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PAPER

The synthesis of double-headed nucleosides by the CuAAC reaction and their effect in secondary nucleic acid structures†

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Four double-headed nucleosides were prepared by the CuAAC reaction. Hereby, a triazole-containing linker connects an additional thymine or adenine to the 2′-position of 2′-deoxyuridine, a thymine to the 5′-position of thymidine and a thymine to the 6′-position of an LNA-thymidine monomer. Whereas no conclusive recognition effects of the additional thymines were found when introduced in LNA or at the 5′-position, both thymine and adenine in the 2′-position were found to stabilise three-way junctions in both dsDNA and DNA : RNA contexts and to give cross-strand interactions in a DNA-duplex, when specifically introduced in a so-called (+1)-zipper motif.

Introduction

Nucleic acids are intensively investigated as scaffolds for supramolecular design for instance by organizing various entities on the surface of the double helix.[1–4] Double-headed nucleosides are synthetic nucleoside analogues with two nucleobases. We and others have recently prepared a series of double-headed nucleosides in order to study the recognition effects of the additional nucleobases in different nucleic acid constructs (Fig. 1).[5–12] A uridine analogue with an additional thymine in the 2′-position, **1**, demonstrated a stabilisation of a three-way junction when incorporated in the center of the junction propably due to the additional stacking introduced by the thymine.[5] On the other hand, a thymidine analogue with an additional thymine in the 5′(*S*)-*C*-position, **3** (B = T), did not stabilize a three-way junction but demonstrated a very selective zipper-interaction in a duplex. With the monomer incorporated in each of the complementary strands with two interspacing unmodified base-pairs (defined as a (−3)-zipper), a large relative stabilisation was seen due to stacking between the two additional thymines across the minor groove.[6] This was observed specifically in the (−3)-zipper motif, and an even stronger contact was recently found when one (and only one) of the two thymines in the zipper was replaced with a phenyl group.[7] Double-headed nucleosides with the additional nucleobase in the 4′-position, **2** (B = T or A) have been introduced into each complementary strand of a duplex but only weak base-base

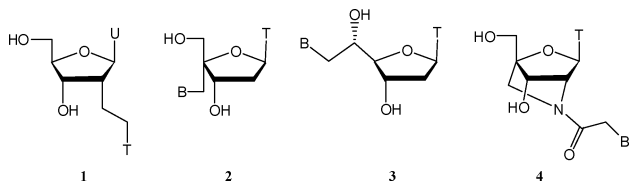

Fig. 1 Double-headed nucleosides. U = uracil-1-yl, T = thymin-1-yl. B = various nucleobases.

interactions have been observed.[8] The same has been observed for double-headed amino-LNA nucleosides **4** (B = T or A).[9]

Since the introduction of Click Chemistry in 2001,[13] a lot of attention has been given to especially one reaction; the Cu(I)-catalyzed alkyne azide cycloaddition (CuAAC).[14,15] This reaction fulfils all the original demands for click chemistry[13] including the high efficiency and specificity, tolerancy of all other functional groups and no side products formed. It is now a well-established reaction[16,17] also in nucleoside/nucleic acid chemistry.[2,18,19] We have recently applied the CuAAC reaction in the study of triazoles stacking in the major groove leading to highly stable DNA : RNA duplexes.[20,21] We have also recently studied a series of double-functionalized nucleosides with triazoles replacing the additional nucleobase in **3**.[7] It was found, however, that the (−3)-zipper contact in the minor groove was smaller when one or both of the additional thymines in the zipper were replaced by either unsubstituted triazoles or triazoles connected directly to a phenyl or a uracil. On the other hand, other contacts in the minor groove, for instance hydrogen-bonds of the latter, were observed.[7]

Herein, we present four new double-headed nucleosides prepared by the CuAAC reaction and placing the additional nucleobases in three different positions; the 2′-position of 2′-deoxyuridine, the 5′(*S*)-*C*-position of thymidine and the 6′(*R*)-position of the LNA-thymidine monomer. All three positions will place the additional nucleobases through a triazolemethylene linker in the minor groove of a duplex.

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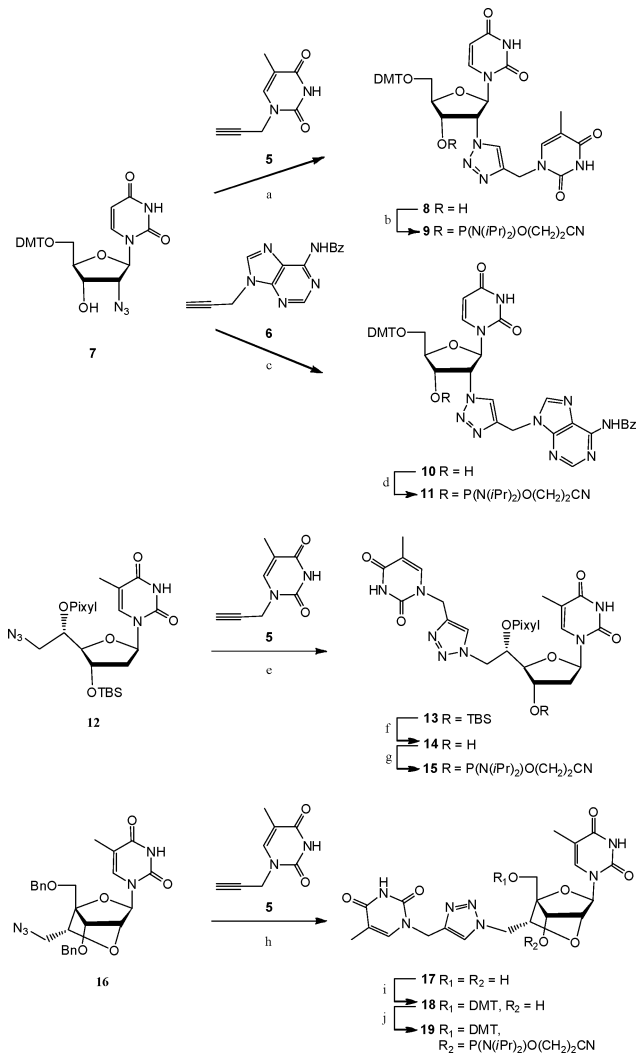
Results and discussion

Chemical synthesis. The CuAAC reaction was used by the combination of three different nucleoside azides and two propargylated nucleobases (Scheme 1). 2′-Azido-2′-deoxyuridine was prepared by a known method based on the nucleophilic opening of 2,2′-anhydrouridine,[22] and protected with the DMT-group to give **7**,[23] which was then reacted in two different cycloadditions. With *N*1-propargylthymine **5**,[24] the CuAAC reaction afforded the double-headed nucleoside **8** in a good yield. Standard phosphitylation gave the phosphoramidite **9** as an appropriate building block for automated oligonucleotide synthesis. With the corresponding propargylated benzoyl-adenine **6**, which was obtained from the known *N*9-propargyladenine[24] by benzoylation, the other double-headed nucleoside **10** was obtained in a high



