Synthesis, Physicochemical and Biochemical Studies of 1′,2′-Oxetane Constrained Adenosine and Guanosine Modified Oligonucleotides, and Their Comparison with Those of the Corresponding Cytidine and Thymidine Analogues

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Abstract: We have earlier reported the synthesis and antisense properties of the conformationally constrained oxetane-C and -T containing oligonucleotides, which have shown effective down-regulation of the proto-oncogene c-myb mRNA in the K562 human leukemia cells. Here we report on the straightforward syntheses of the oxetane-A and oxetane-G nucleosides as well as their incorporations into antisense oligonucleotides (AONs), and compare their structural and antisense properties with those of the T and C modified AONs (including the thermostability and RNase H recruitment capability of the AON/RNA hybrid duplex by Michaelis–Menten kinetic analyses, their resistance in the human serum, as well as in the presence of exo and endonucleases).

Introduction

Antisense oligonucleotides (AONs), based on elegant principle of Watson–Crick base pairing with the complementary mRNA, have been shown to be capable of down-regulation of the genes of interest.1 AONs are being developed as a gene knock-off tool in functional genomics as well as therapeutic agent1, since their first inception in 1978.2 In the clinical perspective, the antisense technology utilizing AONs, which elicit the RNase H cleavage of target RNA for the down-regulation of the disease causing genes (Figure 1), offers advantages in terms of efficiency and dosage compared to those AONs, which offer gene silencing by the steric blockage of the ribosomal read-through.3 The RNase H cleavage efficiency of the AON/RNA hybrid duplex can be best exploited for potential therapeutic usage when the AONs show the adequate nuclease resistance in vivo, sequence specificity, cell deliverability, nontoxicity, and favorable pharmacokinetics.1,4 Chemical modifications are warranted to achieve those goals, however, in most of the cases the nature of modifications hampers one or more of the above-mentioned requirements.4 Since the widely studied phosphorothioate AONs are marred with their low sequence specificity and higher (1–3 orders of magnitude) binding affinity for various cellular proteins, especially the heparin binding proteins, which accounts for many of their reported nonantisense effects,5 the search for alternative chemical routes of nucleoside modification without changing the phosphodiester backbone has been intensified in the past decade.

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In recent times, AONs incorporated with sugar modified nucleosides have been widely pursued as a valuable alternative.  Among these, the AONs modified with North-conformationally (−1° < P < 34°) constrained nucleosides have attracted much attention because of their ability to drive the AON strand of the AON/RNA hybrid duplex to the RNA/ 
RNA type, thereby increasing the target affinity. They can be broadly classified into three groups. The first group comprises of AONs incorporated with various 2′-O-alkyl moieties [−CH3,−CH2−CH2−O−CH3,−CH2−CH2−CH2−NH−etc.] or electron withdrawing functional groups such as the F in the ricigo configuration. The monomer units of 2′-O-alkyl modified compounds exist in solution in the North-South pseudorotational equilibrium (with very little preference for the North-type sugar). However, upon incorporation into the AON and subsequent hybridization with the target RNA, they impart 3′-endo conformation to their sugar moiety and to the neighboring nucleotides in a varying extent. The second group of AONs encompasses nucleic acids containing pyranose derivatives such as the hexitol nucleic acids and their AON/RNA hybrid encompasses nucleic acids containing pyranose derivatives such as the hexitol nucleic acids and their AON/RNA hybrid encompa

The third group of AONs consists of those containing conformationally constrained bicyclic or tricyclic monomer nucleotide units. Because of their conformational preorganization at the monomer level itself, the complete or sometimes partial modification of the AON strand with North-conformationally constrained units would drive the AON/RNA duplex to the rigid RNA/RNA type duplex which results in the loss of the RNase H cleavage efficiency. However, RNase H eliciting capability can partly or fully be regained by adopting various mixmer and gammer strategies utilizing these modifications. In some cases such as in the β-D-LNA modified AONs, a gap size of 8–10 nucleotides was necessary to recruit the effective RNase H cleavage capability of the target RNA. It is likely that use of such a lengthy unmodified phosphodiester oligonucleotide gap could be harmful in view of the endonuclease susceptibility of the AONs.

We have developed novel 1′,2′-oxetane locked nucleosides, [1′-(3′,3′-O-anhydro-β-D-3-picolurranosyl) nucleosides] which have a unique fixed North-East sugar conformation and belong to the third class of the above-mentioned conformationally constrained nucleosides. Even though the Tm of the AON/RNA hybrid drops by ~5–6 °C/oxetane-thymidine (T) modification, the mixmer AON/RNA hybrids incorporated with T units were found to be substrates for RNase H promoted cleavage as good as the native hybrid. However, the incorporation of the oxetane-cytidine (C) moiety into the AONs imparts only ~3 °C loss in Tm per oxetane-modification. The loss of the thermodynamic stability in case of oxetane-T and -C was fully or partly regained by the introduction of the nonotox DPPZ (dipyridophenazine) group at the 3′ end, and it gave additional stability against exonucleases similar to that of the phosphoro-

The endonuclease cleavage of these molecules was reduced significantly and it was proportional to the number of the oxetane modified nucleosides per AON molecule: single modification gave 2-fold protection to the cleavage and double and triple modification gave 4-fold protection compared to that of the native phosphodiester oligonucleotide.

The antisense oligo constructs with the oxetane-C and 3′-DPPZ were found to be nontoxic in K562 human leukemia cells and have been successfully employed to down-regulate the proto-oncogene c-mob in a very efficient manner. The QRT-PCR and Western blotting the “gold standard in antisense
efficacy have shown that rationally designed oxetane-C modified AONs were highly efficient both in diminishing the c-myb mRNA (85% has been reduced) and the c-myb protein (70% of its expression was found to be halted) of the targeted gene. On the basis of the amount of AON uptake after delivery, determined by slot blot, it was apparent that the oxetane modified AONs are 5-6 times more efficient antisense agents than those of the corresponding isosequential phosphorothioate analogue.

The above-mentioned successful antisense design combining the oxetane-C units and 3′-DPPZ group prompted us to investigate the properties of the oxetane-purine modified AONs. Here, we report the synthesis of the oxetane-A[9-(1′,3′-O-anhydro-β-D-psicofuranosyl)adenine] and the oxetane-G[9-(1′,3′-O-anhydro-β-D-psicofuranosyl)guanine] units (Figure 2) as well as the structure, target affinity, RNase H cleavage, exo and endonuclease tolerance of AONs incorporating these units. Michaelis–Menten kinetics of E. coli RNase H1 promoted cleavage has been carried out for the oxetane-A and -G modified AONs (2 and 3)/RNA (13) hybrids, and the kinetic parameters are compared with those of the oxetane-C modified (4) and native AON (1)/RNA (13) hybrids.

Results and Discussions

(A) Binding Affinity of the Oxetane-A and -G Modified AONs to the Target RNA and the Thermodynamic Properties of the Modified AON/RNA Hybrids. When tested for target affinity, the triple oxetane-A modified AON (2)/RNA (13) and the triple oxetane-G modified AON (3)/RNA (13) have exhibited melting temperatures very similar to that of the native AON (1)/RNA (13) hybrid duplex (Figure 2). However, the introduction of the triple C modifications as in AON (4)/RNA (13) causes 4 °C drop in Tm per oxetane-modification while the introduction of the oxetane-T modifications into the AON (10)/RNA (13) hybrid makes it even more unstable showing a Tm drop of 5 °C per oxetane-T modification (Figure 2). This clearly shows that the oxetane-purine units have considerable destabilizing effect on the thermostability of the AON/RNA hybrids compared to those of their purine counterparts. The mixed sequences of the oxetane-A and -G moieties, such as AONs (5-7), have also shown similar target binding affinities close to that of the native AON (1) (Figure 2). It should be noted that Tm enhancement effect of the 3′-DPPZ group which we have observed earlier in the studies of 9mer and 15mer AON/RNA hybrids has been considerably reduced in the case of 20mer AON/RNA hybrids studied here.

