New methylene-bridged hexopyranosyl nucleoside modified oligonucleotides (BHNA): synthesis and biochemical studies

Chuanzheng Zhou and Jyoti Chattopadhyaya*

Department of Bioorganic Chemistry, Box 581, Biomedical Center, Uppsala University, SE-751 23 Uppsala, Sweden
E-mail: jyoti@boc.uu

This paper is dedicated to Professor Harri Lönnberg on the occasion of his 60th anniversary

Abstract

The hyper-constrained nucleoside, methylene-bridged hexopyranosyl nucleoside (BHNA) was incorporated into the antisnese oligonucleotides (AON), which show more preference for binding toward the complementary RNA (T_m loss by ca 5°C) than that with the complementary DNA (T_m loss by 10°C), vis-à-vis corresponding native duplex. The origin of reduction of T_m of the duplexes formed by the BHNA incorporated AON and the complementary RNA or DNA was further investigated by thermal denaturation study with the single-mismatched DNA or RNA, CD spectroscopy, RNase H digestion study, as well as by molecular model building. These studies showed that the introduction of BHNA causes only a limited local conformational perturbation in the AON/RNA heteroduplex, whereas it affects the global conformation in the AON-DNA duplex. BHNA incorporated AONs also show improved stability in the human blood serum, which may prove to have some potential therapeutic application.

Keywords: Conformationally-constrained nucleotide, hexopyranosyl nucleotide, antisense oligonucleotides (AON), nuclease stability, molecular modeling

Introduction

Conformationally-constrained oligonucleotides have attracted considerable interest in the last two decades because they enable us to dictate specific conformational character in to the therapeutically interesting oligonucleotides, thereby allowing to steer their biological properties, as required in the antisense, siRNA or triplex strategy.[1-3] The pentose-sugar moiety in natural nucleos(t)ide is very flexible compared to those of the hexose-based oligos, and consequently can adopt a dynamic equilibrium of several sugar pseudorotamers.[4, 5] It has been however found that the North-type (34º > P> -1º, Φ_m > 30º) or South-type (194º > P > 137º, Φ_m > 48º)

conformation determines the global conformation of DNA (A-form B-form or Z-form) or RNA (A-type), and consequently their physical, chemical and biological properties.[6] We have shown that the purine-rich single-stranded native oligonucleotides[7] can adopt a helical structures that are reminiscent of the native double-stranded counterpart. Appropriate modification of the sugar moiety of oligonucleotide can also potentially assist in their preorganization in to a helical form, and can improve its recognition to the target RNA and/or DNA,[8] which is important in the antisense and siRNA technology. Sugar modifications, so far known, can be classified in to three major types: (1) The 2′-substituion with an electronegative group such as -OMe, F, MOE[9] can drive the pentose sugar part adopting a North-type conformation, which, when introduced to the AON, can induce the modified AON to have higher affinity to the target RNA (2-3ºC/modification). (2) Covalent links between the C2′ and C4′ with alkyloxyl (LNA,[10] ENA[11, 12]) or the carbocylic chain (carba-LNA[13] and carba-ENA[13, 14]), or by a covalent linkage between C1′ and C2′ (such as oxetane[15] or azetidine[16, 17] types of constraints) result in to a conformationally-constrained North-type sugar. Oligonucleotides with these types of covalently constrained nucleosides can also potentially preorganize the single-stranded oligonucleotide in to a helical form, which, in turn, show much improved affinity toward complementary RNA (3-8ºC/modification, with the exception of C1′ and C2′ constrained systems) as well as to DNA[18]; they also show improved resistance toward enzyme degradation.[13, 17] (3) The AONs produced by the replacement of the pentofuranosyl sugar with the hexopyranosyl sugar[19] give thermodynamically stable duplexes with the target DNA or RNA. It is because, first, the six-membered ring system adopt a rigid chair conformation, requiring a less negative entropy change during the duplex formation; and second, the interstrand phosphate distance in the six-membered pyranosyl-modified system is larger than in the natural nucleic acid duplexes giving a relatively less interstrand charge repulsion compared to the native counterpart.[19] Many different types of pyranosyl modified nucleosides have been so far incorporated in to AONs, and their affinity toward complementary RNA or DNA, as well as the biochemical features have been studied by the groups of Herdewijn and Eschenmoser:[2] AONs composed of homo-DNA (Figure 1) is almost linear, and not able to form duplexes with either RNA or DNA[19, 20], While HNA[21, 22], CNA[23] and CeNA[24-26] all have improved affinity toward RNA and show improved nuclease resistance compared to the native counterpart. Amongst these the CeNA modified AONs have attracted more attention because they are able to activate RNase H, resulting in cleavage of the RNA strand in the AON-RNA heteroduplexes.

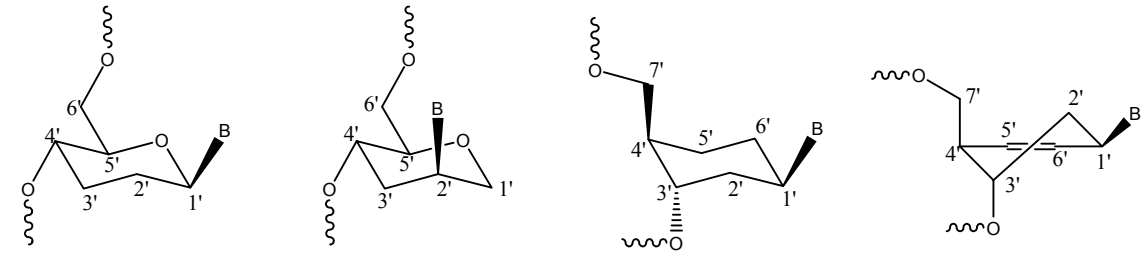


Figure 1. Structures of various pyranosyl nucleoside incorporated in to oligonucleotides.

