Conformation-specific cleavage of antisense oligonucleotide-RNA duplexes by RNase H

Pushpangadan I. Pradeepkumar, Edouard Zamaratski, András Földesi and Jyoti Chattopadhyaya*

Department of Bioorganic Chemistry, Box 581, Biomedical Center, S-75123 Uppsala, University of Uppsala, Sweden. E-mail: jyoti@bioorgchem.uu.se; Fax: +4618-554495; Tel: +4618-4714577

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The North-form (3′-*endo*) constrained 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine block, **T**, was systematically incorporated at various sites, one at a time, into a set of four antisense oligonucleotides (AONs). The hybrids of these AONs with a matched 15mer RNA target were subjected to the RNase H cleavage reaction, and compared with that of the native counterpart, in order to probe how far the local influence of a single North-locked sugar is transmitted in steering conformational changes in the neighbouring nucleotides. It was found that the introduction of a single North-sugar locked **T** nucleotide in the AONs makes up to four of the neighbouring nucleotides at the 5′-end of the modification site resistant to the RNase H cleavage reaction. This suggests that a stretch of 5-nucleotides, including the **T** nucleotide, in the AON strand adopts a North-type conformation, giving a local RNA/RNA type hybrid structure instead of a regular DNA/RNA type duplex structure. Although these 5-nucleotide regions were completely resistant to RNase H promoted hydrolysis, they could serve as the binding site for the enzyme. Interestingly, none of these local adaptations of the RNA/RNA type structure were observable by CD spectroscopy, showing it to be an unsuitable means of monitoring any subtle alteration of the local structure. This work, therefore, constitutes an example of how the engineered conformation of a substrate can be used to exploit the stereochemical sensitivity of an enzyme to map local microscopic conformational changes. The other implication of this work is that it provides a new tool to gather local structural information, which may help to optimize the number of constrained residues which need to be incorporated to induce the antisense strand to adopt either A- or B-type geometry in the hybrid duplex, with or without the loss of RNase H recognition and/or cleavage properties.

Introduction

The recruitment of RNase H, an endogenous enzyme that specifically degrades the target RNA part of DNA/RNA hybrid duplexes, is an important pathway for antisense action, besides translational arrest.[1] To elicit the RNase H activity, the antisense oligonucleotide (AON) part of the AON/RNA hybrid should retain the B-type DNA conformation with a 2′-*endo* sugar (South-type, *S*), while the RNA moiety should retain its A-type helix character with a 3′-*endo* sugar (North-type, *N*).[2] To fulfill these requirements, various modifications of the sugar and nucleobase, as well as of the phosphate backbone, have been investigated, and numerous reports are available about these modified AONs and their antisense action.[3] The AONs with one or more conformationally *N*-[4] or *S*-[5] form locked nucleoside residues have also been an area of considerable interest. The *N*-form constrained nucleoside-containing AONs have been, however, found to be most promising because they exhibit high affinity to the target RNA.[6] Recently, the locked nucleic acid (LNA, in which the sugar moiety is locked in the North conformation) has shown unprecedented affinity towards RNA.[4e,h,j] LNA (and other modifications which have the fixed North-sugar moiety) drive the AON helix to the A-type, resulting in an RNA/RNA-type duplex, which accounts for their higher binding affinity; this, however, leads to the loss of RNase H activity.[7] The introduction of the conformationally constrained (*N*)-methanocarbathymidine (4′,4′a-methano-4′a-carbathymidine) residue in the North-form[4a] has been shown to increase the thermodynamic stability of the AON/RNA duplex, whereas with (*S*)-methanocarbathymidine (1′,4′a-methano-4′a-carbathymidine) in the South-form,[5a] a destabilizing effect was observed. It was later found that the introduction of multiple (*N*)-methanocarbathymidines, although increasing the thermodynamic stability of the AON/RNA duplex, failed to recruit any RNase H activity.[4c]

It is now quite clear[6a,7] that all modifications that lead preferentially to a North-type sugar, including its constrained form, in an RNA-type AON result in the loss of RNase H activity, because they resemble the RNA/RNA duplex, except when they appear at the termini or in the middle in the gapmer-AON.[8]

It has been so far assumed that probably three or four North-type conformational repeats are necessary to enhance the thermal stability of an RNA-type AON/RNA duplex.[6a] Clearly, the introduction of a few North-type constrained nucleosides in an AON is perhaps necessary to enhance the binding specificity to the target RNA, yet their numbers have to be somehow restricted in order to elicit an RNase H response. At this time, however, nobody knows where the balance lies between accomplishing tighter binding and target specificity to the RNA and loosing the conformational tolerance of the RNase H recognition and its substrate specificity, owing to the *local* structural perturbances in an RNA-type AON/RNA hybrid.

We show here, by a combination of CD spectroscopy and RNase H degradation studies on a set of hybrid AON/RNA duplexes (consisting of a series of analogous single bicyclic psiconucleoside-modified AONs (**3**)–(**6**) with fixed North-sugar conformation and the complementary target RNA (**7**)), that the *local structure perturbance upon introduction of a single North-type constrained nucleoside spreads up to 4 nucleotides toward the 5′-end of the modification site,* producing neither any global helical conformational change nor reducing any overall RNase H activity compared to the native hybrid duplex: [(**2**) + (**7**)].