To shed more light on the differential binding affinities of the oxetane modified AONs to the target RNA (13), we have elucidated thermodynamic parameters (Table 1), based on the concentration dependent Tm analysis of the native AON (1)/RNA (13) duplex and those of the triple oxetane-purine (2 and 3) or pyrimidine-modified AONs (4 and 10). It has emerged that the thermodynamic parameters of the triple oxetane-G modified AON (3)/RNA (13) duplex are very close to that of the native AON (1)/RNA (13) duplex. However, ΔF° and ΔS° of the triple oxetane-A modified AON (2)/RNA (13) duplex are slightly lower (5.5 kJ/mol drop in ΔG°) than those of the

Figure 2. Sequences and Tm s of various heteroduplexes containing oxetane-modified AONs and their target RNA. The Tm and the differences in Tm (ΔTm) with respect to that of the native phosphodiester AON (1) are also shown for comparison.
Table 1. Thermodynamic Parameters of 20Mer AON (1–4 and 10)/RNA (13) Hybrids Determined from $T_m^{-1}$ vs In($C_r$) Plots

<table>
<thead>
<tr>
<th>AONs</th>
<th>$T_m$ (°C)</th>
<th>$-\Delta H^\circ$ (kJ/mol)</th>
<th>$-\Delta S^\circ$ (J/Kmol)</th>
<th>$-\Delta G^\circ$ (298 K) (kJ/mol)</th>
<th>$\Delta G^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native 20mer (1)</td>
<td>74</td>
<td>740.4 ± 22.3</td>
<td>2016 ± 64</td>
<td>600.9</td>
<td>139.5</td>
</tr>
<tr>
<td>20–3A (2)</td>
<td>73</td>
<td>714.2 ± 22.5</td>
<td>1946 ± 65</td>
<td>580.2</td>
<td>134.0</td>
</tr>
<tr>
<td>20–3G (3)</td>
<td>74</td>
<td>747.7 ± 13.9</td>
<td>2034 ± 40</td>
<td>606.4</td>
<td>141.3</td>
</tr>
<tr>
<td>20–3C (4)</td>
<td>61</td>
<td>661.5 ± 13.5</td>
<td>1860 ± 40</td>
<td>554.5</td>
<td>107.0</td>
</tr>
<tr>
<td>20–37 (10)</td>
<td>59</td>
<td>646.6 ± 20.5</td>
<td>1853 ± 62</td>
<td>544.5</td>
<td>102.3</td>
</tr>
</tbody>
</table>

The triple oxetane-C modified AON (4) has exerted a large drop in enthalpy of the oxetane-modified pyrimidine AON/RNA hybrids compared to that of the native duplex. Apparently, the large enthalpic destabilization and slight entropic stabilization of the triple oxetane-C modified hybrid duplexes.

Endonuclease, Exonuclease, and Human Serum Stability of Oxetane-Modified AONs. When treated with the endonuclease (DNase 1), the triple oxetane-A (2) and triple oxetane-G (3) modified AONs offered no resistance toward the cleavage by the enzyme (Figure 3, Figure S2 in Supporting Information). The half-life of the AON (7) with the three A and three G units was found to be about 2 h, which is about half of that observed for the triple oxetane-C modified AON (4) studied under similar conditions. These results reveal the high susceptibility of the oxetane-purine modified AONs toward the DNase 1 promoted cleavage.

To date, only very limited information is available for the structural and cleavage characteristics of DNase 1 with single stranded AONs. Although the DNase 1 cleavage has limited sequence specificity, the crystal structure of the DNase 1 with various short oligonucleotide duplexes, has revealed that in addition to the minor groove width and DNA flexibility, the local sequence preference is crucial for the proper alignment of the phosphodiester bond for the cleavage reaction. It has emerged that three nucleotides toward the 5'- and 3'-end of the cleavage site have significant influence on the cleavage properties. On the basis of the cleavage characteristics of various DNA duplexes, Herrera and Chaires found that among these six nucleotides around the cleavage site, the nucleotide sequences at position 3 (denoted as +3) and 2 (denoted as +2) toward the 5'-end (Figure 3) and nucleotide at position 2 (denoted as +2) toward the 3'-end of the cleavage site are very crucial in determining the major cleavage sites and cleavage rates of DNA duplexes by DNase 1. This is because the NH$_2$ of G at position +3 cause steric clash with arginine 41 of the enzyme and hamper the cleavage rate. Tyrosine-76 has shown favorable interactions with T/C moieties at the position +2. At the position +2 toward 3'-end presence of T is highly disfavored.

Our results can be partly explained based on the local sequence requirements described above. In the native AON (1), the cleavage sites have been restricted to the 5'-end of the oligo and the major cleavage site was found to be at G9, which is in accordance with the aforementioned general requirements. In all of the oxetane-modified AONs, the major and minor cleavage sites were observed at one or two nucleotides away from the modified sites. This clearly shows that the oxetane ring in the nucleotides interferes with the catalytic core of the enzyme and prevents the cleavage. Also, the modified residues might alter the flexibility and the geometrical requirements of phosphate required for the cleavage. In the triple A modified AON (2) all the cleavage sites of the native AON (1) are retained because there is no oxetane modification present at those cleavage sites. This explains why the extent of cleavage in the triple A modified AON (2) is very similar to that of the native AON (1). In the triple G modified AON (3) the major cleavage site has been shifted to the T8 instead of G9, which is probably because of the presence of the oxetane-G at G11 position (Figure 3, Figure S2 in Supporting Information). But the oxetane-G at +3 did not affect the cleavage rate. Probably, the enzyme might not have encountered the perturbations caused by the additional oxetane-ring in the sugar, which is being located far away from the nucleobase. However, in the case of pyrimidine-oxetane units, the oxetane ring is close to the pyrimidine ring, which might interfere with the enzyme. This is evident in the triple
oxetane-C modified AON (4), where only minor cleavage was observed at the G9 along with the G11 while the major cleavage was observed at the C3 and it is very close to the 5′-end. However, the rate of cleavage was low at those sites probably owing to the perturbations of the AON-enzyme interaction by the oxetane-C, which is situated just three nucleotide away from the cleavage site. The AON (7) incorporated with oxetane-purines has shown a half-life of the cleavage to be about 2 h. These results lead us to suggest that to get effective endonuclease resistance with the oxetane-purine units, extensive (probably every alternate nucleotide) modifications of the AON strand are needed. This, however, may slow the RNase H recruiting capability (vide infra). Another viable approach can be the use of a mixture of the oxetane-purines and a minimum amount of the oxetane-pyrimidines.

To investigate the tolerance of the oxetane-modifications toward an exonuclease (snake venom phosphodiesterase, SVPDE), we have synthesized an AON (12) with 3 consecutive oxetane-C, -A, and -G units at the 3′-end. Incubation with the SVPDE showed that the presence of the oxetane-modified units at the 3′-end offers resistance toward cleavage: 36% of AON (12) was left after 24 h incubation with the enzyme, while the native PO-AON (1) was completely degraded in less than 2 h of incubation (Figure S3 in Supporting Information). Another viable approach can be the use of a mixture of the oxetane-purines and a minimum amount of the oxetane-pyrimidines.

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(D) RNase H1 Cleavage Pattern of the Complementary RNA and the Extent (%) of RNA Cleavage in Hetero-duplexes Containing Oxetane-A, -G, -C Units and Their DPPZ Conjugates. All of the oxetane-A and -G modified AON (2-7)/RNA (13) hybrids and their DPPZ conjugates (8 and 9) were found to be substrates for the Escherichia coli RNase H1 with varying cleavage potential. In the RNase H1 cleavage pattern of all the oxetane-A and -G modified hybrids, except for the AON (7)/RNA (13), a region of 5 nucleotides in the RNA strand toward the 3′-end from the site opposite to the oxetane modification, was found to be resistant toward RNase H promoted cleavage (Figures 4 and 5). This is presumably owing to the local steric and structural alterations brought about by the modification, thereby preventing the flexibility and accessibility required for the RNase H cleavage. This 5-nucleotide footprint is identical to what was found earlier for the oxetane-T and -C modified AON/RNA hybrids.22-24 The cleavage of the AON (7)/RNA (13) at A11 site was surprising because this cleavage site is located within the anticipated footprint region of the oxetane-A modification (Figure 4).

Our earlier studies of the oxetane-T and -C modified AON/RNA hybrids revealed that the RNase H cleavage potentials of these modified hybrids depend on the concentration of the RNA as a substrate.22b,23,24 This has prompted us to test all our modified AONs at two different substrate (RNA) concentrations. We have deduced the extent of cleavage at high (0.8 μM) and low (0.01 μM) RNA concentrations (Figure 6) under saturation
conditions (the enzyme is completely saturated by the substrate). At the low RNA concentration, the triple oxetane-\(A\) (2) and triple oxetane-\(G\) (3) modified AON/RNA hybrid duplexes and their respective DPPZ counterparts (8) and (9) have shown the extent of the cleavage slightly lower than that of the native AON (1)/RNA (13) hybrid duplex (Figure 6). The mixed \(A\) and \(G\) incorporated AON (5 and 6)/RNA (13) hybrids have also shown similar cleavage characteristics. Exception was found for the AON (7)/RNA (13), incorporated with 6 oxetane–purine moieties, where the extent of cleavage was extremely low (17\% compared to that of the native AON (1)/RNA (13) hybrid duplex. It should be noted that under these conditions the triple oxetane-\(C\) modified AON (4)/RNA (13) hybrid duplex has shown a comparable or slightly better extent of cleavage (78\% compared to that of the native AON (1)/RNA (13) hybrid duplex.)