Scheme 1 *Reagents and conditions*: a, Na ascorbate, $CuSO_4 \cdot 5H_2O$, *t*BuOH, H_2O, pyridine, 72%; b, $NC(CH_2)_2OP(Cl)N(iPr)_2$, DIPEA, DCE, 70%; c, Na ascorbate, $CuSO_4 \cdot 5H_2O$, THF, H_2O, pyridine, 93%; d, $NC(CH_2)_2OP(Cl)N(iPr)_2$, DIPEA, CH_2Cl_2, 62%; e, same as a, 80%; f, TBAF, THF, 91%; g, same as d, 90%; h, ref. 27 (i) Na ascorbate, $CuSO_4 \cdot 5H_2O$, *t*BuOH, H_2O, MW, 75%, (ii) H_2, Pd/C, EtOH, 67%; i, DMT-Cl, CH_3CN, pyridine, 60%; j, same as d, 41%; TBS = *tert*-butyldimethylsilyl, Pixyl = 9-phenylxanthen-9-yl, DMT = 4,4′-dimethoxytrityl.

93% yield. Phosphitylation afforded the phosphoramidite **11**. Recently, other examples of CuAAC reactions with 2′-azido-2′-deoxyuridine have been presented.[25,26] The protected 5′(*S*)-*C*-azidomethylthymidine **12** has been recently prepared by us and used in CuAAC-reactions with a series of small alkynes.[7] Here the reaction with **5** gave the double-headed nucleoside **13** in 80% yield. Desilylation gave **14** and phosphitylation gave the amidite **15**. The branched LNA-derivative **16** has been recently presented by us including also the CuAAC-reaction affording the double-headed nucleoside **17**.[27] Protection with the DMT-group gave **18** and phosphitylation afforded the phosphoramidite **19**.

Preparation and evaluation of oligonucleotides. The four phosphoramidites were introduced into oligonucleotides by standard methodology following the phosphoramidite approach. 1*H*-Tetrazole was used as the coupling reagent for both modified and unmodified 2′-deoxynucleoside phosphoramidites. Extended coupling time of 15–20 min. was applied for the modified amidites affording >80% coupling yields. The oligonucleotide (ON) sequences applied in this study are the same as used in former studies of zipper contacts in duplexes[6,7,12] (Table 1 and 2), and of bulged duplexes and three-way junctions[5,6,28,29] (Table 3), respectively. The modified phosphoramidites, **9**, **11**, **15** and **19** were incorporated to give the modified monomers **K**, **L**, **M** and **N**, respectively.

At first, an 11-mer duplex was studied, in which five centrally placed thymidines can be replaced systematically with the modified monomers (Table 1). When either of the two 2′-modified monomers **K** and **L** was incorporated, this gives the sequences **K1–K5** and **L1–L5**. These sequences were mixed with unmodified complementary sequences and the melting temperatures (T_m) of these modified duplexes were measured in thermal denaturation experiments. These were compared with the T_m of the corresponding unmodified duplex, and the differences in melting temperature (ΔT_ms) are shown in Table 1. As evident from the data, all single incorporations of **K** or **L** demonstrated a rather uniform and large decrease in duplex stability of 8–11 °C. In other words, neither the adenine nor the thymine substituents are very well adapted in the minor groove of the duplex indicating that the 2′-*C*-triazole is unfavourable for duplex formation. A small part of the decrease, however, might be deduced to the uracil replacing the thymine in the core of the duplex.

Hereafter, the modified sequences where mixed with complements also containing the same modification (**K** or **L**) in order to study the possibility for zipper contacts in the minor groove. Hence, the two modifications in each of the complementary strands were placed with 0–3 interspacing base-pairs revealing possible (+1) to (–4)-zippers as shown in Table 1. In the case of (–2) to (–4)-zippers, all melting temperatures were decreased with 19–23 °C indicating a fully additive negative effect for each of the two modifications. Hence, when the changes in T_m were compared to the changes obtained for each of the corresponding single modified duplexes, the changes (defined as $\Delta\Delta T_m$s) were between 0 and –5 °C indicating absolutely no positive contacts between the modifications in the minor groove. When the two modifications were placed on two neighbouring base-pairs giving a possible (–1)-zipper contact, the picture varies between the studied duplexes. Hence the duplex formed between **K5** and **K1** was somewhat more stable than the **K4** : **K2** duplex (with a 5.5 °C difference in T_m), and

Table 1 Hybridisation data for incorporations of monomer **K**, **L**, **M** and **N**

ΔT_m/°C[a] [$\Delta\Delta T_m$]/°C[b]

ON	Duplex	X = K	L	M	N	Zipper	ON	Duplex	X = K	L	M	N
ref.[c]	5′-d(CGC ATA TTC GC) 3′-d(GCG TAT AAG CG)					(+1)	**X5** **X2**	5′-d(CGC A**X**A TTC GC) 3′-d(GCG TA**X** AAG CG)	–8.0 [+7.5]	–5.5 [+11.0]	n.d[d]	n.d[d]
X1	5′-d(CGC ATA TTC GC) 3′-d(GCG **X**AT AAG CG)	–8.0	–8.5	–6.0	+3.0	(–1)	**X5** **X1**	5′-d(CGC A**X**A TTC GC) 3′-d(GCG **X**AT AAG CG)	–14.5 [+1.5]	–14.5 [+2.0]	n.d[d]	n.d[d]
X2	5′-d(CGC ATA TTC GC) 3′-d(GCG TA**X** AAG CG)	–7.5	–8.5	–6.0	+5.0	(–1)	**X4** **X2**	5′-d(CGC ATA **X**TC GC) 3′-d(GCG TA**X** AAG CG)	–20.0 [–2.5]	–17.0 [0.0]	–14.0 [–1.5]	+6.0 [+0.5]
X3	5′-d(CGC ATA T**X**C GC) 3′-d(GCG TAT AAG CG)	–11.0	–10.0	–6.5	+1.5	(–2)	**X3** **X2**	5′-d(CGC ATA T**X**C GC) 3′-d(GCG TA**X** AAG CG)	–21.5 [–3.0]	–19.0 [–0.5]	–14.0 [–1.5]	+9.0 [+2.5]
X4	5′-d(CGC ATA **X**TC GC) 3′-d(GCG TAT AAG CG)	–10.0	–8.5	–6.5	+0.5	(–3)	**X4** **X1**	5′-d(CGC ATA **X**TC GC) 3′-d(GCG **X**AT AAG CG)	–21.0 [–3.0]	–22.0 [–5.0]	–12.0 [+0.5]	+4.5 [+1.0]
X5	5′-d(CGC A**X**A TTC GC) 3′-d(GCG TAT AAG CG)	–8.0	–8.0	n.d[d]	n.d[d]	(–4)	**X3** **X1**	5′-d(CGC ATA T**X**C GC) 3′-d(GCG **X**AT AAG CG)	–22.0 [–3.0]	–22.5 [–4.0]	–12.0 [+0.5]	+2.5 [–2.0]

[a] Differences in melting temperatures as compared to the unmodified duplex; $\Delta T_m = T_{m(duplex)} - T_{m(ref)}$, $T_{m(ref)}$ = 46.0 °C. Melting temperatures (T_m values/°C) were obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) recorded in a medium salt buffer (Na_2HPO_4 (2.5 mM), NaH_2PO_4 (5 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.0 μM concentrations of each strand. **K**, **L**, **M** and **N** correspond to the incorporation of the amidites, **9**, **11**, **15** and **19**, respectively. [b] Differences in melting temperatures as compared to singly modified duplexes; $\Delta\Delta T_m = \Delta T_{m(a:b)} - (\Delta T_{m(a:ref)} + \Delta T_{m(ref:b)})$. [c] Reference sample. [d] Not determined.