Recently, during our effort to functionalize carbocyclic moiety of the carbocyclic-LNA[13, 27], we obtained one unusual bicyclo[2. 2.1] 2′,5′- methylene-bridged hexose nucleoside **1**[28] (BHNA, Scheme 1), which is a member of pyranose sugar family but is more rigid than any other reported constrained nucleoside. Here, we report the incorpoation of this hyper-constrained BHNA nucleoside into AON by the phosphoramidite-based DNA synthesis. We also report their affinity and specificity toward the complementary RNA and DNA, studied by UV melting experiment. We have also measured the CD spectra of their homo and heteroduplexes to evidence their global structure, which was also confirmed by RNase H foot printing experiment. It has also turned out that modification of the AON with the new hyper-modified pyranose-modified molecules, as in **1**, greatly improve human blood serum stability in comparison with the native counterpart.

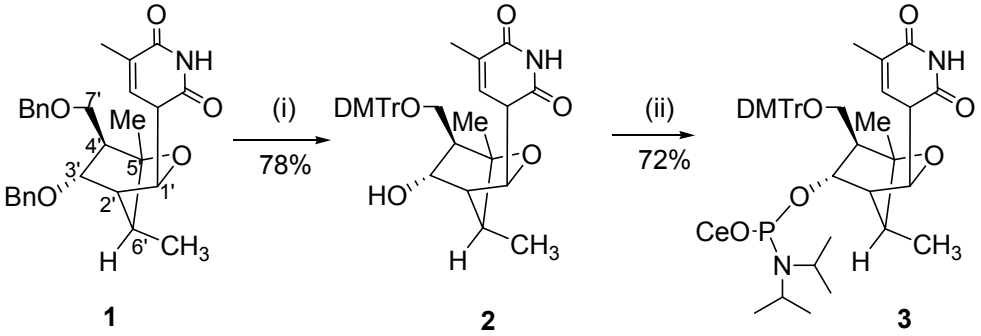
Results

(A) Phosphoramidite synthesis

During our effort to functionalize the carbocyclic moiety of the 5-membered carba-LNA[13] through new synthetic routes[27], we attempted to remove the 6′-hydroxyl group of 6′-hydroxy-6′-methyl-carba-LNA[27], prepared from 6′-hydroxy-carba-LNA[27,28]: The synthetic route to compound **1**[28] starts from 6′-hydroxyl-6′-methyl-carba-LNA[27] (prepared by Chuanzheng Zhou in ref 27), which was obtained by the oxidation of 6′-hydroxy-carba-LNA (prepared by Munir Andaloussi in ref 27) followed by Grignard reaction to give 6′-hydroxy-6′-methyl-carba-LNA[27]. Unexpectedly, a unique stereospecifically rearranged compound **1** (Scheme 1) was obtained[28] during the radical deoxygenation of 6′-methyloxalyl-6′-methyl-carba-LNA in addition to a diastereomeric mixture of C6′-reduced products.[27,28] It turned out that this radical rearrangement only takes place when the tertiary radical is formed from the 5-membered conformationally-constrained 6′-methyloxalyl-6′-methyl-carba-LNA[27]. The synthetic implication and mechanistic studies of this unusual rearrangement will be published elsewhere.[28] Compound **1** was spectroscopically pure and its structural determinations were based upon ^{1}H NMR, ^{13}C NMR,

DEPT, COSY, ^{1}H-^{13}C HMQC and long range HMBC and 1D-nOe experiments as well as by MALDI-TOF mass spectrometry (see supporting information).

Compound **1** was debenzylated using 20% $Pd(OH)_2/C$ and ammonium formate under reflux in methanol for 4h, followed by 5′-dimethoxytritylation to give **2** (two step 78%), which was 3′-phosphitylated to give the phosphoramidite **3** using standard conditions.[29] The ^{31}P NMR spectrum of compound **3** showed that this phosphoramidite was pure (Figure 2), hence was used for oligo-DNA synthesis directly.



Scheme 1. *Reagents and conditions*: (i) a) 20% $Pd(OH)_2/C$, Ammonium Formate, dry methanol, reflux, 3 h; b) DMTr-Cl, dry pyridine, overnight; (ii) 2-cyanoethyl N,N-diisopropylphosphoramidochloridite, DIPEA, dry THF, 2h.

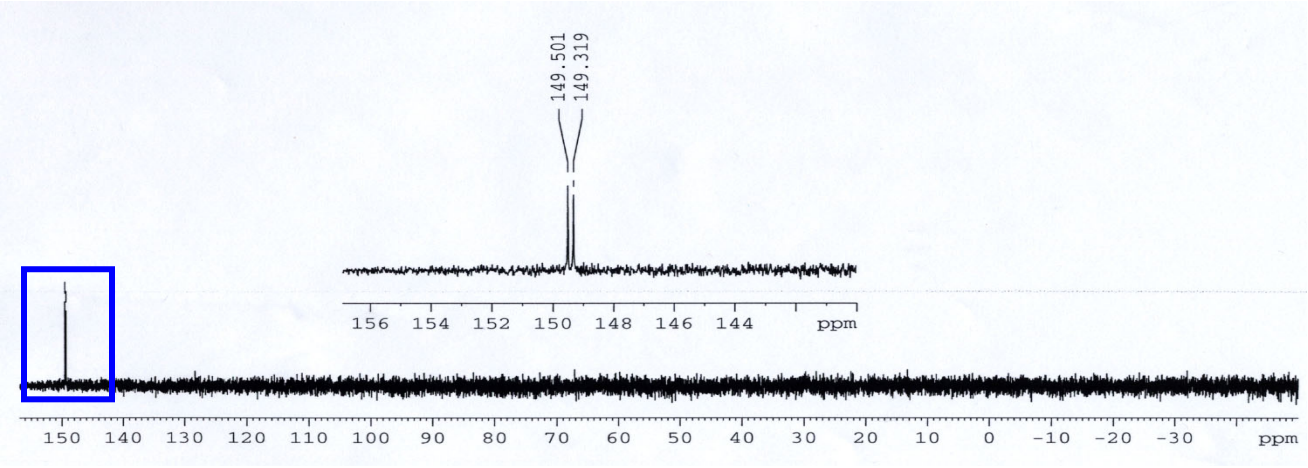


Figure 2. ^{31}P NMR spectrum of phosphoramidite **3**. The region inside the blue frame is expanded and shown in the middle of the figure to evidence the purity.

