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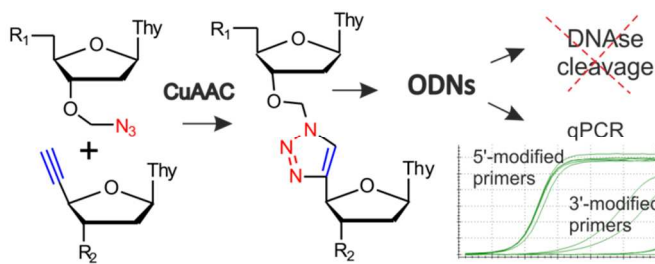
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SYNTHESIS OF TRIAZOLE-LINKED OLIGONUCLEOTIDES WITH HIGH AFFINITY TO DNA COMPLEMENTS AND AN ANALYSIS OF THEIR COMPATIBILITY WITH BIOSYSTEMS

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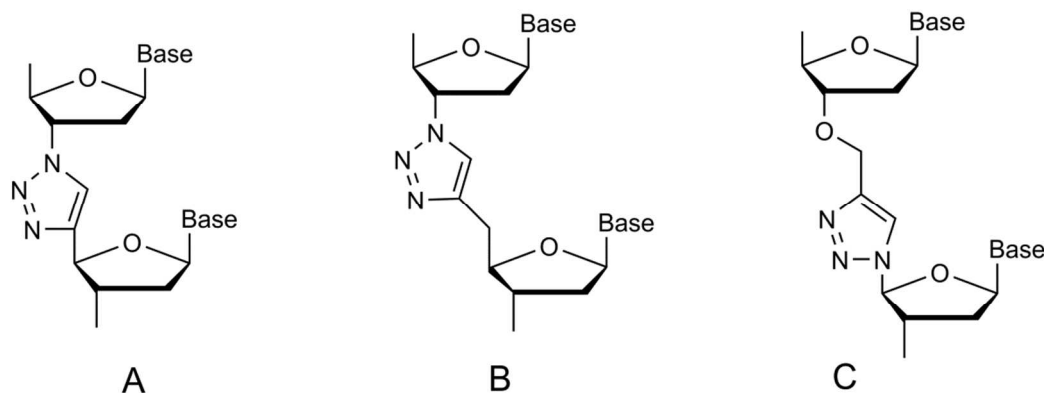
Keywords: nucleosides, oligonucleotide analogs, click chemistry, hybridization

ABSTRACT. New oligonucleotide analogs with triazole internucleotide linkages were synthesized, and their hybridization properties were studied. The analogs demonstrated DNA binding affinities similar to those of unmodified oligonucleotides. The modification was shown to protect 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 analogs are among the most promising triazole DNA mimics characterized to date.

INTRODUCTION

Oligonucleotide (ON) analogs with triazole internucleotide linkages have shown great promise in the field of antisense technology and as DNA hybridization probes.¹⁻⁴ These analogs 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 stabilization⁹. Unfortunately, this stabilization was later shown to be sequence-dependent¹⁰. 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 analogs 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.