**Figure 4.** (A) Autoradiograms of 20% denaturing PAGE, showing the cleavage kinetics of 5'-32P-labeled target RNA (13) by E. coli RNase H1 in the native AON (1)/RNA (13) and the oxetane-\(A\), \(-G\), and \(-C\) modified AON (2–4 and 7)/RNA (13) hybrid duplexes. Lanes 1 to 5 represent the aliquots of the digest taken after 5, 10, 15, 30, and 60 min, respectively. Conditions of the cleavage reaction: RNA (0.8 mM) and AONs (4 mM) in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl\(_2\) and 0.1 mM DTT at 21 °C; 0.8 U of RNase H. Total reaction volume was 30 \(\mu\)L. The % of RNA left intact after 1 h of incubation: 28% for AON (1), 36% for AON (2), 50% for AON (3), 26% for AON (4) and AON for 32% (7). (B) RNase H1 cleavage pattern of hybrid duplexes. The vertical arrows show the RNA cleavage sites and relative length of an arrow shows the relative extent of cleavage at that site.

**Figure 5.** (A) Autoradiograms of 20% denaturing PAGE, showing the cleavage kinetics of 5'-32P-labeled target RNA (13) by E. coli RNase H1 in the native AON (1)/RNA (13) and the oxetane-\(A\), \(-G\), and \(-C\) modified AON (5, 6, 8, and 9)/RNA (13) hybrid duplexes. Lanes 1 to 5 represent the aliquots of the digest taken after 5, 10, 15, 30, and 60 min respectively. Conditions of the cleavage reaction: RNA (0.8 mM) and AONs (4 mM) in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl\(_2\) and 0.1 mM DTT at 21 °C; 0.8 U of RNase H. Total reaction volume was 30 \(\mu\)L. The % of RNA left after 1 h of incubation: 34% for AON (1), 65% for AON (5), 64% for AON (6), 37% for AON (8) and 64% for AON (9). (B) RNase H cleavage pattern of hybrid duplexes. The vertical arrows show the RNA cleavage sites and relative length of an arrow shows the relative extent of cleavage at that site.
hybrids (Figure 6) However, the hybrid duplexes of the triple oxetane-A, -G, and -C modified AON/RNA hybrids are capable of recruiting RNase H1 for reactions under high RNA concentration. Total reaction volume was 30 μL.

These data clearly show that the triple oxetane-A and oxetane-G modified AON/RNA hybrids are capable of recruiting RNase H for reactions under low RNA concentration and 0.8 unit of enzyme for reactions under high RNA concentration. Total reaction volume was 30 μL.

1% than that of the native hybrid duplex (75 ± 1%). These data clearly show that the triple oxetane-A and triple oxetane-G modified AON/RNA hybrids are capable of recruiting RNase H in an efficient manner (52–60% of RNA was cleaved) at low substrate concentrations, and it is important in view of extremely low concentrations of target mRNAs in many infected cells.30

At high substrate concentration (0.8 μM RNA), the hybrid duplexes of the triple oxetane-A modified AON (2) and its DPPZ counterpart (8) showed the extent of cleavage comparable to that of the native (1) and triple C (4) modified AON/RNA hybrids (Figure 6) However, the hybrid duplexes of the triple oxetane-G modified AON (3), its 3’-DPPZ containing counterpart (9) and the AONs (5 and 6) containing both the oxetane-A and oxetane-G units have shown relatively low cleavage by the RNase H (Figure 6). Surprisingly, the AON (7)/RNA (13) hybrid, which had shown very low cleavage at low RNA concentration, showed RNase H cleavage close to that of the native hybrid duplex. This underscores the differential cleavage characteristics of the oxetane-purine modified AON/RNA hybrids at different substrate concentrations.

(E) Michaelis–Menten Kinetics of the Oxetane-A and -G Modified AON/RNA Hybrids and Their Comparison with the Oxetane-C and Native AON/RNA Hybrid Duplexes. To obtain a deeper understanding of the E. coli RNase H1 recognition and cleavage of the oxetane-A and -G modified AON (3 and 4)/RNA (13) hybrids, a detailed Michaelis–Menten kinetics has been carried out and the kinetic parameters were subsequently compared with those of the native (1) and the triple oxetane-C modified AON (4)/RNA (13) hybrids (Table 2, Figures 7 and 8).

It is evident from the kinetic data that the triple oxetane-A modified AON (2)/RNA (13) duplex has a maximum velocity, $V_{\text{max}}$, slightly lower than that of the native (1) and the triple oxetane-C modified AON (4)/RNA (13) hybrids. However, its enzyme binding affinity, $1/K_m$, has been found to be very close to the native yielding an effective enzyme activity, $k_{\text{cat}}/K_m$, close to that of the native. The triple oxetane-G modified AON (3)/RNA (13) has shown a lower $V_{\text{max}}$ and $K_m$ value compared to those of the native, triple oxetane-A and the triple oxetane-C modified AON/RNA hybrids (Table 2, Figures 7 and 8). Thus, the $k_{\text{cat}}/K_m$ value of oxetane-G modified AON (3)/RNA (13) is half of that of the native AON (1)/RNA (13) duplex. Noteworthy the fact that the $V_{\text{max}}$ value appeared to be higher for the triple oxetane-C modified AON/RNA duplex than for the native (Table 2, Figures 7 and 8). This can be attributed to the lower binding affinity ($1/K_m$) and lower $T_m$ of the triple C modified AON/RNA duplex similar to the observations made in our previous studies on various oxetane-C and -T modified 15mer AON/RNA hybrids.23,24 The reason for the lower $V_{\text{max}}$ and high $1/K_m$ of the triple oxetane-G modified AON/RNA duplex is not

1′,2′-Oxetane Constrained Modified Oligonucleotides

Table 2. Kinetic Characteristics of the RNA Cleavage by E. coli RNase H1 of the AON (1–4)/RNA (13) Hybrid Duplexes

<table>
<thead>
<tr>
<th>AONs</th>
<th>T_m (°C)</th>
<th>V_max, 10⁻³ µM min⁻¹</th>
<th>K_m, 10⁻⁴ µM</th>
<th>k_cat min⁻¹</th>
<th>(V_max/K_m), min⁻¹</th>
<th>(k_cat/K_m), µM⁻¹ min⁻¹</th>
<th>relative (k_cat/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native 20mer (1)</td>
<td>74</td>
<td>7.3 ± 0.3</td>
<td>44.4 ± 6.3</td>
<td>24.2 ± 1</td>
<td>0.164</td>
<td>545.1</td>
<td>1</td>
</tr>
<tr>
<td>34-oxetane-modified AON</td>
<td>73</td>
<td>5.8 ± 0.2</td>
<td>38.3 ± 6.4</td>
<td>19.2 ± 0.6</td>
<td>0.15</td>
<td>501.3</td>
<td>0.92</td>
</tr>
<tr>
<td>20–34 (2)</td>
<td>74</td>
<td>1.9 ± 0.1</td>
<td>25.6 ± 4.1</td>
<td>6.3 ± 0.35</td>
<td>0.074</td>
<td>246.1</td>
<td>0.45</td>
</tr>
<tr>
<td>3G-oxetane-modified AON</td>
<td>61</td>
<td>8.1 ± 0.5</td>
<td>70.2 ± 13.8</td>
<td>26.9 ± 1.5</td>
<td>0.11</td>
<td>383.7</td>
<td>0.70</td>
</tr>
<tr>
<td>20–3C (4)</td>
<td></td>
<td></td>
<td></td>
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</table>

a V_max = k_cat/E_0 (E_0 = 0.08 U/30 µL = 2.66 × 10⁻³ U/µL = 3.016 × 10⁻⁴ µM), specific activity = 420 000 U/mg or 2.38 × 10⁻⁶ mg/U = 1.133 86 × 10⁻¹³ mol/unit, MW = 21 000 g/mol.

Scheme 1

![Scheme 1](image)

clear. Clearly, more modified sequences have to be tested before drawing any general conclusions regarding the RNase H recruitment by the oxetane-G and -A modified AON/RNA hybrids.

