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Synthesis and Structure of an α,β-D-CNA Featuring a Noncanonical α/β Torsion Angle Combination within a Tetranucleotide

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Synthesis and structural analysis of a ($S_{C5'}$,R_P)-configured α,β-D-CNA TT within a tetranucleotide composed of four thymidine units showed that the solution structure determined at the dinucleotide level for the α,β-D-CNA with α and β torsional angles constrained to noncanonical values [*gauche*(+), *trans*] was unchanged and imposed a sharp turn in the single-stranded tetranucleotide. The overall structure of the tetranucleotide T_4 has a hairpin loop shape.

Introduction

Conformationally restricted nucleosides were developed essentially because of the important potential applications of antisense oligonucleotides. Therefore, many efforts have been devoted to restrict the furanose ring conformational flexibility in a way to enhance the binding affinity of the DNA analogue.[1–4]

On the basis of the concept of preorganization, it is anticipated that if the conformational states of an oligonucleotide single strand could be locally reduced to those that match the geometry of this strand in the target structure, then an entropic benefit could be expected, which should lead to an overall stabilization of the structure.[5] Conformational preorganization by introduction of structural restriction was successful in DNA analogues such as locked nucleic (LNA) in which the prelocked 3′-*endo* sugar conformation favored its binding with complementary DNA/RNA sequences to therefore stabilize the double helix structure.[6–9]

In other respects, only a few reports deal with the synthesis of conformationally restricted models of others secondary structural elements of DNA or RNA.[10,11] Nevertheless, to date there is no example to illustrate the preorganization concept when applied to stabilize unpaired DNA or RNA structures.

One possible approach to preorganize single-stranded oligonucleotides is to include one or more backbone bonds (α–ζ) within a well-defined ring structure. With this in mind, we have undertaken an experimental program to explore the chemistry of DNA and RNA analogues with a modified backbone that, at least in principle, might favor unusual helical conformations as well as the formation of structurally well-defined nonbase-pair states. Our approach is based on the introduction of the neutral 1,3,2-dioxaphosphorinane ring structure at key positions along the sugar–phosphate backbone (Figure 1). We reasoned that the resulting constrained nucleic acids (termed D-CNAs) could locally adopt either a helical or nonhelical conformation depending on the spatial arrangement of the dioxaphosphorinane system and which backbone bonds are included in its ring structure.[12,13] As a first result in this field, we already reported the synthesis of a covalently constrained ($S_{C5'}$,R_P) α,β-D-CNA TT building unit in which α and β torsions are locked in a noncanonical [*gauche*(+), *trans*] conformation that frequently occurs in protein–DNA complexes and in bulged or loop regions of nucleic acids.[14] This approach was usefully applied to prepare α,β-D-CNA with exceptional binding properties[15] and dinucleotide units with α,β,γ and δ,ε,ζ featuring noncanonical values (α,β,γ-D-CNA and δ,ε,ζ-D-CNA).[16]

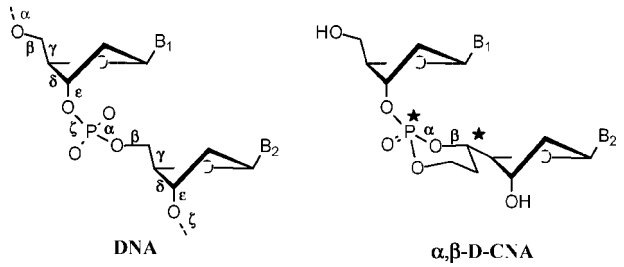

Figure 1. Left: the six backbone torsion angles (labeled α to ζ) of nucleic acids. Right: the α,β-D-CNA unit is a dinucleotide in which α and β are stereocontrolled to canonical or noncanonical values by a dioxaphosphorinane ring structure exhibiting two new asymmetric centers.

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However, when the α,β-D-CNA featuring the noncanonical *gauche*(+) value of α was included within an oligomer, a high destabilization level of the duplex was observed (ΔTm = –9 °C). Strikingly, whereas the destabilizing effect of a mismatched base pair opposite to the α,β-D-CNA is highly pronounced in the homoduplex (DNA/DNA), it became insignificant in the heteroduplex (DNA/RNA), suggesting that the introduced *gauche*(+) distortion could favor an internal loop structure within an A-type duplex structure.[15] Consequently, we decided to explore the intimate conformation of the ($S_{C5'}$,R_P) α,β-D-CNA TT dimer and its behavior within a single-stranded tetranucleotide composed of four thymidine units, a loop motif well-studied in the hairpin structure.[17]

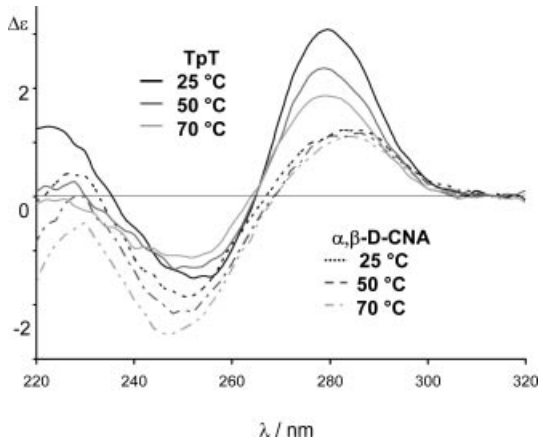


Figure 2. CD spectra of TpT and the ($S_{C5'}$,R_P) α,β-D-CNA TT in 10 mM sodium phosphate (pH 7.0) at 70, 50 and 25 °C.