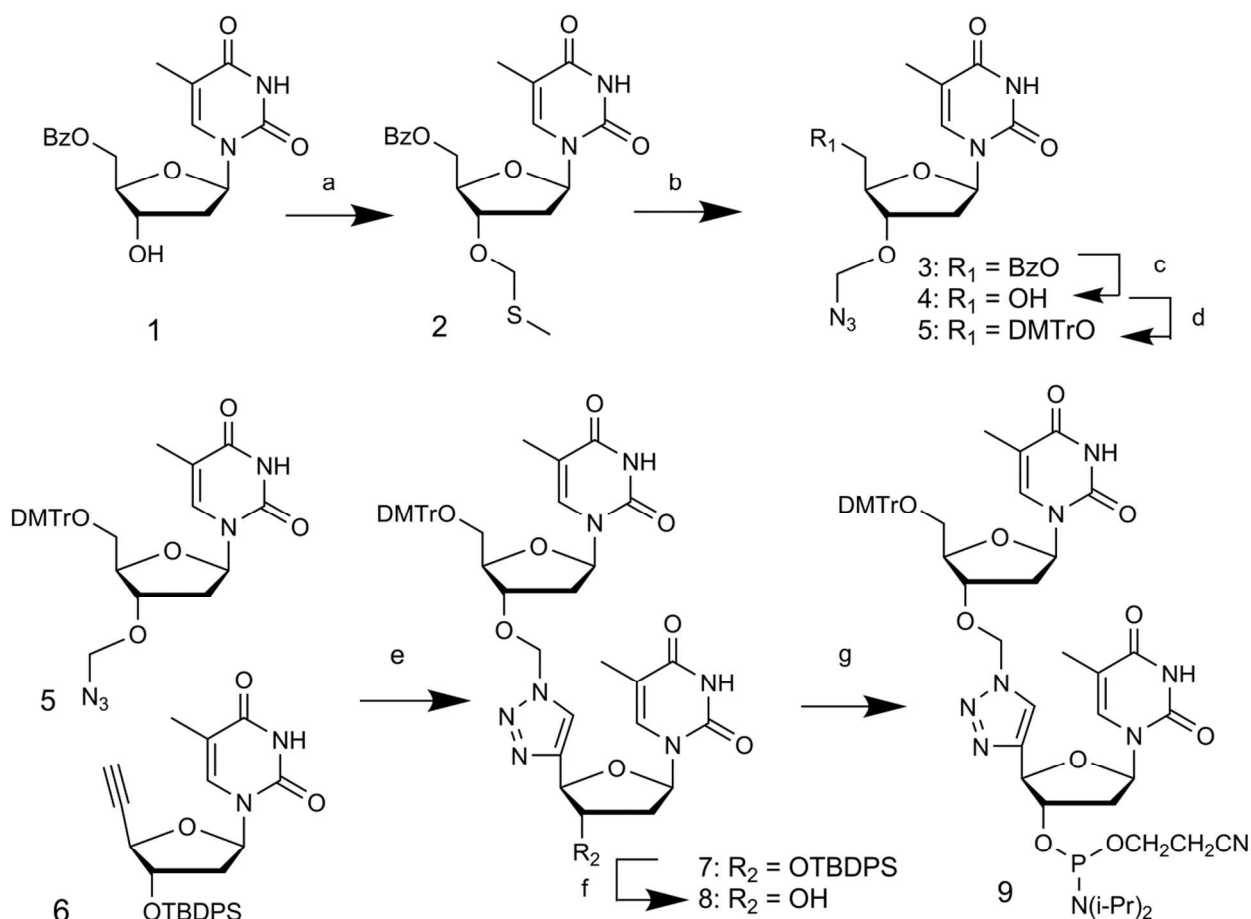


Scheme 1. Fragments of ON analogs with three different types of triazole internucleotide modifications.^{6,9,19}

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 coworkers.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 dimethylsulfoxide and acetic anhydride in the presence of acetic acid. The 3'-*O*-methylthiomethyl derivative (**2**) was obtained in 80% yield.



Scheme 2. Synthesis of the phosphoramidite dinucleoside block with a triazole internucleoside linkage. Reagents and conditions: (a) DMSO, Ac₂O, AcOH; (b) Br₂, LiN₃, CH₃CN; (c) K₂CO₃, MeOH; (d) DMTrCl, pyridine; (e) CuSO₄, sodium ascorbate, CH₂Cl₂/H₂O; (f) TBAF, THF; (g) NCCH₂CH₂OP(Ni-Pr)₂, 1H-tetrazole, pyridine, CH₂Cl₂.

Nucleoside **2** was reacted with molecular bromine followed by lithium azide in dry CH₃CN to produce the 3'-*O*-azidomethyl derivative (**3**) in 83% yield. The treatment of **3** with a K₂CO₃ 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 CuSO₄·5H₂O and 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 1H-tetrazole and pyridine in dry CH₂Cl₂ afforded 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 minutes 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.

