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ARTICLE

Oligonucleotides Containing a *Ribo*-Configured Cyclohexanyl Nucleoside: Probing the Role of Sugar Conformation in Base Pairing Selectivity

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The synthesis and a preliminary evaluation of the pairing properties of *ribo*-cyclohexanyl nucleic acids (*r*-CNA) is herein reported. Incorporation of a single *r*-CNA nucleotide into natural duplexes did not enhance their stability, while a very high pairing selectivity for RNA was found. As deduced by comparative analysis of T_m and NMR data, a relationship between pairing selectivity and conformational preferences of the "sugar" moiety of *r*-CNA (and most generally of six-membered nucleic acids) was suggested.

Introduction

Sugar-modified nucleic acids represent artificial pairing systems devised to resemble, improve or even expand structure, properties and functions of DNA and RNA.[1] Indeed, replacement of natural (deoxy)ribose with either bioisosteric[2-4] or alternative structures[5] typically leads to a dramatic improvement in the pairing potential of the corresponding oligonucleotides, and likewise to greater biostability and bioavailability properties *in vivo*. Accordingly, these systems have found widespread interest and applications, especially in the modern biomedical research, acting either as diagnostic probes, as tools in molecular biology or as gene silencers in functional genomics and human therapy.[6] One the most renowned examples of sugar-modified nucleic acids is represented by the oligonucleotide system composed of (6'→4')-linked 1',5'-anhydro-D-*arabino*-hexitol nucleotides[7] (HNA, *Hexitol Nucleic Acids*; Figure 1). As widely reported, the strong and selective affinity for RNA (ΔT_m/mod up to + 3 °C) relies on a pre-organized conformation of the non-hydrolyzable hexitol ring, closely resembling the C3'-*endo* sugar ring pucker of natural ribonucleotides.[7]

Inspired by this example, a systematic screening of six-membered nucleic acids bearing hexitol derivatives/analogues in the backbone has been carried out over the last years.[2] The analysis of the physicochemical properties of oligonucleotide systems composed of pyranyl (α-HNA,[8] ANA,[9] MNA, F-HNA,[10] cANA[11]), pyranosyl (homoDNA,[12] MANA,[13] DMANA[14]) and carbocyclic (CNA,[15] CeNA,[16] F-CeNA[17]) nucleotides, quite often evaluated in both enantiomeric forms,[15,18-20] has led to the identification of HNA congeners endowed with even superior hybridization properties,[8,10,13,16] thus acting, along with HNA, as excellent candidates for applications in therapy,[21] diagnostics[22] and chemical genetics.[23] In addition, the capacity of six-membered nucleic acids to act as orthogonal pairing systems, adopting double helices even beyond classical A-, B- and Z-type structures, has likewise justified applications in biotechnology,[24] synthetic biology and even in prebiotic chemistry.[15b,19b,25]

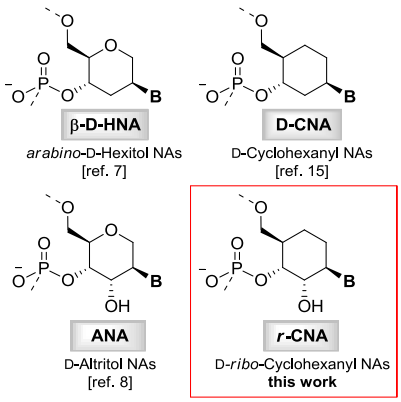

Figure 1. Representative examples of six-membered oligonucleotide systems and *ribo*-configured cyclohexanyl nucleic acids (*r*-CNA).

In this context, we herein introduce a novel synthetic nucleic acid composed of D-*ribo*-configured[26] cyclohexanyl nucleotides (*r*-CNA, ribo-*Cyclohexanyl Nucleic Acids*; Figure 1). The analysis of the pairing properties of *r*-CNA represents an extension of the work on two long studied oligonucleotide systems. On the one hand, the sugar core of *r*-CNA comprises a carbocyclic skeleton with stereogenic centers resembling those of D-altrose. Thus, *r*-CNA can be regarded as a carbocyclic congener of the highly effective, bioactive pairing system composed of 1',5'-anhydro-D-altritol nucleotides (ANA, *Altritol Nucleic Acids*; Figure 1). On the other

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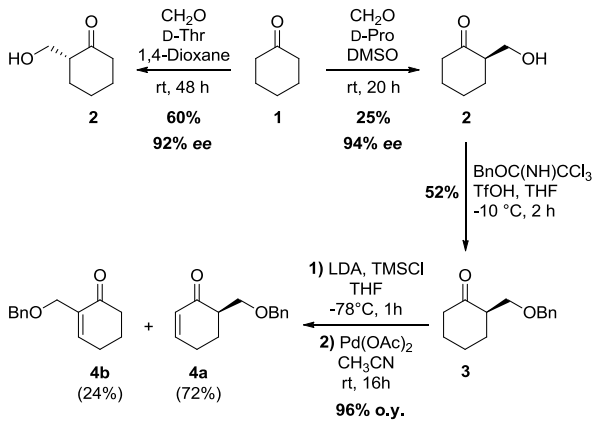
hand, *r*-CNA is a derivative of the oligonucleotide system containing cyclohexanyl nucleotides (CNA, *Cyclohexanyl Nucleic Acids*). Starting from the hybridization versatility of the latter,[27] the capacity by *r*-CNA to pair with both natural and unnatural complements by suitably adopting more than one annealing conformation[15,27] will be likewise analyzed. In this preliminary communication, the synthesis of the nucleoside monomers and an early look at the hybridization properties of *r*-CNA toward natural complements is reported.