Results and Discussion

The 3D structures of the various synthesized D-CNA are important points to determine in order to better understand theirs behaviors and the induced properties within the DNA oligomers. Whereas an X-ray crystal structure determination was available for the ($R_{C5'}$,S_P) α,β-D-CNA TT featuring a canonical value of the torsional angles,[12] the relative spatial arrangement of the three cycles of the ($S_{C5'}$,R_P) α,β-D-CNA was determined by means of NMR spectroscopy and circular dichroism to gain better insight of the relation between the α,β-D-CNA structure and the single stranded oligonucleotide conformation in which it could be introduced.

Conformational Analysis of the ($S_{C5'}$,R_P) α,β-D-CNA TT

To determine the behaviors of the two thymine moieties in the ($S_{C5'}$,R_P) α,β-D-CNA, the CD spectra were measured in phosphate buffer (pH 7.0) at 25, 50, and 70 °C and compared with those of an unmodified thymidine dinucleotide (denoted as TpT in Figure 2). The most striking features are the lower intensity and the very little temperature dependence observed for the ($S_{C5'}$,R_P) α,β-D-CNA of the positive Cotton effect around 280 nm. Whereas TpT showed a decrease in the staking of the thymine bases as the temperature increased, no variation was observed for the ($S_{C5'}$,R_P) α,β-D-CNA. These results could be undoubtedly explained in terms of the relative conformational rigidity of the dioxaphosphorinane structure that does not allow any base stacking. Because our purpose was to create a stable turn in single-stranded DNA oligomers with a control of theirs spatial arrangement, this extreme rigidity was highly suitable.

On the other hand, the solution-state structure determination in terms of sugar puckering and dioxaphosphorinane conformation was established by recording the ^{1}H, ^{1}H-{^{31}P}, 2D COSY ^{1}H/^{1}H, and 2D NOESY NMR spectra at 500 MHz of the ($S_{C5'}$,R_P) α,β-D-CNA TT in deuterated methanol.

The 2′-deoxyribose moieties puckering in solution was assigned by examination of the sugar ring (H/H) coupling constants (Table 1). The small $^2J_{H3'/H4'}$ coupling constants measured and the values of $^2J_{H2'/H3'}$ and $^2J_{H1'/H2'}$ are consistent with the standard C2′-*endo* conformation previously observed in natural 2′-deoxyribose units.[18] It should be mentioned that the neutral phosphoryl moiety in the ($S_{C5'}$,R_P) α,β-D-CNA TT affects the conformation of the 2′-deoxyribose moiety. Generally, the dialkoxyphosphoryloxy group serves as a strong electron-withdrawing group whereas the monoalkoxyphosphoryloxy group behaves as a weakly electron-withdrawing group. Basically, the electronegativity of a substituent attached to the 2′- or 3′-carbon is a key factor that controls the sugar pucker.[19] The 3′-oxygen of the neutral phosphoryl group increases its electronegativity due to the electron-withdrawing effect of the neutral phosphotriester so that the sugar conformation of the 5′-upstream thymidine moiety can be expected to be strongly affected. The determination of the impact that a neutral phosphotriester linkage would display in the conformational north/south equilibrium is particularly important as it is well recognized that this conformational state is of major importance for the DNA structure formation ability.

Table 1. (H/H) coupling constants (Hz) in the ^{1}H NMR spectra (500 MHz) of the ($S_{C5'}$,R_P) α,β-D-CNA TT.

Nucleoside	$J_{H1'/H2'}$		$J_{H2'/H3'}$		$J_{H3'/H4'}$
a (D-CNA up)	5.8	8.4	2.0	6.0	2.0
b (D-CNA low)	6.9	6.9	3.4	6.6	3.0

Like that of the dioxaphosphorinane ($R_{C5'}$,S_P) isomer,[12] the chair conformation is clearly established from the ^{1}H and ^{1}H-{^{31}P} NMR spectra, with no detectable coupling constant between the 5′b-H involved in the dioxaphosphorinane system and phosphorus ($^3J_{5'b\text{-}H/P} < 1$ Hz), which is characteristic of an axial position of this proton.[20] This proton also exhibits a small (1.9 Hz) and a larger (11.9 Hz) coupling constant with the vicinal 6′b-H protons, which corroborates a true chair conformation of the dioxophosphorinane ring.