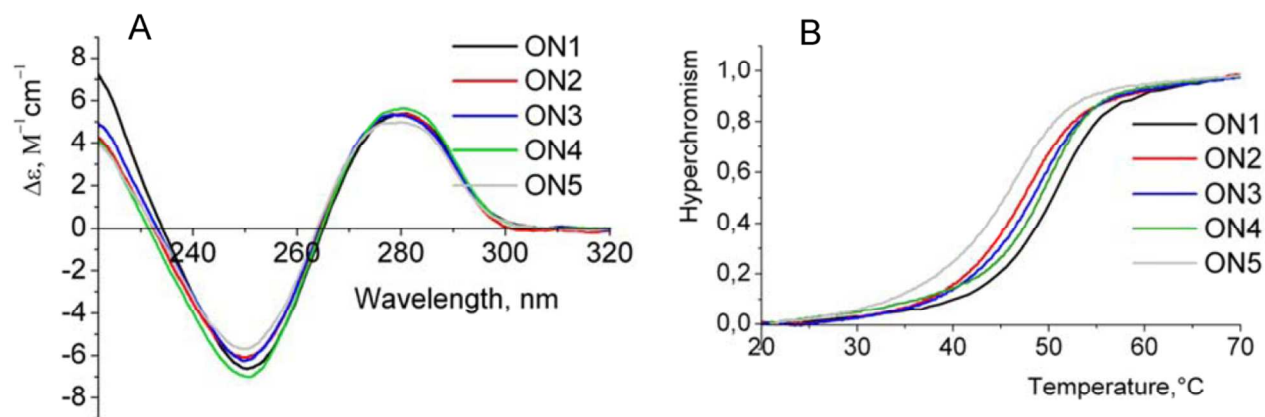


Figure 1. CD spectra (A) and melting curves (B) of the modified duplexes.

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 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 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 analogs are among the most promising triazole-bearing DNA mimics in terms of hybridization properties.

Table 1. Sequences, MALDI-TOF MS data and melting temperatures of the duplexes formed by modified ONs and their unmodified complement (duplex concentration $5 \cdot 10^{-6}$ M).

Code	Sequence of a modified strand (5'→3') ^[a]	m/z,		T_m , $^\circ\text{C} \pm 0.5$, (ΔT_m , $^\circ\text{C}$) ^[b]
		found	(calculated for $[\text{M} + \text{H}]^+$)	
ON1	TTAACTTCTTCACATTC	5072.2	(5071.3)	50.3
ON2	XAACTTCTTCACATTC	5059.3	(5058.4)	50.0 (-0.3)
ON3	TTAACTTCTTCACAXC	5059.1	(5058.4)	48.2 (-2.1)
ON4	TTAACTTCXCACATTC	5058.5	(5058.4)	49.1 (-1.2)
ON5	XAACTTCXCACAXC	5032.3	(5032.5)	45.4 (-4.9)

[a] X –triazole-linked dithymidine fragment. [b] T_m difference between modified and natural duplexes.

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 to the one described here ('iso-triazole'; 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 'iso-triazole' 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 8 nucleotides from the 3' end resulted in a substantial decrease in the product accumulation rate..