(B) Synthesis and thermal denaturation studies of BHNA incorporated AONs

The phosphoramidite **3** was incorporated into a 15mer DNA sequence by solid supported DNA synthesis protocol on Applied Biosystems 392 RNA/DNA synthesizer. Standard DNA synthesis cycle (1 μmol scale) and common reagents[29] were used except for incorporating modified

nucleotides **3** by using a coupling time of 10 minutes. Cleavage of oligos from the support and deprotection was carried out just by treating the solid support with 33% aqueous ammonia at room temperature for 12 h. The fully-deprotected AONs were then purified by 20% denatured PAGE.

The selected 15mer DNA sequence is targeted to the coding region of SV40 large T antigen.[30] Four sequences were synthesized and each sequence has single modification of BHNA but at different sites of the 15mer DNA sequence. The sequences and modification position are shown in Table 1. The integrity of AONs was corroborated by MALDI-TOF mass measurement (see Table 1).

The affinity of AONs 2-5 toward their complementary strands, DNA or RNA, were evaluated by UV melting experiments (Table 1). The melting temperatures (T_m) of duplex between native AON **1** and complementary DNA and RNA were 45.5ºC and 44.7ºC, respectively. The T_ms of AONs **2-5**/DNA duplexes were 5.8–11.5ºC lower than the native counterpart AON **1**/ DNA. The terminal modification in the sequence AON **2** showed however relatively less T_m decrease (-5.8ºC) compared to the modification introduced in the middle of the sequences, AONs **3-5** (-10 to -11 ºC decrease). This sequence-dependent T_m decrease suggests the BHNA probably caused a global conformational change in the AON/DNA duplex.

BHNA also destabilized the AON/RNA duplex, but this effect is much less than in the AON/DNA duplexes, in view of the fact AONs **2-5**/RNA heteroduplexes showed only T_m decrease of -4.3 to –5.5 ºC. Moreover, the middle-modified sequences AONs 3-5 showed a more moderate reduction of T_m (-4.1 to –4.3ºC) compared to the terminal modified sequence AON **2**. These observations suggest the BHNA only cause local structural perturbation in the AON/RNA duplexes, which means that it could be still be a good substrate for RNase H digestion.

In order to validate this hypothesis that BHNA only introduced a local conformational heterogeneity in AON/RNA hybrid duplex, AON **5** was used to target RNAs **2-4** (Table 2) which have one mismatch just opposite the BHNA. The T_m values of duplexes of AON **5**/RNA **2,** AON **5**/RNA **3**, AON **5**/RNA **4** are –6.5, -10.6, -8.9ºC lower respectively than fully-matched duplex AON **5**/RNA **1**, which is very similar to the native counterpart AON **1**, in that AON **1**/RNA **2**, AON **1**/RNA **3**, AON **1**/RNA **4** showed T_m decrease –2.9, -11.5, -9.2ºC respectively than fully-matched duplex AON **1**/RNA **1**. This observation suggested that 1-thyminyl moiety of BHNA can base-pair well with the opposite complementary nucleobase moiety in the AON/RNA hybrid, and confirmed that BHNA can only cause local perturbation in the AON/RNA duplex.

Table 1. Thermal Denaturation (T_m) of native and modified AONs in the duplexes with complementary RNA or DNA[a]

Entry	Sequence	$(MH)^+$		T_m (°C)			
		Calc.	Found	With RNA		With DNA	
				T_m	ΔT_m	T_m	ΔT_m*
AON 1	3′-d(CTT CTT TTT TAC TTC)	4448.7	4449.7	44.7		45.5	
AON 2	3′-d(CT**T** CTT TTT TAC TTC)	4502.7	4503.0	39.2	-5.5	39.7	-5.8
AON 3	3′-d(CTT CT**T** TTT TAC TTC)	4502.7	4503.1	40.6	-4.1	35.2	-10.3
AON 4	3′-d(CTT CTT T**T**T TAC TTC)	4502.7	4502.8	40.4	-4.3	34.0	-11.5
AON 5	3′-d(CTT CTT TTT **T**AC TTC)	4502.7	4503.0	40.4	-4.3	35.0	-10.5

[a] T_m values measured as the maximum of the first derivative of the melting curve (A260 vs temperature) in medium salt buffer (60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM $MgCl_2$) with temperature range 20 to 70 °C using 1 μM concentrations of the two complementary strands. ΔT_m: T_m relative to RNA compliment; ΔT_m*: T_m relative to DNA compliment.

Table 2. T_m (°C) of AON **1** or AON **5** in the duplexes with complementary RNA with or without one base mismatch.[a]

	Complementary RNA : 5′-r(GAA GAA AAA **X**UG AAG)			
	X = A(**RNA 1**,paired)	G(**RNA 2**)	C(**RNA 3**)	U(**RNA 4**)
AON 1	44.7	41.8 (-2.9)[b]	33.2 (-11.5)[b]	35.5 (-9.2)[b]
AON 5	40.4	33.9 (-6.5)[b]	29.8 (-10.6)[b]	31.5 (-8.9)[b]

[a] medium salt buffer is shown in footnote of Table 1. [b] The value in parentheses is ΔT_m which is the difference between mismatched and matched duplexes.

(C) Helical structure of the modified oligonucleotide duplex with DNA and RNA

CD spectroscopy was used to further qualitatively illustrate the nature of helical conformation of AON/DNA vis-a-vis AON/RNA duplexes (Figure 3). Since BHNA causes serious reduction of T_m (ca 10° C reduction for AON/DNA and ca 5° C reduction for AON/RNA) suggest that AON/DNA and AON/RNA conformations for these modified duplexes are likely to be different. It is noteworthy that this reduction of T_m is more marked for the AON/DNA duplexes than for the AON/RNA duplexes. Both AON **2**/RNA and AON **5**/RNA exhibited CD spectra intermediate between the native A-type RNA/RNA and B-type DNA/DNA duplexes, mimicking the natural AON **1**/RNA hybrid duplex, which confirmed again that BHNA did not cause much conformational perturbation in AON/RNA duplex compared to AON/DNA duplex. On the contrary, the positive Cotton bands for AON **2**/DNA and AON **5**/DNA, 279 nm and 278 nm respectively, are blue shifted, and are more intense than the native AON **1**/DNA duplex (281 nm). This result suggests that a single modification of BHNA in AON can modulate the

AON/DNA duplex conformation beyond alteration of the local geometry, it has more global impact in terms of dictating conformational changes than that of the AON/RNA hybrid duplex. Thus the above CD observation actually supports the conclusion obtained form the T_m study, in that the conformational perturbation imparted by BHNA in AON/RNA is relatively local, while in AON/DNA is much more pronounced and affects the structure of the duplex more globally.

