Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 1861

www.rsc.org/obc

PAPER

Parallel-stranded DNA: Enhancing duplex stability by the 'G-clamp' and a pyrrolo-dC derivative†

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Received 20th September 2011, Accepted 5th December 2011
DOI: 10.1039/c2ob06606h

The new pyrrolo-dC derivative **4** tethered with an alkylamino side chain *via* a triazole linker was synthesized. Oligonucleotides containing the G-clamp **3** or the pyrrolo-dC derivative **4** were prepared. Oligonucleotide synthesis and deprotection under standard conditions led to unwanted side product formation. The side product was identified as an acrylonitrile adduct of the aminoalkyl side chain. Changing the synthesis and work-up conditions to fast-deprotection chemistry and performing *β*-elimination of the cyanoethyl group on the solid support yielded pure oligonucleotides. Oligonucleotide duplexes with parallel chain orientation were constructed incorporating dA·dT and isoG$_d$·dC base pairs. Replacement of dC-residues by the G-clamp **3** led to extraordinarily stable duplexes (ΔT_m = +11 °C for two incorporations) in ps DNA, while the pyrrolo-dC derivative **4** behaved like dC. Surprisingly, the G-clamp **3** forms an even more stable base pair with 2′-deoxyisoguanosine in DNA with parallel chain orientation than with 2′-deoxyguanosine in aps DNA.

Introduction

DNA is a polymorphic molecule adopting various conformations such as A-, B- and Z-DNA. Complementary strands of canonical duplex DNA exhibit antiparallel chain orientation (aps DNA), while parallel strand orientation (ps DNA) is realized in triplex, quadruplex and i-motif DNA.[1–3] Parallel stranded duplex DNA was proposed by Pattabiraman already in 1986[4] and represents a unique DNA structure with both sugar-phosphate chains pointing to the same direction.[5,6] Since then, progress has been made in the theoretical and experimental evaluation of ps DNA.[7–9] A series of manuscripts were published by our laboratory and by others reporting on the chemical synthesis and physical properties of ps DNA.[10–12] These studies demonstrated that the spectroscopic properties, enzymatic recognition as well as drug-binding properties of ps DNA are different from native DNA with antiparallel chains.[13–15] Ps DNA has the potential to be used as entirely new base pairing system in synthetic biology and systems chemistry, as well as in chemical evolution or for the construction of DNA nanostructures.[16]

As the dA-dT base pair system shows pairing ambiguity – Watson Crick as well as reverse Watson–Crick pairs (Donohue pairs) can be formed (Fig. 1) – ps DNA was constructed from complementary strands incorporating dA-dT base pairs exclusively. By this, no structural changes of the monomeric units are required and chain orientation is controlled entirely by the sequence. As ps DNA is less stable than canonical DNA with antiparallel chains, protocols were developed to stabilize ps DNA by chemical modification. Our laboratory has introduced tridentate base pairs of dG·isoC$_d$ (2′-deoxyisocytidine) and dC·isoG$_d$ (2′-deoxyisoguanosine, **1**) (Fig. 1) which stabilize ps DNA strongly.[11,17] Consequently, ps DNA contains less stable bidentate dA-dT base pairs and more stable tridentate pairs of either isoG$_d$-dC or isoC$_d$-dG;[10,18] a principle which is comparable to aps DNA. Additionally, ps DNA can be stabilized by modified nucleosides in a similar way as aps DNA. 7-Halogenated 7-deaza-2′-deoxyisoguanosines or 7-substituted 8-aza-7-deaza-2′-deoxyisoguanosines (purine numbering) have been used for this purpose.[19,20]

Earlier, it has been reported that the tricyclic nucleoside analogue **3** and derivatives (Fig. 2),[21–23] named G-clamp, stabilizes dC-dG base pairs within aps DNA extraordinarily when the dC residue is replaced by the nucleoside **3**. This is the result of an additional hydrogen bond formed between the protonated amino group of the side chain as proton donor and the oxo-function of the guanine base as proton acceptor.[21,24–26]

This manuscript reports on the stabilization of ps DNA by the G-clamp **3**. For this, dC-residues within the isoG$_d$·dC base pair

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†Electronic supplementary information (ESI) available: ^{1}H-^{13}C NMR coupling constants, mass spectra of oligonucleotides, melting curves of ps and aps DNA, ^{1}H-, ^{13}C- and ^{31}P-NMR as well as ^{1}H-^{13}C gated-decoupled NMR spectra. See DOI: 10.1039/c2ob06606h

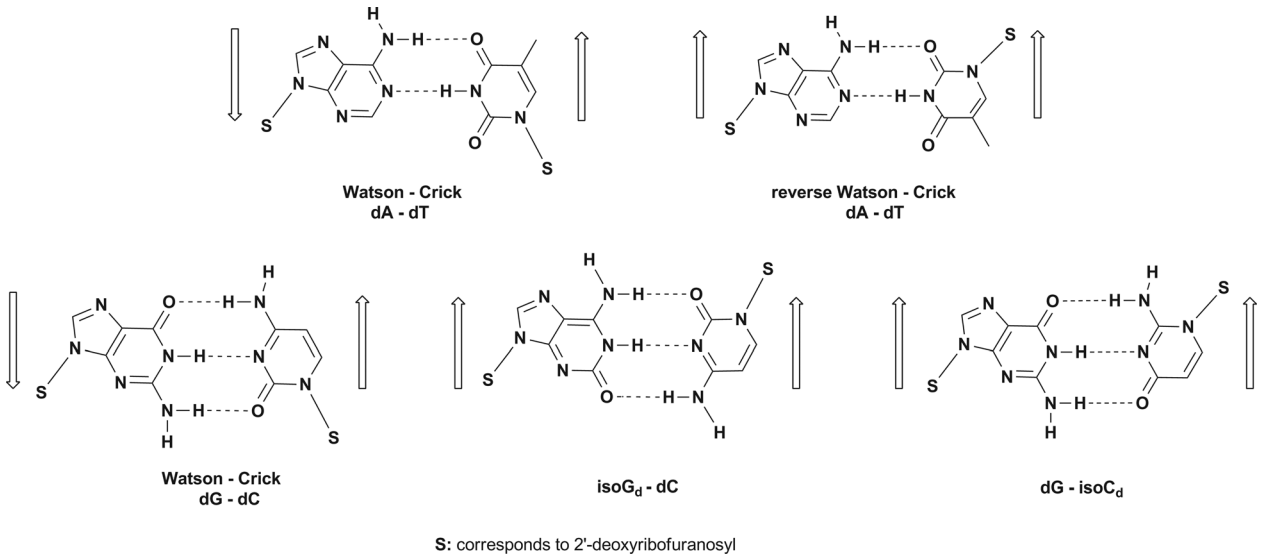


Fig. 1 Base pair motifs of ps and aps DNA; arrows indicate chain orientation.