The observation of a long-range coupling constant between the 4′b-H proton of the lower sugar unit and the phosphorus atom ($^4J_{4'b\text{-}H/P}$ = 5.0 Hz) is indicative of a typical W-shaped P–O5′–C5′–C4′–H4′ junction. This is consistent with a relative orientation of the sugar unit with respect to the dioxaphosphorinane ring with a *gauche*(+) conformation of the torsion angle γ (Figure 3).

The relative position of the upper nucleoside (denoted **a** in Figure 3) in relation to the phosphorus triester linkage can be calculated by the measurement of the coupling constant $^3J_{3'a\text{-}H/P}$ (6.6 Hz), which provides the value of the torsional angle ε (–156°) by use of the empirical equation established by Lankhorst et al.[21] Moreover, the ($S_{C5'}$,R_P) α,β-D-CNA TT 2D NOESY spectrum was acquired at 500 MHz. Although many cross peaks are present, most of them are derived from intraresidual (H/H) interactions indicative of the sugar puckering and the relative position of the thymine bases, except the cross peaks displayed by the 4′a-H with 5′b and 7′b-H that provide information about the spatial geometry between the 5′-terminal and 3′-ter-

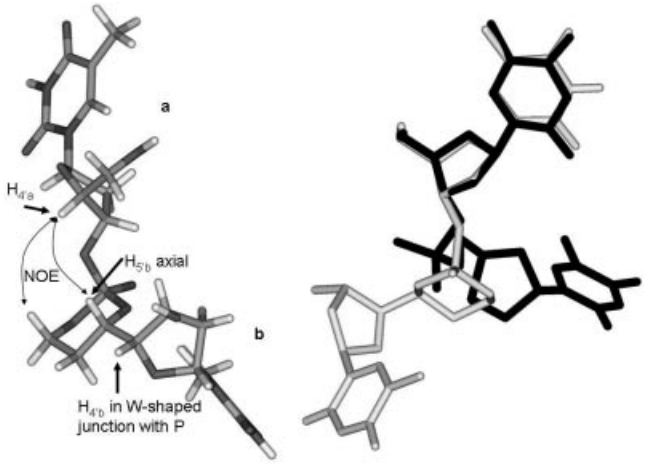
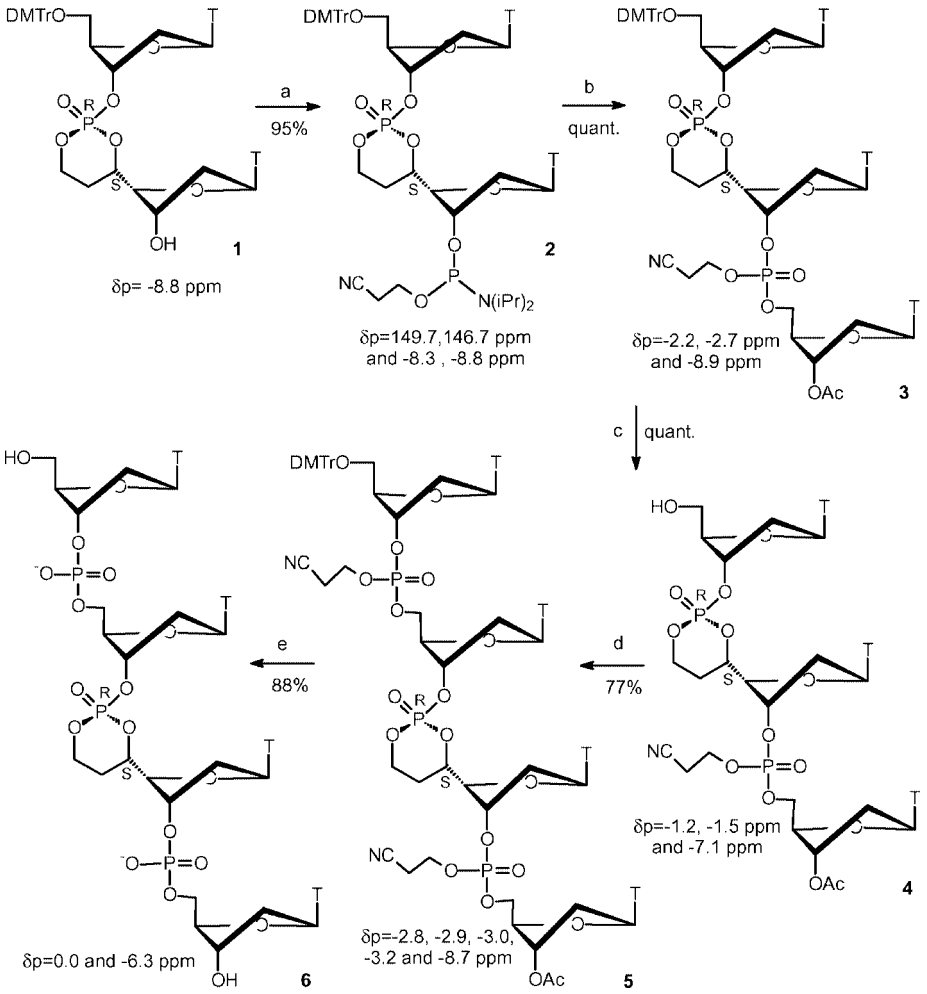


