyl-3′-*O*-(methylthiomethyl)thymidine (2). 5′-*O*-Benzoylthymidine[20] (2.1 g, 6 mmol) was dissolved in a mixture of DMSO (19 mL), acetic acid (4 mL), and acetic anhydride (13 mL). The mixture was stirred for 3 days at room temperature and then poured into cold saturated $NaHCO_3$ (250 mL) and stirred for 2 h more. The precipitate was filtered, washed on the filter with water, air-dried, and recrystallized from a mixture of ethyl acetate and hexane. Compound **2** was obtained as colorless needles (1.95 g, 80%): R_f = 0.31 (solvent system A).

^{1}H NMR (400 MHz, DMSO): δ 11.31 (s, 1H, 3-H), 8.00 (d, ^3J = 7.6 Hz, 2H, *o*-H Bz), 7.68 (t, ^3J = 7.4 Hz, 1H, *p*-H Bz), 7.55 (dd, $^3J_{o,m}$ = 7.7 Hz, $^3J_{p,m}$ = 7.4 Hz, 2H, m-H Bz), 7.41 (s, 1H, 6-H), 6.17 (t, $^3J_{1',2'a}$ = $^3J_{1',2'b}$ = 7.1 Hz, 1H, 1′-H), 4.75 (d, ^2J = 11.5 Hz, 1H, 3′a-OCH_2S), 4.73 (d, ^2J = 11.5 Hz, 1H, 3′b- OCH_2S), 4.59−4.52 (m, 2H, 3′-H and 5′a-H), 4.48 (dd, $^2J_{5'a,5'b}$ = 11.8, $^3J_{5'b,4'}$ = 5.1 Hz, 1H, 5′b-H), 4.28−4.23 (m, 1H, 4′-H), 2.36−2.31 (m, 2H, 2′a-H and 2′b-H), 2.10 (s, 3H, SCH_3), 1.61 (s, 3H, 5-CH_3). ^{13}C NMR (101 MHz, DMSO): δ 165.43 (C═O), 163.47 (4-C), 150.27 (2-C), 135.46 (6-C), 133.50 (*p*-CH, Bz), 129.25 (C, Bz), 129.12 (*o*-CH, Bz), 128.78 (*m*-CH, Bz), 109.75 (5-C), 84.14 (1′-CH), 81.04 (4′-CH), 75.98 (3′-CH), 73.04 (OCH_2S), 64.16 (5′-CH_2), 35.79 (2′-CH_2), 13.19 (CH_3S), 11.78 (5-CH_3). HR MS ESI: m/z 429.1083, calcd for $[C_{19}H_{22}N_2NaO_6S]^+$ m/z 429.1091.

3′-*O*-(Azidomethyl)-5′-*O*-benzoylthymidine (3). To a stirred solution of nucleoside **2** (1.63 g, 4 mmol) in 60 mL of absolute CH_3CN was added Br_2 (0.21 mL, 4 mmol) dropwise at 0 °C. The mixture was stirred upon cooling for 30 min, and then lithium azide (0.98 g, 10 mmol) was added. The mixture foamed after the addition of lithium azide. The resulting suspension was mixed for 10 min upon cooling and then for 2 h at 60 °C. The solvent was evaporated under vacuum, and the residue was dissolved in CH_2Cl_2 (50 mL) and water (50 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by CC in 0−3% of ethanol in CH_2Cl_2 to give dinucleoside **3** as a hard foam (1.35 g, 82.5% yield): R_f = 0.33 (solvent system A).