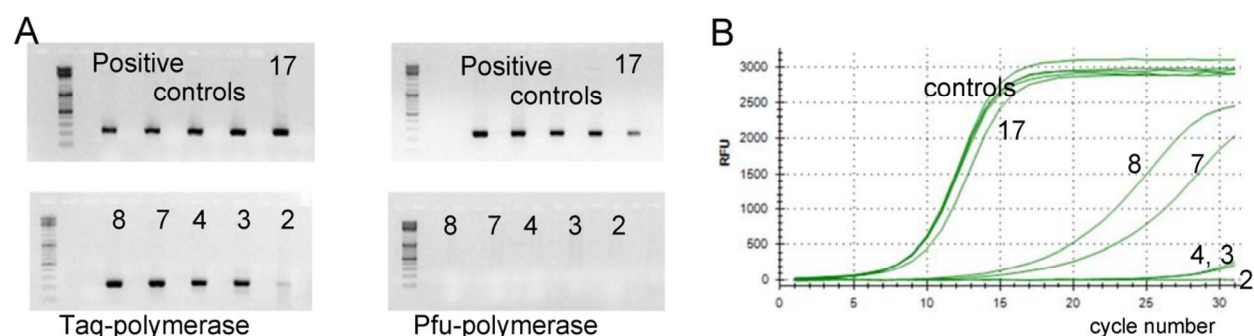


Figure 2. PCR with modified primers. A: Relative sensitivity of the Taq (left) and Pfu (right) polymerases to primer modification. The first 4 lanes of the upper gels are the products of PCR with unmodified primers. The numbers represent the positions of the modifications (number of nucleotides from the 3'-terminus) For primer sequences, see Supplementary data. B: Accumulation kinetics curves obtained during qPCR with Taq polymerase.

Thus, the new triazole-linked ONs are generally usable as PCR primers, but only some polymerases can tolerate the modifications, and the dependency 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 Supplementary. 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 modification¹⁰ (B in Scheme 1) had a similar effect on the rate of enzymatic hydrolysis (see Supplementary data). 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 synthesized 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 analogs are adequate mimics of native DNA with enhanced biostability and may be attractive tools for diagnostics or the investigation of DNA structure.

EXPERIMENTAL

All reagents were commercially available unless otherwise mentioned and used without further purification. 1-(2,5,6-Trideoxy-3-O-(tert-butylidiphenylsilyl)-beta-D-erythro-hex-5ynofuranosyl)-

5-methyluracil (**6**) was synthesized as previously described.¹⁹ All solvents were purchased from Khimmid (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). ¹H NMR and ¹³C 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 {¹H-¹³C} HSQC experiments (see supporting information). The NMR data were processed and presented using MestReNova version 7.0.3 (Mestrelab Research SL, Spain).

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 (N₂ gas laser, 337 nm). The solution of 35g/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 *m/z* 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 x 4.0 mm² Hypersil C18 column with detection at 260 nm. Chromatography of dimethoxytrityl protected ONs was performed using 10-50% gradient of CH₃CN in 0.05 M TEAA. Detritylated oligonucleotides were further purified in 0-25% gradient of CH₃CN 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, pH 7.0, concentration of each duplex being 2.5·10⁻⁶M. Samples were denatured at 95°C for 5 min and slowly cooled to 20°C prior to measurements. 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). The calculation method taking into account 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 and under the same conditions as for the thermal denaturation studies. The CD values ($\Delta\epsilon$) are given per moles 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 μ L of 10 $\times$ buffer in a total reaction volume of 25 μ L with 1 ng of

the DNA template, 0.5 μ M of each primer, 0.2 mM dNTP and 0.5 units of Taq DNA polymerase (Lytech, Russia) or Pfu DNA polymerase (Fermentas, US). 10 $\times$ Taq buffer: 100mM Tris-HCl, 500mM KCl, 15mM MgCl₂, pH 8.3; 10 $\times$ Pfu buffer: 200mM Tris-HCl (pH 8.8 at 25 $^{\circ}$ C), 100mM KCl, 100mM (NH₄)₂SO₄, 20mM MgSO₄, 1.0% Triton[®] X-100 and 1mg/ml nuclease-free BSA. PCR cycling conditions: 95 $^{\circ}$ C (initial denaturation) for 2 min then 35 cycles of 95 $^{\circ}$ C (denaturation) for 15 s, 55 $^{\circ}$ C (annealing) for 20 s and 72 $^{\circ}$ C (extension) for 30 s. The reaction mixture was loaded onto a 2% agarose gel in 1 X 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 was performed using the bundled software.