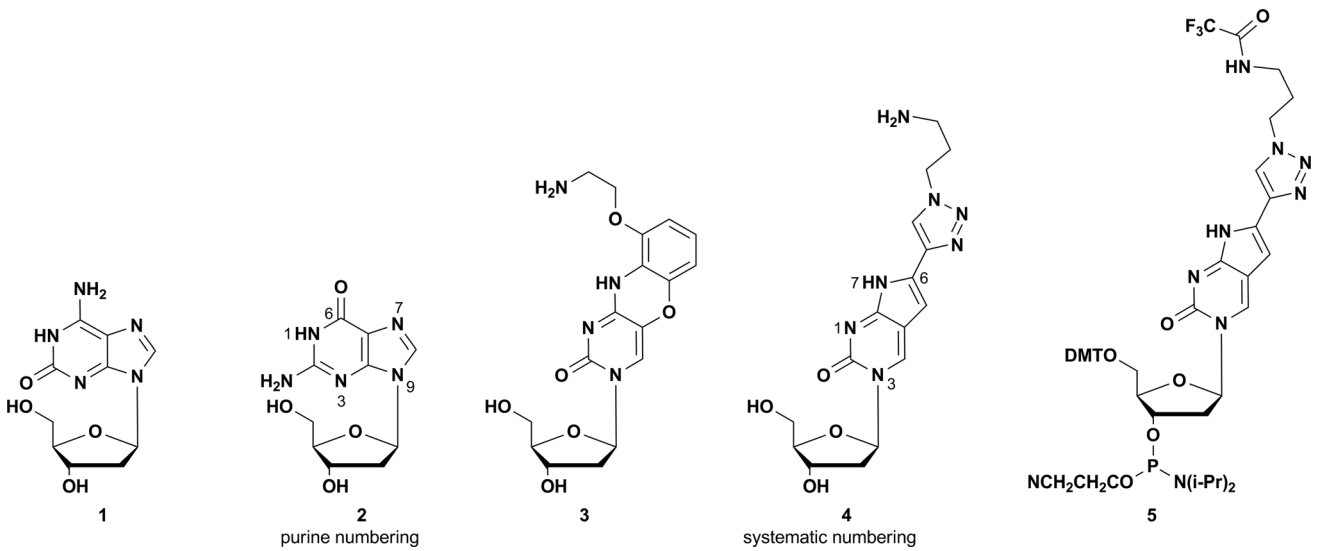


Fig. 2 Structures of nucleosides **1**–**4** and the phosphoramidite building block **5**.

were replaced by the G-clamp **3**. Furthermore, we constructed the pyrrolo-dC derivative **4** as a G-clamp analogue containing the necessary structural elements of the G-clamp **3** but having a different connectivity between the amino group of the side chain and the pyrrolo-dC unit (Fig. 2). The influence of the 'G-clamp' **3** and pyrrolo-dC derivative **4** on ps DNA was compared to that of aps DNA.

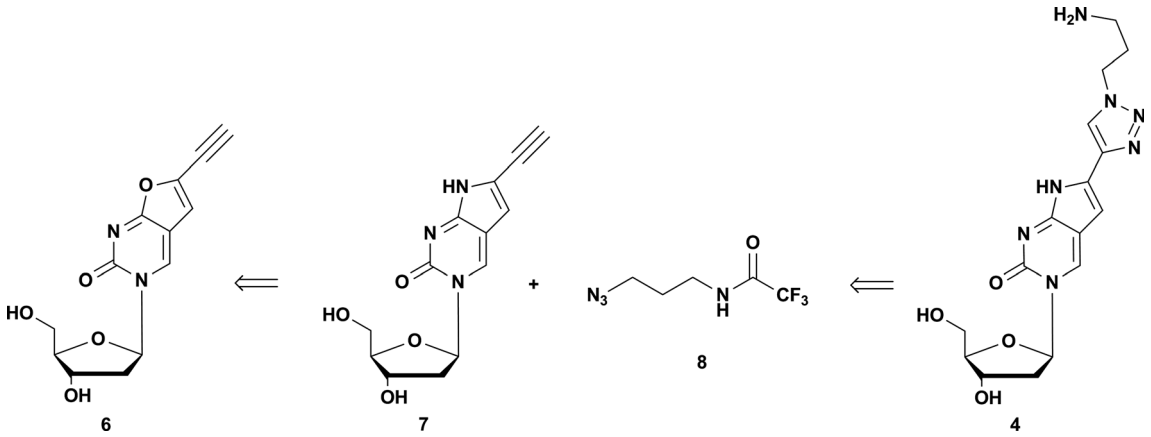
Results and discussion

1. Synthesis of monomers

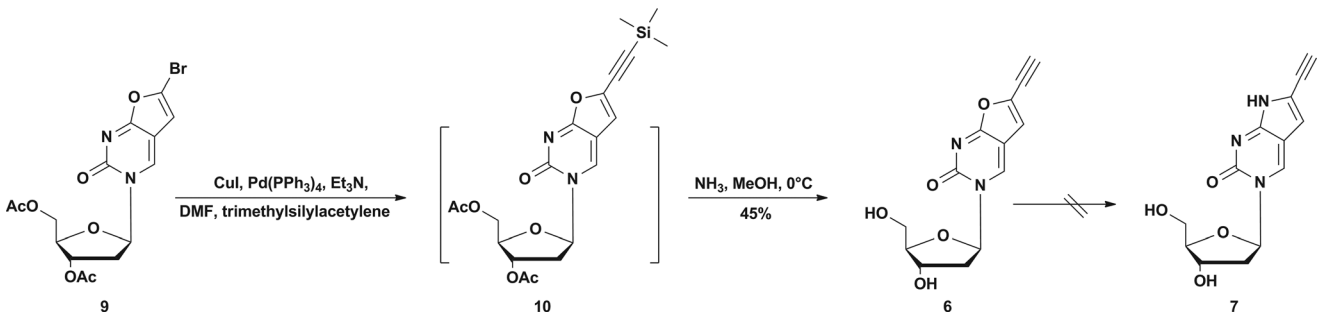
In order to access compound **4**, the application of the copper(I)-catalyzed Huisgen–Sharpless–Meldal 1,3-dipolar cycloaddition ('click' chemistry) was envisaged to connect the side chain with the pyrrolo-dC skeleton *via* a triazole ring which can be formed by click chemistry (Scheme 1).[27,28]

For this, 6-ethynyl pyrrolo-dC (**7**) and the azido side chain precursor *N*-trifluoroacetyl-1-azido-3-aminopropane (**8**) were prepared as click precursor. Usually, pyrrolo-dC derivatives are obtained from corresponding furano-dU precursors, which are accessible from 5-alkynylated dU nucleosides by cyclization with copper iodide followed by the treatment of the obtained furo[2,3-*d*]pyrimidine nucleosides with ammonia.[29–31]

To receive the furano compound **6**, the protected 6-bromo-furano-dU derivative **9** was prepared following a literature protocol.[32] The bromo compound **9** was found to be unstable and turned black after a few days of storage. Thus, it was immediately used in Sonogashira cross-coupling with trimethylsilylacetylene followed by treatment with MeOH/NH_3 to obtain **6** in 45% overall yield. This compound was found to be stable. However, treatment with methanolic or aq. NH_3 to convert the furano into the pyrrolo system (→ **7**) failed. An inseparable mixture of reaction products was formed. So, in our hands



Scheme 1 Retrosynthetic analysis for the synthesis of pyrrolo-dC nucleoside **4**.



