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Triazole-Linked Oligonucleotides with Mixed-Base Sequences: Synthesis and Hybridization Properties

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A new class of backbone-modified oligonucleotide analogs has emerged since the discovery of the Cu^{I}-catalyzed [3+2] azide/alkyne cycloaddition reaction. These are oligonucleotide analogs with 1,4-disubstituted 1,2,3-triazoles as the internucleotide linkages. Of all such analogs known, only the triazole-linked deoxythymidine decamer [$(dT)_{10}$] has been reported to show enhanced binding affinity to complementary DNA. Importantly, it is a fully modified $(dT)_{10}$ analog. To date, sequentially heterogeneous oligonucleotides bearing the same backbone modification have not been described. With the goal of investigating sequence and regularity dependence of the effect of this modification on duplex stability, we have designed partially modified mixed-base oligonucleotides, which can be prepared by using a modified dinucleoside block. In this paper we report the synthesis of a dithymidine phosphoramidite analog with a triazole linker, its use in oligonucleotide synthesis and hybridization data of the resulting oligonucleotide analogs. The effect of single and multiple modifications on stability of mixed-base duplexes is assessed and compared with published data for the oligo(T)/oligo(A) duplex. We also compared the effect of the linker concerned with that of a shorter triazole linker.

Introduction

The discovery of the copper(I) catalysis of the Huisgen 1,3-dipolar cycloaddition reaction by Sharpless and Meldal in 2002 had a marked impact on the field of artificial nucleosides and oligonucleotides. A number of 1,2,3-triazole-containing nucleoside analogs exhibiting antibacterial or antiviral activity have been obtained recently by using a Cu^{I}-catalyzed [3+2] azide/alkyne cycloaddition (CuAAC) reaction as the key step.[1] Furthermore, a new class of backbone-modified oligonucleotides, with 1,4-disubstituted 1,2,3-triazoles as the internucleotide linkages, has emerged in the last decade.[2] These oligonucleotides are regarded as potential posttranscriptional gene-silencing agents and may become an alternative to well-known backbone-modified oligonucleotides, such as locked nucleic acids, phosphorothioates, oligonucleotides with amide internucleotide linkages, and so on.[3–5]

Of all triazole-linked oligonucleotides reported to date, only the deoxythymidine decamer analog reported by Isobe et al. exhibits enhanced binding affinity to complementary DNA.[6] This $(dT)_{10}$ analog is a fully modified oligonucleotide, which can be prepared from a thymidine derivative by repeating functionalization and CuAAC ligation steps. The melting temperature (T_m) of the corresponding duplex is about 40 °C higher than that of the isosequential unmodified duplex. The authors conjecture that this is due to the structural complementarity and the absence of the repulsion between neutral triazole and anionic natural phosphodiester linkages. We believe it would be of interest to clarify whether this remarkable enhancement in duplex stability is sequence-dependent. Because the synthesis of fully modified mixed-base triazole-linked oligonucleotides could be complex – requiring preparation of four different monomers and optimization of oligomerization conditions – we employed a different strategy, based on the use of a modified dinucleoside block.

In this paper, we report the synthesis of a dithymidine phosphoramidite block with a triazole linker, its incorporation in oligonucleotides and hybridization data of modified oligonucleotides. The effect of single and multiple modifications on stability of mixed-base duplexes is assessed and compared with published data for the oligo(T)/oligo(A) duplex. We also compare the effect of the linker concerned with that of a shorter triazole linker, reported in our previous work.[7]

Results and Discussion

We started the synthesis of the triazole-linked dinucleotide with the preparation of acetylenic nucleoside compo-

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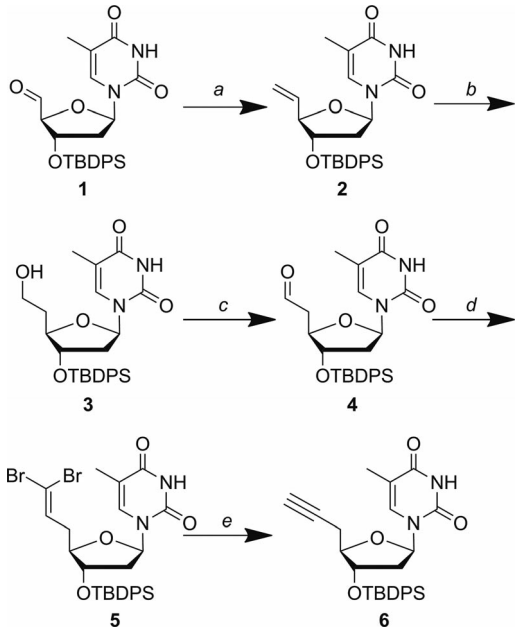
nent **6** from aldehyde **1** (Scheme 1). The latter was readily accessed from 3′-*O*-(*tert*-butyldiphenylsilyl)thymidine[8] by oxidation with *o*-iodoxybenzoic acid.[7] Wittig-type condensation of aldehyde **1** with the ylide derived from methyltriphenylphosphonium bromide in the presence of BuLi in THF afforded terminal olefin **2** in 84% yield. A methyltriphenylphosphonium bromide/BuLi ratio of 1:1 is crucial to obtain a high yield of nucleoside **2**. A lack of BuLi leads to the formation of a polar by-product, presumably similar to the phosphonium salt reported by Matsuda et al.[9], and in the presence of an excess of BuLi β-elimination of the *tert*-butyldimethylsilyloxy group occurs.



