pH-Independent triplex formation: hairpin DNA containing isoguanine or 9-deaza-9-propynylguanine in place of protonated cytosine

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Triplex-forming oligonucleotides (TFOs) containing 2′-deoxyisoguanosine (**1**), 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) as well as the propynylated 9-deazaguanine N^7-(2′-deoxyribonucleoside) **3b** were prepared. For this the phosphoramidites **9a**, **b** of the nucleoside **1** and, the phosphoramidites **19**, **20** of compound **3b** were synthesized. They were employed in solid-phase oligonucleotide synthesis to yield the protected 31-mer oligonucleotides. The deblocking of the allyl-protected oligonucleotides containing **1** was carried out by Pd(0)[PPh_3]$_4$–PPh_3 followed by 25% aq. NH_3. Formation of the 31-mer single-stranded intramolecular triplexes was studied by UV-melting curve analysis. In the single-stranded 31-mer oligonucleotides the protonated dC in the dCH$^+$–dG–dC base triad was replaced by 2′-deoxyisoguanosine (**1**), 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) and, 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleoside) (**3b**). The replacement of protonated dC by compounds **1** and **3b** resulted in intramolecular triplexes which are formed pH-independently and are stable under neutral conditions. These triplexes contain "purine" nucleosides in the third pyrimidine rich strand of the oligonucleotide hairpin.

Introduction

Sequence-specific binding of triplex-forming oligonucleotides (TFOs) with duplex DNA can selectively block gene expression ("antigene strategy").[1–3] Therefore, TFOs are gaining interest for therapeutic applications.[4] In the case of the pyrimidine–purine–pyrimidine motif, triplex formation takes place by the binding of a third pyrimidine strand in the major groove of duplex DNA in a parallel orientation with respect to the purine strand. The recognition of the third strand occurs by the formation of Hoogsteen pairing between dT with the dA–dT pair (dT–dA–dT base triad) and/or of protonated dC with the dG–dC pair (dCH$^+$–dG–dC base triad).[1,2] In the latter the necessary protonation of cytosine limits the use of this method performed at neutral conditions.

A number of modified nucleosides have been synthesized and incorporated into the TFO's to improve the stability of triplexes under physiological conditions. The replacement of cytosine by 5-methylcytosine increases the stability of triplexes but does not alleviate the pH dependence.[5–8] As the pK_a-value of 5-methyl-2′-deoxycytidine (pK_a = 4.5) is similar to that of dC (pK = 4.3) this change cannot affect the protonation significantly. It was already discussed that the higher stability is caused by additional stacking interaction of the hydrophobic methyl group.[6–8] Oligonucleotides containing base modified nucleosides have been intensively investigated for the same purpose.[5,9–12] A number of N^7-glycosylated purine nucleosides (purine numbering is used throughout the results and discussion section), acyclic derivatives or peptide nucleic acid-analogues (PNA) have been also studied.[13–16] Recently, we have reported on the synthesis and triplex formation properties of the N^7-glycosylated 9-deazaguanine 2′-deoxyribonucleoside **3a**[17] which represents a glycosylic bond stable analogue of the N^7-glycosylated guanine nucleoside **4**.[13,18] It was shown that nucleoside **3a** and its 9-halogenated derivatives form stable DNA triplexes when three individual strands are hybridized under neutral conditions.[19]

2′-Deoxyisoguanosine (**1**) and 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) contain the same recognition pattern as the protonated 2′-deoxycytidine or the N^7-gylcosylated 9-deazapurine nucleoside **3a** (Scheme 1). Compounds **1** and **2** have been used as inducer of parallel-stranded (*ps*) DNA.[20,21] In antiparallel-stranded (*aps*) DNA, compounds **1** and **2** form a stable Watson–Crick base pair with 2′-deoxy-5-methylisocytidine (iC_d), which can be incorporated in duplex DNA by polymerases.[21,22] 7-Bromo substituted 7-deaza-2′-deoxyisoguanosine (**2**) exists almost entirely in the keto form in aqueous solution[21] causing a better mismatch discrimination than compound **1** which presents partially the enol form.[23,24] Also, 2′-deoxyisoguanosine (**1**) is known for the formation of DNA quartets and pentameric assemblies.[25]

This manuscript reports on the hairpin triplexes incorporating 2′-deoxyisoguanosine (**1**) or the halogenated 7-deazapurine derivative **2** as well as the N^7-glycosylated 9-deazapurine nucleoside **3b**. According to the presence of a H-atom at the nitrogen-3 it is expected that in all three cases triplexes are already formed under neutral conditions. This is the first report on isoguanine nucleosides (**1** or **2**) to act as the parallel triplex inducers. It was also anticipated that a propynyl group, as in **3b**, could have the same favorable effect on the triplex stability as it has been reported for the duplex formation.[26]

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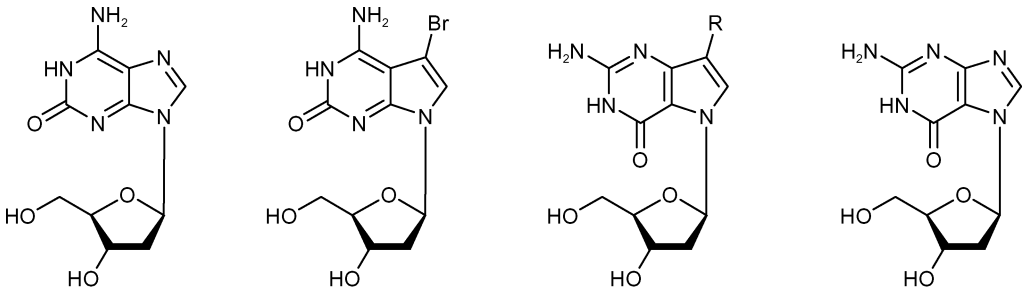

1 **2** **3a** R = H, **b** R = C≡CCH$_3$ **4**

Scheme 1 Structures of 2′-deoxyribonucleosides incorporated into the hairpin triplex.

Results and discussion

Monomers

1. Synthesis of the *O*-allyl-protected 2′-deoxyisoguanosine phosphoramidites 9a, b. The 2-oxo-function of 2′-deoxyisoguanosine (**1**) is more reactive as compared to the 6-oxo-group of 2′-deoxyguanosine.[20] Earlier, the diphenylcarbamoyl (DPC) residue was used for the protection of the 2-oxo-functionality of **1**[20d], while the *N*,*N*-dimethylacetamidine residue was used for the protection of the 6-amino group. As an alternative the allyl residue is now chosen to protect the oxo-function. The allyl residue was selected as it was used to protect the 2-oxo group of 2′-deoxyguanosine,[27] 2′-deoxyxanthosine[28] and the 4-oxo-function of 2′-deoxythymidine[27] as well as for the protection of hexopyranosyl nucleosides.[29] As this group is sensitive to palladium(0) complexes in the presence of nucleophiles and also stable in ammonia, it represents an orthogonal protecting group among the common oxo protecting groups.

For the 6-amino group protection of compound **1** the isobutyryl, benzoyl and *N*,*N*-di-*n*-butylformamidine residues were selected and were compared regarding their applicability in solid-phase synthesis. For that purpose, 2-chloro-2′-deoxyadenosine (**5**) was chosen as a starting material. Treatment of the nucleoside **5** with 1 M $NaOCH_2CH{=}CH_2$ in $HOCH_2CH{=}CH_2$ furnished the 2-allyloxy derivative **6** (79% yield). This compound was used for the evaluation of various amino-protected compounds (**7a–d**). At first the isobutyryl residue was used for the 6-amino protection using the protocol of transient protection.[30] Isobutyryl chloride in the presence of trimethylsilyl chloride furnished **7a**. The benzoylated derivative **7b** was prepared from **6** under the same conditions using benzoyl chloride. In this reaction the bis-benzoylated compound **7c** was isolated as a side product. Treatment of compound **6** with *N*,*N*-di-*n*-butylformamide dimethylacetal in MeOH afforded compound **7d** (see Scheme 2).

Next the stabilities of the 6-amino protecting groups of **7a**, **7b** and **7d** were determined in 25% aq. NH_3 solution at 40 °C. The reaction was followed UV-spectrophotometrically. The half-life values of **7a** ($t_{1/2}$ = 3 min), **7b** ($t_{1/2}$ = 46 min) and **7d** ($t_{1/2}$ = 10.5 min) indicate that the isobutyryl group is too labile for further manipulations. Thus, only compounds **7b**, **d** were converted into the 5′-*O*-dimethoxytrityl-derivatives **8a**, **b** under standard conditions. Treatment of **8a**, **b** with 2-cyanoethyl diispropylphosphoramidochloridite afforded the phosphoramidites **9a**, **b** respectively. All synthetic intermediates were characterized by ^{1}H, ^{13}C and ^{31}P-NMR spectra as well as by elemental analyses (see Table 1 and Experimental section).

Table 1 ^{13}C-NMR chemical shifts [ppm] of 2′-deoxyisoguanosines and 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleosides) measured in [d$_6$]DMSO at 23 °C

Compound	C(2)[a,d] C(2)[b]	C(6)[a,d] C(4)[b]	C(5)[a] C(4a)[b]	C(8)[a] C(6)[b]	C(9)[a] C(7)[b]	C(4)[a] C(7a)[b]	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)
6[e]	157.1	155.9	117.5	139.1	—	150.2	82.7	[c]	70.5	85.4	64.0
7a[e]	160.3	154.5	117.5	141.1	—	150.8	83.7	[c]	70.7	87.9	61.7
7b[e]	160.2	153.7	121.7	141.8	—	151.5	83.6	38.6	70.8	87.9	61.7
7c[e]	159.9	154.4	123.0	144.3	—	151.6	83.8	38.6	70.7	88.1	61.7
7d[e]	161.2	159.0	122.8	140.8	—	153.4	84.2	[c]	71.7	88.5	62.6
8a[e]	160.0	153.3	121.6	141.9	—	151.4	83.6	38.4	70.7	85.9	64.3
8b[e]	161.1	158.7	123.1	141.1	—	153.3	84.2	[c]	71.6	86.6	65.2
11	153.5	154.0	113.2	131.1	98.4	144.3	—	—	—	—	—
14	154.1	154.1	112.5	130.9	99.9	145.7	85.7	[c]	74.8	87.7	64.2
15	154.7	154.8	113.4	129.8	98.8	146.8	85.5	41.2	70.4	87.3	61.6
16	153.8	153.9	112.1	130.9	99.0	145.3	85.6	41.3	70.4	87.2	61.5
17	153.8	153.9	112.2	130.7	99.2	145.3	85.4	[c]	70.1	87.2	63.3
18	155.0	157.1	113.2	129.6	99.1	147.0	85.3	[c]	70.3	86.9	64.0
3b	151.4	154.0	111.1	129.5	99.9	148.5	85.6	41.2	70.4	87.3	61.6

[a] Purine numbering. [b] Systematic numbering. [c] Superimposed by [d$_6$]DMSO. [d] Tentative. [e] Only purine numbering is used.