(F) Synthesis of the Oxetane-G and -G Building Blocks

The oxetane-G and oxetane-G phosphoramidites, 23a and 23b, required for incorporation into AONs (2–9 and 12) were synthesized using a strategy shown in Scheme 1. The synthesis started with the conversion of 6-β-(toluoyl)-1,2,3,4-dipropylpyrene-β-d-psicofuranose (14),22b into 16, a possible key intermediate in the nucleobase coupling, using a reported procedure.23 It is noteworthy that some yields obtained in our hands were significantly lower in certain steps compared to that reported earlier.23 Thus, the transformation from 14 to 15 (Scheme 1) yielded in only 60% compared to the 91% reported.23 Since the attempts to couple the silylated purine nucleobase derivatives with 16 were unsuccessful, we have decided to transform 16 into the bromosugar 17 followed by immediate coupling of the crude 17 with the silylated N°-benzoyladenine and N°-acetyl-O°-diphenylcarbamoylguanine in the presence of SnCl₄ as Lewis acid catalyst. This afforded the β-nucleosides 18a; and yielded 18b (together with the short treatment with TFA to remove the DPC: 25%). The yield of two step transformation from 16 to 18 was 42% compared to 95% reported.31 Along with 18a we got very little amount of α anomers with a lot of nonnucleosidic impurities. This has reduced the overall yield. For 18b we got inseparable mixtures of N°7 α and β nucleosides along with inseparable mixtures of N°9 (both α and β) and N°7 (both α and β) psicocoulenes without the DPC protection. Moreover, there also were non-nucleosidic impurities. Unfortunately, due to the use of repeated columns for purification we could not quantify the yields of all those side products. The β configurations of the products were determined by NOE measurements. NOE observed between H-8 and H-3′, was found to be 2.7%. Such kind of NOE cannot be observed in the α anomers owing to the 3.9 Å distance between H-8 and H-3′. The N°9-connectivity of the base in 18b was evidenced by the chemical shift of C5 of the base at 122.0 ppm in the ¹³C NMR spectra.

which is a characteristic for the N9-isomer. Methanolyis of 18a–b followed by simultaneous protection of 4′ and 6′ hydroxyl groups using 1,3-dichloro-1,1′,3,3′-tetraisopropyl-disiloxane (TIPDSCl2) furnished the precursors, 19a (72%) and 19b (63%), required for the oxetane ring formation. The cyclization was achieved by the treatment of 19a–b with NaHMDS in THF at 4°C, giving 20a (81%) and 20b (83%). The exocyclic amino group of the 20a was protected by phenoxyacetyl group and that of 20b by dimethylformamidine group to furnish 21a (60%) and 21b (91%). The removal of the TIPDS group from 21a–b followed by one-pot dimethoxytritylation afforded 22a (80%) and 22b (83%). Compounds 22a–b were transformed to their corresponding phosphoramidites 23a (60%) and 23b (75%), the building blocks required for the solid-phase synthesis of the modified AONs (2–9 and 12).

Table 3. Proton Chemical Shifts (δ, ppm) and 3JH-H Coupling Constants (Hz) for the Oxetane-C (1a), -T (1b), -A (1c), and -G (1d) (1H NMR spectra were recorded in DMSO-d6 with methanol-d4 at 600 MHz using DMSO-d6 (2.6 ppm) peak as internal standard)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oxetane-A (1c)</th>
<th>Oxetane-G (1d)</th>
<th>Oxetane-C (1a)</th>
<th>Oxetane-T (1b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>5.55 (d, J2 = 7.9 Hz)</td>
<td>5.42 (d, J2 = 8.0 Hz)</td>
<td>5.67 (d, J2 = 8.15 Hz)</td>
<td>5.11 (d, J2 = 8.15 Hz)</td>
</tr>
<tr>
<td>H1*</td>
<td>4.96 (d)</td>
<td>4.88 (d)</td>
<td>4.67 (d)</td>
<td>4.68 (d)</td>
</tr>
<tr>
<td>H1**</td>
<td>5.63 (d, J2 = 3.73 Hz)</td>
<td>5.53 (d, J2 = 3.71 Hz)</td>
<td>5.30 (d, J2 = 3.9 Hz)</td>
<td>5.36 (d, J2 = 3.9 Hz)</td>
</tr>
<tr>
<td>H1H5</td>
<td>3.87±0.01 (m, J2 = 8.59 Hz, J2 = 8.55 Hz, J2 = 5.7 Hz)</td>
<td>4.3±0.02 (m, J2 = 8.55 Hz, J2 = 5.7 Hz)</td>
<td>4.19±0.02 (m, J2 = 8.55 Hz, J2 = 5.7 Hz)</td>
<td>4.19±0.03 (m, J2 = 8.62 Hz, J2 = 5.85 Hz)</td>
</tr>
<tr>
<td>H41H5</td>
<td>9.31 (dd)</td>
<td>3.89 (dd)</td>
<td>3.85 (dd)</td>
<td>3.85 (dd)</td>
</tr>
<tr>
<td>H2H5</td>
<td>3.66 (dd)</td>
<td>3.66 (dd)</td>
<td>3.59 (dd)</td>
<td>3.60 (dd)</td>
</tr>
<tr>
<td>H2H5</td>
<td>8.39 (s, H-2)</td>
<td>-</td>
<td>5.84 (s, H-4)</td>
<td>7.73 (H-3)</td>
</tr>
<tr>
<td>H3H5</td>
<td>8.25 (s, H-8)</td>
<td>7.92 (s, H-8)</td>
<td>7.47 (dd, H-6)</td>
<td>7.42 (dd, 1.28 Hz, H-6)</td>
</tr>
<tr>
<td>CH3</td>
<td>-</td>
<td>-</td>
<td>1.94 (d)</td>
<td>-</td>
</tr>
</tbody>
</table>

The 1H NMR data for the oxetane-C (1a) in D2O and the oxetane-T (1b) in methanol-d4 have been reported in refs 24 and 22b, respectively.

3JH-H coupling (JH-H) in the nomenclature of the oxetane-modified nucleosides of > 8 Hz, along with 3JH-H of > 1.5 Hz, would indicate that the pentose-sugar is locked in the North-type conformation (Figure 9, frame B) and, in contradistinction, a 3JH-H of < 1.0 Hz and 3JH-H of > 8 Hz would finger-point a constrained South-type conformation (the exact limits depend on the electronegativity of the substituents and the substitution pattern). Thus, a 3JH-H of ≈ 0 Hz for the compound 6a alone indicates the South-type structure (Figure 9, frame D) which agrees with its available X-ray structure. The 3JH-H values of the oxetane-modified compounds 1a-1d (Figure 9, frame D) are > 8.6 Hz (Table 3) which leads to conclusion that the sugar moieties of the oxetane-C -T, -A, and -G nucleosides are locked in the North-type conformation. The dependence 3JH-H versus 3JH-H (frame C, Figure 9) shows the combination of these coupling constants for the oxetane-modified nucleosides to be typical for P = 20–60° range which unambiguously points to the North-conformation as opposed to South-type conformation for compound 6a (Figure 9, frames C and D). The 3JH-H and 3JH-H (see numbering in Figure 9 of the oxetane-C, -T, -A, and -G nucleosides have been found to be temperature-independent, thus the 1H NMR data suggest that the sugar moiety of the oxetane-modified nucleosides is locked in the North-type conformation.

Ab Initio Calculations. In an alternative analysis, the ab initio geometry optimizations for each of the oxetane modified nucleosides 1a-1d (Figure 9, frame D) were performed to obtain the geometrical parameters. It was followed by the 0.5 ns MD simulations in the explicit aqueous medium to probe the conformational hyperspace available for these systems. The ab initio calculations utilizing 6-31G* Hartree–Fock geometry optimization by Gaussian 98 program resulted in the H3′-C3′-C4′-H4′ and H4′-C4′-C5′-H5′ torsions to be ~43.4° and ~163.5°, respectively and these values varied only by ±0.4° for the oxetane-A, -G, -C, and -T nucleosides 1a-1d (Table S1 in Supporting Information). The positions of the heavy atoms of sugar moieties of the respective oxetane-modified nucleosides vary slightly between the nucleosides resulting in less than 0.01 Å RMS difference as it can be seen in Figure 10, frame A. The theoretical vicinal coupling constants estimated using these H3′-C3′-C4′-H4′ and H4′-C4′-C5′-H5′ torsions and the generalized Karplus equation agree well with the experimental 3JH-H constants (max RMSd = 0.46 Hz, see Table S1 in the Supporting Information).

References

with the empirical 3
ring within the typical
is firmly restricting the dynamics of the oxetane-fused furanose
oxetane-fused furanose ring is indeed rigid and locked in to
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constants and ab initio obtained values of the respective H3
Supporting Information). Figure 11 shows that a combination
Molecular structures of the oxetane-modified nucleosides shown
Figure 10. Molecular structures of the oxetane-modified nucleosides shown
frames from simulations of the oxetane-modified nucleosides (Figure 10,
every 10 ps) are superimposed from the respective MD
snapshots of the last 100 ps of the MD trajectory (harvested at
30.6
E2
°
torsions correlates well
the conformation based on the values of the sugar torsion from the ab
initio geometries collected in Table 4 for all the oxetane-
modified nucleosides reveals that the furanose ring is locked in the
North-East-type conformation with the phase angle of 40–
43° and the puckering amplitude 35–37°.

Molecular dynamics simulations (details are in the
Experimental section below) clearly demonstrated that the oxetane ring
is firmly restricting the dynamics of the oxetane-fused furanose
ring within the typical North-East-type (16° < P < 56°, 23° < 
φm < 41°) conformation range (Table 4) with the average values
30.6° < P < 36.6° and 31.7° < φm < 32.7° somewhat lower
than the corresponding values from the ab initio geometries
(39.8° < P < 42.8°, 35.1° < φm < 36.6°) (Table 4). Ten
snapshots of the last 100 ps of the MD trajectory (harvested at
every 10 ps) are superimposed from the respective MD
simulations of the oxetane-modified nucleosides (Figure 10,
frames from B1, B2 to E1, E2). This shows that that the oxetane-fused furanose ring is indeed rigid and locked in to
North-East-type conformation (shown in Figure 10, projections
B2–E2) with the average RMSd being max 0.10 ± 0.03 Å
along the trajectories and the major movements are observed for the aglycon part of the oxetane- modified nucleosides (Figure
10, frames from B1, B2 to E1, E2).

