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Graphical abstract

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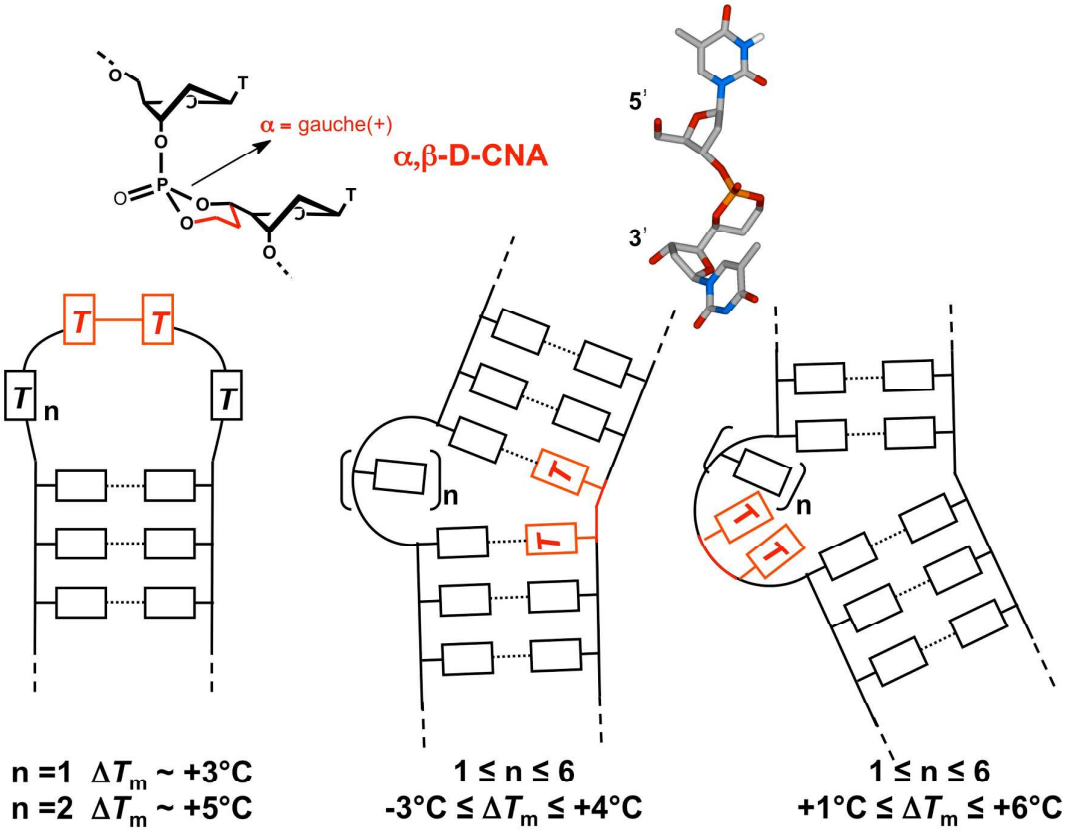

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ARTICLE TYPE

Stabilization of hairpins and bulged secondary structures of nucleic acids by single incorporation of α,β-D-CNA featuring a gauche(+) alpha torsional angle

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Constrained dinucleotide unit featuring a *gauche*(+) alpha torsional angle configuration stabilized DNA hairpin or bulged structures. Large five nucleotides looped hairpin structures can be stabilized up to +5 °C. Depending on the nature of the closing base pair, the increase of hairpin stability can be reached through loop moiety preorganization or stem rearrangement induced on the first two base pairs. With an alpha *gauche*(+) constrain either within or opposite to the bulge, the larger the bulged structures, the better the stabilization.