Scheme 2 Synthesis of 6-ethynyl furano-dU (**6**).

6-ethynyl pyrrolo-dC (**7**) was not accessible by our envisaged 'click' route (Scheme 2). An alternative approach performing the click reaction first on compound **6** followed by the conversion of the furano system into a pyrrolo system failed as well (data not shown).

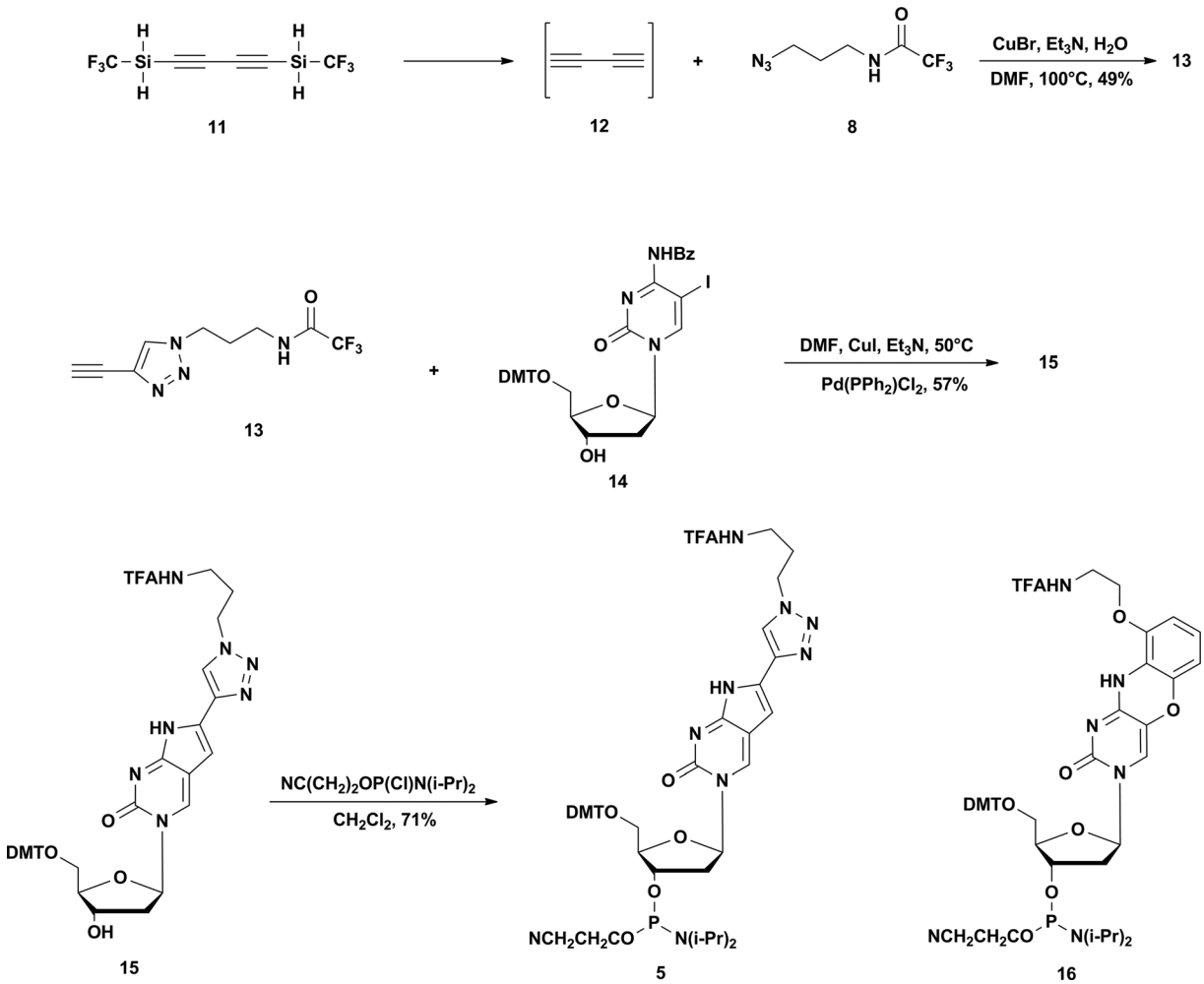
Therefore, we changed our synthesis strategy and made use of the Sonogashira cross-coupling reaction to connect the side chain with the pyrrolo-dC system. Recently, this protocol was used by Hudson and co-workers for the synthesis of PNA-G-clamp derivatives and by the Brown group for the preparation of *N*-methylpyrrolo-dC nucleosides.[25,33] In our synthesis route, cross-coupling was performed between the protected 5-iodo-2′-deoxycytidine **14** with the alkyne **13** to give the DMT-protected pyrrolo-dC nucleoside **15**. In one step, the benzoyl protecting group is cleaved and simultaneously the cross-coupling/annulation reaction proceeds as reported by Ohtsuka.[34] The alkyne *N*-(3-(4-ethynyl-1*H*-1,2,3-triazol-1-yl)propyl)-2,2,2-trifluoroacetamide (**13**) was synthesized following a synthetic protocol of Cuevas and co-workers using the copper(I)-catalyzed click reaction of 1,4-(bis-trifluoromethyl)silyl-buta-1,3-diyne (**11**) with *N*-trifluoroacetyl-1-azido-3-aminopropane (**8**) in the presence of CuBr (0.15 eq.).[35,36] Formation of two triazole moieties by a 'double click' reaction was not observed under these conditions. Apparently, the triple bond of compound **13** is less reactive than that of the deprotected intermediate **12**. So, **12** is consumed rather quickly, while **13** does not react in a second 'click' reaction. Phosphitylation of **15** under standard conditions gave the corresponding phosphoramidite **5** in 71% yield (Scheme 3).

Next, the phosphoramidite of the G-clamp (**16**) was synthesized. Phosphitylation of the DMT precursor[21–23] with 2-cyanoethyl *N*,*N*-diisopropylphosphoramido chloridite resulted in impure material. After flash chromatography (FC), this product formed a yellowish solid foam (90% yield) which could not be further purified by FC. It contained the phosphoramidite **16** (^{31}P-NMR: 148.8 ppm, 148.5 ppm) contaminated with P(III) hydrolysis products (phosphonates; ^{31}P-NMR: 14.59 ppm, 14.50 ppm). Consequently, the crude reaction product was dissolved in a small amount of CH_2Cl_2 and precipitated from a petroleum ether/CH_2Cl_2 mixture resulting in pure material (for details see experimental part and Figures S24–S26, supporting information†).

The synthesized compounds were characterized by elemental analyses as well as ^{1}H- and ^{13}C-NMR. The ^{13}C-NMR chemical shifts are listed in Table 2 (experimental part) and were assigned by ^{1}H-^{13}C gated-decoupled spectra (for ^{1}H-^{13}C-NMR coupling constants, see supporting information†) as well as by DEPT-135 NMR spectra.