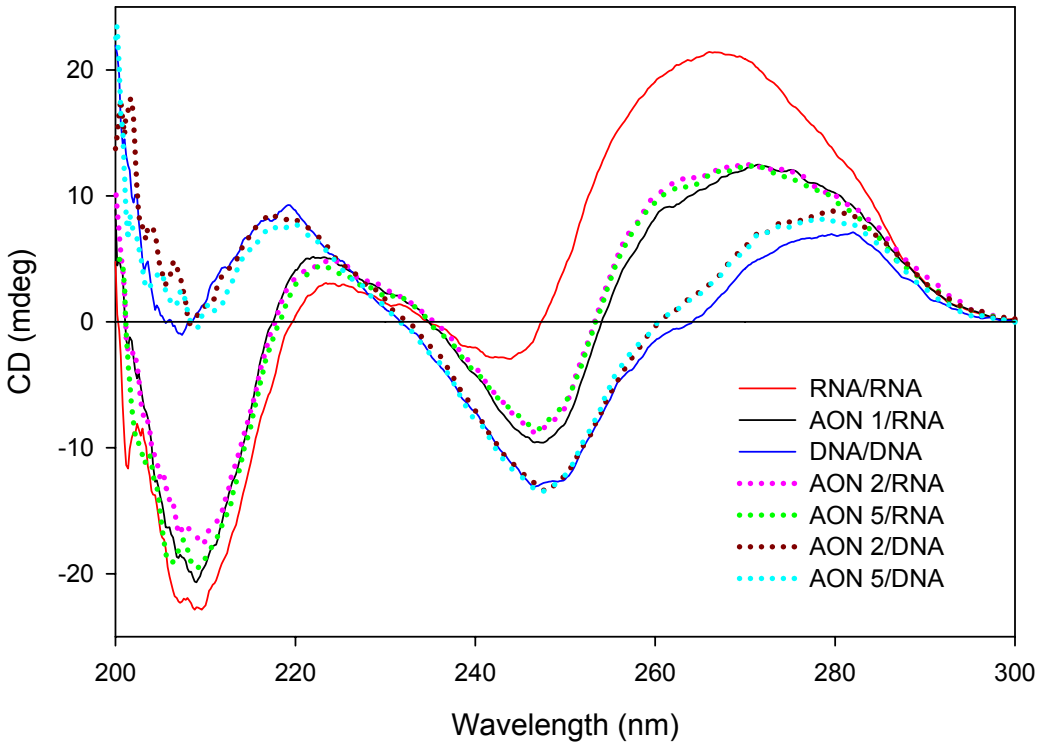


Figure 3. The CD spectra of duplexes formed by AONs **1/2/5** with the complementary RNA or DNA. For comparison, typical B-and A-type spectra are also presented for native DNA/DNA duplex formed by the native AON **1** with complementary DNA. Native RNA/RNA duplex shown is formed by 3′-r(CUU CUU UUU UAC UUC) with complementary RNA. The CD spectra were measured in the same buffer as used for UV melting experiment (Table 1) at 20ºC. The total concentration is 10 μM.

(D) RNase H footprinting AON/RNA duplex

Since the AONs **2-5** can form stable hybrid duplex with complementary RNA, which have comparable conformation as that of the native AON **1**/RNA, the AONs **2-5**/RNA should be good substrate for RNase H and cleave the RNA strand selectively. Here, native AON 1 and modified AONs 2-5 were hybridized with 5′-^{32}P labeled 15mer complementary RNA at 21 ºC, and incubated with *Escherichia coli* RNase H1. Aliquots were taken after 3, 7, 15, 25, 40, 60 min, analyzed by PAGE, and results are shown in Figure 4A.

The cleavage rates were determined by densitometric quantification of gels and subsequently by plotting the uncleaved RNA fraction as a function of time (Figure 4B). The reaction rates were obtained by fitting the degradation curves to single-exponential decay functions.[31] The

relative cleavage rates for AONs **2-5**/RNA duplexes were found to be very similar to that of native counterpart (Figure 4C).

Cleavage pattern of AONs **1-5**/RNA hybrids was also compared to shed light as to how much local structure was disrupted by the BHNA modification (Figure 4D). In the native hybrid duplex AON **1**/RNA, the whole region from A5 to U11 was accessible for RNase H promoted cleavage with a slight preference at the 3′-phosphate of A8. In the case of AON **2**, the modification opposite A3 of the complementary RNA made the region A3-A7 inaccessible for RNase H cleavage while A8 remain the major cleavage site. For AON **3**, the modification opposite A6 of RNA result in loss of RNase H promoted cleavage in the region A6-A10. Instead, U11 and A13 appeared as the major cleavage sites. The AON **4**/RNA hybrid duplex also show unique cleavage pattern toward RNase H incubation. The region of A8-G12 affected by the modification is not accessible for cleavage and the major cleavage sites were shifted to A6 and A13. As expected, the modification opposite A10 of the complementary RNA, as in the AON **5**, result in the absence of any cleavage sites in the region A10-A14, while regaining all the cleavage sites from A5 to A8 present in the native hybrid duplex, and A8 remain the major cleavage site. So cleavage activity of RNase H was suppressed with a region, about 5-6 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 RNase H promoted cleavage has a preference at the middle site A8 of the RNA strand. If A8 is included within the modification region (a stretch of 5 basepairs), the major cleavage site just shift to the edges of the modification region.