(H) Lessons for Future Design of AONs of Therapeutic
Potentials. To show bona fide antisense action the AONs should fulfill broad spectrum of requirements. These include sequence
specificity, high exo and endonuclease resistance, RNase H
recruitment capability, deliverability, low toxicity, and favorable pharmacokinetics. Achieving all of these goals in a balanced
manner by chemical modification of AONs has indeed proven
to be a challenging task. The off-target effects and nonantisense
properties associated with phosphorothioate AONs5 pose serious
corns on its therapeutic utilities. Other backbone modifications
such as PNA,33 N3′−P5′ phosphoramidates34 and morpholino AONs35 were less specific and unable to activate RNase
H degradation of the target RNA. Hence, any potential modifications without altering the phosphodiester backbone of
AONs have been opted as a viable alternative. Among these,
the modifications of the sugar moieties of the nucleosides have
been intensively pursued in recent years. However, the loss of
RNase H action by extensive modifications of carbohydrate
residues such as 2′-O-CH3,9 2′-O-CH2−CH2−O−CH2,9 2′-O-
CH2−CH2−CH2−NH2,7,10 LNA16f etc. in an oligochain limits
the antisense potential of these constructs. The “gapper”
design,7 utilizing such modifications at the ends of the oligo
chain and keeping a long stretches of 6–8 unmodified residues,
was helpful to partly or fully regain the RNase H recruitment.
But such designs will probably be less effective in invoking
the antisense action in vivo considering the endonuclease
vulnerability observed for some of these gapmers.21 A recent
report20 showed the utility of α-L-LNA to partly circumvent
this problem. A state-of-the-art design would consist of a
minimum number of modifications and a maximum antisense
action using a minimum amount of antisense reagent, which
can only be exploited by employing the catalytic cleavage
property of RNase H. Our studies with the oxetane-C modified
mixmer AONs have proven to be in the right direction toward
achieving these goals.26 We have demonstrated recently in a
cellular system the effective down-regulation of the target RNA
using the oxetane-C modified AONs conjugated with the
nontoxic DPPZ group.28 The fully phosphodiestor AONs of such
design were in fact ~5 to 6 times more effective in target
silencing than the corresponding phosphorothioate AONs.26 One
inherent problem with the oxetane-C modified AONs was the
loss of thermodynamic stability associated with the modification,
even though it has partly or fully been regained by the DPPZ
conjugation to short oligomer sequences. The loss of the target
affinity can be bypassed by modifications of AONs by the
oxetane-A and oxetane-G units, as reported here. The oxetane-
modified AONs, unlike many other conformationally
constrained nucleic acids, require only 4 nucleotide gaps to achieve
efficient target cleavage by RNase H. Since the flexibility of
AON/RNA hybrids is perhaps decisive in dictating their RNase
H cleavage rates,3 it is particularly important that three to four
oxetane modifications do not hamper the hybrid flexibility
required for the RNase H cleavage, as it is shown in our studies.
At low RNA concentration (10 nM) both the oxetane-A and -G
modified AONs and their DPPZ conjugates were found to be
good substrates for RNase H with extent of cleavage comparable
to that of the native AON/RNA hybrids. The DPPZ group offers excellent 3'-exonuclease protection and augmented stabilities in the Human serum. However, the vulnerability of the oxetane-A and G modified AONs toward endonucleases might be a drawback. Since we have not checked their tolerance toward cellular endonucleases other than DNase I, it is not possible to comment at this time on their potential nuclease resistance in the cellular systems, although oxetane-C modified AONs were found to be adequately stable in K562 human leukemia cells.26 It is also worth to mention that, there are reports existing in the literature which show the importance of the modifications at the pyrimidine sites in an AON rather than at purines to achieve endonuclease resistance.21c,d Further biological tests are in progress to examine the potential target down-regulation by the oxetane-A and -G modified AONs in cellular systems.

Conclusions

(1) Oxetane-A and -G phosphoramidites were synthesized and incorporated into 20mer AONs and targeted to 20mer RNA, and their antisense properties have been evaluated in vitro.

(2) The molecular structures of the oxetane-C, T, A, and G monomer units have been studied by means of the high-field 1H NMR and theoretical ab initio and MD simulations. The combined experimental and theoretical studies have demonstrated that the oxetane-fused furanose ring is indeed locked in the typical North-East-type conformation with the pseudorotational phase angle (P) and puckering amplitude (φ_ho) for the ab initio optimized geometries (6-31G* HF) varying from 39.8° < P < 42.8°, 35.1° < φ_ho < 36.6° for all four oxetane-modified nucleosides. The last 100 picoseconds (ps) of 0.5 nanosecond (ns) MD simulation starting from the respective ab initio geometries have shown for the oxetane-modified sugars accessible conformational range of P and φ_ho to be 16° < P < 56°, 23° < φ_ho < 41°.

(3) Oxetane-A and -G modified AONs showed their target affinity with complementary RNA, and the thermodynamic parameters to be identical or very close to the native AON/RNA duplex. However, the oxetane-C and oxetane-T modified duplexes showed large enthalpic destabilization and slight entropic stabilization compared to that of the native hybrid duplex. The large drop in enthalpy amounts to 32 to 37 kJ/mol drop in the free energy.

(4) The global helical structure of all the oxetane modified AON/RNA hybrids, as revealed by the CD spectra, was very...
similar to the native AON/RNA duplex showing that the CD failed to detect the local conformational perturbations brought about by the North-East conformationally constrained oxetane modifications.

(5) All of the oxetane-A and oxetane-G modified AON/RNA hybrid duplexes were found to be good substrates for the E. coli RNase H1. In the oxetane-A and G modified AON/RNA hybrids, except for one case, a region of 5 nucleotides in the hybrid duplexes were found to be good substrates for the modifications.

(6) Michaelis–Menten kinetic analysis showed that triple oxetane-A modified AON/RNA duplex has a 10_4 value very close to its counterpart resulting in a 10_4 value being half of that of the native AON/RNA hybrid.

(7) The modification of nontoxic DPPZ group at the 3′ end of the oxetane-A and G modified AONs increases the 10_4 value of the respective AON/RNA duplexes only by 1–2 °C in comparison with their unconjugated counterparts. However, the DPPZ conjugation has helped the AONs to achieve substantial stability against exonuclease (t1/2 more than 24 h) and nuclease in human serum (t1/2 more than 9 h).

(8) Modification of AONs with the oxetane-A and G units failed to offer resistance toward DNase 1 endonuclease degradation in comparison with the oxetane-C modified AONs. Thus, DNase I appeared to have varying tolerance toward the oxetane-purine and oxetane-pyrimidine nucleotides.

(9) This study unravels the pros and cons of the AONs modified with the oxetane-purine and oxetane-pyrimidine nucleosides and provides valuable information regarding the optimal design of AONs having completely natural phosphodiester backbone for the therapeutic applications.

**Experimental Section**

Comparative 13C chemical shifts in ppm (δ scale) of the oxetane nucleosides 1a (C), 1b (T), 1c (A), and 1d (G) are shown in Table S2 in Supporting Information.

2-O-Methyl-3,4,4′-isopropylidene-6-O-(4-toluoyl)-α-D-psicofuranose (15). The sugar 14 (9.5 g, 25 mmol) was treated with 0.4 M methanolic HCl (125 mL overnight). The reaction mixture was cooled, subsequently neutralized with triethylamine, and evaporated. The crude mixture was partitioned between CH2Cl2 and water. The aqueous layer was washed three times with CH2Cl2 and the combined organic layer was dried over MgSO4, filtered, and evaporated. Column chromatography of the residue afforded 15 (5.2 g, 15 mmol, 60%). 1H NMR (270 MHz, CDCl3): 7.96 (d, 2H, J = 8.2 Hz, 2H-1, 4-toluoyl), 7.24 (d, 2H-1, 4-toluoyl), 6.82 (dd, 1H, Jα,α-1 = 5.6 Hz, Jα,α-2 = 1.2 Hz, H-2, H-1), 4.66 (d, H-3), 4.55–4.50 (m, 1H, Jα,α-1 = 7.2 Hz, Jα,α-2 = 1 Hz, H-4, 4.42–4.30 (m, 2H, H-5, H-6), 3.84 (4-CHO, 1.53 g), 3.92 (2H, H1-1′, H-1′), 3.29 (3H, OMe), 2.4-H, 4-toluoyl), 1.53 (3H, OAc), 1.35 (3H, OAc), 13C NMR (67.9 MHz, CDCl3): 166 (C-O, 4-toluoyl), 143.7, 129.5, 128.9, 126.7 (4-toluoyl), 112.9 (C-Me2), 110.1 (C-2), 85.3 (C-3), 84.0 (C-4), 82.0 (C-4′), 64.6 (C-5′), 58.3 (C-1), 48.4 (OCH3), 26.0, 24.7 (CH3, isopropyl), 21.4 (CH3, 4-toluoyl). FAB–HRMS: [MH]+ 353.1624; calculated 353.1602.