Introduction

Constrained nucleotides have been introduced and developed with the aim of increasing duplex formation ability mostly in the context of antisense applications or antigen therapies.[1] Despite of their biological relevant functions, secondary nucleic acid structures such as hairpins, bulges or junctions have received far less attention in terms of designed modified nucleotides able to preorganize and stabilize them.[2]

It is also well established that these disparate structures, which are predisposed to promote a significant local conformational heterogeneity in the sugar—phosphate backbone, play a crucial role in fundamental biological processes where protein—nucleic acid interactions, folding, or catalytic activity are involved. As proposed by few studies, the sugar/phosphate backbone of these unusual motifs exhibit a variety of conformations which markedly differ from the regular conformational states of nucleic acid duplex.[3] Unfortunately, experimental studies aimed at determining the structural and functional implications of such structural deformations are somewhat complicated by the intrinsically transient nature of the corresponding backbone states. Stable structural analogues of these distorted backbone geometries would be very useful in the elucidation of the role that uncanonical conformations play in nucleic acid interactions and folding.

We have engaged a program towards the design and the synthesis of structural analogues of nucleosides in which the sugar/phosphate backbone is constrained at selected positions by a dioxaphosphorinane structure that allowed fixing the values of two or more torsional angles (Fig.1).[4] Among the synthesized Dioxaphosphorinane-Constrained Nucleic Acid dinucleotide (D-CNA), those featuring B-DNA canonical values of the torsional angles showed improved duplex formation ability.[5] The behaviour of these constrained analogues were easy to understand

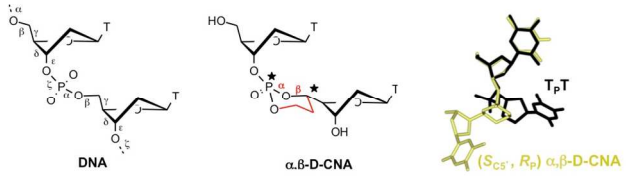

Fig.1. Left: the six backbone torsion angles (labeled α to ζ) of nucleic acids. Middle: the α,β-D-CNA dinucleotide in which α and β are stereocontrolled by a dioxaphosphorinane ring structure. Right: superimposition of X-ray structures of α,β-D-CNA TT featuring an alpha *gauche*(+) configuration (gold)[6] and of unmodified T_pT (black).[7]

in that case according to the preorganization concept[8] and then could be selected as unnatural nucleoside analogs for antisense therapy.[9] However, many of the D-CNAs exhibited conformations that strongly differ with those necessary to fit with the duplex structure (Fig.1).[10] Therefore, as a consequence, the preorganization concept could be extended to nucleic acid secondary structures in which the sugar/phosphate backbone is necessarily distorted to ensure the functional folding.

Bulges and hairpin loops belong to the most important structural motifs in folded nucleic acids that can be of functional importance as ligand recognition elements, folding initiation sites or they may be created in DNA recombination or replication process.[11] These bulged motifs consist of Watson-Crick base paired stem structures and a loop or bulge sequence with unpaired or non-canonical (WC) paired nucleotides made when one or more nucleotide cannot have regular hydrogen bonding with counterparts in the opposite strands.

Similarly to the hairpin loop structure, the directionality of the sugar/phosphate backbone in nucleic acid bulges can be changed rather sharply in both strands of the structure as depicted in a solution structure of a five nucleotides DNA bulge.[12] The bulge

induces a sharp kink in the structure and the torsional stress within the loop of the bulge is located at one specific phosphate while preventing base stacking at the kinking site. Meanwhile on the opposite strand, the kink induced in between the two stems of the structure is also ensured by the phosphate connecting the stem residues still involved in Watson-Crick base pairing. Therefore, according to the preorganization concept,[8] it appeared that a constrained dinucleotide featuring alpha torsional angle deviating from the normal range of -62±15° (*gauche*(-)) observed in B-DNA[13] to a *gauche*(+) conformation (Fig. 1) could play the role of the turning phosphate either in the loop moiety or within the strand opposite to the bulge. Therefore it should be suitable for preorganisation of the single strand and as a consequence should induce stabilization of these secondary structures.

We have already communicated that Dioxaphosphorinane-Constrained Nucleic Acid dinucleotide (D-CNA) featuring non-canonical *gauche*(+) a□□□□ torsional angle value installed within four nucleotides loop moiety of hairpin structures was the first constrained nucleotide analogue able to stabilize the overall secondary structure (ΔTm > +3°C).[14] However, this seminal study showed that the effect was dependant firstly of the position of the strain within the unpaired strand and secondly of the nature of the loop-closing base pair AT or CG.