(E) Blood serum stability of BHNA incorporated AONs

The stability of AONs toward various exo- and endonucleases is necessary in order to develop therapeutic oligonucleotides. Here, the stability of BHNA modified oligonucleotides in human blood serum was studied and compared with native counterpart. Since human blood serum is mainly composed of 3′- exonuclease,[32] the oligonucleotide with a single BHNA modification at position 3 from 3′-end (AON **2**) was choosen to test the BHNA′s stability in human blood serum. Thus, the AON **1** and AON **2** (^{32}P-labeled at the 5′-end) were incubated with human blood serum (male, type AB) at 21˚C. Aliquots were taken out at specific time points and analyzed by 20% denatured PAGE. The gel pictures after autoradiography are shown in Figure 5.

The native AON **1** is not stable in human blood serum and was completely degraded in 4 h. For modified AON **2**, the last nucleotide dC was first removed to give the 14mer as the predominant fragment in 1 h. The 14 mer fragment thus produced was found to be resistant to nucleases up to 24 h. In view of the presence of alkaline phosphatase in blood serum which removed the 5′-end ^{32}P-label gradually, the BHNA incorporated AON should be even more stable than we observed here (24 h). Thus the work described show that the 3′-phosphodiester linkage of BHNA modified AONs is relatively more resistant to nucleases digestion compared to that of native AONs, and the BHNA integrated oligonucleotides could be have potential therapeutic application in the future.

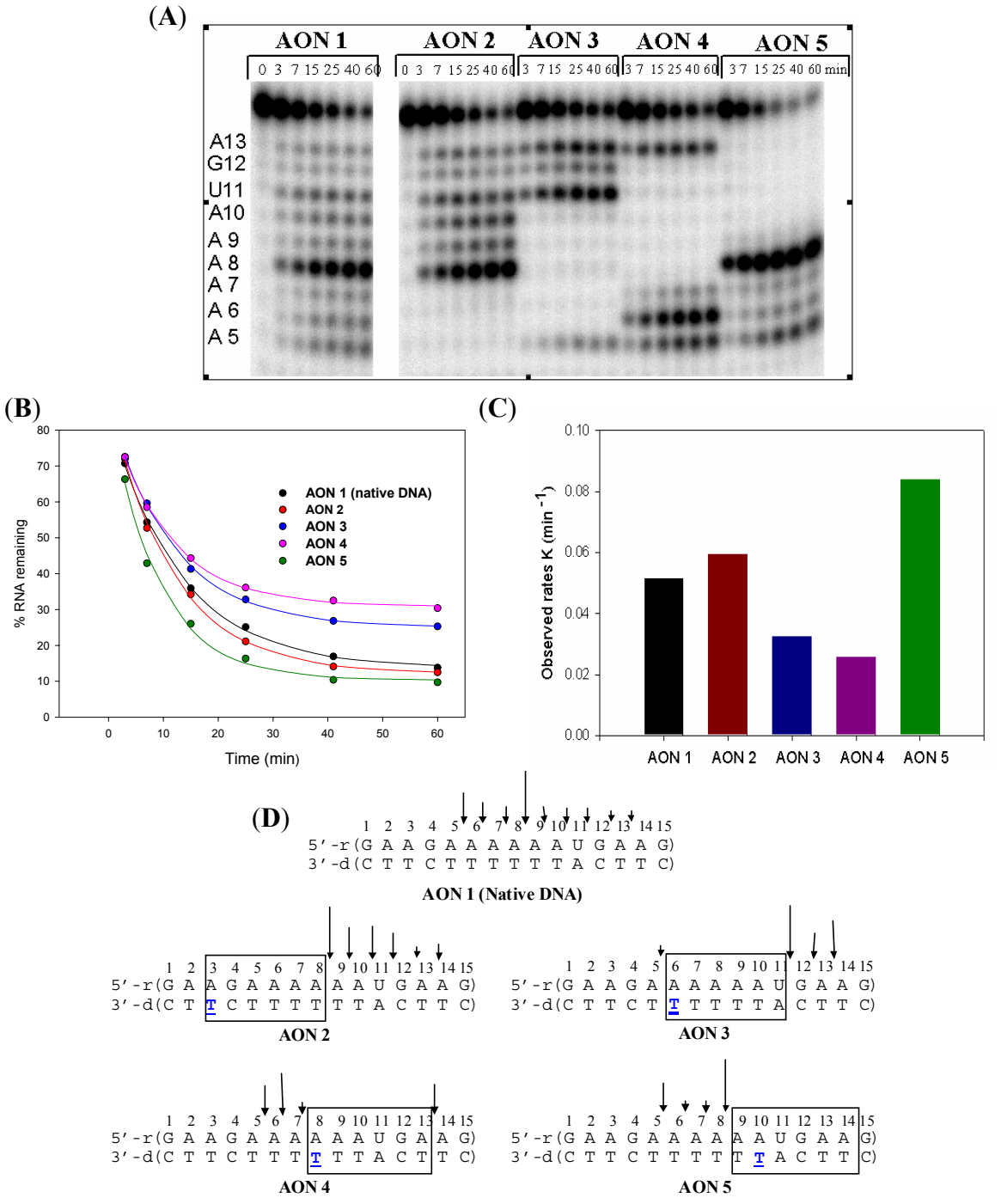


Figure 4. (A) Autoradiograms of 20% denaturing PAGE, showing the cleavage kinetics of 5′-^{32}P-labeled target RNA by E. coli RNase H1 in AON/RNA hybrids after 3, 7, 15, 25, 40 and 60 min of incubation. Conditions of cleavage reactions: RNA (0.1 μM, specific activity 80 000 cpm) and AON (2 μM) in buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C, 0.08 U E. coli RNase H1, total reaction volume 30 μL. (B) Kinetics of cleavage. The target RNA remaining is densitometrically evaluated and plotted as a function of time. (C) Bar plot shows the comparative cleavage rates of

target RNA in different AON/RNA hybrids. (D) RNase H 1 cleavage pattern of AON **1-5**/RNA hybids. Vertical arrows show the cleavage sites, with the relative length of an arrow showing the extent of the cleavage.