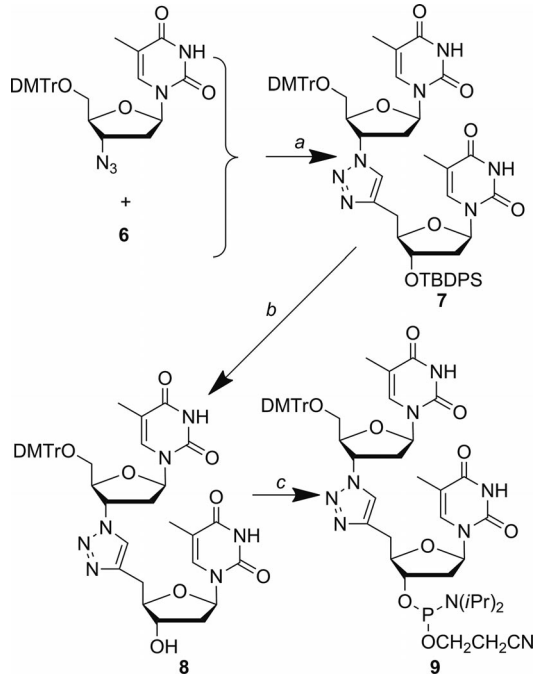
Scheme 1. Synthesis of the acetylenic component of the triazole-linked dinucleotide. Reagents and conditions: (a) $PPh_3CH_3^+Br^-$, BuLi, THF; (b) (*i*) 9-BBN-H, THF; (*ii*) $NaBO_3{\cdot}4H_2O$, THF/H_2O/MeOH (5:2:3); (c) Dess–Martin periodinane, CH_2Cl_2; (d) $PPh_3CHBr_2^+Br^-$, Zn, dioxane, reflux; (e) *n*BuLi, THF, –70 °C.

Compound **2** was subjected to hydroboration with 9-borabicyclo[3.3.1]nonane (9-BBN-H) in THF and subsequent oxidative treatment with $NaBO_3{\cdot}4H_2O$ to give compound **3** in 86% yield. Sodium perborate was chosen as the oxidizing agent, because the conventional reagent, H_2O_2 in alkaline solution, has been reported to cause side reactions with a similar nucleoside.[10] Oxidation of alcohol **3** with *o*-iodoxybenzoic acid gave the corresponding carboxylic acid; however, when Dess–Martin periodinane was used, aldehyde **4** was obtained in 89% yield.

All attempts to convert aldehyde **4** into terminal alkyne **6** by using an Ohira–Bestmann reaction were unsuccessful. Neither a one-pot procedure with in situ generation of dimethyl (1-diazo-2-oxopropyl)phosphonate (Ohira–Bestmann reagent) from tosyl azide and dimethyl (2-oxopropyl)-phosphonate,[11] nor a more complex strategy involving isolation of the intermediate Ohira–Bestmann reagent afforded alkyne **6**. Therefore, formyl → ethynyl group transformation was realized in two steps, through the intermediate dibromoalkene.

Aldehyde **4** was treated with (dibromomethyl)triphenylphosphonium bromide (prepared by Wolkoff's method[12]) in the presence of Zn in dioxane at reflux temperature to give dibromomethylene derivative **5** in 80% yield. Treatment of compound **5** with BuLi in THF afforded acetylenic nucleoside **6** in 77% yield.

Alkyne **6** was coupled with 3′-azido-3′-deoxy-5′-*O*-dimethoxytritylthymidine (Scheme 2) in the presence of $CuSO_4{\cdot}5H_2O$ and sodium ascorbate. The CuAAC reaction was carried out in a two-phase solvent system (H_2O/CH_2Cl_2),[13] which has proven to be favorable for the click coupling of poorly water-soluble compounds, to give dinucleoside **7** in 80% yield. The silyl protecting group was removed by using 0.5 M TBAF in THF. Treatment of dinucleoside **8**, bearing a free hydroxy group, with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite in the presence of 1*H*-tetrazole and pyridine in CH_2Cl_2 afforded target phosphoramidite **9** in 72% yield.



Scheme 2. Synthesis of the phosphoramidite dinucleoside block with a triazole internucleoside linkage. Reagents and conditions: (a) $CuSO_4$, sodium ascorbate, CH_2Cl_2/H_2O; (b) TBAF, THF; (d) $NCCH_2CH_2OP(NiPr_2)_2$, 1*H*-tetrazole, pyridine, CH_2Cl_2.

The structures of nucleosides **2**–**6** and dinucleosides **7**–**9** were ascertained by high-resolution mass spectrometry, and the structures of **7** and **8** were confirmed by COSY NMR spectroscopy. The COSY NMR experiments enabled assignment of signals to the protons of the 5′-deoxy-5′-ethenylthymidine and 3′-amino-3′-deoxythymidine moieties in the ^1H NMR spectra of the dinucleosides. C–H correlation NMR experiments enabled assignment of ^{13}C NMR spectra of the dinucleosides (for COSY and C–H correlation NMR spectra, see Supporting Information).

Dinucleoside block **9** was used directly for solid-phase synthesis of modified oligonucleotides by using standard phosphoramidite protocols. The coupling time was in-

Table 1. MALDI-TOF mass spectra of oligonucleotides and melting temperatures of their duplexes with an unmodified DNA complement (duplex concentration 2.5×10^{-6} M).

Code	Sequence of modified strand (5′→3′)[a]	*m/z* [M + H]$^+$ (calcd.)	T_m [°C ± 0.5] (ΔT_m [°C])[b]
1	TTAACTTCTTCACATTC	5072.2 (5071.32)	50.3
2	**XAACTTCTTCACATTC**	5041.8 (5042.44)	50.4 (+0.1)
3	TTAACTTCTTCACAXC	5042.0 (5042.44)	47.2 (–3.1)
4	TTAACTTCXCACATTC	5042.5 (5042.44)	41.9 (–8.4)
5	**XAACTTCXCACAXC**	4984.1 (4984.58)	37.7 (–12.6)

[a] X: triazole-linked dithymidine fragment. Sequence of a non-modified strand 5′-GAATGTGAAGAAGTTAA-3′. [b] ΔT_m: difference between T_m of modified and natural duplexes.

creased to 15 min for the modified phosphoramidite. No decrease in coupling efficiency was observed (98–99% stepwise coupling yields were achieved for both the modified and unmodified amidites). Along with the modified oligonucleotides, an unmodified complement and unmodified isosequential oligonucleotides were synthesized. All the oligonucleotides were purified by reverse-phase HPLC and characterized by using MALDI-TOF mass spectrometry (Table 1).