Nuclease hydrolysis

ON (0.35 nmole) was dissolved in 15 μ L of a reaction buffer (100 mM Tris-HCl (pH 7.5), 25 mM MgCl₂ and 1 mM CaCl₂). A 5- μ L aliquot was taken as a control, and 1 u of DNaseI (Fermentas, US) in 1 μ L of storage buffer was added to the remaining solution. The mixture was incubated at 37 $^{\circ}$ C for 15 min, then an aliquot was removed and stored in a freezer at -20 $^{\circ}$ C until the end of the reaction, and the mixture was incubated at 37 $^{\circ}$ C for another 15 min. Once all three samples were collected, 1 μ L of formamide was added to each sample. The samples were heated to 95 $^{\circ}$ C, 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-Benzoylthymidine²⁰ (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, then poured into cold saturated NaHCO₃ (250 mL) and stirred for 2 more hours. The

precipitate was filtered, washed on filter with water, air-dried and recrystallized from a mixture of ethylacetate and hexane. Compound **2** was obtained as colorless needles (1.95 g, 80%), R_f 0.31 (Solvent system A).

^1H 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-OCH₂S), 4.73 (d, 2J = 11.5 Hz, 1H, 3'b-OCH₂S), 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, SCH₃), 1.61 (s, 3H, 5-CH₃).

^{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 (OCH₂S), 64.16 (5'-CH₂), 35.79 (2'-CH₂), 13.19 (CH₃S), 11.78 (5-CH₃).

HR MS ESI: m/z 429.1083, calculated for $[\text{C}_{19}\text{H}_{22}\text{N}_2\text{NaO}_6\text{S}]^+ m/z$ 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 abs. CH₃CN, Br₂ (0.21 mL, 4 mmol) was added dropwise at 0°C. The mixture was stirred upon cooling for 30 min, 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 hours at 60°C. The solvent was evaporated under vacuum and the residue was dissolved in CH₂Cl₂ (50 mL) and water (50 mL). The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (2*25 mL). Combined organic layers were washed with brine, dried over Na₂SO₄,

1
2
3 filtered and concentrated. The residue was purified by CC in 0—3% of ethanol in CH₂Cl₂ to give
4
5 dinucleoside **3** as a hard foam (1.35 g, 82.5% yield). R_f = 0.33 (Solvent system A).

6
7
8 ¹H NMR (400 MHz, DMSO): δ = 11.31 (s, 1H, 3-H), 8.01 (d, ³J = 7.2 Hz, 2H, *o*-H Bz), 7.69
9
10 (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
11
12 (t, ³J_{1',2'a} = ³J_{1',2'b} = 7.2 Hz, 1H, 1'-H), 4.92 (d, ²J = 9 Hz, 1H, 3'a-OCH₂N₃), 4.89 (d, ²J = 9 Hz,
13
14 1H, 3'b-OCH₂N₃), 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,
15
16 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-CH₃).

17
18
19 ¹³C NMR (101 MHz, DMSO): δ 165.45 (C=O), 163.49 (4-C), 150.28 (2-C), 135.54 (6-CH),
20
21 133.52 (*p*-CH, Bz), 129.25 (C, Bz), 129.15 (*o*-CH, Bz), 128.79 (*m*-CH, Bz), 109.79 (5-C), 84.00
22
23 (1'-CH), 81.24 (4'-CH), 81.18 (OCH₂N), 78.01 (3'-CH), 64.08 (5'-CH₂), 36.25 (2'-CH₂), 11.78
24
25 (5-CH₃).

26
27
28 HR MS ESI: *m/z* 424.1220, calculated for [C₁₈H₁₉N₅NaO₆]⁺ *m/z* 424.1228.

29 30 31 **3'-O-(Azidomethyl)thymidine (4).**

32
33
34 Finely powdered K₂CO₃ (0.22 g, 1.6 mole) was added to a solution of nucleoside **3** (1.3 g, 3.2
35
36 mmole) in 25 mL of abs. methanol and the suspension was stirred for 12 hours. Dry silica gel (20
37
38 mL) was added to the resulting transparent solution. The mixture was stirred for 10 min and the
39
40 solvent was removed in vacuum. The residue was onto CC in 10 mL of CH₂Cl₂ and eluted with
41
42 2-5% of ethanol in CH₂Cl₂ to give nucleoside **4** as a hard foam (0.9 g, 94.6% yield). R_f = 0.63
43
44 (Solvent system B).