Herein, in order to get insight into the stem rearrangement observed only in the case of hairpin with a CG closing base pair, we studied four thymidine-looped hairpins with stem composed from seven to two base pairs. Then, we present the effect of the *gauche*(+) constrain applied on □lpha torsional angle in larger loop made of five thymidines either with AT or CG closing base pair. Eventually, we introduced the *gauche*(+) torsional stress in or opposite to bulges of various sizes in double stranded oligonucleotides. With these investigations we aim at understanding and determining the rules that govern the use of conformationally constrained nucleotides featuring non-B-DNA conformations within unpaired moieties of nucleic acid secondary structures.

Results and Discussion

In this report $^{i}H_{AB}T_{j}$ and $^{i}H_{AB}T_{j}gp_{x}$ will denote hairpin structures with i: number of stem base pairs ($2 \leq i \leq 7$), AB: nature of the closing base pair (AT or CG), j: number of thymidine within the loop (4 or 5) and x: position of the a□□□□ *gauche*(+) torsional angle constrain (gp) within the loop.

Study of *gauche(+)* constrained D-CNA within T_4 loop on hairpin stem rearrangement

Our preliminary study showed that within T_4-loop of hairpins with CG closing base pair, an a□□□□ *gauche*(+) constrain located in the middle of the unpaired moiety induced a remarkable stabilizing effect of +3.5°C (Table 1, entry 2 *vs* 1) whereas a similar trend was also observed when the constrain was installed close to the 3'-end of the loop with a ΔTm of +2.7°C (Table 1, entry 3). In the former case, the effect could be attributed to a preorganization of the loop because it was known that the structure reversed the directionality of the strand at this specific position by adopting a *gauche*(+) of a□□□□ torsional angle at the so-called turning phosphate.[15] On the other hand the stabilization depicted by shifting the constrain to the 3'-end of the loop was accompanied with a stem rearrangement evidenced by circular dichroism with a displacement of the positive Cotton band from 278 to 262 nm (Figure 2 compare $^{7}H_{CG}T_{4}$ and $^{7}H_{CG}T_{4}gp_{3}$) showing a partial change of the stem from B- to A-type double helix. Therefore, to get insight into the behaviour of the stem towards the torsional stress applied to the sugar/phosphate backbone in these CG-closed loops, we synthesized T4-hairpins with stem from seven to two base pairs. In all these hairpins, we installed the *gauche*(+) torsional stress on α either in the middle of the loop ($^{i}H_{CG}T_{4}gp_{2}$, $2 \leq i \leq 7$) or close to the 3'-end ($^{i}H_{CG}T_{4}gp_{3}$, $2 \leq i \leq 7$). Thermal denaturation curves and circular dichroism spectra were recorded for unmodified and constrained hairpins, results and spectra are reported in Table 1 and Figure 2 respectively. It is noteworthy that all denatured hairpins exhibited the same circular dichroism spectrum with a maximum of the positive Cotton band at about 278 nm (see SI, Fig S7-9).

When shortening the stem length from seven to four base pairs, the melting temperatures (*T*m), determined by UV-spectrophotometry, decreased from 74.0°C to 53.2°C (Table 1, entries 1, 4, 7 and 10). But reducing the stem length to two or three base pairs resulted in the impossibility to observe any transition in the melting curves and therefore disabled the *T*m determination either for unmodified or constrained hairpins.

Interestingly, wherever the *gauche*(+) constrain was applied the shortened hairpins were nicely stabilized as depicted by UV-spectrophotometry for hairpins with stem composed of six to four base pairs. Except for $^{7}H_{CG}T_{4}$ in which the main stabilizing effect was brought with constrain in the middle of the loop (Table 1, entries 2 *vs* 3), the predominant effect upon stabilization was established with the constrain close to the 3'-end of the loop (Table 1, $^{i}H_{CG}T_{4}gp_{2}$ *vs* $^{i}H_{CG}T_{4}gp_{3}$, $6 \leq i \leq 4$, entries 6 *vs* 5, 9 *vs* 8 and 12 *vs* 11) with an induced stem rearrangement as depicted by circular dichroism with an increasing displacement of the main band from 280 to 260 nm (Figure 2 bottom).