Figure 3. Left: Tentative model of the conformation of the ($S_{C5'}$,R_P) α,β-D-CNA TT derived from NMR spectroscopic data. The upper nucleoside is denoted as **a** and the lower as **b**. Right: superimposition of the structure of ($S_{C5'}$,R_P) α,β-D-CNA TT (grey) and an X-ray structure of unmodified TpT (black).[22]



Scheme 1. Reagents and conditions: a) (2-cyanoethyl)(*N*,*N*-diisopropylamino)chlorophosphite (2 equiv.), DIPEA (4 equiv.), THF, room temp; b) 3′-*O*-acetylthymidine (2 equiv.), tetrazole, CH_3CN then collidine, I_2/H_2O; c) 3% TFA in CH_2Cl_2; d) 5′-*O*-dimethoxytrityl-3′-*O*-phosphoramiditethymidine, tetrazole, CH_3CN then collidine, I_2/H_2O; e) (i) 3% TFA in CH_2Cl_2; (ii) 30% NH_4OH, room temp.

minal nucleotide. All these observations are summarized in Figure 3.

The chair conformation of ($S_{C5'}$,R_P) α,β-D-CNA allows the stabilization of an unusual conformation state [α = *gauche*(+), β = *trans*], which greatly differs from the canonical [α = *gauche*(–), β = *trans*] backbone conformation adopted by the B- and A-forms of the double helix (Figure 3). It is likely that once incorporated into the DNA oligomers, ($S_{C5'}$,R_P) α,β-D-CNA might favor the formation of unpaired secondary motifs or induce a significant conformational distortion from the ideal B- or A-form helical geometry.[15]

Synthesis of Tetranucleotide T_4 Including an ($S_{C5'}$,R_P) α,β-D-CNA TT Dimer

To synthesize tetranucleotide T_4 (**6**), readily available diastereopure 5′-*O*-dimetoxytrityl-($S_{C5'}$,R_P) α,β-D-CNA TT **1** was employed as the starting material (Scheme 1).[14] α,β-D-CNA (cyanoethyl)(diisopropylamino)phosphoramidite **2** was prepared in high yield (95%) by action of (cyanoethyl)-(diisopropylamino)chlorophosphite on **1** in anhydrous THF in the presence of Hünig's base. Compound **2** was recovered as a 1:1 mixture of diastereoisomers as depicted by ^{31}P NMR spectroscopy (δ_P = 149.7, 146.7, –8.3, and –8.8 ppm). The lower nucleotide (denoted **d** in Figure 4) was introduced according to standard phosphoramidite technology by coupling **2** with 3′-*O*-acetylthymidine to provide **3** in quantitative yield as a mixture of two diastereoisomers (δ_P = –2.2, –2.7, and –8.9 ppm).[23] Removal of the dimethoxytrityl protecting group occurred in quantitative yield by treatment with trifluoroacetic acid leading to **4** (δ_P = –1.2, –1.5, and –7.1 ppm). The following phosphoramidite coupling step with 5′-*O*-dimethoxytrityl-3′-*O*-phosphoramidite-thymidine proceeded in a reasonable yield (77%) and tetranucleotide **5** was isolated as a mixture of four diastereoisomers (δ_P = –2.8, –2.9, –3.0, –3.2, and –8.7 ppm). Finally, the sequential removal of the protective groups with trifluoroacetic acid and aqueous ammonia provided final compound **6** as a single isomer (δ_P = 0.0 and –6.3 ppm) in 64% overall yield from **1**.

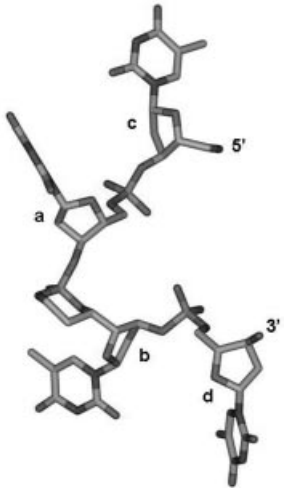


Figure 4. Tentative model derived from NMR spectroscopic data of the conformation of the ($S_{C5'}$,R_P) α,β-D-CNA within a T_4 tetranucleotide.

Structural Assignment of the ($S_{C5'}$,R_P) α,β-D-CNA within Tetranucleotide T_4

To determine the relative spatial arrangement of the four nucleotides within tetranucleotide **6**, ^{1}H, ^{1}H-{^{31}P}, 2D COSY ^{1}H/^{1}H, ^{1}H/^{31}P HMBC, and 2D ROESY NMR spectra were recorded at 500 or 700 MHz in deuterium oxide.