1-O-Methanesulfonyl-2-O-methyl-3,4,4′-acetyl-6-O-(4-toluoyl)-α-D-psicofuranose (16). Compound 15 (5.2 g, 15 mmol) was coevaporated with pyridine three times and dissolved in 105 mL of the same solvent. The mixture was cooled in an ice bath and methanesulfonyl chloride (3.2 mL, 41.3 mmol) was added dropwise to the mixture and the stirring was continued for 15 min at the same temperature. The reaction was kept at 4°C for 12 h, poured into cold saturated NaHCO3 solution and extracted with CH2Cl2. The organic phase was washed with brine, dried over MgSO4, filtered, and evaporated. The residue was dissolved in 60 mL of dry pyridine, acetic anhydride (8.6 mL, 90 mmol) was added and stirred at room temperature for 3 h. The reaction mixture was poured into saturated NaHCO3 solution and extracted with CH2Cl2. The organic phase was dried with brine, dried over MgSO4, filtered, and evaporated. The residue was dissolved in dry pyridine. The mixture was dried over MgSO4, filtered, and evaporated. The residue was dissolved in dry pyridine. 1H NMR (270 MHz, CDCl3): 8.85 (br s, 1H, NH), 8.69 (s, 1H, H-2), 8.20 (s, 1H, H-8), 7.99 (d, J = 7.92 Hz, 2H, 4-toluoyl), 7.67–7.52 (m, 5H, benzoyl), 7.01 (d, 2H, 4-toluoyl), 6.53 (d, Jα,α-1 = 5.32 Hz, 1H, H-3′), 5.61 (d, Jα,α-2 = 3.96 Hz, 1H, H-4′), 4.97–4.79 (m, Jα,α-2 = 11.5, 4H, H-5′, H-1′, H-3′, H-4′), 4.37 (d, Jα,α-2 = 12.6 Hz, Jα,α-2 = 2.97 Hz, H-6′), 2.84 (s, 3H, CH3, mesyl), 2.28 (s, 6H, CH3, 4-toluoyl, CH3, O-acetyl), 2.16 (s, 3H, CH3, O-acetyl). 13C NMR (67.9 MHz, CDCl3): 169.3, 168.5 (C=O, acetly), 165.5 (C=O, 4-toluoyl), 164 (C=O, benzoyl), 152.6 (C-6), 150.1 (C-4), 149.4 (C-2), 144.5 (4-toluoyl), 140.7 (C-8), 133.5, 132.7 (benzoyl) 129.7, 129.2, 129.7, 125.3 (4-toluoyl, benzoyl), 123.0 (C-5′, 94.9 (C-2′), 82.4 (C-5′), 74.8 (C-3′), 71.7 (C-4′), 68.0 (C-1′), 62.8 (C-6′), 37.8 (CH3, mesyl), 21.4 (CH3, 4-toluoyl), 20.3 (CH3, O-acetyl). FAB–HRMS: [MH]+ 682.1767; calculated 682.1819.
9-[1'-O-Methanesulfonyl-4',6'-O-(tetrasopropylsilyloxy-1,3-di-yl]-β-D-psicofuranosyl]adenine (19a). Compound 18a (6.1 g, 9 mmol) was treated with methanolic NH4Cl solution for 48 h at room temperature. The mixture was evaporated, triturated with dichloromethane, coevaporated three times with pyridine and dissolved in 90 mL of the same solvent. The mixture was cooled in an ice bath and 1,3-dichloro-1',3',3'-tetrasopropylsilyloxy (2.88 mL, 9 mmol) was added dropwise. The mixture was stirred at 0 °C for 30 min and at r.t. for 2 h. It was poured into saturated NaHCO3 solution and extracted with CH2Cl2. The organic phase was dried, evaporated and coevaporated with toluene. Column chromatography of the residue afforded compound 19a (4.1 g, 6.47 mmol, 72%). Rf = 0.6 (CH2Cl2/MeOH 94:6 v/v). 1H NMR (270 MHz, CDCl3): 8.34 (s, 1H, H-2), 8.21 (s, 1H, H-8), 5.86 (br s, 2H, NH2), 5.28 (d, Jgem = 11.8 Hz, 1H, H-1'), 4.94 (d, 1H, H-6'), 5.8 (d, Jgem = 4.4 Hz, H-1'), 4.9 (d, 1H, H-1'), 4.41 (dd, Jgem = 1.6 Hz, H-7'), 1.83 (m, 16H, CH2(Si(CH3)3)), 13.2, 12.9, 12.5, 12.4 (Si-CH2(CH3)). FAB–HRMS: [MH]+ 618.2427; calcd 618.2449.

9-[1'-3'-O-Anhydro-4',6'-O-(tetrasopropylsilyloxy-1,3-diy]-β-D-psicofuranosyl]adenine (20a). Compound 19a (3.19 g, 5.2 mmol, 1M solution in THF) was dissolved in THF (100 mL) and cooled in an ice bath. Sodium bis(trimethylsilyl)amide (10 mL, 10 mmol, 1 M solution in THF) was added in dropwise and the stirring was continued for 2 h. The reaction was quenched by adding saturated NaHCO3 and extracted with excess of ethyl acetate. The combined organic phase was dried, filtered, and evaporated. The residue on column chromatography yielded compound 20a (2.2 g, 4.2 mmol, 81%). Rf = 0.5 (ethyl acetate/cyclohexane 93:7 v/v). 1H NMR (270 MHz, CDCl3): 8.28 (s, 1H, H-2), 7.79 (s, 1H, H-8), 5.86 (br s, 2H, NH2), 5.74 (d, Jgem = 3.9 Hz, 1H, H-3'), 5.58 (d, Jgem = 7.8 Hz, 1H, H-1'), 5.01 (d, 1H, H-1'), 4.83 (dd, Jgem = 8.5 Hz, H-4'), 4.49 (dd, Jgem = 1.6 Hz, H-5'), 4.18 (dd, Jgem = 1.6 Hz, H-6'), 3.12 (m, 28H, Si–CH2(CH3)2 and CH2 from 4-Pr).13C NMR (67.9 MHz, CDCl3): 155.5 (C-6), 152.7 (C-2), 148.2 (C-4), 139.4 (C-8), 120.6 (C-5), 94.9 (C-2'), 82.5 (C-3'), 75.3 (C-3'), 69.6 (C-4'), 68.8 (C-1'), 60.6 (C-3'), 75.3 (CH3, mesyl), 17.1, 17.0, 16.9, 16.8 (Si-CH2(CH3)2), 13.3, 12.9, 12.5, 12.4 (Si-CH2(CH3)). FAB–HRMS: [MH]+ 822.2540; calcd 822.2568.

9-[1'-3'-O-Anhydro-4',6'-O-(tetrasopropylsilyloxy-1,3-diy]-β-D-psicofuranosyl]N-(4-phenoxacycl)adenine (21a). To a stirred solution of 20a (763 mg, 1.07 mmol) in 7 mL of CH2Cl2, 2-cyanoethoxy-bis(N,N-diisopropylamino)phosphine (0.62 mL, 1.6 mmol) was added followed by N,N-diisopropylammonium tetradsodium (92 mg, 0.54 mmol) and left for stirring overnight. The reaction mixture was diluted with ethyl acetate, poured into saturated NaHCO3 solution and extracted. The organic layer was washed with saturated brine solution, dried over MgSO4, filtered, and evaporated. The residue on chromatography (40–60% ethyl acetate, cyclohexane + 2% Et3N) afforded compound 21a (1.4 g, 2.1 mmol, 60%). Rf = 0.6 (ethyl acetate/cyclohexane 93:7 v/v). 1H NMR (270 MHz, CDCl3): 8.17 (s, 1H, NH), 9.15 (s, 1H, NH), 7.98 (s, 1H, H-8), 7.67 (s, 1H, H-1'), 5.92 (s, 1H, H-2), 4.82 (s, 1H, H-1'), 4.79 (s, 1H, H-2'), 4.39 (s, 1H, H-1'), 3.79 (s, 3H, CH3), 13.1, 12.9, 12.5, 12.4 (Si-CH2(CH3)). FAB–HRMS: [MH]+ 916.3730; calcd 916.3799.