Table 2 Hybridisation data for crossed zippers with monomer **K** and **L**

ΔT_m/°C[a] [$\Delta\Delta T_m$]/°C[b]

Zipper	ON	Duplex	X/Y = K/L	L/K
(+1)	**X5** X2	5′-d(CGC A**X**A TTC GC) 3′-d(GCG TA**Y** AAG CG)	–5.0 [+11.5]	–6.0 [+9.5]
(–1)	**X5** X1	5′-d(CGC A**X**A TTC GC) 3′-d(GCG **Y**AT AAG CG)	–15.5 [+1.0]	–14.5 [+1.5]
(–1)	**X4** X2	5′-d(CGC ATA **X**TC GC) 3′-d(GCG TA**Y** AAG CG)	–19.0 [–0.5]	–19.5 [–3.5]
(–2)	**X3** X2	5′-d(CGC ATA T**X**C GC) 3′-d(GCG TA**Y** AAG CG)	–21.5 [–2.0]	–20.0 [–2.5]
(–3)	**X4** X1	5′-d(CGC ATA **X**TC GC) 3′-d(GCG **Y**AT AAG CG)	–23.5 [–5.0]	–18.5 [–2.0]
(–4)	**X3** X1	5′-d(CGC ATA T**X**C GC) 3′-d(GCG **Y**AT AAG CG)	–23.5 [–4.0]	–20.5 [–2.5]

[a] [b] See Table 1.

the same, though less pronounced, was valid for the **L5** : **L1** duplex compared to **L4** : **L2**. Nevertheless, the relative gains in T_m (as valid from the $\Delta\Delta T_m$s of up to +2.0 °C) were still too small to conclude for a contact between the 2′-substituents in the duplex, and they might just reflect other sequence specific variations related to, for instance, duplex hydration.

When the possible (+1)-zipper contact was studied, interesting results were revealed. Hence the duplexes **K5** : **K2** and **L5** : **L2** were equally or more stable than either of the corresponding single modified duplexes indicating a stabilising contact between the 2′-substituents. Based on simple modeling showing the two 2′-carbons in opposite positions relative to the duplex core, this contact could be the additional nucleobases partly intercalating in the duplex opposing each other. This is supported by the fact that a larger compensation in the duplex stability was obtained with the adenines of **L** ($\Delta\Delta T_m$ = +11 °C) than with the thymines of **K** ($\Delta\Delta T_m$ = +7.5 °C), as the adenine has a larger surface for stacking than the thymine. In order to investigate the possibility for mixed base-contacts the sequences containing **K** and **L** were also mixed (Table 2). The (+1)-zipper contacts of the **K5** : **L2** and **L5** : **K2** duplexes were comparable to the contacts found for the two adenines with $\Delta\Delta T_m$s of +11.5 and +9.5 °C, respectively. This indicates that the zipper effect is based on stacking and not on any Watson-Crick base-pair formation between A and T. In order to get the full picture of possible A-T contacts, all other combinations of **K** and **L** sequences were also investigated (Table 2). The results were in all cases fully comparable to the results found with either two **K**'s or two **L**'s, that is all $\Delta\Delta T_m$s being negative, despite in one (–1)-zipper being slightly positive, indicating in general no positive base-base contacts in the minor groove.

Hereafter, the monomer **M** containing a CH_2-triazole-CH_2 linked thymine at the 5′(*S*)-*C*-position was incorporated in all the same sequences despite one (**M1–M4**, Table 1). When these were mixed with unmodified complements, the decreases in stability for the single modified duplexes were very uniform with a decrease in T_m of 6–6.5 °C. Hereby, the 5′-substituent is slightly better accommodated in the duplex as compared to the corresponding 2′-substituent, in line with the 5′-position being closer to the rim of the minor groove and away from the duplex core. Compared to other 5′-substituents (analogues of **3**, Fig. 1), the thermal penalty paid for the thymine-methylene-triazole substituent is comparable to other substituted triazoles[7] and to the thymine as introduced by our first monomer **3** (B = T), which gave T_ms of around –5 °C.[6] When the modified sequences **M1–M4** were mixed, no zipper contacts were found, as indicated by very neutral $\Delta\Delta T_m$s of –1.5 or +0.5 °C. This is completely opposite the situation found for **3** (B = T), for which a very strong (–3)-zipper was found ($\Delta\Delta T_m$s around +6–7 °C),[6] but parallel to the results found for an unsubstituted triazole[7] or a thymine on an extended linker **3** (B = T-CH_2).[12]

Table 3 Hybridization data for bulged duplexes and three-way junctions[a]

T_m/°C (ΔT_m)/°C

			X =				
X6	5′- d(GCT CAC **X**CT CCC A),		T	TT	**K**	**L**	**M**
DNA	3′- d(CGA GTG AGA GGG T)		52.5	n.d.[b]	47.0 (–5.5)	47.5 (–5.0)	48.0 (–4.5)
RNA	3′- r(CGA GUG AGA GGG U)		61.0	n.d.[b]	60.0 (–1.0)	59.5 (–1.5)	58.0 (–3.0)
DNA, A-bulge	3′- d(CGA GTG AAG AGG GT)		43.0	53.5	43.0 (0.0/–10.5)	46.0 (+3.0/–7.5)	39.5 (–3.5/–14.0)
RNA, A-bulge	3′- r(CGA GUG AAG AGG GU)		50.5	60.5	49.5 (–1.0/–11.0)	50.5 (0.0/–10.0)	47.5 (–3.0/–13.0)
DNA, GA -bulge	3′- d(CGA GTG AGA GAG GGT)		43.5	42.5	40.0 (–3.5/–2.5)	41.5 (–2.0/–1.0)	39.0 (–4.5/–3.5)
RNA, GA-bulge	3′- r(CGA GUG AGA GAG GGU)		50.5	50.5	49.5 (–1.0/–1.0)	49.5 (–1.0/–1.0)	48.0 (–2.5/–2.5)
DNA hairpin	3′- d(CGA GTG ACC CGC GTT TTC GCG AGA GGG T)	0 mM Mg^{2+}	24.5	26.0	28.0(+3.5/+2.0)	28.5 (+4.0/+2.5)	24.0 (–0.5/–2.0)
		5 mM Mg^{2+}	34.5	35.5	35.0 (+0.5/–0.5)	36.0 (+1.5/+0.5)	32.0 (–2.5/–3.5)
		10 mM Mg^{2+}	38.0	38.5	37.5 (–0.5/–1.0)	38.0 (0.0/–0.5)	34.5 (–3.5/–4.0)
RNA haripin	3′- r(CGA GUG ACG CGU UUU CGC GAG AGG GU)	0 mM Mg^{2+}	37.0	37.5	41.5 (+4.5/+4.0)	39.5 (+2.5/+2.0)	38.0 (+1.0/+0.5)
		5 mM Mg^{2+}	44.5	n.d.[b]	n.d.[b]	46.0 (+1.5)	n.d.[b]
		10 mM Mg^{2+}	47.0	47.5	49.0 (+2.0/+1.5)	47.5 (+0.5/0.0)	47.0 (0.0/–0.5)