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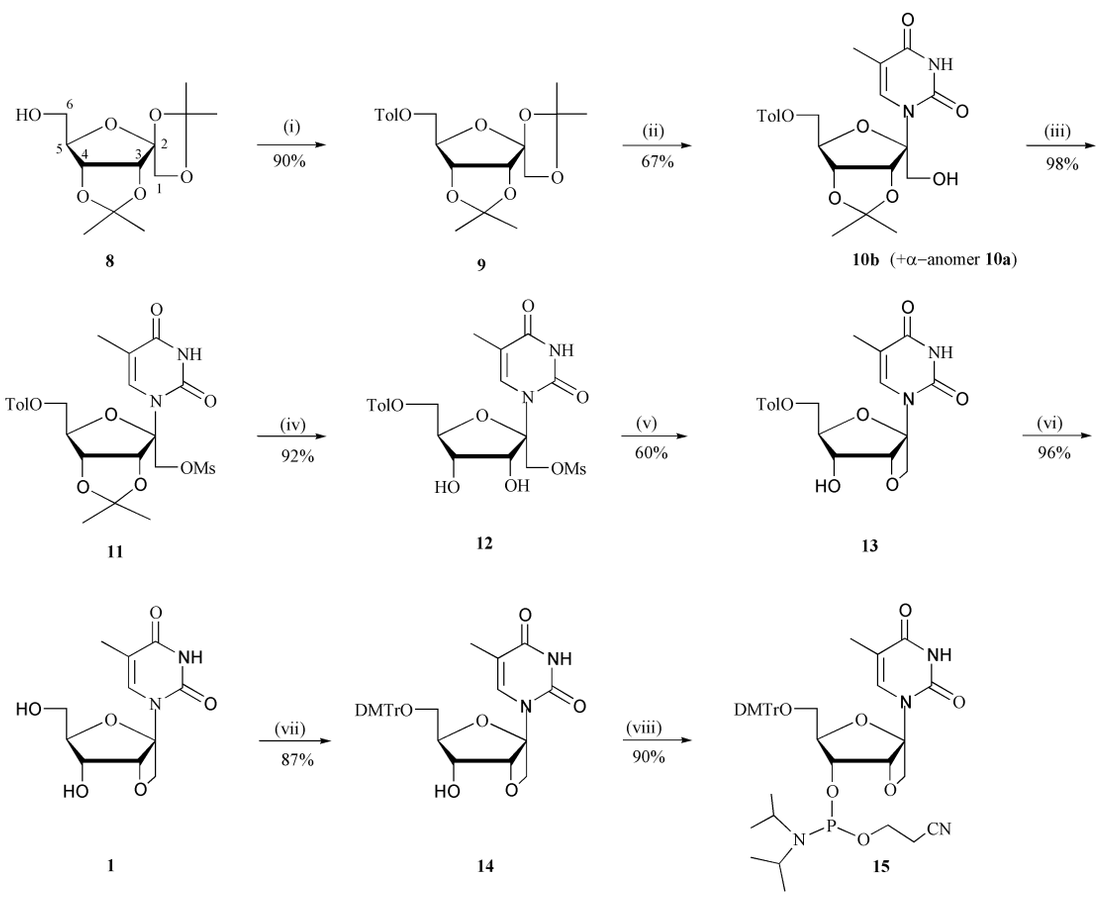
Table 1 The T_ms and thermodynamic parameters of the duplexes formed by the RNA target (**7**) and the AONs (**2**)–(**6**)

AONs (**2**)–(**6**)/ RNA (**7**) hybrids	T_m/°C	ΔT_m/°C	$-\Delta H°$/ kJ mol^{-1}	$-\Delta S°$/e.u.	$-T\Delta S°$/ kJ mol^{-1}	$-\Delta G°_{298}$/ kJ mol^{-1}	$\Delta\Delta G°_{298}$/ kJ mol^{-1}
Native AON (**2**)	44.5		494.3 (±32.4)	1.44 (±0.10)	429.1	65.2	
AON (**3**)	37.7	−6.8	413.1 (±39.7)	1.21 (±0.13)	359.9	53.2	12.0
AON (**4**)	39.5	−5	501.6 (±27.6)	1.48 (±0.09)	442.1	59.5	5.7
AON (**5**)	39.7	−4.8	465.2 (±14.5)	1.37 (±0.05)	408.2	57.0	8.2
AON (**6**)	39.3	−5.2	437.9 (±44.3)	1.28 (±0.14)	381.4	56.5	8.7



Scheme 1 *Reagents and conditions*: (i) 4-toluoyl chloride, pyridine, rt, overnight; (ii) silylated base, TMSOTf, acetonitrile, 4 °C, 1 h, rt, 18 h; (iii) MsCl, pyridine, 4 °C, overnight; (iv) 90% aq. CF_3COOH, rt, 20 min.; (v) NaH, DMF, 4 °C, 9 h; (vi) methanolic NH_3, rt, 2 d; (vii) DMTrCl, pyridine, rt, overnight; (viii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphonamidite, *N*,*N*-diisopropylethylamine, acetonitrile, rt, 2 h.

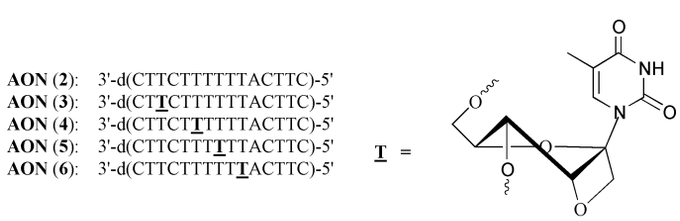


Fig. 1 Sequences of the AONs containing the conformationally restricted nucleoside **T**, their native counterpart and their RNA target.

Results and discussion

We have synthesized a bicyclicpsiconucleoside, 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine (compound **1** in Scheme 1), in which the sugar is fixed in the 3′-*endo* conformation ($J_{4',5'} = 8.4$ Hz).[9] This 1′,2′-oxymethylene bridged nucleoside has been systematically incorporated at different sites (Fig. 1) in a set of test antisense sequences [AON (**3**)–(**6**)] targeted to the coding region [oligo-RNA (**7**)] of the SV-40 large T antigen.[10] All oligodeoxynucleotides have been prepared by the phosphonamidite approach[11] on an automated DNA/RNA synthesizer and deprotected at room temperature to give the AONs (**2**)–(**6**) (**CAUTION**: opening of the oxetane ring in **T** takes place upon ammonia treatment at 55 °C).

The thermal meltings (T_ms) and the thermodyanamic properties[12] of the native and modified-AON/RNA hybrid duplexes are shown in Table 1. It can be seen that the T_ms of the modified-AON (**3**)–(**6**)/RNA (**7**) hybrid duplexes are 4.8–6.8 °C lower than the native counterpart, (**2**) + (**7**). Interestingly, the T_m differences of the AONs (**4**)–(**6**)/RNA (**7**), having modifications around the middle are within 5 ± 0.2 °C whereas modification at the 3′-terminus, in AON (**3**), results in a T_m reduction of 6.8 °C. It is noteworthy that a slight gain in enthalpy is well compensated by a loss of entropy owing to the restricted

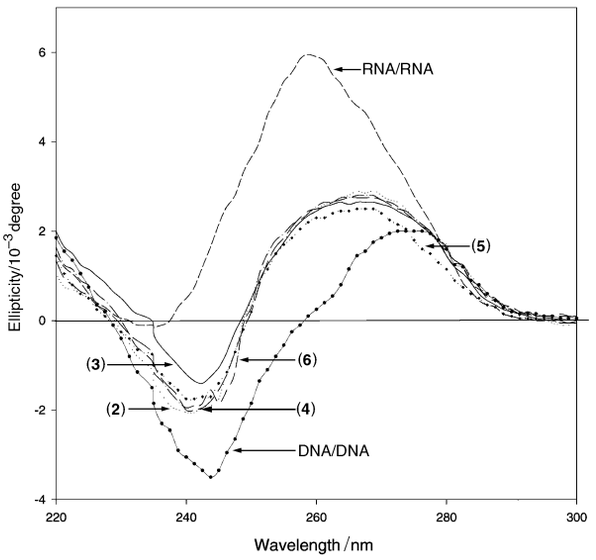


Fig. 2 CD spectra of the duplexes formed by the AONs (**2**)–(**6**) and the RNA target (**7**). For comparison, typical B-type and A-type spectra are presented: DNA/DNA duplex formed by AON (**2**) and complementary DNA: 5′-d(GAAGAAAAAATGAA)-3′, and RNA/RNA duplex formed by self-complementary 17mer RNA: 5′-r(UAACAUGUUUGGACUCU)-3′.