9-[1'-3'-O-Anhydro-4',6'-O-(4-toluoyl)-β-D-psicofuranosyl]N2-acyethylguanine (18b). The sugar 16 (4.7 g, 10 mmol) was brominated using the same procedure as used for 18a. After being kept in an oil pump for 30 min, the crude bromosugar was dissolved in dry CH2Cl2 (60 mL) and added to silylated N2-acyetyl-O6-diphenylcarbamoylguanine. [The silylation was carried out by heating N2-acyetyl-O6-diphenyl carbamoylguanine (5.8 g, 15 mmol) with N,O-bis(trimethylsilyl)acetamide (30 mL) in (CH2)3N (75 mL) at 80 °C for 30 min. The mixture was evaporated, coevaporated with dry toluene and dried on an oil pump for 20 min.] The mixture was cooled in an ice bath and SnCl2 (2.5 mL) was added. After being stirred in an ice bath for 20 min, the reaction mixture was heated at 70 °C for 3 h and continued stirring at r.t. overnight. The reaction mixture was poured into saturated NaHCO3 solution and passed through diatomaceous earth. It was extracted with CH2Cl2 and the combined organic phase was washed with brine, dried over MgSO4, filtered, and evaporated. The residue on chromatography afforded DPC protected nucleoside, which was then treated with 90% trifluoroacetic acid in water for 30 min to remove the DPC group. The reaction mixture was evaporated, co-evaporated with toluene and a quick column furnished 18b (1.6 g, 2.5 mmol, 25%). Rf = 0.5 (CH2Cl2/MeOH 90:10 v/v). 1H NMR (270 MHz, CDCl3): 12.01 (s, 1H, NH), 9.59 (s, 1H, NH), 7.98 (s, 1H, H-8), 6.77 (d, J = 8.16 Hz, 2H, 4-tolyl), 7.20 (d, 4-tolyl), 6.56 (d, J = 4.82 Hz, 1H, H-3), 5.52 (dd, JH-2, H-2' = 7.42 Hz, JH-1, H-1' = 2.72 Hz, H-2, H-6), 4.86 (d, Jgem = 11.38 Hz 1H, H-1'), 4.7–4.64 (m, 3H, H-5', H-1', H-6'), 4.53 (dd, Jgem = 12.8 Hz, H-1', H-1' = 4.33 Hz, 1H, H-6').
2.87 (s, 3H, CH₃, mesyl), 2.38 (s, 3H, CH₃, 4-toluoyl), 2.27(s, 3H, CH₃, O-acetyl), 2.2 (s, 3H, CH₃, O-acetyl), 2.05 (s, 3H, CH₃, N-acetyl).

¹³C NMR (67.9 MHz, CDCl₃): 172.0 (C-O, C-6), 169.1 (C-O, N₂-acetyl), 168.8 (C=O, 4-toluoyl), 165.8, 155.2 (C-6), 147.0 (C-2), 146.8 (C-4), 144.4 (4-toluoyl), 136.8 (C-8), 129.2, 129.0, 125.6 (4-toluoyl), 122.0 (C-5), 94.4 (C-2'), 80.0 (C-5'), 73.3 (C-3'), 69.7 (C-4'), 67.0 (C-1'), 61.6 (C-6), 37.7 (CH₃, mesyl), 24.1 (CH₃, O-acetyl), 21.4 (CH₃, 4-toluoyl), 20.2 (CH₃, O-acetyl) 20.1 (CH₃, N-acetyl). FAB–HRMS: [MH⁺] 636.1667; calc 636.1564.

9-[(1′-Methanesulfonyl)-4′,6′-(tetraisopropylsilyl)oxa-1,3-diyl]−β-p-isofuranosylguanine (19b). Compound 18b (2.2 g, 3 mmol) was deprotected and reacted with 1,3-dichloro-1′,1′,3′-tetraisopropylsilyl oxide (1.1 mL, 3.5 mmol) using the same procedure described for compound 19a. After workup and chromatography afforded 19b (1.4 g, 2.2 mmol, 63% yield). Rf = 0.6 (CH₂Cl₂/MeOH 90:10 v/v).¹³C NMR (270 MHz, CDCl₃, DMSO-d₆): 83.4 (s, 1H, CH), 59.2 (d, J₁₃₋₅ = 3.9 Hz, 1H, H-1'), 5.39 (d, J₁₋₃ = 3.9 Hz, 1H, H-3'), 4.95 (d, J₁₋₃, H-3 = 3.9 Hz, 1H, H-4'), 4.44 (dt, J₁₋₃ = 3.9 Hz, H-3 = 3.9 Hz, 1H, H-5'), 4.13 (dd, 2H, H-6', H-6''), 1.18–1.09 (m, 28 H, Si-CH₂(CH₃)₃); FAB–HRMS: [MH⁺]+ 634.2432; calc 634.2401.

9-[(1′-O-4′,6′-(tetraisopropylsilyl)oxa-1,3-diyl)−β-p-isofuranosyl]guanine (20b). Compound 19b (1.4 g, 2.2 mmol) was cyclized using the procedure described as for 20a. After workup and chromatography of the residue furnished 20b (990 mg, 1.8 mmol, 83% yield). Rf = 0.6 (CH₂Cl₂/MeOH 88:12 v/v).¹³C NMR (270 MHz, DMSO-d₆): 7.85 (s, 1H, H-8), 6.32 (s, 2H, NH₂), 5.70 (d, J₁₋₃, H-3 = 3.9 Hz, 1H, H-1'), 5.16 (d, J₁₋₃, H-3 = 3.9 Hz, 1H, H-4'), 4.42 (dd, J₁₋₃, H-3 = 3.9 Hz, H-4 = 3.9 Hz, 1H, H-5'), 4.13 (2H, H-6', H-6''), 1.20–1.10 (m, 28 H, Si-CH₂(CH₃)₃); FAB–HRMS: [MH⁺]+ 638.2525; calc 638.2517. A small amount of 20b was deprotected to get 9′-[(1′-O-4′,6′-(tetraisopropylsilyl)oxa-1,3-diyl)−β-p-isofuranosyl]guanine (1d) and the data are shown in Table 3 and in Table S2 in Supporting Information. FAB–HRMS: [MH⁺]+ 296.0990; calc 296.0997.

9-[(1′-O-4′,6′-bis(tetraisopropylsilyl)oxa-1,3-diyl)−β-p-isofuranosyl]N²-(N,N-dimethylamino)ethylene glyamine (21b). To a stirred solution of compound 20b (733 mg, 1.4 mmol) in dry MeOH (13 mL), N,N-dimethyleformamide dimethylacetal (0.9 mL, 6.8 mmol) was added and allowed to stir at r.t. overnight. The mixture was evaporated and concentrated under reduced pressure. The residue was dissolved in CH₂Cl₂ (2 mL) and precipitated from hexane at 0.6 mmol to give phosphoramide (II) containing oxetane-O, N-2-anhydro-6′-O-(4′,4′-di-methoxytrityl)-β-p-isofuranosyl)guanine (23b). Compound 22b (392 mg, 0.6 mmol) was converted to the corresponding phosphoramide using the conditions described for 23a. Workup and chromatography (80%–100% CH₂Cl₂, cyclohexane + 2% Et(N) furnished 23b (341 mg, 0.44 mmol, 73%). Rf = 0.6 (CH₂Cl₂/MeOH 94:6 v/v). The compound was dissolved in CH₂Cl₂ (2 mL) and precipitated from hexane at −40 °C.

1′³C NMR (109.4 MHz, CDCl₃): 157.0 (C-1), 157.5 (C-1'), 154.7, 154.2 (C-2), 154.1 (C-2'), 153.8 (C-3), 153.7 (C-3'), 86.3 (C-4'), 76.3 (C-3'), 70.9 (C-1'), 70.7 (C-4'), 61.1 (C-6), 37.5 (OMs), 18.2, 18.1, 17.7, 17.1 (Si-CH₂(CH₃)), 14.9, 14.5, 14.2, 14.1 (Si−CH₂(CH₃)); FAB–HRMS: [MH⁺]+ 858.3817; calc 853.3802.

Synthesis, Deprotection and Purification of Oligonucleotides. All oligonucleotides were synthesized using automated DNA/RNA synthesizer by Applied Biosystems, model 392. For modified AONs containing oxetane-A, G, C, and T units, fast deprotecting phosphoramides (nucleobases were protected using the following groups: Ac for C, P, Ar—PAC for G, and PAC for A) were used. The protected AONs were deprotected at room temperature by the treatment of aqueous NaOH for 24 h. For the 3′,5′-DPPZ conjugated AONs, a CPG support containing DPPZ was used. All AONs and the target RNA were purified by 20% polyacrylamide/7M urea) PAGE, extracted with 0.3 M NaOAc, desalted with C18-reverse phase catridges and their purity (greater than 95%) was confirmed by PAGE. MALDI-MS analysis: AON (2) [MH⁺] = 6128.37; calc = 6127.01; AON (3) [MH⁺] = 6127.18; calc = 6127.01; AON (4) [MH⁺] = 6127.86; calc = 6127.01; AON (5) [MH⁺] = 6129.72; calc = 6127.01; AON (6) [MH⁺] = 6127.44; calc = 6127.01; AON (7) [MH⁺] = 6211.51; calc = 6211.04; AON (8) [MH⁺] = 6735.68; calc = 6736.48; AON (9) [MH⁺] = 6737.88; calc = 6736.48; AON (12) [MH⁺] = 6178.81; calc = 6178.02.