^{1}H NMR (400 MHz, DMSO): δ 11.31 (s, 1H, 3-H), 8.01 (d, ^3J = 7.2 Hz, 2H, *o*-H Bz), 7.69 (t, ^3J = 7.2 Hz, 1H, p-H Bz), 7.53 (t, $^3J_{o,m}$ = $^3J_{p,m}$ = 7.2 Hz, 2H, *m*-H Bz), 7.40 (s, 1H, 6-H), 6.18 (t, $^3J_{1',2'a}$ = $^3J_{1',2'b}$ = 7.2 Hz, 1H, 1′-H), 4.92 (d, ^2J = 9 Hz, 1H, 3′a-OCH_2N_3), 4.89 (d, ^2J = 9 Hz, 1H, 3′b- OCH_2N_3), 4.59−4.50 (m, 2H, 3′-H and 5′a-H), 4.48 (dd, $^2J_{5'a,5'b}$ = 12.0, $^3J_{5'b,4'}$ = 6.8 Hz, 1H, 5′b-H), 4.29−4.26 (m, 1H, 4′-H), 2.41−2.37 (m, 2H, 2′a-H and 2′b-H), 1.61 (s, 3H, 5-CH_3). ^{13}C NMR (101 MHz, DMSO): δ 165.45 (C═O), 163.49 (4-C), 150.28 (2-C), 135.54 (6-CH), 133.52 (*p*-CH, Bz), 129.25 (C, Bz), 129.15 (*o*-CH, Bz), 128.79 (*m*-CH, Bz), 109.79 (5-C), 84.00 (1′-CH), 81.24 (4′-CH), 81.18 (OCH_2N), 78.01 (3′-CH), 64.08 (5′-CH_2), 36.25 (2′-CH_2), 11.78 (5-CH_3). HR MS ESI: m/z 424.1220, calcd for $[C_{18}H_{19}N_5NaO_6]^+$ m/z 424.1228.

3′-*O*-(Azidomethyl)thymidine (4). Finely powdered K_2CO_3 (0.22 g, 1.6 mol) was added to a solution of nucleoside **3** (1.3 g, 3.2 mmol) in 25 mL of absolute methanol, and the suspension was stirred for 12 h. Dry silica gel (20 mL) was added to the resulting transparent solution. The mixture was stirred for 10 min, and the solvent was removed under vacuum. The residue was purified by CC in 10 mL of CH_2Cl_2 and eluted with 2−5% of ethanol in CH_2Cl_2 to give nucleoside **4** as a hard foam (0.9 g, 94.6% yield): R_f = 0.63 (solvent system B).

^{1}H NMR (400 MHz, DMSO): δ 11.28 (s, 1H, 3-H), 7.68 (s, 1H, 6-H), 6.14 (t, $^3J_{1',2'a}$ = 8.0 Hz, $^3J_{1',2'b}$ = 6.8 Hz, 1H, 1′-H), 5.14 (dd, $^3J_{5'OH,5'a}$ = $J_{5'OH,5'b}$ = 5.2 Hz, 1H, 5′−OH), 4.86 (s, 2H, 3′-OCH_2N), 4.39−4.35 (m, 1H, 3′-H), 3.97−3.94 (m, 1H, 4′-H), 3.63−3.58 (m, 2H, 5′a-H and 5′b-H), 2.30−2.22 (m, 2H, 2′a-H and 2′b-H),1.79 (s, 3H, 5-CH_3). ^{13}C NMR (101 MHz, DMSO): ^{13}C NMR (101 MHz, DMSO) δ 163.59 (4-C), 150.35 (2-C), 135.85 (6-CH), 109.46 (5-C), 84.59 (1′-CH), 83.67 (4′-CH), 80.93 (O-CH_2-N), 78.31 (3′-CH), 61.13 (5′-CH_2), 36.53 (2′-CH_2), 12.14 (5-CH_3). HR MS ESI: m/z 320.0962, calcd for $[C_{11}H_{15}N_5NaO_5]^+$ m/z 320.0965.

3′-*O*-(Azidomethyl)-5′-*O*-(4,4′-dimethoxytrityl)thymidine (5). Nucleoside **3** (0.85 g, 2.9 mmol) was evaporated with absolute pyridine (2 × 5 mL) and dissolved in 5 mL of absolute pyridine. Dimethoxytrityl chloride (1.19 g, 3.5 mmol) was added, and the mixture was stirred for 12 h at room temperature. Then 0.2 mL of water was added, and the mixture was stirred for 10 min more and

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evaporated under vacuum. The residue was dissolved in 50 mL of chloroform, washed with water (20 mL) and saturated $NaHCO_3$ (20 mL), dried over Na_2SO_4, and concentrated. The residue was purified by CC in 3% ethanol in CH_2Cl_2 to give nucleoside **5** (1.6 g, 92% yield) as a hard foam: R_f = 0.48 (solvent system A).