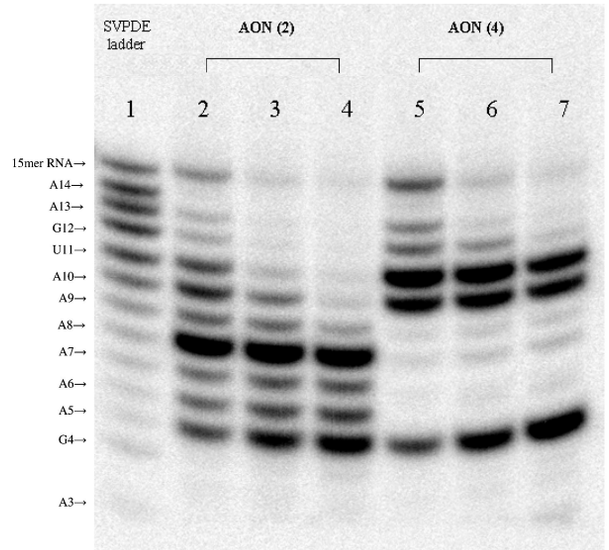


Fig. 3 RNase H hydrolysis of the target RNA (**7**) when hybridized to the AONs (**2**) (native) and (**4**): Lane 1 represents the snake venom phosphodiesterase ladder of the target RNA; Lanes 2, 3 and 4 correspond to the cleavage reaction promoted by the native AON (**2**), after 30, 60 and 120 min, respectively; Lanes 5, 6 and 7 correspond to the cleavage reaction promoted by the AON (**4**), after 30, 60 and 120 min, respectively.

motion of the constrained **T** block in the hybrid duplexes of the AONs (**3**)–(**6**) and the target RNA, resulting in an overall loss of free energy (7–12 kJ mol^{-1}) of stabilization (Table 1) relative to the native AON (**2**)/RNA (**7**) duplex at 298 K. This may suggest that the 1′,2′-oxymethylene bridged nucleoside moiety (**1**) has introduced a local conformational heterogeneity[6a] in the AON/RNA hybrid structure (*vide infra*) (*Note*: a mismatch in a DNA/RNA duplex normally results in a 12–18 °C loss of T_m).[4e]

Fig. 2 shows superimpositions of the CD spectra of all four single modified AONs (**3**)–(**6**) along with the native AON (**2**)/RNA, typical RNA/RNA and DNA/DNA duplexes.[13] All the modified AON/RNA hybrid duplexes (**3**)–(**6**) exhibited CD spectra intermediate between those of the native A-type RNA/RNA and B-type DNA/DNA duplexes, mimicking those of the natural AON (**2**)/RNA hybrid duplex. The spectra of the modified AONs (**3**)–(**6**)/RNA and the native AON/RNA hybrid duplexes had a positive band at 263–267 nm, a negative band at 240–245 nm and a crossover point at 247–250 nm. These data suggested that the single modification, the incorporation of the conformationally constrained **T** nucleotide in various sites of the AONs (**3**)–(**6**), did not alter the global helical conformation of the resulting hybrid compared to the native DNA/RNA counterpart. This is also an indication of the potential ability of the AON (**3**)–(**6**)/RNA hybrids to recruit RNase H (see below) as does the native structure.

This also seems to indicate that there are no significant conformational differences on average, that affect base stacking in the AON (**3**)–(**6**)/RNA hybrids. If a single nucleotide is *N*-type, then the resultant spectral change would indeed be small, but if 5 nucleotides became *N*-type, one might well expect a larger difference in the CD spectrum, towards the A-form. On the other hand, it is not clear how much the sugar conformation has to be altered to the *N*-type, or near *N*-type, in all four nucleotides next to the constrained **T** to alter the base stacking and helicity of the hybrid duplex. So the apparent absence of a sugar-induced co-operative effect (although observable by the RNase H assay) on the conformation of the stacked helices shows the insensitive nature of CD (only reflecting base stacking geometry). The lack of CD spectroscopic evidence presented, therefore, either argues against base-stacking induced conformational changes orchestrated by the sugar modification, or else change of the sugar puckers simply does not induce any alteration of the base stacking geometry, meaning that the sugar

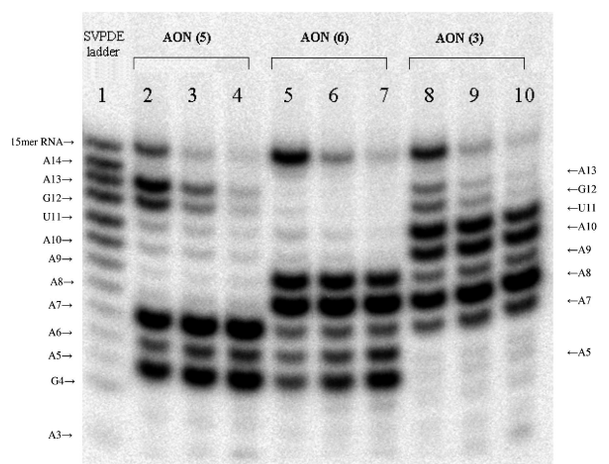


Fig. 4 RNase H hydrolysis of the target RNA (**7**) when hybridized to the AONs (**3**), (**5**) and (**6**): Lane 1 represents the snake venom phosphodiesterase ladder of the target RNA; Lanes 2, 3 and 4 correspond to the cleavage reaction promoted by the AON (**5**), after 30, 60 and 120 min, respectively; Lanes 5, 6 and 7 correspond to the cleavage reaction promoted by the AON (**6**), after 30, 60 and 120 min, respectively; Lanes 8, 9 and 10 correspond to the cleavage reaction promoted by the AON (**3**), after 30, 60 and 120 min, respectively.

pucker change is uncoupled from the base-stacking. It is also possible that a subtle change of hydration characteristics (not detectable by CD) in the minor groove might inhibit the Mg^{2+} hydrate complex of RNase H acting on the neighboring nucleotides of the **T** residue in the AON (**3**)–(**6**)/RNA hybrids.