Modified oligonucleotides and their unmodified counterpart were hybridized to ssDNA. Thermal stability of the duplexes was examined by monitoring the change in hyperchromicity at 260 nm (Figure 1). Sequences and melting temperatures of the duplexes are shown in Table 1.

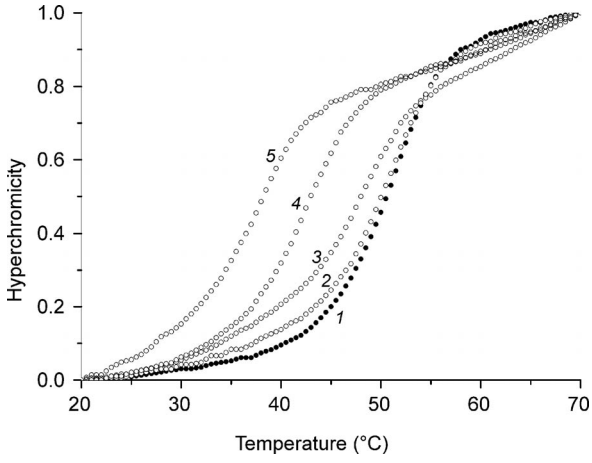


Figure 1. Melting curves of the modified and unmodified duplexes. Legends represent duplex code numbers (see Table 1). Temperature gradient: 0.2 °C/min.

As evident from Table 1 the impact of the 5′-terminal modification on oligonucleotide hybridization is insignificant. The 3′-terminal modification destabilizes the duplex to a certain extent (ΔT_m = –3.1 °C), and the middle-strand modification leads to a drastic loss in duplex stability (ΔT_m = –8.4 °C). Average ΔT_m per modification is –4.0 °C (calculated as a sum of all ΔT_m values divided by the total number of modifications).

To understand what changes in oligonucleotide geometry underlie the destabilization, we performed molecular-modeling studies on a modified backbone fragment in a duplex. We designed 12 initial structures differing in the position of the triazole ring relative to the duplex axis. Computational minimization of the energy of these structures resulted in two main minima. The most stable conformer is displayed in Figure 2. As evident from Figure 2, duplex distortion may arise from the lesser twist of the triazole linker than the phosphordiester one. The results imply that the introduction of a triazole linker concerned results in a longer helix pitch. This agrees with the conclusion Isobe et al. made based on their molecular-modeling studies.[6]

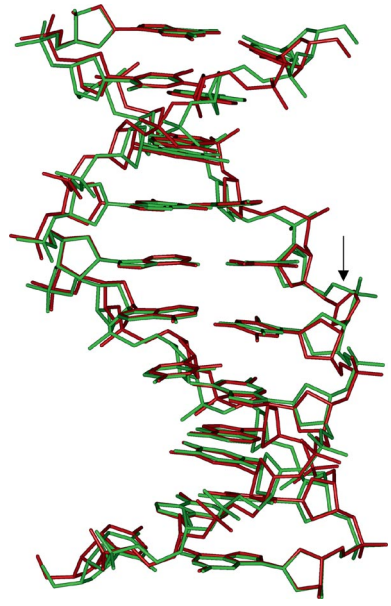


Figure 2. Superimposition of optimized conformations of a natural duplex fragment (green strands) and a modified one (red strands). The arrow points to the triazole internucleotide linker. Modified strand sequence: 5′-CATGTtriazoleTCATG-3′.

Overall duplex conformation is rather close to a classical β-helix in our case. CD spectra of modified oligonucleotides hybridized to complementary ssDNA confirm that the former adopt β-form geometry upon formation of the duplex (Figure 3).

Our hybridization results are generally consistent with those obtained earlier for mixed-sequence oligonucleotide analogs with shorter (four-atom) triazole linkers.[7] Both the four-atom (C3′ to C5′) linker described in our previous work and the five-atom linker discussed herein tend to destabilize duplexes. The above triazole linkers have a length closest to that of natural phosphodiester linkages. Further linker elongation – introduction of an additional methylene group on one side of a triazole or imidazole ring – is known

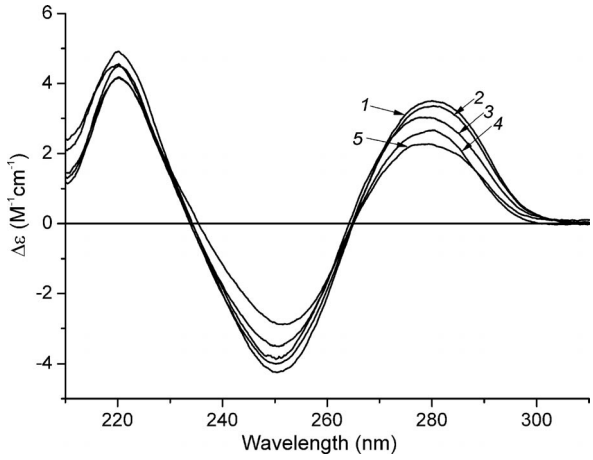


Figure 3. CD spectra of the modified and unmodified duplexes. Legends represent duplex code numbers (see Table 1).

to decrease duplex stability.[14] In view of hybridization data reported by Isobe et al.,[6] five-atom linkers were regarded as the most promising.

Unfortunately, the results of the present work do not confirm this and are not in accord with the hybridization data obtained for the fully modified $(dT)_{10}$ analog. We suggest that distinct geometry of oligo(T)/oligo(A) DNA fragments[15] may be somewhat responsible for the outstanding stability of the regular triazole-modified decamer duplex.

Conclusions

We have reported the synthesis of the dinucleoside phosphoramidite block with a triazole internucleoside linkage. Surprisingly, incorporation of this block in sequentially heterogeneous oligonucleotides was disadvantageous for their hybridization ability, whereas the fully modified oligo-(dT) analog is known to bind tightly to a complementary DNA fragment. Thus, we believe that the effect of this modification is likely to be highly dependent on the regularity of the oligonucleotide structure. The phosphoramidite block we have described is a useful intermediate for further synthesis and investigation of triazole-functionalized oligonucleotides.