[a] Melting temperatures (T_m values/°C) obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) recorded in a medium salt buffer (Na_2HPO_4 (2.5 mM), NaH_2PO_4 (5 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.0 μM concentrations of each strand. In brackets, differences in melting temperatures as compared to **X** = T/TT. **K**, **L** and **M** correspond to the incorporation of the amidites, **9**, **11** and **15**, respectively. [b] Not determined.

Apparently, the triazole itself cannot induce any positive stacking contact, and with a methylene in the linker, the flexibility removes any significant effect of the thymines. Like with other 5′-functionalized nucleosides,[6,7,12] we also studied multiple incorporations of **M** but found no melting temperatures indicating any compensating effects of having more than one monomer in the strand (see ESI†).

Finally, the monomer **N** with the same triazole-thymine substituent in the 6′(*R*)-position of LNA was incorporated in four sequences **N1**–**N4**. LNA (*locked nucleic acid*) monomers induce a large increase in thermal stability when introduced in the nucleic acid duplexes,[30] and in general increases in T_m of 2–5 °C can be expected in a DNA-duplex, though both larger and smaller values have been reported.[31] With monomer **N** incorporated, increases in T_m were indeed observed with ΔT_ms of +0.5–5.0 °C (Table 1). The variation, however, indicates some sequence specific interactions of the 6′-substituent. When monomer **N** was incorporated twice in the duplex as in the four combinations of the four single modified sequences, the increases in T_m were, as expected, of +2.5–9.0 °C corresponding to +1.3–4.5 °C for each monomer. When recalculated as $\Delta\Delta T_m$ values between –2 and +2.5 °C, however, these indicated no important contacts in the minor groove. On the other hand, the large substituent at the 6′-position was generally well accommodated in a duplex showing that the azide **16** can be a key compound in new series of derivatised LNA's.

The monomers **K**, **L** and **M** were also investigated in another sequence context in order to investigate the effect of the additional nucleobase in bulged duplexes and three-way junctions (TWJs). Hence, three 13-mer sequences with the monomer placed in the central position, **K6**, **L6** and **M6** were prepared and mixed with different complementary strands (Table 3). First, a fully complementary DNA-sequence was applied and the effects on the standard duplex were hereby investigated. The three monomers led to decreases in thermal stability compared to the unmodified duplex of 5.5, 5.0 and 4.5 °C, respectively, which were somewhat smaller decreases as compared to the other sequences studied (Table 1). However, this is expectable due to the difference in sequence length. With an RNA complement, the decreases in T_m were smaller with ΔT_ms of only –1, –1.5, and –3 °C for **K**, **L** and **M**, respectively. Hence, especially the 2′-modifications are much better accommodated in a DNA : RNA duplex than in dsDNA.

Hereafter, the hybridisation of the modified sequences with complementary bulged DNA and RNA sequences were investigated. With an additional adenine opposite **K** in a DNA-duplex, no change in thermal stability was observed when compared to an unmodified A-bulged DNA duplex. This indicates that the 2′-substituent is accommodated into the bulged region but that no positive influence from the additional thymine is obtained. With monomer **L** in the bulge, a stabilisation of the bulge of 3.0 °C was observed indicating some effect of the additional adenine. This could again be due to a stacking effect from the larger purine. In contrary, monomer **M** demonstrated a decrease in thermal stability of the modified A-bulge (ΔT_m = –3.5 °C) in accordance with results of other nucleoside analogues with 5′-*C*-substituents.[6,12] When comparing the melting temperatures of the three bulged duplexes with regular duplexes, that is with two thymidines instead of **K**, **L** or **M**, however, large decreases of 10.5, 7.5 and 13 °C were seen. This demonstrates that these double-headed nucleosides, like **1** and all studied analogues of **3**, do not behave as dinucleotides. With complementary RNA, the results were similar with decreases in thermal stability, ΔT_ms, of the modified A-bulged duplexes of 0 °C for **L**, –1 °C for **K** and –3 °C for **M**. With larger GA bulges, slightly larger decreases in thermal stability were observed with ΔT_ms varying between –1 and –4.5 °C, the decreases being larger with a DNA than with an RNA complement, and with monomer **M** as compared to monomers **K** and **L**.

Finally, the effect of double-headed nucleosides in a TWJ was studied by mixing the modified sequences **K6**, **L6** and **M6** with DNA and RNA strands with standard stable hairpin sequences flanked by single stranded regions being complementary to the modified sequences. As in our former studies,[6,12] the DNA strand contains an additional CC-bulge as compared to the RNA strand in order to form a more well-defined secondary structure. In the DNA : DNA TWJ, both monomers **K** and **L** were found to induce increases in thermal stability of around 4 °C when compared to the unmodified TWJ with a thymidine in the same position.

Compared to a larger TWJ with two centrally placed thymidines, the increases with **K** and **L** were only slightly smaller (ΔT_ms of +2–2.5 °C). These results indicated a substantial stabilising effect from the 2′-substituents (the triazole, the additional base or the combination of the two) most probably from a stacking on one of the three stems in the TWJ. By the addition of Mg^{2+}, the stability of the TWJ increased but the relative stabilising effect of the modification decreased to give neutral T_ms with a 10 mM Mg^{2+} concentration. With monomer **M** in the TWJ, no stabilisation was found without Mg^{2+} in the solution and relative destabilisation was observed by the addition of Mg^{2+}. In the DNA : RNA TWJ, the stabilisation was even higher with ΔT_ms of +4.5 °C for **K**, +2.5 °C for **L** and +1 °C for **M**, decreasing to ΔT_ms of +2 °C for **K**, +0.5 °C for **L** and a neutral 0 °C for monomer **M** by a 10 mM Mg^{2+} concentration. The stabilisation of the DNA : RNA TWJ found with the double-headed nucleoside monomer **K** is hereby more pronounced than earlier found for any other of the double-headed nucleosides studied in this sequence, including the other 2′-modified monomer **1**.[5]

Discussion

The preparation of four new double-headed nucleosides by a CuAAC reaction demonstrated the convenience of this method for introducing new functionalities, herein additional nucleobases, into nucleosides. Especially, the use of the 2′-azido-2′-deoxyuridine is convenient as the double-headed nucleosides were obtained in high yields in a small number of steps. Thus, the phosphoramidite **9** is obtained in 25% overall yield in only 5 linear steps from uridine. Also the 5′-modified double-headed nucleoside phosphoramidite **15** was conveniently obtained through the azide **12**, which itself needed 7 synthetic steps from thymidine.[7,32] The LNA-azide derivative **16** was more difficult to obtain using 15 linear steps,[27] but in all cases, the CuAAC reaction was very efficient in introducing the additional nucleobase.