The maximum of ΔTm +4.1°C observed for $^{4}H_{CG}T_{4}gp_{3}$ (Table 1, entry 12) led us to speculate that the *gauche(+)* constrain, due to the shortening of the hairpin stem, became more efficient on the stem rearrangement and as a consequence on the hairpin stabilization.

Table 1: Thermal melting temperatures [°C] of α,β-D-CNA within T_4 looped hairpin structures with shortened stems

Entry	Name	Sequence (5'-3')[a]	*T*m [°C][b]	Δ*T*m [°C]
1	$^{7}H_{CG}T_4$	AGGATCC*TTTT*GGATCCT	74.0	-
2	$^{7}H_{CG}T_4gp_2$	AGGATCC*T**TT***TGGATCCT	77.5	+3.5
3	$^{7}H_{CG}T_4gp_3$	AGGATCC*TT**TT***GGATCCT	76.7	+2.7
4	$^{6}H_{CG}T_4$	GGATCC*TTTT*GGATCC	69.1	-
5	$^{6}H_{CG}T_4gp_2$	GGATCC*T**TT***TGGATCC	71.2	+2.1
6	$^{6}H_{CG}T_4gp_3$	GGATCC*TT**TT***GGATCC	71.8	+2.7
7	$^{5}H_{CG}T_4$	GATCC*TTTT*GGATC	63.0	-
8	$^{5}H_{CG}T_4gp_2$	GATCC*T**TT***TGGATC	65.0	+2.0
9	$^{5}H_{CG}T_4gp_3$	GATCC*TT**TT***GGATC	65.1	+2.1
10	$^{4}H_{CG}T_4$	ATCC*TTTT*GGAT	53.2	-
11	$^{4}H_{CG}T_4gp_2$	ATCC*T**TT***TGGAT	56.2	+2.9
12	$^{4}H_{CG}T_4gp_3$	ATCC*TT**TT***GGAT	57.3	+4.1
13	$^{3}H_{CG}T_4$	TCC*TTTT*GGA	nd	-
14	$^{3}H_{CG}T_4gp_2$	TCC*T**TT***TGGA	nd	nd
15	$^{3}H_{CG}T_4gp_3$	TCC*TT**TT***GGA	nd	nd
16	$^{2}H_{CG}T_4$	CC*TTTT*GG	nd	-
17	$^{2}H_{CG}T_4gp_2$	CC*T**TT***TGG	nd	nd
18	$^{2}H_{CG}T_4gp_3$	CC*TT**TT***GG	nd	nd

[a] **TT** denotes a ($S_{C5'}$, R_P) α,β-D-CNA-modified TT dinucleotide and italic character denotes nucleotide within the loop. [b] Melting temperatures (average *T*m values on three times experiments ± 1°C) were measured as the maximum of the first derivate of the UV melting curve (OD_{260} *vs* temperature, 5-80 °C, 0.5 °C/min) which was recorded at concentration of 5 µM in sodium phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) and EDTA (1 mM).

Underlying this work was the question (hypothesis) that the torsional stress imposed by the modified D-CNA within the loop could be responsible of the transition from B- to A-type duplex form of a few base pairs of the stem conferring a higher stability to the hairpin structure.

Whereas UV-thermal denaturation studies were useless for the determination of the melting temperature either for the constrained or unmodified small hairpins $^{3}H_{CG}T_4$ and $^{2}H_{CG}T_4$, circular dichroism appeared to be able to provide information on the number of base pairs of the stem impacted by the torsional stress imposed by the D-CNA within the loop.

CD spectra of $^{3}H_{CG}T_4$ and $^{3}H_{CG}T_4gp_2$ (Figure 2, blue curves, up and middle) exhibited maxima at 276 and 277 nm, respectively whereas the maximum of the positive band was depicted at 267 nm for $^{3}H_{CG}T_4gp_3$ (Figure 2, down, blue curve). In the latter case the maximum was displaced to 267 nm with a shoulder around 280 nm in a broad band and therefore it can be speculated that the three base pairs were not all in A-form. Interestingly, CD spectrum of $^{2}H_{CG}T_4gp_3$ (Figure 2, down, grey curve) exhibited a narrow band centered at 264 nm while $^{2}H_{CG}T_4$ and $^{2}H_{CG}T_4gp_2$ had maxima at about 271 and 273,5 nm, respectively.