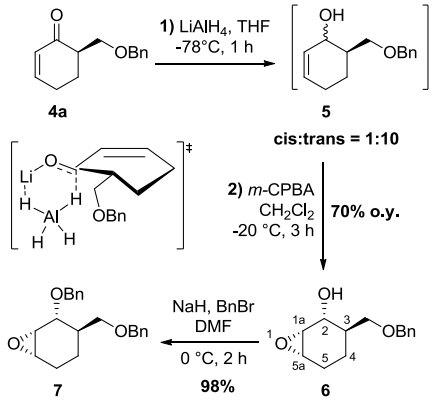
Results and Discussion

The access to nucleoside monomers of *r*-CNA was devised through an asymmetric route enabling the enantio- and diastereoselective installation of four adjacent stereocenters from commercially available cyclohexanone (**1**) (Schemes 1-3). The synthesis started with the organocatalytic, asymmetric α-hydroxymethylation of **1** (Scheme 1), required to set the steric series[26] of D-cyclohexanyl nucleoside monomers. According to Chen and Chai,[28] treatment of **1** with aqueous formaldehyde and catalytic D-proline provided, after 20 h at rt, the 2-(*R*)-hydroxymethyl cyclohexanone (**2**) with satisfying enantioselectivity[29] (94% *ee*), albeit with a low yield (25%). Conversely, in line with Takabe's conditions,[30] the same reaction, performed using D-threonine as catalyst, provided **2** with roughly the same optical purity (92% *ee*) but with a much higher yield (60%). After benzylation of the primary alcohol ($BnOC(NH)CCl_3$/TfOH, 52%), α,β-unsaturated ketone **4** from benzyl ether **3** was obtained by a two-steps procedure, relying on the transient formation of "kinetic" trimethylsilyl enol ether of **3** (LDA/TMSCl), followed by a $Pd(OAc)_2$-mediated double bond rearrangement (Scheme 1). Unexpectedly, the formation of two regioisomeric compounds, i.e. the expected **4a** and the achiral **4b** (96% o.y.; **4a**:**4b** = 3:1) was detected, the latter apparently deriving from the "thermodynamic" trimethylsilyl enol ether of **3**. However, even more unexpected, the side reaction did not alter the optical purity of cyclohexenone **4a** (Scheme 1).



Scheme 1. *En route* to D-*ribo*-configured cyclohexanyl nucleosides: synthesis of chiral cyclohexenone **4a**.

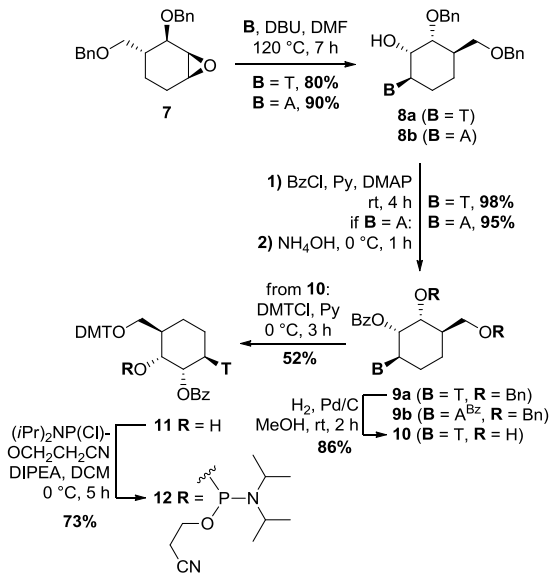
Looking for a stereoselective strategy enabling the installation of three contiguous stereocenters on cyclohexenone **4a**, carbonyl group reduction followed by epoxidation of the resulting allylic alcohol was devised (Scheme 2). Out of a wide variety of reducing reagents tested [$NaBH_4$, $(AcO)_3BH$, $LiBH_4$, DIBAL-H, Red-Al, $(MeO)_3AlH$], only use of $LiAlH_4$ provided stereoselective ketone reduction of **4a**, mainly leading to the *trans* isomer of **5** (cis:trans = 1:10). The high level of selectivity observed in this case can be explained as result of the preferential attack to the axial face of the ketone group of **4a** by the "small" nucleophile AlH_4^- (leading to a thermodynamically favored transition state)[31] (Scheme 2). Epoxidation of the allylic alcohol **5** was then accomplished by treatment of the latter with *m*-chloroperoxybenzoic acid (*m*-CPBA), obtaining the sole epoxide **6** with (1a*S*,5a*S*) configuration at the newly formed stereocenters (70% yield from **4a**). Benzylation of the *sec*-alcohol **6** was achieved under standard conditions (NaH/BnBr), providing **7** in an excellent 98% yield (Scheme 2).



Scheme 2. Synthesis of oxirane **7**.

With the key intermediate **7** in hand, the synthesis of carbocyclic nucleosides was eventually carried out as described for similar substrates[18b] (Scheme 3). Epoxide ring opening (DBU) using unprotected thymine or adenine (taken as model nucleobases) smoothly provided the corresponding protected nucleoside analogues **8a-b** in very good yields (**B** = T, 80%; **B** = A, 90%). Benzoylation of the last ones under standard conditions (BzCl/DMAP) then gave esters **9a-b** (**B** = T, 98%; **B** = A, 95%). Unfortunately, benzyl group removal of adenosine **9b** (Pd/C, H_2) provided an inseparable mixture of deprotected nucleosides; not totally unexpected,[32] ^{1}H NMR analysis suggested the formation of migration products of the Bz group. Conversely, under the same conditions the thymidine analogue **9a** was deprotected to give the sole diol **10** in 86% yield. Standard nucleoside chemistry involving regioselective 6'-*O*-dimethoxytritylation of **10** (DMTCl, 52%) and 4'-*O*-phosphitylation of **11** [$(i\text{-Pr})_2NP(Cl)OCH_2CH_2CN$/DIPEA, 73%] eventually furnished phosphoramidite **12** to be used for incorporation studies.

Assembly of **cT***-containing oligonucleotides (**cT*** = D-*ribo*-cyclohexanyl thymidine) was accomplished by using the common phosphoramidite method on a solid support.[7] Modified DNA and RNA sequences composed of 9-13-mer oligonucleotides were obtained; deconvoluted ESI-MS analysis showed all oligonucleotides to be of correct molecular weight (Table 1).



Scheme 3. Synthesis of D-*ribo*-cyclohexanyl phosphoramidite nucleoside **12**.

Hybridization studies were aimed at analyzing the stability perturbation of DNA and RNA duplexes produced by the incorporation of single **cT*** units. The hybridisation strength was studied by thermal denaturation experiments, which were typically determined at 260 nm in 0.1 or 1 M NaCl buffer with KH_2PO_4 (20 mM, pH 7.5) and EDTA (0.1 mM) at a concentration of 4 μM for each strand.

Table 1. ESI-MS data of oligonucleotides containing cyclohexanyl nucleotides.