2. Oligonucleotide synthesis and duplex stabilization of ps and aps DNA

2.1. Synthesis of oligonucleotides. Oligonucleotides were synthesized using the phosphoramidite of isoG$_d$, as well as the standard phosphoramidite building blocks following the synthesis protocol for 3′-*O*-(2-cyanoethyl)phosphoramidites.[37] This method failed for oligonucleotides incorporating the G-clamp **3**

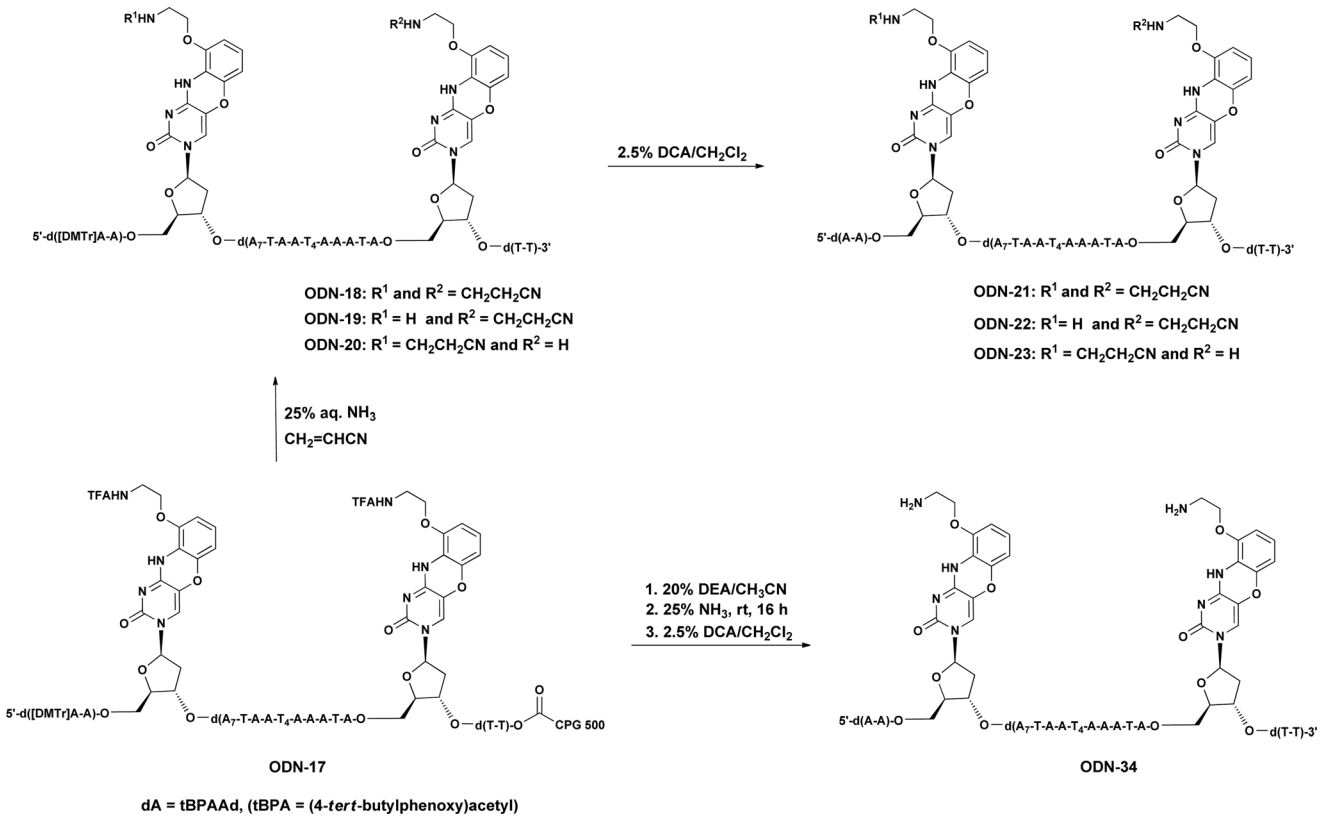


Scheme 3 Synthesis of phosphoramidite building block **5** and structure of phosphoramidite **16**.

or the pyrrolo-dC derivative **4**. Also modified protocols described in the literature resulted in a mixture of products. Careful inspection of our data showed that an acylation of the side chain with a stable protecting group introduced by the capping reagent might occur. Therefore, TAC-chemistry [(4-*tert*-butylphenoxy)acetyl as amino protecting group] has to be applied. This protocol avoids this side reaction. An even more severe side reaction is the Michael addition of the side chain amino group to acrylonitrile (Scheme 4), which is formed by *β*-elimination of the phosphate protecting groups.

Scheme 4 shows the Michael adduct formation of oligonucleotide clamp derivatives with acrylonitrile (adducts ODNs **18–20**) and the final compounds after detritylation (impurity ODNs **21–23**). The HPLC profiles of the clamp modified oligonucleotides show a large impurity peak eluted just after the expected oligonucleotide (Fig. 3a, c). The faster migrating product is the expected oligonucleotide while the second peak contains an oligonucleotide with a mass of +55 demonstrating the modification (mono-acrylonitrile adduct formation). Depending on the number of G-clamp incorporations and the work-up conditions, multiple additions of acrylonitrile are possible. In our experiments, a bis-adduct (ODN-**21**) was also isolated showing an even lower mobility. Consequently, the CPG bound oligonucleotide ODN-**17** was washed with 20% DEA/CH_3CN resulting in the deblocking of the cyanoethyl protecting groups from the immobilized oligonucleotide. Simultaneously, the formed acrylonitrile was removed by the washing procedure (for details see experimental part). Finally, the CPG-bound oligonucleotide ODN-**17** was treated with aq. NH_3 at r.t. overnight, thereby the amino protecting groups were removed and the oligonucleotide was cleaved from the solid support (Fig. 3b, d). Detritylation and purification were performed as described in the experimental part. To avoid side reactions of the side chain, all oligonucleotides containing the G-clamp **3** or the pyrrolo-dC derivative **4** were synthesized and worked-up by this protocol.

2.2. Stabilization of ps and aps oligonucleotide duplexes by the G-clamp 3 and its derivative 4. As we wanted to evaluate the influence of the G-clamp **3** and the pyrrolo-dC derivative **4** on duplex stability of ps DNA and to compare our findings with those of aps DNA, it was necessary to synthesize a set of different oligonucleotides. For this purpose, three 25-mer sequences were selected which were originally reported by Jovin *et al.*[38] The composition of the complementary strands (ODN-**25** or ODN-**26**) was altered to change the orientation of the resulting duplexes from aps to ps DNA (ps **24**·**26** and aps **24**·**25**). Ps and aps oligonucleotide duplexes were modified at defined positions to evaluate the influence of the modifications on duplex stability.



Scheme 4 Deprotection and purification protocol of oligonucleotides incorporating the G-clamp **3**.