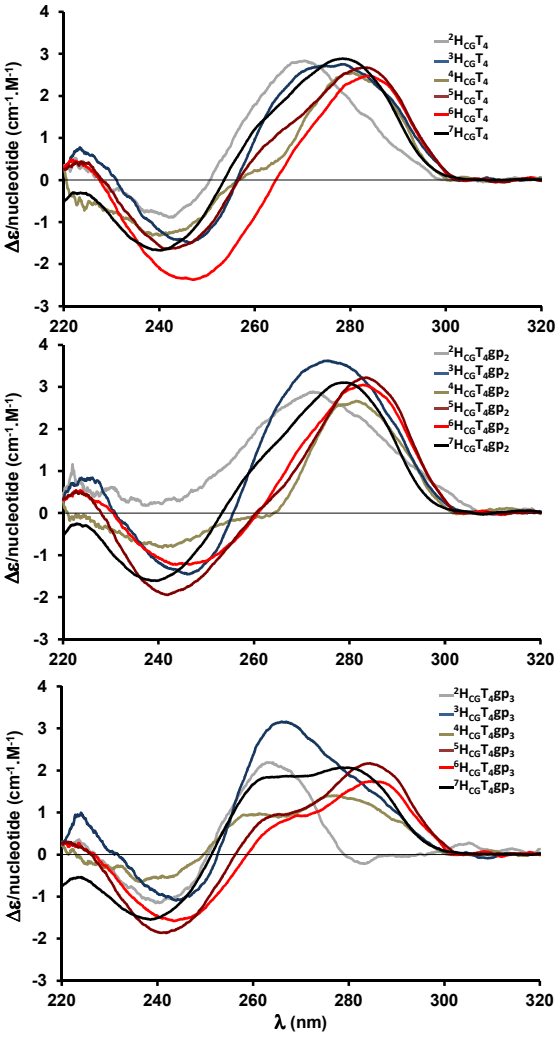

Fig.2 CD spectra of hairpins consisting of loop with four thymidine residues and stem from seven to two base pairs in sodium phosphate buffer (10 mM, pH 7.0, NaCl (100 mM), and EDTA (1 mM), T = 25°C). Up: unmodified hairpins. Middle: alpha in *gauche*(+) conformation in central position of the loop. Down: alpha in *gauche*(+) conformation in 3' end position of the loop.

These observations made on the CD spectra of short hairpins, together with melting measurements of longer ones, helped us to propose that the torsional stress brought by the D-CNA modification in the 3'-end of the loop was accommodated by a conformational change of the two closing base pairs from a B- to A-duplex form providing an effective stabilization of the hairpin structure independently of a preorganization effect.

Thermostability of *gauche(+)* constrained D-CNA within T_5 hairpins

It was of interest to investigate the concept of stabilization of

hairpin structure by means of conformational restriction within the unpaired moiety, in larger five-looped hairpins. We choose to study hairpins with the same stem sequences as the T_4-looped hairpins previously studied with an increment of one thymidine in the loop. Therefore two different T_5-looped hairpins were investigated that differ in the nature of the loop-closing base pair AT or CG in which the alpha *gauche*(+) constrain has been installed once in all possible position within the loop and denoted $^{6}H_{AT}T_5gp_i$ ($1 \leq i \leq 5$) and $^{7}H_{CG}T_5gp_i$ ($1 \leq i \leq 4$). Thermal melting temperatures determined by UV melting curves analysis of unmodified and constrained hairpins are reported in Table 2.