Sequence	MS calcd	MS found
5′-r(GCGU)-**cT***-r(UGCG)-3′	3010.5	3010.5
5′-d(GCGT)-**cT***-d(TGCG)-3′	2910.5	2910.6
5′-d(CACCG)-**cT***-d(TGCTACC)-3′	3896.7	3896.8
5′-d(CGCA)-**cT***-d(ACGC)-3′	2848.5	2848.6

In early studies, the incorporation of *r*-CNA nucleotides into DNA duplexes was considered (Table 2). Replacement of a natural thymidine (**T**) with a *ribo*-cyclohexanyl thymidine at the **X** position of the DNA sequence 5'-d(CACCG**X**TGCTACC)-3' reduced significantly the thermal stability of the corresponding duplex (ΔT_m -11.6 °C). Additionally, single mismatches in this sequence (**cT***–T, **cT***–G and **cT***–C) resulted into a further drop in the T_m values. As an example, we found a ΔT_m value of –5.5 °C (–10.4 °C in the natural duplex) as a result of the **cT***–T mismatch; similarly, the **cT***–G and **cT***–C mismatches provided a drop in the stabilities of the corresponding duplexes of –3.9 °C (**T**–G: ΔT_m –6.8 °C) and –6.7 °C (**T**–C: ΔT_m –12.9 °C).

Thermal stability studies of duplexes containing other modified DNA sequences [5′-d(CACCG)-**cT***-d(TGCTACC)-3′ and 5′-d(CGCA)-**cT***-d(ACGC)-3′] gave broad melting curves, suggesting formation of multiple complexes/transitions (data not shown). The incorporation of a **cT*** at the **X** position of the short RNA sequence 5'-r(GCGU**X**UGCG)-3' was then explored (Table 3). Despite the presence of the slightly more stabilizing T base, replacement of a natural uridine also led to a reduction in the thermal stability of the resulting duplex, providing a drop in the T_m value of the corresponding duplex of 5.1 °C (entry 2). Conversely, when melting experiments were performed at higher salt concentration (1 M NaCl), incorporation into the matched sequence led to a slight increase in the thermal stability of the duplex (T_m 52.0 °C; ΔT_m + 0.4 °C; entry 3). Considering other six-membered nucleic acids such as HNA (entry 4), ANA (entry 5), MANA (*3-O-Methyl Altritol Nucleic Acids*) (entry 6) and DMANA (*Di*-O-*Methyl Altropyranosyl Nucleic acids*) (entry 7), incorporated in the same *ds*-RNA sequence, binding affinity followed the order: MANA>ANA~HNA>DMANA>*r*-CNA.

Table 2. T_m values (°C) of DNA duplexes [5'-d(CACCG**X**TGCTACC)-3'/3'-d(GTGGC**Y**ACGATGG)-5'].[a]

Y	A		T		G		C	
X	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
T	57.1	-	46.7	−10.4	50.3	−6.8	44.2	−12.9
cT*	45.5	-11.6	40.0	−5.5	41.6	−3.9	38.8	−6.7

[a]Measures were taken at 260 nm in 0.1 M NaCl, 20 mM KH_2PO_4 (pH 7.5), 0.1 mM EDTA.

It's noteworthy that the introduction of single mismatches in this sequence resulted in considerable decreases of ΔT_m values. As an example, we found a ΔT_m value of –16.9 °C (–16.5 °C in the natural duplex) as a result of the **cT***–U mismatch; the **cT***–C mismatch even produced a drop in the thermal stability of the corresponding duplex down to –23.0 °C (**U**–C: ΔT_m –19.6 °C). In Py-Py mismatches (**cT***-C and **cT***-U), selectivity was even higher than that previously exhibited by the above six-membered nucleic acids, selectivity order being: *r*-CNA>DMANA>MANA~ANA>HNA. Conversely, under Wobble[33] conditions (**T**/**U**-G), selectivity order was the same as binding affinity: MANA>ANA>HNA>DMANA>*r*-CNA. Unexpectedly, in both mismatched sequences the melting profile of RNA exhibited a much closer similarity to *r*-CNA than to all the other oligonucleotide systems (Table 3).

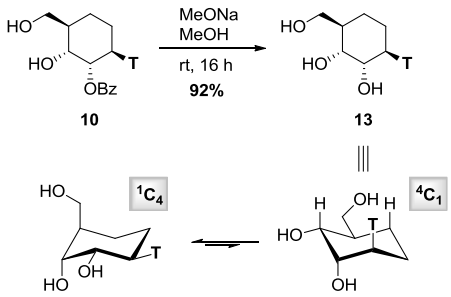
Table 3. T_m values (°C) of complementary RNA duplexes [5'-r(GCGU**X**UGCG)/5'-r(CGCA**Y**ACGC)].[a]

	Y	A		U		G		C	
	X	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
1	**U**	51.6	-	35.1	−16.5	47.3	−4.3	32.0	−19.6
2	**cT**	46.5	−5.1	29.6	−16.9	41.0	−5.5	23.5	−23.0
3	**cT**	52.0[b]	+0.4	37.7[b]	−14.3	47.9[b]	−4.1	31.0[b]	−21.0
4	**hU**[c,d]	54.6	+3.0	48.2	-6.4	44.5	-10.1	39.4	-15.2
5	**aU**[d,e]	54.4	+2.8	47.3	-7.1	43.6	-10.8	39.4	-15
6	**mU**[d,f]	55.0	+3.4	47.8	-7.2	42.3	-12.7	39.8	-15.2
7	**dmU**[d,g]	50.6	−1.0	40.4	-10.2	42.5	-8.1	33.3	-17.3

[a]Measures were taken at 260 nm, in 0.1 M NaCl, 20 mM KH_2PO_4 (pH 7.5), 0.1 mM EDTA (unless otherwise specified). [b]Determined in 1 M NaCl. [c]Incorporation of an HNA nucleotide. [d]Data taken from ref. 14. [e]Incorporation of an ANA nucleotide. [f]Incorporation of a MANA nucleotide. [g]Incorporation of a DMANA nucleotide.