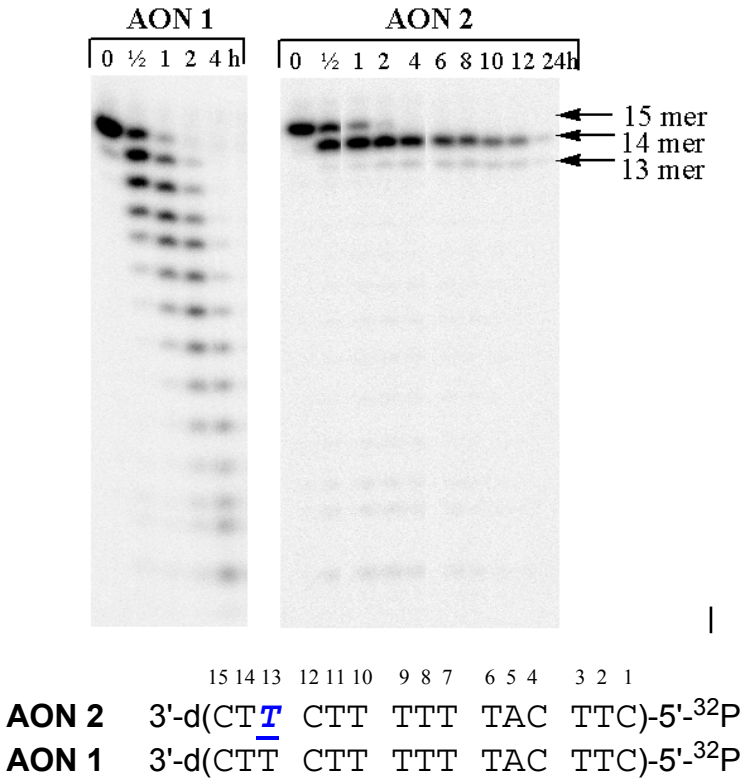


Figure 5. Autoradiograms of 20% denatured PAGE showing the stability of 5′-^{32}P-labeled AON **1** and AON **2** in human blood serum. Time points are taken after 0, ½, 1, 2, 4, 6, 8, 10, 12, 24h of incubation at 21˚C (see Experimental section for details). ***T*** represents BHNA.

Discussion

Mismatch study suggests that BHNA are well basepaired with the opposite nucleobase in the AON/RNA hybrid duplex. The CD spectra also showed the BHNA modified AON/RNA adopt a conformation comparable to that of the native AON/RNA duplex, which means that BHNA can only cause limited local distortion in the AON/RNA duplex, which was also confirmed by the molecular model of AON/RNA duplex. The model of A-type duplex d(TTT)/r(AAA) and d(TTT)/ r(AAA) obtained by Amber geometry optimization are shown in Figure 6D-F. Overlaying the model of native d(TTT)/r(AAA) with that of d(T**T**T)/ r(AAA) has shown that in the A-type duplex (although BHNA modification in d(T**T**T) changes the conformation of backbone to some extent) the base of BHNA still stacks between neighboring nucleobases and is coplanar with the complementary base of the opposite strand, forming perfect hydrogen bonds.

Thus, the limited local perturbation imparted by BHNA may contribute, but only partly, to the T_m loss (ca -5°C) in AON/RNA duplex. Another possible reason for this big T_m loss may be the hydrophobic character of the BHNA. Extensive hydration of individual hydrogen bond acceptors and donors in oligoucleotides generally increase thermodynamic stability of the corresponding duplexes. The unusually extensive hydration pattern in LNA or 2′-MOE integrated duplex potentially contribute substantially to the stability of the duplex. In the RNA•RNA or DNA•RNA duplex, a water network can be formed in the minor groove by interaction with the 2′-OH, with the heterobase atom, O3′, and O5′, O4′, phosphate anion oxygen of adjacent nucleotides at the 3′ end directly or through water bridge. As shown in the model, in the d(T**T**T)/ r(AAA) duplex, the hydrophobic methylene bridge and two methyl groups of BHNA are located in the minor groove and would seriously disturb the hydration network in the minor groove. The hydrogen bonding network around the sugar-phosphate backbone can also be disturbed considerably. It is quite likely that this change of the hydrogen bonding pattern maybe the major reason for the unusual T_m loss (ca -5°C) in AON/RNA.

Comparing the model of B-type duplex d(TTT)/d(AAA) and d(T**T**T)/ d(AAA) (Figures 6A-C) show that the modification of BHNA lead to a much more significant conformational perturbation in AON/DNA duplex. The base of BHNA points at the major groove, and stereochemically not favored to form any hydrogen bond with the base of the opposite nucleotide. This perturbation stretches along the phosphate backbone and affect the conformation of neighboring nucleotides. This model offers a plausible explanation why BHNA causes a serious T_m (ca -10°C) loss and global conformational change in AON/DNA duplex compared to the native DNA/DNA duplex.

The BHNA integrated AON/RNA duplex elicits RNase H, but no RNA cleavage can however be detected in a five to six nucleotide gap starting from the corresponding 3′-end of the modification site in the AON strand. This is very similar to what we have observed earlier with other North-type conformationally constrained systems such as oxetane[15] azitidine[16, 17] or aza-ENA[12] based AONs/RNA duplexes. This can be attributed to the local conformational change from DNA•RNA type to the RNA•RNA type conformation, which is not a substrate to RNase H. This local conformational change for a stretch of 5-6 nucleotides is however not detectable either by CD spectra or by the molecular model. Both of them show that the modified-AON/RNA adopt a perfect conformation similar to that of the native DNA/RNA duplex.