In comparison with $^{6}H_{AT}T_4$ and $^{7}H_{CG}T_4$, $^{6}H_{AT}T_5$ and $^{7}H_{CG}T_5$ were less stable by 2 and 6°C, respectively underlining the lower stability of larger loop hairpin structures. Specifically and as the main result, D-CNA can significantly stabilize T_5-looped DNA hairpins by +4.0 and +5.0°C (Table 2, entries 4 and 10) showing

Table 2: Thermal melting temperatures [°C] of α,β-D-CNA within T_5 looped hairpin structures

Entry	Name	Sequence (5'-3')[a]	*T*m [°C][b]	Δ*T*m [°C]
1	$^{6}H_{AT}T_5$	ATCCTA*TTTTT*TAGGAT	50.0	-
2	$^{6}H_{AT}T_5gp_1$	ATCCTA***TT***TTTTAGGAT	49.0	-1.0
3	$^{6}H_{AT}T_5gp_2$	ATCCTA*T****TT****TTTAGGAT	52.0	+2.0
4	$^{6}H_{AT}T_5gp_3$	ATCCTA*TT****TT****TTAGGAT	54.0	+4.0
5	$^{6}H_{AT}T_5gp_4$	ATCCTA*TTT****TT****TAGGAT	49.5	-0.5
6	$^{6}H_{AT}T_5gp_5$	ATCCTA*TTTT****TT****AGGAT	44.0	-6.0
7	$^{7}H_{CG}T_5$	AGGATCC*TTTTT*GGATCCT	68.0	-
8	$^{7}H_{CG}T_5gp_1$	AGGATCC***TT***TTTGGATCCT	68.3	+0.3
9	$^{7}H_{CG}T_5gp_2$	AGGATCC*T****TT****TTGGATCCT	69.8	+1.8
10	$^{7}H_{CG}T_5gp_3$	AGGATCC*TT****TT****TGGATCCT	73.0	+5.0
11	$^{7}H_{CG}T_5gp_4$	AGGATCC*TTT****TT****GGATCCT	70.6	+2.6

[a] **TT** denotes an ($S_{C5'}$, R_P) α,β-D-CNA-modified TT dinucleotide and italic character denotes nucleotide within the loop. [b] Melting temperatures (average *T*m values on three times experiments ± 1°C) were measured as the maximum of the first derivate of the UV melting curve (OD_{260} *vs* temperature, 20-90°C, 0.5 °C/min) which was recorded at concentration of 5 µM in sodium phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) and EDTA (1 mM).

that an appropriate torsional constrain is more effective on more flexible loop (compare Table 1, entry 2 and Table 2 entry 10).

The modulation of the overall stability of the T_5-looped hairpins when replacing one phosphodiester internucleotidic linkage by the rigid dioxaphosphorinane structure was dependent of the nature of the loop closing base pair as previously observed for their T_4-looped analogues.

When installed at the 5'- or 3'-end of the AT closed loop, the *gauche*(+) constrain slightly destabilize the hairpin (Table 2, entries 2 and 5) whereas this constrain was not tolerated at the junction position between the stem and the loop, inducing a strong loss in thermal stability of -6 °C (Table 2, entry 6). These results showed that T_5-loop ($^{6}H_{AT}T_5gp_i$) behave similarly to their T_4 counterparts ($^{6}H_{AT}T_4gp_i$)[10] with two favourable positions for the constrain in the middle of the loop providing an improvement of the thermal stability of +2 and +4 °C (Table 2, entries 3 and 4). All the CD spectra recorded for $^{6}H_{AT}T_5gp_i$ were identical to those of the unmodified $^{6}H_{AT}T_5$ without any difference neither in the shape nor in the value of the λmax of the positive Cotton band at 269 nm (see SI, S5).

The results obtained for $^{7}H_{CG}T_5gp_i$ ($1 \leq i \leq 4$) were in line with those of $^{j}H_{CG}T_4gp_i$ ($2 \leq j \leq 7$; $1 \leq i \leq 3$) outlining that the torsional stress induced always a notable stabilization with a maximum of +5 °C when the modification was placed in a central position of the loop (Table 2, entry 10). From this main stabilizing position, when shifting to the 5'-end of the loop the Δ*T*m decreased to +1.8 and +0.3°C (Table 2, entry 8 and 9) as previously depicted for $^{j}H_{CG}T_4gp_i$. The rather important stabilization measured (Δ*T*m = +2.6°C) when the D-CNA was installed at the 3'-end of the loop (Table 2, entry 11) was accompanied by stem rearrangement identified by an additional shoulder at 260 nm to the major positive Cotton band at 280 nm within the dichroic spectrum of $^{7}H_{CG}T_5gp_4$ (see SI, S6).