Looking for a rationale linking the observed results, we noticed that in all nucleoside intermediates **8-10** as well as in the unprotected thymidine **13** (obtained by 3'-*O*-benzoyl group removal of **10**, Scheme 4), the cyclohexanyl core adopted sugar conformations with equatorially oriented nucleobases, as revealed

by ^{1}H NMR analysis of the vicinal coupling constants ($^{3}J_{H,H}$) and especially observing the large $^{3}J_{1',2'}$ values[34] (e.g., **13**: J = 10.0 Hz) and likewise the small $^{3}J_{4',5'}$ values (e.g., **13**: J = 2.7 Hz). As already observed for similar substrates,[15] the conformational preference of the cyclohexanyl ring is the result of 1,3-diaxial strains occurring in the $^{4}C_{1}$ form among the nucleobase and the vicinal hydrogen atoms (Scheme 4). The relationship between pairing affinity of a nucleic acid and conformational preferences of the corresponding sugar moiety has been already extensively pointed out.[2,11,14] Accordingly, the unsatisfactory melting profile of *r*-CNA could be due to the energetic penalty required for a forced conformational change from the non-hybridizing $^{1}C_{4}$ form to the RNA-mimicking $^{4}C_{1}$ chair (Scheme 4). In addition, we reasoned that the same arguments could also explain the high pairing selectivity of *r*-CNA under mismatching conditions. Indeed, in the absence of a complementary nucleotide promoting this conformational change, the presence of an *r*-CNA nucleotide adopting the $^{1}C_{4}$ chair is expected to destabilize the corresponding RNA duplex.



Scheme 4. Conformational preferences of *ribo*-cyclohexanyl thymidine **13**.

In support of this hypothesis, a comparative conformational analysis of the "sugar" backbones of the six-membered nucleic acids reported in Table 3 was performed, providing a rough estimation of the relative abundances of the main conformers as deduced by the analysis of the $^{3}J_{H,H}$ coupling constants of the corresponding nucleoside monomers (Table 4). It was fairly interesting to observe that only in the case of HNA the sugar backbone adopted an almost pure $^{4}C_{1}$ conformation, as deduced by the close proximity of $J_{1a',2'}$, $J_{2',3a'}$ and $J_{4',5'}$ values with those expected theoretically.[35,36] In all remaining cases, significant deviations from canonical ^{3}J values belonging to $^{4}C_{1}$ chair conformations occurred (Table 4). For instance, the $^{3}J_{1a',2'}$ and $^{3}J_{2',3a'}$ values of MANA, ANA and DMANA nucleosides (MANA: 3.9 and 4.8 Hz, respectively; ANA: 4.0 and 5.6 Hz) were intermediate between those expected for the pure $^{4}C_{1}$ ($J_{1a',2'}$ = 1.8 Hz; $J_{2',3a'}$ = 3.3 Hz) and $^{1}C_{4}$ ($J_{1a',2'}$ = 11.6 Hz; $J_{2',3a'}$ = 10.3 Hz) forms. As indicated by the broadness of some NMR signals, this suggests the occurrence of a mixture of the two (or more) conformers including those bearing equatorially-oriented nucleobases. In the case of DMANA, the conformational equilibrium was only slightly more displaced to the $^{1}C_{4}$ form. The sugar moiety of *r*-CNA, having the highest $J_{1a',2'}$ and $J_{2',3a'}$ values (both 10.0 Hz) as well as the smallest $J_{4',5'}$ value (2.7 Hz), was estimated to adopt a sugar conformation fairly close to the pure $^{1}C_{4}$ form (theoretical: $J_{1a',2'}$ = 12.4 Hz; $J_{2',3a'}$ = 10.2 Hz; $J_{4',5'}$ = 3.4 Hz).

Table 4. Experimentally and theoretically determined coupling constant values ($^{3}J_{H,H}$, Hz) of nucleoside building blocks of various sugar-modified nucleic acids.[a]

Nucleoside	$^{3}J_{1'a,2'}$			$^{3}J_{2',3'a}$			$^{3}J_{4',5'}$		
	Expected ($^{4}C_{1}$)	Expected ($^{1}C_{4}$)	Measured	Expected ($^{4}C_{1}$)	Expected ($^{1}C_{4}$)	Measured	Expected ($^{4}C_{1}$)	Expected ($^{1}C_{4}$)	Measured
HNA[b,c]	1.8	11.5	**2.4**[d]	3.0	12.5	**4.4**[d]	9.2	2.2	**7.5**[d]
MANA	1.8	11.6	**3.9**[e]	3.4	10.4	**4.8**[f]	9.2	2.2	ND
ANA[b,c]	1.8	11.6	**4.0**	3.4	10.4	**5.6**	9.2	2.2	ND
DMANA	2.2	9.5	**4.5**[f]	3.8	11.0	**5.5**[d,f]	9.3	2.2	**5.1**[f,g]
***r*-CNA**	3.2	12.4	**10.0**[g]	3.3	10.2	**10.0**[g]	10.0	3.4	**2.7**[g]

[a]Experimentally determined *J* values were estimated either from high-resolution 1D NMR analysis or by double quantum-filtered COSY (DQF-COSY) experiments. All NMR spectra were obtained in DMSO-d_6. The theoretical *J* values were obtained by applying the Haasnoot-de Leeuw-Altona equation.[37] [b]Synthesized according to ref. 18. [c]Thymidine analogue was chosen in place of the corresponding uridine because of the slightly better resolution of NMR signals. [d]An error of up to ±0.5 Hz was estimated due to signal broadness. [e]Taken from ref. 13b. [f]Compared to literature data, resolution enhancement by Gaussian multiplication was achieved in this case. [g]An error of up to ±0.2 Hz was estimated due to signal broadness.