45
46
47 ¹H 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,
48
49 ³J_{1',2'b} = 6.8 Hz, 1H, 1'-H), 5.14 (dd, ³J_{5'OH,5'a} = ³J_{5'OH,5'b} = 5.2 Hz, 1H, 5'-OH), 4.86 (s, 2H, 3'-
50
51 OCH₂N), 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),
52
53 2.30-2.22 (m, 2H, 2'a-H and 2'b-H), 1.79 (s, 3H, 5-CH₃).

¹³C NMR (101 MHz, DMSO): ¹³C 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-CH₂-N), 78.31 (3'-CH), 61.13 (5'-CH₂), 36.53 (2'-CH₂), 12.14 (5-CH₃).

HR MS ESI: *m/z* 320.0962, calculated for [C₁₁H₁₅N₅NaO₅]⁺ *m/z* 320.0965.

3'-O-(Azidomethyl)-5'-O-(4,4'-dimethoxytrityl)thymidine (5).

Nucleoside **3** (0.85 g, 2.9 mmole) was evaporated with abs. pyridine (2x5mL) and dissolved in 5mL of abs. pyridine. Dimethoxytrityl chloride (1.19 g, 3.5 mmole) was added and the mixture was stirred for 12 hours at room temperature. Then 0.2 mL of water was added, the mixture was stirred for 10 more minutes and evaporated in vacuum. The residue was dissolved in 50 mL of chloroform, washed with water (20 mL) and saturated NaHCO₃ (20 mL), dried over Na₂SO₄, and concentrated. The residue was purified by CC in 3% of ethanol in CH₂Cl₂ to give nucleoside **5** (1.6 g, 92 % yield) as a hard foam. *R_f* = 0.48 (Solvent system A).

¹H 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, ³*J*_{1',2'a} = ³*J*_{1',2'b} = 7.2 Hz, 1H, 1'-H), 4.85 (d, ²*J* = 9 Hz, 1H, 3'a-OCH₂N), 4.82 (d, ²*J* = 9 Hz, 1H, 3'b-OCH₂N), 4.49 (br.s, 1H, 3'-H), 4.04 (d, ³*J*_{3',4'} = 3.2 Hz, 1H, 4'-H), 3.74 (s, 6H, CH₃O DMTr), 3.27 (br.s, 2H, 5'CH₂), 2.37 (br.s, 2H, 2'CH₂), 1.50 (s, 3H, 5-CH₃).

¹³C 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 (OCH₂N), 78.00 (3'-CH), 63.34 (5'-CH₂), 54.98 and 54.79 (CH₃O DMTr), 36.59 (2'-CH₂), 11.64 (5-CH₃).

HR MS ESI: *m/z* 622.2271, calculated for [C₃₂H₃₃N₅NaO₇]⁺ *m/z* 622.2272.

1-(5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-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'-ethynylthymidine¹⁹ (0.4 g, 0.8 mmol) in dichloromethane (5 mL) and water (5 mL), CuSO₄·5 H₂O (30 mg, 0.13 mmol) and sodium ascorbate (130mg, 0.7 mmol) were added. The resulting solution was stirred for 3 hours 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 NaHCO₃ (20 mL), dried over Na₂SO₄, and concentrated. The residue was purified by CC in 1% of ethanol in CH₂Cl₂ to give dinucleoside **7** as a hard foam (0.77 g, 88/% yield). *R*_f = 0.74 (Solvent system B).