^{1}H NMR (400 MHz, DMSO): δ 11.31 (s, 1H, 3-H), 7.50 (s, 1H, 6-H), 7.45−6.88 (m, 13 H, ArH DMTr), 6.16 (t, $^3J_{1',2'a} = {}^3J_{1',2'b}$ = 7.2 Hz, 1H, 1′-H), 4.85 (d, ^2J = 9 Hz, 1H, 3′a-OCH_2N), 4.82 (d, ^2J = 9 Hz, 1H, 3′b- OCH_2N), 4.49 (br.s, 1H, 3′-H), 4.04 (d, $^3J_{3',4'}$ = 3.2 Hz, 1H, 4′-H), 3.74 (s, 6H, CH_3O DMTr), 3.27 (br s, 2H, 5′CH_2), 2.37 (br.s, 2H, 2′CH_2), 1.50 (s, 3H, 5-CH_3). ^{13}C NMR (101 MHz, DMSO) δ 163.52 (4-C), 158.14 (*p*-C, PhOMe), 150.25 (2-C), 144.55 (C, Ph), 135.54 (6-CH), 135.26 and 135.17 (C, PhOMe), 129.63 (*o*-CH, PhOMe), 127.82 (*o*-CH, Ph), 127.58 (*m*-CH, Ph), 126.75 (*p*-CH, Ph), 113.19 (*m*-CH, PhOMe), 109.62 (5-C), 85.95 (C, DMTr), 83.71 (1′-CH), 82.71 (4′-CH), 81.03 (OCH_2N), 78.00 (3′-CH), 63.34 (5′-CH_2), 54.98 and 54.79 (CH_3O DMTr), 36.59 (2′-CH_2), 11.64 (5-CH_3). HR MS ESI: *m/z* 622.2271, calcd for $[C_{32}H_{33}N_5NaO_7]^+$ *m/z* 622.2272.

1-(5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl-*O*-methyl)-4-(3′-*O*-(*tert*-butyldiphenylsilyl)-5′-deoxythymidin-5′-yl)-1*H*-1,2,3-triazole (7). To a stirred solution of compound **5** (0.54 g, 0.9 mmol) and 3′-*O*-*tert*-butyldiphenylsilyl-5′-deoxy-5′-ethynylthymidine[19] (0.4 g, 0.8 mmol) in dichloromethane (5 mL) and water (5 mL) were added $CuSO_4·5H_2O$ (30 mg, 0.13 mmol) and sodium ascorbate (130 mg, 0.7 mmol). The resulting solution was stirred for 3 h at room temperature. The reaction mixture was diluted with dichloromethane (25 mL) and brine (25 mL). The organic layer was separated, washed with water (20 mL) and saturated $NaHCO_3$ (20 mL), dried over Na_2SO_4, and concentrated. The residue was purified by CC in 1% ethanol in CH_2Cl_2 to give dinucleoside **7** as a hard foam (0.77 g, 88/% yield): R_f = 0.74 (solvent system B).