Experimental Section

General: All reagents were commercially available unless otherwise mentioned and used without further purification. 3′-Azido-3′-deoxythymidine was a kind gift from the Association AZT (Russia). Dess–Martin reagent was prepared according to a published procedure.[16] All solvents were purchased from Khimmid (Russia). Dioxane was dried with sodium hydroxide and distilled, CH_2Cl_2 was distilled from phosphorus pentoxide, pyridine was distilled from calcium hydride, and THF was distilled from lithium aluminium hydride prior to use. Flash column chromatography was performed on silica gel Kieselgel 60 (0.040–0.063 mm, Merck, Germany). TLC was performed on silica gel Kieselgel 60 F_{254} precoated plates (Merck) with detection by UV light with the following solvent systems (compositions expressed as v/v): ethanol/CH_2Cl_2, 1:95 (A), 1:49 (B), 1:25 (C), 1:14 (D). ^1H and ^{13}C NMR spectra were recorded with a Bruker AMXIII-400 NMR spectrometer (Germany). Abbreviations used: ethT, 5′-deoxy-5′-ethenylthymidine moiety; aT, 3′-amino-3′-deoxythymidine moiety; DMTr, 4,4′-dimethoxytrityl. The signals were assigned to protons by using COSY experiments. The NMR spectroscopic data were processed and are presented by using MestReNova version 7.0.3 (Mestrelab Research SL, Spain). MALDI-TOF mass spectra were acquired with a Bruker Microflex mass spectrometer (Bruker, Germany) in a positive ion linear mode (+20 kV). Oligonucleotides were desalted with mZipTipC18 pipette tips (Millipore, USA) before MALDI-TOF mass spectra analysis. Each spectrum was acquired with 200 laser shots (N_2 gas laser, 337 nm) with a solution of 35 g/mL of 3-hydroxypicolinic acid with dibasic ammonium citrate as the matrix. HRMS (ESI) data were acquired with 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 m/z = 50–3000 Da, scanning frequency 1 scan/s). Internal calibration was done by using Electrospray Calibration Solution (Fluka). Oligonucleotides were synthesized with an Applied Biosystems 3400 DNA synthesizer (USA) by using standard phosphoramidite protocols and purified by using preparative-scale reverse-phase HPLC on a 250 mm × 4.0 mm Hypersil C18 column with detection at 260 nm. Chromatography of dimethoxytrytil-protected oligonucleotides was performed with a 10–50% gradient of CH_3CN in 0.05 M triethylammonium acetate. Detritylated oligonucleotides were further purified in a 0–25% gradient of CH_3CN in triethylammonium acetate buffer. Melting curves of the duplexes were recorded with a Shimadzu UV 160-A spectrophotometer (Japan) and with a thermostated cell in 20 mM sodium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, pH = 7.0, 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 analysis. Duplex absorbance at 260 nm was measured as a function of temperature. Data was recorded every 0.5 °C from 20 to 70 °C. Thermodynamic parameters of duplex formation were obtained by performing nonlinear regression analysis by using DataFit version 9.0.059 (Oakdale Engineering, USA). A calculation method taking into account the temperature dependence of the UV absorbance of duplexes and single strands was applied. Circular dichroism (CD) spectra were obtained with a Jasco J-715 spectropolarimeter at 20 °C. Samples were annealed in the same buffer and under the same conditions as for the thermal denaturation studies. The CD values ($\Delta\varepsilon$) are given per mol of nucleotides. Conformational analysis of the modified duplex fragment was performed by molecular mechanics calculations by using the HyperChem. 8.0.9 Amber force field (HyperCube, Inc., USA). The double-stranded B-DNA (5′-CATGTTCATG-3′) was generated by using the nucleic acid feature in the database of HyperChem. The phosphate linker in the TT fragment was cut off, and a triazole linker, constructed by using a model building option of HyperChem, was inserted manually. Partial charges on triazole linker atoms were calculated by using the ab initio method (6-31G**). We obtained 12 initial structures by performing geometry optimizing with the restrained C4′–C5′–C4(triazole)–C5(triazole) with a dihedral angle ranging from 0 to 270° through 30° increments. A distance-dependent dielectric constant was used. After the restraint was removed, the geometry of 12 initial structures was further optimized.

3′-*O*-*tert*-Butyldiphenylsilyl-5′-deoxy-5′-methylenethymidine (2): To a suspension of methyltriphenylphosphonium bromide (0.97 g, 2.72 mmol) in THF (10 mL), a solution of butyllithium (1.8 mL, 1.6 M in hexane) was added. The mixture was stirred at room tem-