As expected, we observed by examination of the sugar ring (H/H) coupling constants that both of the 2′-deoxyribose moieties are puckered in the standard C2′-*endo* conformation previously observed in natural 2′-deoxyribose units (Table 2).[18] Interestingly, there is no change in the coupling constants of the upper nucleoside sugar moiety of the ($S_{C5'}$,R_P) α,β-D-CNA (denoted **a** in Figure 4), whereas for the lower nucleoside sugar moiety (denoted **b**) the smaller $^2J_{H3'/H4'}$ and the different $^2J_{H1'/H2'}$ coupling constants measured are indicative of a more pronounced south conformation. This is consistent with the substitution of the free 3′-hydroxy functionality of the ($S_{C5'}$,R_P) α,β-D-CNA by a phosphoryl group in tetranucleotide **6**.

Table 2. (H/H) coupling constants (Hz) in the ^{1}H NMR spectra (700 MHz) of tetranucleotide **6** (n.d. = not determined).

Nucleoside	$J_{H1'/H2'}$		$J_{H2'/H3'}$		$J_{H3'/H4'}$
c (upper)	6.2	7.6	3.2	7.4	3.3
a (D-CNA up)	5.9	8.0	2.0	6.0	2.1
b (D-CNA low)	6.2	8.7	3.1	6.4	2.0
d (lower)	6.8	6.8	n.d.	n.d.	n.d.

We took advantage of the typical chemical shift of the phosphorus atom involved in the dioxaphosphorinane ring (δ_P = –6.3 ppm) in contrast with those of the phosphate groups (δ_P = 0.0 ppm) to record a ^{1}H/^{31}P HMBC NMR spectrum (Figure 5). As a consequence, we easily visualized which protons exhibit scalar coupling with phosphorus atoms.

Owing to the puckering of the sugar, the fact that the chair conformation of the dioxaphosphorinane ring is not altered within the tetranucleotide, and that there is no detectable coupling constant between 5′b-H (lower nucleoside of the α,β-D-CNA, denoted **b** in Figure 4) and the phosphorus atom ($^3J_{5'b\text{-}H/P} \approx 0$ Hz; which corresponds to a clean axial position of this proton) a true chair conformation of the six-member ring is expected. This feature was corroborated by examination of the ^{1}H NMR spectrum in comparison with the ^{1}H-{^{31}P} spectrum, with no change in the signal for 5′b-H in these spectra. Another important indication provided by the ^{1}H/^{31}P HMBC NMR spectrum is that the previously observed coupling constant between the 4′b proton (lower nucleoside of the α,β-D-CNA) and the phosphorus atom at the dinucleotide level was still present in the tetranucleotide structure ($^4J_{4'b\text{-}H/P}$ = 5.0 Hz). Neither the 5′ and 3′ substitution, nor the change in the solvent (methanol

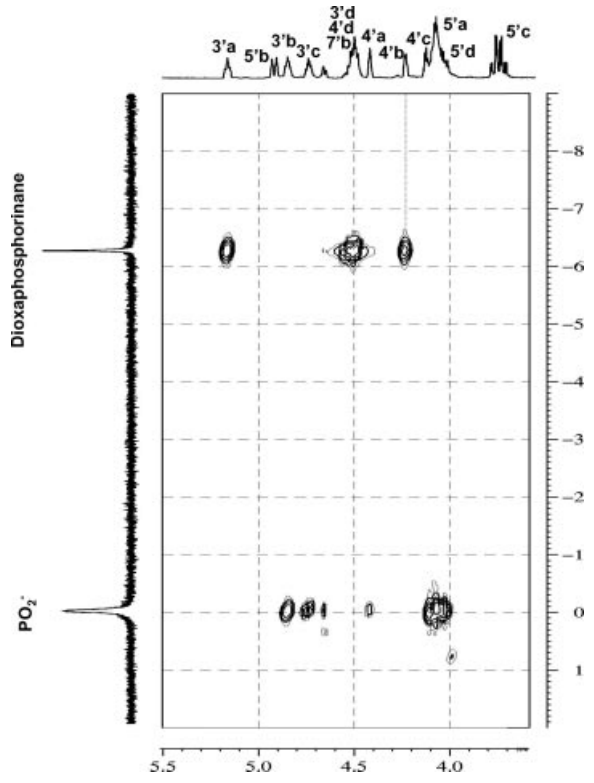


Figure 5. $^{1}H/^{31}P$ HMBC NMR spectrum of tetranucleotide **6** showing the long range coupling between phosphorus of the dioxaphosphorinane ring and the 4′b-H.

to water) had an influence on the relative position of nucleoside **b** with respect to the dioxaphosphorinane ring. Therefore, the local geometry of the phosphorus linkage is maintained in the [α = *gauche*(+), β = *trans,* γ = *gauche*(+)] conformation.