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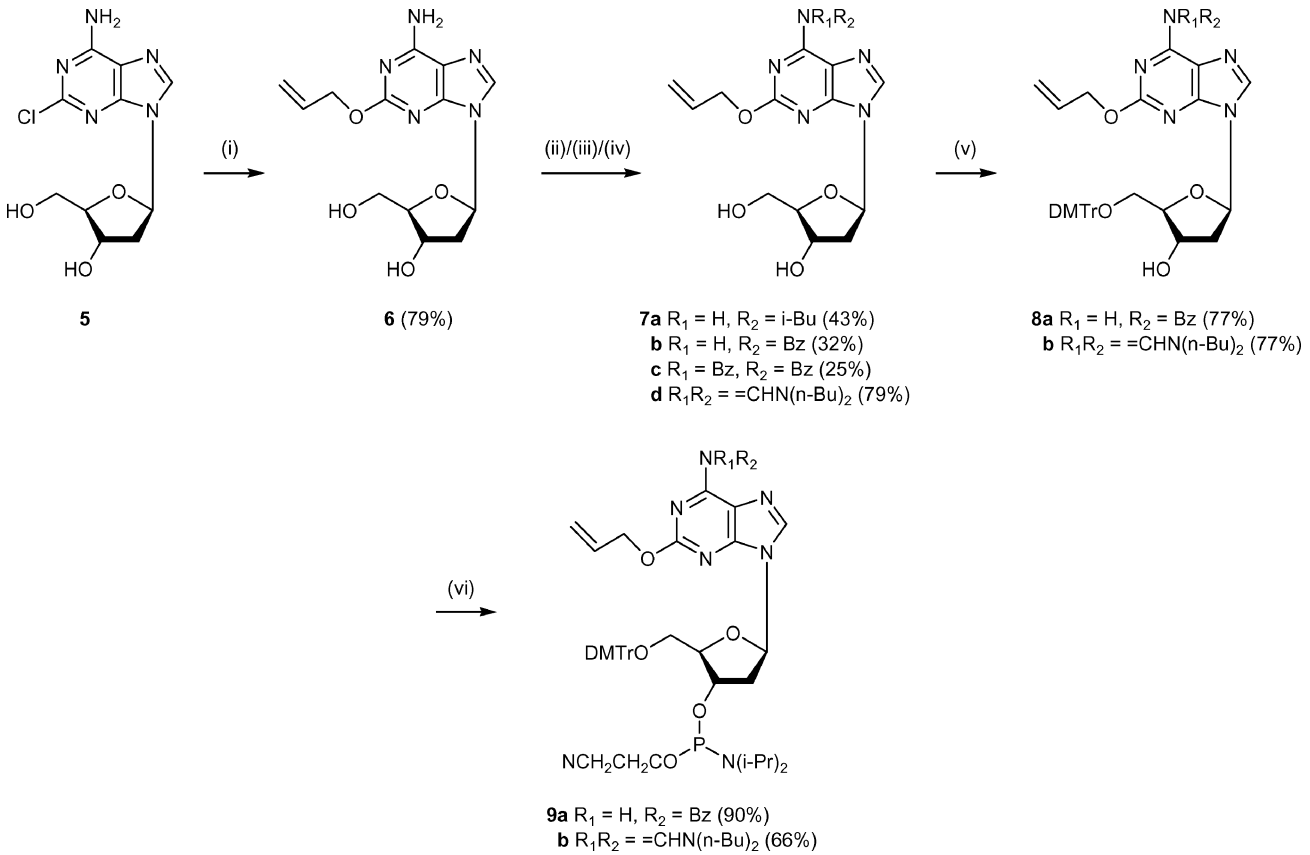
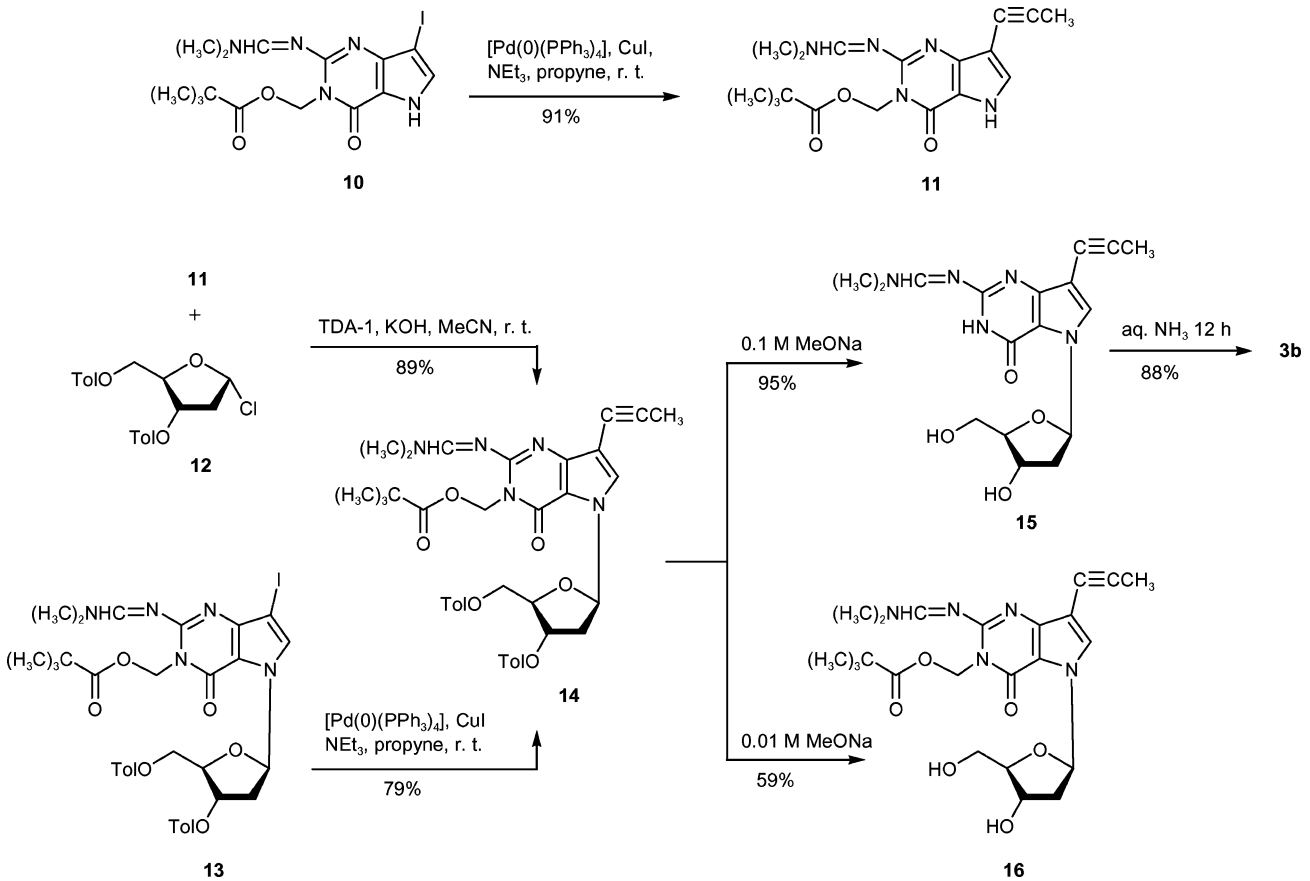


Scheme 2 Reagents and conditions: (i) 1 M $CH_2{=}CHCH_2ONa/CH_2{=}CHCH_2OH$, 60 °C; (ii) i-BuCl, Me_3SiCl, pyridine, r.t.; (iii) BzCl, Me_3SiCl, pyridine, r.t.; (iv) *N*,*N*-di-*n*-butylformamide dimethylacetal, MeOH, 40 °C; (v) DMTr-Cl, pyridine, r.t., 4 h; (vi) 2-cyanoethyl-diisopropylphosphoramido chloridite, $(i\text{-}Pr)_2NEt$, CH_2Cl_2, r.t., 30 min.

2. Synthesis of the 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleoside) 3b and conversion into the phosphoramidites 19 and 20. For the preparation of the phosphoramidite building blocks **19** and **20** of the 9-propynyl substituted 9-deazaguanine N^7-(2′-deoxyribonucleoside) **3b**, the fully protected 9-iodo-9-deazaguanine (**10**) (2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-7-iodo-3-[(pivaloyloxy)methyl]-pyrrolo[3,2-*d*]pyrimidin-4-one) was used as starting material.[17] Two different routes employing the palladium-catalyzed *Sonogashira* cross-coupling reaction[26] were used to incorporate the propynyl group into the nucleoside **3b**. (i) In the first route, the fully protected key intermediate **10** was treated with $[(PPh_3)_4Pd(0)]$–CuI in the presence of triethylamine in anhydrous DMF. The solution was saturated with propyne at room temperature which yielded compound **11** (91%). Then, the stereoselective nucleobase-anion glycosylation was employed on compound **11**. The reaction was performed with the protected nucleobase **11**, 2-deoxy-3,5-di-*O*-(*p*-totuoyl)-*α*-D-*erythro*-pentofuranosyl chloride (**12**) in MeCN with powdered KOH–TDA-1 to generate the nucleobase anion. The fully protected N^7-β-D-nucleoside **14** was obtained in a stereoselective way in 89% yield (see Scheme 3 and Experimental section). (ii) In the second route the cross-coupling reaction was performed on the fully protected nucleoside **13**.[17] A solution of **13** was treated with $[(PPh_3)_4Pd(0)]$–CuI–triethylamine in anhydrous DMF and saturated with propyne furnishing **14** in 79% yield.