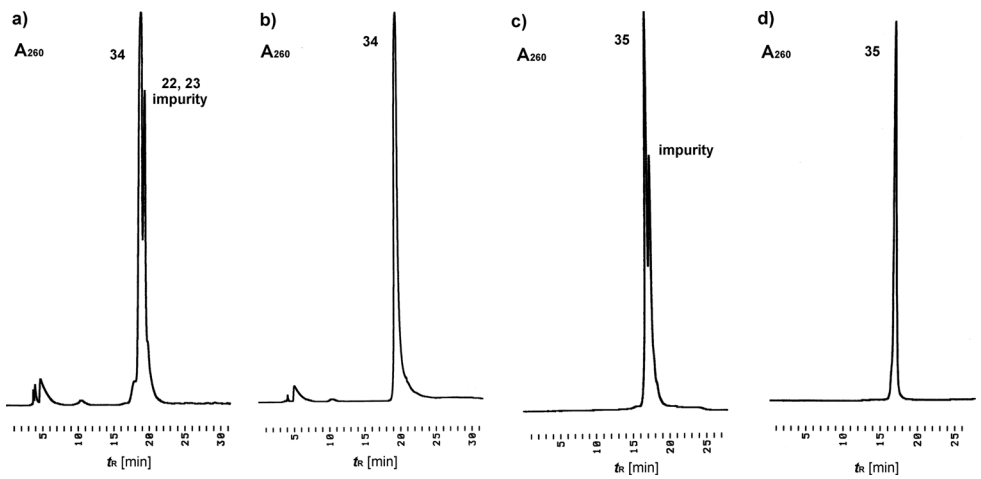


Fig. 3 HPLC (RP-18) profiles measured at 260 nm of crude detritylated oligomers (a) ODN-**34** containing **3** without deblock/washing step. (b) ODN-**34** containing **3** with deblock/washing step. (c) ODN-**35** containing **4** without deblock/washing step. (d) ODN-**35** containing **4** with deblock/washing step. The following gradient system was used: MeCN (A) and 0.1 M (Et_3NH)OAc (pH 7.0) (B). Gradient: 0–20 min 0–20% A, 20–25 min 20% A in B, 25–30 min 20–0% A in B with a flow rate of 0.8 cm^3 min^{-1}.

However, first the influence of the G-clamp **3** on aps duplexes was investigated under our experimental conditions.[21] For that, oligonucleotides 5′-d(TCTC**X**CTCTC) (**27**: **X** = dC, **28**: **X** = **3**, **29**: **X** = **4**) and 3′-d(AGAGGGAGAGAAAAA) (**30**) were synthesized. Under our conditions, the incorporation of one G-clamp residue **3** (**28**·**30**: T_m = 63 °C) led to a T_m-increase of 16 °C compared to the parent duplex **27**·**30** (T_m = 47 °C), which is similar to the stabilization (+18 °C) of a closely related duplex reported by Matteucci *et al.*[21] The pyrrolo-dC **4** increased the T_m by only 5 °C (**29**·**30**: T_m = 52 °C). Moreover, to exclude participation of nitrogen-7 in hydrogen bonding and to prove base pair motif **I**, we chose ODN-**30** to replace one dG (**2**) residue by 7-deaza-2′-deoxyguanosine (c^7G_d) and obtained 3′-d(AGAGc^{7}G$_d$-GAGAGAAAAA) (**31**).[39] As the T_m value of **28**·**31** was only slightly decreased (T_m = 60 °C) compared to the parent duplex **28**·**30** (T_m = 63 °C), nitrogen-7 of 2′-deoxyguanosine (**2**) is not expected to participate in the formation of the fourth hydrogen bond. Instead, the oxo group of dG is the acceptor side as

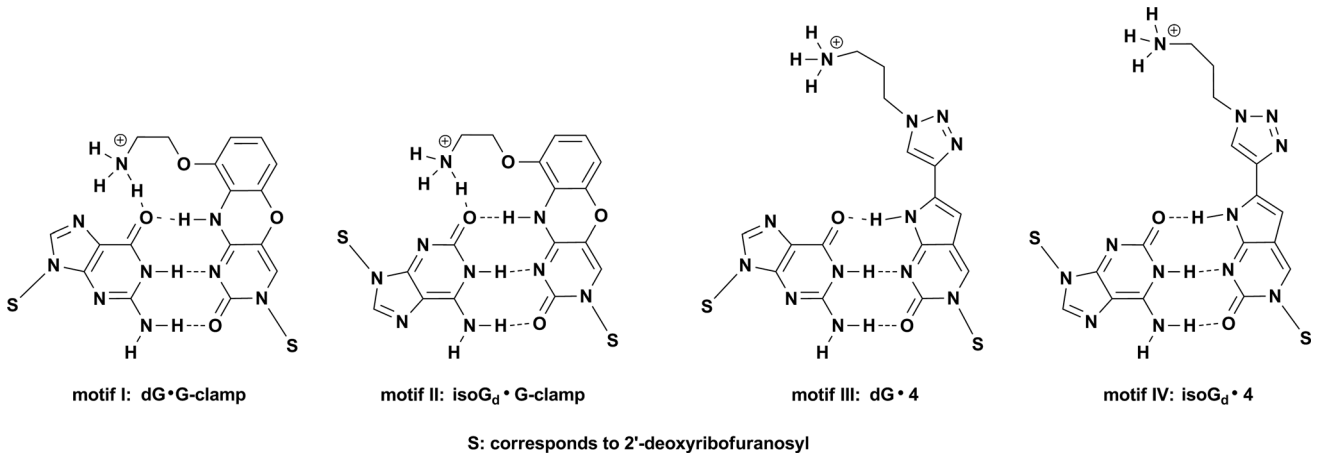


Fig. 4 Possible base pair motifs of the G-clamp **3** and pyrrolo-dC derivative **4** in ps and aps DNA.

Table 1 T_m values of ps- and aps-DNA duplexes containing the G-clamp **3**, the pyrrolo-dC derivative **4** as well as isoG$_d$ (**1**)[a,b]

ps Duplexes	T_m [°C]	ΔT_m[c] [°C]	aps Duplexes	T_m [°C]	ΔT_m[d] [°C]
5′-d(AAAAAAAAAATAATTTTAAATATTT)-3′(**24**) 5′-d(TTTTTTTTTTATTAAAATTTATAAA)-3′(**26**)	42	—	5′-d(AAAAAAAAAATAATTTTAAATATTT)-3′(**24**) 3′-d(TTTTTTTTTTATTAAAATTTATAAA)-5′(**25**)	56	—
5′-d(AA**C**AAAAAAATAATTTTAAATA**C**TT)-3′(**32**) 5′-d(TT**1**TTTTTTTATTAAAATTTAT**1**AA)-3′(**33**)	47	—	5′-d(AA**C**AAAAAAATAATTTTAAATA**C**TT)-3′(**32**) 3′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-5′(**37**)	60	—
5′-d(AA**3**AAAAAAATAATTTTAAATA**3**TT)-3′(**34**) 5′-d(TT**1**TTTTTTTATTAAAATTTAT**1**AA)-3′(**33**)	58	+11	5′-d(AA**3**AAAAAAATAATTTTAAATA**3**TT)-3′(**34**) 3′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-5′(**37**)	65	+5
5′-d(AA**4**AAAAAAATAATTTTAAATA**4**TT)-3′(**35**) 5′-d(TT**1**TTTTTTTATTAAAATTTAT**1**AA)-3′(**33**)	48	+1	5′-d(AA**4**AAAAAAATAATTTTAAATA**4**TT)-3′(**35**) 3′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-5′(**37**)	61	+1
5′-d(AA**C**AAAAAAATAATTTTAAATA**C**TT)-3′(**32**) 5′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-3′(**36**)	39	−3			
5′-d(AA**3**AAAAAAATAATTTTAAATA**3**TT)-3′(**34**) 5′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-3′(**35**)	43	+1			
5′-d(AA**4**AAAAAAATAATTTTAAATA**4**TT)-3′(**35**) 5′-d(TT**G**TTTTTTTATTAAAATTTAT**G**AA)-3′(**36**)	39	−3			