A consequence of using the CuAAC method over other ways of introducing the additional nucleobases is, of course, the 1,4-disubstituted 1,2,3-triazole moiety, which should also be accomodated in the nucleic acid secondary structures. Our recent studies on unsubstituted 5′(*S*)-*C*-triazolemethyl-thymidines have shown relatively low decreases in duplex stability (ΔT_ms between –1 °C and –5.8 °C in sequences as found in Table 1),[7] indicating that the triazole itself is reasonably well accommodated in the 5′-position. Therefore, the overall decreases in duplex stability induced by monomer **M** is due to the entire substituent, probably by disturbance of hydration of the duplex, and not specifically due to the triazole. On the other hand, it seems obvious that a 2′-*C*-triazole substituent is having a much more detrimental effect on duplex stability due to its position deep in the minor groove. This is evident from the loss in thermal stability of 5–11 °C with **K** and **L** incorporated in DNA duplexes. On the other hand, the double-headed nucleoside **1** also gave a drop in T_m of 4 °C for the DNA duplex, when incorporated into the **X6** sequence,[5] indicating a smaller negative contribution from the triazole itself, and in the corresponding DNA : RNA duplex, monomer **1** gave a ΔT_m of –1 °C,[5] which is the same as obtained herein with **K** and **L**. Hence the triazole as well as the additional nucleobase is much better accomodated in a DNA : RNA duplex. Concerning the behaviour of the LNA double-headed nucleoside monomer **N**, increases in DNA duplex stabiliy due to the conformationally restricted LNA-monomer[30,31] was found as expected, and apparently, the substituent is well-accommodated in the minor groove.

Concerning the study of zipper contacts in the minor groove, that is the (–1) to (–4)-zippers of Table 1 and 2, no strong and specific contacts were detected for any of the monomers **K**–**N**. Thus, the monomer **3** (B = T)[6] is still the only of our 5′-functionalized nucleoside analogues that is able to form a strong and very specific (–3)-zipper contact as indicated by large compensations of the drop in T_m otherwise induced by the modifications with $\Delta\Delta T_m$s of 6–7 °C. On the other hand, the (+1)-zipper contacts obtained with **K** and **L** are very interesting indicating a large compensation for the decrease in duplex stability otherwise obtained with single modifications. Hence, the overall drop in T_m of duplexes with (+1)-zippers of **L** and **K** or 2x**L** is only around 5 °C. From simple modelling, this effect must be due to an intercalation of the substituents into the duplex core and eventually an extension of the duplex. Apparantly, this extension gives room for the 2′-connected triazoles in the minor groove. The zipper contact is, on the other hand apparently not a hydrogen-bonding interaction as two adenines form a contact that is equally strong as a cross-zipper with an adenine and a thymine. The effect, therefore, seems to be due to stacking alone. This result shows a short way to an extended nucleic acid duplex structure with a normal back-bone made from simple 2′-modifications. This can be important in future nanoscale design of artificial nucleic acid structures.

For the stabilisation of a TWJ, it is clear, that the 2′-modified monomers **K** and **L** demonstrate a significantly larger effect than the 5′-modified monomer **M**. This is in line with the positioning of the 2′-substituents pointing towards the core of the complexes and in line with our earlier results demonstrating stabilisations of a TWJ with **1** but not with **3** (B = T or T-CH_2).[5,6,12] Most interestingly, and in contrary to **1**,[5] the stabilisaton of a TWJ is most pronounced when the target is an RNA-sequence. This gives the method and the monomers **K** and **L** a therapeutic potential following antisense strategies[33] with oligonucleotides containing these double-headed nucleosides targeting RNA with a preference for secondary structures. Finally, it is also interesting that the double-headed nucleoside with an adenine, **L**, in contrary to **K**, is able to stabilise an A-bulged duplex. This indicates that the additional adenine is stacking in the complex.

Conclusion

The CuAAC is a very convenient method for introducing additional nucleobases into nucleosides and oligonucleotides. The triazole groups are not hampering the duplex structures significantly, and the additional nucleobases have been found to increase the affinity of the oligonucleotides for secondary nucleic acid structures like for instance an RNA-hairpin structure. Furthermore, an extended duplex with additional nucleobases stacking has been found. Hereby, the use of double-headed nucleosides can be an important tool in the construction of artificial nucleic acid structures.

Experimental section

All commercial reagents were used as supplied. Reactions were carried out under argon or nitrogen when anhydrous solvents

were used. Column chromatography was performed with Silica gel 60 (particle size 0.040–0.063 μm, Merck). NMR spectra were recorded on a Varian Gemini 2000 spectrometer or a Bruker Advance III 400 spectrometer. Values for δ are in ppm relative to tetramethylsilane as an internal standard or 85% H_3PO_4 as an external standard. Assignments of NMR-signals are based on 2D spectra and follow standard nucleoside convention. ESI mass spectra were performed on an Thermo Finnigan TSQ 700 spectrometer.