To check its ability to elicit RNase H, the 5′-^{32}P labeled 15mer RNA target (**7**) was hybridized with the complementary AONs (**2**)–(**6**) and incubated with *Escherichia coli* RNase H1 at 21 °C. Aliquots were taken after 30, 60 and 120 min, analysed by PAGE, and the extent of the hydrolysis was estimated from the residual full length RNA left after a given incubation time. The cleavage sites and the products were identified by comparison with the partial snake venom phosphodiesterase digest ladder of the target RNA. Results are shown in Figs. 3–5: after RNase digestion for 60 min, the PAGE showed that all the modified AONs (**3**)–(**6**) were hydrolysed at similar rates compared to the native counterpart (**2**). The target RNA digestion

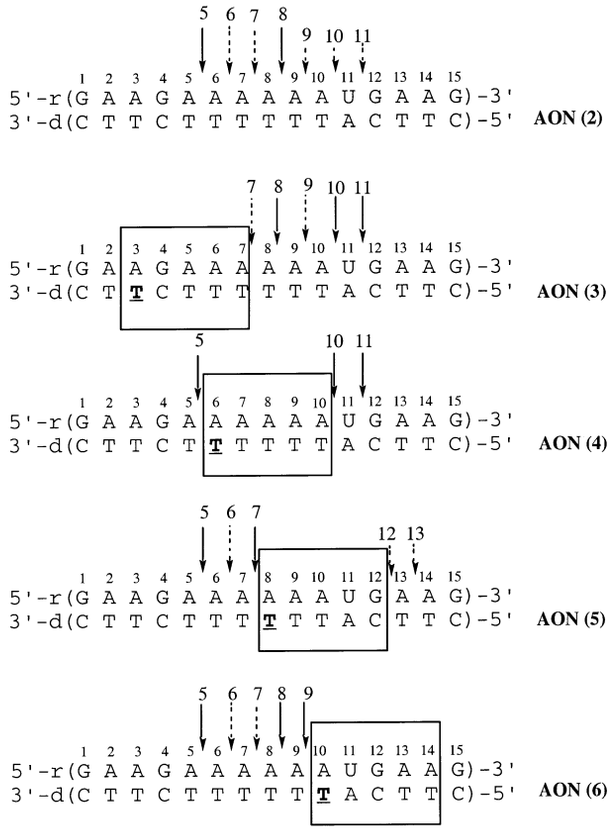


Fig. 5 RNase H cleavage pattern of the hybrid duplexes formed by the AONs (**2**)–(**6**) and the RNA target (**7**). Solid and dashed arrows represent the major and the minor cleavage sites, respectively, at complete degradation (2 h of incubation). Short arrows demonstrate the sites accessible for cleavage at the initial reaction times (30 min of incubation). Boxes represent the parts of the RNA sequence insensitive to RNase H cleavage as the result of the AON modification.

was complete after 120 min for the native AON/RNA as well as for the modified AON/RNA hybrids, despite the fact that the AONs (**3**)–(**6**)/RNA (**7**) had 4.8–6.8 °C lower T_ms than the native counterpart (**2**).

Comparison (see Fig. 5) of the cleavage sites of the AON/RNA hybrids with those of the native counterpart sheds light on how local structural differences dictate the substrate specificity, recognition and cleavage properties of degradation by RNase H:

(i) In the native hybrid duplex, [(**2**) + (**7**)], the major cleavage sites (after 120 min) were found to be at the 3′-phosphate of A5 (*i.e.* A5–A6) and A8 (*i.e.* A8–A9) (shown by arrow in Fig. 5), and the minor cleavage sites were at the 3′-phosphate of A6 and A7. However after digestion for 30 min, longer 9–11mer RNA species were detected as a result of cleavage at A9, A10 and U11, which were later degraded further to shorter fragments. This shows that the whole region from A5 to U11 in the RNA of the native duplex was accessible for RNase H promoted cleavage.

(ii) In the case of AON (**3**), the modification opposite A3 of the complementary RNA, made the region A3–A7 inaccessible for RNase H cleavage, which resulted in the loss of the cleavage sites at A5 and A6. Instead major sites at A10 and U11 appeared, in addition to the preserved sites A7, A8 and A9. This shift of the cleavage site shows that the A3–A7 region, although inaccessible to the cleavage, can serve as a binding site for the enzyme.

(iii) A modification opposite A6 of RNA in the AON (**4**) resulted in complete loss of RNase H promoted cleavage in the region A6–A10; the only sites accessible for cleavage were at A5, A10 and U11, situated on the edges of the A6–A10 region. This shows that there are at least two binding sites available in this hybrid, allowing cleavage of the RNA both at the A5 site as well as the A10/U11 sites.

(iv) This kind of RNase H recognition of the structural perturbances was also found in the cleavage behaviour of the AON (**5**)/RNA hybrid (modification opposite A8 site in RNA) where further shift of the cleavage sites was observed. The cleavage sites after 120 min were found to be at A5, A6, and A7. However after digestion for 30 min, cleavage at G12 and A13 was revealed, showing that the region A13–G15 is accessible for cleavage, which again proves that the A8–G12 region affected by the modification is not accessible for cleavage, but can serve as a binding site for the enzyme.

(v) As expected, the AON modification opposite A10 of the complementary RNA, as in the AON (**6**), resulted in the absence of any cleavage sites in the region A10–G15, while regaining all the cleavage sites from A5–A9 present in the native hybrid duplex. Interestingly, the AON (**6**) retains the major cleavage sites of the native duplex, while the 5 basepairs from A10 to A14 were insensitive to RNase H promoted cleavage.

That there was no detectable difference in the hydrolysis rates of the native and modified AON/RNA duplexes shows that the enzyme binds to the region affected by the modification almost as well as to the native hybrid, although the former has both globally and locally a DNA/RNA type structure and the latter produces a local distortion giving an RNA/RNA type structure. However, absolutely no cleavage of the RNA occurred within the modified region (spanning a stretch of 5 basepairs) although the enzyme can bind to it, which means that the structural requirements of enzyme binding and catalytic cleavage are very different.

The above results suggest the following: (a) that the cleavage activity of the enzyme was suppressed within a region, about 5 basepairs long, towards the 3′-end of the RNA in the AON/RNA hybrid, starting from the base opposite the modified **T** nucleotide in the AON strand. The neighboring sites beyond this 5 basepair region were fully susceptible to hydrolysis by the enzyme. (b) That the binding site and the cleavage site in the substrate–enzyme complex are different [14] and that the binding site is at least five nucleotides away towards the 5′-end of the RNA from the cleavage site. (c) It is also interesting to note that the structural requirements for substrate binding and substrate cleavage by RNase H appear to be different. RNase H could bind to the A3–A7 region in the AON (**3**)/RNA duplex to produce hydrolysis at A7, the cleavage at position A5 (which is present in the native duplex) is absent.

One of the interesting aspects of this work is how the *conformational transmission* from the **T** residue to the neighbouring nucleotides in the AONs controls the enzyme recognition and function. The structural distortions caused by the constrained North-type sugar conformation of **T** in any of the AONs (**3**)–(**6**) makes the neighbouring 4 basepairs at the 5′-end of the AON strand (*i.e.*, the 3′-end of the RNA strand) completely inactive to the catalytic cleavage reaction, although good for enzyme binding, showing how a conformationally constrained nucleotide can enforce the neighbouring nucleotide structure locally to adopt a specific RNA/RNA duplex type conformation [13] (compared to the normal B-DNA/A-RNA type conformation). This work, therefore, is a direct example of how the conformation of a substrate can be used to exploit the stereochemical sensitivity of an enzyme to map local microscopic conformational change. The second implication of this work is that the above information may help to optimize the number of constrained residues that need to be incorporated to induce the antisense strand to adopt either A- or B-type geometry in the hybrid duplex, with or without the loss of RNase H recognition and/or cleavage properties.