^{1}H NMR (400 MHz, DMSO): δ 11.37 (s, 1 H, 3-H), 11.26 (s, 1 H, 3-H), 8.16 (s, 1 H, 6′-H dmT), 7.57 (d, ^4J = 1.3 Hz, 1 H, 6-H dmT), 7.49 (1 H, d, ^4J = 1.1 Hz, 6-H aT), 7.50−6.83 (m, 23 H, ArH DMTr and TBDPS), 6.42 (t, $^3J_{1',2'a(2'b)}$ = 7.5 Hz, 1 H, 1′-H dmT), 6.24 (dd, $^3J_{1',2'a}$ = 6.5 Hz, $^3J_{1',2'b}$ = 7.9 Hz, 1 H, 1′-H, aT), 5.84 (d, ^2J = 11.0 Hz, 1H, 3′a-OCH_2, aT), 5.78 (d, ^2J = 11.0 Hz, 1H, 3′b-OCH_2, aT), 5.07 (d, $^3J_{4',3'}$ = 2.7 Hz, 1 H, 4′-H, dmT), 4.61−4.52 (m, 2 H, 3′-H dmT and 3′-H aT), 3.93 (td, $^3J_{3',4'}$ = 2.9 Hz, $^3J_{4',5'a(5'b)}$ = 3.3 Hz, 1 H, 4′-H, aT), 3.72 (s, 6 H, CH_3O DMTr), 3.15 (dd, $^3J_{4',5'a}$ = 3.3 Hz, $J_{5'a,5'b}$ = 10.9 Hz, 1H, 5′a-H, aT), 3.10 (dd, $^3J_{4',5'b}$ = 3.3 Hz, $J_{5'a,\ 5'b}$ = 10.9 Hz, 1H, 5′b-H, aT), 2.40−2.22 (m, 3 H, 2′-CH_2 dmT and 2′a-H aT), 2.23−2.15 (m, 1 H, 2′b-H, aT), 1.64 (d, ^4J = 1.3 Hz, 3 H, 5-CH_3, dmT), 1.42 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3, aT), 1.01 (s, 9 H, CH_3 *t*-Bu). ^{13}C NMR (101 MHz, DMSO): δ 163.5 (4-C, dmT and aT), 158.1 (*p*-C PhOMe DMTr), 150.4 and 150.2 (2-C, dmT and aT), 145.6 (5′-C, dmT), 144.5 (C, Ph DMTr), 135.8 (6-CH, aT and dmT), 135.1 (C, PhOMe DMTr), 135.1 and 135.0 (*o*-CH, Ph_2Si), 132.6 and 132.4 (C, PhSi), 130.0 and 129.9 (*p*-CH, Ph_2Si), 129.6 (*o*-CH, PhOMe DMTr), 127.84 (*m*-CH, Ph_2Si), 127.75 (*o*-CH, Ph DMTr), 127.5 (*m*-CH, Ph DMTr), 126.7 (*p*-CH, Ph DMTr), 124.0 (6′-CH, dmT), 113.2 (*m*-CH, PhOMe DMTr), 109.8 (5-C, dmT), 109.7 (5-C, aT), 86.1 (C, DMTr), 84.3 (1′-CH, dmT), 83.6 (1′-CH, aT), 82.8 (4′-CH, aT), 79.9 (4′-CH, dmT), 78.7 (3′-CH, aT), 76.9 (3′-CH, dmT), 76.6 (OCH_2N), 63.5 (5′-CH_2, aT), 55.0 (CH_3O DMTr), 38.9 (2′-CH_2, dmT), 36.7 (2′-CH_2, aT), 26.6 (CH_3, t-Bu), 18.5 (C, t-Bu), 12.0 (5-CH_3, dmT), 11.5 (5-CH_3, aT). HR MS ESI: *m/z* 1096.4252, calcd for $[C_{59}H_{63}N_7NaO_{11}Si]^+$ *m/z* 1096.4247.

1-(5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl-*O*-methyl)-4-(5′-deoxythymidin-5′-yl)-1*H*-1,2,3-triazole (8). To a stirred solution of dinucleoside **7** (0.77 g, 0.7 mmol) in dry THF (1.4 mL) was added a 1 M solution of tetrabutylammonium fluoride in dry THF (1.4 mL). The mixture was stirred for 2 h at room temperature, diluted with saturated $NaHCO_3$ (50 mL), and extracted with $CHCl_3$ (3 × 40 mL). The combined organic layers were washed with water, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by CC in 6% ethanol in CH_2Cl_2 + 0.1% TEA to give dinucleoside **8** as a hard foam (0.58 g, 99% yield): R_f = 0.43 (solvent system B).