The deprotection of compound **14** was carried out with 0.1 M NaOMe–MeOH at r.t. for 1 h resulting in the removal of the *p*-toluoyl groups of the sugar moiety as well as the pivaloyloxymethyl group of the nucleobase. The [(*N*,*N*-dimethylamino)methylidene]amino protecing group is stable under those conditions. Compound **15** was isolated in almost quantitative yield (95%). When the deprotection was performed on **14** with 0.01 M NaOMe–MeOH at r.t. for 45 min, only the *p*-toluoyl groups were removed furnishing fully nucleobase-protected compound **16** in good yield (59%). Both, the partially base protected nucleoside **15** as well as the fully base protected nucleoside **16**, were used for further manipulations. In order to test the suitability of the protecting groups, their hydrolysis was studied. The complete removal of all protecting groups of **15** was accomplished in aq. NH_3 (25%) in a sealed vessel at 60 °C for 12 h furnishing the nucleoside **3b** (88%) (see Scheme 3 and Experimental section). To assure the stability and applicability of the formamidine protecting group on the oligonucleotide level the half-life of the formamidine deprotection was determined in 25% aq. NH_3 at 40 °C UV-spectrophotometrically. The half-life of compound **15** was 25 min which is longer than that of the non-functionalized nucleoside **3a** (12 min). According to this, the 9-propynyl substituent increases the stability of the amino protecting group similar to the 9-halogen substituents.[19]

As the conformational freedom of the glycosylic bond could be restricted in compound **3b**, ^{1}H-NOE difference spectra were measured in [d_6]DMSO resulting in the NOE's on H–C(6) (η = 1.1%) and H_α–C(2′) (η = 5.4%) when irradiation of H–C(1′) was performed. Thus compound **3b** prefers the *high-syn*-conformation



Scheme 3 The route for the synthesis of 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleoside) **3b**.

at the *N*-glycosylic bond, which is similar to the 9-halogenated derivatives.[17] According to the calibration graph for the estimation of the *syn* and *anti* conformer populations of β-D-nucleosides,[31] compound **3b** gave 55% of an *anti*-rotamer population. The NOE on H–C(4′) (η = 1.5%) when irradiating H–C(1′) also confirms the β-D-configuration of nucleoside **3b**. The conformation of the sugar moiety of nucleoside **3b** was studied on the basis of vicinal [^{1}H,^{1}H] coupling constants, using the program PSEUROT (version 6.3).[32,33] According to the coupling constants J (1′,2′) = 6.71, J (1′,2″) = 6.46, J (2′,3′) = 6.57, J (2″,3′) = 4.18, and J (3′,4′) = 4.00 it is concluded that the 9-propynyl substituent of **3b** has a similar influence on the conformation as the 9-halogeno substituents. The $N \Leftrightarrow S$ equilibrium of the sugar conformation of **3b** is biased towards *N* conformation (63% *S*) compared to non-functionalized nucleoside **3a** (67% *S*).[17]

Next, the UV-spectrum of compound **3b** was measured in 0.1 M sodium phosphate buffer (pH 7.0). The 9-propynyl substituted nucleoside **3b** shows a bathochromic shift compared to the non-functionalized nucleoside with three distinct maxima at 235, 277 and 302 nm.[17] The pK_a values for nucleoside **3b** were also determined in the same buffer solution. The pK_a values for nucleoside **3b** are 4.0 and 10.1, while compound **3a** shows slightly higher pK_a values (4.9 and 10.3).[17]

In order to access oligonucleotides, intermediates **15** and **16** were converted into phosphoramidites. For this, they were treated with 4,4′-dimethoxytrityl chloride in anhydrous pyridine furnishing the 4,4′-dimethoxytrityl derivatives **17** and **18** respectively (Scheme 4). Subsequent treatment of **17** and **18** with 2-cyanoethyl diisopropylphosphoramido chloridite yielded the phosphoramidites **19** and **20**. All synthetic intermediates described above were characterized by ^{1}H, ^{13}C and ^{31}P-NMR spectra as well as by elemental analyses (see Table 1 and Experimental section). According to Table 1 the C-9 signals (purine numbering is used) of all propynyl substituted synthetic intermediates were shifted upfield compared to the unsubstituted nucleoside.[17]

Oligonucleotides

1. Synthesis and characterization. Oligonucleotide synthesis was carried out on solid phase with an ABI 392-08 synthesizer at a 1 μmol scale employing phosphoramidite chemistry (Applied Biosystems, Weiterstadt, Germany) using the phosphoramidites **9a**, **b** of 2′-deoxyisoguanosine (**1**) and the phosphoramidites **19** and **20** of 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleoside) **3b**. The syntheses of oligonucleotides **21–29** were performed employing the DMT-on mode. The deallylation of oligomers containing **1** was performed on solid support bound oligonucleotides using Pd(0)[PPh$_3$]$_4$ in the presence of PPh$_3$, HCOOH–*n*-butylamine (1 : 1) as scavenger in anhydrous THF[26] (for details see Experimental section). The excess of the reagent was removed with 5 M aq. *N*,*N*-diethyldithiocarbamate (DDTC). The solid support bound oligonucleotides obtained by the allyl-depotection were then deprotected in 25% aq. NH$_3$ at 60 °C. The DMT-protected oligomers were purified by reversed-phase HPLC. The

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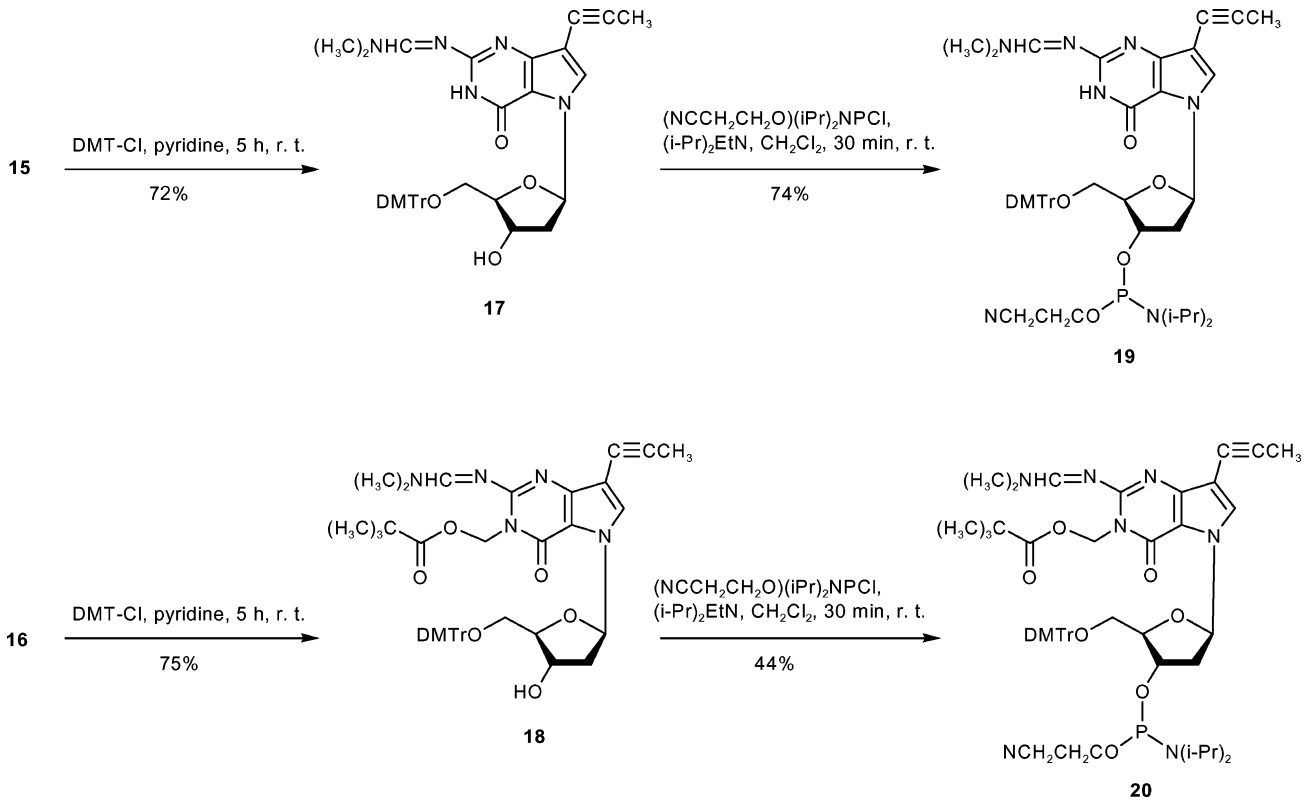


Scheme 4 Synthesis of the phosphoramidites **19** and **20** of 9-deaza-9-propynylguanine N^7-(2′-deoxyribonucleoside) **3b**.

DMT-residues were removed with 2.5% dichloroacetic acid in CH_2Cl_2 followed by RP-18 HPLC purification. The oligonucleotides **21–29** (Table 3) were characterized by MALDI-TOF mass spectrometry. 2′-Deoxyisoguanosine is sensitive in MALDI-TOF spectrometry. In the MALDI-TOF spectra of oligonucleotides **22** and **26** containing nucleoside **1**, in addition to the main peak, mass peaks with low intensity corresponding to depurination were also observed. Therefore, the composition of the oligonucleotides containing 2′-deoxyisoguanosine (**1**) was determined by enzymatic analysis (see Fig. 1 and Experimental section). Fig. 1a, b shows the HPLC profiles of the digest of oligomer **26** measured at 260 and 290 nm. This was necessary as compound **1** has its main absorbance at 292 nm. Oligomers containing nucleosides **2**, **3a** and **3b** were synthesized under the same conditions as nucleoside **1** using the corresponding phosphoramidites. MALDI-TOF spectra of these oligonucleotides show correct masses (see Table 2 and Experimental section).