The measured coupling constants between the 3′-protons and their related phosphorus ($^{3}J_{3'c\text{-}H/P}$ = 4.7 Hz, $^{3}J_{3'a\text{-}H/P}$ = 6.6 Hz, and $^{3}J_{3'b\text{-}H/P}$ = 7.8 Hz, respectively) provided us with the ε torsion angles values according to the Lankhorst's relation (ε_c = –166°, ε_a = –156°, and ε_b = –125°, respectively). Whereas $^{3}J_{3'\text{-}H/P}$ coupling constants of 2–4 Hz are observed for the B_I-type duplex,[24] several loop phosphates show somewhat larger coupling constants of 5.5–6 Hz due to atypical values of ε at these loop positions without a corresponding change in ζ.[17b]

In the 2D ROESY NMR spectrum of **6**, the excepted cross peak resulting from 5′b-H of the lower nucleoside **b** and the 4′a-H proton of the upper nucleoside **a** of the α,β-D-CNA can be seen, and all cross peaks are derived from intraresidual (H/H) interactions (Figure 6). Therefore, the first observation is in agreement with the conformation observed for the ($S_{C5'}$,R_P) α,β-D-CNA dinucleotide whereas the other one suggests the conformation in which the distance between the adjacent nucleotides are relatively long and therefore correspond to the relative conformation of the unmodified nucleotides.

All together, these data helped us to propose a tentative model for the structure of tetranucleotide **6** in which the ($S_{C5'}$,R_P) α,β-D-CNA might induce a stable turn in the single-stranded oligonucleotide (Figure 4).

In all the described models of the hairpin loop structure, the sharp turn that is present is denoted as a "turning phosphate".[17] Depending on the stem base composition of the hairpin, the position of the turning phosphate within the loop can differ for four-membered structures whereas it seems to be located close to the 5′-top of the stem in the three-member ones. Another common feature of these loops is that the [α = *gauche*(+), β = *trans,* γ = *gauche*(+)] phosphodiester conformation at the turning phosphate is responsible for the sharp turn that the backbones take.

We can therefore propose, according to the observed structure conformation of the ($S_{C5'}$,R_P) α,β-D-CNA within the T_4 tetranucleotide, that this α,β-D-CNA building unit should be a good candidate to prepare stabilized hairpin structures as a turning phosphate mimetic.

Conclusions

Structural analysis of an α,β-D-CNA in which the backbone torsional angles (α, β) are simultaneously locked in a dioxaphosphorinane ring structure in the noncanonical [*gauche*(+), *trans*] conformation, indicates that the dinucleotide unit is highly rigid and exhibits a shape remarkably

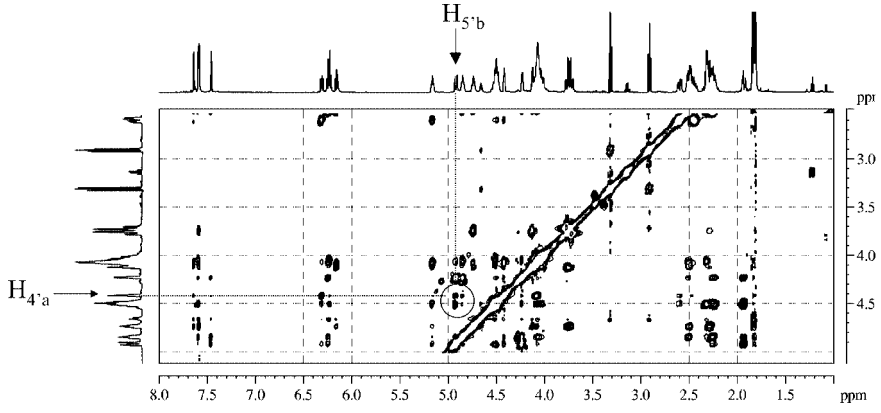


Figure 6. 2D ROESY NMR spectroscopic data of tetranucleotide **6**, showing the NOE effect between the axial proton 5′b-H of the dioxaphosphorinane ring and the 4′a-H proton of the upper nucleoside of the α,β-D-CNA.

different from that of an unmodified dimer in terms of relative orientation of deoxysugars and bases moieties.

When incorporated into a tetranucleotide, the α,β-D-CNA induced a sharp bend into the structure, providing a hairpin loop shape to the structure of the single-stranded oligonucleotide.

We therefore are disposing of a building unit featuring the noncanonical value *gauche*(+) of the α torsional angles, providing the unique opportunity to preorganize single-stranded oligonucleotide to explore its capability to stabilize unpaired structures of nucleic acids such as bulges or hairpin loops.