^{1}H NMR (400 MHz, DMSO): δ 11.32 (s, 1 H, 3-H), 11.28 (s, 1 H, 3-H), 8.39 (s, 1 H, 6′-H, dmT), 7.66 (d, ^4J = 1.0 Hz, 1 H, 6-H, dmT), 7.46 (1 H, d, ^4J = 0.8 Hz, 6-H, aT), 7.38−6.84 (m, 13 H, ArH DMTr), 6.34 (t, $^3J_{1',2'a(2'b)}$ = 7.1 Hz, 1 H, 1′-H dmT), 6.14 (t, $^3J_{1',2'a(2'b)}$ = 6.2 Hz, 1 H, 1′-H, aT), 5.86 (d, ^2J = 11.0 Hz, 1H, 3′a-OCH_2, aT), 5.84 (d, ^2J = 11.0 Hz, 1H, 3′b-OCH_2, aT), 5.57 (d, ^3J = 4.5 Hz, 1H, 3′-OH, dmT), 4.93 (d, $^3J_{4',3'}$ = 2.9 Hz, 1 H, 4′-H, dmT), 4.61−4.56 (m, 1 H, 3′-H, aT), 4.47 - 4.41 (m, 1 H, 3′-H dmT), 3.98 (td, $^3J_{3',4'}$ = 3.0 Hz, $^3J_{4',5'a(5'b)}$ = 3.9 Hz, 1 H, 4′-H, aT), 3.73 (s, 6 H, CH_3O DMTr), 3.20 (dd, $^3J_{4',5'a}$ = 3.9 Hz, $J_{5'a,5'b}$ = 10.9 Hz, 1H, 5′a-H, aT), 3.12 (dd, $^3J_{4',5'b}$ = 3.9 Hz, $J_{5'a,5'b}$ = 10.9 Hz, 1H, 5′b-H, aT), 2.47−2.39 (m, 1 H, 2′a-H, dmT), 2.35−2.21 (m, 3 H, 2′-CH_2 aT and 2′b-H dmT), 1.67 (d, ^4J = 1.3 Hz, 3 H, 5-CH_3, dmT), 1.58 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3, aT). ^{13}C NMR (101 MHz, DMSO): δ 163.51 (4-C, aT), 163.47 (4-C, dmT), 158.1 (*p*-C PhOMe DMTr), 150.4 and 150.2 (2-C, dmT and aT), 146.4 (C, Ph DMTr), 144.5 (5′-C, dmT), 135.9 (6-CH, dmT), 135.3 (6-CH, aT), 135.3 (C, PhOMe DMTr), 129.6 (*o*-CH, PhOMe DMTr), 127.9 (*o*-CH, Ph DMTr), 127.5 (*m*-CH, Ph DMTr), 126.7 (*p*-CH, Ph DMTr), 123.9 (6′-CH, dmT), 113.2 (*m*-CH, PhOMe DMTr), 109.7 (5-C, dmT), 109.5 (5-C, aT), 86.0 (C, DMTr), 84.3 (1′-CH, dmT), 83.7 (1′-CH, aT), 82.7 (4′-CH, aT), 79.9 (4′-CH, dmT), 78.4 (3′-CH, aT), 76.5 (OCH_2N), 74.1 (3′-CH, dmT), 63.5 (5′-CH_2, aT), 55.9 (CH_3O DMTr), 38.7 (2′-CH_2, dmT), 36.5 (2′-CH_2, aT), 12.1 (5-CH_3, dmT), 11.5 (5-CH_3, aT). HR MS ESI: *m/z* 858.3076, calcd for $[C_{43}H_{45}N_7Na\ O_{11}]^+$ *m/z* 858.3069.

1-(5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl-*O*-methyl)-4-(3′-deoxy-3′-((2-cyanoethoxy)(diisopropylamino)-phosphinooxy)-5′-deoxythymidin-5′-yl)-1*H*-1,2,3-triazole (9). Dinucleoside **8** (0.59 g, 0.7 mmol) and tetrazole (98 mg, 1.4 mmol) were evaporated with absolute pyridine (2 × 5 mL) and dissolved in absolute CH_sCN. The mixture was stirred over molecular sieves for 30 min at room temperature. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-phosphoramidite (324 μL, 1.4 mmol) was added. The mixture was stirred for 3 h at room temperature and then poured into 50 mL of cold saturated $NaHCO_3$ and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layers were washed with water, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by CC in 2−3% ethanol in CH_2Cl_2 + 0.1% TEA to give amidite **9** as a hard foam (0.42 g, 74% yield): R_f = 0.45 (solvent system B).