Synthesis of 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine (1)

The title compound (**1**) was prepared from 1,2:3,4-di-*O*-

isopropylidene-β-D-psicofuranose (**8**) (Scheme 1), which was synthesized from D-fructose.[15] Protection of **8** with a 4-toluoyl group gave **9**, which was coupled with *O,O*-bis(trimethylsilyl)thymine, in the presence of TMSOTf as Lewis acid and acetonitrile as solvent to furnish an (1 : 1) anomeric mixture of the protected psiconucleosides **10a** and **10b** in 67% yield.[16] These were separated by careful column chromatography and the stereochemistry of C2′ in **10** was confirmed by means of NOE measurements. Methanesulfonylation of the β-anomer **10b** afforded the 1′-mesylate **11** (98%). Deprotection of the isopropylidine group using 90% aqueous CF_3COOH yielded **12** (92%). The oxetane ring formation was achieved by treatment of **12** with NaH in DMF at 0 °C for 9 h to give **13** (60%). Removal of the 4-toluoyl group from **13** furnished the desired 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine (**1**),[17] which was converted into the phosphonamidite building block **15** (90%) *via* the 6′-*O*-(4,4′-dimethoxytrityl) derivative **14**. The phosphonamidite **15** was then used for incorporation of the **T** residue into AONs (**3**)–(**6**).

Experimental

All solvents were dried and distilled according to the reported procedures.[18] Chromatographic separations were performed on Merck G60 silica gel. Thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60 F_{254} glass-backed plates developed in the following dichloromethane–methanol mixtures: (A) 98 : 2 (v/v), (B) 95 : 5 (v/v), (C) 93 : 7 (v/v) and (D) 90 : 10 (v/v). ^{1}H-NMR spectra were recorded on JEOL GX 270 (if nothing else is indicated) and Bruker DRX 600 spectrometers at 270.1 MHz and 600 MHz, respectively, using TMS (0.0 ppm) or methanol (3.4 ppm) as internal standards. ^{13}C-NMR spectra were recorded on a JEOL GX 270 spectrometer at 67.9 MHz using the central peak of $CDCl_3$ (76.9 ppm) as an internal standard. ^{31}P-NMR spectra were recorded on a JEOL GX 270 spectrometer at 109.4 MHz using 85% phosphoric acid as an external standard. Chemical shifts (δ scale) are reported in ppm. Thermal denaturation experiments were performed on a PC computer interfaced with a Perkin-Elmer UV/VIS spectrophotometer Lamda 40 with PTP-6 peltier temperature contoller. The CD spectra were recorded using a JASCO J41-A spectropolarimeter. Fast deprotecting phosphonamidites were purchased from Amersham Pharmacia Biotech (Sweden). T4 polynucleotide kinase, *E. coli* RNase H (5units/5 μL) and [γ-^{32}P] ATP were purchased from Amersham Pharmacia Biotech (Sweden), phosphodiesterase I from *Crotalus adamanteus* venom was purchased from SIGMA.

Preparation of the phosphonamidite (15)

6-*O*-(4-Toluoyl)-1,2 : 3,4-di-*O*-isopropylidene-D-psicofuranose (9). The psicofuranose (**8**) (5.9 g, 22.5 mmol) was coevaporated with pyridine 3 times and dissolved in 100 ml of the same solvent. The solution was cooled in an ice bath and 4-toluoyl chloride (3.3 ml, 24.8 mmol) was added dropwise under a nitrogen atmosphere, with continued stirring at the same temperature for 2 h. Saturated sodium bicarbonate solution was added and stirring was continued for a further 2 h; the mixture was then extracted with CH_2Cl_2. The organic phase was washed with brine and dried over $MgSO_4$ and evaporated, then coevaporated with toluene. Recrystallisation from methanol furnished **9** (7.7 g, 20.2 mmol, 90%). R_F: 0.75 (System A). ^{1}H-NMR ($CDCl_3$): 7.9 (d, J = 8 Hz, 2H, 4-toluoyl), 7.3 (d, J = 7.9 Hz, 2H, 4-toluoyl), 4.8 (d, $J_{3,4}$ = 5.7 Hz, 1H, H-4), 4.7 (d, 1H, H-3), 4.48–4.35 (m, 3H, H-5, H-6, H-6′), 4.33 (d, $J_{1,1'}$ = 9.7 Hz, 1H, H-1), 4.1 (d, 1H, H-1′), 2.41 (s, 3H, CH_3, 4-toluoyl), 1.46 (s, 3H), 1.44 (s, 3H), 1.35, 1.33 (s, 2 × 3H, CH_3, isopropyl); ^{13}C-NMR ($CDCl_3$): 166.3 (C=O, 4-toluoyl); 143.7, 129.8, 128.9, 126.8 (4-toluoyl); 133.6, 112.7, 111.6; 85.2 (C-3); 82.9 (C-5); 82.3 (C-4); 69.7 (C-1); 64.5 (C-6); 26.4, 26.2, 24.8 (CH_3, isopropyl); 21.2 (CH_3, 4-toluoyl).

1-[3′,4′-*O*-Isopropylidene-6′-*O*-(4-toluoyl)-α-D-psicofuranosyl]thymine (10a) and 1-[3′,4′-*O*-isopropylidene-6′-*O*-(4-toluoyl)-β-D-psicofuranosyl]thymine (10b). Thymine (3.7 g, 29.6 mmol) was suspended in hexamethyldisilazane (35 ml) and trimethylchlorosilane (5.6 ml) was added. The reaction mixture was stirred at 120 °C under a nitrogen atmosphere for 16 h. The volatile material was evaporated and the residue was kept on an oil pump for 20 min. The sugar **9** (7.0 g, 18.5 mmol) was dissolved in dry acetonitrile and added to the persilylated nucleobase. The mixture was cooled to 4 °C and trimethylsilyl trifuromethanesulfonate (4.3 ml, 24 mmol) was added dropwise under a nitrogen atmosphere. After being stirred at 4 °C for 1 h, the mixture was stirred at room temperature for 18 h. Saturated NH_4Cl was added to the reaction mixture and with stirring for 30 min. The organic layer was decanted and the aqueous layer was extracted 3 times with diethyl ether. The combined organic phase was washed first with saturated sodium bicarbonate solution and then with brine, then dried over $MgSO_4$, filtered and evaporated. The resultant oil was carefully chromatographed using 0–3% MeOH–CH_2Cl_2 yielding **10a** and **10b** (5.5 g, 12.3 mmol, 67%).