Therefore, these results showed that the D-CNA behaviour within hairpin structures remained the same independently of the loop and stem sizes.

Thermostability of *gauche(+)* constrained D-CNA within bulges

In order to study further the impact of D-CNA on partially unpaired DNA structures, we engaged the study of bulges. In these structures, each strand is submitted to a torsional stress that is increasing with the size of the bulged region. However, in the bulged moiety the change of the strand orientation is rather limited when compared with monomolecular hairpins, since there is not a total reversion of the directionality of the strand.

Two kinds of bulged structures have been prepared from one D-CNA modified strand 5'-GATTTGCATA***TT***CATGAG (***TT*** denotes an ($S_{C5'}$, R_P) α,β-D-CNA-modified TT dinucleotide). The modification was installed within growing bulges from one to six bases by using complementary strands of 17 to 12 bases in which bases were removed one by one from the central region (Table 3). Similarly, to generate bulges opposite to the modification, complementary strands of 19 to 24 bases in which bases were added one by one in the central region (Table 4), were used.

In comparison with the fully matched duplex in which the D-CNA induced a strong loss in thermal stability of -5.5°C,[16] the alpha *gauche*(+) torsional stress in single nucleotide bulge was already stabilizing by +1°C (Table 3, entry 1 *vs* 2). Increasing the size of the bulge from two to four unpaired nucleotides resulted in moderately stabilized structures with Δ*T*m around +2°C when compared with their corresponding unmodified bulges (Table 3, entries 3-5). Larger bulged moieties of five and six nucleotides took advantage of the D-CNA and exhibited higher thermal stability with Δ*T*m of +4 and +6 °C respectively to the their unconstrained counterparts (table 3 entries 6 and 7). To date these are the only results reported on effective large stabilization by means of preorganized nucleotide installed in unpaired moiety of

very unstable bulged secondary DNA structures.

Table 3: Thermal melting temperatures [°C] of α,β-D-CNA within loop of bulged structures

Entry	Target oligonucleotide 3'→5'	*T*m(Δ*T*m) [°C][a] 5'-GATTTGCATATxTCATGAG	
		x=PO_2-	x=D-CNA g^+
1	CTAAACGTATAAGTACTC	57.0	52.6 (-4.4)
2	CTAAACGTAT AGTACTC	49.0	50.0 (+1.0)
3	CTAAACGTATGTACTC	43.0	45.1 (+2.1)
4	CTAAACGTAGTACTC	39.3	41.2 (+1.9)
5	CTAAACGTATACTC	36.4	39.0 (+2.4)
6	CTAAACGTTACTC	26.0	30.0 (+4.0)
7	CTAAACGTACTC	25.0	31.0 (+6.0)

[a] Melting temperatures (average *T*m values on three times experiments ± 1°C) were measured as the maximum of the first derivate of the UV melting curve (OD_{260} *vs* temperature, 20-90 °C, 0.5 °C/min) which was recorded at concentration of 5 µM in sodium phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) and EDTA (1 mM).

As it was known that bulges induced a sharp kink with the degree of kinking increasing in roughly equal increment as the number of bases in the bulge increased from one to six[17], it appeared that *T*m measurement of growing bulges opposite to the strand in which the alpha *gauche*(+) torsional stress was applied by means of the D-CNA modification, would give insight on how such structures can be preorganized and as a consequence stabilized.

In these constructions all different bulge loops were flanked by the same helices (11 and 7 bases pairs) that share the D-CNA at their junction (position 7-8 on the constant strand). It could be then postulated that the variation of melting temperature between the various bulges would reflect the ability of the whole structures to accommodate the loop-induced kink. The results of thermal denaturation experiments obtained for unmodified and D-CNA-constrained bulges are summarized in Table 4. The main observation was that all bulges exhibited similar thermal stability with *T*m around 44 ± 1°C except for the single nucleotide bulge structure that had a slightly higher *T*m of 46.5°C (Table 4, entries 1 *vs* 2-6). It appeared that the alpha *gauche*(+) torsional stress within strand opposite to the bulge moiety was not well tolerated by bulged structures with one to three unpaired nucleotides with Δ*T*m increasing with the number of unpaired nucleotides (Table 4, entries 1-3). A four nucleotides bulged structure was insensitive to the D-CNA modification with Δ*T*m = +0.3 °C (Table 4, entries 4). Finally, in comparison with unmodified five and six nucleotides bulges, those featuring the alpha *gauche*(+) constrain were more stable by +2 and +4.2°C, respectively (Table 4 entries 5 and 6). It was tempting to correlate the preorganization of the strand including the D-CNA that imposed a change in its directionality with the kink of the overall structure.