^{31}P NMR (162 MHz, DMSO): δ 150.84, 150.51 ppm. ^{1}H NMR (400 MHz, DMSO; chemical shifts of the diastereomers are separated by semicolons): δ 11.26 (br.s, 2 H, 3-H aT and dmT), 8.44; 8.42 (s, 1 H, 6′-H, dmT), 7.64; 7.63 (s, 1 H, 6-H, dmT), 7.45 (1 H, s, 6-H, aT), 7.38−6.85 (m, 13 H, ArH DMTr), 6.36; 6.33 (t, $^3J_{1',2'a(2'b)}$ = 8.0 Hz, 1 H, 1′-H dmT), 6.14 (t, $^3J_{1',2'a(2'b)}$ = 6.5 Hz, 1 H, 1′-H, aT), 5.95−5.77 (m, 2H, 3′-OCH_2), 5.15; 5.04 (d, $^3J_{4',3'}$ = 2.8 Hz, 1 H, 4′-H, dmT), 4.79 - 4.66 (m, 1 H, 3′-H, dmT), 4.62 - 4.55 (m, 1 H, 3′-H, aT), 3.99 (td, $^3J_{3',4'}$ = 3.0 Hz, $^3J_{4',5'a,(5'b)}$ = 3.9 Hz, 1 H, 4′-H, aT), 3.78−3.62 (m, 2H, $POCH_2$), 3.73 (s, 6 H, CH_3O DMTr), 3.62−3.47 (m, 2H, CH *i*-Pr), 3.22 (dd, $^3J_{4',5'a}$ = 3.4 Hz, $J_{5'a,5'b}$ = 10.4 Hz, 1H, 5′a-H, aT), 3.13 (dd, $^3J_{4',5'a}$ = 3.9 Hz, $^2J_{5'a,5'b}$ = 10.5 Hz, 1H, 5′b-H, aT), 2.75; 2.70 (t, ^3J = 4.6 Hz, 2 H, CH_2CN), 2.64−2.56 (m, 1 H, 2′a-H, dmT), 2.44−2.35 (m, 1 H, 2′b-H dmT), 2.35−2.15 (m, 2 H, 2′-CH_2, aT), 1.70; 1.67 (s, 3 H, 5-CH_3, dmT), 1.41 (s, 3 H, 5-CH_3, aT), 1.13; 1.09 (d, ^4J = 5.9 Hz, 3 H, CH_3 *i*-Pr). ^{13}C NMR (101 MHz, DMSO): δ 163.5 (4-C, dmT and aT, 158.2 (*p*-C PhOMe DMTr), 150.4 and 150.3 (2-C, dmT and aT), 145.7 (C, Ph DMTr), 144.5 (5′-C, dmT), 135.9 (6-CH, dmT), 135.3 (6-CH, aT), 134.9 (C, PhOMe DMTr), 129.6 (*o*-CH, PhOMe DMTr), 127.84 (*m*-CH, Ph_2Si), 127.79 (*o*-CH, Ph DMTr), 127.6 (*m*-CH, Ph DMTr), 126.7 (*p*-CH, Ph DMTr), 122.9 (6′-CH, dmT), 118.8 (CN, CH_2CN), 113.2 (*m*-CH, PhOMe DMTr), 109.7 (5-C, dmT and aT), 86.1 (C, DMTr), 84.3 (1′-CH, dmT), 83.5 (1′-CH, aT), 82.7 (4′-CH, aT), 76.6 (4′-CH, dmT and 3′-CH, aT), 76.4 (OCH_2N), 63.5 (5′-CH_2, aT), 58.4 ($POCH_2$), 42.6 (CH, *i*-Pr), 55.0 (CH_3O DMTr), 37.9 (2′-CH_2, dmT), 36.6 (2′-CH_2, aT), 24.3 (CH_3, *i*-Pr), 19.6 (CH_2, CH_2CN), 12.1 (5-CH_3, dmT), 11.5 (5-CH_3, aT). HR MS ESI: *m/z* 1058.4144, calcd for $[C_{52}H_{62}N_9NaO_{12}P]^+$ *m/z* 1058.4148.

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■ ASSOCIATED CONTENT

Supporting Information

Tables and figures giving sequences of PCR primers and MALDI-TOF MS data, an electrophoretic analysis of DNase I cleavage of native and triazole-modfied ONs, sequences, MALDI-TOF MS data, T_m values, and UV-melting profiles of duplexes with mismatches, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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