perature for 2 h and then cooled to 0 °C. A solution of 1-(3-*O*-*tert*-butyldiphenylsilyl-2-deoxy-β-D-*erithro*-pentadialdo-1,4-furanosyl)-5-methyluracil (**1**) (650 mg, 1.36 mmol) in THF (10 mL) was added dropwise. The mixture was stirred at room temp. for 18 h before being poured into a saturated solution of NH_4Cl (30 mL) and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL), dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography with ethanol and CH_2Cl_2 (1:100, v/v) as eluents to give compound **2** as a colorless foam (0.54 g, 84% yield). R_f = 0.56 (solvent system A). ^{1}H NMR (400 MHz, DMSO): δ = 11.27 (s, 1 H, 3-H), 7.63–7.43 (m, 10 H, Ph_2Si), 7.34 (d, ^4J = 1.1 Hz, 1 H, 6-H), 6.23 (t, ^3J = 7.0 Hz, 1 H, 1′-H), 5.74 (ddd, $^3J_{5',6'a(trans)}$ = 17.1 Hz, $^3J_{5',6'b(cis)}$ = 10.6 Hz, $^3J_{5',4'}$ = 7.1 Hz, 1 H, 5′-H), 5.12–5.05 (m, 2 H, 6′a-H and 6′b-H), 4.29–4.22 (m, 2 H, 3′-H and 4′-H), 2.16–2.11 (m, 2 H, 2′a-H, 2′b-H), 1.74 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3), 1.05 (s, 9 H, CH_3 *t*Bu) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.5 (4-C), 150.4 (2-C), 135.9 (5′-CH), 135.8 (6-CH), 135.3, 135.2 (4 × *o*-CH, Ph), 131.9 (2 × C, Ph), 130.0 (4 × *p*-CH, Ph), 127.88, 1127.85 (4 × *m*-CH, Ph), 117.6 (6′-CH_2), 109.67 (5-C), 87.1 (1′-CH), 83.9 (4′-CH), 76.4 (3′-CH), 38.2 (2′-CH_2), 26.6 (3 × CH_3, *t*Bu), 18.6 (C, *t*Bu), 12.0 (5-CH_3) ppm. HRMS: calcd. for $C_{27}H_{32}N_2O_4Si$ [M + Na]$^+$ 499.2024; found 499.2010.

3′-*O*-*tert*-Butyldiphenylsilyl-5′-deoxy-5′-(hydroxymethyl)thymidine (3): A solution of compound **2** (0.47 mg, 0.99 mmol) in THF (4 mL) was cooled to 0 °C, and 9-BBN-H (1.46 g, 5.97 mmol, 6 equiv.) was added. The reaction mixture was allowed to warm slowly and stirred at 20 °C for 20 h. The reaction mixture was then cooled to 0 °C, and methanol (5.1 mL) was added dropwise. When gas evolution had ceased, water (7.7 mL) was added, followed by $NaBO_3$·4H_2O (5.04 g, 24 mmol, 24 equiv.). The resulting mixture was stirred vigorously at room temperature for 30 h and filtered to remove the precipitate. The filtrate was diluted with ethyl acetate (50 mL) and washed with brine (2 × 25 mL). The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography with ethanol and CH_2Cl_2 (2:100, v/v) as eluents to give compound **3** as a colorless foam (0.42 g, 86% yield). R_f = 0.43 (solvent system B). ^{1}H NMR (400 MHz, DMSO): δ = 11.25 (s, 1 H, 3-H), 7.66–7.40 (m, 10 H, Ph_2Si), 7.32 (d, ^4J = 1.1 Hz, 1 H, 6-H), 6.20 (dd, $^3J_{1',2'a}$ = 8.0 Hz, $^3J_{1',2'b}$ = 6.2 Hz, 1 H, 1′-H), 4.41 (t, ^3J = 5.0 Hz, 1 H, 6′-OH), 4.26–4.21 (m, 1 H, 3′-H), 3.97 (ddd, $^3J_{3',4'}$ = 2.7 Hz, $^3J_{4',5'a}$ = 6.0 Hz, $^3J_{4'\ 5'b}$ = 7.3 Hz, 1 H, 4′-H), 3.40–3.29 (m, 2 H, 6′-CH_2), 2.13–2.06 (m, 1 H, 2′a-H), 2.03 (ddd, $^3J_{1',2'b}$ = 6.2 Hz, $^3J_{2'b,3'}$ = 2.8 Hz, $^2J_{2'a,2'b}$ = 13.3 Hz, 1 H, 2′b-H), 1.75 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3), 1.57–1.50 (m, 2 H, 5′-CH_2), 1.04 (s, 9 H, CH_3 *t*Bu) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.5 (4-C), 150.4 (2-C), 135.8 (6-CH), 135.2 (4 × *o*-CH, Ph), 132.9 and 132.8 (2 × C, Ph), 129.9 (4 × *p*-CH, Ph), 127.9 (4 × *m*-CH, Ph), 109.6 (5-C), 83.6 (1′-CH), 83.5 (4′-CH), 76.1 (3′-CH), 57.3 (6′-CH_2), 38.2 (2′-CH_2), 36.1 (5′-CH_2), 26.6 (3 × CH_3, *t*Bu), 18.6 (C, *t*Bu), 12.0 (5-CH_3) ppm. HRMS: calcd. for $C_{27}H_{34}N_2O_5Si$ [M + Na]$^+$ 517.2129; found 517.2122.