Experimental Section

5′-*O*-Dimethoxytrityl-3′-*O*-[(cyanoethyl)(diisopropylamino)phosphoramidite]-α,β-D-CNA (S_C,R_P) (2): To a solution of 5′-*O*-dimethoxytrityl α,β-D-CNA (S_C, R_P) **1**[14] (920 mg, 1.06 mmol) in anhydrous THF (10 mL) was added, at room temperature under an argon atmosphere diisopropylethylamine (732 μL, 4.24 mmol) and (2-cyanoethyl)(*N*,*N*-diisopropylamino)chlorophosphite (500 mg, 2.12 mmol). After 30 min of stirring the diisopropylethylamine hydrochloride was filtered off, and the reaction mixture was diluted with ethyl acetate saturated with argon. The organic layer was washed with a cold aqueous solution of K_2CO_3 (10%), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude material was submitted to silica gel chromatography (1% Et_3N in ethyl acetate). α,β-D-CNA phosphoramidite **2** (1.07 g, 1.0 mmol, 95%) was recovered (mixtures of diastereoisomers) as a white foam after careful removal of the solvent under high vacuum for 24 h. R_f = 0.20 (1% Et_3N in AcOEt). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 164.0, 163.9, 158.8, 150.8, 150.7, 150.6, 144.0, 136.4, 135.1, 135.0, 130.1, 128.1, 128.0, 127.3, 121.2, 118.1, 117.8, 113.3, 111.9, 111.3, 87.3, 86.5, 86.1, 85.9, 80.0, 79.6, 79.5, 78.9, 78.8, 72.9, 72.7, 72.5, 68.4, 63.4, 60.4, 58.4, 58.3, 58.2, 58.1, 57.8, 57.7, 5.3, 45.3, 38.8, 38.5, 38.2, 29.7, 28.1, 24.6, 24.5, 24.4, 22.9, 22.8, 21.4, 20.5, 20.4, 20.1, 20.0, 12.5, 11.6 ppm. ^{31}P NMR (81 MHz, $CDCl_3$): δ = 149.7, 146.6, –8.3, –8.8 ppm. MS (ES): *m*/*z* = 1075.5 $[M + H]^+$, 1097.5 $[M + Na]^+$, 1113.0 $[M + K]^+$.

5′-*O*-Dimethoxytrityl-3′-*O*-(acetyl)-$T_{P*}T_{PCE}T$ (3): Compound **2** (120 mg, 0.12 mmol), thymidine $O_{3'}$-acetyl (65 mg, 0.23 mmol), and freshly sublimed tetrazole (39.2 mg, 0.56 mmol) were dissolved with anhydrous acetonitrile (1.5 mL) and stirred for 20 min at room temp. After the addition of collidine (34 μL, 0.26 mmol), the intermediate phosphite was oxidized with iodine (0.1 M solution in THF/H_2O, 2:1) until the dark brown color persisted. The reaction mixture was diluted with ethyl acetate and washed with an aqueous solution of sodium thiosulfate (15%) to remove excess of iodine. The organic layer was washed with water and brine, and the solvent was removed in vacuo. The crude material was chromatographed on silica gel (ethyl acetate/methanol, 9:1). After evaporation of the solvent, compound **3** (mixture of diastereoisomers) was recovered as a white foam (145 mg, 100%). R_f = 0.22 (10% MeOH in AcOEt). ^{31}P NMR (81 MHz, $CDCl_3$): δ = –2.2, –2.7, –8.9 ppm. MS (ES): *m*/*z* = 1275.4 $[M + H]^+$, 1296.5 $[M + Na]^+$, 1313.9 $[M + K]^+$.

3′-*O*-(Acetyl)-$T_{P*}T_{PCE}T$ (4): Compound **3** (140 mg, 0.11 mmol) was dissolved in a solution of trifluoroacetic acid (3%) in dichloromethane (3 mL) at room temp. After 5 min, the red solution was evaporated to dryness. The crude material was dissolved in THF and purified by silica gel chromatography. It was first eluted with ethyl acetate to remove the dimethoxytrityl residue and then with ethyl acetate/methanol (4:1) to collect trinucleotide **4**, which was obtained as a white foam after evaporation of the solvent (107 mg, quantitative yield). R_f = 0.20 (20% MeOH in AcOEt). ^{31}P NMR (81 MHz, $CDCl_3$): δ = –1.2, –1.5, –7.1 ppm. MS (ES): *m*/*z* = 972.6 $[M + H]^+$, 993.6 $[M + Na]^+$, 1010.4 $[M + K]^+$.

5′-*O*-Dimethoxytrityl-3′-*O*-(acetyl)-$T_{PCE}T_{P*}T_{PCE}T$ (5): Compound **4** (140 mg, 0.144 mmol), thymidine $O_{3'}$-phosphoramidite (214 mg, 0.29 mmol), and freshly sublimed tetrazole (0.1 g, 1.43 mmol) were dissolved with anhydrous acetonitrile (3 mL) and stirred for 20 min at room temp. After the addition of collidine (88 μL, 0.67 mmol), the intermediate phosphite was oxidized with iodine (0.1 M solution in THF/H_2O, 2:1) until the dark brown color persisted. The reaction mixture was diluted with ethyl acetate and washed with an aqueous solution of sodium thiosulfate (15%) to remove excess iodine. The organic layer was washed with water and brine, and the solvent was removed in vacuo. The crude material was chromatographed on silica gel (acetate/methanol, 4:1). After evaporation of the solvent, compound **5** (mixture of diastereoisomers) was recovered as a white foam (181 mg, 77%). R_f = 0.15 (20% MeOH in AcOEt). ^{31}P NMR (81 MHz, $CDCl_3$): δ = –2.8, –2.9, –3.0, –3.2, –8.7 ppm. MS (ES): *m*/*z* = 1632.0 $[M + H]^+$, 1654.1 $[M + Na]^+$, 1671.0 $[M + K]^+$.