¹H 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, ⁴*J* = 1.3 Hz, 1 H, 6-H dmT), 7.49 (1 H, d, ⁴*J* = 1.1 Hz, 6-H aT), 7.50 – 6.83 (m, 23 H, ArH DMTr and TBDPS), 6.42 (t, ³*J*_{1',2'a(2'b)} = 7.5 Hz, 1 H, 1'-H dmT), 6.24 (dd, ³*J*_{1',2'a} = 6.5 Hz, ³*J*_{1',2'b} = 7.9 Hz, 1 H, 1'-H, aT), 5.84 (d, ²*J* = 11.0 Hz, 1H, 3'a-OCH₂, aT), 5.78 (d, ²*J* = 11.0 Hz, 1H, 3'b-OCH₂, aT), 5.07 (d, ³*J*_{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, ³*J*_{3',4'} = 2.9 Hz, ³*J*_{4',5'a(5'b)} = 3.3 Hz, 1 H, 4'-H, aT), 3.72 (s, 6 H, CH₃O DMTr), 3.15 (dd, ³*J*_{4',5'a} = 3.3 Hz, *J*_{5'a, 5'b} = 10.9 Hz, 1H, 5'a-H, aT), 3.10 (dd, ³*J*_{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'-CH₂ dmT and 2'a-H aT), 2.23-2.15 (m, 1 H, 2'b-H, aT), 1.64 (d, ⁴*J* = 1.3 Hz, 3 H, 5-CH₃, dmT), 1.42 (d, ⁴*J* = 1.1 Hz, 3 H, 5-CH₃, aT), 1.01 (s, 9 H, CH₃ *t*-Bu).

¹³C NMR (101 MHz, DMSO): ¹³C 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, Ph₂Si),

132.6 and 132.4 (C, PhSi), 130.0 and 129.9 (*p*-CH, Ph₂Si), 129.6 (*o*-CH, PhOMe DMTr), 127.84 (*m*-CH, Ph₂Si), 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 (OCH₂N), 63.5 (5'-CH₂, aT), 55.0 (CH₃O DMTr), 38.9 (2'-CH₂, dmT), 36.7 (2'-CH₂, aT), 26.6 (CH₃, *t*-Bu), 18.5 (C, *t*-Bu), 12.0 (5-CH₃, dmT), 11.5 (5-CH₃, aT).

HR MS ESI: *m/z* 1096.4252. Calculated for [C₅₉H₆₃N₇NaO₁₁Si]⁺ *m/z* 1096.4247.

1-(5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-*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), 1M solution of tetrabutylammonium fluoride in dry THF (1.4 mL) was added. The mixture was stirred for 2 hours at room temperature, diluted with saturated NaHCO₃ (50 mL) and extracted with CHCl₃ (3×40 mL). Combined organic layers were washed with water, dried over Na₂SO₄, filtered and concentrated. The residue was purified by CC in 6% of ethanol in CH₂Cl₂ + 0.1% TEA to give dinucleoside **8** as a hard foam (0.58 g, 99% yield). *R*_f = 0.43 (Solvent system B).

¹H 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, ⁴*J* = 1.0 Hz, 1 H, 6-H, dmT), 7.46 (1 H, d, ⁴*J* = 0.8 Hz, 6-H, aT), 7.38 – 6.84 (m, 13 H, ArH DMTr), 6.34 (t, ³*J*_{1',2'a(2'b)} = 7.1 Hz, 1 H, 1'-H dmT), 6.14 (t, ³*J*_{1',2'a(2'b)} = 6.2 Hz, 1 H, 1'-H, aT), 5.86 (d, ²*J* = 11.0 Hz, 1H, 3'a-OCH₂, aT), 5.84 (d, ²*J* = 11.0 Hz, 1H, 3'b-OCH₂, aT), 5.57 (d, ³*J* = 4.5 Hz, 1H, 3'-OH, dmT), 4.93 (d, ³*J*_{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, ³*J*_{3',4'} = 3.0 Hz, ³*J*_{4',5'a(5'b)} = 3.9 Hz, 1 H, 4'-H, aT), 3.73 (s, 6 H, CH₃O DMTr), 3.20 (dd, ³*J*_{4',5'a} = 3.9 Hz, *J*_{5'a, 5'b} = 10.9 Hz, 1H, 5'a-H, aT), 3.12 (dd, ³*J*_{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'-CH₂ aT and 2'b-H dmT), 1.67 (d, ⁴J = 1.3 Hz, 3 H, 5-CH₃, dmT), 1.58 (d, ⁴J = 1.1 Hz, 3 H, 5-CH₃, aT).