3′-*O*-*tert*-Butyldiphenylsilyl-5′-deoxy-5′-formylthymidine (4): Nucleoside **3** (1.3 g, 2.6 mmol) was dissolved in CH_2Cl_2 (10 mL), and Dess–Martin periodinane (2.2 g, 5.2 mmol) was added. The resulting suspension was stirred at room temperature. After 3 h, the reaction mixture was diluted with ethyl acetate (60 mL), poured into a cold solution of $Na_2S_2O_3$·5H_2O (8.5 g, 34 mmol) in saturated $NaHCO_3$ (60 mL), and shaken for 5 min. The reaction mixture was allowed to cool to room temperature and filtered. The organic layer was separated, washed with $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL), dried with Na_2SO_4 and concentrated to provide aldehyde **4** as a white foam (1.14 g, 89% yield, >95% pure by ^{1}H NMR). Aldehyde **4** was used without further purification in the next reaction step. R_f = 0.59 (solvent system B). ^{1}H NMR (400 MHz, DMSO): δ = 11.26 (s, 1 H, 3-H), 9.49 (dd, $^3J_{6',5'a}$ = 2.6 Hz, $^3J_{6',5'b}$ = 1.3 Hz, 1 H, 6′-H), 7.66–7.40 (m, 10 H, Ph_2Si), 7.33 (d, ^4J = 1.1 Hz, 1 H, H6), 6.21 (dd, $^3J_{1',2'a}$ = 7.9 Hz, $^3J_{1',2'b}$ = 6.4 Hz, 1 H, 1′-H), 4.35–4.27 (m, 2 H, 3′-H, 4′-H), 2.58 (ddd, $^3J_{5'a,4'}$ = 8.3 Hz, $^3J_{5'a,6'}$ = 2.6 Hz, $^2J_{5'a,5'b}$ = 16.5 Hz, 1 H, 5′a-H), 2.42 (ddd, $^3J_{5'b,4'}$ = 4.3 Hz, $^3J_{5'b,6'}$ = 1.3 Hz, $^2J_{5'a,5'b}$ = 16.5 Hz, 1 H, 5′b-H), 2.16 (ddd, $^3J_{1',2'a}$ = 7.9 Hz, $^3J_{2'a,3'}$ = 5.8 Hz, $^2J_{2'a,2'b}$ = 13.9 Hz, 2 H, 2′a-H), 2.09 (ddd, $^3J_{1',2'b}$ = 6.4 Hz, $^3J_{2'b,3'}$ = 2.7 Hz, $^2J_{2'a,2'b}$ = 13.9 Hz, 2 H, 2′b-H), 1.74 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3), 1.05 (s, 9 H, CH_3, *t*BuSi) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 200.8 (6′-CH), 163.5 (4-C), 150.4 (2-C), 135.2 (6-CH), 135.12 (4 × *o*-CH, Ph), 132.7 (2 × C, Ph), 130.1 and 130.0 (4 × *p*-CH, Ph), 127.9 (4 × *m*-CH, Ph), 109.8 (5-C), 84.2 (1′-CH), 80.8 (4′-CH), 75.6 (3′-CH), 46.3 (5′-CH_2), 37.9 (2′-CH_2), 26.6 (3 × CH_3, *t*Bu), 18.6 (C, *t*Bu), 11.9 (5-CH_3) ppm. HRMS: calcd. for $C_{27}H_{32}N_2O_5Si$ [M + Na]$^+$ 515.1973; found 515.1972.

3′-*O*-*tert*-Butyldiphenylsilyl-5′-deoxy-5′-(2,2-dibromoethenyl)-thymidine (5): To a stirred solution of nucleoside **4** (0.89 g, 1.8 mmol) and (dibromomethyl)triphenylphosphonium bromide (1.85 g, 3.59 mmol) in dioxane (4 mL), Zn (260 mg, 3.95 mmol) was added. The reaction mixture was stirred at reflux temperature for 3 h, then cooled to room temperature and filtered. The filtrate was concentrated, the residue dissolved in CH_2Cl_2 (50 mL) and washed with brine (30 mL). The organic layer was separated, dried with Na_2SO_4 and concentrated. Purification by flash column chromatography with ethanol/CH_2Cl_2 (1–3:100, v/v) as eluent afforded compound **5** as a colorless foam (0.93 g, 80% yield). R_f = 0.46 (solvent system A). ^{1}H NMR (400 MHz, DMSO): δ = 11.28 (s, 1 H, 3-H), 7.72–7.41 (m, 10 H, Ph_2Si), 7.32 (d, ^4J = 1.0 Hz, 1 H, 6-H), 6.43 (t, $^3J_{5',6'}$ = 7.1 Hz, 1 H, 6′-H), 6.19 (dd, $^3J_{1',2'a}$ = 7.8 Hz, $^3J_{1',2'b}$ = 6.5 Hz, 1 H, 1′-H), 4.32–4.22 (m, 1 H, 3′-H), 3.99–3.88 (m, 1 H, 4′-H), 2.21–2.13 (m, 3 H, 2′a-H and 5′-CH_2), 2.10 (ddd, $^3J_{1',2'b}$ = 6.5 Hz, $^3J_{3',2'b}$ = 3.2 Hz, $^2J_{2'a,2'b}$ = 13.5 Hz, 1 H, 2′b-H), 1.76 (d, ^4J = 1.0 Hz, 3 H, 5-CH_3), 1.05 (s, 9 H, CH_3, *t*Bu) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.5 (4-C), 150.3 (2-C), 136.0 (6-CH), 135.1 (4 × *o*-CH, Ph), 134.8 (6′-CH), 132.7 (2 × C, Ph), 130.1 (4 × *p*-CH, Ph), 127.94 and 127.92 (4 × *m*-CH, Ph), 109.7 (5-C), 90.1 (7′-C), 84.1 (1′-CH), 83.1 (4′-CH), 75.2 (3′-CH), 38.1 (2′-CH_2), 36.2 (5′-CH_2), 26.6 (3 × CH_3, *t*Bu), 18.5 (C, *t*Bu), 12.0 (5-CH_3) ppm. HRMS: calcd. for $C_{28}H_{32}Br_2N_2O_4Si$ [M + Na]$^+$ 669.0390; found 669.0390.

3′-*O*-*tert*-Butyldiphenylsilyl-5′-deoxy-5′-ethynylthymidine (6): Nucleoside **5** (0.73 g, 1.13 mmol) was dissolved in THF (15 mL), and butyllithium (4 mL, 1.6 M in hexane) was added dropwise at –70 °C. The reaction mixture was stirred at –70 °C for 30 min and allowed to warm to room temperature. Saturated NH_4Cl (30 mL) was added and the resulting mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were washed with water (50 mL), dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography with ethanol/CH_2Cl_2 (1:100, v/v) as eluent to give compound **6** as a colorless foam (0.73 g, 77% yield). R_f = 0.39 (solvent system A). ^{1}H NMR (400 MHz, DMSO): δ = 11.28 (s, 1 H, 3-H), 7.65–7.44 (m, 10 H, Ph_2Si), 7.43 (d, ^4J = 1.1 Hz, 1 H, 6-H), 6.26 (dd, $J_{1',2'a}$ = 8.1 Hz, $J_{1',2'b}$ = 6.2 Hz, 1 H, 1′-H), 4.36–4.32 (m, 1 H, 3′-H), 4.00–3.96 (m, 1 H, 4′-H), 2.84 (t, $^4J_{7',5'a(5'b)}$ = 2.6 Hz, 1 H, 7′-H), 2.38 (ddd, $^3J_{5'a,4'}$ = 6.4 Hz, $^4J_{5'a,7'}$ = 2.6 Hz, $^2J_{5'a,5'b}$ = 17.1 Hz, 1 H, 5′a-H), 2.25 (ddd, $^3J_{5'b,4'}$ = 5.5 Hz, $^4J_{5'b,7'}$ = 2.6 Hz, $^2J_{5'a,5'b}$ = 17.1 Hz, 1 H, 5′b-H), 2.14–2.08 (m, 2 H, 2′a-H, 2′b-H), 1.74 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3), 1.05 (s, 9 H, CH_3, *t*BuSi) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.5 (4-C), 150.3 (2-C), 135.5 (6-CH), 135.24 and