Preparation of 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-(4-(thymin-1-ylmethyl)-1,2,3-triazole-1-yl)uridine (8). A solution of nucleoside **7**[23] (191 mg, 0.33 mmol), *N*1-propargylthymine **5**[24] (75 mg, 0.46 mmol), sodium ascorbate (42 mg, 0.21 mmol) and $CuSO_4 \cdot 5H_2O$ (17 mg, 0.07 mmol) in a mixture of *t*-BuOH, H_2O and pyridine (5 : 5 : 1 v/v, 5.5 mL) was stirred at room temperature for 19 h and a color change from yellow to green was observed. CH_2Cl_2 (15 mL) was added and the resulting mixture was washed with a saturated aqueous solution of $NaHCO_3$ (10 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash column chromatography (0–10% MeOH and 1% Et_3N in CH_2Cl_2) to afford the product **8** (176 mg, 72%) as a white solid; R_f 0.36 (10% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.50–10.35 (br m, 2H, NH), 8.31 (s, 1H, H-5(triazole)), 7.84 (d, 1H, J = 8.2 Hz, H-6(U)), 7.42 – 7.17 (m, 10H, Ar, H-6(T)), 6.83 (d, 4H, J = 8.6 Hz, Ar), 6.57 (d, 1H, J = 6.1 Hz, H-1′), 5.46 (t, 1H, J = 5.8 Hz, H-2′), 5.34 (d, 1H, J = 8.1 Hz, H-5(U)), 5.00–4.75 (m, 2H, CH_2N), 4.79 (t, 1H, J = 4.6 Hz, H-3′), 4.46 (m, 1H, H-4′), 3.76 (s, 6H, 2 × OCH_3), 3.53 (s, 2H, H-5′), 1.79 (s, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.1, 163.6 (C-4(U), C-4(T)), 158.9 (Ar), 151.6, 151.0 (C-2(U), C-2(T)), 144.3 (Ar), 142.0 (C-4(triazole)), 141.0 (C-6(T)), 139.6 (C-6(U)), 135.3, 135.1, 130.3, 128.3, 128.2, 127.4 (Ar), 126.4 (C-5(triazole)), 113.5 (Ar), 111.1 (C-5(T)), 103.3 (C-5(U)), 87.5 (CAr_3), 86.1 (C-1′), 85.3 (C-4′), 71.2 (C-3′), 66.5 (C-2′), 63.0 (C-5′), 55.4 (OCH_3), 43.5 (CH_2N), 12.2 (CH_3(T)); HRMS-ESI m/z 758.2565 $[MNa]^+$: calcd ($C_{38}H_{37}N_7O_9Na^+$) 758.2545.

Preparation of 3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphinyl)-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-(4-(thymin-1-ylmethyl)-1,2,3-triazole-1-yl)uridine (9). Nucleoside **8** (144 mg, 0.20 mmol) was coevaporated with anhydrous DCE (3 × 4 mL) and dissolved in the same solvent (2.0 mL). *N*,*N*-diisopropylethylamine (0.40 mL, 2.30 mmol) and *N*,*N*-diisopropylamino-2-cyanoethylphosphinochloridite (0.09 mL, 0.40 mmol) were added and the reaction mixture was stirred at room temperature for 2 h. Ethanol (1.0 mL) and CH_2Cl_2 (15 mL) were added and the resulting solution was washed with a saturated aqueous solution of $NaHCO_3$ (10 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 8 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0 – 5% MeOH and 0.5% pyridine in CH_2Cl_2) to afford the product **9** (129 mg, 70%) as a white foam; R_f 0.54 (10% MeOH in CH_2Cl_2); ^{31}P NMR (162 MHz, $CDCl_3$) δ 151.9, 149.4 (Ratio 5 : 3); HRMS-ESI m/z 958.3623 $[MNa]^+$: calcd ($C_{47}H_{54}N_9O_{10}PNa^+$) 958.3624.

Preparation of 2′-(4-(*N*6-benzoyladenine-9-ylmethyl)-1,2,3-triazole-1-yl)-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine (10). A solution of *N*6-benzoyl-*N*9-propargyladenine **6**[24] (120 mg, 0.43 mmol) in a mixture of THF, H_2O and pyridine (6 : 3 : 1 v/v, 6.0 mL) was stirred at room temperature. A solution of sodium ascorbate (43 mg, 0.22 mmol) and $CuSO_4 \cdot 5H_2O$ (15 mg, 0.06 mmol) in H_2O (0.25 mL) was added to the reaction mixture. Nucleoside **7**[23] (206 mg, 0.36 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. CH_2Cl_2 (15 mL) was added and the resulting mixture was washed with a saturated aqueous solution of $NaHCO_3$ (15 mL), brine (15 mL), H_2O (15 mL) and aqueous EDTA [a suspension of EDTA (208 mg, 0.71 mmol) in H_2O (16 mL) neutralized with 25% aqueous NH_3 (0.35 mL)]. The combined aqueous phases were extracted with CH_2Cl_2 (2 × 10 mL) and the combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0 – 6% MeOH and 0.5% pyridine in CH_2Cl_2) to afford the product **10** (285 mg, 93%) as a white foam; R_f 0.34 (10% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (s, 1H, NH(U)), 11.18 (s, 1H, NH(A)), 8.76 (s, 1H, H-2(A)), 8.55 (s, 1H, H-8(A)), 8.24 (s, 1H, H-5(triazole)), 8.05 (d, 2H, J = 7.4 Hz, Bz), 7.81 (d, 1H, J = 8.1 Hz, H-6(U)), 7.64 (t, 1H, J = 7.3 Hz, Bz), 7.55 (t, 2H, J = 7.6 Hz, Bz), 7.44 – 7.21 (m, 9H, Ar), 6.93 – 6.88 (m, 4H, Ar), 6.41 (d, 1H, J = 4.5 Hz, H-1′), 5.79 (d, 1H, J = 5.7 Hz, 3′-OH), 5.63 (s, 2H, CH_2N), 5.52 (dd, 1H, J = 4.7, 6.7 Hz, H-2′), 5.46 (dd, 1H, J = 1.9, 8.1, H-5(U)), 4.52 (q, 1H, J = 6.5 Hz, H-3′), 4.22 (m, 1H, H-4′), 3.74 (s, 6H, 2 × OCH_3), 3.38 – 3.26 (m, 2H, H-5′); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.6 (C═O(Bz)), 162.9 (C-4(U)), 158.1 (Ar), 152.2, 151.6, 150.1 (C-2(A), C-4(A), C-6(A)), 150.2 (C-2(U)), 144.7 (Ar), 144.5 (C-8(A)), 141.7 (C-4(triazole)), 140.7 (C-6(U)), 135.4, 135.2 (Ar), 133.4, 132.4 (Bz), 129.8 (Ar), 128.4 (Bz), 127.9, 127.7, 126.8 (Ar), 125.2 (C-5(A), C-5(triazole)), 113.3 (Ar), 102.0 (C-5(U)), 87.3 (C-1′), 85.9 (CAr_3), 83.3 (C-4′), 68.9 (C-3′), 64.7 (C-2′), 62.9 (C-5′), 55.0 (OCH_3), 38.2 (CH_2N); HRMS-ESI m/z 849.3093 $[MH]^+$: calcd ($C_{45}H_{40}N_{10}O_8H^+$) 849.3103.

Preparation of 2′-(4-(*N*6-benzoyladenine-9-ylmethyl)-1,2,3-triazole-1-yl)-3′-*O*-(*P*-2-cyanoethyl-*N*,*N*-diisopropylaminophosphinyl)-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl) uridine (11). Nucleoside **10** (255 mg, 0.30 mmol) was coevaporated with anhydrous CH_2Cl_2 (2 × 8 mL) and dissolved in the same solvent (4.0 mL). *N*,*N*-diisopropylethylamine (0.52 mL, 2.99 mmol) and *N*,*N*-diisopropylamino-2-cyanoethylphosphinochloridite (0.10 mL, 0.45 mmol) were added and the reaction mixture was stirred at room temperature for 4 h. Ethanol (1.5 mL) and CH_2Cl_2 (15 mL) were added and the resulting solution was washed with a saturated aqueous solution of $NaHCO_3$ (10 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 8 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0–5% MeOH and 0.5% pyridine in CH_2Cl_2) to afford the product **11** (195 mg, 62%) as a white foam; R_f 0.47 (10% MeOH in CH_2Cl_2); ^{31}P NMR (162 MHz, DMSO-d_6) δ 149.9, 148.2 (Ratio 3 : 4); HRMS-ESI m/z 1049.4174 $[MH]^+$: calcd ($C_{54}H_{57}N_{12}O_9PH^+$) 1049.4182.