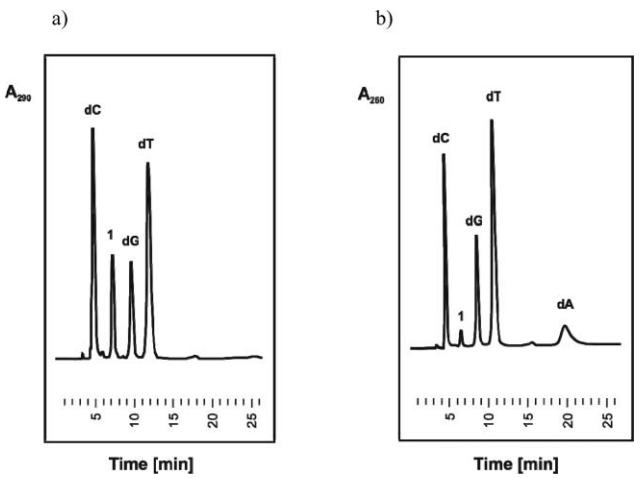


Fig. 1 Reversed-phase HPLC profiles of the hydrolysis products of the oligomer **26** containing 2′-deoxyisoguanosine (**1**) by snake-venom phosphodiesterase followed by alkaline phosphatase at 290 nm (a) as well as at 260 nm (b) (solvent gradient system [A: 0.1 M $(Et_3NH)OAc$ (pH 7.0)] with flow rate 0.7 ml min^{-1}).

2. pH-independent formation of hairpin triplexes. Up to now, we have studied triplex formation with systems containing three separate strands.[19] Now we are investigating triplex formation on the hairpin triplex **21** being related to a 31-mer hairpin.[10] Compared to a triplex formed by three individual oligonucleotides, such an intramolecular triplex is more stable. The oligonucleotide **21** forms a double hairpin with a triplex core (see Fig. 2). In the single-stranded oligomer **21** a triplex is formed *via* the hairpin duplex between 5′-$dG_1 \rightarrow dG_7$-3′ and 3′-$dC_{19} \rightarrow dC_{13}$-5′ with five dT residues in the loop. A third strand 5′-$dC_{25} \rightarrow dC_{31}$-3′ folds back and binds in a parallel orientation to 5′-$dG_1 \rightarrow dG_7$-3′ by Hoogsteen pairing with the second loop of another five dT residues (see Fig. 2).

At first we incorporated the 9-deazaguanine N^7-(2′-deoxyribonucleoside) **3a**[19] which resembles the recognition site of a protonated 2′-deoxycytidine in position **X** and in positions **X** and **Y** of the oligonucleotide **21**. As the propynyl group is a standard residue to stabilize DNA-duplexes[26] we reasoned that these favorable properties can also be seen on triplexes. The results

Table 2 Molecular masses (MH^+) of the oligonucleotides measured by MALDI-TOF mass spectrometry

Oligonucleotides	MH^+ (calc.)	MH^+ (found)
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**3a**TC C] (**24**)	9411	9412
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**3a**T**3a**C] (**28**)	9450	9450
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**3b**TC C] (**25**)	9451	9453
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**3b**T**3b**C] (**29**)	9526	9528
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**2**TC C] (**23**)	9489	9489
5′-d[GAA GAG GTT TTT CCT CTT CTT TTT CTT**2**T**2**C] (**27**)	9608	9608

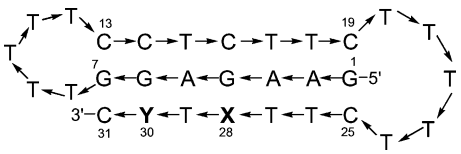
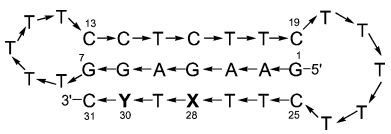


Fig. 2 31-Mer triplex-forming hairpin **21** containing dCH^+–dG–dC base triads (**X**, **Y** = dC); symbols in the figure represent 2′-deoxyribonucleosides.

of this investigation are outlined in Table 3. The unmodified intramolecular triplex-forming oligonucleotide hairpin **21** shows two thermal transitions. The lower T_m (39 °C) results from the melting of the 3′-terminus formed by Hoogsteen pairing (triplex melting) and the higher one (T_m = 65 °C) represents the dissociation of the Watson–Crick pairs (duplex melting) (Table 3) (motif **I**). According to Table 3, the replacement of **X** by N^7-glycosylated 9-deazaguanine 2′-deoxyribonucleoside (**3a**) (hairpin **24**) in the triplex forming hairpin **21** does not change the triplex stability (T_m = 39 °C) and the duplex melting remains the same at pH 6.5 (65 °C). The replacement of **X** by the N^7-glycosylated 9-deaza-9-propynylguanine 2′-deoxyribonucleoside **3b** (hairpin **25**) enhances the stability of the triplex extraordinarily by 6 °C for one incorporation (T_m = 45 °C) at pH 6.5 with duplex melting remaining at 65 °C (Table 3). At pH 8.0 oligonucleotides **24** and **25** with a single incorporation of **3a** and **3b** respectively as well as the unmodified oligonucleotide **21** do not form triplexes and only duplex melting was observed (65 °C). The reason for this is the absence of the protonation at the remaining dC_{25}, dC_{30} (=**Y**) and dC_{31} residues. They cannot form Hoogsteen pairs which provide the necessary stability for triplex formation (see Fig. 2 and Table 3).

Table 3 T_m Values of the single-stranded hairpin triplexes containing 2′-deoxyisoguanosine (**1**), 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) and, 9-deazaguanine N^7-(2′-deoxyribonucleosides) (**3a**, **b**)[a,b]



Oligonucleotide	X	Y	T_m/°C pH 6.5	T_m/°C pH 8
21	dC	dC	39/65	—[c]/65
22	**1**	dC	44/65	—[c]/66
23	**2**	dC	43/64	—[c]/65
24	**3a**	dC	39/65	—[c]/65
25	**3b**	dC	45/65	—[c]/65
26	**1**	**1**	48/65	44/65
27	**2**	**2**	46/65	—[c]/65
28	**3a**	**3a**	44/65	39/65
29	**3b**	**3b**	48/65	42/65

[a] Measured at 260 nm in 10.5 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-3-(ethansulfonic acid)] with 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine. [b] The concentration of single strand was 2 μM at 260 nm. [c] Not detected.

The replacement of both—**X** (dC_{28}) and **Y** (dC_{30})—residues by the non-functionalized nucleoside **3a** (hairpin **28**) increases the stability of the triplex to 44 °C at pH 6.5. The introduction of the 9-propynyl derivative **3b** in the place of **X** and **Y** (hairpin **29**) further enhances the triplex stability to 48 °C. The hairpin oligonucleotides **28** and **29** with two incorporations of **3a** and **3b** respectively now form stable triplexes at pH 8.0. At pH 8.0 for hairpin **28** containing two **3a** residues, a T_m value of 39 °C is observed and the hairpin **29** with two N^7-glycosylated 9-propynyl-9-deazaguanine 2′-deoxyribonucleoside (**3b**) residues leads to a stepwise enhancement of the triplex stability (T_m value 42 °C) without affecting duplex melting (T_m = 65 °C). Thus, the 9-propynyl group of the N^7-glycosylated 9-deazaguanine 2′-deoxyribonucleoside (**3b**) is well accommodated into the triplex structure. The stabilizing effect corresponds to the stabilization of 5-propynylated pyrimidine nucleosides or 7-substituted 7-deazapurine nucleosides in DNA duplexes.[34,26]

Comparatively to the protonated 2′-deoxycytidine (data see Table 3) the phosphodiester backbone should be distorted when 9-deazaguanine replaces cytosine. This results from the glycosylation position of the "purine" bases of **3a** or **3b** in which the glycosylation site is more distant from the recognition site than in the pyrimidine nucleoside dC. Nevertheless, even in sequence motifs formed by alternating purine and pyrimidine bases triplex formation is stabilized. From that it is concluded that other compounds containing a purine base in the third strand of a pyrimidine rich oligonucleotide can also show such favorable properties.

2′-Deoxyisoguanosine (**1**) shows the same donor acceptor pattern as the protonated 2′-deoxycytidine. Thus, we became interested to study the triplex forming capability of the nucleoside **1**; its congener 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) was included in this study as we want to see the effect of the 7-substituents on the dCH^+–dG–dC base triad. According to Table 3, in the triplex forming hairpin **21** the replacement of **X** (dC_{28}) by 2′-deoxyisoguanosine (**1**) (hairpin **22**) increases the stability of the triplex and shows a T_m value of 44 °C while the duplex melting remains the same at pH 6.5 (65 °C). The replacement of **X** (dC_{28}) by 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) (hairpin **23**) stabilizes the triplex to the same level with a T_m value of 43 °C at pH 6.5 (Table 3). The stabilization of triplexes caused by the incorporation of **1** and **2** is higher than that of the triplexes formed by N^7-glycosylated 9-deazaguanine 2′-deoxyribonucleoside **3a** but similar to its 9-propynyl substituted derivative **3b**. At pH 8.0 the oligonucleotides **22** and **23** with one modified residue of **1** and **2**

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respectively again do not form triplexes and only duplex melting was observed (65 °C). The typical triplex melting curves and their derivatives are shown in Fig. 3. In hairpin **21** the replacement of both **X** and **Y** by 2′-deoxyisoguanosines (**1**) (hairpin **26**) increases the stability of the triplex to 48 °C at pH 6.5 while the replacement of 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) (hairpin **27**) slightly decreases the stability of the triplex (T_m = 46 °C). The hairpin oligonucleotide **26** with two incorporations of **1** now form stable triplexes at pH 8.0. At pH 8.0 the hairpin **26** containing two **1**-residues shows a T_m value of 44 °C, while in the case of the hairpin **27** containing two compound **2**-residues, no triplex formation was observed. This might be due to the effect of the 7-bromo substituent which increases the acidic character of the 6-amino group. This effect of the increase in acidic character might be more when compound **2** is a part of a polymeric chain which can force the deprotonation of the 6-amino proton and hence it may prevent the base pair formation under alkaline conditions. Thus, 2′-deoxyisoguanosine (**1**) mimics the protonated dC in dCH$^+$–dG–dC base triads and thereby forming triplexes pH independently while 7-bromo-7-deaza-2′-deoxyisoguanosine (**2**) forms triplexes only under slightly acidic conditions. We suggest the base pair motif **II** and **III** for **1**/**2**–dG–dC base triads which are relatively similar to the base pair motif suggested for nucleoside **3b** (motif **IV**).[19] Base pair motifs **II**, **III** and **IV** resemble the dCH$^+$–dG–dC base triad (see Fig. 4). The enhancement of triplex stability might be due to the increased surface area of the purine moiety which can increase stacking interactions within the triplex even though in a distorted triplex structure.