There is a substantial agreement between binding affinity of an oligonucleotide system and $^4C_1$ character of the corresponding sugar backbone as found by NMR analysis. In this context, *r*-CNA reasonably acts as the weakest pairing system due to the largest $^1C_4$ character of its "sugar" moiety. However, as already observed the superior hybridization potential of MANA and ANA[9] over HNA (not in line with their conformational preferences) points to the occurrence of further stabilizing elements, such as the formation of intra- and/or interstrand hydrogen bondings[38] or the more hydrophilic character of the sugar moiety (affecting the hydration spine of the duplex).[2,39] Full agreement between conformational preferences and pairing selectivities in Py-Py mismatches was conversely found (selectivity increases with the $^1C_4$ character of the sugar backbone). The close pairing similarity between *r*-CNA and RNA can be interpreted assuming that, in the absence of a complementary nucleotide, destabilizing sugar conformations ($^1C_4$ and C2'-*endo*) are adopted in both cases. Regarding Wobble mismatches, the inverted selectivity can be judged as a consequence of pairing **T**/**U**-G interactions achieved by *r*-CNA and RNA, apparently not occurring (or only to a limited extent) by the remaining oligonucleotide systems. While it is well established that RNA adopts a C3'-*endo* sugar ring pucker under Wobble conditions,[40] in this case the comparable melting behavior of RNA and *r*-CNA could rather be explained by similarities involving other conformational parameters.[40]

Conclusions

A preliminary investigation into the pairing properties of a new oligonucleotide system composed of D-*ribo*-cyclohexanyl nucleotides (*r*-CNA) has been herein reported. The synthesis of the nucleoside monomers has been accomplished in 10 steps by a diastereo- and enantioselective route starting from commercial cyclohexanone. When incorporated into a *ds*-RNA, the presence of a *r*-CNA nucleotide did not improve duplex stability (ΔT_m up to +0.4 °C). On the other hand, the pairing selectivity in Py-Py mismatches (ΔT_m down to –23 °C) was considerably higher than that exhibited by both RNA and all other six-membered nucleic acids evaluated thus far. The limited hybridization potential of this oligonucleotide system can be interpreted as a consequence of the energetic penalty for a forced conformational change from the $^1C_4$ chair (which cyclohexanyl nucleosides predominantly adopt at monomeric level) to the $^4C_1$ form (the RNA-mimicking chair conformation). Similarly, it has been suggested that in the absence of a complementary nucleotide promoting the inversion $^1C_4 \rightarrow {}^4C_1$, the *r*-CNA nucleotide in the $^1C_4$ form destabilizes the corresponding RNA duplex, thus explaining the high pairing selectivity. Beyond the potential applications of this system (e.g., in the detection of single base mismatches), the analysis of the pairing properties of *r*-CNA acting as model system may provide new structural elements in the design of novel, highly active and selective oligonucleotides.

Experimental Section

Nucleoside synthesis. General methods and materials. All chemicals and solvents were purchased with the highest degree of purity and used without further purification. All moisture-sensitive reactions were performed under nitrogen atmosphere using oven-dried glassware. Reactions were monitored by TLC (precoated silica gel plate F254, Merck) and the products were detected by exposure to ultraviolet radiation, iodine vapor and chromic mixture. Column chromatography: Merck Kieselgel 60 (70-230 mesh); flash chromatography: Merck Kieselgel 60 (230-400 mesh). NMR spectra were recorded on NMR spectrometers operating at 200, 300, 400, 500 and 600 MHz, using $CDCl_3$ solutions unless otherwise specified. Combustion analyses were performed using a CHNS analyzer. Exact mass measurements were performed with a quadrupole/orthogonal acceleration time-of-flight tandem mass spectrometer (qTOF2, Micromass, Manchester, UK) fitted with a standard electrospray ionization (ESI) interface. Exact mass measurements were performed with a quadrupole/orthogonal acceleration time-of-flight tandem mass spectrometer (qTOF2, Micromass, Manchester, UK) fitted with a standard electrospray ionization (ESI) interface.

Compound 2. *Method A*: to a solution of cyclohexanone (**1**) (10 g, 0.1 mol) in DMSO (300 mL), d-proline (1.2 g, 0.01 mol) and formaldehyde (4.0 mL, 0.05 mol, solution 37% by volume in H_2O) were sequentially added at room temperature. After 16 h, the mixture was extracted with EtOAc and washed with brine. The organic layer was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. Chromatography of the crude residue over silica gel (hexane:EtOAc = 7:3) gave the pure **2** (1.8 g, 29% yield, 94% *ee*) as a colourless oil. *Method B*: to a solution of **1** (1 g, 10 mmol) in 1,4-dioxane (10 mL), d-threonine (71 mg, 0.6 mmol) was added. After stirring for 1 h at 25 °C, formaldehyde (160 µL, 2 mmol, solution 37% by volume in H_2O) was added. The reaction mixture was stirred for 48 h at rt, then the volatiles were removed under reduced pressure. Chromatography of the crude residue over silica gel (hexane:EtOAc = 7:3) provided the pure **2** (153 mg, 60% yield, 92% *ee*). ^1H and ^{13}C NMR data were fully in agreement with those reported earlier.[28]

Compound 3. To a cooled (-10 °C) solution of alcohol **2** (0.5 g, 3.9 mmol) in anhydrous CH_2Cl_2 (40 mL), a solution of freshly prepared[41] $BnOC(NH)CCl_3$ (3.9 mmol) in anhydrous THF (52 mL) was added. After 5 min, TfOH (0.09 mL, 0.1 mmol) was added dropwise to the reaction mixture. After 3 h, TLC monitoring showed complete disappearance of the starting

product. The mixture was then quenched with 0.1 M NaOH until neutral and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and solvent evaporated under reduced pressure. Chromatography of the crude residue over silica gel (hexane:Et_2O = 8:2) afforded the pure **3** (0.5 g, 52% yield). ^{1}H NMR (200 MHz): δ 1.38–1.58 (m, 1H), 1.60–1.75 (m, 2H), 1.83-1.97 (m, 1H). 2.00-2.18 (m, 1H), 2.20-2.41 (m, 3H), 2.58–2.75 (m, 1H), 3.42 (dd, *J* = 9.5, 7.4, 1H), 3.82 (dd, *J* = 9.5, 5.1, 1H), 4.52 (d, *J* = 11.9, 1H), 4.57 (d, *J* = 11.9, 1H), 7.28-7.40 (m, 5H). ^{13}C NMR (75 MHz): ppm 24.7, 27.6, 31.4, 42.0, 50.8, 69.3, 73.2, 127.4, 127.5, 128.2, 138.3, 211.5. Anal. Calcd for $C_{14}H_{18}O_2$: C 77.03, H 8.31. Found: C 77.17, H 8.26.