UV Melting Experiments. Determination of the Tm of the AON/RNA hybrids was carried out in the following buffer: 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM MgCl₂. Absorbance was monitored at 260 nm in the temperature range from 20 °C to 90 °C using UV spectrophotometer equipped with Peltier temperature programmer with the heating rate of 1 °C per minute. Prior to measurements, the samples (1 μM of AON and 1 μM RNA mixture) were preannealed by heating to 90 °C for 5 min followed by slow cooling to 4 °C and 30 min equilibration at this temperature.

Thermodynamic Calculations from the UV Experiments. The thermodynamic parameters characterizing the helix-to-coil transition for the DNA/RNA hybrids were obtained from Tm measurements over the concentration range from 0.6 to 16 μM (total strand concentration). Values of 1/ΔTm were plotted versus ln[C(R)/4] and ΔΔTm and ΔΔS were calculated from slope and intercept of fitted line: 1/ΔTm = (R/ΔΔS)[ln(C(R)/4)] + ΔΔS/ΔΔTm, where S reflects the sequence symmetry of the s (S = 1) or non-self-complementary strands (S = 4).

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 duplex concentration of 5 μM in 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM MgCl₂. All the spectra were measured at 5 °C and each spectrum is an average of 5 experiments from which CD value of the buffer was subtracted.

Theoretical Calculations. The structural parameters of the oxetane-modified nucleosides and conformational hyperspace available for the compounds have been determined by the ab initio geometry optimizations followed by the 0.5 ns molecular dynamics simulations. The
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geometries optimizations of the oxetane-modified nucleosides have been carried out by the Gaussian 98 program package\textsuperscript{15} at the Hartree–Fock level using 6-31G* basis set. The atomic charges and optimized geometries of the oxetane-C (1a), -T (1b), -A (1c), and -G (1d) were then used for the Amber force field parameters employed in the MD simulations. The protocol of this MD simulations is based on Cheatham–Kollman’s\textsuperscript{47} procedure employing modified version of Amber 1994 force field as it is implemented in Amber 7 program package.\textsuperscript{46} The TIP3P water model was used to introduce explicit solvent molecules in the MD calculations. Periodic boxes containing 1048, 1125, 1084, and 1122 water molecules were created around the oxetane-C (1a), -T (1b), -A (1c), and -G (1d), respectively, extending 12.0 Å from these molecules in all three dimensions.

\textsuperscript{3}P Labeling of Oligonucleotides. The oligoribonucleotide, oligodeoxyribonucleotides were 5'-end labeled with \textsuperscript{3}P using T4 polynucleotide kinase, [\gamma-\textsuperscript{3}P]ATP and standard procedure.\textsuperscript{48} Labeled AONs and RNA were purified by 20% denaturing PAGE and specific activities were measured using Beckman LS 3800 counter.

Kinetics of RNase H Hydrolysis. (A) Calibration of RNase H Concentration Based on its Cleavage Activity. The source of RNase H1 (obtained from Amersham Bioscience) was Escherichia coli containing clone of RNase H gene. The solutions of 20mM AON (1/ RNA (13) duplex: [AON] = 10\textsuperscript{−8} M, [RNA] = 10\textsuperscript{−7} M (the first control substrate) and 20mM AON (3/ RNA (13) duplex: [AON] = 10\textsuperscript{−6} M, [RNA] = 10\textsuperscript{−7} M (the second control substrate) in a buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl\textsubscript{2}, 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT) at 21 °C in 30 μL of the total reaction volume have been used as standard substrates to calibrate the amount of RNase H actually used. The percentage of RNA cleavage was monitored by gel electrophoreses as a function of time (1–5 min), with using of 0.08 U of RNase H, to give the initial velocity. Thus, the initial velocity of the RNase H cleavage reaction, 5.4171 \times 10\textsuperscript{−3} μM/min (for the first standard substrate control) or 2.0869 \times 10\textsuperscript{−3} μM/min (for the second standard substrate control) under the above condition corresponds to 0.08 units of enzyme in 30 μL of the total reaction mixture. These are based on 9 independent experiments. Since Michaelis equation suggests that the initial velocity of the reaction linearly depends on the enzyme concentration, therefore, using the initial velocity for the first standard substrate control or the second standard substrate control corresponding to 0.08 units/30 μL concentration of the RNase H, two correlation coefficients were found by dividing the observed experimental initial velocity by the standard initial velocity of 5.4171 \times 10\textsuperscript{−3} or 2.0869 \times 10\textsuperscript{−3} μM/min. Then, the real enzyme concentration was as well as the initial velocity in each experiment was corrected using average value of the correlation coefficients of both the standard substrate controls, which were used to calibrate the initial velocity of the RNase H promoted cleavage reaction for each substrate presented in this work.

(B) RNA Concentration Dependent Experiments. \textsuperscript{3}P-Labeled RNA (0.01 to 0.8 μM, specific activity 50 000 cpm) with AONs (4 μM) were incubated with 0.08 units of RNase H in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl\textsubscript{2} and 0.1 mM DTT at 21 °C. Total reaction volume was 30 μL. 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 1.5 h equilibration at 21 °C. After 1–7 min, aliquots (3 μL) were mixed with stop solution (6 μL), containing 0.05 M disodium salt of the ethylenediamine-

tetraacetic acid (EDTA), 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol in 95% formamide, and subjected to 20% 7 M urea denaturing gel electrophoresis. The kinetic parameters \( K_{m} \) and \( V_{\text{max}} \) were obtained from \( v_{0} vs. [S] \) plots obeying Michaelis–Menten equation: \( v_{0} = V_{\text{max}}[S]/K_{m} + S_{0} \). Values of \( K_{m} \) and \( V_{\text{max}} \) at this method were determined directly from \( v_{0} vs. [S] \) plots by using of correlation Program, where correlation equation was: \( v = ax/(b + x) \) where \( a = V_{\text{max}} \) and \( b = K_{m} \). Since the \( V_{\text{max}} \) = \( E_{0} \cdot k_{\text{cat}} \) (\( E_{0} \) = initial enzyme concentration), and for all of our RNA concentration dependent kinetics, the \( E_{0} \) was identical, hence \( V_{\text{max}} \) is proportional to \( k_{\text{cat}} \). In other words, the \( k_{\text{cat}} \) can be understood by comparing simply the \( V_{\text{max}} \).

Exonuclease Degradation Studies. Stability of the AONs toward 3’-exonucleases was tested using snake venom phosphodiesterase from Crotalus adamanteus. All reactions were performed at 3 μM DNA concentration (5’-end \textsuperscript{3}P labeled with specific activity 50 000 cpm) in 56 mM Tris-HCl (pH 7.9) and 4.4 mM MgCl\textsubscript{2} at 22 °C. Exonuclease concentration of 35 ng/μL was used for digestion of oligonucleotides. Total reaction volume was 14 μL. Aliquots were taken at 1, 2, 4, 8 and 24 h and quenched by addition of the same volume of 50 mM EDTA in 95% formamide. Reaction progress was monitored by 20% denaturing (7 M urea) PAGE and autoradiography.

Endonuclease Degradation Studies. Stability of AONs toward endonuclease was tested using DNase 1 from bovine pancreas. Reactions were carried out at 0.9 μM DNA concentration (5’-end \textsuperscript{3}P labeled with specific activity 50 000 cpm) in 100 mM Tris-HCl (pH 7.5) and 10 mM MgCl\textsubscript{2} at 37 °C using 20 unit of DNase 1. Total reaction volume was 22 μL. Aliquots were taken at 1, 2, 4, 8 and 12 h and quenched with the same volume of 50 mM EDTA in 95% formamide. They were resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by autoradiography.

Stability Studies in Human Serum. AONs (6 μL) at 2 μM concentration (5’-end \textsuperscript{3}P labeled with specific activity 90 000 cpm) were incubated in 30 μL of human serum (male AB) at 22 °C (total reaction volume was 36 μL). AONs (3 μL) were taken at 0, 15 and 30 min, 1, 2 and 9 h, and quenched with 6 μL of solution containing 8 M urea and 50 mM EDTA, resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by autoradiography.

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Supporting Information Available: (1) General experimental procedures; (2) Figure S1: CD spectra of AON (1–4, 7, and 10)/RNA (13) duplexes; (3) Figure S2: PAGE analysis of the DNase I degradation of AON (1)–(4), (7), and (11); (4) Figure S3: PAGE analysis of the snake venom phosphodiesterase (SVPDE) degradation of AONs (1), (7), (12), (8), (9) and (11); (5) Figure S4: PAGE analysis of the degradation of AONs (1), (7), (12), (8), (9) and (11) in human serum; (6) Table S1: Experimental and calculated vicinal 1J,HH coupling constants; (7) Table S2: Carbon chemical shifts in ppm (δ scale) of the oxetane nucleosides 1a (C), 1b (T), 1c (A), and 1d (G); (8) Figures S4–S16; 13C NMR spectra of compounds 15–22; (9) Figures S17–S18: 31P NMR spectra of compounds 23a–b; (10) Figure S19: 13C NMR spectrum of oxetane-A (1c); (11) Figure S20: 13C NMR spectrum of oxetane-G (1d). This material is available free of charge via the Internet at http://pubs.acs.org.

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