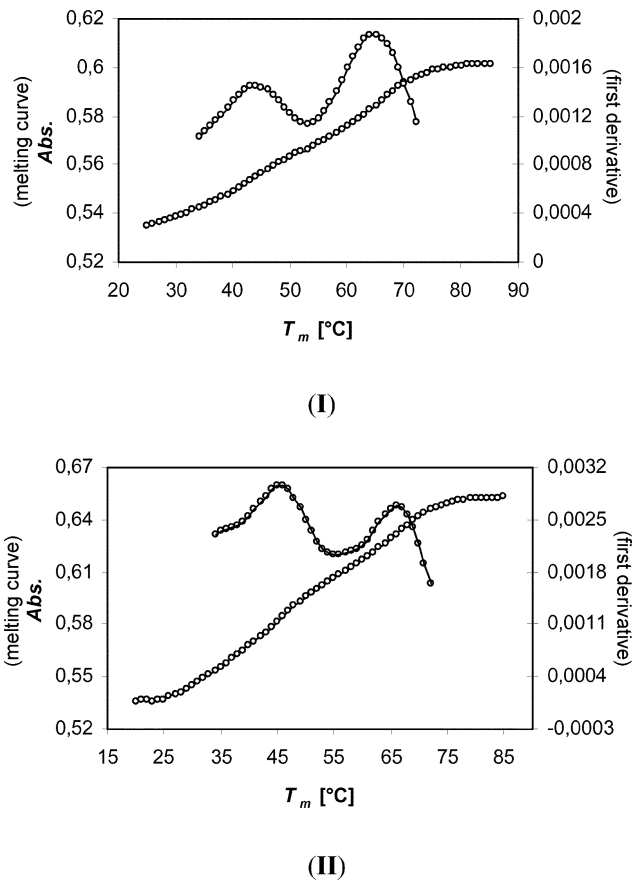

Fig. 3 UV-melting curves and their first derivatives of triplexes: (**I**) hairpin **22** containing nucleoside **1** and, (**II**) hairpin **27** containing compound **2**. Melting temperatures were measured in 10.5 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine (pH 6.5) with 2 μM concentration of hairpin oligonucleotides at 260 nm.

Conclusion and outlook

2′-Deoxyisoguanosine (**1**) which mimics the recognition site of protonated dC is a very strong inducer of triplex formation. It forms triplexes without protonation already under neutral conditions and avoids charge repulsion in the third strand. Similar but slightly altered properties are observed for the brominated 7-deaza-2′-deoxyisoguanosine **2** as a constituent of a triplex forming oligonucleotide. The same favourable properties were found when 9-deazapurine N^7-(2′-deoxyribonucleoside) **3b** bearing a 9-propynyl group is incorporated in the third strand of triplex DNA. Both deazapurine compounds allow the incorporation of reporter groups in the third strand in a sterically non-demanding position. It should be noted that the modification described above represents the incorporation of a "purine" nucleoside in the pyrimidine rich strand of an oligonucleotide triplex.

Experimental

General

All chemicals were purchased from Aldrich, Sigma, or Fluka (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Solvents were of laboratory grade. Thin layer chromatography (TLC): aluminium sheets, silica gel 60 F_{254}, 0.2 mm layer (VWR, Germany). Column flash chromatography (FC): silica gel 60 (VWR, Germany) at 0.4 bar; sample collection with an UltraRac II fractions collector (LKB Instruments, Sweden). UV spectra: U-3200 spectrometer (Hitachi, Tokyo, Japan); λ_{max} (ε) in nm. CD spectra: Jasco 600 (Jasco, Japan) spectropolarimeter with thermostatically (Lauda RCS-6 bath) controlled 1 cm cuvettes. NMR spectra: Avance-250 or AMX-500 spectrometers (Bruker, Karlsruhe, Germany), at 250.13 MHz for ^{1}H and ^{13}C; δ in ppm relative to Me_4Si as internal standard or external 85% H_3PO_4 for ^{31}P. The J values are given in Hz. Elemental analyses were performed by Mikroanalytisches Laboratorium Beller (Göttingen, Germany). The melting temperatures were measured with a Cary-100 Bio UV–VIS spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller. The temperature was measured continuously in the reference cell with a Pt-100 resistor.

Oligonucleotides

The oligonucleotide syntheses were carried out on an ABI 392-08 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) at 1 μmol scale using the phosphoramidites following the synthesis protocol for 3′-cyanoethylphosphoramidites (user manual for the 392 DNA synthesizer, Applied Biosystems, Weiterstadt, Germany). The coupling efficiency was always higher than 97%.

(i) Deprotection of *O*-allyl-protected oligonucleotides using Pd(0) complex followed by 25% aq. NH_3. After the completion of the oligonucleotide synthesis the columns were washed with argon-flushed THF and disassembled. The supported oligonucleotides were treated at room temperature with a THF solution of

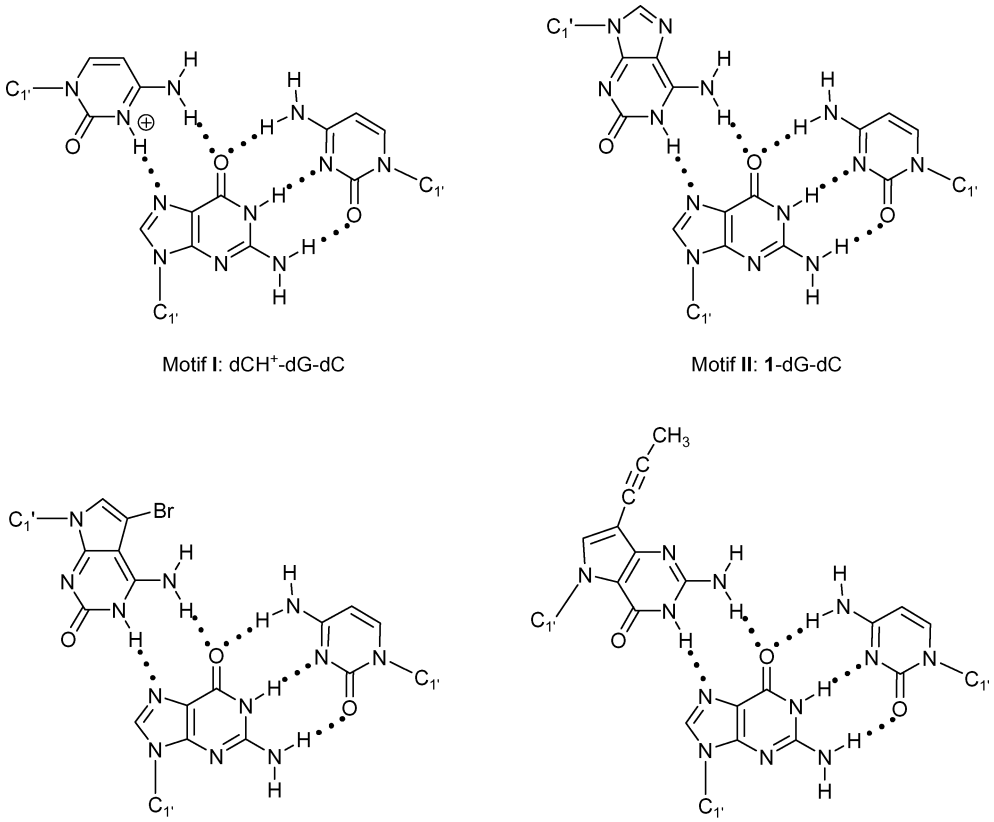

Fig. 4 Triplex motifs formed by the protonated 2′-deoxycytidine with dG–dC (motif **I**) and **1**, **2** and **3b** with dG–dC (motif **II**, **III** and **IV** respectively).

$Pd(0)[PPh_3]_4$ (≈3 equiv./allylic group), PPh_3 (≈25 equiv./allylic group) and excess of allyl scavenger HCOOH–*n*-butylamine (1 : 1) (1.5 ml) for 1 h at r.t.[27,35] This was centrifuged and the solution was discarded. The supports were then washed with THF, acetone, 5 M aq. *N*,*N*-diethyldithiocarbamate (DDTC) and finally with distilled water (two times each). The deprotection of other base protecting groups was achieved by the treatment of 25% NH_3 for 14–16 h at 60 °C.

(ii) Purification of oligonucleotides. The 5′-dimethoxytrityl oligomers were purified by reversed-phase HPLC (RP-18) with the following solvent gradient system [A: 0.1 M $(Et_3NH)OAc$ (pH 7.0)–MeCN 95 : 5; B: MeCN]: 3 min, 20% B in A, 12 min, 20–50% B in A and 25 min, 20% B in A with a flow rate of 1.0 ml min^{-1}. The solvent was evaporated to dryness in a Speed-Vac evaporator and the residue was treated with 2.5% $CHCl_2COOH$ in CH_2Cl_2 for 5 min at 0 °C to remove the 4,4′-dimethoxytrityl residues. The detritylated oligomers were purified by reversed phase HPLC with the gradient: 0–20 min, 0–20% B in A with a flow rate of 1.0 ml min^{-1}. The oligomers were desalted (RP-18, silica gel) and lyophilized on a Speed-Vac evaporator to yield colorless solids which were frozen at −24 °C.