[a] Measured at 260 nm in 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Na-cacodylate (pH 7.0) with 5 μM + 5 μM single-strand concentration. [b] The T_m values were determined from the melting curves using the software MELTWIN, version 3.0. [c] ΔT_m was calculated as $T_m^{\text{modified duplex}} - T_m^{\text{unmodified duplex}}$ using **24**·**26** or **32**·**33** as comparison. [d] ΔT_m was calculated as $T_m^{\text{modified duplex}} - T_m^{\text{unmodified duplex}}$ using **24**·**25** or **32**·**37** as comparison.

Table 2 ^{13}C-NMR chemical shifts (δ) of the pyrrolo-dC analogue **4** and its intermediates[a]

Cpd	C(2)[b]	C(4)[b]	C(4a)[b]	C(5)[b]	C(6)[b]	C(7a)[b]	C1′	C2′	C3′	C4′	C5′	=CH	CH_2	C=O	CF_3
6	153.7	140.0	104.6	111.3	135.5	170.3	88.0	41.2	69.5	88.3	60.6	—	—	—	—
13	—	—	—	—	—	—	—	—	—	—	—	128.6, 128.4	47.4, 36.5, 28.6	156.4	115.9
15	153.8	136.0	108.7	96.2	139.1	158.2	86.8	41.5	69.4	85.8	62.9	127.8, 129.7	47.4, 36.7, 28.9	156.2	115.9

[a] Measured in [d_6]DMSO at 298 K. [b] Systematic numbering.

already suggested by Matteucci *et al.* (motif **I**, Fig. 4).[21] These results showed that the G-clamp **3** represents a useful tool to strengthen the dG·dC base pair in aps DNA.

We expect that the G-clamp **3** should be able to form a base pair with 2′-deoxyisoguanosine (isoG$_d$, **1**) in a reverse Watson–Crick pair as indicated in motif **II** (Fig. 4). In this regard, the capability of the clamps **3** and compound **4** to stabilize ps DNA was studied next using the 25-mer duplexes shown in Table 1. For that, two dG·dC as well as two isoG$_d$·dC base pairs were incorporated first, and then the dC residues were replaced by the clamp **3** or pyrrolo-dC **4** (Table 1).

From Table 1 the following conclusions can be drawn: (i) ps oligonucleotide duplexes (25-mers) formed by dA·dT base pairs are less stable (**24**·**26**: T_m = 42 °C) than corresponding aps

duplexes (**24**·**25**: T_m = 56 °C), a phenomenon which was already reported earlier for related duplexes by our laboratory and by others.[17,18] (ii) The incorporation of two dG·dC base pairs in antiparallel duplexes (**32**·**37**, ΔT_m = +4 °C) or $isoG_d$·dC base pairs in parallel duplexes (**32**·**33**, ΔT_m = +5 °C) results in increased duplex stability. These findings are in line with earlier reports on parallel DNA containing dA·dT and $isoG_d$·dC base pairs.[18,40] (iii) The introduction of two G-clamp residues (**3**) replacing dC in the $isoG_d$·dC base pair (ps DNA) or dG·dC base pair (aps DNA) leads to an extraordinary duplex stabilization in ps DNA (**34**·**33**: ΔT_m = +11 °C) and aps DNA (**34**·**37**: ΔT_m = +5 °C). This strong stabilization of the G-clamp **3** in ps DNA was unexpected and will be discussed below on the basis of base pair motifs. (iv) The incorporation of pyrrolo-dC derivative **4** instead of dC in the $isoG_d$·dC base pair (ps DNA) or in the dG·dC base pair (aps DNA) almost did not alter the T_m-value. It should be noted that all duplexes described above were composed of complementary base pairs.

Subsequently, duplex stability was studied for ps DNA containing two mismatches (dG·dC pairs). It has been reported earlier, that the dG·dC base pair represents a mismatch in ps DNA.[40] From Table 1, it can been seen that the ps duplex **32**·**36** with two dG residues located opposite to dC is slightly destabilized (T_m = 39 °C) compared to the parent ps duplex **24**·**26** (T_m = 42 °C). A similar effect is observed for the pyrrolo-dC derivative **4** (**35**·**36**: T_m = 39 °C), while a tendency of stabilization is observed when the G-clamp **3** is replacing dC (**34**·**35**, T_m = 43 °C).

Due to the change of the strand orientation from aps to ps DNA together with the change of the donor–acceptor pattern of the nucleobase (dG → $isoG_d$), the base pair motif **IV** (G-clamp·$isoG_d$) in ps duplexes closely resembles that of the base pair motif **I** (G-clamp·dG) in aps duplexes. Both base pairs (motifs **I** and **II**) form a fourth hydrogen bond with the ammonium group of the side chain as proton donor and the oxo group of the nucleobase as acceptor. However, it should be noted that the amino as well as the oxo group of $isoG_d$ and dG can behave differently in their donor and acceptor properties. This results from their positions in the pyrimidine moiety of the nucleobase which affects the electronic properties and reactivity of the substituents, supported by the fact that it is necessary to protect the oxo function of $isoG_d$ in DNA synthesis. Moreover, keto-enol tautomerism is observed for the 2-oxo group of $isoG_d$ but not for the 6-oxo group of dG which leads to base pairing ambiguity of the modified nucleoside.

Concerning the base pairing pattern (motifs **III** and **IV**) of the pyrrolo-dC derivative **4**, it can be concluded that **4** forms a perfectly matched base pair with $isoG_d$ in ps duplexes and with dG in aps duplexes, but in both cases the fourth hydrogen bond is lacking. Consequently, this compound does not act as a 'G-clamp', but instead compound **4** offers the opportunity to use the 'free' amino group as labeling site at a position not disturbing the DNA duplex structure.

Conclusion and outlook

The novel pyrrolo-dC analogue **4** tethered with an alkylamino side chain was synthesized and converted into a phosphoramidite building block. DNA with parallel chain orientation was constructed incorporating dA·dT and $isoG_d$·dC base pairs. dC residues were replaced by the clamp **3** or pyrrolo-dC derivative **4**. Special deprotection conditions were used to avoid formation of clamp acrylonitrile adducts during oligonucleotide deprotection. When dC was replaced by the G-clamp **3**, extraordinary stable parallel stranded duplexes were formed, while the pyrrolo-dC moiety **4** behaved like dC. The G-clamp **3**·$isoG_d$ base pair of parallel stranded DNA resembles the G-clamp·dG base pair formed in aps DNA with even higher efficiency in ps than in aps DNA. The stabilizing property of the G-clamp **3** will improve the application of ps DNA in chemical biology and for the construction of DNA nanostructures.