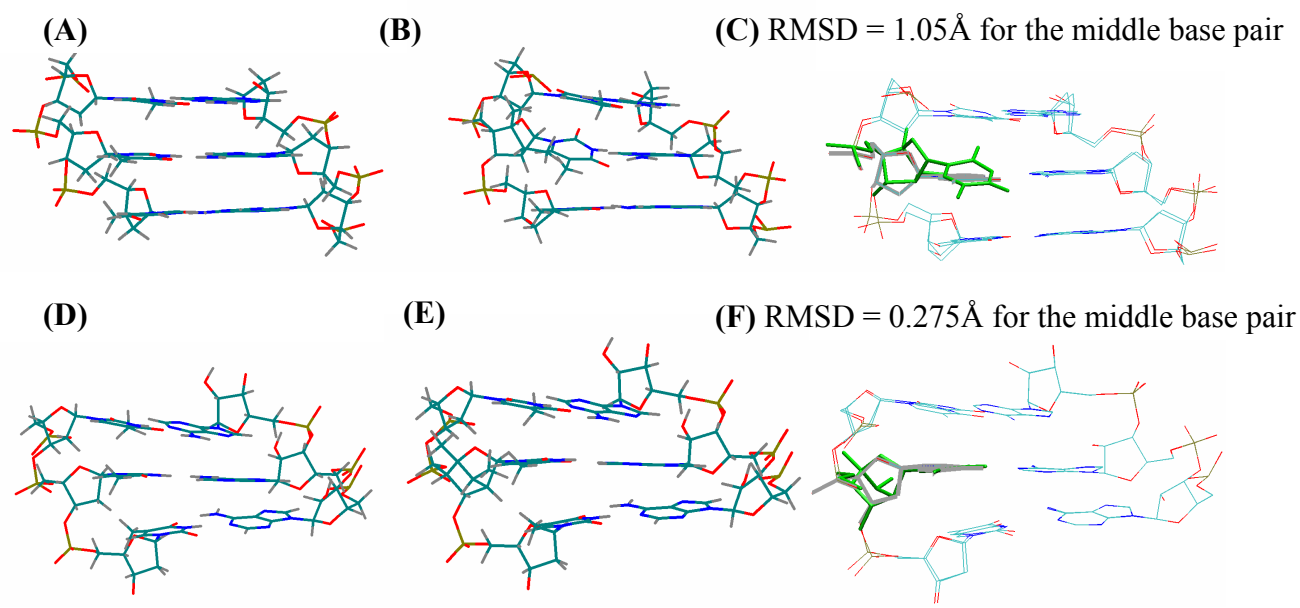


Figure 6. Model of B-type d(TTT)/d(AAA) duplex (panel A) and d(T<u>T</u>T)/d(AAA) (panel B), Model of A-type d(TTT)/r(AAA) duplex (panel D) and d(T<u>T</u>T)/r(AAA) (panel E). Panel C shows an overlay structures in panels A and B (RMSD difference ≈ 1.05Å). Panel F shows an overlay structures in panels D and E (RMSD difference ≈ 0.029). <u>T</u> represents BHNA.

Conclusions and Implications

(1) The hyper-constrained nucleoside, methylene-bridged hexose (BHNA) was purified and incorporated into antisnese oligonucleotides (AON).
(2) The BHNA incorporated AON show preference toward complementary RNA than complementary DNA, although both AON/RNA (ca -5°C) and AON/DNA (ca -10°C) duplex have lower T_m compared to the native DNA/RNA and DNA/DNA duplex.
(3) Thermal denaturation study with mismatch and CD spectra of AON/RNA suggest BHNA can only cause limited local conformational perturbation in AON/RNA duplex, which was also confirmed by RNase H digestion.
(4) BHNA causes a global conformation perturbation, whereas its hydrophobic character contributing to the serious T_m loss in AON/DNA duplex.
(5) BHNA incorporated AONS have much improved stability in human blood serum and so could have potential therapeutic application in the future.

Experimental Section

General Procedures. Chromatographic separations were performed on Merck G60 silica gel. Thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60 F254 glass-backed plates. ^{1}H NMR spectra were recorded at 500 and 600 MHz respectively, using TMS (0.0 ppm) as internal standards. ^{13}C NMR spectra were recorded at 125.7 MHz and 150.9 MHz respectively, using the central peak of CDCl3 (76.9 ppm) as an internal standard. ^{31}P NMR spectra were recorded at 202.5 MHz. Chemical shifts (δ scale) are reported in ppm. The nucleobase of the A, C have been protected using fast deprotecting phenoxyacetyl (PAC) and acetyl (AC) groups, respectively. MALDI-TOF mass spectra were recorded in positive ion mode for oligonucleotides and other compounds. All compounds were spectroscopically pure and their structural determinations were based upon ^{1}H NMR, ^{13}C NMR, COSY, HETCORR experiments as well as by MALDI-TOF mass spectrometry.

(1S, 3R, 4R, 5S, 6R, 7S)-5-Benzyloxy-6-benzyloxmethyl-1, 7-dimethyl-3-(thymin-1-yl)-2-oxa-bicyclo[2.2.1]heptane (1). (compound **1** was prepared by a two-step procedure from 6′-hydroxyl-6′-methyl-carba-LNA[27] by radical deoxygenation at C6′[28]) ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.23 (1H, brs, H3), 7.35-7.25 (m, 11H), 5.52 (d, $J_{1',\ 2'}$ = 3.1 Hz, H1'), 4.57-4.45 (m, 4H, BnC$\underline{H}_2$), 3.58 (dd, $J_{7',\ 4'}$ = 6.9 Hz, J_{gem} = 9.5 Hz, 1H, H7'), 3.36 (s, 1H, H2'), 3.31 (dd, $J_{7'',\ 4'}$ = 6.9 Hz, J_{gem} = 9.5 Hz, 1HH7''), 3.28 (d, $J_{3',\ 4'}$ = 3.2 Hz, 1H, H3'), 2.47 (m, 1H, H6'), 2.19 (m, 1H, H4'), 1.78 (s, 3H, T-Me), 1.37 (s, 3H, 5'-Me), 1.12 (d, $J_{6',\ 6'Me}$ = 6.9 Hz, 3H, 6'-Me). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 163.5 (C4), 149.7 (C2), 138.03, 138.01, 136.9 (C6), 128.6-127.3 (aromatic), 109.4 (C5), 89.7 (C5'), 88.0 (C1'), 77.2 (C3'), 73.1, 70.8 (CH_2Bn), 68.0 (C7'), 56.6 (C4'), 49.8 (C2'), 45.5 (C6'), 15.4 (5'Me), 12.4 (T-Me), 8.8 (6'-Me). MALDI-TOF *M/S*: $[M+H]^+$ found 477.5, calcd 476.2.