The enzymatic analysis of the oligonucleotides containing 2′-deoxyisoguanosine (**1**) was performed as described by Seela and Shaikh[26b] with snake-venom phosphodiesterase (EC 3.1.15.1, *Crotallus adamanteus*) and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli* from *Roche Diagnostics GmbH*, Germany) in 0.1 M Tris-HCl buffer (pH 8.3), which was carried out on reversed-phase HPLC by gradient: 0.1 M $(Et_3NH)OAc$ (pH 7.0) with flow rate 0.7 ml min^{-1}. Quantification of the constituents was made on the basis of the peak areas, which were divided by the extinction coefficients of the nucleosides [(ε_{260}): dT 8800, dC 7300, dA 15400, dG 11700, **1** 4300). The molecular masses of the modified oligonucleotides were determined by MALDI-TOF *Biflex-III* mass spectrometry (Bruker Saxonia, Leipzig, Germany) with 3-hydroxypicolinic acid (3-HPA) as a matrix (see Table 3). UV-Melting curves of the single-stranded intramolecular triplexes (single strand concentration 2 μmol at 260 nm) were measured in the buffer solution containing 10.5 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-3-(ethansulfonic acid)] with 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine.

2-Allyloxy-6-amino-9-[2-deoxy-β-D-*erythro*-pentofuranosyl]-9*H*-purine (6). A mixture of 2-chloro-2′-deoxyadenosine (**5**)[36] (748 mg, 2.6 mmol) and 1 M $CH_2{=}CHCH_2ONa/CH_2{=}CHCH_2OH$ solution (17 ml) was stirred at 60 °C for 2 h. After cooling, the reaction mixture was evaporated to dryness. The residue was co-evaporated with MeOH (10 ml) and then applied to FC (silica gel, column 3 × 14 cm, CH_2Cl_2–MeOH 95 : 5 to 70 : 30), yielding **6** (628 mg, 79%). (Found: C, 50.67; H, 5.71; N, 22.56%. $C_{13}H_{17}N_5O_4$ requires C, 50.81; H, 5.57; N, 22.79%); TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.20; λ_{max} (MeOH)/nm 267 (ε/dm^3 mol^{-1} cm^{-1} 11 900); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 2.24 (1 H, m, 2′-H_α), 2.72 (1 H, m, 2′-H_β), 3.52–3.59 (2 H, m, 5′-H_2), 3.86 (1 H, m, 4′-H), 4.41 (1 H, m, 3′-H), 4.77 (2 H, m, CH_2O), 4.99 (1 H, t, *J* 5.7, 5′-H), 5.22, 5.39 (2 H, 2d, $H_2C{=}$), 5.27

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(1 H, d, *J* 3.7, 3′-OH), 6.05 (1 H, m, =CH), 6.24 (1 H, t, *J* 6.7, 1′-H), 7.27 (2H, bs, NH_2), 8.13 (1 H, s, 8-H).

2-Allyloxy-6-isobutyrylamino-9-[2-deoxy-β-D-*erythro*-pentofuranosyl]-9*H*-purine (7a). To a solution of compound **6** (337 mg, 1.1 mmol) in anhydrous pyridine (6 ml) was added trimethylchlorosilane (1.0 ml, 7.7 mmol). After the reaction mixture was stirred for 30 min, isobutyryl chloride (0.4 ml, 3.7 mmol) was added and the stirring was continued for another 3 h. The reaction was then cooled in an ice bath and H_2O (1.2 ml) was added. After 5 min 25% aq. NH_3 (1.0 ml) was added and the reaction was stirred for 20 min at r.t. The mixture was evaporated and co-evaporated twice with MeOH. The resulting residue was applied to FC (silica gel, column 2 × 12 cm, CH_2Cl_2–MeOH, 9 : 1). The main zone yielded **7a** as a colorless foam (180 mg, 41%). TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.50; λ_{max} (MeOH)/nm 250 (ε/dm^3 mol^{-1} cm^{-1} 6900) and 279 (8400); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si): 0.99–1.18 (6 H, m, 2 CH_3), 2.39 (1 H, m, 2′-H_α), 2.82 (1 H, m, 2′-H_β), 2.94 (1 H, m, CH), 3.55 (1 H, m, 5′-H), 3.62 (1 H, m, 5′-H), 3.90 (1 H, m, 4′-H), 4.45 (1 H, m, 3′-H), 4.90 (2 H, m, CH_2O), 5.29 (1 H, m, 5′-OH), 5.27, 5.42 (2 H, 2 m, H_2C=), 5.35 (1 H, d, 3′-OH), 6.10 (1 H, m, =CH), 6.39 (1 H, m, 1′-H), 8.63 (1 H, s, 8-H), 10.52 (1 H, s, NH).

2-Allyloxy-6-benzoylamino-9-[2-deoxy-β-D-*erythro*-pentofuranosyl]-9*H*-purine (7b) and 2-allyloxy-6-(dibenzoyl)amino-9-[2-deoxy-β-D-*erythro*-pentofuranosyl]-9*H*-purine (7c). As described for **7a** with **6** (337 mg, 1.1 mmol), anhydrous pyridine (6 ml), Me_3SiCl (0.65 ml, 5.0 mmol), benzoylchloride (0.44 ml, 3.7 mmol), H_2O (1.2 ml) and 25% aq. NH_3 solution (1.0 ml). FC (silica gel, column 2 × 12 cm, CH_2Cl_2–MeOH 98 : 2 to 95 : 5): the slow migrating zone, **7b** (145 mg, 32%); the fast migrating zone, **7c** (142 mg, 25%). **7b**: (Found: C, 58.48; H, 5.33; N 17.01%. $C_{20}H_{21}N_5O_5$ requires C, 58.39; H, 5.15; N, 17.02%); TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.42; λ_{max} (MeOH)/nm 234 (ε/dm^3 mol^{-1} cm^{-1} 17 600) and 296 (14 100); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 2.30 (1 H, m, 2′-H_α), 2.78 (1 H, m, 2′-H_β), 3.47–3.66 (2 H, m, 5′-H), 3.87 (d, *J* 5.2, 4′-H), 4.44 (1 H, m, 3′-H), 4.85 (2 H, d, *J* 5.2, CH_2O), 4.99 (1 H, t, *J* 5.5, 5′-OH), 5.25, 5.40 (2 H, 2 m, H_2C=), 5.33 (1 H, d, *J* 4.1, 3′-OH), 6.08 (1 H, m, =CH), 6.35 (1 H, t, *J* 6.7, 1′-H), 7.50–8.02 (5 H, m, arom. H), 8.44 (1 H, s, 8-H), 11.10 (1 H, s, NH). **7c**: (Found: C, 63.06; H, 5.05; N 13.35%. $C_{27}H_{25}N_5O_6$ requires C, 62.91; H, 4.89; N, 13.58%); TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.48; λ_{max} (MeOH)/nm 246 (ε/dm^3 mol^{-1} cm^{-1} 21 700) and 295 (11 900); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 2.33 (1 H, m, 2′-H_α), 2.75 (1 H, m, 2′-H_β), 3.52 (2 H, m, 5′-H), 3.85 (1 H, m, 4′-H), 4.40 (1 H, m, 3′-H), 4.56 (2 H, d, *J* 5.3, CH_2O), 4.91 (1 H, t, *J* 5.5, 5′-OH), 5.10–5.34 (3 H, m, H_2C= and 3′-OH), 5.81 (1 H, m, =CH), 6.32 (1 H, t, *J* 6.6, 1′-H), 7.45–7.81 (10 H, m, arom. H), 8.55 (1 H, s, 8-H).

2-Allyloxy-6-benzoylamino-9-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-9*H*-purine (8a). The compound **7b** (330 mg, 0.8 mmol) was dried by repeated co-evaporation from anhydrous pyridine and then dissolved in anhydrous pyridine (5 ml). 4,4′-Dimethoxytrityl chloride (312 mg, 0.9 mmol) was added at room temperature and the solution was stirred for 18 h. To the reaction methanol (5 ml) was added. After 5 min the solution was poured into 5% aqueous $NaHCO_3$ (15 ml), extracted with CH_2Cl_2 (4 × 10 ml). The combined organic phase was dried over Na_2SO_4. After evaporation of the solvent, the residue was applied to FC (silica gel, column 2 × 14 cm, CH_2Cl_2–MeOH, 97 : 3 containing traces of Et_3N) to give **8a** as a colorless foam (441 mg, 77%). (Found: C, 68.81; H, 5.61; N, 9.76%. $C_{41}H_{39}N_5O_7$ requires C, 68.99; H, 5.51; N, 9.81); TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.37; λ_{max} (MeOH)/nm 234 (ε/dm^3 mol^{-1} cm^{-1} 35 600) and 294 (13 700); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 2.38 (1 H, m, 2′-H_α), 2.92 (1 H, m, 2′-H_β), 3.16–3.28 (2 H, m, 5′-H), 3.72 (6 H, s, 2 CH_3O), 4.02 (1 H, m, 4′-H), 4.51 (1 H, m, 3′-H), 4.70, 4.76 (2 H, 2m, CH_2O), 5.22–5.39 (3 H, m, H_2C= and 3′-OH), 6.05 (1 H, m, =CH), 6.41 (1 H, t, *J* 6.3, 1′-H), 6.76–8.03 (18 H, m, arom. H), 8.37 (1 H, s, 8-H), 11.10 (1 H, s, NH).

2-Allyloxy-6-benzoylamino-9-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-9*H*-purine 3′-[*O*-(2-cyanoethyl)-diisopropylphosphoramidite] (9a). A solution of compound **8a** (214 mg, 0.3 mmol) in dichloromethane (8.7 ml) was flushed with argon and kept under an argon atmosphere. Then chloro(2-cyanoethoxy)(diisopropylamino)phosphine (0.27 ml, 1.21 mmol) and diisopropylethylamine (0.21 ml, 1.2 mmol) were added under stirring at room temperature. The stirring was continued for 2.5 h and the solution was poured into 5% aq. $NaHCO_3$ soln (9 ml). It was extracted with CH_2Cl_2 (3 × 8 ml). The combined organic phase was dried over Na_2SO_4, filtered and evaporated to give an oily residue. The residue was dissolved in CH_2Cl_2 and a white powder (**9a**) (247 mg, 90%) was isolated from precipitation into cyclohexane (200 ml). TLC (silica gel, CH_2Cl_2–AcOEt–Et_3N, 45 : 45 : 10): R_f 0.66; ^{31}P-NMR ($CDCl_3$): δ 149.4, 149.6.