Preparation of 3′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-pixyl-5′(*S*)-*C*-(4-(thymin-1-ylmethyl)-1,2,3-triazol-1-yl)methylthymidine (13). To a stirred solution of compound **12**[7] (500 mg, 0.75 mmol)

and *N*1-propargylthymine **5**[24] (164 mg, 1.00 mmol) in a mixture of *t*-BuOH, H_2O and pyridine (5 : 5 : 2 v/v, 12 mL) was added sodium ascorbate (37 mg, 0.19 mmol) and $CuSO_4 \cdot 5H_2O$ (25 mg, 0.10 mmol) and the mixture was stirred at room temperature for 18 h. A second portion of *N*1-propargylthymine (164 mg, 1.00 mmol), sodium ascorbate (37 mg, 0.19 mmol) and $CuSO_4 \cdot 5H_2O$ (25 mg, 0.10 mmol) was added and the mixture was stirred for another 3 h. Ethyl acetate (30 mL) was added and the mixture was washed with water (30 mL). The aqueous phase was extracted with ethyl acetate (3 × 30 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (33–100% ethyl acetate and 0.2% pyridine in petrolium ether) affording the product **13** (500 mg, 80%) as a colorless foam; R_f 0.26 (ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.60–9.30 (br s, 2H, NH), 7.72 (s, 1H, H-6), 7.48–7.30 (m, 10H, H-5(triazole), Ar), 7.26 (s, 1H, H-6), 7.13–6.98 (m, 4H, Ar), 6.23 (dd, 1H, J = 4.8, 9.6 Hz, H-1′), 4.95, 4.73 (AB, 2H, J = 15.0 Hz, CH_2N), 4.15 (m, 1H, H-6′), 3.74 (m, 1H, H-5′), 3.52 (dd, 1H, J = 3.9, 13.5 Hz, H-6′) 3.25 (d, 1H, J = 4.5 Hz, H-3′), 3.19 (br s, 1H, H-4′), 2.06 (s, 3H, CH_3), 2.04–1.75 (m, 5H, H-2′, CH_3), 0.66 (s, 9H, $C(CH_3)_3$), –0.22 (s, 3H, $Si(CH_3)_2$), –0.36 (s, 3H, $Si(CH_3)_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.3, 164.1 (C-4), 152.2, 152.0 (Ar), 150.9, 150.4 (C-2), 146.1, 141.9, 140.1, 135.5 (C-6, C-4(triazole), Ar), 131.3, 130.9, 130.8, 130.6, 128.1, 127.7, 127.6, 124.8, 124.1, 124.0, 122.9, 122.3, 117.6, 117.2 (Ar, C-5(triazole)), 111.3, 111.2 (C-5), 86.4 (C-4′), 84.8 (C-1′), 78.8 (CAr_3), 73.3 (C-3′), 71.9 (C-5′), 50.1 (C-6′), 42.7 (CH_2N), 40.9 (C-2′), 25.6 ($C(CH_3)_3$), 17.7 ($C(CH_3)_3$), 12.9, 12.4 (CH_3), –4.7, –4.8 ($Si(CH_3)_2$); HRMS-ESI m/z 854.3324 $[MNa]^+$: calcd ($C_{44}H_{49}N_7O_8SiNa^+$) 854.3304.

Preparation of 5′-*O*-pixyl-5′(*S*)-*C*-(4-(thymin-1-ylmethyl)-1,2,3-triazol-1-yl)methylthymidine (14). A solution of compound **13** (450 mg, 0.54 mmol) dissolved in anhydrous THF (6 mL) was added a 1.0 M solution of TBAF in THF (1 mL, 1.00 mmol) and stirred at room temperature for 20 h. The mixture was poured into ethyl acetate (40 mL) and the mixture was washed with a 10% aqueous solution of $NaHCO_3$ (40 mL). The aqueous phase was extracted with ethyl acetate (3 × 4 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (10% MeOH and 0.2% pyridine in CH_2Cl_2) to afford the product **14** (353 mg, 91%) as a colorless foam; R_f 0.32 (10% MeOH in CH_2Cl_2); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.37 (s, H, NH), 11.30 (s, H, NH), 7.76–7.27 (m, 16H, H-6, H-5(triazole), Ar), 7.11–6.88 (m, 4H, Ar), 5.93 (t, 1H, J = 7.2 Hz, H-1′), 5.00 (d, 1H, J = 4.5 Hz, OH-3′), 4.83 (s, 2H, CH_2N), 4.25 (dd, 1H, J = 9.3, 15.3 Hz, H-6′), 3.77–3.69 (m, 2H, H-5′, H-6′), 3.49 (m, 1H, H-3′), 3.17 (1H, t, J = 2.7 Hz, H-4′), 1.93–1.86 (m, 5H, H-2′, CH_3), 1.73 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.2, 163.7 (C-4), 151.0, 150.9, 150.6, 150.2, 147.2, 142.3, 140.9, 135.4 (Ar, C-2, C-4(triazole), C-6), 130.9, 130.8, 130.3, 130.1, 127.8, 127.0, 124.2, 123.7, 123.6, 122.5, 122.0, 116.6, 116.2 (Ar, C-5(triazole)), 109.6, 108.8 (C-5), 85.0 (C-1′), 83.6 (C-4′), 77.1 (CAr_3), 71.9 (C-5′), 70.0 (C-3′), 49.6 (C-6′), 42.0 (CH_2N), 39.7 (C-2′), 12.3, 11.9 (CH_3); HRMS-ESI m/z 740.2437 $[MNa]^+$: calcd ($C_{38}H_{35}N_7O_8Na^+$) 740.2439.

Preparation of 3′-*O*-(*P*-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphinyl)-5′-*O*-pixyl-5′(*S*)-*C*-(4-(thymin-1-ylmethyl)-1,2,3-triazol-1-yl)methylthymidine (15). To a stirred solution of nucleoside **14** (300 mg, 0.42 mmol) in anhydrous CH_2Cl_2 (5 mL) was added *N*,*N*-diisopropylethylamine (350 μL, 2.00 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (300 μL, 1.35 mmol) and the mixture was stirred at room temperature for 3 h. CH_2Cl_2 (30 ml) was added and the mixture was washed with a saturated aqueous solution of $NaHCO_3$ (30 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 30 mL) and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (0-25% acetone and 0.2% pyridine in CH_2Cl_2) to afford the product **15** (345 mg, 90%) as a colorless foam; R_f 0.28 (25% acetone in CH_2Cl_2); ^{31}P NMR (75 MHz, $CDCl_3$) δ 152.07, 151.13; HRMS-ESI m/z 940.3541 $[MNa]^+$: calcd ($C_{47}H_{52}N_9O_9PNa^+$) 940.3518.