(1S, 3R, 4R, 5S, 6R, 7S)- 6-(4,4′-Dimethoxytrityloxymethyl)-5-hydroxyl -1,7-dimethyl-3-(thymin-1-yl)-2-oxa-bicyclo[2.2.1]heptane (2). To a solution of compound **1** (53 mg, 0.11 mmol) in dry methanol (3mL) was added 20% $Pd(OH)_2$/C (168 mg) and ammonium formate (423 mg, 6.7 mmol) and reflux for 1.5h. The suspension was filtered over celite bar and organic phase was evaporated. The residue was co-evaporated twice with dry pyridine and dissolved in the same solvent. 4,4′-Dimethoxytrityl chloride (42 mg, 0.12 mmol) was added and stirred overnight at room temperature. Then the solvent was removed and residue was diluted with DCM, washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and subjected to chromatography on silica gel (methanol in dichloromethane containing 1% pyridine, 0.5-2%, v/v) to give **2** (52 mg, 78%). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.92 (broad, 1H, H3), 7.16-7.38 (m, 10H), 6.78-6.81 (m, 4H), 5.45 (d, $J_{1',\ 2'}$ = 3.0 Hz, 1H, H1′), 3.52 (d, $J_{1',\ 2'}$ = 2.8 Hz, 1H, H1′), 3.18 (dd, $J_{7',\ 4'}$ = 6.9 Hz, J_{gem} = 9.1 Hz, 1H, H7′), 3.13-3.09 (m, 2H, H7′, H3′), 2.48 (m, 1H, H6′), 2.42 (broad, 1H, 3′-OH), 2.09 (m, 1H, H4′), 1.64 (s, 3H, T-Me), 1.55 (s, 3H, 5′-Me), 1.10 (d, $J_{6',\ 6'Me}$ = 6.9 Hz, 3H, 6′-Me). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 163.7, 158.5, 144.8, 136.2, 135.9, 130.0, 129.9, 128.2, 128.1, 127.8, 113.1, 109.8, 90.1, 88.2 (C1′), 86.4, 70.4 (C2′), 61.9 (C7′), 58.8

(C4′), 55.2(C3′), 53.0 (C6′), 45.0, 15.8(5′-Me), 12.2 (T-Me), 8.8 (6′-Me). MALDI-TOF *M/S*: $[M+Na]^+$ found 621.2, calcd 621.3.

(1S, 3R, 4R, 5S, 6R, 7S)- 5-(2-(Cyanoethoxy(diisopropylamino)phosphinoxy))-6-(4,4′-dimethoxytrityloxymethyl)-1,7-dimethyl-3-(thymin-1-yl)-2-oxa-bicyclo[2.2.1] heptane (3). Compound **2** (51 mg, 0.085 mmol) was dissolved in dry THF (2 mL) and DIPEA (0.058 mL , 0.34 mmol) was added. 2-cynoethyl-*N, N*-diisopropyl phosphoramidochloridite (0.058 ml, 0.25 mmol) was added dropwise to the solution under N_2. After stirring of 1h at r.t., the reaction was quenched with ethanol and stirred further 10 min. Then diluted with ethyl acetate and washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and and subjected to chromatography on silica gel (ethyl acetate in cyclohexane containing 1% Et_3N, 20-40%,v/v) to give **2** (54 mg, 72%). ^{31}P-NMR (202.5 MHz, $CDCl_3$): δ 149.5, 149.3. MALDI-TOF *M/S*: $[M+Na]^+$ found 799.4, calcd 798.4.

UV melting experiments. Determination of the T_m of the AON/RNA hybrids or AON/DNA duplex was carried out in the following buffer: 60 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.8 mM $MgCl_2$. Absorbance was monitored at 260 nm in the temperature range from 15 °C to 70 °C using an UV spectrophotometer equipped with a Peltier temperature programmer with the heating rate of 1 °C per minute. Prior to measurements, the samples (1 μM of AON and 1μM complementary DNA or RNA mixture) were preannealed by heating to 80 °C for 5 min followed by slow cooling to 20°C and 30 min equilibration at this temperature. The value of T_m is the average of two or three (if error of the first two measurement is more than 0.2 °C, the third measurement was carried out) independent measurements.

CD experiments. CD spectra were recorded from 300 to 200 nm in 0.2 cm path length cuvettes. Spectra were obtained with a AON/RNA or AON/DNA duplex concentration of 10 μM in 60 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.8 mM $MgCl_2$. All the spectra were measured at 20 °C and each spectrum is an average of 5 experiments from which CD value of the buffer was subtracted.

^{32}P Labeling of oligonucleotides. The oligoribonucleotides and oligodeoxyribonucleotides were 5′-end labeled with ^{32}P using T4 polynucleotide kinase, [γ-^{32}P]ATP, and the standard protocol. Labeled AONs and the target RNA were purified by QIAquick Nucleotide Removal Kit, and specific activities were measured using a Beckman LS 3801 counter.

RNase H digestion assay. Target 0.1 μM RNA (specific activity 80 000 cpm) and a 20-fold excess of AON were incubated in a buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C in the presence of 0.08 U *E. coli* RNase H. Prior to the addition of the enzyme, reaction components were preannealed in the reaction buffer by heating at 80 °C for 5 min followed by slow cooling to 21°C and 30 min equilibration at this temperature. Total reaction volume was 30 μL. Aliquots of 3 μL were

removed after 3, 7, 15, 25, 40, and 60 min, and the reactions were terminated by mixing with stop solution (4 μL) containing 0.05 M EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanole in 80% formamide. The samples were subjected to 20% 7 M urea PAGE and visualized by autoradiography.

Stability studies in human serum. AONs at 2 μM concentration (5′-end ^{32}P labeled with specific activity 80 000 cpm) were incubated in 30μl of human blood serum (male AB) at 21 °C (total reaction volume was 36 μL). Aliquots (3 μL) were taken at 0, 30 min, 1, 2, 6, 8, 10, 12, 24, 36, 48 h, and quenched with 4 μL of solution containing 0.05 M EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanole in 80% formamide, resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by autoradiography.

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