2-Allyloxy-6-{(di-*n*-butylaminomethylidene)amino}-9-[2-deoxy-β-D-*erythro*-pentofuranosyl]-9*H*-purine (7d). To a solution of compound **6** (600 mg, 1.95 mmol) in MeOH (15 ml), *N*,*N*-di-*n*-butylformamide dimethylacetal (1 gm, 4.95 mmol) was added. The mixture was stirred at 40 °C for 2 h and evaporated to dryness. The resulting residue was applied to FC (silica gel column, 3 × 12 cm, CH_2Cl_2–MeOH, 9 : 1). The main zone yielded **7d** as a colorless foam (687 mg, 79%). (Found: C, 58.99; H, 7.84; N, 18.71%. $C_{22}H_{34}N_6O_4$ requires C, 59.17; H, 7.67; N, 18.82%); TLC (silica gel, CH_2Cl_2–MeOH, 8 : 2): R_f 0.45; λ_{max} (MeOH)/nm 239 (ε/dm^3 mol^{-1} cm^{-1}10 100), 266 (6400) and 318 (28 800); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 0.91 (6 H, m, 2 CH_3), 1.31 (4 H, m, 2 CH_2), 1.58 (4 H, m, 2 CH_2), 2.22 (1 H, m, 2′-H_α), 2.72 (1 H, m, 2′-H_β), 3.42 (4 H, m, 2 CH_2N), 3.56 (2 H, t, *J* 6.59, 6.79, 5′-H), 3.84 (1 H, m, 4′-H), 4.40 (1 H, m, 3′-H), 4.81 (2 H, m, OCH_2), 5.00 (1 H, t, 5′-OH), 5.25–5.42 (3 H, m, =CH_2 and 3′-OH), 6.04 (1 H, m, =CH), 6.28 (1 H, t, *J* 5.92, 6.19, 1′-H), 8.24 (1 H, s, 8-H), 8.88 (1 H, s, HC=N).

2-Allyloxy-6-{(di-*n*-butylaminomethylidene)amino}-9-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-9*H*-purine (8b). Compound **7d** (500 mg, 1.12 mmol) was dried by repeated co-evaporations from anhydrous pyridine (5 ml × 2) and dissolved in anhydrous pyridine (5 ml). The solution was treated with 4,4′-dimethoxytrityl chloride (440 mg, 1.3 mmol) at r.t. under stirring for 4 h. The mixture was poured into 5% aq. $NaHCO_3$ solution (25 ml) and extracted with CH_2Cl_2 (3 × 30 ml). The combined organic phase was dried over Na_2SO_4, evaporated and the residue was applied to FC (silica gel, column 3 × 12 cm,

stepwise gradient: CH_2Cl_2–$(CH_3)_2CO$ 95 : 5, 9 : 1 and 3 : 1). The main zone gave **8b** as a colorless foam (650 mg, 78%). (Found: C, 69.02; H, 7.10; N, 11.19%. $C_{43}H_{52}N_6O_6$ requires C, 68.96; H, 7.00; N, 11.22%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.32; λ_{max} (MeOH)/nm 234 (ε/dm^3 mol^{-1} cm^{-1} 27 700), 266 (6400) and 317 (25 700); δ_H (250.13 MHz; [d$_6$]DMSO; Me_4Si) 0.92 (3 H, m, CH_3), 1.32 (4 H, m, 2 CH_2), 1.58 (4 H, m, 2 CH_2), 2.31 (1 H, m, 2′-H$_\alpha$), 2.86 (1 H, m, 2′- H$_\beta$), 3.42 (4 H, m, 2 CH_2N), 3.58 (2 H, t, *J* 6.59, 6.79, 5′H), 3.96 (1 H, m, 4′-H), 4.46 (1 H, m, 3′H), 4.68 (2 H, m, OCH_2), 5.29 (2 H, m, =CH_2), 5.35 (1 H, d, *J* 3.20, 3′-OH), 6.01 (1 H, m, =CH), 6.32 (1 H, t, *J* 5.92, 6.19, 1′-H), 6.70–6.80 (m, arom. H) and 7.16–7.32 (m, arom. H), 8.16 (1 H, s, 8-H), 8.87 (1 H, s, HC=N).

2-Allyloxy-6-{(di-*n*-butylaminomethylidene)amino}-9-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-9*H*-purine 3′-(2-cyanoethyl)-diisopropylphosphoramidite(9b). To a solution of compound **8b** (300 mg, 0.4 mmo) in anhydrous CH_2Cl_2 (5 ml) was added *N*,*N*-diisopropylethylamine (116 µl, 0.66 mmol) and chloro(2-cyanoethoxy)(diisopropylamino)phosphine (130 µl, 0.58 mmol) while stirring under an argon atmosphere. The stirring was continued for 30 min and the reaction was quenched with 30 ml CH_2Cl_2. An aqueous solution of 5% $NaHCO_3$ was added, the aqueous phase was extracted with CH_2Cl_2 (30 ml × 3). The combined organic phase was dried over Na_2SO_4, evaporated and the residue was applied to FC (silica gel, column 3 × 8 cm, CH_2Cl_2–$(CH_3)_2CO$ 95 : 5). Evaporation of the main zone afforded compound **9b** as a colorless foam (250 mg, 66%). TLC: (silica gel, CH_2Cl_2–acetone, 95 : 5): R_f 0.54, 0.46; ^{31}P-NMR ($CDCl_3$): δ 150.19, 149.88.

2-{[(Dimethylamino)methylidene]amino}-3,5-dihydro-3-[(pivaloyloxy)methyl]-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (11). To a solution of 2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-7-iodo-3-[(pivaloyloxy)methyl]-4*H*-pyrrolo[3,2-*d*]-pyrimidin-4-one (**10**) (1.5 g, 3.37 mmol)[17] in anhydrous DMF (10 ml) tetrakis(triphenylphosphine)palladium(0) [$(PPh_3)_4Pd(0)$] (348 mg, 0.33 mmol), CuI (204 mg, 1.07 mmol) and triethylamine (840 µl, 5.98 mmol) were added while stirring. The sealed suspension was saturated with propyne at 0 °C and stirred at r.t. for 24 h. The solvent was evaporated *in vacuo*, the reaction mixture dissolved in MeOH (5 ml) and adsorbed on silica gel (4 g). The resulting powder was subjected to FC (silica gel, column 15 × 3 cm, CH_2Cl_2–MeOH, 95 : 5). From the main zone, compound **11** was isolated as a colorless solid (1.103 mg, 91%). (Found: C, 60.32; H, 6.40; N, 19.43%. $C_{18}H_{23}N_5O_3$ requires C, 60.49; H, 6.49; N, 19.59%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.38; λ_{max} (MeOH)/nm 223 (ε/dm^3 mol^{-1} cm^{-1} 26 000), 266 (25 200) and 308 (16 600); δ_H (250.13 MHz; [d$_6$]DMSO; Me_4Si) 1.07 (9 H, s, 3 CH_3), 2.01 (3 H, s, CH_3), 2.96, 3.15 (6 H, 2s, 2 NCH_3), 6.16 (2 H, s, OCH_2), 7.43 (1 H, d, *J* 2.97, 8-H), 8.49 (1 H, s, N=CH), 12.03 (1 H, s, NH).

5-[2-Deoxy-3,5-di-*O*-(*p*-toluoyl)-β-D-*erythro*-pentofuranosyl]-{[2-(dimethylamino)methylidene]amino}-3,5-dihydro-3-[(pivaloyloxy)-methyl]-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (14). *Method A*: To a suspension of powdered KOH (140 mg, 2.50 mmol) and TDA-1 (tris[2-(2-methoxyethoxy)ethyl]amine, 46 µl, 0.14 mmol) in anhyd. MeCN (10 ml) was added compound **11** (725 mg, 2.03 mmol) while stirring at r.t. The stirring was continued for another 10 min and 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride (**12**) (970 mg, 2.50 mmol) was added in portions. After 30 min insoluble material was filtered off and the solvent was evaporated. The resulting foam was applied to FC (silica gel, column, 12 × 3 cm, CH_2Cl_2–$(CH_3)_2CO$, 98 : 2). A colorless foam **14** was isolated from the main zone (1.28 gm, 89%). *Method B*: To a solution of compound **13** (850 mg, 1.06 mmol)[17] in anhydrous DMF (6 ml), tetrakis(triphenylphosphine)palladium(0) [$(PPh_3)_4Pd(0)$] (116 mg, 0.1 mmol), CuI (68 mg, 0.36 mmol) and triethylamine (280 µl, 1.99 mmol) were added. The sealed suspension was saturated with propyne at 0 °C and stirred at r.t. for 24 h. The solvent was evaporated to dryness. The residue was dissolved in MeOH (4 ml), adsorbed on silica gel (2 g) and subjected to FC (silica gel, column 15 × 3 cm, CH_2Cl_2–$(CH_3)_2CO$, 98 : 2). From the main zone, compound **14** was isolated as a colorless foam (598 mg, 79%). (Found: C, 65.98; H, 6.03; N, 9.92%. $C_{39}H_{43}N_5O_8$ requires C, 65.99; H, 6.11; N, 9.87%); TLC (silica gel, CH_2Cl_2–$(CH_3)_2CO$, 95 : 5): R_f 0.75; λ_{max} (MeOH)/nm 235 (ε/dm^3 mol^{-1} cm^{-1} 43 100), 266 (23 300) and 312 (16 600); δ_H (250.13 MHz; [d$_6$]DMSO; Me_4Si) 1.06 (9 H, s, 3 CH_3), 2.01 (3 H, s, CH_3), 2.36, 3.38 (6 H, 2s, 2 OCH_3), 2.66–2.83 (2 H, m, 2′-H), 2.96, 3.15 (6 H, 2 s, 2 NCH_3), 4.48–4.60 (2 H, m, 5′-H and 4′-H), 5.63 (1 H, m, 3′-H), 6.14 (2 H, s, OCH_2), 6.98 (1 H, t, *J* 6.7 Hz, 1′-H), 7.30–7.37 (4 H, m, arom. H), 7.81–7.92 (6 H, m, arom. H and 6-H), 8.50 (1 H, s, N=CH).