Preparation of (1*S*,3*R*,4*R*,6*R*,7*S*)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-3-(thymin-1-yl)-6-(4-(thymin-1-ylmethyl)-1,2,3-triazol-1-yl)methyl-2,5-dioxabicyclo[2.2.1]heptane (18). To a stirred solution of nucleoside **17**[27] (100 mg, 0.2 mmol) in a mixture of anhydrous CH_3CN and pyridine (1 : 1 v/v, 4 mL), was added 4,4′-dimethoxytrityl chloride (104 mg, 0.31 mmol). The mixture was stirred at room temperature for 16 h and then quenched by the addition of methanol (1 mL). The mixture was concentrated under reduced pressure, and the residue was dissolved in CH_2Cl_2 (40 mL). The organic phase was washed with water (8 mL) and a saturated aqueous solution of $NaHCO_3$ (8 mL). The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (3–5% EtOH in chloroform) to afford the product **18** (97 mg, 60%) as a white solid; R_f 0.39 (10% CH_3OH in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.30–10.10 (br m, 2H, NH), 8.00 (s, 1H, H-5(triazole)), 7.60 (s, 1H, H-6), 7.42–7.17 (m, 10H, Ar, H-6), 6.85–6.77 (m, 4H, Ar), 5.73 (s, 1H, H-1′), 4.82–4.47 (m, 7H, H-2′, H-3′, H-6′, 2 × CH_2N), 3.74 (s, 6H, 2 × OCH_3), 3.55 (AB, 2H, J = 8.4, 10.2 Hz, H-5′), 2.15 (br s, 1H, OH), 1.75 (s, 3H, CH_3), 1.59 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.8, 164.5 (C-4), 158.8 (Ar), 151.4, 150.5 (C-2), 144.4, 142.2, 140.6 (C-6, C-4(triazole)), 135.3, 135.2, 134.6, 130.2, 128.2, 127.3 (Ar), 125.5 (C-5(triazole)), 113.5 (Ar), 111.2, 110.8 (C-5), 88.2, 87.2, 86.8, (CAr_3, C-1′, C-4′), 79.8 (C-6′), 79.1 (C-3′), 72.1 (C-2′), 58.4 (C-5′), 55.4, 55.3 (OCH_3), 51.0 (CH_2N), 42.8 (CH_2N), 12.6, 12.2 (CH_3); HRMS-ESI m/z 814.2261 $[MNa]^+$: calcd ($C_{42}H_{43}N_7O_{10}Na^+$) 814.2807.

Preparation of (1*S*,3*R*,4*R*,6*R*,7*S*)-7-(*P*-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphinyl)oxy-1-(4,4′-dimethoxytrityl)-oxymethyl-3-(thymin-1-yl)-6-(4-(thymin-1-ylmethyl)-1,2,3-triazol-1-yl)methyl-2,5-dioxabicyclo[2.2.1]heptane (19). To a stirred solution of nucleoside **18** (58 mg, 0.073 mmol) in anhydrous CH_2Cl_2 (1.0 mL) was added *N*,*N*-diisopropylethylamine (55 μL, 0.31 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (49 μL, 0.22 mmol), and the mixture was stirred at room temperature for 1$\frac{1}{2}$ h. The mixture was diluted with CH_2Cl_2 (30 mL) and washed with a saturated aqueous solution of $NaHCO_3$ (5 mL), water (5 mL) and brine (5 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (30–50% acetone and 0.5% pyridine in CH_2Cl_2) to give the product **19** (30 mg, 41%) as a white foam; R_f 0.68 (10% CH_3OH in CH_2Cl_2); ^{31}P NMR ($CDCl_3$, 300 MHz)

δ 151.08, 150.96; HRMS-ESI *m/z* 1014.3904 $[MNa]^+$: calcd $(C_{51}H_{60}N_9O_{11}Na)^+$ 1014.3886.

Synthesis of oligodeoxynucleotides

Oligonucleotide synthesis was carried out on an automated DNA synthesizer following the phosphoramidite approach. Synthesis of oligonucleotides was performed on a 0.2 μmol scale by using the phosphoramidites **9**, **11**, **15** and **19** as well as the corresponding commercial 2-cyanoethyl phosphoramidites of the natural 2′-deoxynucleosides. The synthesis followed the regular protocol for the DNA synthesizer. For modified phosphoramidites, however, a prolonged coupling time of 15 or 20 min was used. 1*H*-Tetrazole was used as the activator and coupling yields for 2-cyanoethyl phosphoramidites were >98% for **11**, >90% for **9**, >85% for **15** and >60% for **19**. The 5′-*O*-DMT-ON oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at 55 °C for 16 h, which also removed the protecting groups. The oligonucleotides were purified by reversed-phase HPLC on a Waters 600 system using a X_{terra} prep MS C_{18}; 10 μm; 7.8 × 150 mm column; Buffer A: 0.05 M Triethyl Ammonium Acetate pH 7.4. Buffer B: MeCN–H_2O (1 : 1). Program used: 2 min 100% A, 100–30% A over 38 min, 10 min 100% B, 10 min 100% A. All oligonucleotides were detritylated by treatment with an 80% aqueous solution of acetic acid for 20 min, quenched with a aqueous solution of sodium acetate (3 M, 15 μL) and then added sodium perchlorate (5 M, 15 μL) followed by acetone (1 mL). The resulting mixture was precipitated over night at −20 °C. After centrifugation 12000 rpm, 10 min at 4 °C, the supernatant was removed and the pellet washed with cold acetone (2 × 1 mL) and dried for 30 min under reduced pressure, and dissolved in pure water (500 or 1000 μL). The concentration was determined by UV at 260 nm, and the purity confirmed by IC analysis. MALDI-TOF-MS $[M - H]^-$ confirmed the constitution (see ESI†).

Thermal denaturation experiments

Extinction coefficients of the modified oligonucleotides were estimated from a standard method counting the additional nucleobases as additional thymidine/adenosine. UV melting experiments were thereafter carried out on a UV spectrometer. Samples were dissolved in a medium salt buffer containing 2.5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 100 mM NaCl, and 0.1 mM EDTA at pH = 7.0 with 1.0 μM concentrations of the two complementary sequences. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 5 or 10 to 75 or 80 °C at a rate of 0.5 or 1.0 °C min^{-1} by means of a Peltier temperature programmer. The melting temperature was determined as the local maximum of the first derivatives of the absorbance *vs.* temperature curve. The melting curves were found to be reversible. All determinations were averages of at least duplicates within ± 0.5 °C.

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