Compound 4a. *n*-BuLi (1.6 M in hexane, 2.87 mL, 4.59 mmol) was added to a solution of freshly distilled DIPA (0.74 mL, 5.24 mmol) in anhydrous THF (9.48 mL), stirred at -78 °C under nitrogen atmosphere. After 20 min, a solution of **3** (0.95 g, 4.37 mmol) in anhydrous THF (7.0 mL) was added dropwise to the mixture. After 1 h, TMSCl [10 mL, previously distilled and deacidified with a solution of freshly dried triethylamine (TEA) in anhydrous THF] was added and the resulting mixture was stirred for 1 h at –78 °C. After control TLC indicated complete conversion of the starting ketone into the corresponding trimethylsilyl enol ether, the solvent was evaporated under reduced pressure and the mixture filtered on a celite pad washing with pentane (50 mL). To a stirring solution of the crude trimethylsilyl enol ether in anhydrous CH_3CN (65 mL), $Pd(OAc)_2$ (981 mg, 4.37 mmol) was added at rt and the reaction was stirred at the same temperature for 16 h. The mixture was then filtered on a celite pad washing with CH_2Cl_2. The filtrate was washed with brine. The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure affording a crude residue (2.1 g) which chromatography over silica gel gave the pure **4a** (0.68 g, 72% yield starting from **3**). The formation of the regioisomeric **4b** (0.23 g, 24%) was also observed (**4a**:**4b** = 3:1, 96% o.y.). Data for compound 4a: oily; ^{1}H NMR (200 MHz): δ 1.82-2.04 (m, 2H), 2.22-2.44 (m, 2H), 2.57-2.76 (m, 2H), 3.61 (dd, *J* = 7.6, 9.5, 1H), 3.86 (dd, *J* = 4.1, 9.5, 1H), 4.55 (s, 2H), 6.02 (dt, *J* = 1.8, 10.1, 1H), 6.90-7.01 (m, 1H), 7.26-7.38 (m, 5H). ^{13}C NMR (75 MHz): ppm 25.3, 26.0, 47.1, 69.1, 73.3, 127.6, 128.4, 129.8, 138.4, 150.3, 199.5. Anal. Calcd for $C_{14}H_{16}O_2$: C 77.75, H 7.46. Found: C 77.85, H 7.41.

Compound 6. To a solution of **4a** (0.2 g, 0.92 mmol) in anhydrous THF (4.5 mL), stirred at -78 °C and under nitrogen atmosphere, $LiAlH_4$ (9.0 mg, 0.23 mmol) was added. After 1 h, the mixture was cooled at 0 °C, diluted with EtOAc (7.5 mL) and H_2O (7.5 mL). After 15 min, the solution was washed with aq NH_4Cl extracted with EtOAc. The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. To a cooled (-20 °C) solution of the crude **5** (0.20 g, *cis*/*trans*: 1/10) in anhydrous CH_2Cl_2 (5 mL), stirred under nitrogen atmosphere, *m*-CPBA (0.16 g, 0.92 mmol) was added in one portion. After 3 h, the mixture was warmed to room temperature, washed with brine until neutral and extracted with CH_2Cl_2. The collected organic phases were dried (Na_2SO_4) and evaporated under reduced pressure. Flash chromatography of the crude residue (hexane/EtOAc = 8:2), afforded the pure **6** (151 mg, 70% yield). Oily; ^{1}H NMR (300 MHz): δ 1.02–1.19 (m, 2H), 1.38–1.52 (m, 1H), 1.80-1.98 (m, 2H), 3.33 (bs, 2H), 3.42-3.62 (m, 2H), 3.91 (bd, *J* = 9.2, 1H), 4.55 (s, 2H), 7.27-7.48 (m, 5H). ^{13}C NMR (75 MHz): ppm 22.2, 23.1, 37.4, 54.7, 56.0, 72.1, 73.1, 73.5, 127.5, 127.6, 128.3, 137.7. Anal. calcd for $C_{14}H_{18}O_3$: C 71.77, H 7.74. Found: C 71.65, H 7.77.

Compound 7. To a cooled (0 °C) solution of alcohol **6** (0.08 g, 0.35 mmol) in anhydrous DMF (3 mL) under nitrogen atmosphere, BnBr (54 µL, 0.46 mmol) and NaH (14 mg, 0.35 mmol; 60% suspension in mineral oil) were added sequentially. After 2 h, the mixture was quenched with MeOH, washed with brine until neutral and extracted with AcOEt. The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. Chromatography of crude residue over silica gel (hexane/EtOAc = 9:1) gave the pure **7** (0.11 g, 98% yield). Oily; ^{1}H NMR (300 MHz): δ 1.16-1.45 (m, 2H), 1.57-1.73 (m, 1H), 1.78-2.06 (m, 2H), 3.28 (bt, *J* = 3.3, 1H), 3.34 (bs, 1H), 3.46-3.56 (m, 2H), 3.78 (dd, *J* = 1.0, 9.6, 1H), 4.42 (d, *J* = 12.2, 1H), 4.48 (d, *J* = 12.2, 1H), 4.61 (d, *J* = 11.7, 1H), 4.73 (d, *J* = 11.7, 1H), 7.27-7.38 (m, 10H). ^{13}C NMR (125 MHz): ppm 22.3, 24.2, 35.9, 53.4, 54.3, 70.5, 70.6, 72.7, 75.6, 127.2, 127.6, 128.0, 128.1, 138.2, 138.3. Anal. calcd for $C_{21}H_{24}O_3$: C 77.75, H 7.46. Found: C 77.67, H 7.49.