5-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)-methylidene]amino}-3,5-dihydro-7-prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (15). A solution of **14** (1 g, 1.40 mmol) in 0.1 M NaOMe in MeOH (50 ml) was stirred for 1 h at r.t. The reaction mixture was cooled and neutralized with 5% acetic acid in MeOH. Silica gel was added (4 g) and the solvent was evaporated. It was applied to FC (silica gel, column 14 × 3 cm, CH_2Cl_2–MeOH, 9 : 1). The main zone afforded **15** as a colorless solid (480 mg, 95%). (Found: C, 58.80; H, 5.74; N, 19%. $C_{17}H_{21}N_5O_4$ requires C, 56.82; H, 5.89; N, 19.49%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.20; λ_{max} (MeOH)/nm 226 (ε/dm^3 mol^{-1} cm^{-1} 25 800), 266 (23 300) and 300 (19 200); δ_H (250.13 MHz; [d$_6$]DMSO; Me_4Si) 2.03 (3 H, s, CH_3), 2.21–2.28 (2 H, m, 2′-H), 3.00, 3.14 (6 H, 2 s, 2 NCH_3), 3.51 (2 H, m, 5′-H), 3.79 (1 H, m, 4′-H), 4.28 (1 H, m, 3′-H), 4.94 (1 H, t, *J* 5.53 Hz, 5′-OH), 5.21 (1 H, d, *J* 3.80 Hz, 3′-OH), 6.85 (1 H, t, *J* 6.75 Hz, 1′-H), 7.80 (1 H, s, 6-H), 8.50 (1 H, s, N=CH), 11.25 (1 H, s, NH).

5-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)-methylidene]amino}-3,5-dihydro-3-[(pivaloyloxy)methyl]-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (16). A solution of compound **14** (400 mg, 0.56 mmol) in 0.01 M NaOMe–MeOH (20 ml) was stirred for 45 min at r.t. The reaction mixture was cooled in an ice bath and neutralized with 5% acetic acid in MeOH. Silica gel (2 g) was added and the solvent was evaporated to dryness. The resulting powder was applied to FC (silica gel, column 14 × 2 cm, CH_2Cl_2–MeOH, 98 : 2). The main zone afforded compound **16** as a colorless solid (158 mg, 59%). (Found: C, 58.26; H, 6.70; N, 14.61%. $C_{23}H_{31}N_5O_6$ requires C, 58.34; H, 6.60; N, 14.79%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.36; λ_{max} (MeOH)/nm 218 (ε/dm^3 mol^{-1} cm^{-1} 41 500), 267 (38 400) and 312 (29 100); δ_H (250.13 MHz; [d$_6$]DMSO; Me_4Si) 1.05 (9 H, s, 3 CH_3), 1.99 (3 H, s, CH_3), 2.20–2.25 (2 H, m, 2′-H), 2.94, 3.13 (6 H,

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2 s, 2 NCH_3), 3.50 (2 H, m, 5′-H), 3.76 (1 H, m, 4′-H), 4.25 (1 H, m, 3′-H), 4.94 (1 H, t, *J* 4.94 Hz, 5′-OH), 5.21 (1 H, d, *J* 3.65 Hz, 3′-OH), 6.12 (2 H, s, OCH_2), 6.80 (1 H, t, *J* 6.45 Hz, 1′-H), 7.85 (1 H, s, 6-H), 8.47 (1 H, s, N=CH).

2-Amino-5-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,5-dihydro-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (3b). Compound **15** (150 mg, 0.42 mmol) was stirred in 25% aq. NH_3 (25 ml) at 60 °C for 12 h in a closed bottle. The solvent was evaporated under vacuum and the residue was dissolved in water (50 ml) and chromatographed on *SERDOLIT AD-4* resin. The salt was washed with distilled water for 2 h and the product was collected with MeOH–water (1 : 5). The main zone afforded **3b** as a colorless solid (112 mg, 88%). It was crystallized from MeOH (m.p. 195–196 °C). (Found: C, 55.24; H, 5.14; N, 18.46%. $C_{14}H_{16}N_4O_4$ requires C, 55.26; H, 5.30; N, 18.41%); TLC (silica gel, CH_2Cl_2–MeOH, 9 : 1): R_f 0.25; λ_{max} (0.1M sodium phosphate buffer pH 7.0)/nm 235 (ε/dm^3 mol^{-1} cm^{-1} 27 000), 277 (5700) and 302 (5900); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 1.98 (3 H, s, CH_3), 2.20–2.30 (2 H, m, 2′-H), 3.51 (2 H, m, 5′-H), 3.76 (1 H, m, 4′-H), 4.26 (1 H, m, 3′-H), 4.92 (1 H, m, 5′-OH), 5.18 (1 H, d, *J* 2.85 Hz, 3′-OH), 6.08 (2 H, s, NH_2), 6.73 (1 H, t, *J* 6.32 Hz, 1′-H), 7.71 (1 H, s, 6-H), 10.68 (1 H, s, NH).

5-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (17). Compound **15** (170 mg, 0.47 mol) was dried by repeated co-evaporation with anhydrous pyridine (2 × 3 ml) and dissolved in pyridine (4 ml). After the addition of 4,4′-dimethoxytrityl chloride (170 mg, 0.50 mmol), the solution was stirred for 2 h at r.t. CH_2Cl_2 (30 ml) was added and washed with 5% aq. $NaHCO_3$ solution (25 ml). The aqueous layer was extracted with CH_2Cl_2 (25 × 2 ml). The combined organic phase was dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to FC (silica gel, column 12 × 2 cm, CH_2Cl_2–$(CH_3)_2CO$, 95 : 5). The main zone afforded compound **17** as a colorless foam (225 mg, 72%). (Found: C, 68.79; H, 5.91; N, 10.39%. $C_{38}H_{39}N_5O_6$ requires C, 68.97; H, 5.94; N, 10.58%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.27; λ_{max} (MeOH)/nm 231 (ε/dm^3 mol^{-1} cm^{-1} 40 600), 266 (25 300) and 301 (18 300); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 1.99 (3 H, s, CH_3), 2.24 (1 H, m, 2′-H_α), 2.34 (1 H, m, 2′-H_β), 2.99 (3 H, s, NCH_3), 3.12 (5 H, m, NCH_3 and 5′-H), 3.72 (6 H, s, 2 OCH_3), 3.88 (1 H, m, 4′-H), 4.27 (1 H, m, 3′-H), 5.31 (1 H, d, *J* 4.07 Hz, 3′-OH), 6.84 (5 H, m, 1′-H and arom. H), 7.23–7.38 (9 H, m, arom. H), 7.61 (1 H, s, 6-H), 8.49 (1 H, s, N=CH), 11.28 (1 H, s, NH).

5-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-3-[(pivaloyloxy)methyl]-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (18). Compound **16** (170 mg, 0.36 mmol) was dried by repeated co-evaporation with anhydrous pyridine (2 × 3 ml) and dissolved in pyridine (4 ml). After the addition of 4,4′-dimethoxytrityl chloride (135 mg, 0.39 mmol), the solution was stirred for 2 h at r.t. and CH_2Cl_2 (30 ml) was added and washed with 5% aq. $NaHCO_3$ solution (25 ml). The aqueous layer was extracted with CH_2Cl_2 (25 × 2 ml), dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to FC (silica gel, column 12 × 2 cm, CH_2Cl_2–$(CH_3)_2CO$, 95 : 5). The main zone afforded compound **18** as a colorless foam (210 mg, 76%). (Found: C, 68.16; H, 6.37; N, 9.01%. $C_{44}H_{49}N_5O_8$ requires C, 68.11; H, 6.37; N, 9.03%); TLC (silica gel, CH_2Cl_2–MeOH, 95 : 5): R_f 0.42; λ_{max} (MeOH)/nm 231 (ε/dm^3 mol^{-1} cm^{-1} 41 100), 269 (28 700) and 313 (19 800); δ_H (250.13 MHz; [d_6]DMSO; Me_4Si) 1.09 (9 H, s, 3 CH_3), 2.01 (3 H, s, CH_3), 2.24 (1 H, m, 2′-H_α), 2.38 (1 H, m, 2′-H_β), 2.98 (3 H, s, NCH_3), 3.16 (5 H, m, NCH_3 and 5′-H), 3.73 (6 H, s, 2 OCH_3), 3.91 (1 H, m, 4′-H), 4.29 (1 H, m, 3′-H), 5.29 (1 H, d, *J* 4.25 Hz, 3′-OH), 6.17 (2 H, s, OCH_2), 6.84 (5 H, m, 1′-H and arom. H), 7.20–7.39 (9 H, m, arom. H), 7.72 (1 H, s, 6-H), 8.52 (1 H, s, N=CH).

5-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one 3′-[2-cyanoethyl-diisopropylphosphoramidite] (19). To a solution of compound **17** (145 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (5 ml), *N*,*N*-diisopropylethylamine (DIPEA) (70 µl, 0.40 mmol) and 2-cyanoethyl-diisopropylphosphoramido chloridite (84 µl, 0.37 mmol) were added under an argon atmosphere. After stirring for 30 min, 5% aq. $NaHCO_3$ solution was added, and it was extracted with CH_2Cl_2 (10 ml × 2). The organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was subjected to FC (silica gel, CH_2Cl_2–acetone, 9 : 1). The main zone afforded compound **19** as a colorless foam (140 mg, 74%). TLC (silica gel, CH_2Cl_2–$(CH_3)_2CO$, 8 : 2): R_f 0.6; ^{31}P-NMR ($CDCl_3$): δ 149.79, 150.12.

5-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}-3,5-dihydro-3-[(pivaloyloxy)methyl]-7-(prop-1-ynyl)-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one 3′-[2-cyanoethyl-diisopropylphosphoramidite] (20). As described for **19** with compound **18** (215 mg, 0.28 mmol), *N*,*N*-diisopropylethylamine (DIPEA) (40 µl, 0.23 mmol) and 2-cyanoethyl-diisopropylphosphoramido chloridite (50 µl, 0.22 mmol) in anhydrous CH_2Cl_2 (5 ml). FC (silica gel, CH_2Cl_2–acetone, 9 : 1) afforded compound **20** as a colorless foam (120 mg, 44%). TLC (silica gel, CH_2Cl_2–$(CH_3)_2CO$, 8 : 2): R_f 0.7; ^{31}P-NMR ($CDCl_3$): δ 149.79, 150.12.

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