$T_PT_{P*}T_PT$ (6): Compound **5** (145 mg, 0.089 mmol) was treated with a solution of trifluoroacetic acid (3%) in dichloromethane (2 mL) at room temp. After 5 min, the red solution was evaporated to dryness. The crude material was dissolved in dichloromethane/methanol (9:1) and purified by silica gel chromatography. It was first eluted with ethyl acetate to remove the dimethoxytrityl residue and then with ethyl acetate/methanol (4:1) to collect the 3′-*O*-acetylcyanoethylphosphate protected tetranucleotide obtained as a white foam after evaporation of the solvent. This material was treated with 30% aqueous ammonia solution (5 mL) for 12 h at room temp. After evaporation, the tetranucleotide was purified by reverse phase HPLC [Hyperprep C18, 20×250 mm, (TEAA 50 mM, pH 7.0)/acetonitrile: 88:12]. Removal of the solvent provided **6** as a white foam (92 mg, 88%). HPLC [Hyperprep C18, 8 μm, 4.6×250 mm, (TEAA 50 mM, pH 7.0)/acetonitrile: 88:12]: t_R = 14.90 min. ^1H NMR (700 MHz, D_2O): δ = 7.63 (bd, 1 H, 6a-H), 7.58 (bd, 1 H, 6d-H), 7.57 (bd, 1 H, 6c-H), 7.44 (bd, 1 H, 6b-H), 6.30 (dd, *J* = 5.9 and 8.0 Hz, 1 H,1′a-H), 6.22 (dd, *J* = 6.2 and 8.7 Hz, 1 H, 1′b-H), 6.21 (t, *J* = 6.8 Hz, 1 H, 1′d-H), 6.14 (dd, *J* = 6.2 and 7.6 Hz, 1 H, 1′c-H), 5.15 (m, *J* = 2.0, 2.1, 6.0 Hz, $J_{H/P}$ = 6.6 Hz, 1 H, 3′a-H), 4.91 (dt, *J* = 1.9, 2.0, 11.7 Hz, 1 H, 5′b-H), 4.83 (m, *J* = 2.0, 3.1, 6.4 Hz, $J_{H/P}$ = 7.8 Hz, 1 H, 3′b-H), 4.72 (m, *J* = 3.2, 3.3, 7.4 Hz, $J_{H/P}$ = 4.7 Hz, 1 H, 3′c-H), 4.51–4.47 (m, 4 H, 3′d-H, 7′b-H, 4′d-H), 4.40 (m, *J* = 2.1 and 3.1 Hz, 1 H, 4′a-H), 4.22 (m, *J* = 2.2, 3.0 Hz, $J_{H/P}$ = 5.0 Hz, 1 H, 4′b-H), 4.11 (m, *J* = 3.3, 3.5, 4.4 Hz, 1 H, 4′c-H), 4.07–3.99 (m, 4 H, 5′a-H, 5′d-H), 3.75 (A part of an ABX system, *J* = 3.5, 12.5 Hz, 1 H, 5′c-H), 3.71 (B part of an ABX system, *J* = 4.5, 12.5 Hz, 1 H, 5′c-H), 2.58 (A part of an ABX system, *J* = 1.9, 8.0, 14.5 Hz, 1 H, 2′a-H), 2.49 (A part of an ABX system, *J* = 3.0, 6.1, 14.2 Hz, 1 H, 2′b-H), 2.48 (A part of an ABX system, *J* = 3.2, 6.1, 14.0 Hz, 1 H, 2′c-H), 2.43 (B part of an ABX system, *J* = 5.8, 7.3, 14.5 Hz, 1 H, 2′a-H), 2.32–2.21 (m, 6 H, 2′d-H, 2′c-H, 2′b-H, 6′b-H), 1.83 (d, *J* = 1.1 Hz, 3 H, a-Me), 1.81 (d, *J* = 1.1 Hz, 3 H, d-Me), 1.80 (d, *J* = 1.1 Hz, 3 H, c-Me), 1.79 (d, *J* = 1.1 Hz, 3 H, b-Me) ppm. ^{13}C NMR (125.8 MHz, D_2O): δ = 166.5, 166.4, 166.3, 151.8, 151.7, 151.6, 137.8, 137.6, 137.3, 136.9, 112.0, 111.9, 111.6, 111.5, 85.9, 85.8, 85.6, 85.4, 85.2, 85.1, 83.9, 81.3, 78.6, 76.1, 75.5, 70.6, 69.4, 65.3, 64.9, 61.3, 38.7, 38.0, 37.8, 37.7, 35.5, 27.5, 16.0, 11.8 ppm. ^{31}P

NMR (202.3 MHz, D_2O): δ = 0.0, –6.3 ppm. MS (ES): *m*/*z* = 1179.6 $[M + H]^+$, 1201.8 $[M + Na]^+$, 1217.7 $[M + K]^+$.

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