Nucleoside synthesis: general procedure. To a solution of epoxide **7** (0.10 g, 0.31 mmol) in anhydrous DMF (3 mL), stirred at room temperature under nitrogen atmosphere, the nucleobase (0.71 mmol) was added at room temperature. After 15 min, DBU (0.11 µL, 0.71 mmol) was added to the mixture. Temperature was heated to 120 °C and the mixture was stirred at the same temperature for 7 h; afterwards the solution was cooled to room temperature, washed with NH_4Cl until neutral and extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. Chromatography of the crude residue over silica gel (EtOAc/hexane = 1:1) provided the pure nucleoside (**8a**: **B** = T; 0.12 g, 80% yield; **8b**: **B** = A; 0.13 g, 90% yield). Data for **8a**: amorphous solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.61-2.03 (m, 7H), 2.37 (d, *J* = 11.5, 1H), 2.41-2.48 (m, 1H), , 3.57 (d, *J* = 10.4, 1H), 3.62 (d, *J* = 10.4, 1H), 3.88 (bt, *J* = 10.9, 1H), 3.98 (bs, 1H), 4.38-4.63 (m, 4H), 4.70 (d, *J* = 11.7, 1H), 6.75 (s, 1H), 7.30-7.45 (m, 10H), 7.93 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): ppm 12.3, 22.2, 25.9, 36.4, 57.8, 69.3, 71.0, 71.7, 73.5, 79.8, 110.5, 127.7, 127.9, 128.4, 128.5, 137.2, 137.7, 137.8, 151.4, 163.2. Anal. calcd for $C_{26}H_{30}N_2O_5$: C 69.31, H 6.71, N 6.22. Found: C 69.21, H 6.74, N 6.24. Data for **8b**: white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.72 (bd, *J* = 13.8, 1H), 1.88-1.96 (m, 1H) 1.97-2.06 (m, 1H), 2.25 (qd, *J* = 4.0, 13.1, 1H), 2.51-2.62 (m, 1H), 3.63 (dd, *J* = 6.4, 9.4, 1H), 3.76 (dd, *J* = 7.0, 9.4, 1H), 4.04 (bt, *J* = 2.5, 1H), 4.32 (dd, *J* = 3.0, 10.4, 1H), 4.45-4.68 (m, 4H), 4.72 (d, *J* = 11.4, 1H), 5.60 (s, 2H), 7.28-7.42 (m, 10H), 7.62 (s, 1H), 8.20 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): ppm 21.5, 27.0, 37.6, 57.5, 69.3, 70.1, 72.2, 73.0, 79.4, 119.3, 127.5, 127.6, 127.8, 128.0, 128.2, 128.9, 138.7, 139.0, 141.1, 149.9, 152.0, 156.0. Anal. calcd for $C_{26}H_{29}N_5O_3$: C 67.95, H 6.36, N 15.24. Found: C 67.84, H 6.39, N 15.30.

Compound 9a. To a stirring solution of **8a** (0.10 g, 0.23 mmol) in anhydrous pyridine (1 mL), BzCl (276 µL) and DMAP (3 mg, 0.023 mmol) were added at room temperature. After 4

h, the solvent was evaporated under reduced pressure; the resulting residue was washed with brine at pH 7 and extracted with EtOAc. Purification of the crude by chromatography over silica gel (EtOAc/hexane = 1:1) gave the pure **9a** (125 mg, 98% yield). Amorphous solid; ^{1}H NMR (500 MHz): δ 1.69-1.80 (m, 4H), 1.82-1.92 (m, 1H), 2.09-2.20 (m, 2H), 2.37 (bs, 1H), 3.65 (dd, *J* = 5.7, 9.6, 1H), 3.68 (dd, *J* = 6.1, 9.6, 1H), 4.15 (bt, *J* = 2.3, 1H), 4.51-4.62 (m, 4H), 5.16 (bs, 1H), 5.62 (dd, *J* = 3.1, 11.2, 1H), 6.89 (bd, *J* = 1.1, 1H), 7.14-7.60 (m, 11H), 7.66 (d, *J* = 8.2, 2H), 7.98 (d, *J* = 8.2, 2H). ^{13}C NMR (125 MHz): ppm 12.0, 21.9, 26.1, 38.2, 54.5, 71.1, 72.1, 73.5, 77.3, 110.4, 127.2, 127.6, 127.9, 128.1, 128.4, 128.6, 129.1, 129.4, 129.8, 131.2, 133.0, 134.3, 136.8, 137.7, 137.9, 150.8, 162.3, 165.4. Anal. calcd for $C_{33}H_{34}N_2O_6$: C 71.46, H 6.18, N 5.05. Found: C 71.56, H 6.16, N 5.03.

Compound 10. A solution of benzyl ether **9a** (0.19 g, 0.34 mmol) in MeOH (10 mL) was purged of oxygen by bubbling nitrogen for 15 min. 10% Pd/C (0.05 g) was added and the mixture was exposed to H_2 (20 psi) while stirring for 2 h. The suspension was filtered off and the filter cake was rinsed with further MeOH (4×25 mL). The combined filtrate was then concentrated under reduced pressure, to give the pure **10** (129 mg, 86% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.53-1.64 (m, 2H), 1.69 (s, 3H), 1.89-1.92 (m, 1H), 1.92-2.06 (m, 2H), 3.55-3.69 (m, 2H), 4.20 (bs, 1H), 4.63 (bt, *J* = 5.1, 1H), 5.00 (bt, *J* = 10.2, 1H), 5.15-5.24 (m, 2H), 7.48 (t, *J* = 7.6, 2H), 7.63 (t, *J* = 7.6, 1H), 7.78 (s, 1H), 7.85 (d, *J* = 7.6, 2H), 11.12 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): ppm 12.3, 20.9, 25.5, 44.4, 50.8, 60.8, 68.0, 73.6, 109.3, 125.7, 128.7, 129.4, 130.2, 137.8, 151.7, 164.0, 165.4. Anal. calcd for $C_{19}H_{22}N_2O_6$: C 60.95, H 5.92, N 7.48. Found: C 60.83, H 5.93, N 7.51.