Experimental section

General methods

All chemicals and solvents were of laboratory grade as obtained from commercial suppliers and were used without further purification. Thin-layer chromatography (TLC) was performed on TLC aluminum sheets covered with silica gel 60 F254 (0.2 mm).

Flash column chromatography (FC): silica gel 60 (40–60 μM) at 0.4 bar. UV spectra: λ_{max} in nm, ε in $dm^3\ mol^{-1}\ cm^{-1}$. NMR spectra: measured at 300.15 MHz for ^1H, 75.48 MHz for ^{13}C and 121.52 MHz for ^{31}P. The J values are given in Hz and δ in ppm. For NMR spectra measured in [d_6]DMSO, the chemical shift of the solvent peak was set to 2.50 ppm for ^1H NMR and 39.50 ppm for ^{13}C NMR. Reversed-phase HPLC was carried out on a 250 × 4 mm RP-18 LiChrospher 100 column with a HPLC pump connected with a variable wavelength monitor, a controller and an integrator. Gradients used for HPLC chromatography: A = MeCN; B = 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN, 95 : 5. Conditions: (I) 3 min 15% A in B, 12 min 15–50% A in B, and 5 min 50–10% A in B, flow rate 0.7 $cm^3\ min^{-1}$; (II) 0–25 min 0–20% A in B, flow rate 0.7 $cm^3\ min^{-1}$. The molecular masses of oligonucleotides were determined by LC-ESI-TOF mass spectrometry or by MALDI-TOF mass spectrometry in the linear negative or linear positive mode with 3-hydroxypicolinic acid (3-HPA) as a matrix. The detected masses were in line with the calculated values (Table S2, supporting information†).

Synthesis, purification and characterization of oligonucleotides not containing compounds 3 and 4

The syntheses of oligonucleotides were performed on a DNA synthesizer at a 1 μmol scale (trityl-on mode) using the phosphoramidite of $isoG_d$, as well as the standard phosphoramidite building blocks following the synthesis protocol for 3′-*O*-(2-cyanoethyl)phosphoramidites.[37] After cleavage from the solid support, the oligonucleotides were deprotected in 25% aqueous ammonia solution for 12–16 h at 60 °C. The purification of the "trityl-on" oligonucleotides was carried out on reversed-phase HPLC (RP-18 column; gradient system I). The purified "trityl-on" oligonucleotides were treated with 2.5% of $Cl_2CHCOOH$/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 (gradient II). The oligomers were desalted on a short column using distilled water for elution of salt, while the oligonucleotides were eluted with H_2O/MeOH (2 : 3). Then, the

solvent was evaporated using a SpeedVac evaporator to yield colorless solids which were frozen at −24 °C. The molecular masses of the oligonucleotides were determined by LC-ESI-TOF mass spectrometry or by MALDI-TOF mass spectrometry in the linear negative or positive mode (for spectra, see Figures S1–S5, supporting information†).

Synthesis, purification and characterization of oligonucleotides containing compounds 3 and 4

Oligonucleotides containing the G-clamp **3** or pyrrolo-dC derivative **4** were synthesized using the building blocks of dA, dG and dC bearing (4-*tert*-butylphenoxy)acetyl groups (TAC-chemistry) for amino protection. After completion of solid-phase synthesis, the oligonucleotides were kept on the CPG-support. A syringe was adapted to the CPG-column, and a solution of 20% diethylamine in CH_3CN (30 cm^3) was continuously flushed through the support over a period of 15 min at rt. Thereby the cyanoethyl residues bound to the phosphate groups were cleaved and at the same time the resulting acrylonitrile was washed out continuously. Then, the CPG was washed with CH_3CN (5 cm^3), dried by a stream of air and transferred to a vial. 25% aq. NH_3 was added and the mixture was kept for 16 h at rt to remove the amino protecting groups and to cleave the oligonucleotides from the solid support. Next, the mixture was centrifuged and the supernatant containing the oligonucleotides was further purified and detritylated as described above for the standard oligonucleotides.

Melting experiments

The melting temperature curves were measured with a UV-vis spectrophotometer equipped with a thermoelectrical controller. The temperature was measured continuously in the reference cell with a Pt-100 resistor with a heating rate of 1 °C min^{-1}, and the absorbance at 260 nm was recorded as a function of the temperature. The data of duplex formation were calculated by the MeltWin (version 3.0) program using the curve fitting of the melting profiles according to a two-state model.[41]

3-(2-Deoxy-*β*-D-*erythro*-pentofuranosyl)-6-ethynyl-furo[2,3-*d*]pyrimidin-2(3*H*)-one (6)

To a mixture of 6-bromo-3-(3,5-di-*O*-acetyl-2-deoxy-*β*-D-*erythro*-pentofuranosyl)-furo[2,3-*d*]pyrimidin-2(3*H*)-one (**9**)[32] (500 mg, 1.20 mmol), $Pd(PPh_3)_4$ (139 mg, 0.12 mmol) and Et_3N (0.35 cm^3, 2.4 mmol) in DMF (10 cm^3) was added trimethylsilylacetylene (1.66 cm^3, 12 mmol). The reaction mixture was purged with N_2 and stirred in the dark for 4 h. The volatiles were removed, the residue was adsorbed on silica gel and purified by FC (CH_2Cl_2/EtOAc 4 : 1) to afford a yellow solid, which was dissolved in saturated NH_3/MeOH (30 cm^3) cooled at 0 °C and stirred for 3 h. The solvent was removed, the residue was adsorbed on silica gel and purified by FC (CH_2Cl_2/MeOH 20 : 1) to afford compound **6** as a white solid (150 mg, 45%). (Found: C, 56.73; H, 4.20; N, 10.10. $C_{13}H_{12}N_2O_5$ requires C, 56.52; H, 4.38; N, 10.14%); TLC (silica gel, CH_2Cl_2/MeOH 9 : 1) R_f 0.30; λ_{max} (MeOH)/nm 341 (ε/dm^3 mol^{-1} cm^{-1} 10 300), 263 (15 000); δ_H (300 MHz; [d_6]DMSO; Me_4Si) 2.03–2.12 (1 H, m, 2′-H_α), 2.37–2.45 (1 H, m, 2′-H_β), 3.57–3.71 (2 H, m, 2x 5′-H), 3.91–3.95 (1 H, m, 4′-H), 4.19–4.24 (1 H, m, 3′-H), 5.05 (1 H, s, H–C≡H), 5.14 (1 H, t, J = 5.1 Hz, 5′-OH), 5.30 (1 H, d, J = 4.2 Hz, 3′-OH), 6.12 (1 H, s, 1′-H), 7.19 (1 H, s, 5-H), 8.90 (1 H, s, 4-H).