(**10a**): R_F: 0.5 (System B). ^{1}H-NMR ($CDCl_3$): 8.8 (s, 1H, NH), 7.95 (d, J = 8.2 Hz, 2H, 4-toluoyl), 7.5 (s, 1H, H-6), 7.28 (d, J = 8.4 Hz, 2H, 4-toluoyl), 5.22 (d, $J_{3',4'}$ = 5.9 Hz, 1H, H-3′), 4.83 (t, $J_{4',5'}$ = 4.7 Hz, 1H, H-4′), 4.71 (dd, J_{gem} = 13.1 Hz, $J_{5',6'}$ = 7 Hz, 1H, H-6′), 4.55–4.38 (m, 2H, H-5′, H-6″), 4.29 (dd, J_{gem} = 11.8 Hz, $J_{1',1'OH}$ = 7.9 Hz, 1H, H-1′), 3.79 (dd, $J_{1'',1'OH}$ = 6.7 Hz, 1H, H-1″), 3.34 (t, 1H, 1′-OH), 2.43 (s, 3H, 4-toluoyl), 1.92 (s, 3H, CH_3), 1.39, 1.34 (s, 2 × 3H, CH_3); ^{13}C-NMR ($CDCl_3$): 166.6 (C=O, 4-toluoyl); 164.1 (C-4); 150 (C-2); 144.3 (4-toluoyl); 135.1 (C-6); 129.6, 129.2, 126.2 (4-toluoyl); 113.8 (C-5); 108.9 (*C*-Me_2); 99.7 (C-2′); 83.7 (C-5′); 82.5 (C-3′); 80.7 (C-4′); 65.1 (C-1′); 63.7 (C-6′); 27, 25.3 (CH_3, isopropyl); 21.5 (O*C*H_3); 12.5 (CH_3, C-5 CH_3). 1D Diff. NOE shows 1.6% NOE enhancement for H6–H5′ and no other NOEs between the other endocyclic-sugar protons and H6, which are present in the β-anomer (see below).

(**10b**): R_F: 0.45 (System B). ^{1}H-NMR ($CDCl_3$): 9.2 (s, 1H, NH), 7.71 (d, J = 8.2 Hz, 2H, 4-toluoyl), 7.5 (s, 1H, H-6), 7.18 (d, J = 7.9 Hz, 2H, 4-toluoyl), 5.44 (d, $J_{3',4'}$ = 6.2 Hz, 1H, H-3′), 4.87 (d, 1H, H-4′), 4.85–4.82 (m, 1H, H-5′), 4.65 (dd, J_{gem} = 12.6 Hz, $J_{5',6'}$ = 2.4 Hz, 1H, H-6′), 4.3–4.2 (m, $J_{5',6''}$ = 3.7 Hz, 2H, H-6″ and H-1′), 3.8 (dd, $J_{1'',1'OH}$ = 6.4 Hz, J_{gem} = 12.4 Hz, 1H, H-1″), 3.27 (t, 1H, 1′-OH), 2.4 (s, 3H, CH_3, 4-toluoyl), 1.6 (s, 1H, CH_3, thymine), 1.56, 1.4 (s, 2 × 3H, CH_3, isopropyl); ^{13}C-NMR ($CDCl_3$): 165.6 (C=O, 4-toluoyl); 164.3 (C-4); 150.1 (C-2); 144.6 (4-toluoyl); 137.3 (C-6); 129.2, 128.9, 125.9 (4-toluoyl); 113.4 (C-5); 108.6 (*C*-Me_2), 101.2 (C-2′); 86.1 (C-3′); 83.4 (C-5′); 81.7 (C-4′); 64.2 (C-6′); 63.7 (C-1′); 25.6, 24.1 (CH_3, isopropyl); 21.4 (CH_3, 4-toluoyl); 11.9 (CH_3, thymine). 1D Diff. NOE shows 0.21% NOE enhancement for H6–H6′, 0.08% NOE for H6–H3′ and 0.4% NOE for H6–H4′. These are consistent with the β-anomer.

1-[1′-*O*-Methylsulfonyl-3′,4′-*O*-isopropylidene-6′-*O*-(4-toluoyl)-β-D-psicofuranosyl]thymine (11). Compound **10b** (1.6 g, 3.5 mmol) was coevaporated with pyridine three times and dissolved in 25 ml of the same solvent. The mixture was cooled in an ice bath and methanesulfonyl chloride (0.75 ml, 9.7 mmol) was added dropwise to the mixture, with continued stirring for 15 min at the same temperature. The reaction was kept in at 4 °C for 12 h, then poured into cold saturated sodium bicarbonate solution and extracted with CH_2Cl_2. The organic phase was washed with brine, dried over $MgSO_4$, filtered and evaporated, then coevaporated with toluene giving compound **11** (1.89 g, 3.5 mmol, 98%). R_F: 0.7 (System B). ^{1}H-NMR ($CDCl_3$): 7.75 (d, J = 8.3 Hz, 1H, 4-toluoyl), 7.38 (d, J = 1.3 Hz, 1H, H-6), 7.22 (d, J = 8.4 Hz, 1H, 4-toluoyl), 5.39 (d, $J_{3',4'}$ = 6 Hz, 1H, H-3′), 4.96 (d, J_{gem} = 11.4 Hz, 1H, H-1′a), 4.94–4.88 (m, 2H, H-4′ and H-5′), 4.7 (dd, J_{gem} = 12.6 Hz, $J_{5',6'}$ = 2.5 Hz,

1H, H-6′), 4.39 (d, 1H, H-1″), 4.3 (dd, $J_{5',6''}$ = 3.4 Hz, 1H, H-6″), 2.98 (s, 3H, CH_3, OMs), 2.4 (s, 3H, CH_3, 4-toluoyl), 1.7, 1.66 (s, 2 × 3H, CH_3, isopropyl); ^{13}C-NMR ($CDCl_3$): 165.7 (C=O, 4-toluoyl); 162.9 (C-4); 150.2 (C-2); 145.1 (4-toluoyl); 135.5 (C-6); 129.1, 128.7, 125.6, (4-toluoyl); 114.2 (C-5); 110.1 (*C*-Me_2); 98.3 (C-2′); 87.1 (C-3′); 84.2 (C-5′); 81.7 (C-4′); 69.9 (C-1′); 64.1 (C-6′); 37.4 (CH_3, 4-toluoyl); 25.8, 24.3 (CH_3, isopropyl); 21.3 (CH_3, mesyl); 12.3 (CH_3, thymine).