Compound 11. To a cooled (0 °C) solution of diol **10** (118 mg, 0.31 mmol; previously coevaporated in anhydrous pyridine) in anhydrous pyridine (5 mL), stirred under nitrogen atmosphere, dimethoxytrityl chloride (0.130 g, 0.38 mmol, 1.2 eq.) was added in one portion. After being stirred for 3 h, methanol (0.2 mL) was added; then the mixture was partitioned between dichloromethane (2 x 30 mL) and saturated $NaHCO_3$ solution (2 x 15 mL). The organic layer was dried (Na_2SO_4), concentrated under reduced pressure and the resulting solution (3 mL) was precipitated twice in 100 mL of cold hexane (-10°C) containing 1 mL of diisopropyl ether, to afford the pure **11** (0.11 g, 52% yield) as a white powder. ^{1}H NMR (500 MHz): δ 1.60-1.75 (m, 3H), 1.81 (s, 3H), 2.07-2.15 (m, 1H), 2.38-2.50 (m, 1H), 3.22-3.33 (m, 2H), 3.80 (s, 6H), 4.41 (s, 1H), 5.15-5.23 (m, 2H), 3.94 (m, 1H), 4.33 (m, 2H); 4.92 (m, 1H), 6.69 (bs, 1H), 6.87 (d, *J* = 8.8, 2H), 6.88 (d, *J* = 8.8, 2H), 7.22-7.27 (m, 1H), 7.33 (t, *J* = 8.1, 2H), 7.36-7.43 (m, 6H), 7.49 (d, *J* = 7.3, 2H), 7.54 (t, *J* = 7.3, 1H), 7.94 (d, *J* = 7.2, 2H), 8.29 (s, 1H). ^{13}C NMR (125 MHz): ppm δ 2.3, 21.2, 26.1, 40.2, 52.3, 55.2, 62.4, 70.2, 72.6, 86.4, 111.0, 113.2, 126.9, 127.9, 128.3, 128.6, 129.0, 129.1, 129.7, 130.1, 133.5, 135.9, 136.2, 144.6, 151.0, 158.5, 163.1, 165.2. ESMS calcd for $C_{40}H_{40}N_2O_8Na_1$ $[M+Na]^+$: 699.2682, found: 699.2688; calcd for $C_{40}H_{39}N_2O_8$ $[M-H]^-$: 675.2706, found: 675.2720.

Compound 12. To a solution of nucleoside **11** (0.11 g, 0.16 mmol) in anhydrous CH_2Cl_2 (5 mL), kept at 0 °C under argon atmosphere, freshly dried diisopropylethylamine (0.09 mL, 0.5 mmol) and 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite (0.07 mL, 0.30 mmol) were added while the solution turned light orange. After stirring for 3 h, the reaction was incomplete and an additional amount of both reagents was added. The reaction mixture was stirred further at 0 °C for 2 h; then saturated aqueous $NaHCO_3$ (1 mL) was added. The solution was stirred for another 10 min and partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic layer was washed with brine (3 x 30 mL) and the aqueous phases were back extracted with CH_2Cl_2 (30 mL). The collected organic layers were dried (Na_2SO_4) and the solvent was removed under reduced pressure. Flash chromatography of the crude residue (hexane/acetone/TEA = 62/36/1) gave a foam which was dissolved in CH_2Cl_2 (2 mL) and precipitated in cold hexane (100 mL, -30°C) containing 1% of diisopropyl ether, to afford the desired phosphoramidite **12** (0.11 g, 73% yield) as a white powder. The nucleoside was dried under vacuum and stored overnight under nitrogen at -20°C. ESMS calcd for $C_{49}H_{58}N_4O_9P$ $[M+H]^+$: 877.3941, found 877.3947; ^{31}P NMR: δ 149.08, 150.29.

Compound 13. A small amount of compound **10** (10 mg) was debenzoylated by treatment of the latter with an excess of MeONa in anhydrous MeOH (0.5 mL). The resulting mixture was stirred at room temperature for 16 h; afterwards the solvent was removed under reduced pressure. Chromatography of the crude residue (CH_2Cl_2/MeOH = 85:15) gave the pure **13** (83% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.31-1.75 (m, 4H), 1.80-1.94 (m, 1H), 3.31-3.52 (m, 2H), 3.70 (bd, *J* = 10.8, 1H), 4.00 (bt, *J* = 2.4, 1H), 4.47 (bt, *J* = 10.8, 1H), 4.52 (bs, 2H), 4.62 (bs, 1H), 7.59 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): ppm 12.3, 20.9, 25.9, 43.6, 56.0, 60.7, 68.1, 70.9, 108.6, 139.3, 151.7, 164.2.

Oligonucleotide synthesis. Oligonucleotide assembly was performed with an Expedite DNA synthesizer (Applied Biosystems) using the phosphoramidite approach. The RNA sequence was deprotected and cleaved from the solid support by treatment with methylamine (40% in water) and concentrated aqueous ammonia (1:1, 2 h, 30 °C). Following gel filtration on a NAP-25 column (Sephadex G25-DNA grade; Pharmacia) with water containing 20% of ethanol, the eluent was lyophilized and the residue was treated with 1 mL of triethylamine trihydrofluoride solution (mix of 1.5 mL NMP, 0.75 mL TEA and 1 mL of TEA·3HF) for 3 h at 55 °C. The mixture was cooled and 1 mL of ammonium acetate 1.5 M was added for neutralization followed by gel filtration on a NAP-25 column and lyophilization. DNA oligomers were deprotected and cleaved from the solid support by treatment with concentrated aqueous ammonia for 1.5 h at rt and 1.5 h at 55 °C, followed by gel filtration on a NAP-25 column. All crude mixtures were analyzed by using a Mono-Q HR 5/5 anion exchange column, after which purification was achieved by using a Mono-Q HR 10/10 column (Pharmacia) with the following gradient system: A=10 mM to 600 mM $NaClO_4$ in Tris-HCl buffer containing 15% of acetonitrile, pH 7.4. The low-pressure liquid chromatography system consisted of a Merck–Hitachi L-6200A intelligent pump, a Mono-Q HR 10/10 column

(Pharmacia), Uvicord SII 2138 UV detector (Pharmacia-LKB) and a recorder. The product containing fraction was desalted on a NAP-25 column and lyophilized.

LC-MS analysis. Oligonucleotides were dissolved at a concentration of 100 µM in H_2O. Samples (500 nL) were injected on a reverse phase column (C18 PepMap 0.5 x15 mm, Dionex) and eluted with a *N*,*N*-dimethylaminobutane/1,1,3,3,3-hexafluoro-2-propanol and acetonitrile system at a flow rate of 12 µL/min. Spectra were acquired using an orthogonal acceleration/time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) in negative ion mode and subsequently deconvoluted using the MaxEnt algorithm (MassLynx 3.4, Micromass, Manchester, UK).

UV studies. Oligonucleotide concentrations were determined in water at 80 °C to eliminate stacking interactions using extinction values at 260 nm as reported before (C 7100, G 12180, T 8560, U 9660, A 15060; same for deoxynucleotides or ribonucleotides). For the modified thymine building block the same value was used. Tm determinations at 260 nm were done in the buffers as reported in the manuscript and T_m is determined as the first derivative of the sigmoidal curve obtained. The average of 3 runs is reported.

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