¹³C 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 (OCH₂N), 74.1 (3'-CH, dmT), 63.5 (5'-CH₂, aT), 55.9 (CH₃O DMTr), 38.7 (2'-CH₂, dmT), 36.5 (2'-CH₂, aT), 12.1 (5-CH₃, dmT), 11.5 (5-CH₃, aT).

HR MS ESI: *m/z* 858.3076, calculated for [C₄₃H₄₅N₇ Na O₁₁]⁺ *m/z* 858.3069.

1-(5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-*O*-methyl)-4-(3'-deoxy-3'-((2-cyanoethoxy)(diisopropylamino)phosphinoxy)-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 abs. pyridine (2×5 mL) and dissolved in abs. CH₃CN. The mixture was stirred over molecular sieves for 30 min at room temperature. 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoramidite (324 μL, 1.4 mmol) was added. The mixture was stirred for 3 hours at room temperature, then poured into 50 mL of cold saturated NaHCO₃ (50 mL) and extracted with CH₂Cl₂ (3×40 mL). Combined organic layers were washed with water, dried over Na₂SO₄, filtered and concentrated. The residue was purified by CC in 2—3% of ethanol in CH₂Cl₂ + 0.1% TEA to give amidite **9** as a hard foam (0.42 g, 74% yield). *R*_f = 0.45 (Solvent system B).

³¹P NMR (162 MHz, DMSO): δ = 150.84, 150.51 ppm.

¹H NMR (400 MHz, DMSO): δ = 11.26 (br.s, 2 H, 3-H aT and dmT), 8.44; 8.42¹ (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, ³*J*_{1',2'a(2'b)} = 8.0 Hz, 1 H, 1'-H dmT), 6.14 (t, ³*J*_{1',2'a(2'b)} = 6.5 Hz, 1 H, 1'-H, aT), 5.95-5.77 (m, 2H, 3'-OCH₂), 5.15; 5.04 (d, ³*J*_{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, ³*J*_{3',4'} = 3.0 Hz, ³*J*_{4',5'a(5'b)} = 3.9 Hz, 1 H, 4'-H, aT), 3.78-3.62 (m, 2H, POCH₂), 3.73 (s, 6 H, CH₃O DMTr), 3.62-3.47 (m, 2H, CH *i*-Pr), 3.22 (dd, ³*J*_{4',5'a} = 3.4 Hz, *J*_{5'a, 5'b} = 10.4 Hz, 1H, 5'a-H, aT), 3.13 (dd, ³*J*_{4',5'a} = 3.9 Hz, ²*J*_{5'a, 5'b} = 10.5 Hz, 1H, 5'b-H, aT), 2.75; 2.70 (t, ³*J* = 4.6 Hz, 2 H, CH₂CN), 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'-CH₂, aT), 1.70; 1.67 (s, 3 H, 5-CH₃, dmT), 1.41 (s, 3 H, 5-CH₃, aT), 1.13; 1.09 (d, ⁴*J* = 5.9 Hz, 3 H, CH₃ *i*-Pr).

¹³C 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, Ph₂Si), 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, CH₂CN), 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 (OCH₂N), 63.5 (5'-CH₂, aT), 58.4 (POCH₂), 42.6 (CH, *i*-Pr), 55.0 (CH₃O DMTr), 37.9 (2'-CH₂, dmT), 36.6 (2'-CH₂, aT), 24.3 (CH₃, *i*-Pr), 19.6 (CH₂, CH₂CN), 12.1 (5-CH₃, dmT), 11.5 (5-CH₃, aT).

HR MS ESI: *m/z* 1058.4144, calculated for [C₅₂H₆₂N₉NaO₁₂P]⁺ *m/z* 1058.4148.

¹ Here and further chemical shifts of the diastereomers are separated by semicolons

ASSOCIATED CONTENT

Supporting Information. 1. Sequences of PCR primers and MALDI-TOF MS data; 2. Electrophoretic analysis of DNase I cleavage of native and triazole-modified ONs; 3. Sequences, MALDI-TOF MS data, T_m values and UV-melting profiles of duplexes with mismatches. 4. NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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