1-[1′-*O*-Methylsulfonyl-6′-*O*-(4-toluoyl)-β-D-psicofuranosyl]-thymine (12). Compound **11** (1.9 g, 3.5 mmol) was stirred with 10.5 ml of 90% CF_3COOH in water for 20 min at room temperature. The reaction mixture was evaporated and coevaporated with pyridine. Chromatography of the residue furnished **12** (1.58 g, 3.3 mmol, 92.5%). R_F: 0.3 (System B). ^{1}H-NMR ($CDCl_3$ + CD_3OD): 7.75 (d, J = 8.3 Hz, 1H, 4-toluoyl), 7.52 (d, J = 1.24 Hz, 1H, H-6), 7.2 (d, J = 8.4 Hz, 1H, 4-toluoyl), 4.81 (d, J_{gem} = 11.6 Hz, 1H, H-1′), 4.76 (d, $J_{3',4'}$ = 5.3 Hz, 1H, H-3′), 4.75 (dd, J_{gem} = 12.6 Hz, $J_{5',6'}$ = 3.5 Hz, 1H, H-6′), 4.62 (dt, $J_{5',6''}$ = 2.5 Hz, 1H, H-5′), 4.58 (d, 1H, H-1′), 4.41 (dd, $J_{4',5'}$ = 3 Hz, 1H, H-4′), 4.33 (dd, 1H, H-6″), 2.98 (s, 3H, CH_3, OMs), 2.4 (s, 3H, CH_3, 4-toluoyl), 1.73 (s, 3H, CH_3, thymine); ^{13}C-NMR ($CDCl_3$ + CD_3OD): 165.9 (C=O, 4-toluoyl); 163.8 (C-4); 151.7 (C-2); 144.9 (4-toluoyl); 136.3 (C-6); 129.2, 129, 126.1 (4-toluoyl); 110.4 (C-5); 97 (C-2′); 83.9 (C-5′); 79.8 (C-3′); 72.2 (C-4′); 69.3 (C-1′); 63 (C-6′); 37.5 (CH_3, 4-toluoyl); 21.3 (CH_3, mesyl); 11.9 (CH_3, thymine).

1-[1′,3′-*O*-Anhydro-6′-*O*-(4-toluoyl)-β-D-psicofuranosyl]-thymine (13). To a stirred solution of 80% NaH (171 mg, 5.7 mmol) in 15 ml of DMF in an ice bath, a solution of compound **12** (1.3 g, 2.6 mmol) in 15 ml of DMF was added dropwise. The reaction mixture was stirred at the same temperature for 9 h, quenched with 10% acetic acid solution in water and evaporated. The residue was coevaporated with xylene and on chromatography yielded **13** (602 mg, 1.55 mmol, 60%). R_F: 0.5 (System C). ^{1}H-NMR ($CDCl_3$): 7.93 (d, J = 8.1 Hz, 2H, 4-toluoyl), 7.25 (d, J = 7.9 Hz, 2H, 4-toluoyl), 6.81 (s, 1H, H-6), 5.47 (d, $J_{3',4'}$ = 3.9 Hz, 1H, H-3′), 5.15 (d, J_{gem} = 7.9 Hz, 1H, H-1′), 4.79–4.72 (m, J_{gem} = 12.3 Hz, $J_{6',5'}$ = 2.55 Hz, 2H, H-1′ and H-6′), 4.55–4.42 (m, $J_{6'',5'}$ = 2.9 Hz, $J_{4',5'}$ = 8 Hz, 3H, H-4′, H-5′, H-6″), 2.4 (s, 3H, CH_3, 4-toluoyl), 1.8 (s, 3H, CH_3, thymine); ^{13}C-NMR ($CDCl_3$): 166.6 (C=O, 4-toluoyl); 164.3 (C-4); 149.2 (C-2); 143.8 (4-toluoyl); 135.1 (C-6); 129.5, 128.8, 126.5 (4-toluoyl); 111.6 (C-5); 90.9 (C-2′); 87.3 (C-3′); 80.9 (C-5′); 78.1 (C-1′); 70.3 (C-4′); 63 (C-6′); 21.2 (CH_3, 4-toluoyl); 11.8 (CH_3, thymine).

1-(1′,3′-*O*-Anhydro-β-D-psicofuranosyl)thymine (1). Compound **13** (570 mg, 1.5 mmol) was dissolved in methanolic ammonia (50 ml) and stirred at room temperature for 2 days. The solvent was evaporated and the residue, on chromatography, afforded **1** (378 mg, 1.4 mmol, 96%) R_F: 0.3 (System D) ^{1}H-NMR (CD_3OD, 600 MHz): 7.38 (d, J = 1.25 Hz, 1H, H-6), 5.58 (d, $J_{3',4'}$ = 3.8 Hz, 1H, H-3′), 5.33 (d, J_{gem} = 8.1 Hz, 1H, H-1′), 4.9 (d, 1H, H-1″), 4.46–4.41 (m, $J_{4',5'}$ = 8.4 Hz, $J_{5',6'}$ = 2.2 Hz, $J_{5',6''}$ = 5.24 Hz, 2H, H-4′ and H-5′), 4.11 (dd, J_{gem} = 12.4 Hz, 1H, H-6′), 3.9 (dd, 1H, H-6″), 2.1 (s, 1H, CH_3, thymine); ^{13}C-NMR (CD_3OD): 166.8 (C-4); 151.7 (C-2); 138.4 (C-6); 112.7 (C-5); 93.2 (C-2′); 89.3 (C-3′); 85.3 (C-5′); 79.9 (C-1′); 71.9 (C-4′); 62.7 (C-6′); 12.1 (CH_3, thymine).

1-[1′,3′-Anhydro-6′-*O*-(4,4′-dimethoxytrityl)-β-D-psicofuranosyl]thymine (14). To a solution of **1** (353 mg, 1.3 mmol) in anhydrous pyridine (6 ml) was added 4,4′-dimethoxytrityl chloride (DMTrCl; 510 mg, 1.5 mmol), and the mixture was stirred at room temperature overnight. Saturated $NaHCO_3$ solution was added and the mixture extracted with dichloromethane. The organic phase was washed with brine, dried over $MgSO_4$, filtered and evaporated. Column chromatography of the residue afforded **14** (647 mg, 1.13 mmol, 87%). R_F: 0.5 (System B). ^{1}H-NMR ($CDCl_3$): 7.4–7.1 (m, 12H, aromatic DMTr and H-6), 6.85–6.82 (m, 4H, aromatic DMTr), 5.4 (d, $J_{3',4'}$ = 4.1 Hz, 1H, H-3′), 5.13 (d, J_{gem} = 7.9 Hz, 1H, H-1′), 4.76 (d, 1H, H-1″), 4.35 (dd, $J_{4',5'}$ = 8.3 Hz, 1H, H-4′), 4.28–4.21 (m, $J_{5',6'}$ = 2.5 Hz, $J_{5',6''}$ = 4.7 Hz, 1H, H-5′), 3.98 (dd, J_{gem} = 12.4 Hz, 1H, H-6′), 3.81 (dd, 1H, H-6″), 3.8 (s, 6H, OCH_3, DMTr), 1.92 (s, 3H, CH_3, thymine); ^{13}C-NMR ($CDCl_3$): 164.23, 158.1 (C-4); 149.5, 144.5 (C-2); 135.9, 135.3, 129.8, 128.9, 127.9, 127.5, 126.4, 112.8 (DMTr); 111.6 (C-5); 90.9 (C-2′); 87.6 (C-3′); 83.6 (C-5′); 78.2 (C-1′); 69.7 (C-4′); 60.8 (C-6′); 54.9 (DMTr); 11.9 (CH_3, thymine).