N-(3-(4-Ethynyl-1*H*-1,2,3-triazol-1-yl)propyl)-2,2,2-trifluoroacetamide (13)

In a 25 cm^3 round-bottom flask *N*-trifluoroacetyl-1-azido-3-aminopropane[36,42] (1.15 g, 5.87 mmol) (**8**), 1,4-bis(trifluoromethyl) silyl)buta-1,3-diyne (2.02 g, 8.20 mmol) (**11**), CuBr (126 mg, 0.88 mmol), Et_3N (1.6 cm^3, 11.74 mmol) and H_2O (0.2 cm^3, 11.74 mmol) in DMF (4 cm^3) were heated at 100 °C for 4 h. The reaction mixture was diluted with ethyl acetate (20 cm^3) and washed with sat. aq. NH_4Cl solution. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by FC (silica gel, CH_2Cl_2/EtOAc 4 : 1) to afford compound **13** (708 mg, 49%) as a white solid. (Found: C, 43.93; H, 3.65; N, 22.61. $C_9H_9F_3N_4O$ requires C, 43.91; H, 3.68; N, 22.76%); TLC (silica gel, CH_2Cl_2/EtOAc 2 : 1) R_f 0.41; δ_H (300 MHz; [d_6]DMSO; Me_4Si) 2.04–2.11 (2 H, m, CH_2), 3.19 (2 H, t, J = 6.9 Hz, CH_2), 4.38–4.43 (3 H, m, CH, CH_2), 8.46 (1 H, s, ═CH), 9.48 (1 H, s, NH).

3-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-6-{[1*H*-(1′,2′,3′-triazol-4′-yl)propyl]-2-trifluoroacetamide}-2-oxo-3,7-dihydro-2*H*-pyrrolo[2,3-*d*]pyrimidine (15)

A mixture of compound **13** (500 mg, 2.03 mmol) and 5-iodo-5′-*O*-(dimethoxytrityl)- 4-*N*-benzoyl-2′-deoxycytidine (516 mg, 0.68 mmol) (**14**), $Pd(PPh_3)Cl_2$ (48 mg, 0.068 mmol), CuI (26 mg, 0.14 mmol) in Et_3N (8 cm^3) and DMF (4 cm^3) was heated at 50 °C for one day. Then, the solvent was evaporated, and the residue was purified by FC (silica gel, CH_2Cl_2/MeOH 20 : 1) to afford compound **15** (300 mg, 57%) as a yellow foam. (Found: C, 60.71; H, 5.10; N, 12.48. $C_{39}H_{38}F_3N_7O_7$ requires C, 60.54; H, 4.95; N, 12.67%); TLC (silica gel, CH_2Cl_2/MeOH 9 : 1) R_f 0.36; λ_{max} (MeOH)/nm 360 (ε/dm^3 mol^{-1} cm^{-1} 8 300) and 238 (41 500); δ_H (300 MHz; [d_6]DMSO; Me_4Si) 2.08–2.21 (3 H, m, 2′-H, CH_2), 2.42–2.46 (1 H, m, 2′-H), 3.25–3.27 (3 H, m, 5′-H, CH_2), 3.30–3.33 (1 H, m, 5′-H), 3.71 (6 H, s, 2x OCH_3), 3.98–4.03 (1 H, m, 4′-H), 4.39–4.48 (3 H, m, 3′-H, CH_2), 5.39–5.44 (1 H, m, 3′-OH), 5.98 (1 H, s, 6-H), 6.26 (1 H, t, J = 6.0 Hz, 1′-H), 6.88–7.43 (13 H, m, arom-H), 8.38 (1 H, s, ═CH), 8.61 (1 H, s, 4-H), 9.54 (1 H, s, NH), 11.83 (1 H, s, CONH).

3-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-6-{[1*H*-(1′,2′,3′-triazol-4′-yl)propyl]-2-trifluoroacetamide}-2-oxo-3,7-dihydro-2*H*-pyrrolo[2,3-*d*]pyrimidine 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)] phosphoramidite (5)

To the solution of compound **15** (280 mg, 0.36 mmol) in dry CH_2Cl_2 (10 cm^3), (i-Pr)$_2$EtN (0.12 cm^3, 0.72 mmol) and

2-cyanoethyl *N*,*N*-diisopropylphosphoramido chloridite (0.11 cm^3, 0.47 mmol) were added. The solution was stirred at rt for 30 min. The solution was diluted with CH_2Cl_2 (40 cm^3) and washed with 5% aq. $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2 (2 × 20 cm^3). The combined organic phase was dried (Na_2SO_4), filtered, and evaporated. The residue was purified by FC (column 15 × 5 cm, CH_2Cl_2/MeOH 20 : 1). Compound **5** was obtained as a yellow foam (249 mg, 71%). TLC (silica gel, CH_2Cl_2/acetone 4 : 1): R_f 0.5; δ_P (121.52 MHz; $CDCl_3$; H_3PO_4) 149.1, 148.9.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-9-(2-trifluoroacetamidoethoxy)-1,3-diaza-2-oxophenoxazine 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)] phosphoramidite (16)

As described above, the DMT compound of the G-clamp[21–23] (5.0 g, 6.45 mmol) was dissolved in dry CH_2Cl_2 (20 cm^3), (i-Pr)$_2$EtN (2.12 cm^3, 12.72 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramido chloridite (2.12 cm^3, 9.05 mmol). The solution was stirred at rt for 30 min. The solution was diluted with CH_2Cl_2 (70 cm^3) and washed with 5% aq. $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2 (2 × 40 cm^3). The combined organic phase was dried (Na_2SO_4), filtered, and evaporated. The residue was purified by FC (column 15 × 5 cm, CH_2Cl_2/acetone 4 : 1). Compound **16** was obtained as crude phosphoramidite as a yellow foam (5.7 g, 90%). δ_P (300 MHz; $CDCl_3$; H_3PO_4) 148.8, 148.5 (65%); 14.592, 14.505 (35%). The crude product was dissolved in a small amount of CH_2Cl_2 (~ 5 cm^3) and precipitated by dropwise addition of petroleum ether/CH_2Cl_2 (15 : 1). The supernatant was decanted, and the resulting precipitate was washed with PE/CH_2Cl_2 (15 : 1, 3 × 10 cm^3), dissolved in CH_2Cl_2 (20 cm^3), transferred to a round bottom flask and evaporated to dryness to yield **16** as a yellowish foam (3.5 g, 55%). δ_P (121.52 MHz; $CDCl_3$; H_3PO_4) 148.8, 148.5. (for spectrum, see supporting information, Figure S26†). TLC (silica gel, EtOAc/CH_2Cl_2 4 : 1): two fluorescent spots R_f 0.6, 0.7.

Acknowledgements

We thank S. S. Pujari and H. Mei for the measurements of the NMR spectra, N. Q. Tran for oligonucleotide synthesis, and Dr Uhlmann, Coley Pharmaceuticals GmbH for ESI mass spectra and Dr R. Thiele from Roche Diagnostics, Penzberg, for the measurement of the MALDI spectra. We gratefully acknowledge financial support by ChemBiotech, Münster, Germany.

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