The larger the bulge, the more important the kink and therefore the alpha *gauche*(+) constrain, that deviated from the canonical value by 120°, fitted with this requirement only when at least four nucleotides were unpaired.

Table 4: Thermal melting temperatures [°C] of α,β-D-CNA opposite to loop in bulged structures

Entry	Target oligonucleotide 3'→5'	*T*m(Δ*T*m) [°C][a] 5'-GATTTGCATATxTCATGAG	
		x=PO_2-	x=D-CNA g^+
1	T CTAAACGTATA AGTACTC	52.0	46.5 (-5.5)
2	TC CTAAACGTATA AGTACTC	47.2	44.2 (-3.0)
3	TCT CTAAACGTATA AGTACTC	45.0	43.6 (-1.4)
4	TCTC CTAAACGTATA AGTACTC	43.0	43.3 (+0.3)
5	TCTCT CTAAACGTATA AGTACTC	42.0	44.0 (+2.0)
6	TCTCTC CTAAACGTATA AGTACTC	39.0	43.2 (+4.2)

[a] Melting temperatures (average *T*m values on three times experiments ± 1°C) were measured as the maximum of the first derivate of the UV melting curve (OD_{260} *vs* temperature, 20-90 °C, 0.5 °C/min) which was recorded at concentration of 5 µM in sodium phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) and EDTA (1 mM).

The stabilizing effect observed could then be attributed to the lower torsional stress applied to the helices flanking the internal loop in the overall bulged structure due to entropic gain provived by the D-CNA.

Conclusions

Systematic studies of unusual DNA structures deviating from the double-helical DNA set the ground for understanding the principles of nucleic acid architecture and the possible chemical modifications to favour their folding and stability. We reported here how D-CNA dinucleotide featuring the non canonical alpha *gauche*(+) constrain were suitable for the preorganization and stabilization of hairpins and bulged structures of nucleic acids. We showed that the gain in thermal stability could be due either to the loop structuration or the closing base pairs rearrangement within T_4-looped hairpins. Modified T_5-looped hairpins behave similarly and were nicely stabilized with Δ*T*m up to +5 °C. Eventually, to the best of our knowledge, we reported for the first time enhanced thermal stability of bulged structures by means of torsional constrain on the sugar/phosphate backbone. We showed that an alpha *gauche*(+) constrain was always positive when applied within the loop of 1 to 6 nucleotides bulges whereas when located opposite to the unpaired moiety it could be a

stabilizing element for bulges reaching four unpaired nucleotides. Among their inertness towards enzymatic degradation such as snake venon phosphodiesterase as expected for phosphotriesters[18], D-CNAs within single stranded DNA oligonucleotides, showed their potential as terminators in polymerase chain reactions[19] and recently they proved to be efficient during allele selective silencing when included into antisens oligonucleotides directed against the huntingtin protein.[20] In this report we went further in the understanding of how D-CNA as structural distorted nucleotides analogues behave within oligonucleotides that can fold into secondary structures. Since theses non-canonical torsional angle conformations can be observed in protein/DNA complex, researches are in progress about the modulation of integrase activity with D-CNA modified four-way junctions in recombination biochemical process and results will be published in due course. D-CNAs could be suitable elements for the modulation of these interactions and eventually be the bases for the elaboration of decoys or stabilised synthetic functional DNAs such as aptamers or aptazymes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: phosphoramidite and ODN synthesis, thermal denaturation curves and *T*m vs [salt] plots. Circular dichroism spectra. See DOI: 10.1039/b000000x/

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