135.21 (4×*o*-CH, Ph), 132.7 and 135.6 (2×C, Ph), 130.1 (4×*p*-CH, Ph), 127.9 (4×*m*-CH, Ph), 109.7 (5-C), 84.0 (1′-CH), 83.8 (4′-CH), 80.3 (6′-C), 75.1 (3′-CH), 73.1 (7′-CH), 38.1 (2′-CH_2), 26.7 (3×CH_3, *t*Bu), 22.2 (5′-CH_2), 18.5 (C, *t*Bu), 12.4 (5-CH_3) ppm. HRMS: calcd. for $C_{28}H_{32}N_2O_4Si$ [M + Na]$^+$ 511.2024; found 511.2017.

4-[3′-*O*-(*tert*-Butyldiphenylsilyl)-5′-deoxythymidin-5′-yl]-1-[3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl]-1*H*-1,2,3-triazole (7): To a stirred solution of compound **6** (0.37 g, 0.76 mmol) and 3′-amino-3′-deoxy-5′-*O*-dimethoxytritylthymidine (0.47 g, 0.83 mmol) in CH_2Cl_2 (2 mL) and water (2 mL), $CuSO_4·5H_2O$ (19 mg, 0.08 mmol) and sodium ascorbate (49 mg, 0.23 mmol) were added. The resulting solution was stirred at room temp. for 2 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and brine (10 mL). The organic layer was separated, dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography with ethanol/CH_2Cl_2 (1:100, v/v) as eluent to give dinucleoside **7** as a hard foam (0.64 g, 80% yield). R_f = 0.63 (solvent system C). ^{1}H NMR (400 MHz, DMSO): δ = 11.37 (s, 1 H, 3-H, ethT), 11.26 (s, 1 H, 3-H, aT), 7.91 (s, 1 H, 7′-H, ethT), 7.64 (d, ^4J = 1.2 Hz, 1 H, 6-H, ethT), 7.54 (d, ^4J = 1.1 Hz, 1 H, 6-H, aT), 7.61–6.81 (m, 23 H, ArH, DMTr, TBDPS), 6.39 (t, $^3J_{1′,2′a(2′b)}$ = 6.5 Hz, 1 H, 1′-H, aT), 6.24 (dd, $^3J_{1′,2′a}$ = 6.3 Hz, $^3J_{1′,2′b}$ = 7.9 Hz, 1 H, 1′-H, ethT), 5.49–5.38 (m, 1 H, 3′-H, aT), 4.39–4.33 (m, 1 H, 3′-H, ethT), 4.27–4.21 (m, 1 H, 4′-H, aT), 4.15 (td, $^3J_{3′,4′}$ = 2.4 Hz, $^3J_{4′,5′a(5′b)}$ = 6.7 Hz, 1 H, 4′-H, ethT), 3.72 (s, 6 H, CH_3O, DMTr), 3.29–3.24 (m, 2 H, 5′-CH_2, aT), 2.82–2.73 (m, 2 H, 5′-CH_2, ethT), 2.75–2.63 (m, 2 H, 2′-CH_2, aT), 2.13–2.03 (m, 2 H, 2′-CH_2, ethT), 1.73 (d, ^4J = 1.2 Hz, 3 H, 5-CH_3, aT), 1.60 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3, ethT), 1.00 (s, 9 H, CH_3, *t*Bu) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.6 (4-C, ethT), 163.5 (4-C, aT), 158.1 (2×*p*-C, PhOMe, DMTr), 150.3 (2-C, ethT, aT), 144.5 (6′-C, ethT), 143.1 (C, Ph, DMTr), 136.2 (6-CH, ethT), 135. 9 (6-CH, aT), 135.1 (4×*o*-CH, Ph, TBDPS), 135.1 (2×C, PhOMe, DMTr), 132.8 and 132.7 (2×C, Ph, TBDPS), 129.9 (2×*p*-CH, Ph, TBDPS), 129.6 (4×*o*-CH, PhOMe, DMTr), 127.8 (4×*m*-CH, Ph, TBDPS), 127.74 (2×*o*-CH, Ph, DMTr), 127.68 (2×*m*-CH, Ph, DMTr), 126.7 (*p*-CH, Ph, DMTr), 122.1 (7′-CH, ethT), 113.1 (4×*m*-CH, PhOMe, DMTr), 109.8 (5-C, ethT), 109.6 (5-C, aT), 85.9 (C, DMTr), 85.5 (4′-CH, ethT), 84.0 (1′-CH, ethT), 83.9 (1′-CH, aT), 82.2 (4′-CH, aT), 75.3 (3′-CH, ethT), 63.0 (5′-CH_2, aT), 59.1 (7′-CH, ethT), 54.9 (2×CH_3, DMTr), 38.1 (2′-CH_2, ethT), 37.0 (2′-CH_2, aT), 29.1 (5′-CH_2, ethT), 26.6 (3×CH_3, *t*Bu, TBDPS), 18.5 (C, *t*Bu, TBDPS), 12.0 (5-CH_3, aT), 11.8 (5-CH_3, ethT) ppm. HRMS: calcd. for $C_{59}H_{63}N_7O_{10}Si$ [M + Na]$^+$ 1080.4298; found 1080.4276.