1-{1′,3′-Anhydro-4′-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-6′-*O*-(4,4′-dimethoxytrityl)-β-D-psicofuranosyl}thymine (15). To a stirred solution of **14** (529 mg, 0.9 mmol) in THF (5 ml), 0.8 ml of *N*,*N*-diisopropylethylamine was added under a nitrogen atmosphere with stirring at room temperature for 10 min. To this solution 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphonamidite (0.4 ml, 1.8 mmol) was added with continued stirring for 2 h. The reaction was quenched with methanol (3 ml) and the mixture was dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated. Chromatography of the residue (30–40% EtOAc, cyclohexane + 2% Et_3N) furnished **15** (632 mg, 0.81 mmol, 90%). R_F: 0.5 (System B). The compound was dissolved in CH_2Cl_2 (3ml) and precipitated from hexane at −40 °C. ^{31}P-NMR ($CDCl_3$): 150.55; 150.46.

Synthesis, deprotection and purification of oligonucleotides

All oligonucleotides were synthesizesd on 1 μmol scale with an 8-channel Applied Biosystems 392 DNA/RNA synthesizer. Synthesis and deprotection of the AONs, as well as the RNA target, were performed as previously described.[19] For modified AONs fast depropecting amidites were used and they were deprotected by room temperature treatment with NH_4OH for 16 h. All AONs were purified by reversed-phase HPLC eluting with the following systems: A (0.1 M triethylammonium acetate, 5% MeCN, pH 7) and B (0.1 M triethylammonium acetate, 50% MeCN, pH 7). The RNA target was purified by 20% 7 M urea polyacrylamide gel electrophoresis and its purity, and that of all the AONs (greater than 95%) was confirmed by PAGE. Representive data from MALDI-MS analysis: AON (**4**) [M − H]$^-$ 4478.7; calcd 4478; RNA target (**7**) [M − H]$^-$ 4918.1; calcd 4917.1.

UV Melting experiments

Determination of T_ms of the AON/RNA hybrids were carried out in the same buffer as for RNase H degradation: 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM $MgCl_2$ and 2 mM DTT. Absorbance was monitored at 260 nm in the temperature range 20 to 60 °C with a heating rate of 1 °C per minute. Prior to the measurement the samples (1 : 1 mixture of AON and RNA) were preannealed by heating to 80 °C for 4 min followed by slow cooling to 20 °C and 30 min equilibration at this temperature.

Thermodynamic calculations from UV experiments

The thermodynamic parameters characterizing helix-to-coil transition for the DNA/RNA hybrids were obtained from T_m measurements over the concentration range from 2 to 10 μM (total strand concentration, C_T). Values of $1/T_m$ were plotted *versus* (C_T/4) and $\Delta H°$ and $\Delta S°$ parameters were calculated from slope and intercept of fitted line: $1/T_m = (R/\Delta H°)\ln(C_T/s) + \Delta S°/\Delta H°$, where s reflects the sequence symmetry of the self (s = 1) or nonself-complementary strands (s = 4).

CD Experiments

CD spectra were measured from 300 to 220 nm in a 0.2 cm path

length cuvette. Buffer conditions were same as for the UV and RNase H experiments. The total strand concentration used was 2.5 μM and spectra were recorded at 21 °C. Each spectrum was an average of two scans with the buffer blank subtracted, which was recorded at the same scan speed.

^{32}P Labeling of RNA

The target RNA was 5′-end labeled with ^{32}P using T4 polynucleotide kinase, [γ-^{32}P]ATP using a standard procedure.[20] Labeled RNA was purified by 20% denaturing PAGE and specific activitiy was measured using a Beckman LS3801 counter.

RNase H digestion assays

DNA/RNA hybrids (0.8 μM) consisting of a 1 : 1 mixture of AON and the target RNA (specific activity 50 000 cpm) were digested with 0.6 U of RNase H in 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM $MgCl_2$ and 2 mM DTT at 21 °C. Total reaction volume was 26 μL. Aliquots (7 μL) were taken at 30, 60 and 120 min and the reaction was stopped by addition of an equal volume of 20 mM EDTA in 95% formamide. RNA cleavage products were resolved by 20% polyacrylamide denaturing gel electrophoresis and visualized by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics phosphorImager.

Conclusions

(1) Incorporation of the bicyclic psiconucleoside **1**, in which the sugar moiety is fixed in the North-form, into AONs (**3**)–(**6**) resulted in decreased thermodynamic stability of the corresponding duplexes with target RNA (**7**) compared to the native counterpart.

(2) CD Experiments failed to detect any local structure alterations in the modified AON/RNA hybrid duplexes, and showed high similarity between their CD spectra and that of the native counterpart.

(3) All AON/RNA hybrids formed by the modified AONs (**3**)–(**6**) were found to be substrates for RNase H and showed hydrolysis to a comparable extent (after 120 min of incubation) with that of the native counterpart, in spite of their lower thermodynamic stability.

(4) The introduction of the single conformationally constrained nucleotide **T** caused local conformational perturbances in the AON/RNA hybrids spanning 4 nucleotides towards the 5′-end of the AON, which was clearly reflected in the RNase H hydrolysis pattern of all the duplexes studied.

(5) Although not susceptible to cleavage, the structurally distorted 5 basepair region of the AON/RNA hybrids can serve as a binding site for the enzyme. This means that structural requirements for substrate binding and substrate cleavage are different.

(6) The binding site and the cleavage site in the substrate–enzyme complex are different and the binding site distant by at least five nucleotides toward the 5′-direction of the RNA from the cleavage site.

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13 After the completion of this work (*Tetrahedron Lett.*, 2000, **41**, 8601), we have come across the work of Bondensgaard *et al.* (Aug 4th issue of *Chem. Eur. J.*, 2000, **6**, No. 15, 2687) on the structural studies of LNA/RNA duplexes in which the 3′-nucleotide flanking the modification site adopts a clear N-type conformation (NMR); these workers however conclude that overall A-type conformation exists in the AON strand of the duplex stretching only one nucleotide both at the 3′- and 5′-ends of the LNA nucleotide modified site, which means that this part of the duplex takes up a more A-RNA/A-RNA type helix. This contradicts our present observations, in which we see a local structural change spanning *only* at the 5′-end of the modified nucleotide of the AON strand and the 3′-end of the complementary RNA strand. Our present work allows us to predict that one would perhaps observe a similar absence of RNase H cleavage at those comparable sites of the LNA/RNA duplexes owing to the double stranded RNA/RNA conformation.
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