1-[3′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl]-4-(5′-deoxythymidin-5′-yl)-1*H*-1,2,3-triazole (8): To a stirred solution of dinucleoside **7** (0.59 g, 0.56 mmol) in THF (1.7 mL), a solution of tetrabutylammonium fluoride (1.7 mL, 1 M in THF) was added. The mixture was stirred at room temperature for 2 h, diluted with saturated $NaHCO_3$ (50 mL) and extracted with $CHCl_3$ (3×40 mL). The combined organic layers were washed with water, dried with Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography with ethanol/CH_2Cl_2/TEA (1:100:0.1, v/v/v) as eluent to give dinucleoside **8** as a hard foam (0.45 g, 98% yield). R_f = 0.47 (solvent system D). ^{1}H NMR (400 MHz, DMSO): δ = 11.35 (s, 1 H, 3-H, ethT), 11.25 (s, 1 H, 3-H, aT), 8.06 (s, 1 H, 7′-H, ethT), 7.64 (d, ^4J = 1.1 Hz, 1 H, 6-H, ethT), 7.43 (d, ^4J = 1.2 Hz, 1 H, 6-H, aT), 7.36–6.80 (m, 13 H, ArH, DMTr), 6.40 (t, $^3J_{1′,2′a\ (2′b)}$ = 6.5 Hz, 1 H, 1′-H, aT), 6.15 (t, $J_{1′,2′a(2′b)}$ = 6.9 Hz, 1 H, 1′-H, ethT), 5.55–5.45 (m, 1 H, 3′-H, aT), 5.32 (d, ^3J = 4.3 Hz, 1 H, 3′-OH, ethT), 4.36–4.23 (m, 1 H, 4′-H, aT), 4.25–4.18 (m, 1 H, 3′-H, ethT), 4.00–3.92 (m, 1 H, 4′-H, ethT), 3.73 (s, 6 H, CH_3O, DMTr), 3.30–3.28 (m, 2 H, 5′-CH_2, aT), 3.08 (dd, $^3J_{4′,5′a}$ = 5.3 Hz, $^2J_{5′a,5′b}$ = 15.1 Hz, 1 H, 5′a-H, ethT), 2.95 (dd, $^3J_{4′,5′b}$ = 7.6 Hz, $^2J_{5′a,5′b}$ = 15.1 Hz, 1 H, 5′b-H, ethT), 2.80–2.72 (m, 2 H, 2′-CH_2, aT), 2.18 (dt, $^3J_{1′,2′a(3′)}$ = 6.7 Hz, $^2J_{2′a,2′b}$ = 13.6 Hz, 1 H, 2′a-H, ethT), 2.08 (ddd, $^3J_{1′,2′b}$ = 6.4 Hz, $^3J_{2′b,3′}$ = 3.8 Hz, $^2J_{2′a,2′b}$ = 13.6 Hz, 1 H, 2′b-H, ethT), 1.77 (d, ^4J = 1.2 Hz, 3 H, 5-CH_3, aT), 1.60 (d, ^4J = 1.1 Hz, 3 H, 5-CH_3, ethT) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 163.6 (4-C, ethT, aT), 158.1 (2×*p*-C, PhOMe, DMTr), 150.4 (2-C, ethT) 150.3 (2-C, aT), 144.5 (6′-C, ethT), 143.6 (C, Ph, DMTr), 136.2 (6-CH, ethT), 136.0 (6-CH, aT), 135.2 (2×C, PhOMe, DMTr), 129.6 (4×*o*-CH, PhOMe, DMTr), 127.8 (2×*o*-CH, Ph, DMTr), 127.6 (2×*m*-CH, Ph, DMTr), 126.7 (*p*-CH, Ph, DMTr), 122.3 (7′-CH, ethT), 113.2 (4×*m*-CH, PhOMe, DMTr), 109.8 (5-C, ethT), 109.6 (5-C, aT), 86.0 (4′-CH, ethT), 85.9 (C, DMTr), 83.9 (1′-CH, ethT), 83.6 (1′-CH, aT), 82.2 (4′-CH, aT), 75.4 (3′-CH, ethT), 63.0 (5′-CH_2, aT), 59.2 (7′-CH, ethT), 55.0 (2×CH_3, DMTr), 38.2 (2′-CH_2, ethT), 37.0 (2′-CH_2, aT), 29.3 (5′-CH_2, ethT), 12.0 (5-CH_3, aT), 11.8 (5-CH_3, ethT) ppm. HRMS: calcd. for $C_{43}H_{45}N_7O_{10}$ [M + Na]$^+$ 842.3120; found 842.3126.

4-{3′-Deoxy-3′-[(2-cyanoethoxy)(diisopropylamino)phosphanyloxy]-5′-deoxythymidin-5′-yl}-1-[3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl]-1*H*-1,2,3-triazole (9): Dinucleoside **8** (0.35 g, 0.43 mmol) and tetrazole (45 mg, 0.64 mmol) were dissolved in pyridine (5 mL), and the solvent was removed in vacuo. This operation was repeated twice. The residue was dissolved in of CH_2Cl_2 (5 mL). The mixture was stirred in the presence of molecular sieves at room temp. for 30 min, and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite (148 μL, 0.64 mmol) was added. The resulting mixture was stirred at room temp. for a further 30 min, then poured into cold saturated $NaHCO_3$ (50 mL) and extracted with CH_2Cl_2 (3×40 mL). The combined organic layers were washed with water, dried with Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography with ethanol/CH_2Cl_2/TEA (2–3:100:0.1, v/v/v) as eluent to give amidite **9** as a hard foam (0.31 g, 72% yield). R_f = 0.26 (solvent system C). ^{31}P NMR (162 MHz, DMSO): δ = 150.29, 150.02 ppm. HRMS: calcd. for $C_{52}H_{62}N_9O_{11}P$ [M + Na]$^+$ 1042.4199; found 1042.4188.

Supporting Information (see footnote on the first page of this article): COSYDQF and C-H correlation NMR spectra, MALDI-TOF mass spectra and conformational analysis details.

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